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Caracterização funcional do gene *VviAGL11* durante a morfogênese da semente em videira e seu potencial uso biotecnológico

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SEMENTE

Compositores: Almir Eduardo Melke Sater / Paulo Jorge Simões Correa Filho

Atirei minha semente na terra onde tudo dá
Chuva veio de repente carregou levou pro mar
Quando as águas foram embora plantei sonhos no chão
Mais demora minha gente ter na horta um verde puro
Ou dar fruto bem maduro um pomar

Meu adubo foi amor
Esperança o regador
Bem na hora da colheita
Lá se vai a ilusão
Foi geada e a seca me
Queimando a floração

Me doeu a impotência diante da sorte má
Então eu fiz paciência bem maior do que o azar
Convoquei os meus duendes pra fazer mutirão
Logo um toque de magia passou de mão em mão

Esse ano com certeza
Desengano vai ter fim
Natureza tem seus planos
Mas não sabe ser ruim
Tão seguro quanto o ar
Ser mais quente no verão
Da semente sai futuro
Nem que seja temporão

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LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

% - percentagem

AG - gene *AGAMOUS*

AGL11 – gene *AGAMOUS-LIKE 11*

AP1 – gene *APETALA 1*

AP2 – gene *APETALA 2*

AP3 – gene *APETALA 3*

AtAGL11 - gene *AGAMOUS-LIKE* de *Arabidopsis thaliana*

AVAG2 - gene *AGAMOUS 2* de *Asparagus*

BPC1 - gene *BASIC PENTACYSTEINE 1*

BS - gene *B SISTER*

cDNA – DNA complementar (do inglês, *complementary DNA*)

Col-0 - *Arabidopsis thaliana* acesso Columbia-0

DNA – ácido desoxirribonucleico (do inglês, *deoxyribonucleic acid*)

DAF – dias após a fecundação

DEF - gene *DEFICIENS*

DNase – desoxirribonuclease

EgMADS1 - gene *MADS-Box 1* de *Eustoma grandiflorum*

ESTs – etiquetas ou marcas de sequências expressas (do inglês, *Expressed Sequence Tags*)

FAO – Organização das Nações Unidas para Agricultura e Alimentação (do inglês, *Food and Agriculture Organization of the United Nations*)

FBP7 - gene codificador da *FLORAL BINDING PROTEIN 7*

FBP11 - gene codificador da *FLORAL BINDING PROTEIN 11*

GFP - proteína verde fluorescente (do inglês *Green Fluorescent Protein*)

GUS - β-glicuronidase

INDEL - mutação de inserção ou deleção de nucleotídeos (do inglês *Insertion or Deletion*)

LMADS2 – gene *MADS-Box 2* de *Lilium*

Mcm1 - gene *minichromossome maintenance 1*

MAS - seleção assistida por marcadores (do inglês, *marker assisted selection*)

mRNA – RNA mensageiro (do inglês, *messenger RNA*)

NT - não tratadas

OGM - Organismos Geneticamente Modificados

OIV - Organização Internacional da Uva e do Vinho (do francês, *Organization Internacional de la Vigne et du Vin*)

OsMADS13 - gene *MADS-Box 13* de *Oryza sativa*

OX- superexpressão (do inglês, *overexpression)*

pb – par(es) de bases

PCR – reação em cadeia da DNA polimerase (do inglês, *polymerase chain reaction*)

PhalAG2 - gene *AGAMOUS 2* de *Phalaenopsis*

PI - gene *PISTILATTA*

QTL- locus de característica quantitativa (do inglês, *quantitatve trait locus*)

RNA – ácido ribonucleico (do inglês, *ribonucleic acid*)

RNAi - RNA de interferência

RT-qPCR – PCR quantitativa precedida de transcrição reversa (do inglês, *reverse transcription-quantitative PCR*)

SdI - inibidor do desenvolvimento da semente (do inglês *Seed Development Inhibitor*)

SEP1 – gene *SEPALLATA 1*

SEP2 - gene *SEPALLATA 2*

SEP3 - gene *SEPALLATA 3*

SEP4 - gene *SEPALLATA 4*

SHP1 - gene *SHATTERPROOF 1*

SHP2 - gene *SHATTERPROOF 2*

SNP- polimorfismo de nucleotídeo único (do inglês *Single Nucleotide Polymorphism*)

SOC - gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANCE 1*

SRF - gene *SERUM RESPONSE FACTOR*

SSR- repetição de sequência única (do inglês *Single Sequence Repeat)*

STK - gene *SEEDSTICK*

stk - mutante de *A. thaliana* que não expressa o gene *STK/AtAGL11*

T1 - primeira geração transgênica (do inglês, *First Filial Generation of Transgenic)*

TYLCV- Vírus do Amarelamento e Enrolamento da Folha do Tomateiro (do inglês, *Tomato Yellow Leaf Curl Virus*)

VviAGL11 - gene *AGAMOUS-LIKE 11* de *Vitis vinifera* (*VvAG3*)

WT - tipo-selvagem (do inglês, *wild-type*)

ZAG2 - gene *AGAMOUS 2* de *Zea mays*

ZMM1 - gene *MADS-BOX 1* de *Zea mays* (*ZMADS1*)

RESUMO

A ausência de semente em videira, também chamada de apirenia, é amplamente apreciada pelo mercado consumidor de uvas de mesa. No entanto, os mecanismos moleculares que controlam a morfogênese da semente não são totalmente compreendidos. Por este trabalho, buscou-se caracterizar funcionalmente o gene candidato *VviAGL11*, avaliando seu papel na morfogênese de sementes de *Vitis vinifera*. Dados prévios permitiram determinar o padrão de expressão de *VviAGL11* na camada da endotesta da casca da semente, que precisa alongar e aumentar o número de células para que haja a lignificação e a determinação do tamanho final da semente. No presente estudo, a função de *VviAGL11* foi avaliada por meio de sua expressão ectópica no mutante de *seedstick (AGL11)* de *Arabidopsis thaliana*, o que restaurou o fenótipo e confirmou o papel direto deste gene no desenvolvimento da semente, sugerindo que a depleção de sua expressão é responsável pelo desenvolvimento errôneo da camada de endotesta da semente, culminando no fenótipo de apirenia típico. Além disso, a função de *VviAGL11* foi avaliada em videira com o uso de plasmídeos vegetais. Os resultados permitiram demonstrar que a alta expressão de *VviAGL11* na cultivar apirênica Linda, após tratamento, está relacionada com a presença de pequenas sementes que não foram encontradas nas amostras-controle não tratadas. Além disso, cachos de 'Italia' e 'Ruby' tratados com o plasmídeo de silenciamento *VviAGL11* mostraram diminuição da expressão desse gene, número reduzido de sementes e aumento do número de traços de sementes. Em conjunto, os resultados confirmam que *VviAGL11* é um importante regulador da morfogênese de sementes em videira. Em adição, populações segregantes para ausência de sementes foram testadas com cinco marcadores SSR, dos quais três marcadores microssatélites mostraram-se relacionados à ausência de sementes e poderiam ser usados com 100% de eficiência em um haplótipo para seleção assistida. Ao mesmo tempo, nove marcadores do tipo *SNP*s e *INDEL*s foram desenvolvidos com base na sequência do alelo de *VviAGL11* associado à ausência de sementes em *V. vinifera*. Para os marcadores VvAGL11_KASP_2, VvAGL11_KASP_3, VvAGL11_KASP_8 e VvAGL11_KASP_9, polimorfismos foram observados segregando em indivíduos apirênicos genotipados, confirmando sua associação com a ausência de sementes e sugerindo seu uso na estratégia de seleção assistida rápida e eficaz de videiras apirênicas.

ABSTRACT

Grapevine seedlessness, also known as apyreny, is widely appreciated by the table grape's market. Nevertheless, the molecular mechanisms that control seed morphogenesis are not fully understood. This study aimed to characterize the function of the candidate gene *VviAGL11*, evaluating its role in *Vitis vinifera* seed morphogenesis. Previous data allowed us to determine the *VviAGL11* expression pattern in the endotesta layer of the seed coat, which needs to elongate and increase in cell number to accomplish seed lignification and final seed size. In the present study *VviAGL11* function was evaluated by its ectopic expression in *Arabidopsis thaliana seedstick (AGL11)* mutant background, which restored the phenotype and confirmed the direct role of this gene in seed development, suggesting that depletion of its expression is responsible for the erroneous development of the endotesta layer of the seed, therefore culminating in the typical seedless phenotype. Furthermore, we evaluated *VviAGL11* function in grapevine with the use of plant plasmids. The results showed that a high expression of *VviAGL11* in the seedless cultivar Linda, after treatment, was related with the presence of small seeds that were not found in untreated control samples. Additionally, seeded 'Italia' and 'Ruby' bunches treated with a *VviAGL11*-silencing plasmid showed decreased gene expression, reduced number of seeds and increased number of seed traces. Taken together, the results confirm that *VviAGL11* is a key master regulator of seed morphogenesis in grapevine. Moreover, segregating populations for seedlessness were tested with five SSR markers of which three microsatellite markers were proven to be related with seedlessness and could be used with 100% efficiency in a haplotype for assisted selection. Additionally, nine unique *SNP*s and *INDEL*s markers were developed based on *VviAGL11* allele associated with the absence of seeds in *V. vinifera*. For the markers VvAGL11_KASP_2, VvAGL11_KASP_3, VvAGL11_KASP_8 and VvAGL11_KASP_9, polymorphisms were observed segregating in genotyped seedless individuals, confirming their seedlessness association and suggesting their use in fast and effective assisted selection strategy for seedlessness grapevines.

1 INTRODUÇÃO

1.1 A CULTURA DA VIDEIRA

A videira (*Vitis* sp. L.) é uma das frutíferas mais cultivadas mundialmente (MARINVAL, 1997; OIV, 2016). Seu uso antrópico possui origens antigas, datando em mais de 8.000 anos, nos quais antigas civilizações manejaram seu cultivo inicialmente na área mediterrânea visando a produção de vinho e, posteriormente, propagando a viticultura mundialmente devido à adaptabilidade de diferentes variedades a diversos solos e condições climáticas (LACIRIGNOLA & DIGIARO, 1999; THIS *et al.,* 2006). Na atualidade, a uva é a 4ª fruta mais produzida no mundo com 75,7 milhões de toneladas de frutos produzidos em 2016, sendo que a Europa é responsável por 40% da produção mundial, seguida pela Ásia (31 %) e pela América (20 %) (OIV, 2016). O grande apelo comercial desta fruta de clima temperado é justificado pelo consumo de suas bagas em diversos produtos como vinhos (48 %), consumo de uvas de mesa *in natura* (36 %), produção de uvas passas (8 %) e produtos de menor impacto como sucos, geleias, etanol, vinagre, óleo de sementes, ácido tartárico e fertilizantes (FASOLI *et al.,* 2012; OIV, 2016). A viticultura é uma atividade tradicional em países de clima temperado e, recentemente, tem ganhado significância em diversas regiões de clima subtropical e tropical. Essa característica abre perspectivas para uma grande expansão na viticultura tropical, principalmente para a produção de uvas de mesa, sendo que o Brasil ocupa o 6° lugar na produção mundial neste quesito (FAO, 2010; OIV, 2016).

Programas de melhoramento de videiras têm focado esforços na geração de cultivares com determinadas características organolépticas como sabor moscatel, suculência e crocância (LAHOGUE *et al.,* 1998; CAMARGO *et al.,* 2005; FANIZZA *et al.,* 2005). Além disso, o tamanho das bagas e a ausência de sementes, denominada apirenia, são as principais características apreciadas por consumidos de uvas de mesa (VAROQUAUX *et al.,* 2000; CABEZAS *et al.,* 2006). Nas últimas duas décadas, a comercialização mundial de uva *in natura* teve um aumento considerável, com a produção mundial de uva de mesa passando de 25 % da produção total de uvas em 2000, para 36 % em 2014, contabilizando ~27 milhões de toneladas por ano, segundo dados da Organização Internacional da Uva e do Vinho (OIV, do francês, *Organisation Internationale de la Vigne et du Vin*) e da Organização das Nações Unidas para Agricultura e Alimentação (FAO, do inglês, *Food and Agriculture Organization of the United Nations*). Porém, a produção de uvas para essa finalidade cresceu a taxas anuais

próximas de 13 %, não acompanhando a demanda do mercado consumidor (FAO, 2012, OIV, 2016). Essa expansão comercial gerou oportunidades de crescimento aos países produtores de uvas de mesa e criou competitividade entre os mercados exportadores das melhores uvas apirênicas (LAZZAROTTO & FIORAVANÇO, 2012). A compreensão dos mecanismos genéticos e moleculares controladores da apirenia em uvas de mesa possui grande relevância econômica uma vez que, em países altamente consumidores dessa fruta como os Estados Unidos da América e países europeus, as uvas apirênicas já dominam o mercado de uvas de mesa (NACHTIGAL, 2003; DE MELO, 2015).

1.2 MORFOGÊNESE DE SEMENTES E APIRENIA

1.2.1 Desenvolvimento Morfológico de Sementes

O táxon das Angiospermas é distinto dos demais táxons vegetais por possuir importantes características autapomórficas e, entre elas, a presença de flores e frutos. O nome "angiosperma" deriva da palavra grega *angeion*, que significa 'vaso" ou "recipiente", e *sperma,* que significa "semente" (RAVEN *et al.,* 2007). Neste táxon, as sementes encontram-se encerradas no interior de frutos que, por sua vez, são provenientes do desenvolvimento do ovário após a fecundação. Para a formação da semente e do fruto, é necessário que ocorra a dupla fecundação. Nela, o grão de pólen (gametófito masculino) germina em um pistilo que se desenvolve em um tubo no qual dois gametas masculinos são movidos em direção ao saco embrionário (gametófito feminino) que contém em particular a oosfera e os núcleos polares, além das células antípodas. Uma vez que o tubo polínico tenha atingido o saco embrionário, um dos gametas masculinos funde-se com a oosfera para formar um zigoto, e um gameta masculino funde-se aos núcleos polares, fusão tripla, formando um tecido poliploide que se desenvolve no endosperma (JENSEN, 1964; JENSEN & FISHER, 1968).

As sementes das angiospermas são estruturas especializadas que promovem a proteção do embrião, garantindo, assim, a propagação e a manutenção de suas espécies. A semente é composta por tegumentos, endosperma e embrião (BERGER *et al.,* 2006). Os tegumentos desenvolvem-se na casca da semente que se torna responsável por proteger o endosperma e o embrião de situações adversas até que haja condições favoráveis para a

germinação. O endosperma acumula os nutrientes necessários para o desenvolvimento inicial do embrião, e será consumido pelo esporófito em crescimento tanto antes da maturação da semente quanto depois dela. Já o embrião divide-se em duas partes, a radícula e o cotilédone, que germinarão para a formação de uma nova planta (RAVEN *et al.,* 2007). O endosperma e o embrião são ambos de origem tanto materna quanto paterna pois são gerados pela dupla fecundação, sendo o primeiro triplóide, proveniente da fecundação da célula central homodiplóide, e o segundo diploide, originado da fecundação da célula ovo haploide. Diferentemente, a casca da semente, formada pelas camadas de tegumento, possui apenas origem materna (BERGER *et al.,* 2006).

Na planta modelo *Arabidopsis thaliana*, o desenvolvimento da semente é dividido em duas fases distintas (Figura 1). A primeira é caracterizada pela proliferação e crescimento do endosperma, que aumenta o tamanho da semente em cerca de 100 vezes e traz grande contribuição para o seu tamanho final (VAROQUAUX *et al.,* 2000). Em contraste, o crescimento do embrião ocorre durante a segunda fase, quando há a expansão do endosperma (JURGENS & MAYER, 1994). Mais detalhadamente, nos primeiros cinco dias, as duas camadas de células do tegumento externo do óvulo (Figura 1, a, superior, 1,2) e as três camadas de células do tegumento (a, superior, 3-5) sofrem um período de crescimento, visível de (a) para (b). Após essa etapa, as células de camadas individuais diferenciam-se (c) em tipos de células especializadas incluindo endotélio (5), paliçada (2) e epiderme (1), um processo quase completo dez dias após a fecundação (d). No estádio de maturação da semente, em torno de quinze dias após a fecundação (e), todas as células das camadas da casca estão mortas e foram esmagadas juntas, exceto pela epiderme, cuja forma é mantida por uma parede celular secundária espessa (HAUGHN & CHAUDHURY, 2005, Figura 1). Por meio da observação das fases de formação da semente, é possível verificar que o seu crescimento é ditado pelo desenvolvimento conjunto do endosperma e dos tegumentos (GARCIA *et al.,* 2005). Em adição, as camadas da casca da semente possuem um papel relevante na determinação do tamanho final da semente, que derivam de sua capacidade de alongamento, gerando, assim, um forte efeito materno neste processo (BERGER *et al*. 2006).

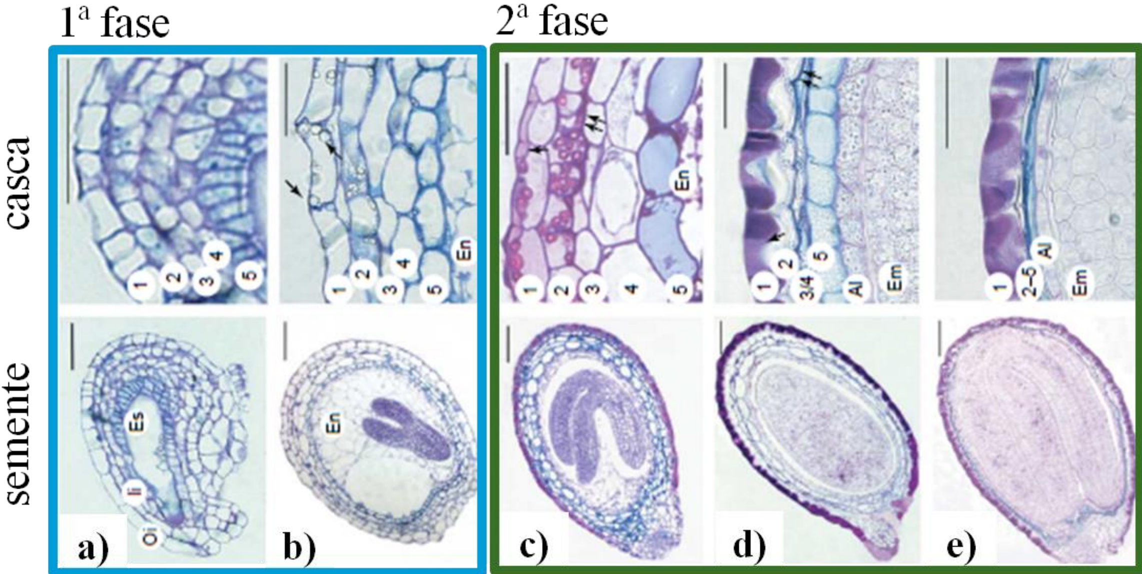

Figura 1. Tegumentos do óvulo em desenvolvimento para a formação da casca da semente, após a fecundação. O painel inferior mostra o desenvolvimento de sementes inteiras de *A. thaliana* enquanto que o painel superior mostra em detalhe as camadas da casca da semente. Sementes dois dias após a fecundação (DAF) (a); cinco DAF (b); sete DAF (c); dez DAF (d) e quinze DAF (e). Setas simples indicam plastídios contendo amido (b), mucilagem no apoplasto (c) ou formação de parede celular secundária na epiderme (d). Camadas de células do tegumento externo do óvulo (1 e 2 em a-b); camadas de células do tegumento (3, 4 e 5 em a-b); células de camadas individuais se diferenciam em tipos de células especializadas incluindo endotélio (5 em d-e), paliçada (2 em d-e) e epiderme (1 em d-e). As setas duplas indicam a parede celular secundária da camada paliçada (c, d). Abreviaturas: Al, aleurona do endosperma; Em, embrião; En, endosperma; Es, saco embrionário; Ii, tegumento interno; Oi, tegumento externo. Escala= 80 mm (b – e, inferior); 40 mm (a, inferior, a – e, superior). Adaptado de Haugh & Chaudhury (2005).

Apesar da relevância econômica da videira e do interesse em variedades com características diferenciadas quanto à produção e ao tamanho das sementes, poucos estudos abordaram as fases de desenvolvimento e a importância das camadas da semente de uva. A semente de videira (*V. vinifera*) possui uma forma específica de pera, com cortes transversais em formato de trígono (CADOT *et al.,* 2006). O entorno da semente é coberto por uma camada de cutícula que se localiza acima de uma camada de epiderme. O tegumento externo pode ser dividido em exotesta e endotesta, que são a camada externa e interna,

respectivamente, do tegumento externo (MALABARBA *et al.,* 2017). Já o tegumento interno possui três camadas que envolvem o endosperma e por sua vez o embrião (Figura 2, PRATT, 1971). A cor da semente modifica-se ao longo de seu amadurecimento, iniciando com coloração verde até marrom escuro quando em maturação. Isso ocorre devido ao endurecimento da casca e da concomitante oxidação dos compostos fenólicos (RIBEREAU-GAYON & PEYNAUD, 1980).

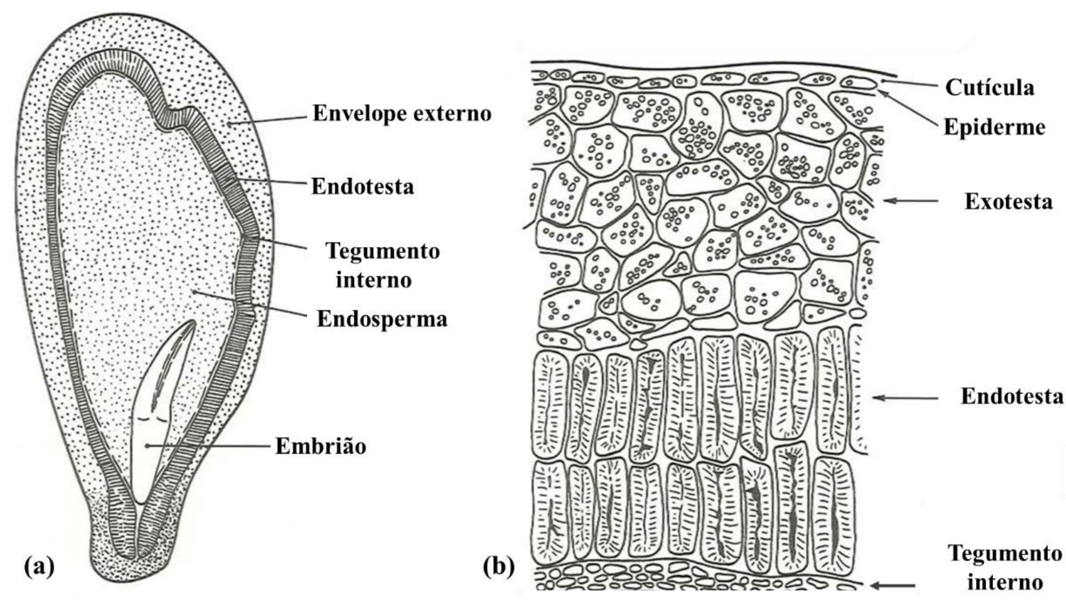

Figura 2. Ilustração da semente de videira. (a) A semente madura apresentada em corte longitudinal, no qual são visualizados o envelope externo, seguido do tegumento externo e interno. A porção central é composta pelo embrião circundado pelo endosperma. (b) Ilustração em detalhe das camadas da casca da semente composta por uma camada de cutícula, uma camada de epiderme seguida pelo tegumento externo, formado pela exotesta e pela endotesta. Abaixo destes, iniciam-se as camadas de tegumento interno. Adaptado de Pratt (1971).

Para que haja o completo desenvolvimento da semente, é necessária a coordenação temporal e espacial de crescimento entre os diferentes tecidos, além da nutrição homogênea de todos os seus componentes (UNGRU *et al.,* 2010). As rotas de passagem de nutrientes e de moléculas como proteínas, mRNAs e micro-RNAs para as diversas camadas da semente ainda não são completamente compreendidas, porém estudos mostram que o fluxo de nutrientes ocorre do tegumento externo para o interno, de forma centrípeta, passando através

de apoplastos para o endosperma e para o embrião (STADLER *et al.,* 2005). A ausência parcial ou total do desenvolvimento de alguma camada da semente pode causar o desenvolvimento errôneo ou a inviabilidade dessa estrutura, um processo denominado de apirenia.

1.2.2 Apirenia

O cultivo de diversas espécies vegetais base da alimentação humana e animal como aveia, arroz, soja e milho depende da produção de grãos (FAO, 2018). Todavia, o mercado consumidor de frutas visa a geração de frutos com características sensoriais necessariamente desconectadas da semente, sendo de grande apreciação a ausência parcial ou total de sementes (EGLI, 1998). A apirenia caracteriza-se pela produção de frutos sem sementes, com traços de sementes ou com sementes reduzidas em número e tamanho (VAROQUAUX *et al.,* 2000). Uma planta apirênica deve possuir ao menos uma das características descritas acima, porém plantas completamente sem sementes ou que apresentem apenas traços de sementes são preferíveis pelos consumidores, sendo consideradas cultivares elite (VAROQUAUX *et al.,* 2000). A geração de frutos apirênicos pode ocorrer por meio de duas formas diferentes de apirenia: por partenorcarpia ou por estenoespermocarpia. Esses processos são caracterizados a partir do estádio fenológico no qual a semente ou o óvulo se encontra quando há o encerramento de seu desenvolvimento normal (EMERSHAD *et al.,* 1989). Na partenocarpia, a semente não se desenvolve pois não há fecundação. Todavia, o ovário ainda é capaz de se desenvolver em fruto, apenas a partir do desenvolvimento dos tecidos maternos, o que gera a formação de frutos com ausência total de sementes (STOUT, 1936; BOUQUET & DANGLOT, 1996). Já no processo de estenoespermocarpia ocorre a produção de frutos com traços de semente pois há fecundação e desenvolvimento inicial da semente. Entretanto, a semente cessa seu desenvolvimento após duas semanas, acarretando na morte do embrião ainda imaturo devido à ausência ou má formação do endosperma (STOUT, 1936; BOUQUET & DANGLOT, 1996; VAROQUAUX *et al.,* 2000; MEJÍA *et al.,* 2011).

Frutos estenoespermocárpicos são altamente comercializados e é o caso para diversas cítricas como, por exemplo, laranja, limão e tangerinas, além de tomates, melões, melancias e uvas (VARDI *et al.,* 2008; WANG *et al.,* 2012). Atualmente, diversas variedades de uvas apirênicas são produzidas no mundo, cada qual sendo escolhida por sua adaptabilidade às

condições locais. No Brasil, a produção de uvas de mesa utilizadas principalmente para consumo *in natura* está baseada nas cultivares Itália, Ruby, Benitaka, Brasil e Red Globe, que são uvas pirênicas, e nas cultivares Sultanine, Centennial Seedless, Superior Seedless, Catalunha e Crimson Seedless, que são uvas apirênicas (PROTAS, 2005). Dentre estas, destaca-se a cultivar estenoespermocárpica Sultanine que, devido à sua boa adaptação de cultivo ao cenário europeu e americano, foi amplamente utilizada em cruzamentos visando a geração de novas cultivares apirênicas e, por consequência, tornando-se a cultivar modelo para o estudo da estenoespermocarpia em videira (ADAM-BLONDON *et al.,* 2001; DI GENOVA *et al.,* 2014). A cultivar Sultanine origina frutos com traços de sementes pouco desenvolvidos e macios, imperceptíveis ao consumidor, como é possível observar na Figura 3, sendo fortemente apreciada no mercado de uvas de mesa (STOUT, 1936). Essa cultivar é uma mutante somática natural, proveniente da Pérsia da Ásia Menor, atualmente áreas do Irã e da Turquia, onde era conhecida como ‘Oval Kechmish’. Em 1872, foi iniciado seu cultivo em Marysville, Califórnia, EUA por Willian Thompson do qual descende seu nome mais comum, ‘Thompson Seedless’ (CHRISTENSEN, 2000).

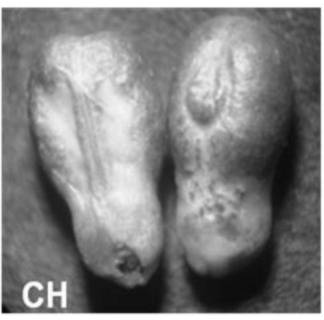

Figura 3. Comparação entre a uma semente com desenvolvimento normal e uma baga estenoespermocárpica. Durante o estádio de maturação dos frutos, ‘Chardonnay’ (CH) apresenta sementes de tamanho adequado e lignificadas enquanto que ‘Sultanine’ (SU) (baga) contém apenas o traço de uma semente. Semente de ‘Chardonnay’ com aumento de 40X. Escala de 1 cm em baga de ‘Sultanine’.

1.3 MECANISMOS MOLECULARES DA MORFOGÊNESE DE SEMENTES

1.3.1 Os genes MADS-box

A identificação e a compreensão dos mecanismos genéticos e moleculares que coordenam o desenvolvimento de espécies com ausência de sementes são essenciais para a obtenção de culturas comerciais de alta qualidade e competitividade no mercado (CAMARGO *et al.,* 2005). Diversos são os componentes que regem o desenvolvimento de flores e frutos e muitos estudos de caráter molecular tem foco no desenvolvimento dos órgãos florais e no processo de transição floral (SMACZNIAK *et al.,* 2012). Neste contexto, a família gênica *MADS-box* é constituída por mais de cem genes em plantas. Em *A. thaliana*, são 107 genes, muitos deles atuando na determinação da identidade dos meristemas florais além de serem importantes para o desenvolvimento da raiz, o controle da floração, a arquitetura floral, o desenvolvimento do pólen e o desenvolvimento de frutos e de sementes (PARENICOVÁ *et al.,* 2003; SMACZNIAK *et al.,* 2012; GRAMZOW & THEIßEN, 2013).

Essa família de fatores de transcrição é conhecida pelo seu domínio MADS de ligação ao DNA, sendo que seu nome é um acrónimo derivado de quatro genes membros dessa família gênica: **M**CM1 de *Saccharomyces cerevisiae*, **A**GAMOUS (AG) de *A. thaliana*, **D**EFICIENS (DEF) de *Antirrhinum majus*, e **S**RF de *Homo sapiens* (SCHWARZ-SOMMER *et al.,* 1990). Mesmo estando presente em diversos organismos eucarióticos, os primeiros genes *MADS-box* isolados foram genes homeóticos de desenvolvimento floral (SCHWARZ-SOMMER *et al.,* 1990; CAUSIER *et al.,* 2010). Os fatores de transcrição MADS-box contêm domínios de ligação ao DNA e de dimerização conservados em diversas espécies (PELLEGRINI *et al.,* 1995; HUANG *et al.,* 2000). Os genes *MADS-box* são divididos em dois grupos tendo-se por base a estrutura de seus domínios proteicos, denominadas de tipo I e de tipo II (SMACZNIAK *et al.,* 2012). Outra característica importante é que os genes *MADS-box* formam subfamílias distintas e altamente conservadas do ponto de vista filogenético, sendo que genes da mesma subfamília geralmente possuem funções relacionadas (THEIßEN *et al.,* 2001; BECKER & THEIßEN, 2003; GRAMZOW & THEIßEN, 2015). Um exemplo é o gene SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) envolvido no controle da floração em eudicotiledoneas, que possui um ortólogo mesmo em arroz, distantemente aparentado (SAMACH *et al.,* 2000).

1.3.2 Os genes MADS-box de determinação floral

Dentre os genes MADS-box mais estudados e caracterizados então os genes do tipo II, que são genes homeóticos que atuam principalmente no desenvolvimento floral, na embriogênese e no desenvolvimento do fruto (SMACZNIAK *et al.,* 2012; THEIßEN *et al.,* 2016). Este grupo é caracterizado por sua estrutura denominada *MIKC*, na qual a porção N-terminal é composta pelo domínio MADS (M) de ligação ao DNA, seguido por dois domínios essenciais para a interação com proteínas, as porções I (*intervening*) e K (*keratin-like*). O domínio C-terminal é responsável pela formação e atividade de complexos proteicos e pela regulação transcricional (KAUFMANN *et al.,* 2005). O domínio MADS consiste em um motivo de 56 resíduos que formam um par alfa-hélices superenroladas antiparalelas, embaladas contra uma folha-beta dupla antiparalela (Figura 4). A folha-beta deste motivo também está envolvida em interações e dimerizações proteicas (LUSCOMBE *et al.,* 2000).

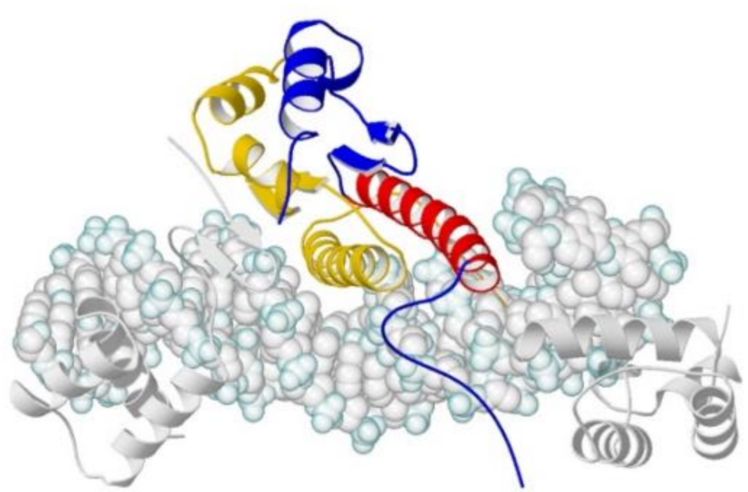

Figura 4. Conformação proteica dos motivos de ligação ao DNA das proteínas MADS-box. Uma folha-beta antiparalela e duas alfa-hélices adjacentes fornecem a interface de dimerização. As alfa-hélices na face oposta da folha divergem do centro do local de ligação para as aberturas principais adjacentes. A molécula de DNA é dobrada em direção à proteína. O motivo de ligação ao DNA está em vermelho. A proteína MADS se liga como um dímero, sendo que um monômero está colorido de azul e o outro de amarelo. O DNA é mostrado como um modelo de preenchimento de espaço. Adaptado de LUSCOMBE *et al.* (2000).

Com base nas características estruturais destes domínios, os genes *MADS-box* do tipo *MIKC* foram ainda divididos em *MIKC*^{C} (forma canônica) e *MIKC**. Este último apresenta alterações no domínio K, possivelmente pela duplicação de éxons desta região, e forma uma rede de interação de proteínas responsável pela maturação do pólen (ADAMCZYK & FERNANDEZ, 2009).

Nas últimas três décadas, os genes do tipo *MIKC*^{C} foram amplamente estudados em *A. thaliana*. Eles compõem o "Modelo de Desenvolvimento Floral ABCDE", e estudos mais recentes demonstram sua maneira de atuação na forma do "Modelo dos Quartetos Florais" conforme apresentado na Figura 5 (THEIßEN, 2001; PARENICOVÁ *et al.,* 2003; SMACZNIAK *et al.,* 2012). Em ambos os modelos, que são complementares e não divergentes, os genes são divididos em classes, compostas pelas subfamílias as quais pertencem. A identidade de sépalas é determinada por genes da classe A (*APETALA1 - AP1*), enquanto que os genes da classe A juntamente com genes da classe B (*APETALA3 - AP3* - e *PISTILATTA - PI*) determinam a identidade das pétalas. Por sua vez, genes da classe B somados aos genes da classe C (*AG*) são responsáveis pela formação de estames. Os genes da classe C possuem papel na identidade de carpelos (LOHMANN & WEIGEL, 2002) que, quando combinados com os genes da classe D (*SEEDSTICK (STK) - SHATTERPROOF1 (SHP1) - SHATTERPROOF2 (SHP2)*), atuam no desenvolvimento dos óvulos (COLOMBO *et al.,* 1995). As proteínas das classes A, B, C e D são dependentes de proteínas da classe E (*SEPALATTA1 - SEP2 - SEP3 - SEP4*) para possuírem atividade na formação do Modelo de Quartetos Florais de transcrição (THEIßEN & SAEDLER, 2001; DORNELAS & DORNELAS, 2005). Os quartetos florais são, portanto, complexos de alta ordem, normalmente em junções tetraméricas formadas por dois dímeros que podem ser homogêneos ou heterogêneos (THEIßEN *et al.,* 2016). Os complexos quaternários das proteínas MADS-box ligam-se a duas sequências de DNA consenso, denominadas CArG-box ($CC(A/T)_6GG$), que podem estar separadas por até 300 pb (LIU *et al.,* 2008). Desta forma, há a geração de um *loop* de DNA entre os dois sítios CArG-box (THEIßEN, 2001; THEIßEN & SAEDLER, 2001; MENDES *et al.,* 2013) (Figura 5).

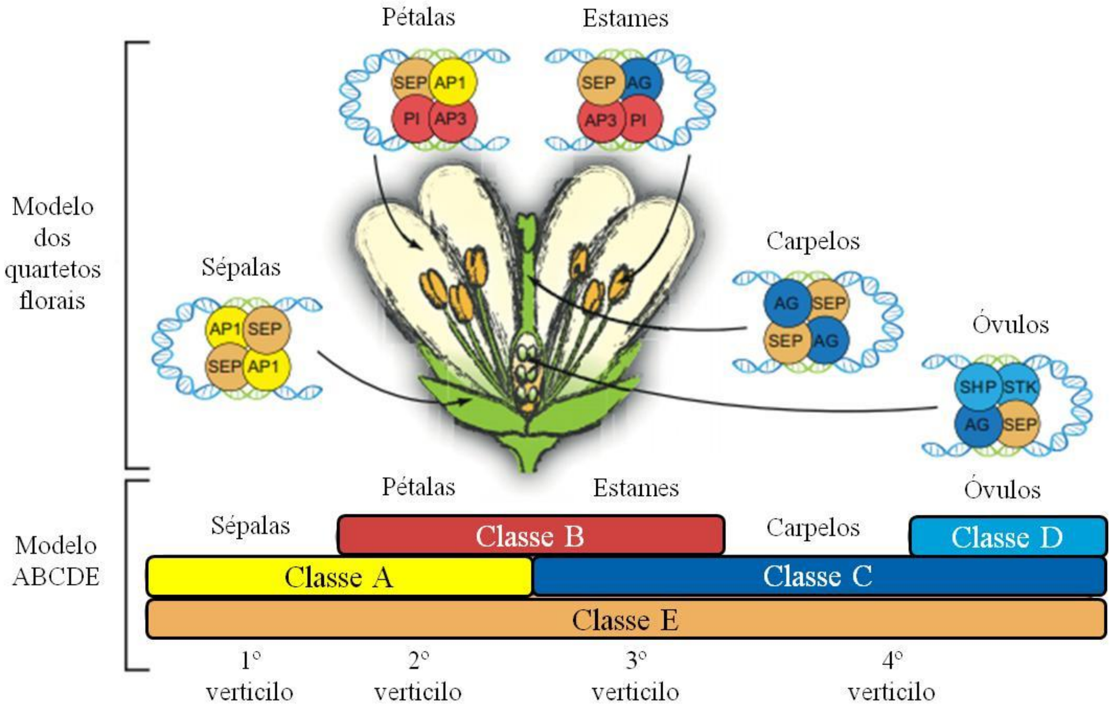

Figura 5. Modelo dos Quartetos Florais e Modelo ABCDE de determinação da identidade dos órgãos florais por proteínas *MIKC*^{C} em *A. thaliana*. Os genes *MIKC*^{C} da classe A (*API*) determinam a formação de sépalas; genes da classe B (*AP3* e *PI*), juntamente com genes da classe A, são necessários para a formação de pétalas. Os genes da classe B também são necessários para a formação de estames junto com os genes da classe C (*AG*) que, por sua vez, são necessários para a formação de carpelos. Os genes da classe D (*STK*, *SHP1* e *SHP2*) são responsáveis pelo desenvolvimento de óvulos. Todas as classes (A, B, C, D) necessitam de ao menos um dos genes da classe E (*SEP1*, *SEP2*, *SEP3* e *SEP4*) para realizarem sua função. As proteínas dos genes *MIKC*^{C} formam complexos quaternários que se ligam em regiões CArG-box dos genes-alvo (painel superior). Adaptado de THEIßEN *et al.* (2016).

A interação entre os complexos quaternários e as sequências CArG-box são essenciais para a atividade desses fatores de transcrição. O genoma vegetal contém inúmeras sequências CArG-box que são devidamente reconhecidas por diferentes conjuntos de proteínas MADS-box atuando na transcrição de genes-alvo distintos (GRAMZOW & THEIßEN, 2013). Acrescido a isso, a formação das diferentes interações proteicas gera inúmeras combinações de multimerização de proteínas que aumentam a diversificação de ligação dos complexos MADS-box ao DNA e, consequentemente, promovem a transcrição

de genes de diferentes rotas metabólicas (MELZER & THEIßEN, 2009; SMACZNIAK *et al.,* 2012).

1.3.3 Genes *MIKC*^{C} responsáveis pelo desenvolvimento de óvulo e semente

O fato dos genes *MADS-box* serem conservados em diversas espécies vegetais e atuarem no desenvolvimento floral os torna excelentes candidatos para o melhoramento de plantas com foco em produção de grãos e de frutos com fenótipos de interesse agronômico (THEIßEN *et al.,* 2016; SCHILLING *et al.,* 2018). Neste quesito encontram-se os genes *MIKC*^{C} das classes C e D. Esses genes formam a subfamília monofilética AG, pertencendo a clados irmãos uma vez que foram gerados por um evento de duplicação ocorrido no início da evolução das angiospermas (KRAMER *et al*. 2004). Essa família é distinta pela presença de dois motivos na região C-terminal de suas proteínas conhecidos como motivos AGI e AGII, os quais atuam na formação de complexos quaternários das proteínas MADS-box (YUN *et al.,* 2004; SONG *et al.,* 2006).

Os genes *FBP7* e *FBP11* (*FLORAL BINDING PROTEIN 7* e *11)* de *Petunia hybrida*, expressos especificamente nos óvulos, foram os primeiros genes *MADS-box* caracterizados da classe D por meio da geração de duplos mutantes de *A. thaliana* com expressão ectópica, o que levou à formação de óvulos no local de pétalas e sépalas (ANGENENT *et al.,* 1995; COLOMBO *et al.,* 1995; ANGENENT & COLOMBO 1996; COLOMBO *et al.,* 1997). Genes de outras espécies que tendem a especificar a identidade dos tecidos da quarta camada da flor foram propostos como ortólogos aos genes *FBP7* e *FBP11*, como *AtAGL11* (*STK*) em *A. thaliana* (ROUNSLEY *et al.,* 1995; PINYOPICH *et al.,* 2003), *ZAG2* e *ZMM1* em *Zea mays* (SCHMIDT *et al.,* 1993; THEIßEN *et al.,* 1995), *OsMADS13* em *Oryza sativa* (LOPEZ-DEE *et al.,* 1999), *PhalAG2* em *Phalaenopsis* sp. (SONG *et al.,* 2006), *AVAG2* em *Asparagus* sp. (YUN *et al.,* 2004), *LMADS2* em *Lilium* sp., *EgMADS1* em *Lisianthus* sp. (TZENG *et al*. 2002), *TAGL11* em *Lycopersicum esculentum* (BUSI et al., 2003) e *VviAGL11* em *V. vinifera* (FAVARO *et al.,* 2003; PINYOPICH *et al.,* 2003; REVERS *et al.,* 2010, MEJÍA *et al.,* 2011).

Um dos genes mais bem caracterizados quanto a seu papel no desenvolvimento da semente é o gene *AtAGL11,* também denominado *SEEDSTICK (STK),* de *A. thaliana.* Esse gene é responsável pela identidade do óvulo, redundantemente com os outros genes da classe D, *SHATTERPROOF1* e *SHATTERPROOF2* (*SHP1, SHP2*). O gene *STK* é uniformemente

expresso nos tegumentos dos óvulos e ao longo do funículo nas fases iniciais de desenvolvimento da semente, enquanto que, na fase de maturação, é fortemente expresso no funículo e fracamente nos tegumentos (ROUNSLEY *et al.,* 1995; PINYOPICH *et al.,* 2003; MATIAS-HERNANDEZ *et al.,* 2010). O mutante *seedstick* (*stk*) de *A. thaliana* apresenta sementes diminutas, além de um conjunto de fenótipos defectivos na geração de sementes e na formação da síliqua (PINYOPICH *et al.,* 2003; BRAMBILLA *et al.,* 2007). Na Figura 6 estão exemplificados os fenótipos diferenciais de *stk* em comparação a plantas de *A. thaliana* Columbia-0 de fenótipo selvagem (WT). Além de demonstrar diminuição no tamanho das sementes, há o aumento da espessura do funículo (Figura 6d-e). Isso ocorre devido ao gene *STK* também atuar no controle da expansão e da divisão celular do funículo, tornando essa estrutura mais larga e espessa na ausência de sua expressão (ROUNSLEY *et al.,*1995; PINYOPICH *et al.,* 2003). Outra característica são as síliquas de menor comprimento e com apenas metade da quantidade normal de sementes (Figura 6a). O mutante *stk* também apresenta o fenótipo de não abscisão das sementes (Figura 6f), provavelmente pela estrutura diferenciada do funículo que as mantém fortemente ligadas à rafe (PINYOPICH *et al.,* 2003).

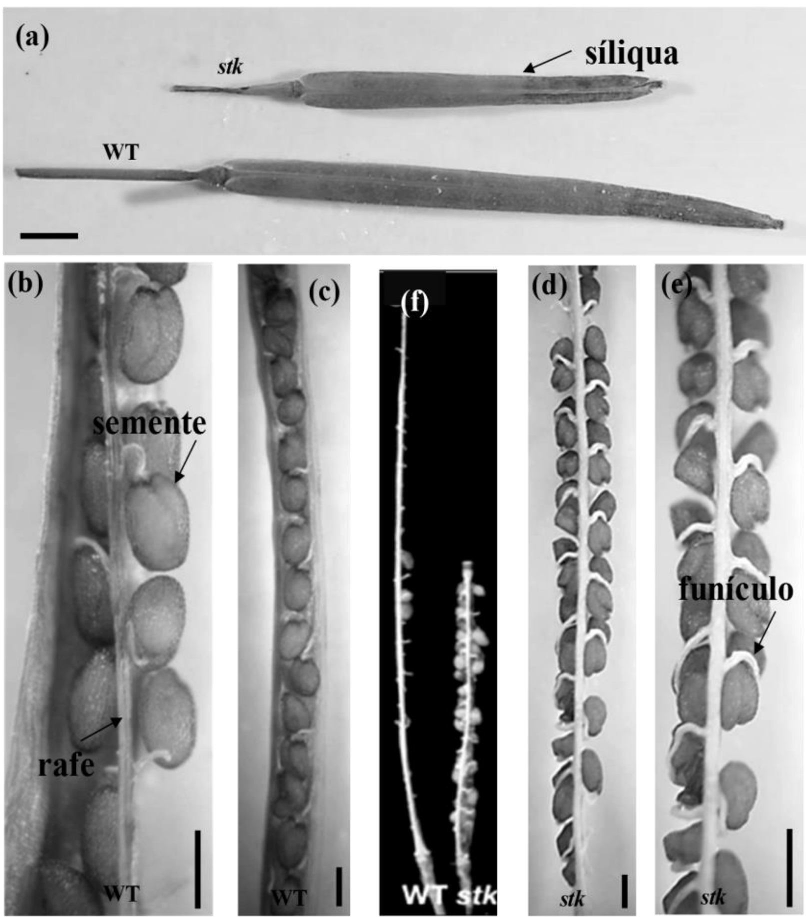

Figura 6. Comparação entre *A. thaliana* Col-0 selvagem e *A. thaliana* Col-0 mutante para o gene *AtAGL11* (*stk*). (a) Diferença entre os tamanhos de síliquas; (b-c) formato e tamanho das sementes WT e espessura e comprimento dos funículos WT; (d-e) formato e tamanho das sementes *stk* e espessura e comprimento dos funículos *stk*; (f) diferença entre WT e *stk* em relação à abscisão das sementes da rafe. Escala = 1 mm (a), 0,5 mm (b-c, d-e). Imagem f copiada de PINYOPICH *et al.* (2003).

O padrão de expressão espaço-temporal dos genes *MIKC*^{C} demonstra a necessidade de uma regulação fina para a transcrição desses genes em tecidos específicos e, muitas vezes, por curtos períodos durante o desenvolvimento de óvulos e sementes. Estudos da expressão tecido-específica do gene *AG* confirmaram a dependência de elementos *cis* presentes no seu segundo íntron, conhecido por sua grande extensão (3 kb) (DEYHOLOS & SIEBURTH, 2000; HONG *et al.*, 2003). O mesmo ocorre com o gene *AtAG11*, regulado por elementos *cis* localizados em sua região promotora e em seu grande primeiro íntron (1,3 kb). Esses elementos regulatórios são alvo da proteína BASIC PENTACYSTEINE 1 (BPC1) que, ao se ligar a *AtAGL11*, gera uma mudança conformacional no DNA que auxilia na formação do precomplexo de ativação da transcrição deste gene (KOOIKER *et al.,* 2005).

Além do interesse na compreensão do controle da expressão de genes *MADS-box* das classes C e D, estudos mais recentes aprofundaram-se na atuação desses genes homeóticos em processos metabólicos. O estudo de MIZZOTTI *et al.* (2014) sugere que o gene *STK* está envolvido no acúmulo de metabólitos secundários pela regulação direta da transcrição do gene BANYULS/ANTHOCYANIDIN REDUCTASE (BAN/ANR), que converte antocianinas em seus correspondentes 2,3-cis-flavan-3-ols, atuando, assim, na rota de síntese de proantocianidinas na casca das sementes, o que as torna mais rígidas.

1.4 CONTROLES GENÉTICO E MOLECULAR DA APIRENIA EM VIDEIRA

Com o aumento da comprovação funcional de genes responsáveis pelo desenvolvimento de semente em plantas modelos como *A. thaliana*, estudos de ortologia gênica em espécies vegetais de interesse agronômico tornaram-se foco em programas de melhoramento genético (MATIAS-HERNANDEZ *et al.,* 2010). Em frutíferas, o interesse pela geração de variedades apirênicas gerou esforços para a compreensão do processo de

morfogênese de sementes em videira, tomateiro, laranjeira, limoeiro, entre outros (VAROQUAUX *et al.,* 2000).

Nas variedades de videira estenoespermocárpicas, tanto de *V. vinifera* como de *Vitis labrusca*, são identificados diferentes graus de tamanho de sementes, o que demonstra o caráter quantitativo dessa forma de apirenia (BOUQUET & DANGLOT, 1996). O modelo genético proposto para o controle dessa característica sugere que alelos recessivos de três *loci* independentes são regulados por um alelo dominante em um único *locus*, o qual inibiria o desenvolvimento da semente (BOUQUET & DANGLOT, 1996). Carecendo de maiores informações moleculares como o sequenciamento completo do genoma de *V. vinifera* ou de genes candidatos, muitos pesquisadores focaram seus esforços na busca de marcadores moleculares que pudessem ser utilizados como alternativa biotecnológica para a detecção do fenótipo de apirenia prematuramente, utilizando-os em estratégias de seleção assistida por marcadores (MAS, do inglês, *Marker Assisted Selection*) em populações segregantes (LAHOGUE *et al.,* 1998; ADAM-BLONDON *et al.,* 2001; MEJÍA *et al.,* 2007; MEJÍA *et al.,* 2011). A partir da produção de mapas genéticos de videira e de estudos genéticos com populações geradas a partir de variedades de videira pirênicas (doadora materna) e apirênicas (doador paterno), foram detectados *QTLs* para tamanho de baga, peso e tamanho de sementes em *V. vinifera* (LODHI *et al.,* 1995; DALBO *et al.,* 2000; DOLIGEZ *et al.,* 2002; RIAZ *et al.,* 2003; ADAM-BLONDON *et al.,* 2004; FISCHER *et al.,* 2004; CABEZAS *et al.,* 2006, MEJÍA *et al.,* 2007). Os *QTLs* detectados para estas características estão localizados na porção distal do grupo de ligação 18, explicando de 50 a 70% da variância fenotípica para estes caracteres (DOLIGEZ *et al.,* 2002; FANIZZA *et al.,* 2005; CABEZAS *et al.,* 2006; MEJÍA *et al.,* 2007; COSTANTINI *et al.,* 2008; REVERS *et al.,* 2010). Posteriormente, este *locus* foi denominado *SdI* (Inibidor do desenvolvimento da semente, do inglês, *Seed development Inhibitor*) (BOUQUET & DANGLOT, 1996; LAHOGUE *et al.,* 1998; MEJÍA *et al.,* 2011). Na região do *locus SdI* encontra-se o marcador molecular SSR (repetição de sequência simples, do inglês, *Simple Sequence Repeat*) VMC7F2 que é altamente polimórfico e capaz de identificar videiras apirênicas com ~95 % de eficiência (COSTANTINI *et al.,* 2008).

Atualmente, *V. vinifera* possui seu genoma completamente sequenciado (http:// http://genomes.cribi.unipd.it/grape/) em sua forma quase homozigota e, também, em sua forma altamente heterozigota, ambas obtidas a partir da cultivar Pinot Noir (PN40024; JAILLON *et al.,* 2007; VELASCO *et al.,* 2007). Os genes de *V. vinifera* foram inicialmente

nomeados com o prefixo ‘Vv’, porém foram recentemente renomeados com o prefixo ‘Vvi’ para evitar confusão de nomenclatura com a bactéria *Vibrio vulnificus*, que teve seu genoma publicado anteriormente ao de videira (GRIMPLET *et al.,* 2016). O conjunto de dados genômicos disponíveis auxilia no entendimento da função de seus mais de 23 mil genes e em estudos de desenvolvimento de variedades por métodos não tradicionais.

Em relação à morfogênese de sementes, os genes *MADS-box* foram caracterizados em videira quanto à presença e a estrutura dos domínios codificados. Na classe de genes *MIKC*^{C}, a subfamília AG é composta por três membros, dois deles mais similares a *AG*, *VviAG1 e VviAG2,* sendo classificados como classe C, e um terceiro, *VviAGL11* ou *VviAG3,* mais similar a *AGL11/STK,* classificado como classe D (BOSS *et al.,* 2001; BOSS *et al.,* 2002; DÍAZ-RIQUELME *et al.,* 2009; GRIMPLET *et al.,* 2016).

1.4.1 O gene *VviAGL11*

Interessantemente, o gene *AGAMOUS-LIKE 11* de *V. vinifera* (*VviAGL11,* acesso no genoma em http://genomes.cribi.unipd.it/grape/: VIV_18s0041g01880) foi mapeado *in silico* no mesmo *contig* que o *locus SdI* e, também, colocalizado com o marcador molecular SSR VMC7F2 que se encontra a montante de *VviAGL11*, mais especificamente a 463 pb de seu códon de início de tradução (COSTANTINI *et al.,* 2008). *VviAGL11* também foi descrito por BOSS *et al.* (2002) como *VvMADS5* e por DÍAZ-RIQUELME *et al.* (2009) como *VvAG3.*

VviAGL11 foi proposto como um possível ortólogo do gene controlador da identidade do óvulo em *A. thaliana, AtAGL11/STK*, possuindo 75 % de similaridade (Figura 7; FAVARO *et al.,* 2003; PINYOPICH *et al.,* 2003). Estudos iniciais da avaliação da expressão de *VviAGL11* demonstram um acúmulo de transcritos em tecidos florais e em frutos de videira, enquanto que há repressão de sua transcrição em tecidos vegetais como raiz, ramo, folha, gavinha e gema (DÍAZ-RIQUELME *et al.,* 2009). Posteriormente, estudos de MEJÍA *et al.* (2011, 2016) observaram que transcritos de *VvAGL11* são 25 vezes mais abundantes no estádio de fruto em comparação com os estádios de prefloração e floração em genótipos de videira sem sementes. Adicionalmente, o gene *VviAGL11* possui expressão acentuada em genótipos homozigotos correlacionada com a presença do alelo de 198 pb do marcador VMC7F2 (MEJÍA *et al.,* 2011).

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)


AtAGL11         MGRGKIEIKRIENSTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSTRGRLYEYANN
VviAGL11        MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
                *************:***********************************:***:***:**

AtAGL11         NIRSTIERYKKACSDSTNTSTVQEINAAYYQQESAKLRQQIQTIQNSNRNLMGDSLSSLS
VviAGL11        NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
                **.***:*****.***** .:. **** ************** :*****:******:**:

AtAGL11         VKELKQVENRLEKAISRIRSKKHELLLVEIENAQKREIELDNENIYLRTKVAEVERYQQH
VviAGL11        VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
                ******:*****..*:***********.***  *******:**.:*****:***** **

AtAGL11         HHQMVSGSEINAIEALASRNYFAHSIMTAGSGSGNGGSYSDPDKKILHLG
VviAGL11        N--MVSTHEFNAIQALVSRNFFQPNMIEGGS-----TGYPLPDKKVLHLG
                :  ***  *:***:**.***:*  .:: .**      .*. ****:****
```

Figura 7. Alinhamento entre as sequências proteicas deduzidas dos genes *AGL11* de *A. thaliana* e de *V. vinifera*. O símbolo de asterisco (*) indica a posição onde há manutenção de resíduos conservados; dois pontos (:) quando há modificação porém com conservação entre os resíduos de forte similaridade de características químicas; enquanto que o ponto simples (.) indica a modificação entre resíduos com fracas similaridades químicas. Alinhamento realizado com o *software online* MUSCLE 3.8. Códigos de acesso: AtAGL11 GenBank NM100640; VviAGL11 GenBank KM401845.

Um estudo anterior ao início da realização da presente tese de doutorado caracterizou as variações alélicas do gene *VviAGL11* por meio de sequenciamento alelo-específico. Os resultados permitiram mostrar que a cultivar pirênica Chardonnay apresenta duas isoformas transcritas com alta similaridade ao genoma referência de videira (Pinot Noir), enquanto que, na cultivar apirênica Sultanine, uma das isoformas de expressão identificada possui mutações únicas, sendo denominada de alelo mutante (SU mut; MALABARBA, 2014; MALABARBA *et al.,* 2017). Também foi observado um grande acúmulo de transcritos em sementes de ‘Chardonnay’ de 2, 4 e 6 semanas em comparação com tecidos de flor e polpa. Contudo, na cultivar Sultanine, a expressão de *VviAGL11* foi extremamente baixa em todos os estádios e tecidos analisados. Pelo estudo realizaram-se análises morfoanatômicas para comparar as sementes e os traços de sementes, característicos das cultivares Chardonnay e Sultanine, respectivamente. Foram identificadas alterações anatômicas que sugerem a não diferenciação da camada interna do tegumento externo, a endotesta (ET) da casca da semente, uma vez que essa camada não apresentou o padrão de diferenciação normal em ‘Sultanine’ (MALABARBA, 2014; MALABARBA *et al.,* 2017). Além disso, por meio da

técnica de hibridização *in situ,* foi observado que o padrão de expressão espaço-temporal de *VviAGL11* é majoritariamente encontrado na casca de sementes de ‘Chardonnay’ com 2 e 4 semanas de desenvolvimento, especificamente na camada dupla da endotesta (ET). Nenhum sinal de hibridização foi observado em ‘Sultanine’ (MALABARBA, 2014; MALABARBA *et al.,* 2017).

Nesse contexto, a presente tese de doutorado é resultado de pesquisas conduzidas para testar a seguinte hipótese: “O gene *MADS-box VviAGL11* é crítico para o controle da morfogênese das sementes de videira”. O conjunto de dados apresentados anteriormente permitem sugerir que a ausência da expressão de *VviAGL11* é responsável pelo desenvolvimento errôneo das sementes de videira. Esta lacuna molecular aparenta gerar a extrema diminuição do tamanho final das sementes devido à não diferenciação da camada ET da casca das sementes, provocando o cessar de seu alongamento e duplicação. Deste modo, com a não diferenciação da camada ET, o endosperma também não teria desenvolvimento normal, interrompendo seu crescimento, causando a posterior morte do embrião e levando à formação de um traço de semente (MALABARBA, 2014; MALABARBA *et al.,* 2017).

A avaliação da ortologia entre os genes *AtAGL11* e *VviAGL1,* somada à caracterização funcional de *VviAGL11* em modelo heterólogo e em videiras, teve o propósito de avaliar se o gene *VviAGL11* é essencial para a morfogênese de sementes e auxiliar na compreensão do processo de apirenia em *V. vinifera*. Este conjunto de resultados poderá ser útil no desenvolvimento de novas variedades de uvas de mesa que atendam às exigentes demandas do mercado de consumo nacional e internacional.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Caracterizar funcionalmente o gene *VviAGL11* visando uma maior compreensão de seu papel durante a morfogênese das sementes em videira.

2.2 OBJETIVOS ESPECÍFICOS

a. Elucidar a função do gene *VviAGL11* em modelo heterólogo e avaliar a ortologia entre *VviAGL11* e *AtAGL11*.

Racionalização: caracterizar a funcionalidade das isoformas de expressão de *VviAGL11* por meio de complementação do mutante *stk* (*seedstick*-*Atagl11*) de *Arabidopsis thaliana*;

b. Elucidar a função de *VviAGL11* em *Vitis*.

Racionalização: caracterizar funcionalmente *VviAGL11* por meio da superexpressão do gene em cultivares apirênicas e do silenciamento de sua expressão em cultivares pirênicas;

c. Desenvolver ferramentas biotecnológicas para a seleção assistida de videiras apirênicas.

Racionalização: identificar um haplótipo de marcadores moleculares associado e desenvolver e avaliar novos marcadores moleculares candidatos baseados em polimorfismos do alelo *VviAGL11* de 'Sultanine' (mut) com potencial para a seleção assistida da apirenia em videira.

Os resultados dos estudos relativos ao objetivo específico **a** foram publicados na forma de artigo e estão apresentados no **Capítulo I**. O conjunto de resultados do objetivo **b**

foi publicado e está apresentado no **Capítulo II**. Finalmente, os dados referentes ao item **c** compõem o **Capítulo III**.

Na sessão de anexos da presente tese encontra-se o manuscrito que aborda a caracterização de genes de resistência ao míldio da videira, que estão colocalizados com o gene *VviAGL11* (Anexo I). Esta parte do trabalho foi realizada adjacentemente aos estudos apresentados nos capítulos que compõem a presente tese.

Durante o período de doutoramento, e considerando a experiência adquirida em genética, fisiologia e biologia molecular de *A. thaliana* e *V. vinifera*, foi oportunizada pela CAPES a realização de doutorado sanduíche nos laboratórios de pesquisa coordenados pelo Dr. Axel Mithöfer do *Department of Bioorganic Chemistry*, *Max Planck Institute for Chemical Ecology*, em Jena, Alemanha. As atividades de pesquisa realizadas durante os doze meses de doutorado sanduíche culminaram na publicação de dois artigos científicos, os quais se encontram anexos ao final desta tese, anexo II e III. Textos introdutórios a cada um destes artigos estão apresentados junto aos mesmos.

3 CAPÍTULO I

The MADS-box gene *AGAMOUS-like 11* is essential for seed morphogenesis in grapevine

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RESEARCH PAPER

The MADS-box gene *Agamous-like 11* is essential for seed morphogenesis in grapevine

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Abstract

Despite the wide appreciation of seedless grapes, little is known about the molecular mechanisms that drive the stenospermocarpic seedless-type phenotype in grapevine. In order to address the molecular mechanisms that control seedlessness in grapevine, our study aimed to characterize *VviAGL11*, a class D MADS-box transcription factor gene that has been proposed as the major candidate gene involved in *Vitis vinifera* seed morphogenesis. *VviAGL11* allelic variations in seeded and seedless grapevine cultivars were determined, and its correlations with allele-specific steady-state mRNA levels were investigated. *VviAGL11* relative expression was significantly higher in seeds at 2, 4, and 6 weeks after fruit set, whereas in the seedless grape its transcript levels were extremely low in all stages analyzed. *In situ* hybridization revealed transcript accumulation specifically in the dual endotesta layer of the seeds, which is responsible for elongation and an increase of cell number, a necessary step to determine the lignification and the final seed size. No hybridization signals were visible in the seedless grapevine tissues, and a morphoanatomical analysis showed an apparent loss of identity of the endotesta layer of the seed traces. Ectopic expression of *VviAGL11* in the Arabidopsis *SEEDSTICK* mutant background restored the wild-type phenotype and confirmed the direct role of VviAGL11 in seed morphogenesis, suggesting that depletion of its expression is responsible for the erroneous development of a highly essential seed layer, therefore culminating in the typical apirenic phenotype.

Key words: Apireny, grapevine, *in situ* hybridization, seedlessness, Sultanine, *VviAGL11*.

Introduction

Seedlessness is one of the most appreciated features in table grapes (*Vitis vinifera*) along with enlarged fruit size, both being the main goals in breeding programs (Varoquaux *et al.*, 2000; Cabezas *et al.*, 2006). In the past two decades, the worldwide

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market for *in natura* grapes increased ~26% per year, while the production of such grapes grew at a 13% annual rate (FAO, 2014; OIV, 2014). In grapevine, the absence of seeds, also called apireny, can occur by two distinct biological processes. One is known as parthenocarpy, when fecundation does not occur and there is no seed formation (used in production of raisins). The second process is called stenospermocarpy, in which fertilization takes place to form the fruit but is followed by embryo abortion due to the cessation of endosperm development, normally after 4 weeks of fruit growth. The consequence of stenospermocarpy is the production of berries with reduced seed size, known as seed traces (Bouquet and Danglot, 1996; Mejía *et al.*, 2011). The Sultanine cultivar is the main donor of the stenopermocarpy phenotype among breeding programs worldwide, and most of the commercial table grape varieties descend from this cultivar (Di Genova *et al.*, 2014).

In grapevine, several studies have shown that the presence of a dominant allele in the region of the *Seed Development Inhibitor* (*SdI*) locus, located at the distal portion of chromosome 18, has been responsible for 50–90% of the total phenotypic variance of seedlessness (Bouquet and Danglot, 1996; Cabezas *et al.*, 2006; Mejía *et al.*, 2007; Costantini *et al.*, 2008; Doligez *et al.*, 2013). *In silico* analysis allowed the identification of a MADS-box transcription factor gene *AGAMOUS-LIKE 11* (*VvAGL11*; Vv18s0041g01880; Boss *et al.*, 2002), renamed *Vv1AGL11* by Grimplet *et al.* (2014), that mapped to the same locus contig *SdI* (Costantini *et al.*, 2008). The same work also co-located *VviAGL11* with the molecular microsatellite marker VMC7F2, a highly polymorphic microsatellite marker capable of an efficiency of up to 95% in the detection of seedlessness in grapes (Costantini *et al.*, 2008).

The transcription factors of the MADS-box family have extremely important roles in the development of plants by controlling floral organ identity (Smaczniak *et al.*, 2012). In a study covering the MADS-box family in grapevine, it was demonstrated that *VviAGL11* is induced in floral and fruit tissues and it is repressed in roots, branches, leaves, buds, and tendrils (Díaz-Riquelme *et al.*, 2012). More recently another study showed that *VviAGL11* is 25 times more expressed in fruits at the pea size stage compared with flower stages, in seedless grapevines homozygous genotypes, whereas in heterozygous genotypes an intermediate level of *VviAGL11* expression was observed (Mejía *et al.*, 2011).

The *VviAGL11* gene was proposed, by our group and others, as a strong candidate gene involved in the absence of seeds in grapevine (Costantini *et al.*, 2008; Mejía *et al.*, 2011; Revers *et al.*, 2014; Ocarez and Mejía, 2016). This MADS-box gene was shown to be a possible ortholog of *AGL11* (*STK*) of *Arabidopsis thaliana*, which acts on the identity and control of ovule and seed development (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003; Díaz-Riquelme *et al.*, 2012). The *AtAGL11* mutant *SEEDSTICK* (*stk*) presents a reduced number and size of seeds (Pinyopich *et al.*, 2003; Brambilla *et al.*, 2008).

In this work, we identified several polymorphisms in the *VviAGL11* allele sequences in the apirenic genotype background including mutations in the intronic regions and a set of single nucleotide polymorphisms (SNPs) in the coding region of the gene. *VviAGL11* transcript levels were 65-fold higher in grapevine seeds compared with apirenic grapevine fruits. Spatial and temporal analyses of expression patterns in reproductive tissues demonstrate correlation between abnormal development of a specific seed layer and reduction of *VviAGL11* expression. Additionally, *VviAGL11* ectopic expression restored silique and seed development to the wild-type-like standards in the Arabidopsis *stk* mutant, confirming its essential role in seed morphogenesis.

Materials and methods

Plant material

Grapevine samples were harvested from cv. Chardonnay and cv. Sultanine plants located at Embrapa Grape and Wine experimental areas in Bento Gonçalves, Rio Grande do Sul, Brazil (29°09'48"S, 51°31'42"W, and 616 m altitude). The cultivar plants were at the reproductive stage (6–8 years old), planted on a pergola trellising system and managed with conventional annual pruning and control of pests and diseases. Both cultivars were grafted on Paulsen 1103 rootstock and the phenological scale was followed as described by Baggiolini (1952) (Supplementary Table 2 at *JXB* online). The 'Chardonnay' cultivar was chosen for comparison with the 'Sultanine' cultivar because of similar phenological development. For the *VviAGL11* transcriptional profile, 'Chardonnay' (seeded) and 'Sultanine' (seedless) organ and tissue samples were harvested at the stages of flowers at pre-anthesis, fruit set, and fruits at 2, 4, and 6 weeks after fruit set in 2010/2011. For *in situ* hybridization and morphological assays, 'Chardonnay' and 'Sultanine' samples were harvested as described by Baggiolini (1952; Supplementary Table S2) at H1, flowers at pre-anthesis, fruit set, and fruits at 2, 4, and 6 weeks after fruit set in the 2012/2013 harvest.

Arabidopsis thaliana plants, the wild type (ecotype Columbia) and *stk* mutant, were grown at 21 °C under short-day (8 h light/16 h dark) or long-day (16 h light/8 h dark) conditions. *stk* mutant seeds were kindly provided by L. Colombo (Universitá degli Studi di Milano, Italy).

Sequencing

Genomic DNA was extracted from leaves following the protocol of Lefort and Douglas (1999). Primer sets were designed to amplify sequences in the promoter and in exon/intron regions of *VviAGL11* (see Supplementary Table S1) in order to sequence the whole gene. Overlapping gene fragments were amplified by a primer walking strategy employing six primer pairs that allowed the assembly of six contigs (Fig. 1). Each projected contig contains 300–500 bp of overlapping sequence. PCR amplifications were performed with 10–25 ng of grapevine genomic DNA using Platinum® Pfx DNA polymerase (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. RACE (rapid amplification of cDNA ends) was performed with the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) as recommended by the manufactor. PCR products were cloned into pGEM®-T Easy Vector (Promega, Madison, WI. USA) following the manufacturer's instructions. Plasmid DNA was purified by the boiling miniprep protocol (Sambrook *et al.*, 1987). All PCR products were sequenced with T7 (GTAATACGACTCACTATAGGG) and SP6 (TACGATTTAGGTGACACTATAG) primers and with internal sequence primers (Supplementary Table S1) in an ABI Prism® 310 Genetic Analyser (Applied Biosystems) using standard sequencing protocols described by Falavigna *et al.* (2014). Sequence analysis was carried out with DNA Sequencing Analysis Software v5 (Applied Biosystems) and MEGA7 software (http://www.megasoftware.net/home). The six contigs were assembled manually and

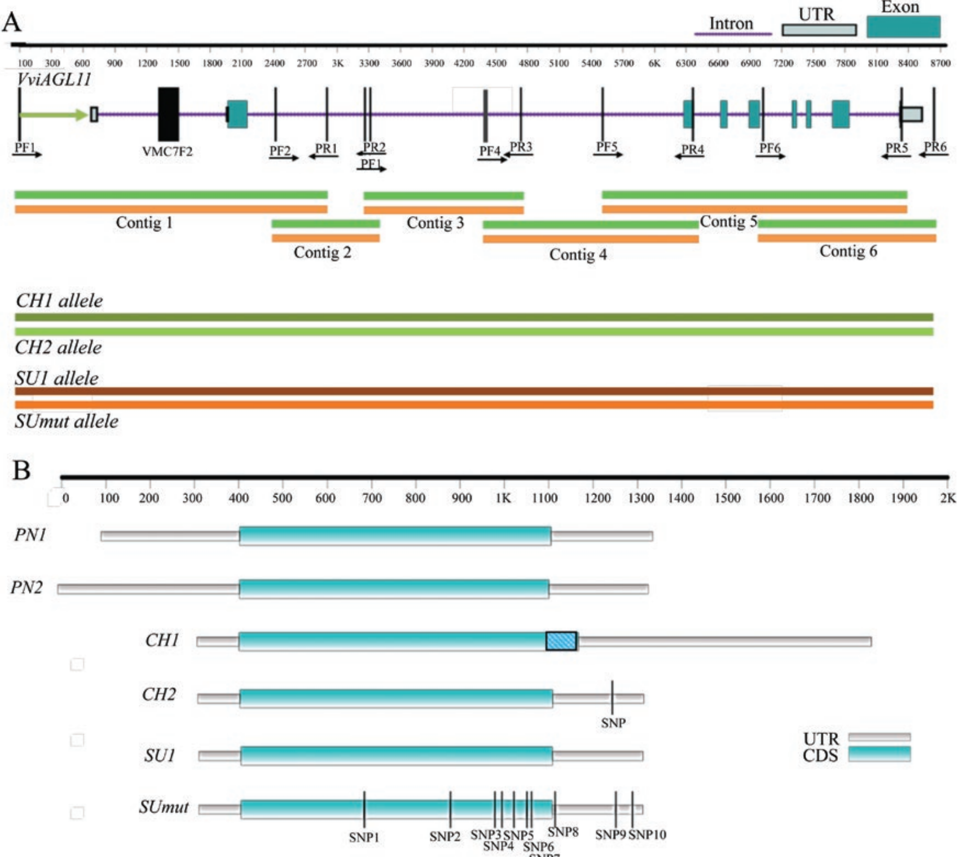

Fig. 1. *VviAGL11* allelic features. (A) Structural representation of *VviAGL11*. DNA structure is composed of a promoter region, 5'-UTR, eight introns, eight exons, and 3'-UTR. The black lines represent the localization of the primers used for allele amplification. Below, the contigs for each cultivar and their assembly, to produce 'Sultanine' and 'Chardonnay' *VviAGL11* alleles. (B) Comparison between *VviAGL11* mRNA alleles. *PN1* and *PN2* represent mRNA versions of *VviAGL11* from the Pinot Noir reference cultivar (VIT_218s0041g01880.1 and VIT_218s0041g01880.2, respectively). 'Chardonnay' allele 1 (*CH1*), 'Chardonnay' allele 2 (*CH2*), and 'Sultanine' allele 1 (*SU1*) are compared with the 'Sultanine' mutant allele (*SUmut*). The box in *CH1* represents the 20 amino acids derived from the 5' alternative splice. SNPs are represented by black stripes (GenBank KM401845, KM401846, KM401847, and KM401848). (This figure is available in colour at *JXB* online.)

with Codon Code Aligner 6.0. (www.codoncode.com/). The contigs from the same alleles were identified by specific polymorphisms in overlapping sequences. The online program Fancy Gene v1.4 (http://bio.ieo.eu/fancygene/) was used to draw the gene and mRNA representations (Fig 1A, B). Sequences were compared with the grapevine reference ('Pinot Noir' PN40024) genome and with the whole-genome sequence from cv. Sultanine (Di Genova *et al.*, 2014).

Identification of putative CArG sequences

Genomic regions located 5 kb upstream of the *VviAGL11* ATG start codon, 377 bp downstream of the stop codon, and exons and introns were analyzed to identify CArG-Box sequences with the New Place Bioinformatic Program 26.0 available at the Sogo website (http://sogo.dna.affrc.go.jp).

RNA extraction

Total RNA samples were extracted from frozen material (~200 mg) by LiCl precipitation using the protocol of Zeng and Yang (2002) scaled to 2 ml microcentrifuge tubes. Each sample extraction was performed in triplicate, and their volumes were pooled before the LiCl precipitation step. Genomic DNA in total RNA samples was removed using the TURBO DNA-free Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA integrity and quantity were monitored by agarose gel electrophoresis and spectrophotometric quantitation, respectively.

Quantitative reverse transcription–PCR (RT–qPCR)

cDNAs were synthesized using the GeneAmp RNA PCR Core Kit (Applied Biosystems) according to the manufacturer's instructions. Gene-specific primer pairs were designed for the two alleles of the *VviAGL11* gene (*VviAGL11F* 5'-CACTTAATGGGTGATTCCTTGGC-3', *VviAGL11R* 5'-AGCAACTCATGCTTCTTCGACC-3'; and *VvAGL3F* 5'-ATTGTTCATCTGGGCATTTCG-3', *VvAGL3R* 5'-GGAGATGAAGTTGGCGGATA-3') and evaluated by Oligo Analyzer 3.1 (IDT, http://www.idtdna.com), with the standards settings of 0.2 µM oligo concentration, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. The reproductive tissues evaluated were divided into initial stages of development, represented by flowers at pre-anthesis and fruit set, and by fruit stages at 2, 4, and 6 weeks of development after fruit establishment (fruit set). Seeds were dissected from the pulp in 'Chardonnay' berry samples and the whole berry was evaluated in 'Sultanine' samples. RT–qPCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems). SYBR Green (Invitrogen, Carlsbad, CA, USA) was used to monitor dsDNA synthesis and ROX (Invitrogen) was employed as a passive fluorescence reference. Each biological sample was analyzed in technical quadruplicates. Cycling consisted of one step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and finished by a dissociation curve between 60 °C and 95 °C. The specificity of PCR amplifications was assessed by the presence of a single peak in melting curves, visualization of single amplification products of the expected size in 1% ethidium

bromide-stained agarose gel electrophoresis, and sequencing of the amplicons. Primer efficiency was calculated by LinRegPCR (version 11.0, Ruijter *et al.*, 2009). Mean relative gene expression was calculated by the method of Pfaffl (2001) employing *ACTIN* (GenBank EC969944) as reference gene (Reid *et al.*, 2006). For the evaluation of *A. thaliana* transformants with the *VviAGL11* coding region, RNA was obtained from leaves, flowers, and siliques, with the same protocols as described above. The expression of *VviAGL11* was analyzed by a specific pair of primers (VviAGL11OX_F 5'-AACAAACGAATCTCAAGCAATCAAGC-3' and VviAGL11SOX_R 5'-TGATCTCGATCTTTCCTCTCCCC-3'). Relative enrichment of *VviAGL11* transcripts was calculated normalizing the amount of mRNA against an *ACTIN* fragment (AtACT2 RTqPCR For 5'-GACCTTGCT GGACGTGACCTTAC-3' and AtACT2 RTqPCR Rev 5'-GTAGTCAACAGCAACAAAGGAGAGC-3', Locus AT3G18780).

Seed morphoanatomical assay

Seeds and seed traces were transferred into a fixation solution under vacuum (Mcdowell and Trump, 1976). Thereafter, samples were dehydrated in an increasing gradient of ethanol as described by Gabriel (1982), and embedded in 2-hydroxyethyl methacrylate resin according to Gerrits and Smid (1983). The 5 µm sections were obtained in a Leica RM 2255 microtome. The metachromatic reagent Toluidine Blue O (Feder and O'brien, 1968) was used to determine seed structure. For the staining procedure, slides with sections were submerged in the reagent for 1 min, washed with water, and dried on a plate at 45 °C. The count of the number of cells in the endotesta (ET) layer was performed with support of ZEN microscope software from Zeiss.

In situ *hybridization analysis*

'Chardonnay' and 'Sultanine' samples were fixed in 4% formaldehyde for 16 h. Around 20 samples of the same stage were used from each cultivar. To submerge the samples, a vacuum was applied (~600 mmHg) for 20 min. The material was stored under refrigeration (4–10 °C) after fixation. Thereafter, the tissues were dehydrated in an ethanol series and embedded in paraffin. Longitudinal and transversal sections (8–10 µm) were prepared with a Leica RM 2255 (Leica, Wetzlar, Germany) microtome and mounted on silanized microscope slides. Gene-specific sense and antisense probes corresponded to 185 nucleotides of the 3'-untranslated region (UTR) of the *VviAGL11* gene, which were amplified using VvAG3F (5'-ATTGTTCATCTGGGCATTTCG-3') and VvAG3R (5'-GGAGATGAAGTTGGCGGATA-3') as primers. Hybridizations were performed with non-radioactive probes (Dornelas *et al.*, 2000). Labeled probes were generated by digoxigenin (DIG) labeling using T7 or SP6 RNA polymerase of the DIG RNA Labeling Kit (Roche, Basel, Switzerland). The hybridization signal was detected by a colorimetric assay in which an anti-DIG antibody coupled with alkaline phosphatase and NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine) served as a substrate. After the detection of the hybridization signals by immunostaining, slides were washed, dehydrated, and mounted using Entellan® (Merck, product accession code 107961). We performed *in situ* hybridization analysis for two consecutive years.

Functional complementation analysis in Arabidopsis

In order to evaluate the competence of *VviAGL11* to complement the *stk* phenotype, *stk* plants were transformed with overexpression plasmids carrying the *VviAGL11* coding region. Two constructs were generated: (i) pVviAGL11-OX, an overexpression plasmid harboring the *VviAGL11* 'Chardonnay' allele; and (ii) pVviAGL11-OX^{mut}, an overexpression plasmid harboring the *VviAGL11* 'Sultanine' mutant allele (*SUmut*). Both constructions had the same coding region size (672 bp) besides the SNPs in the *SUmut* allele (Supplementary Fig. S2). *VviAGL11* was amplified as described previously and cloned into pENTER/D-TOPO (Invitrogen). The PCR product was obtained with the primers *VviAGL11CDSF* 5'-CACCATGGGGAGAGGAAAGATCG-3' and *VviAGL11CDSR* 5'-TTACCCGAGATGGAGGACCTTCTTATC-3'. Employing the Gateway® LR Clonase TM II Enzyme Mix recombination system (Invitrogen), the insert was transferred into the pH7WG2D vector (Karimi *et al.*, 2002). The T-DNA fragment also encodes a visible selection marker [green fluorescent protein (GFP)] under the control of the promoter RolD (Karimi *et al.*, 2002). Final constructs were verified by sequencing and used to transform thermocompetent *Agrobacterium tumefaciens* strain EHA105. The floral dip method (Clough and Bent, 1998) was used to transform *stk* plants, and T_1 seeds were initially germinated in Phytagel™ BioReagent culture medium (Sigma, St Louis, MO, USA) and selected for hygromycin resistance (25 µg ml^{-1}). Two-week-old putative transformants were screened for GFP fluorescence, and the T-DNA insertion of the *VviAGL11* alleles on T_1 plants was analyzed by PCR using specific primers targeting the plasmid promoter and the *VviAGL11* coding region (TransF 5'-CTGGGAACTACTCACACATTA-3' and TransR 5'-CATTTGGAGAGGACTCCG-3'). Only T_1 lineages positive for these three steps of selection were used for the phenotype assay: (i) silique length; (ii) seed number in siliques; (ii) seed size; (iv) funiculus thickness; and (v) funiculus length. For each plant, five well-developed siliques at the same stage were used for measurements/counting, and the final score for each plant was composed of their average. Columbia-0 and *stk* plants were used as controls for all measurements. Statistical analysis was performed using Prism 5.1 with one-way ANOVA and Tukey test.

Accession numbers

The nucleotide sequences reported in this paper have been submitted to GenBank under the accession numbers KM401845, KM401846, KM401847, and KM401848.

Results

Sequencing of the VviAGL11 *locus*

To distinguish the structural organization of the *VviAGL11* gene in apirenic and seeded grapevines, allelic variations were amplified by PCR with specific primers from genomic DNA extracted from the apirenic 'Sultanine' and from the seeded 'Chardonnay' as previously described. A total of six contigs were assembled for each *VviAGL11* allele as shown in Fig. 1A. The complete *VviAGL11* allelic sequences constituted by their promoter, coding, and intragenic regions (eight exons and eight introns) were sequenced. After allele-specific isolation and sequencing, we identified two alleles for each cultivar. Because 'Chardonnay' is a direct offspring of Pinot Noir, we were able make comparisons with the 'Pinot Noir' (PN40024) sequence in the publicly available genome database (http://genomes.cribi.unipd.it/gb2/gbrowse/public/vitis_vinifera/).

The two *VviAGL11* alleles from 'Chardonnay' showed 99% sequence identity to the corresponding 'Pinot Noir' genome sequence for *VviAGL11*. One allele from 'Sultanine' also exhibited 99% identity with PN40024. However, the second 'Sultanine' *VviAGL11* allele, linked to the molecular microsatellite marker VMC7F2 198 bp allele (Costantini *et al.*, 2008), presented a different group of polymorphisms when

compared with all the other alleles. This allele was named as *SUmut* ('Sultanine' mutant allele) and contains a group of exclusive polymorphisms composed of 28 INDELs (insertions/deletions) and 105 SNPs (Supplementary Fig. S1).

The sequence alignment of the four *VviAGL11* alleles sequenced within this work, from the PN40024 reference genome and from the newest sequences of the *VviAGL11* locus from 'Sultanine' described by Di Genova *et al.* (2014) is presented in Supplementary Fig. S1. Among the observed alterations in *SUmut*, two SNPs give rise to two amino acid substitutions (R590L and T628A) as shown in Supplementary Fig. S2. We also searched the sequences for MADS-box protein complex-binding sites (CArG-box sequences: perfect CArG-boxes and CArG-boxes with one mismatch deduced by a probability matrix; Smaczniak *et al.*, 2012). We were able to identify 10 putative CArG-box sequences within the *VviAGL11* gene structure, one located in the promoter region, eight in the second intron, and one in the 3'-UTR. The seventh CArG-box putative sequence of 'Sultanine' *VviAGL11 mut* has a nucleotide modification (Supplementary Fig. S1, black arrow).

Allele-specific transcription profiles of VviAGL11 *during fruit development*

The 3'- and 5'-RACE experiments allowed the identification of two distinct mRNA alleles of *VviAGL11* in apirenic ('Sultanine') when compared with seeded ('Chardonnay') grapevines. Two amplicons of different sizes were obtained from 'Chardonnay' and one amplicon was obtained from 'Sultanine'. After sequencing them, it was possible to confirm the specificity of the amplicons and to characterize each allele transcript present in both cultivars.

The results revealed that 'Chardonnay' has two alleles of *VviAGL11* mRNA. The allele isoform, named *CH1*, spans 1454 bp and contains a 732 bp coding region. This alternative form is longer and has an increased coding region of 60 nucleotides (20 amino acids when compared with the *VviAGL11* gene model in the PN40024 reference genome (Jaillon *et al.*, 2007). The second Chardonnay allele, named *CH2*, spans 958 bp and contains an ORF of 672 bp. 'Sultanine' also has two transcript alleles of the same total length (958 bp) and coding sequence (672 bp), respectively. The differences between the two *VviAGL11* alleles derived from 'Sultanine' are the presence of 10 SNPs found in the mutant allele (*SUmut*). The coding regions of *CH2* and 'Sultanine' allele 1 (*SU1*) are identical to those of the *VviAGL11* gene predicted in the Pinot Noir reference genome (Jaillon *et al.*, 2007); however, none of the 'Sultanine' alleles is identical to the 'Pinot Noir' gene due to the different 5'-UTR sizes, as shown in Fig. 1B.

To evaluate the relative expression of *VviAGL11* alleles during fruit development, RT–qPCR analysis with mRNA samples extracted from the reproductive organs of 'Chardonnay' and 'Sultanine' at critical fruit development stages was performed. Two sets of primers were used in this approach in order to differentiate *VviAGL11* mRNA isoforms. The first *VviAGL11* primer set was designed to amplify the longer allele (*CH1*) (see the Materials and methods). The second pair of primers was designed to anneal in the first (forward) and in the second (reverse) exons that allowed us to amplify simultaneously the two *VviAGL11* alleles (the shorter *CH1* and longer *CH2*). Considering that in 'Sultanine' the alleles are differentiated only by 10 SNPs, we employed primers flanking the first and second exons, covering both alleles (*SU1* and *SUmut*).

The results showed that *VviAGL11* relative expression was similarly low in all stages sampled from the 'Sultanine' variety (Fig. 2). 'Chardonnay' flowers and berry pulp also presented low transcription levels of *VviAGL11* for isoforms *CH1* and *CH2*. RT–qPCR with primers designed for the intronic region was also performed with 'Sultanine' mRNA samples, and a minor level of expression was detected, probably due to residual pre-mRNA in the samples. Nevertheless, seeds of 'Chardonnay' from fruits at 2, 4, and 6 weeks after fruit set exhibited much higher accumulation of *VviAGL11 CH2* transcripts, 15-fold higher than *CH2* transcripts when compared with berries at fruit set and 65-fold higher when compared with 'Sultanine' whole berries. The 'Chardonnay' *CH1* allele relative expression fluctuated from 5- to 11-fold higher throughout seed stages compared with fruit set (Fig. 2).

Morphoanatomical analysis of seeds from apirenic and seeded grapevines

Grapevine seeds have a particular shape resembling a pear, being triangular in cross-section (Fig. 3A, B). The seeds are composed of a cuticle, an epidermis, two layers making up the outer integument (exotesta and ET), both forming the seed coat at the ripening stage, and three inner integument layers that are positioned around the endosperm and embryo (Ribereau-Gayon and Peynaud, 1980). The structure of the seeds from 'Chardonnay' and seed traces from 'Sultanine' was found to be very different in shape, size, and hardness (Fig. 3C). For that reason, a morphoanatomical analysis was conducted to better characterize the development of endosperm, embryo, and seed layers of both cultivars at 2, 4, and 6 weeks of fruit development.

A normal and complete development of the endosperm and the embryo was observed in 'Chardonnay' seeds after 6 weeks of fruit development. 'Sultanine' seed traces showed an undeveloped endosperm, and no embryo could be distinguished at any stage. When analyzing the integuments, three layers of inner integuments were observed in the seeds at the initial stage of fruit development in both varieties (Fig. 3D, E), although only two layers were present at later stages in 'Chardonnay' (Fig. 3F, H) and in 'Sultanine' (Fig. 3G, I).

The epidermis covers the outer integument in both varieties. Parenchyma cells are the main constituent of the outer integument, and cell layers are thicker in the ventral face of the seed. At the ripening stage there is an increase in parenchyma cell volume in 'Chardonnay', but the number of cells does not change. We could also observe the lignification process of the seed (Fig. 3D, F, H). However, in 'Sultanine' seed traces, this layer had no visible development (Fig. 3E, G, I).

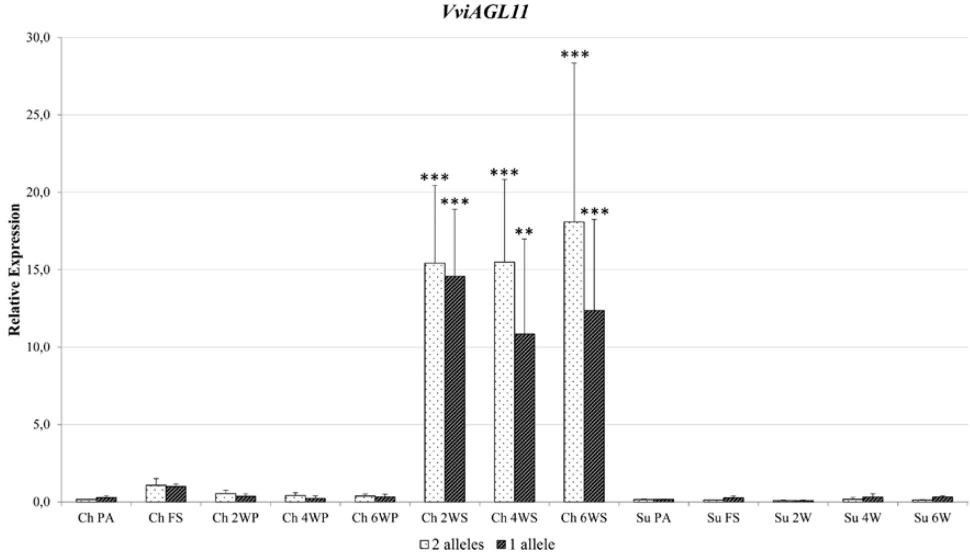

Fig. 2. Relative gene expression of *VviAGL11* alleles from 'Chardonnay' and 'Sultanine' via RT–qPCR. Two alleles correspond to *CH1* and *CH2*, and also *SU1* and *SUmut*. One allele corresponds to *CH1*. The developmental stages sampled are shown on the *x*-axis: PA, pre-anthesis flower; FS, fruit set; 2WP-4WP-6WP, pulp from fruits at 2, 4, and 6 weeks after fruit set; and 2WS-4WS-6WS, 'Chardonnay' seed stages. 'Sultanine' stages evaluated at pre-anthesis, fruit set, and fruits at 2, 4, and 6 weeks after fruit set (2W-4W-6W). 'Chardonnay' fruit set was used as reference. The relative expression expressed on the *y*-axis was calculated by the method of Pfaffl (2001). The SD is shown for each stage and sample. Asterisks indicate statistical significance between 'Chardonnay' alleles in seed stages compared with other stages evaluated for the 'Chardonnay' and 'Sultanine' alleles (Tukey test: $^{**}P<0.01$, $^{***}P<0.001$).

The most interesting morphoanatomical results were found in the ET layer, the inner layer of the outer integument. After 2 weeks of fruit development, one layer of rectangular palisade cells was observed in the seeds from both varieties (Fig. 3D, E). After 4 weeks, two layers were observed in 'Chardonnay' ET seeds (Fig. 3F) after a periclinal division, but this duplication was not observed in 'Sultanine' seed traces (Fig. 3G). After 6 weeks of fruit development, 'Chardonnay' seeds showed a sclerified and thicker ET (Fig. 3H), unlike 'Sultanine' seed traces that exhibited the same structures as its initial stage of development, with no lignification of this integument (Fig. 3I). The number of cells making up the ET layer was higher in 'Chardonnay', demonstrating a periclinal development. In contrast, this characteristic was not observed in 'Sultanine' (Fig. 4).

Spatial and temporal accumulation of VviAGL11 *transcripts*

In situ hybridization (ISH) with the *VviAGL11* complementary (antisense) probe reveals no expression at the stages H1 (Fig. 5B), pre-anthesis flower (Fig. 5C), fruit set (Fig. 5D), and at 6 weeks of fruit development (Fig. 5J) in 'Chardonnay'. *VviAGL11* transcripts exhibited a high accumulation in seeds after 2 (Fig. 5E, F) and 4 weeks (Fig. 5G–I) of development in 'Chardonnay'. The ISH signal was clearly visible in the ET layer of the seeds (Fig. 5E, I). In 'Sultanine', no gene expression was observed at reproductive tissue/organ or stage of development (Fig. 6A–F). The ISH slides with the antisense probe were compared with slides hybridized with the sense *VviAGL11* probe as the control in the analysis of 'Chardonnay' and 'Sultanine' samples (Supplementary Figs S3 and S4, respectively). At the time of ISH assays, the transcript isoforms were not known and the probe used for hybridization was designed according to the *VviAGL11* gene predicted model (GSVIVT01025945001) available at http://www.genoscope.cns.fr. Therefore, the whole probe (185 bp) hybridizes with the *CH1* mRNA isoform while 38 nucleotides still hybridize with *CH2*, *SU1*, and *SUmut* mRNA isoforms. Therefore, despite the differences within the genomic organization of the locus, the probe employed in the ISH assays is still capable of generating reliable data.

Functional analysis of VviAGL11

In order to confirm the role of *VviAGL11* as a key gene for seed morphogenesis in grapevine, we generated Arabidopsis *stk* mutant plants overexpressing *VviAGL11*. Two constructs were made with (i) a *VviAGL11* 'Chardonnay' allele (*VviAGL11 CH*); and (ii) a *VviAGL11* 'Sultanine' mutant allele (*VviAGL11 SU*). The 'Sultanine' mutant allele (*SUmut*) was used to evaluate the importance of the two SNP mutations conferring amino acid substitutions (*VviAGL11* R590L and T628A; Supplementary Fig. S2). Thirty-six well-developed plants were used for transformation with each construct, including controls. The reporter gene contained in the plasmid construct (GFP) was visualized in 3-week-old plants (Supplementary Fig. S5). The GFP-positive selected T_1 plants were tested by PCR for the presence of the complementation construct and the presence of the *VviAGL11* alleles, and resulted in 16 independent lineages transformed with *VviAGL11 CH* and 21 with *VviAGL11 SU*. In general, transformed plants had an increase of 12-fold in *VviAGL11* expression, compared with the wild type (Fig. 7C). Remarkably, an *stk* plant complemented with *VviAGL11* from the 'Sultanine' mutant allele presented a relative expression of 400-fold.

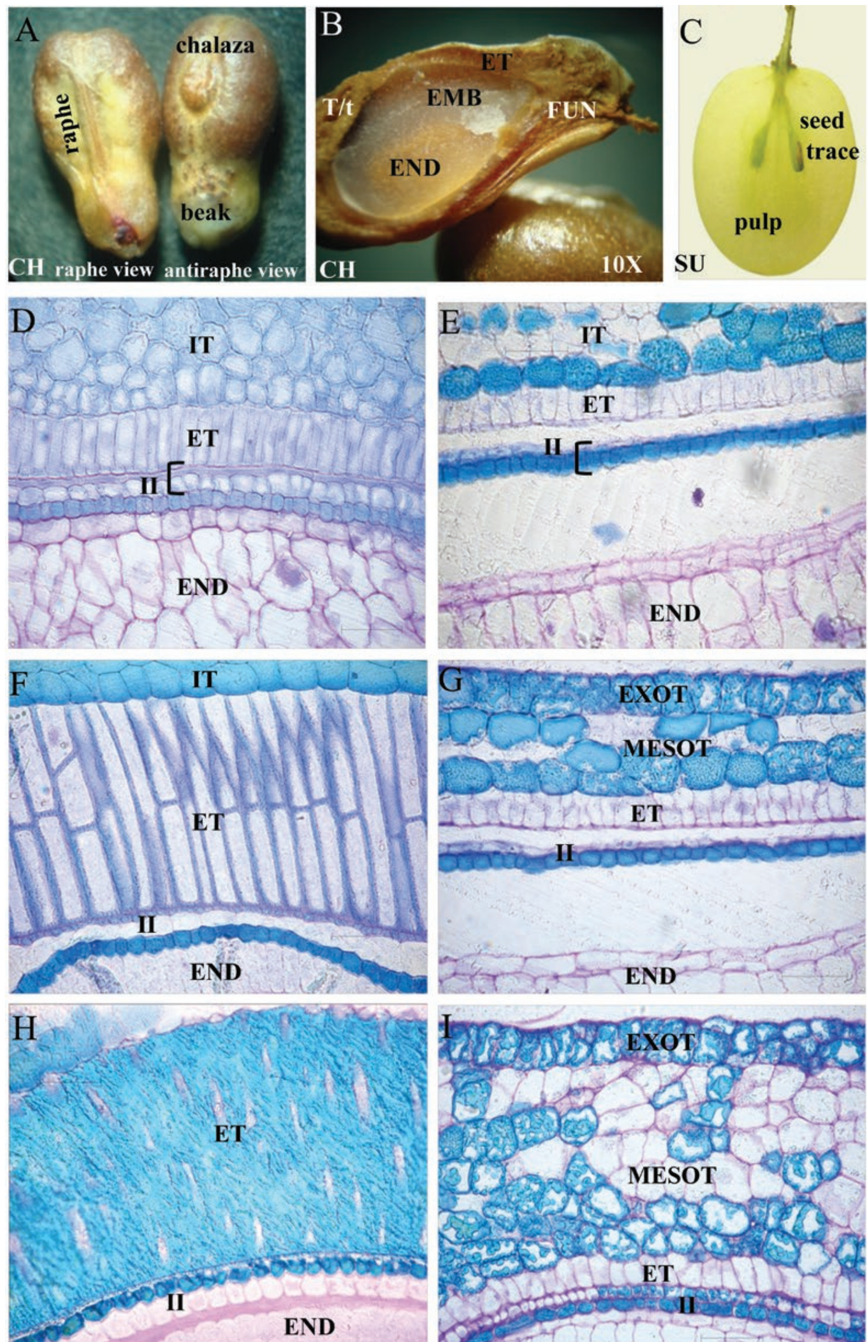

Fig. 3. Seeds and seed traces in *V. vinifera*. (A) Mature 'Chardonnay' (CH) seeds at their raphe and antiraphe view. (B) CH seed longitudinal section, with the view exhibiting the testa/tegmen (T/t), endotesta (ET), endosperm (END), embryo (EMB), and funiculus (FUN). (C) Mature 'Sultanine' (SU) berry showing its seed traces. Grapevine seed structure development from 2 to 6 weeks after fruit set (D–I). Light micrographs of grape seed cross-sections were stained with Toluidine Blue O. The cross-sections in the median area of the seeds of 'Chardonnay' and seed traces of 'Sultanine' enable the visualization of two defined integuments. 'Chardonnay' seed: (D) 2 weeks, (F) 4 weeks, and (H) 6 weeks. 'Sultanine' seed trace: (E) 2 weeks, (G) 4 weeks, and (I) 6 weeks. Endosperm (END), exotesta (EXOT), mesotesta (MESOT), endotesta (ET), inner integument (II), integuments (IT). Scale bar=200 µm.

The Arabidopsis *stk* mutant presents five distinct phenotypes when compared with the wild type: (i) the number of seeds is reduced to half; (ii) the size of the seeds is diminished (including many seed abortions); (ii) the silique length is reduced; (iv) the funiculus shows a thicker conformation; and (v) the funiculus shows an elongated conformation (Pinyopich *et al.*, 2003; Matias-Hernandez *et al.*, 2010). The siliques of T_1 lines present reduced seed abortion, increased seed size, silique length enlargement, and modifications in the funiculus resembling the wild-type phenotype (Fig. 7C). Among the *VviAGL11 CH*-transformed plants, six showed wild-type-like developed siliques and increased seed number, eight demonstrated wild-type seed size, seven plants showed reduction of the funiculus thickness, and eight showed a shortened funiculus (Fig. 7A, D). Among the *VviAGL11 SU* plants, nine presented wild type-like developed siliques, six plants showed increased seed number, 10 had wild-type seed size, 10 plants presented reduced

funiculus thickness, and nine showed a shortened funiculus (Fig. 7B, D).

These results demonstrate that the ectopic expression of *VviAGL11* is capable of restoring silique and seed morphogenesis in the Arabidopsis *stk* background and that very similar genes have a relatively well conserved function *in planta*, gathering evidence to support an evolutionary orthology relationship between *AtAGL11* and *VviAGL11*.

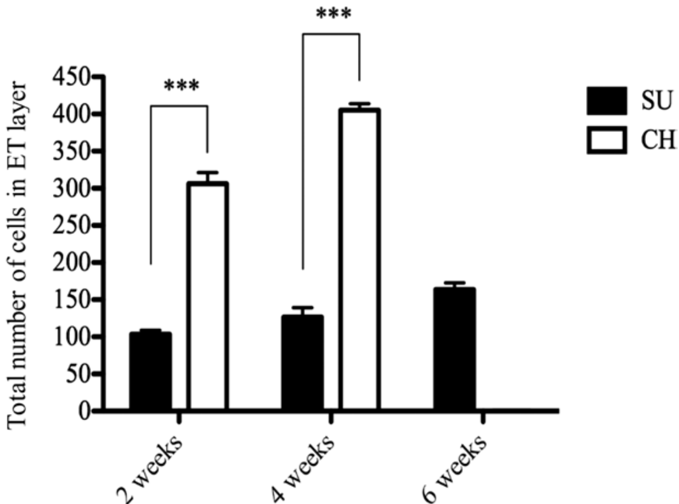

Fig. 4. Analysis of the endostesta (ET) layer of the seed of 'Chardonnay' and the seed trace of 'Sultanine'. The SD is shown for each stage and sample. Asterisks indicate statistical significance between the number of cells in the ET layer of 'Chardonnay' compared with 'Sultanine' (two-way ANOVA with Bonferroni post-test to compare replicate means by row. ***P<0.001).

Discussion

Spontaneous somatic variants have been selected through grapevine domestication, creating new varieties and cultivars with interesting traits such as berry size, taste, color, and seedlessness (This *et al.*, 2006). A collection of reports in the literature indicated *VviAGL11* as the major candidate involved in the absence of seeds (Costantini *et al.*, 2008; Mejía *et al.*, 2011; Revers *et al.*, 2014; Ocarez and Mejía, 2016). Despite that, no specific and direct functional evidence was reported concerning the role of *VviAGL11* during seed development in grapevine. Therefore, our approach was to explore a combination of methods such as *de novo* sequencing, gene expression, and ISH, combined with anatomical examination and complementation analysis of the Arabidopsis *stk* mutant to gather evidence to address the function of *VviAGL11* during seed morphogenesis.

De novo sequencing based on a PCR strategy of *VviAGL11* allelic variants from 'Sultanine' and 'Chardonnay' was performed, allowing the identification of a group of polymorphisms that allowed the identification of four *VviAGL11* alleles (Fig. 1; Supplementary Fig. S1). No transposon or large insertion or deletion sequence was found in 'Sultanine' that could explain the gene loss of transcription observed in previous data (Mejía *et al.*, 2011). We found two amino

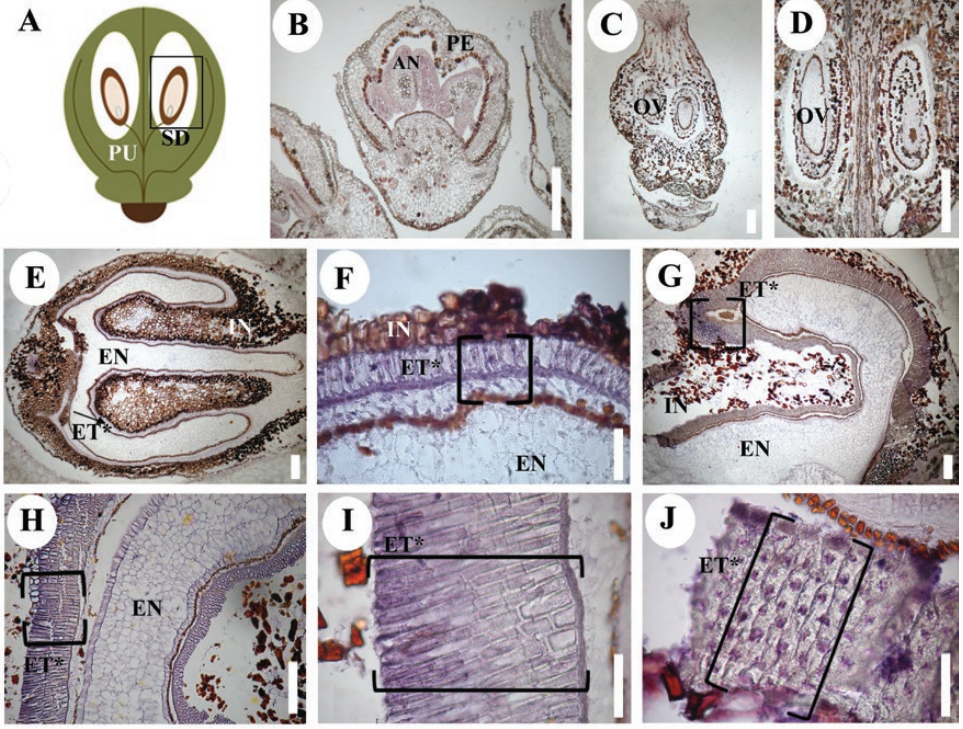

Fig. 5. *In situ* hybridization of *VviAGL11* in 'Chardonnay' reproductive tissues and organs, from flowers to seeds after 6 weeks of fruit development. (A) Schematic berry. No hybridization signal is visible in (B) H1 flower stage, (C) pre-anthesis flower stage, and (D) fruit set flower stage. *VviAGL11* expression is detectable in the endotesta of (E and F) seeds at 2 weeks of development and (G–I) seeds at 4 weeks of development (black arrows). No clear hybridization signal is visible in seeds at 6 weeks of development (J) when compared with the control (Supplementary Fig. S3H). Anther (an), endosperm (en), endotesta (et), integuments (it), petal (pe), pulp (pu), seed (sd). Scale bar=200 µm.

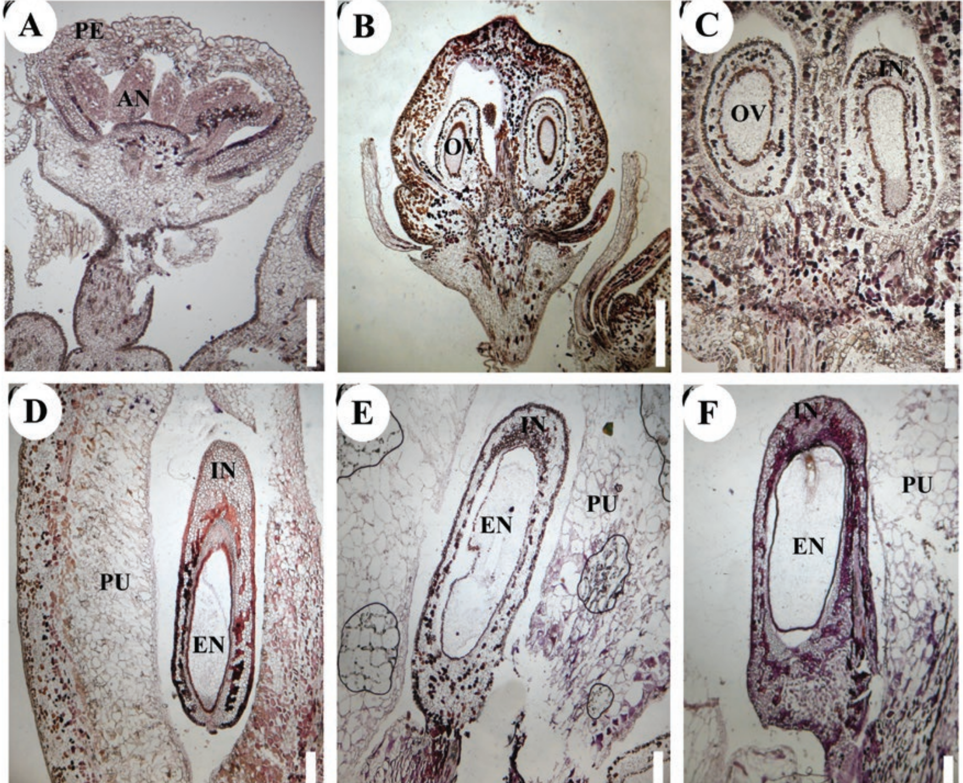

Fig. 6. *In situ* hybridization of *VviAGL11* in 'Sultanine' reproductive tissues and organs, from flowers to fruits and seed traces after 6 weeks of development. No hybridization signal is visible in (A) H1 flower stage, (B) pre-anthesis flower stage, (C) fruit set stage, and in seed traces from fruits of (D) 2, (E) 4, and (F) 6 weeks of development when compared with control slides (Supplementary Fig. S4). Anther (an), endosperm (en), endotesta (et), integuments (it), petal (pe), pulp (pu). Scale bar=200 μm.

acid modifications in 'Sultanine's' *VviAGL11* coding region (Supplementary Fig. S2). Arginine and threonine were substituted by two more hydrophobic amino acids, leucine and alanine, respectively. These amino acid substitutions were localized in the C-terminal portion of the VviAGL11 protein, a well-known region responsible for activity and ternary complex formation (Favaro *et al.*, 2003; Matias-Hernandez *et al.*, 2010). Furthermore, 17 INDELs and 53 additional SNPs were identified in the second intron of the 'Sultanine' mutant allele (Supplementary Fig. S1). The second intron of MADS-box AG (AGAMOUS) subfamily members is recognized by the presence of a quite large (*AtAG*. ~3 kb; *AtAGL11*, ~1.8 kb; and *VviAGL11*, ~4 kb) region with important *cis*-elements that may control tissue-specific expression of AG and AG-like genes (Deyholos and Sieburth, 2000; Hong and Hamaguchi, 2003). Interestingly, MADS-box proteins form complexes of high order, often in tetrameric junctions made by two dimers that bind to two DNA consensus sequences called CArG-boxes [CC(A/T)$_6$GG] separated by up to 300 bp (Liu *et al.*, 2008). A CArG-box modification was observed in the 'Sultanine' mutant (*SUmut*) allele, in the seventh putative sequence. This *cis*-element is only 84 bp distant from its putative partner CArG-box, which would suggest their relevance in *VviAGL11* gene regulation (Supplementary Fig. S1).

The transcription profile of *VviAGL11* isoforms provided complete data for each expression isoform during the development of flowers, fruits, and seeds. 'Sultanine' alleles presented a low level of expression in all organs and stages of development, including berries, while the 'Chardonnay' alleles were expressed at very low levels in the berry pulp, but highly expressed in seeds (Fig. 2). Of the two 'Chardonnay' alleles, *VviAGL11-CH1* possesses a larger coding region, caused by alternative splicing, resulting in 20 additional amino acids. This alternative splicing is characterized as a 5' splice site and occurs in 7.5% of the Arabidopsis genome (Eckardt, 2013). The sequence of the *CH1* allele transcript was also found in a 'Cabernet Sauvignon' cDNA library derived from early stages of berry development (GenBank CB974197.1). This could cause conformational changes in the C-terminal portion of VviAGL11 protein, yet no abnormal seed morphogenesis is observed in Chardonnay or Cabernet Sauvignon cultivars. Thus it is clear that these 20 additional amino acid residues are not relevant to seed development.

The relative expression of the *CH1* allele in seeds showed a fluctuation between 5- and 11-fold when compared with fruit set (Fig. 2). These results suggest that *VviAGL11* indeed has a major role in seed development. Without its expression (transcript accumulation) during early fruit developmental stages, there is no normal seed formation.

The interaction of proteins encoded by MADS-box genes of classes A, B, C, D, and E forms the widely accepted model of floral quartets (Theissen *et al.*, 2000; Dornelas and Dornelas, 2005). The AGAMOUS subfamily of *MADS-box* genes is known to have redundant functions in *A. thaliana*, as is the case for *AtAGL11* which is the major gene involved in the regulation of ovule development along

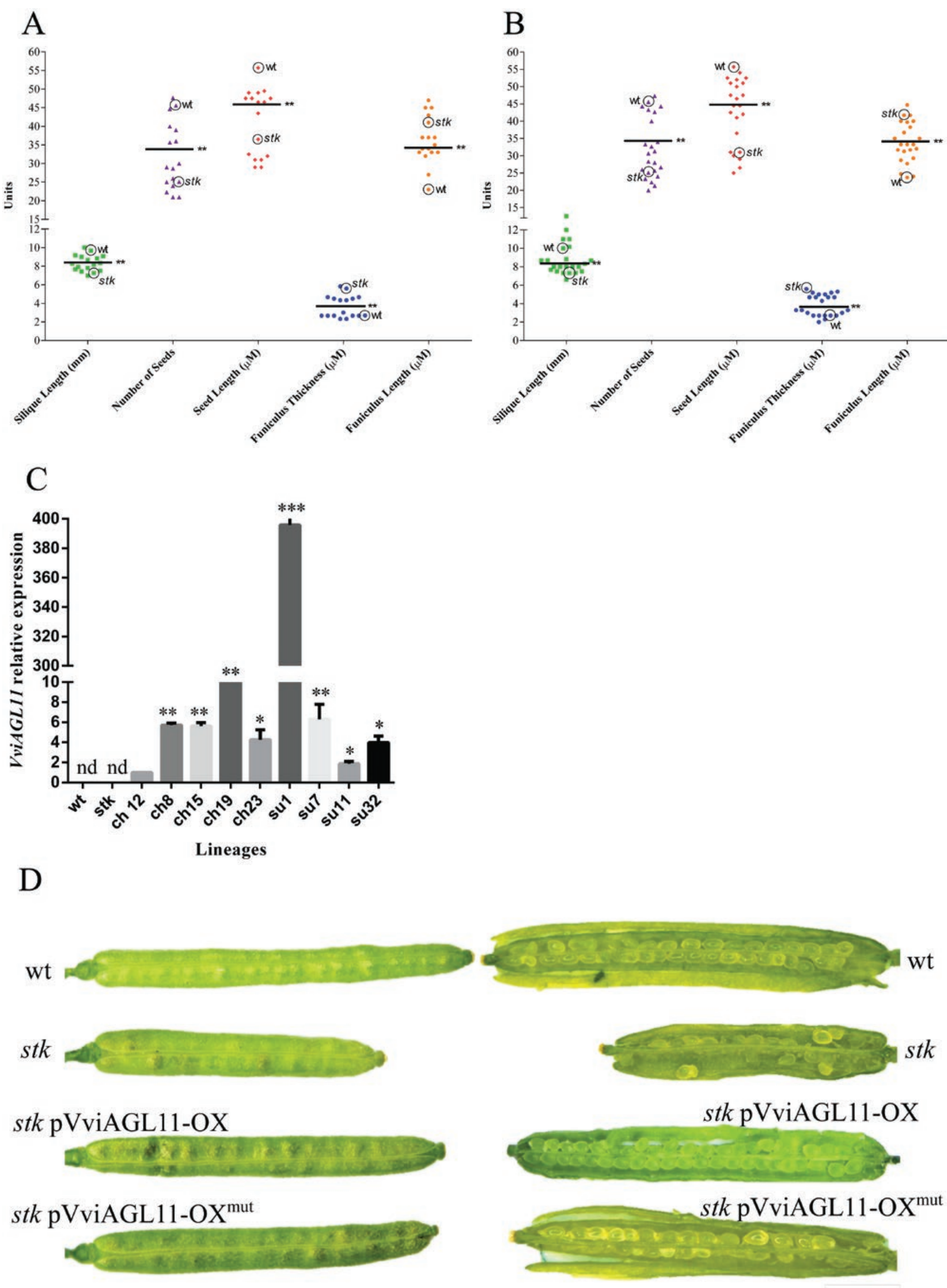

Fig. 7. Complementation analysis in the Arabidopsis *stk* mutant. (A) pVviAGL11-OX T_1 distribution by phenotype. (B) pVviAGL11-OX^{mut} T_1 distribution by phenotype. (C) *VviAGL11* relative expression analyzed in wild-type (wt), *stk*, and *stk*-transformed plants with *VviAGL11* constructions. (D) Comparison between controls and *stk*-complemented constructions. A wild-type silique showing full seed set (wt); siliques of *stk* plants containing aborted ovules and aborted seeds; siliques of *stk* pVviAGL11-OX plants complemented with the genomic region of the *VviAGL11* gene from Chardonnay, and siliques of *stk* pVviAGL11-OX^{mut} plants complemented with the genomic region of the *VviAGL11* gene from Sultanine. For each plant, five well-developed siliques at the same stage were evaluated, and the final score was composed of their average. Columbia-0 and *stk* plants were used as controls for measurements. Scale bar=2 mm.

with other two genes of class D, *SHATERPROOF1* and *SHATTERPROOF2* (Colombo *et al.*, 1995; Pinyopich *et al.*, 2003). Our evidence together with data from the literature support that the *VviAGL11* gene can be considered as an ortholog of *AtAGL11*; however, no gene has been identified yet as orthologous to *SHP1* or *SHP2* in

grapevine (Díaz-Riquelme *et al.*, 2012). These data suggest that *VviAGL11* might be the unique class D gene present in grapevine. Therefore, a possible model of the floral quartet could be proposed to be formed by *VvAG1* and *VvAG2* (*AGAMOUS* genes), *VviAGL11* (*VvAG3*), and one of the *SEPALLATA* genes of grapevine (*VvSEP1*, *VvSEP2, VvSEP3*, or *VvSEP4*) as previously described by Boss *et al.* (2002) and by Díaz-Riquelme *et al.* (2012). Additional studies of the VviAGL11 protein, such as employing fluorescence resonance energy transfer (FRET) (Sekar and Periasamy, 2003), are alternatives to confirm this hypothesis.

Previous studies have described grapevine seed anatomy as well as the changes of internal layers in the process of seed ripening; however, no study has demonstrated detailed morphological aspects in seeds of stenospermocarpic grapevines, especially seed traces (Pratt, 1971; Ribereau-Gayon and Peynaud, 1980; Cadot *et al.*, 2006). We compared normal seeds from 'Chardonnay' with seed traces from 'Sultanine', and the results showed that the most substantial difference was at the ET of the seed coat, which did not develop normally in 'Sultanine' seed traces, preserving its initial features until the complete maturation of the fruits. As expected, 'Chardonnay' seeds developed correctly with the elongation and cell duplication of the ET cells through periclinal divisions along with normal endosperm growth (Fig. 3). Our complete analysis of seed development showed that grapevine seeds have only two integuments, outer and inner (Figs 3, 5, 6), instead of three integument layers as previously described (Pratt, 1971; Cadot *et al.*, 2006).

Garcia *et al.* (2005) have previously shown that there is a strong maternal effect of the integuments in the control, and determination of final seed size, due to the elongation of these layers, and, in the case of absence of elongation, there is a compensation effect promoted by the duplication of these layers. Furthermore, the nutrient flux in seeds occurs in a centripetal way, from the outer integument to the inner, going through apoplasts to the endosperm and to the embryo (Stadler and Lauterbach, 2005). Formation of seed traces is likely to originate due to disruption of normal development of seed tissues. It is possible that the erroneous formation of the ET seed layer that occurs in stenospermocarpic grapevines could interfere in seed nutrient intake and cause the degeneration of some tissues during seed development, such as the endosperm. Therefore, the crosstalk between seed coat and endosperm could be compromised by the defects on the ET, affecting the normal development of the seed (Figueiredo and Köhler, 2014).

The accumulation of transcripts of *VviAGL11* was localized in the ET layer of 'Chardonnay' seeds at 2 and 4 weeks (Fig. 5E–I) of development, while no signal of transcripts was detected in 'Sultanine' seed traces (Fig. 6). When 'Chardonnay' seeds at 6 weeks after fruit set were examined, it was assumed that no hybridization signals was detected (Fig. 5J). However, *VviAGL11* expression at this stage cannot be excluded because RT–qPCR data indicate that at least until 6 weeks after fruit set *VviAGL11* is still expressed in seed tissues (Fig. 2). Unambiguous interpretation of the hybridization signals (Fig. 5J) during this stage was difficult to confirm probably due to the initiation of the lignification process that will be part of the mature and hard seed coat (Haughn and Chaudhury, 2005). A large amount of tannins and phenolic compounds turn brown, tending to darken throughout treatments during slide processing, giving a very strong background, which may impede clear signal identification in grapevine ISH analysis from tissues prone to lignification (Fernandez *et al.*, 2007; Colas *et al.*, 2010).

The ISH data are in agreement with the RT–qPCR transcriptional profile, where 'Sultanine' shows almost no expression of *VviAGL11* in the stages evaluated. The particular accumulation of *VviAGL11* transcripts in a specific layer of the seed coat in 'Chardonnay', combined with the morphological differences in this same layer when comparing 'Chardonnay' and 'Sultanine', suggests that this gene is essential for the elongation and duplication of the ET of the seed coat. Furthermore, seed growth is dictated by the coordinated development of endosperm and integuments and, hence, a spatial and temporal co-ordination of endosperm and integument growth is critical for normal seed development (Garcia *et al.*, 2005). These results showed the similarities between the expression pattern of *AtAGL11* and *VviAGL11* in the fruit tissues, both being expressed in the ovule integuments. *AtAGL11*, however, has a spatial–temporal expression in the funiculus and in the ovule integuments mainly before fertilization, while *VviAGL11* expression is observed in ovule integument after fertilization (Mejía *et al.*, 2011; Fig. 3).

The overexpression of *AtAGL11* in the Arabidopsis *AGAMOUS* mutant (*ag*) promotes homeotic conversions of sepals into carpeloid structures, achieving ovule development (Favaro *et al.*, 2003). More recently, Mizzotti *et al.* (2014) expressed *AtAGL11* in the mutant *stk* background and observed the complete complementation of the wild-type phenotype, with normal seeds and correct seed abscission. In order to drive the expression of *VviAGL11*, the *Cauliflower mosaic virus* (CaMV) 35S promoter instead of the endogenous promoter of *AtAGL11* was used in the constructions. The *stk* transformants (T_1 generation) with pVviAGL11-OX and pVviAGL11-OX^{mut} restored the wild-type phenotype with both constructions (Fig. 7). The number and seed size increased, the silique achieved its normal size, and the typical morphology of the funiculus was also restored (Fig. 7D). Studies with the *stk* single mutant demonstrated that the funiculus is longer and thicker, wherein *AtAGL11* is also responsible for controlling cell expansion and cell division in this structure (Pinyopich *et al.*, 2003). *VviAGL11* overexpression in *stk* plants demonstrates that the ectopic expression of this gene was able to restore *stk* to the wild-type phenotypes for siliques, seeds, and funiculus (Fig. 7C). These results show that *VviAGL11* is responsible for the control of seed morphogenesis in a similar manner to Arabidopsis *AtAGL11*, providing evidence of an orthology relationship between them. The differences in the coding region conferred by *VviAGL11 SUmut* (*SUmut*) had no effect during silique and seed development in the transformed T_1 *stk* mutant plants.

The results showing that *VviAGL11* is down-regulated in the apirenic genetic background despite having a wild-type-like

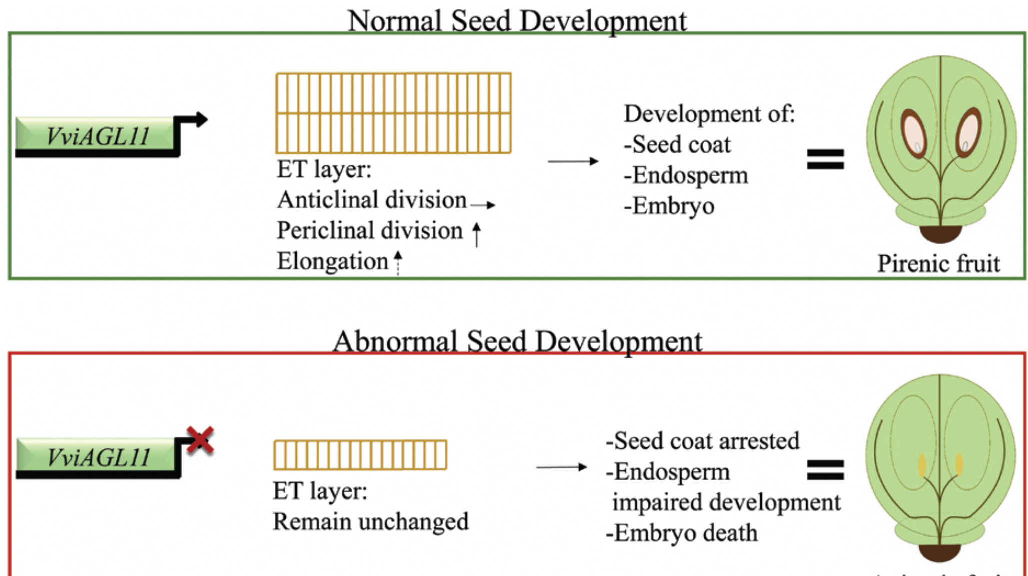

Fig. 8. The effect of *VviAGL11* on grapevine seed development. When *VviAGL11*expression is increased in the ovule after fertilization, the endotesta (ET) layer undergoes several modifications such as cell elongation,and cell duplication in anticlinal and periclinal directions. The correct development of this layer directs the further development of the seed coat, the endosperm, and, consequently, the embryo. Therefore, the seeds have a normal development, resulting in a seeded fruit. However, when *VviAGL11* expression remains low, the ET layer remains without the expected modifications, affecting the development of the seed coat and the endosperm, and causing embryo death. Thus, the final seed have an abnormal development, referred to as seed trace, resulting in an apirenic (seedless) fruit. (This figure is available in colour at *JXB* online.)

allele raises questions about its regulation and indicates that the mutant allele (*VviAGL11 SUmut*) has a dominant effect in the resulting phenotype, excluding the possibility that the seedless phenotype might be caused by variations in its promoter region as previously proposed by Mejía *et al*. (2011). Based on the general gene structure of the MADS-box genes reported in the literature and the data gathered in this work, our hypothesis is that the CArG-boxes present in the second intron may play a role in *VviAGL11* regulation. In addition, based on the genetic data obtained from sequencing of the *VviAGL11* alleles and in the transcription profile of this gene, we suggest that VviAGL11 protein may self-regulate *VviAGL11* transcription. *AtAGL11* direct targets are VDD (VERDANDI), BAN (BANYULS/ANTHOCYANIDIN REDUCTASE), ABS (ARABIDOPSIS B SISTER), and EGL3 (ENHANCER OF GLABRA3) genes, but unfortunately there are still no data referring to or testing this class D gene self-regulation (Matias-Hernandez *et al.*, 2010; Mizzotti *et al.*, 2014). Experiments involving the functional study of the polymorphisms between 'Chardonnay' and 'Sultanine' and analysis of VviAGL11 proteins, derived from different alleles of *VviAGL11*, are under way in order to test the *VviAGL11* self-regulation hypothesis.

We propose that the lack of increased expression of *VviAGL11* during berry development is responsible for the abnormal development of the seeds. The extremely small final seed size results from the non-differentiation of the ET seed layer, causing the cessation of elongation, division, and duplication of this layer and, consequently, of the lignified seed coat. Moreover, with the loss of identity of the ET layer, the endosperm ceases its growth, causing embryo death and seed trace formation (Fig. 8). Our results are in agreement with the hypothesis of Bouquet and Danglot (1996) for seed development, in which the inheritance of seedlessness in grapevine is based on a complex system whereby the expression of three independently inherited recessive genes is controlled by a dominant major regulator gene from a single dominant locus named *SdI*. The previous evidence reported by Mejía *et al*. (2011), Revers *et al.* (2014), and Ocarez and Mejía (2016) is now reinforced by the data gathered in this work, allowing affirmation that the *SdI* locus phenotype effect is derived from the *VviAGL11* function during seed morphogenesis. Taken together, our results provide functional evidence to show that *VviAGL11* transcription is essential for seed morphogenesis in grapevine during berry development. Despite the valuable literature dedicated to seedlessness in grapevine, our work presents new and solid data towards the understanding of the molecular mechanism driving grapevine seed development with high potential in agriculture use in the generation of seedless grape varieties.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Complete alignment of *VviAGL11* alleles.

Fig. S2. Alignment of the coding regions of *VviAGL11* alleles.

Fig. S3. *In situ* hybridization of the *VviAGL11* sense probe (control) in 'Chardonnay'.

Fig. S4. *In situ* hybridization of the *VviAGL11* sense probe (control) in 'Sultanine'.

Fig. S5. Plants of *Arabidopsis thaliana* under GFP evaluation.

Table S1. Primers designed for the amplification and sequencing of *VviAGL11*.

Table S2. Stages of development of grapevine reproductive organs.

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Author contributions

LFR and JM conceived the original screening and research plans; LFR, VB, MCD, MMP, GP, and JEAM supervised the experiments; JM and VB performed most of the experiments; MCD, MLG, and JEAM provided technical assistance to JM and VB; JM, VB, LFR, and MCD designed the experiments and analyzed the data; JM conceived the project and wrote the article with contributions of all the authors; LFR supervised and complemented the writing.

References

Baggiolini M. 1952. Stades repères de la vigne. Revue Romande d'Agriculture, de Viticulture et d'Arboriculture **8,** 4–6.

Boss PK, Sensi E, Huab C, Davies C, Thomas MR. 2002. Cloning and characterisation of grapevine (*Vitis vinifera* L.) MADS-box genes expressed during inflorescence and berry development. Plant Science **162,** 887–895.

Bouquet A, Danglot Y. 1996. Inheritance of seedlessness in grapevine (*Vitis vinifera* L.). Vitis **35,** 35–42.

Brambilla V, Kater M, Colombo L. 2008. Ovule integument identity determination in Arabidopsis. The Plant Cell **3,** 246–247.

Cabezas JA, Cervera MT, Ruiz-García L, Carreño J, Martínez-Zapater JM. 2006. A genetic analysis of seed and berry weight in grapevine. Genome **49,** 1572–1585.

Cadot Y, Miñana-Castelló MT, Chevalier M. 2006. Anatomical, histological, and histochemical changes in grape seeds from *Vitis vinifera* L. cv *Cabernet franc* during fruit development. Journal of Agricultural and Food Chemistry **54,** 9206–9215.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. The Plant Journal **16,** 735–743.

Colas S, Jacquens L, Manteau S, Devy J, Conéjéro G, Clément C, Baillieul F, Mazeyrat-Gourbeyre F, Monti-Dedieu L. 2010. Expression analysis in grapevine by in situ hybridization and immunohistochemistry. In: Delrot S, Medrano H, Or E, Bavaresco L, Grando S, eds. Methodologies and Results in Grapevine Research, Dordrecht, The Netherlands: Springer: 361–374.

Colombo L, Franken J, Koetje E, van Went J, Dons HJ, Angenent GC, van Tunen AJ. 1995. The petunia MADS box gene FBP11 determines ovule identity. The Plant Cell **7,** 1859–1868.

Costantini L, Battilana J, Lamaj F, Fanizza G, Grando MS. 2008. Berry and phenology-related traits in grapevine (Vitis vinifera L.): from quantitative trait loci to underlying genes. BMC Plant Biology **8,** 38.

Deyholos MK, Sieburth LE. 2000. Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. The Plant Cell **12,** 1799–1810.

Díaz-Riquelme J, Grimplet J, Martínez-Zapater JM, Carmona MJ. 2012. Transcriptome variation along bud development in grapevine (*Vitis vinifera* L.). BMC Plant Biology **12,** 181.

Di Genova A, Almeida AM, Muñoz-Espinoza C, *et al*. 2014. Whole genome comparison between table and wine grapes reveals a comprehensive catalog of structural variants. BMC Plant Biology **14,** 7.

Doligez A, Bertrand Y, Farnos M, *et al*. 2013. New stable QTLs for berry weight do not colocalize with QTLs for seed traits in cultivated grapevine (*Vitis vinifera* L.). BMC Plant Biology **13,** 217.

Dornelas MC, Dornelas O. 2005. From leaf to flower: revisiting Goethe's concepts on the 'metamorphosis' of plants. Brazilian Journal of Plant Physiology **17,** 335–343.

Dornelas MC, Van Lammeren AA, Kreis M. 2000. *Arabidopsis thaliana* SHAGGY-related protein kinases (AtSK11 and 12) function in perianth and gynoecium development. The Plant Journal **21,** 419–429.

Eckardt NA. 2013. The plant cell reviews alternative splicing. The Plant Cell **25,** 3639.

Falavigna V da S, Porto DD, Buffon V, Margis-Pinheiro M, Pasquali G, Revers LF. 2014. Differential transcriptional profiles of dormancy-related genes in apple buds. Plant Molecular Biology Reporter **32,** 796–813.

FAO. Food and Agriculture Organization of the United Nations. http://www.fao.org/

Favaro R, Pinyopich A, Battaglia R, Kooiker M, Borghi L, Ditta G, Yanofsky MF, Kater MM, Colombo L. 2003. MADS-box protein complexes control carpel and ovule development in Arabidopsis. The Plant Cell **15,** 2603–2611.

Feder N, O'brien TP. 1968. Plant microtechnique, some principles and new methods. American Journal of Botany **55,** 123–142.

Fernandez L, Torregrosa L, Terrier N, Sreekantan L, Grimplet J, Davies C, Thomas MR, Romieu C, Ageorges A. 2007. Identification of genes associated with flesh morphogenesis during grapevine fruit development. Plant Molecular Biology **63,** 307–323.

Figueiredo DD, Köhler C. 2014. Signalling events regulating seed coat development. Biochemical Society Transactions **42,** 358–363.

Gabriel B. 1982. Biological scanning electron microscopy. New York: Van Nostrand Reinhold Co.

Garcia D, Fitz Gerald JN, Berger F. 2005. Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis. The Plant Cell **17,** 52–60.

Gerrits PO, Smid L. 1983. A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. Journal of Microscopy **132,** 81–85.

Grimplet J, Adam-Blondon AF, Bert PF, *et al*. 2014. The grapevine gene nomenclature system. BMC Genomics **15,** 1077.

Haughn G, Chaudhury A. 2005. Genetic analysis of seed coat development in Arabidopsis. Trends in Plant Science **10,** 472–477.

Hong RL, Hamaguchi L, Busch MA, Weigel D. 2003. Regulatory elements of the floral homeotic gene AGAMOUS identified by phylogenetic footprinting and shadowing. The Plant Cell **15,** 1296–1309.

Jaillon O, Aury JM, Noel B, *et al*. 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature **449,** 463–467.

Karimi M, Inzé D, Depicker A. 2002. GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends in Plant Science **7,** 193–195.

Lefort F, Douglas GC. 1999. An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species Acer, Fraxinus, Prunus and Quercus. Annals of Forest Science **56,** 259–263.

Liu C, Chen H, Er HL, Soo HM, Kumar PP, Han JH, Liou YC, Yu H. 2008. Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis. Development **135,** 1481–1491.

Matias-Hernandez L, Battaglia R, Galbiati F, Rubes M, Eichenberger C, Grossniklaus U, Kater MM, Colombo L. 2010. VERDANDI is a direct target of the MADS domain ovule identity complex and affects embryo sac differentiation in Arabidopsis. The Plant Cell **22,** 1702–1715.

Mcdowell EM, Trump BR. 1976. Histological fixatives for diagnostic light and electron microscopy. Archives of Pathology and Laboratory Medicine **1000,** 405–414.

Mejía N, Gebauer M, Muñoz L, Hewstone N, Muñoz C, Hinrichsen P. 2007. Identification of QTLs for seedlessness, berry size, and ripening

date in a seedless × seedless table grape progeny. American Journal of Enology and Viticulture. **58,** 499–507.

Mejía N, Soto B, Guerrero M, *et al*. 2011. Molecular, genetic and transcriptional evidence for a role of VvAGL11 in stenospermocarpic seedlessness in grapevine. BMC Plant Biology **11,** 57.

Mizzotti C, Ezquer I, Paolo D, *et al*. 2014. SEEDSTICK is a master regulator of development and metabolism in the Arabidopsis seed coat. PLoS Genetics **10,** e1004856.

Ocarez N, Mejía N. 2016. Suppression of the D-class MADS-box AGL11 gene triggers seedlessness in fleshy fruits. Plant Cell Reports **35,** 239–254.

OIV. 2014. Statistiques du secteur vitivinicole mondial. International Organisation of Vine and Wine.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research **29,** e45.

Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF. 2003. Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature **424,** 85–88.

Pratt C. 1971. Reproductive anatomy in cultivated grapes. A review. American Journal of Enology and Viticulture **22,** 91–109.

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST. 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biology **6,** 27.

Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research **37,** e45.

Revers LF, Welter LJ, Irala PB, Silva DC, Lampe VS, Oliveira PRD, Garrido L. 2014. Co-localization of QTLs for seedlessness and downy mildew resistance in grapevine. International Conference on Grapevine Breeding and Genetics **10,** 449–456.

Ribereau-Gayon J, Peynaud E. 1980. Sciences et techniques de la vigne. Tome I. Biologie de la vigne, sols de vignobles. Paris: Dunod.

Sambrook J, Fritsch EF, Maniatis T. 1987. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sekar RB, Periasamy A. 2003. Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. Journal of Cell Biology **160,** 629–633.

Smaczniak C, Immink RG, Angenent GC, Kaufmann K. 2012. Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. Development **139,** 3081–3098.

Stadler R, Lauterbach C, Sauer N. 2005. Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in Arabidopsis seeds and embryos. Plant Physiology **139,** 701–712.

Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Münster T, Winter KU, Saedler H. 2000. A short history of MADS-box genes in plants. Plant Molecular Biology **42,** 115–149.

This P, Lacombe T, Thomas MR. 2006. Historical origins and genetic diversity of wine grapes. Trends in Genetics **22,** 511–519.

Varoquaux F, Blanvillain R, Delseny M, Gallois P. 2000. Less is better: new approaches for seedless fruit production. Trends in Biotechnology **18,** 233–242.

Zeng Y, Yang T. 2002. RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. Plant Molecular Biology Reporter **20,** 417.

Supplementary Material

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)


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                        ************************************************************

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su_Di Genova            GAAGGCATGAACGAAGTTGATTACTGAARATATTAGCAACATTACTAGGGTTTTGGCGGA
CH2                     GAAGGCATGAACGAAGTTGATTACTGAAGATATTAGCAACATTACTAGGGTTTT---GGA
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su_wt                   GAAGGCATGAACGAAGTTGATTACTGAAGATATTAGCAACATTACTAGGGTTTT---GGA
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                        **************************** *************************   ***

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su_Di Genova            ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
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VIT_218S0041G01880.2    GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTGTGGTGGGAATTTGGAACT
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CH1                     GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTGTGGTGGGAATTTGGAACT
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su_Di Genova            GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
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CH1                     GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
                        ************************************************************

su_mut                  TTCGGAAGTCTTTTTCTTCCTCAACCC------CTCTCTCTCTCTCTCTCTTTGAAGAAG
su_Di Genova            TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
CH2                     TTCGGAAGTCTTTTTCTTCCTCAACCCCTCTCTCTCTCTCTCTCTCTCTCTTTGAAGAAG
VIT_218S0041G01880.2    TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
su_wt                   TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
CH1                     TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
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su_mut                  TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--CTC
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su Di Genova         TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTCCTCTC
CH2                  TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC-----
VIT_218S0041G01880.2 TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--TTC
su_wt                TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--TTC
CH1                  TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--TTC
                     *******************************************************

su_mut               TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
su Di Genova         TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
CH2                  -----TTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCA
VIT_218S0041G01880.2 TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
su_wt                TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
CH1                  TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
                          ******************************************************

su_mut               TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCCCTCTCCCTCCCTCTCTCTCTCTCT
su Di Genova         TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCTCT
CH2                  TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--------CTCCCTCTCCCTCTCT
VIT_218S0041G01880.2 TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCTCT
su_wt                TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCTCT
CH1                  TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCTCT
                     ************************************        * * ***** ******

su_mut               CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTCTCTCTCTCTTTCA
su Di Genova         CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
CH2                  CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTC------------TCA
VIT_218S0041G01880.2 CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
su_wt                CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
CH1                  CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
                     *********************************************            ***

su_mut               TATCCGTCTCACATCTCTCCATTGTGGTATATATATATATAAGGACTAAACACAAGTCTTAA
su Di Genova         TATCCGTCTCACATCTCTCCATTGTGGTATATATATATATAAGGACTAAACACAAGTCTTAW
CH2                  TATCCGTCTCACATCTCTCCATTGTGGTATATATATATATAAGGACTAAACACAAGTCTTAA
VIT_218S0041G01880.2 TATCCGTCTCACATCTCTCCATTGTGGTATATATATATATAAGGACTAAACACAAGTCTTAA
su_wt                TATCCGTCTCACATCTCTCCATTGTGGTATATATATATATAAGGACTAAACACAAGTCTTAA
CH1                  TATCCGTCTCACATCTCTCCATTGTGGTATATATATATATAAGGACTAAACACAAGTCTTAA
                     ***********************************************************

su_mut               GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
su Di Genova         GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
CH2                  GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
VIT_218S0041G01880.2 GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
su_wt                GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
CH1                  GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
                     ************************************************************

su_mut               AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
su_Di Genova         AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
CH2                  AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
VIT_218S0041G01880.2 AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
su_wt                AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
CH1                  AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
                     ************************************************************

su_mut               CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
su_Di Genova         CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
CH2                  CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
VIT_218S0041G01880.2 CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
su_wt                CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
CH1                  CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
                     ************************************************************

su_mut               TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
su_Di Genova         TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
CH2                  TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
VIT_218S0041G01880.2 TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
su_wt                TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
CH1                  TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
                     ************************************************************

su_mut               TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
su_Di Genova         TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
CH2                  TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
VIT_218S0041G01880.2 TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
su_wt                TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
CH1                  TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
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su_mut                     TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
su_Di Genova               TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
CH2                        TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
VIT_218S0041G01880.2       TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
su_wt                      TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
CH1                        TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
                           ************************************************************

su_mut                     CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
su_Di Genova               CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
CH2                        CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
VIT_218S0041G01880.2       CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
su_wt                      CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
CH1                        CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
                           ************************************************************

su_mut                     TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
su_Di Genova               TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
CH2                        TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
VIT_218S0041G01880.2       TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
su_wt                      TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
CH1                        TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
                           ************************************************************

su_mut                     AGTTTGATTATTTTGCCTTTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTCTCAG
su_Di Genova               AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTCTCAG
CH2                        AGTTTGATTATTTTGCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
VIT_218S0041G01880.2       AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
su_wt                      AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
CH1                        AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
                           ************** ** ************************************* ****

su_mut                     TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAAGGGAAAAAGCCAGAATTTT
su_Di Genova               TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGARGGGAAAAAGCCAGAATTTT
CH2                        TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAAGGGAAAAAGCCAGAATTTT
VIT_218S0041G01880.2       TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAGGGGAAAAAGCCAGAATTTT
su_wt                      TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAGGGGAAAAAGCCAGAATTTT
CH1                        TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAGGGGAAAAAGCCAGAATTTT
                           **************************************** *******************

su_mut                     TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
su_Di Genova               TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
CH2                        TTTTCCTGCGTTCTGGTCTTAAGCTTCTTATCCATATCCTAGGTTTATGGGAAATGTGTT
VIT_218S0041G01880.2       TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
su_wt                      TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
CH1                        TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
                           **************************** *********** *******************

su_mut                     AAGGGTCATGGGGTTTTTGAGAAATGTG----GAGAGAGAGAGAGAGAGAGAGAGAGAGA
su_Di Genova               AAGGGTTATGGGGTTTTTGAGAAATGTG----GAGAGAGAGAGAGAGAGAGAGAGAGAGA
CH2                        AAGGGTCATGGGGTTTTTGAGAAATGTGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
VIT_218S0041G01880.2       AAGGGTTATGGGGTTTTTGAGAAATGTG----------------------GAGAGAGAGA
su_wt                      AAGGGTTATGGGGTTTTTGAGAAATGTG----------------------GAGAGAGAGA
CH1                        AAGGGTTATGGGGTTTTTGAGAAATGTG----------------------GAGAGAGAGA
                           ****** *********************                      **********

su_mut                     GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
su_Di Genova               GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
CH2                        GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
VIT_218S0041G01880.2       GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
su_wt                      GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
CH1                        GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
                           ************************************************************

su_mut                     GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
su_Di Genova               GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
CH2                        GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
VIT_218S0041G01880.2       GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
su_wt                      GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
CH1                        GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
                           ************************************************************

su_mut                     TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
su_Di Genova               TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
CH2                        TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
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VIT_218S0041G01880.2 TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
su_wt                TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
CH1                  TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
                     ************************************************************

su_mut               CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
su_Di Genova         CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
CH2                  CCAAGGTCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
VIT_218S0041G01880.2 CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
su_wt                CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
CH1                  CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
                     ***** ******************************************************

su_mut               CCTATTTTTCAAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGA-----
su_Di Genova         CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
CH2                  CCTATTTTTCAAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
VIT_218S0041G01880.2 CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
su_wt                CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
CH1                  CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
                     ********** ********************************************

su_mut               --TCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
su_Di Genova         CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
CH2                  CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACAAATTTTTTCGGTAGATCTTC
VIT_218S0041G01880.2 CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
su_wt                CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
CH1                  CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
                       ************************************** *******************

su_mut               TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCATTTT-----TT
su_Di Genova         TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAWTTT-----TT
CH2                  TCTCACAAATTACATGCATTATTACCCTTCATGTGTCACTCATACTTCAATTTTTTTATT
VIT_218S0041G01880.2 TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAATTTT----TT
su_wt                TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAATTTT----TT
CH1                  TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAATTTT----TT
                     ******************************** **************** ***     **

su_mut               TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
su_Di Genova         TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
CH2                  TTTTATTTTTAAAGCTTGATCAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
VIT_218S0041G01880.2 TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
su_wt                TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
CH1                  TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
                     **** ************** ****************************************

su_mut               CAGTTTCATAAAGAGATGCTTTGGTAATTTTTTCTAATATTTTCAGTTGGTGATATAATA
su_Di Genova         CAGTTTCATAAAGAGATGCTTTGGTAAWTTTTTCTAATATTTTCAGTTGGTGATATAATA
CH2                  CAGTTTCATAAAGAGATGCTTTGGTAATTTTTTCTAATATTTTCAGTTGGTGATATAATA
VIT_218S0041G01880.2 CAGTTTCATAAAGAGATGCTTTGGTAAATTTTTCTAATATTTTCAGTTGGTGATATAATA
su_wt                CAGTTTCATAAAGAGATGCTTTGGTAAATTTTTCTAATATTTTCAGTTGGTGATATAATA
CH1                  CAGTTTCATAAAGAGATGCTTTGGTAAATTTTTCTAATATTTTCAGTTGGTGATATAATA
                     *************************** ********************************

su_mut               AAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
su_Di Genova         WAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
CH2                  TAACAATGTATAGTAGTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
VIT_218S0041G01880.2 TAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
su_wt                TAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
CH1                  TAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
                     ************** ********************************************

su_mut               GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
su_Di Genova         GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
CH2                  GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
VIT_218S0041G01880.2 GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
su_wt                GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
CH1                  GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
                     ************************************************************

su_mut               CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
su_Di Genova         CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
CH2                  CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
VIT_218S0041G01880.2 CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
su_wt                CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
CH1                  CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
                     ************************************************************
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su_mut             GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
su_Di Genova       GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
CH2                GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
VIT_218S0041G01880.2 GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
su_wt              GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
CH1                GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
                   ************************************************************

su_mut             CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
su_Di Genova       CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
CH2                CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
VIT_218S0041G01880.2 CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
su_wt              CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
CH1                CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
                   ************************************************************

su_mut             ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
su_Di Genova       ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
CH2                ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACTGGCAATTAAT
VIT_218S0041G01880.2 ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
su_wt              ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
CH1                ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
                   ************************************************* **********

su_mut             GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
su_Di Genova       GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
CH2                GGTTTTATATTGATGAGATTAATATCATTAGAAGTTGCAGAACCCTAATGTTGTTAAAGC
VIT_218S0041G01880.2 GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
su_wt              GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
CH1                GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
                   **************************** *********************** *******

su_mut             AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
su_Di Genova       AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
CH2                AAAGAAAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
VIT_218S0041G01880.2 AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
su_wt              AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
CH1                AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
                   **** *******************************************************

su_mut             ACTGCAGATTATGATTTTTTACGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
su_Di Genova       ACTGCAGATTATGATTTTTTAYGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
CH2                ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
VIT_218S0041G01880.2 ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
su_wt              ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
CH1                ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
                   ********************* **************************************

su_mut             ATTACATGGGTACCTGCTTTGATATTTATGAT-ATTTTCTTTCATCAAAATTTAATTAAT
su_Di Genova       ATTACATGGGTACCTGCTTTGATATTTATGAT-ATTTTCTTTCATCAAAATTTAATTAAT
CH2                ATTACATGGGTACCTGCTTTGATATTTATGAT-ATTTTCTTTCATCAAAATTTAATTAAT
VIT_218S0041G01880.2 ATTACATGGGTACCTGCTTTGATATTTATGATAATTTTCTTTTATCAAAATTTA---AAT
su_wt              ATTACATGGGTACCTGCTTTGATATTTATGATAATTTTCTTTTATCAAAATTTA---AAT
CH1                ATTACATGGGTACCTGCTTTGATATTTATGATAATTTTCTTTTATCAAAATTTA---AAT
                   ******************************** ********* ***********   ***

su_mut             GAAGGAAAATGCAGATAATATTCTGACATTTCATCAGGCATCTGCTGATGAATCTGAGAA
su_Di Genova       GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
CH2                GAAGGAAAATGCAGATAATATTCTGACATTTCATCAGGCATCTGCTGATGAATCTGAGAA
VIT_218S0041G01880.2 GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
su_wt              GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
CH1                GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
                   *********************************** ************************

su_mut             AACAATGTCTTCTTCAAGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
su_Di Genova       AACAATGTCTTCTTCAAGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
CH2                AACAATGTCTTCTTCATGATTTTTCACTAATTGCATCTTTGACTGATATAAGGAAAATAT
VIT_218S0041G01880.2 AACAATTTCTTCTTCATGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
su_wt              AACAATTTCTTCTTCATGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
CH1                AACAATTTCTTCTTCATGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
                   ****** ********* *************** ***************************

su_mut             GAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
su_Di Genova       SAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
CH2                CAGAAAGAACACTTTACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
VIT_218S0041G01880.2 CAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
su_wt              CAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
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CH1                    CAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
                        ************ **********************************************

su_mut                 TTTTTATTGTATGTTTAGTGCATTAAAA-----AAAAATTATAATGAAAAAATAAAAATA
su_Di Genova           TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
CH2                    TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
VIT_218S0041G01880.2   TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
su_wt                  TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
CH1                    TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
                       ****************************     ***************************

su_mut                 AAATA-GCAAAAAAAACCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTACTGA
su_Di Genova           AAATAGGAAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
CH2                    AAATAGGAAAAAAAAACCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTACTGA
VIT_218S0041G01880.2   AAATAGGAAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
su_wt                  AAATAGGAAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
CH1                    AAATAGGAAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
                       ***** * ******** ************************************** ****

su_mut                 TATGCATGCAAGGGAAGACAATCTAGGCAACAGCCAGTTAGGGTTTCTTGTCCTAGCTAG
su_Di Genova           TATGCATGCAAGGGAAGACAATCTAGGCAACARCCAGTTAGGGTTTCTTGTCCTAGCTAG
CH2                    TATGCATGCAAGGGAAGACAATCTAGGCAACAGCCAGTTAGGGTTTCTTGTCCTAGCTAG
VIT_218S0041G01880.2   TATGCATGCAAGGGAAGACAATCTAGGCAACAACCAGTTAGGGTTTCTTGTCCTAGCTAG
su_wt                  TATGCATGCAAGGGAAGACAATCTAGGCAACAACCAGTTAGGGTTTCTTGTCCTAGCTAG
CH1                    TATGCATGCAAGGGAAGACAATCTAGGCAACAACCAGTTAGGGTTTCTTGTCCTAGCTAG
                       ******************************** ***************************

su_mut                 TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
su_Di Genova           TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
CH2                    TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
VIT_218S0041G01880.2   TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
su_wt                  TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
CH1                    TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
                       ************************************************************

su_mut                 ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
su_Di Genova           ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
CH2                    ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
VIT_218S0041G01880.2   ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
su_wt                  ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
CH1                    ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
                       ************************************************************

su_mut                 TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
su_Di Genova           TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
CH2                    TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
VIT_218S0041G01880.2   TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
su_wt                  TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
CH1                    TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
                       ************************************************************

su_mut                 GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
su_Di Genova           GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
CH2                    GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
VIT_218S0041G01880.2   GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
su_wt                  GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
CH1                    GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
                       ************************************************************

su_mut                 TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
su_Di Genova           TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
CH2                    TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
VIT_218S0041G01880.2   TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
su_wt                  TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
CH1                    TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
                       ************************************************************

su_mut                 CCCATTCTCATTTAAAGTGCAGTAAAGTGATCACCTTTCACTGCTCTTTGTTTTTTTCAC
su_Di Genova           CCCATTCTCATTTAAAGTGCAGTAAAGTGATCASCTTTCACTGCTCTTTGTTTTTTTCAC
CH2                    CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
VIT_218S0041G01880.2   CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
su_wt                  CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
CH1                    CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
                       ********************************* **************************

su_mut                 TTGGTGTGGGCAATTTGTGGATATCAAA--TCAGAAATGGGACGGTGTACATCAGCACCC
su_Di Genova           TTGGTGTGGGCAATTTGTGGATATCAAA--TCAGAAATGGGACGGTGTACATCAGCACCC
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CH2                  TTGGTGTGGGCAATTTGTGGATATCAAA--TCAGAAATGGGACGGTGTACATCAGCACCC
VIT_218S0041G01880.2 TTGGTGTGGGCAATTTGTGGATATCAAATCTCAGAAATGGGACGGTGTACATCAGCACCC
su_wt                TTGGTGTGGGCAATTTGTGGATATCAAATCTCAGAAATGGGACGGTGTACATCAGCACCC
CH1                  TTGGTGTGGGCAATTTGTGGATATCAAATCTCAGAAATGGGACGGTGTACATCAGCACCC
                     ****************************  ******************************

su_mut               ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
su_Di Genova         ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGMTCAA
CH2                  ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
VIT_218S0041G01880.2 ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
su_wt                ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
CH1                  ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
                     ******************************************************* ****

su_mut               TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCCCGATTTCTCCCTT
su_Di Genova         TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCCCGATTTCTCCCTT
CH2                  TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
VIT_218S0041G01880.2 TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
su_wt                TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
CH1                  TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
                     **********************************************  ************

su_mut               GTTCAAGGAAAATTGAAGATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
su_Di Genova         GTTCAAGGAAAATTGAAGATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
CH2                  GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
VIT_218S0041G01880.2 GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATCGAT
su_wt                GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
CH1                  GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
                     ***************** ************************************** ***

su_mut               CTTACATGAGATTTTACTTTTTTGTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
su_Di Genova         CTTACATGAGATTTTACTTTTTTGTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
CH2                  CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
VIT_218S0041G01880.2 CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
su_wt                CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
CH1                  CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
                     ***************** ***** ************************************

su_mut               AAATACTTGTTTTTACCCTTAATCAATTAACAAACAAAAAAATAAAATGAAAAAAAAGAC
su_Di Genova         AAATACTTGTTTTTACCCTTAATCAATTAACAAACAAAAAAATAAAATGAAAAAAAAGAC
CH2                  AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAAATGA-AAAATG-AAAAAAAGAC
VIT_218S0041G01880.2 AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAAATGA-AAAATG-AAAAAAAGAC
su_wt                AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAAATGA-AAAATG-AAAAAAAGAC
CH1                  AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAAATGA-AAAATG-AAAAAAAGAC
                     ********************************** ****  * ****** **********

su_mut               CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------------TGGGGGTGGGGGGG
su_Di Genova         CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------------TGGGGGTGGGGGGG
CH2                  CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------TGGGGGTGGGGGTGGAGGGG
VIT_218S0041G01880.2 CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCATGGGGGTGGGGGTGGGGGTGGAGGGG
su_wt                CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------TGGGGGTGGGGGTGGAGGGG
CH1                  CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------TGGGGGTGGGGGTGGAGGGG
                     **********************************            ********* ****

su_mut               AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTTTA
su_Di Genova         AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTWTA
CH2                  AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
VIT_218S0041G01880.2 AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
su_wt                AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
CH1                  AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
                     ********************************************************* **

su_mut               TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
su_Di Genova         TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
CH2                  TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
VIT_218S0041G01880.2 TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
su_wt                TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
CH1                  TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
                     ************************************************************

su_mut               TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
su_Di Genova         TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
CH2                  TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
VIT_218S0041G01880.2 TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
su_wt                TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
CH1                  TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
                     ************************************************************
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su_mut               ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
su_Di Genova         ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
CH2                  ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
VIT_218S0041G01880.2 ATATATGAGAGAAAAATAGAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
su_wt                ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
CH1                  ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
                     ****************** *****************************************

su_mut               TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCATTTTTTTTTTGTTTCTTTTTTTTC
su_Di Genova         TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCATTTTTTTTTTGTTTCTTTTTTTTC
CH2                  TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
VIT_218S0041G01880.2 TCAAGCTTTTTTTATTTATTTTTTA-CTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
su_wt                TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
CH1                  TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
                     *************************  ********* ***********************

su_mut               TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
su_Di Genova         TCTCTCTTAAATTTTYGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
CH2                  TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
VIT_218S0041G01880.2 TCTCTCTTAAATTTTCGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
su_wt                TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
CH1                  TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
                     *************** ********************************************

su_mut               GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
su_Di Genova         GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
CH2                  GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
VIT_218S0041G01880.2 GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
su_wt                GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
CH1                  GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
                     ************************************************************

su_mut               AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
su_Di Genova         AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
CH2                  AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
VIT_218S0041G01880.2 AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
su_wt                AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
CH1                  AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
                     ************************************************************

su_mut               TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
su_Di Genova         TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
CH2                  TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
VIT_218S0041G01880.2 TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
su_wt                TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
CH1                  TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
                     ************************************************************

su_mut               TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
su_Di Genova         TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACY
CH2                  TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
VIT_218S0041G01880.2 TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACT
su_wt                TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
CH1                  TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
                     *********************************************************** 

su_mut               GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
su_Di Genova         GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
CH2                  GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
VIT_218S0041G01880.2 GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
su_wt                GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
CH1                  GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
                     ************************************************************

su_mut               AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
su_Di Genova         AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
CH2                  AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
VIT_218S0041G01880.2 AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
su_wt                AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
CH1                  AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
                     ************************************************************

su_mut               TTAAATAAACTCTAAGATGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
su_Di Genova         TTAAATAAACTCTAAGATGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
CH2                  TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
VIT_218S0041G01880.2 TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
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su_wt                TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
CH1                  TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
                     ***************** ******************************************

su_mut               ACATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATTATCCCT
su_Di Genova         GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
CH2                  GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
VIT_218S0041G01880.2 GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
su_wt                GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
CH1                  GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
                      **************************************************** ******

su_mut               TTCTACAGAACTTGTCGATTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
su_Di Genova         TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
CH2                  TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
VIT_218S0041G01880.2 TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
su_wt                TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
CH1                  TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
                     *** ** ********** ******************************************

su_mut               ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
su_Di Genova         ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
CH2                  ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
VIT_218S0041G01880.2 ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
su_wt                ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
CH1                  ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
                     ************************************************************

su_mut               AACCTTTGAAATTAATAAATGGTGGGATATGTG--AGAGGTGCAAAGTCTTGGGTTTGAT
su_Di Genova         AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTTGAT
CH2                  AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
VIT_218S0041G01880.2 AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
su_wt                AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
CH1                  AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
                     *********************************  *********************  **

su_mut               TACCATCATTAAGAATCCCTGGATTACTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
su_Di Genova         TACCATCATTAAGAATCCCTGGATTACTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
CH2                  TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
VIT_218S0041G01880.2 TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
su_wt                TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
CH1                  TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
                     ************************* **********************************

su_mut               CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGGT
su_Di Genova         CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGGT
CH2                  CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGGT
VIT_218S0041G01880.2 CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGTT
su_wt                CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGTT
CH1                  CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGTT
                     ********************************************************** *

su_mut               TATCAAAAAAAAAAACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAATTGAG
su_Di Genova         TATCAAAAAAAAAAACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAKTGAG
CH2                  TAT----AAAAAACACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
VIT_218S0041G01880.2 TAT--AAAAAACACACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
su_wt                TAT------AAAAAACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
CH1                  TAT--AAAAAACACACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
                     ***      ** * ***************************************** ****

su_mut               AATAAGGTAGAGAAGATGTTTTTTCC------TAGGTACTCTAGGGTTTTGTGATAATAG
su_Di Genova         AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
CH2                  AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
VIT_218S0041G01880.2 AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
su_wt                AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
CH1                  AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
                     **************************      ****************************

su_mut               ATTTGGATTTTTTTCCCCCAATAATTCAAATTATTAATTCAATTATAATAAATATAAAAA
su_Di Genova         ATTTGGATTTTTTTCCCCCAATAATTCAAATTATTAATTCAATTATAATAAATATAAAAA
CH2                  ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
VIT_218S0041G01880.2 ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
su_wt                ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
CH1                  ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
                     ************** *********** *********************************

su_mut               TCAATGAGAAATTCCATGGAAAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
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su_Di Genova          TCAATGAGAAATTCCATGGAAAACCCAAATCAAATGTTGCAATATAGWTCAAGATTAGCA
CH2                   TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
VIT_218S0041G01880.2  TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
su_wt                 TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGTTCAAGATTAGCA
CH1                   TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
                      ******************* *************************** ************

su_mut                TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
su_Di Genova          TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
CH2                   TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
VIT_218S0041G01880.2  TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
su_wt                 TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
CH1                   TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
                      ************************************************************

su_mut                CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACTCCTGCATCTAGTAATTAGA
su_Di Genova          CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACWCCTGCATCTAGTAATTAGA
CH2                   CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
VIT_218S0041G01880.2  CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
su_wt                 CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
CH1                   CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
                      **************************************** *******************

su_mut                GATGAAACAGACATTATTTGTATTATGTAATCTATTTTACGAACAAAATTTTAAAAATGC
su_Di Genova          GATGAAACAGACATTATTTGTATTATGTAATCTATTTTACGAACAAAATTTTAAAAATGC
CH2                   GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
VIT_218S0041G01880.2  GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
su_wt                 GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
CH1                   GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
                      ********* ***************************** ********************

su_mut                TTTAATAAGTAGTGAGGATGACTATGATATTTCAAAAAAAATTATTAATTAAAAAAATTT
su_Di Genova          TTTAATAAATAGTGAGGATGACTATGATATTTCAAAAAAAATTATTAATTAAAAAAATTT
CH2                   TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
VIT_218S0041G01880.2  TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
su_wt                 TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
CH1                   TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
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su_mut                ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTACATGTCCTTAAAATCACTT
su_Di Genova          ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
CH2                   ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
VIT_218S0041G01880.2  ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
su_wt                 ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
CH1                   ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
                      ***************************************** *** **************

su_mut                ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATTAAAAGAAAA-----AAAAATT
su_Di Genova          ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAATAAAATTT
CH2                   ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAATAAAATTT
VIT_218S0041G01880.2  ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAATAAAATTT
su_wt                 ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAATAAAATTT
CH1                   ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAATAAAATTT
                      ************************************** *********     **** **

su_mut                GTACAAACCATCCAGTTTAGTACTGGTCCGAACCCCCTTCTAGCTCAAAAGGTCAATAGG
su_Di Genova          GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
CH2                   GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
VIT_218S0041G01880.2  GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
su_wt                 GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
CH1                   GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
                      ******* ******************* ************ **************** **

su_mut                ATTGGACTAGGATCCGGTCAATAGTTCGATTGATTAAATCGATCAATT-GGTCCGATGTT
su_Di Genova          ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
CH2                   ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
VIT_218S0041G01880.2  ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
su_wt                 ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
CH1                   ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
                      ************** ****** *************     ******** ******** **

su_mut                TAAAACATTACAAATAACTTATCTTATCTGAGTTTTGATGCATCTCATACATGCAAAGTA
su_Di Genova          TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATACATGCAAAGCA
CH2                   TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATTCATGCAAAGCA
VIT_218S0041G01880.2  TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATACATGCAAAGCA
su_wt                 TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATACATGCAAAGCA
CH1                   TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATTCATGCAAAGCA
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su_mut                     GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
su_Di Genova               GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
CH2                        GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
VIT_218S0041G01880.2       GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
su_wt                      GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
CH1                        GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
                           ************************************************************

su_mut                     ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
su_Di Genova               ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
CH2                        ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
VIT_218S0041G01880.2       ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
su_wt                      ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
CH1                        ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
                           ************************************************************

su_mut                     ATACAGTTGCAGAGTGGCAGTGCTCTCCATTGTTCTTTCTCTTGCTTTTCAGTGGTGCAA
su_Di Genova               ATACAGTTGCAGAGTGGCAGTGCTCTYCATTGTTCTTTCTCTTGCTTTTCAGTGGTGCAA
CH2                        ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
VIT_218S0041G01880.2       ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
su_wt                      ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
CH1                        ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
                           ************************** ****************************** **

su_mut                     TCCATGAGAATCTGAACCCTCTGGCAGTGTCTGAAAAAGGGTGGTCATAGCAGTACAACC
su_Di Genova               TCCATGAGAATCTGAACCCTCTGGCAGTGTCTGAAAAAGGGTGGTCATAGCAGTACAACC
CH2                        TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
VIT_218S0041G01880.2       TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
su_wt                      TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
CH1                        TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
                           ****************** ********************** ******************

su_mut                     AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
su_Di Genova               AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
CH2                        AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
VIT_218S0041G01880.2       AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
su_wt                      AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
CH1                        AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
                           ************************************************************

su_mut                     GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
su_Di Genova               GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
CH2                        GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
VIT_218S0041G01880.2       GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
su_wt                      GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
CH1                        GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
                           ************************************************************

su_mut                     CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGTAGAAACCAAGA
su_Di Genova               CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGTAGAAACCAAGA
CH2                        CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
VIT_218S0041G01880.2       CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
su_wt                      CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
CH1                        CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
                           ************************************************ ******** **

su_mut                     CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
su_Di Genova               CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
CH2                        CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
VIT_218S0041G01880.2       CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
su_wt                      CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
CH1                        CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
                           ************************************************************


su_mut                     TAGGGTCACTGTGCATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
su_Di Genova               TAGGGTCACTGTGYATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
CH2                        TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
VIT_218S0041G01880.2       TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
su_wt                      TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
CH1                        TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
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su_mut              GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
su_Di Genova        GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
CH2                 GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
VIT_218S0041G01880.2 GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
su_wt               GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
CH1                 GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
                    ************************************************************

su_mut              CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
su_Di Genova        CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
CH2                 CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
VIT_218S0041G01880.2 CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
su_wt               CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
CH1                 CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
                    ************************************************************

su_mut              TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATCATATGTAATATTTCTATCCAT
su_Di Genova        TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATCATATGTAATATTTCTATCCAT
CH2                 TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
VIT_218S0041G01880.2 TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
su_wt               TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
CH1                 TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
                    **************************************  ************* ******

su_mut              TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
su_Di Genova        TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
CH2                 TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
VIT_218S0041G01880.2 TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
su_wt               TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
CH1                 TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
                    ************************************************************

su_mut              CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
su_Di Genova        CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
CH2                 CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
VIT_218S0041G01880.2 CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
su_wt               CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
CH1                 CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
                    ************************************************************

su_mut              TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
su_Di Genova        TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
CH2                 TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
VIT_218S0041G01880.2 TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
su_wt               TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
CH1                 TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
                    ************************************************************

su_mut              TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
su_Di Genova        TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
CH2                 TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
VIT_218S0041G01880.2 TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
su_wt               TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
CH1                 TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
                    ************************************************************

su_mut              GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
su_Di Genova        GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
CH2                 GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
VIT_218S0041G01880.2 GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
su_wt               GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
CH1                 GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
                    ************************************************************

su_mut              TTAGCCATAAACAAATTTGTTAAAATCATTATTCTACTTTCTTTTTTCTTTTTGGATTAT
su_Di Genova        TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
CH2                 TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
VIT_218S0041G01880.2 TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
su_wt               TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
CH1                 TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
                    **************** *************** ***************************

su_mut              CCCATTGAACTTTCTCGAATTCTGTCGTTGAACATAGTATTACCAGCAAGAATCAGCAAA
su_Di Genova        CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
CH2                 CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
VIT_218S0041G01880.2 CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
su_wt               CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
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CH1                    CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
                       ************************* ************* ********************

su_mut                 GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
su_Di Genova           GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
CH2                    GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
VIT_218S0041G01880.2 GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
su_wt                  GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
CH1                    GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
                       ************************************************************

su_mut                 TTTTTCTCACATGTAATTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
su_Di Genova           TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
CH2                    TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
VIT_218S0041G01880.2 TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
su_wt                  TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
CH1                    TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
                       ************* * ********************************************

su_mut                 TGTCTTTAACTAGGTTTTTGGCATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
su_Di Genova           TGTCTTTAACTAGGTTTTTGRCATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
CH2                    TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACCTAACG
VIT_218S0041G01880.2 TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
su_wt                  TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
CH1                    TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
                       ******************** ********************************* *****

su_mut                 ATCTTAATTAGGTATGTTAAGACTTAAGAGCAGGTTATACTAAGACATTTTGTCTGTGTT
su_Di Genova           ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
CH2                    ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
VIT_218S0041G01880.2 ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
su_wt                  ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
CH1                    ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
                       *********** ***************** ****** ***********************

su_mut                 TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
su_Di Genova           TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
CH2                    TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
VIT_218S0041G01880.2 TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
su_wt                  TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
CH1                    TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
                       ************************************************************

su_mut                 GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACAC--ATA
su_Di Genova           GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACAC--ATA
CH2                    GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
VIT_218S0041G01880.2 GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
su_wt                  GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
CH1                    GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
                       *******************************************************  * *

su_mut                 CACTAATTCTAATCTGGCTGGGCATTTGACTTTGTGACTTAAATGAAATTGAAAAAAAAA
su_Di Genova           CACTAATTCTAATCTGGCTGGGCATTTGACTTTGTGACTTAAATGAAATTGAAAAAAAAA
CH2                    CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
VIT_218S0041G01880.2 CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
su_wt                  CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
CH1                    CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
                       ****** ************* ******** **** ************* ** ********

su_mut                 ATGGTATCATTCACTGATTA---AATGATACATGAAGATTGAATTAATTTACGATACTGC
su_Di Genova           ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
CH2                    ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
VIT_218S0041G01880.2 ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
su_wt                  ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
CH1                    ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
                       ********************   *** **** ***** ************* ***** **

su_mut                 TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
su_Di Genova           TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
CH2                    TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
VIT_218S0041G01880.2 TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
su_wt                  TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
CH1                    TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
                       ************************************************************

su_mut                 TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
su_Di Genova           TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
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CH2                  TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
VIT_218S0041G01880.2 TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
su_wt                TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
CH1                  TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
                     ************************************************************

su_mut               AAAGCCTATCTTTTTTCCTTTGTTCTAATGTTGTGCTTGTTCTAATGCAATAGCATGAGT
su_Di Genova         AAAGCCTATCTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
CH2                  AAAGCCTATCTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
VIT_218S0041G01880.2 AAAGCCTATCTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
su_wt                AAAGCCTATCTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
CH1                  AAAGCCTATCTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
                     ******************************* *********** ****************

su_mut               TGCTGTTGGCTGAGATTGAATACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
su_Di Genova         TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
CH2                  TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
VIT_218S0041G01880.2 TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
su_wt                TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
CH1                  TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
                     ******************* ****************************************

su_mut               TTCCATGTTGGTTCCCTTCTTTTATGATTTTACCAGAATTCTTTATCGAGCATGTGTTAA
su_Di Genova         TTCCATGTKGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
CH2                  TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
VIT_218S0041G01880.2 TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
su_wt                TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
CH1                  TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
                     ******** ********************* *********************  * ****

su_mut               TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
su_Di Genova         TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
CH2                  TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
VIT_218S0041G01880.2 TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
su_wt                TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
CH1                  TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
                     ************************************************************

su_mut               AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
su_Di Genova         AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
CH2                  AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
VIT_218S0041G01880.2 AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
su_wt                AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
CH1                  AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
                     ************************************************************

su_mut               CAGTTCAGTTGAACTTGTTAGTGTATATATTCCACAATTGATTGAGTTATCATTACTTAA
su_Di Genova         CAGTTCAGTTGAACTTGTTAGTGTATATATTCCACAATTGATTGAGTTATCATTACTTAA
CH2                  CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
VIT_218S0041G01880.2 CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
su_wt                CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
CH1                  CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
                     *******************************  ************* *************

su_mut               TTTTTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
su_Di Genova         TTTTTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
CH2                  TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
VIT_218S0041G01880.2 TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
su_wt                TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
CH1                  TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
                     ** *********************************************************

su_mut               CATTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTGTCAACAC
su_Di Genova         CAYTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
CH2                  CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
VIT_218S0041G01880.2 CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
su_wt                CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
CH1                  CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
                     ** ************************************************* *******

su_mut               ATGAGTTTAATGCCATCCAGGCATTAGTTTCTCTCAATTTCTTTCAGCCCAATATGATTG
su_Di Genova         ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCTCAATTTCTTTCAGCCCAATATGATTG
CH2                  ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
VIT_218S0041G01880.2 ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
su_wt                ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
CH1                  ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
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su_mut               AAGGTGGATCCGCAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
su_Di Genova         AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
CH2                  AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
VIT_218S0041G01880.2 AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
su_wt                AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
CH1                  AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
                     * ********* ************************************************

su_mut               ATCTTCCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
su_Di Genova         ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
CH2                  ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
VIT_218S0041G01880.2 ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
su_wt                ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
CH1                  ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
                     ***** ******************************************************

su_mut               GCACATAAATCTCCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
su_Di Genova         GCACATAAATCWCCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
CH2                  GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
VIT_218S0041G01880.2 GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
su_wt                GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
CH1                  GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
                     *********** ************************************************

su_mut               ATGAATAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
su_Di Genova         ATGAAYAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
CH2                  ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
VIT_218S0041G01880.2 ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
su_wt                ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
CH1                  ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
                     ***** ******************************************************

su_mut               CTTCGTCTTTTAGAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
su_Di Genova         CTTCGTCTTTTARAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
CH2                  CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
VIT_218S0041G01880.2 CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
su_wt                CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
CH1                  CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
                     ************ ***********************************************

su_mut               TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTAT
su_Di Genova         TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTAT
CH2                  TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
VIT_218S0041G01880.2 TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
su_wt                TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
CH1                  TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
                     ********************************************************** *

su_mut               GATGTGTATGACGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
su_Di Genova         GATGTGTATGACGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
CH2                  GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
VIT_218S0041G01880.2 GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
su_wt                GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
CH1                  GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
                     *********** ************************************************

su_mut               TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
su_Di Genova         TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
CH2                  TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
VIT_218S0041G01880.2 TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
su_wt                TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
CH1                  TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
                     ************************************************************

su_mut               GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
su_Di Genova         GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
CH2                  GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
VIT_218S0041G01880.2 GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
su_wt                GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
CH1                  GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
                     ************************************************************

su_mut               GGAGGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
su_Di Genova         GGRAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
CH2                  GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
VIT_218S0041G01880.2 GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
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su_wt                  GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
CH1                    GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
                       ** ********************************************************

su_mut                 CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
su_Di Genova           CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
CH2                    CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
VIT_218S0041G01880.2   CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
su_wt                  CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
CH1                    CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
                       ************************************************************

su_mut                 CTTGGTATTTATCAGCTAGCAACCAGTACTGTTTTGTATAACATTATGACATATAATATA
su_Di Genova           CTTGGTATTTATCMGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
CH2                    CTTGATATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
VIT_218S0041G01880.2   CTTGGTATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
su_wt                  CTTGGTATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
CH1                    CTTGGTATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
                       **** ******** *********************************** **********

su_mut                 GCCTATTAAAAT
su_Di Genova           GCCTATTAAAAT
CH2                    GCCTATTAAAAT
VIT_218S0041G01880.2   GCCTATTAAAATATACAATATTCCTAATCT
su_wt                  GCCTATTAAAAT
CH1                    GCCTATTAAAATATACAATATTCCTAATC
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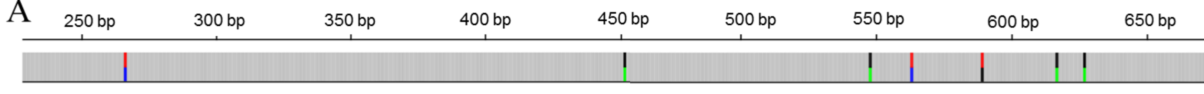
Supplementary Fig. 1. Multiple sequence alignment of *VviAGL11* alleles. The sequences presented in the alignment are *VviAGL11* from PN40024 (VIT_218S0041G01880.2), *VviAGL11* 'Chardonnay allele 1' (*CH1*), *VviAGL11* 'Chardonnay allele 2' (*CH2*), *VviAGL11* 'Sultanine' mutant allele (*SUmut*), *VviAGL11* 'Sultanine wt allele' (SU *wt*) and the sequence from 'Sultanine' reported by Di Genova *et al.* (2014). Underlined sequences indicate the SSR marker VMC7F2 position, UTRs are indicated in blue, exons are indicated in red, putative CArG-Box sequences are indicated in green, SNPs in the coding region are highlighted in grey and the black arrow indicates the CArG-Box mutation. Y equals pyrimidine (C or T), R equals purine (A or G) and W equals A or T bases.

A



B CLUSTAL multiple sequence alignment by MUSCLE (3.8)

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su_mut          MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
CH1             MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
CH2             MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
su_wt           MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
VIT             MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
su_Di           MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRVYEYSNN
                ************************************************************

su_mut          NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
CH1             NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
CH2             NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
su_wt           NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
VIT             NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
su_Di           NIKSTIDRYKKASSDSTNGGSTMEINAQYYQQESAKLRQQIQMLQNSNRHLMGDSLASLT
                ************************************************************

su_mut          VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
CH1             VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
CH2             VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
su_wt           VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
VIT             VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
su_Di           VKELKQLENRLERGITRIRSKKHELLLAEIEYLQKREIELENESVYLRTKIAEVERLQQA
                ************************************************************

su_mut          NMVSTHEFNAIQALVSLNFFQPNMIEGGSAGYPLPDKKVLHLG
CH1             NMVSTHEFNAIQALVSRNFFQPNMIEGGSTGYPLPDKKVLHLG
CH2             NMVSTHEFNAIQALVSRNFFQPNMIEGGSTGYPLPDKKVLHLG
su_wt           NMVSTHEFNAIQALVSRNFFQPNMIEGGSTGYPLPDKKVLHLG
VIT             NMVSTHEFNAIQALVSRNFFQPNMIEGGSTGYPLPDKKVLHLG
su_Di           NMVSTHEFNAIQALVSINFFQPNMIEGGSTGYPLPDKKVLHLG
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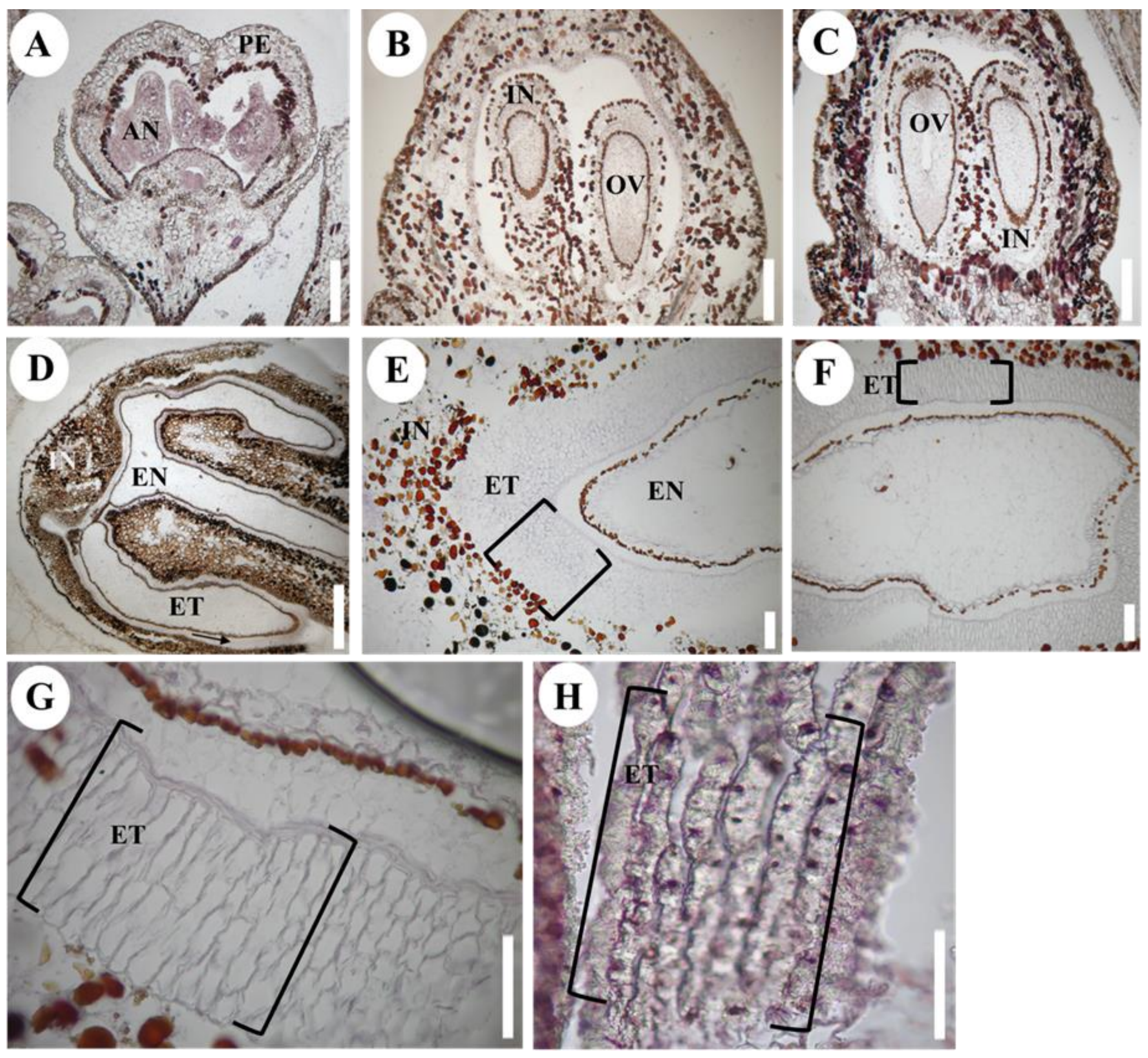
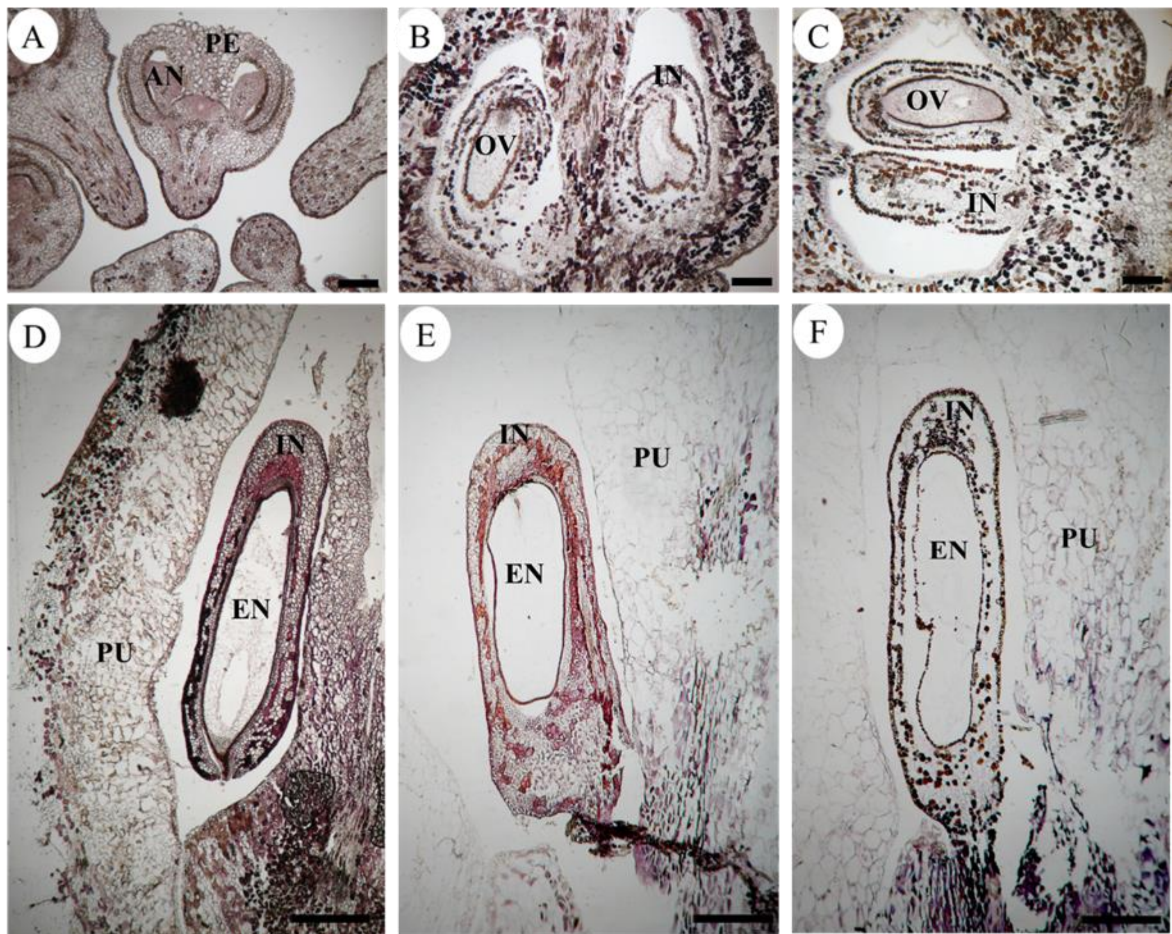
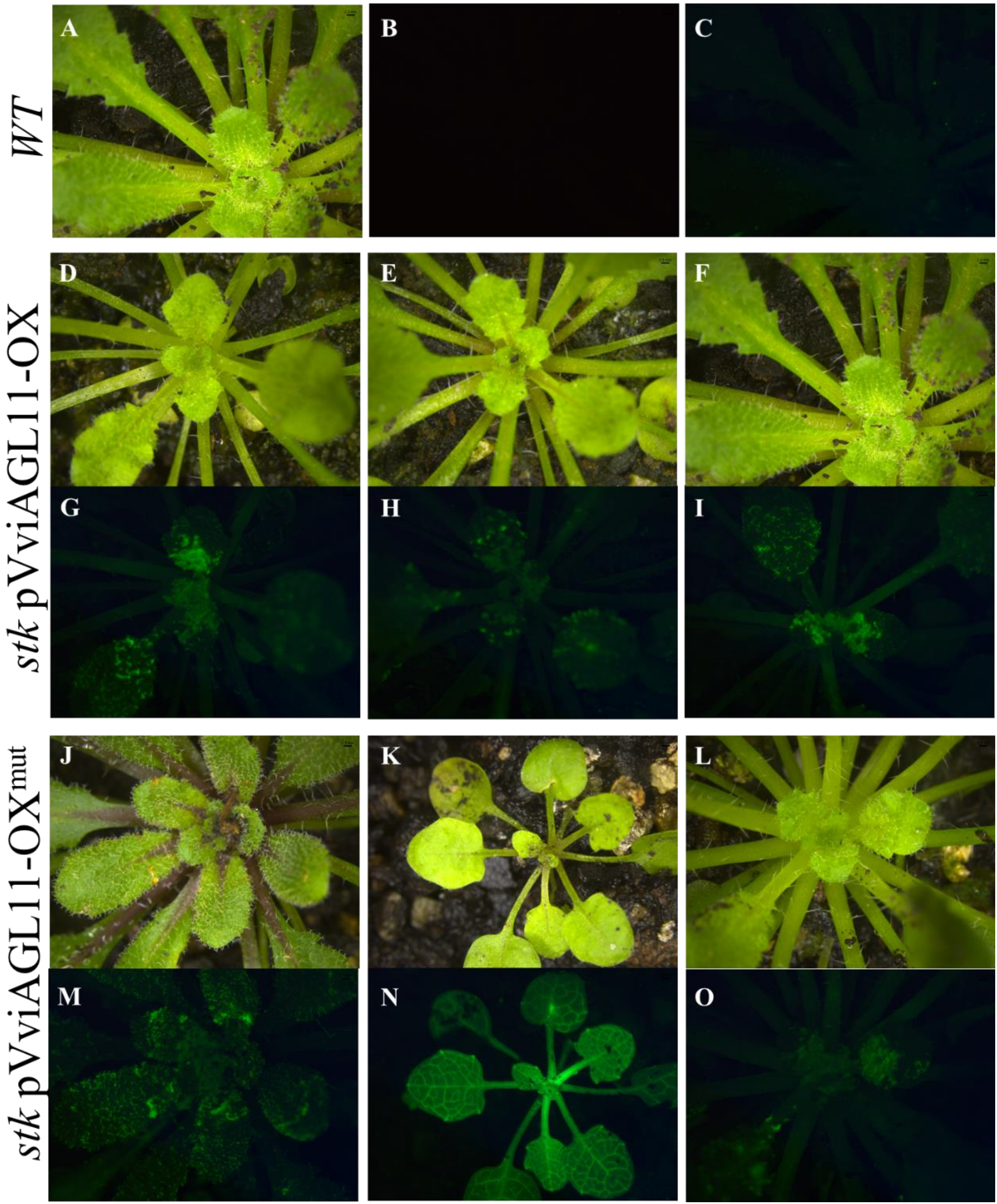
Supplementary Fig. 2. Alignment of thesequences segments from *VviAGL11* alleles. **(A)** Alignments of the alleles from grapevine genome (VIT-PN40024), ‘Chardonnay’ (both *VviAGL11* alleles CH1 and CH2), ‘Sultanine’ wild-type like and mutant alleles (su_wt and su_mut) and ‘Sultanine’ sequence reported by Di Genova *et al.* (2014) (su_Di). Colors stripes represent SNPs (A-green, C-blue, T-red, G-black). **(B)** Protein sequence alignment showing the two amino acid modifications marked in red.



Supplementary Fig. 3. *In situ* hybridization of *VviAGL11* sense probe (control) in 'Chardonnay'. H1 flower stage **(A)**, pre-anthesis flower stage **(B)**, fruit set flower stage **(C)**, 2 weeks **(D)**, 4 weeks **(E)** and 6 weeks **(F)** of fruit development. Anther (an), endosperm (en), endotesta (et), integuments (it), ovule (ov), petal (pe), pulp (pu). Scale bar: 200 µm.



Supplementary Fig. 4. *In situ* hybridization of *VviAGL11* sense probe (control) in 'Sultanine'. H1 flower stage **(A)**, pre-anthesis flower stage **(B)**, fruit set flower stage **(C)**, 2 weeks **(D)**, 4 weeks **(E)** and 6 weeks **(F)** of fruit development. Anther (an), endosperm (en), endotesta (et), integuments (it), ovule (ov), petal (pe), pulp (pu). Scale bar: 200 µm.



Supplementary Fig. 5. Plants of *Arabidopsis thaliana* under GFP evaluation. WT plant (bright field (A); GFP filter for 1 second of exposition (B); GFP filter for 3 seconds of exposition (C)). *stk* plants transformed with pVviAGL11-OX from 'Chardonnay' (bright field (D-F); GFP filter for 1 second of exposition (G-I)). *stk* plants transformed with pVviAGL11-OX^{mut} from 'Sultanine' (bright field (J-L); GFP filter for 1 second of exposition (M-O)).

Supplementary Table 1. Primers designed for the amplification and sequencing of *VviAGL11.*

VviAGL11- PF1	CCA CTG ATA TGG ATT GAT TTG CC
VviAGL11- PR1	GGA CAG TGG AAT ACA GAT TC
VviAGL11- PF2	TAG GAA GGG ATT ACA TGG G
VviAGL11- PR2	GAT GGC ATT GAG CAA ACA
VviAGL11- PF3	CCC ATT ACA TCT TTG TGT GG
VviAGL11- PR3	AAA CCC CAC TGT GAT AGG
VviAGL11- PF4	GGA TGG TGT CAT AAT GAT AGG
VviAGL11- PR4	GAG TTT GTA TGG AGA ATA GCA G
VviAGL11- PF5	GCA AAG CAG TTA TTG AAA GC
VviAGL11- PF6	TGC CCA AGT AAG AAA ACT TC
VviAGL11- PR5	GGC GGA TAT TTT CTC CCA TC
VviAGL11- PR6	TGA TTA TCA ACA CCA AAC ATG GC
SEQ_AGL11_R_ 1	CAT CAG CAG ATG CCA GAT
SEQ_AGL11_F_ 1	CAA TAA ACC TAA CAG TGT AGT GAA C
SEQ_AGL11_F_ 2	CTA GGC AAC AAC CAG TTA G
SEQ_AGL11_R_ 2	ATA AGG AAG ACT TCT CCC C
SEQ_AGL11_R_ 3	CTT TTT CTC CTA CTT TTC CTC
SEQ_AGL11_F_3	CGA TCT TAC ATG AGA TTT TAC C
SEQ_AGL11_R_ 4	TTG GAG CAT CGT CTT AGA G
SEQ_AGL11_F_ 4	GAGGAAAAGTAGGAGAAAAAG
SEQ_AGL11_R_ 5	GAA ATA TCA TAG TCA TCC TCA C
SEQ_AGL11_F_ 5	TAA GGT GTC GAA GCC ATG
SEQ_AGL11_R_ 6	TGA GTT TCT TTG TGT GGA C
SEQ_AGL11_F_ 6	GAG AGC CTA TTT GGG ATA AC
SEQ_AGL11_R_ 7	GGA TTT GGA TTG ATT CAT TAA C
SEQ_AGL11_F_ 7	AGG AGA AAC CAG GAC AAG
SEQ_AGL11_R_ 8	CAA ATG CTC AGC CAG ATT AG
SEQ_AGL11_R_ 8	CGG TAT CAA CTG TTG TGC
SEQ_AGL11_F_ 9	GGT AAC GAC ACC TAG ACA C
SEQ_AGL11_R_ 9	GCT TGA AAT GTC TCA TTA AGC
SEQ_AGL11_F_ 10	CGT ATA TCT CCG AAC CAA G
SEQ_AGL11_F_ 11	CTC GGG TAC ACT CAT CTT TC
SEQ_AGL11_F_ 12	TTC AAG TTC GAC CTA ATT CCT GG
SEQPromo_AGL11_R_ 1	GAT CTC GAT CTT TCC TCT CCC
SEQPromo_AGL11_F_ 1	CCT CAC CCT ATT TTT CGA ACA AC
SEQPromo_AGL11_R_ 2	GAG ATC TGA ATG ATG ATA GAC TGA G
SEQPromo_AGL11_F_ 2	CTT GAG GGG AAA AAG CCA G
SEQPromo_AGL11_R_ 3	CAT AAA CCC AGG ATA TGG ATG AG
SEQPromo_AGL11_F_ 3	TTG CCT ACA TAT GAA CAT CTG C
SEQPromo_AGL11_R_ 4	ATT TGG AGA GAT GGG CAC TG
SEQPromo_AGL11_F_ 4	AAA GTC ACT TTC TAC ATC TGC AC
SEQPromo_AGL11_R_ 5	GGA GAG ATG TGA GAC GGA TAT G
SEQPromo_AGL11_F_ 5	AGA AGA AAG TTT GCA GTT TGT G
SEQPromo_AGL11_R_ 6	CAA AGT CGG ATA CAA GAC ATA GTA G

Supplementary Table 2. Stages of development of grapevine reproductive organs sampled for the evaluation of *VviAGL11* transcriptional profile (except H1) and for the *in situ* hybridization experiment. Dates of harvesting, the phenological stage according to Baggiolini (1952), grapevine cultivars and a representative picture of each sample are represented.

Dates	Phenological stage (Baggiolini, 1952)	Sample	Cultivar
09/11/12	**H1**	Flowers at inicial development	Chardonnay
08/30/12			Sultanina
10/08/12	**Pre anthesis**	Flowers before anthesis	Chardonnay
10/08/12			Sultanina
10/22/12	**Fruit-set**	Flowers in anthesis	Chardonnay
10/08/12			Sultanina
11/05/12	**2 weeks of development**	Fruits (Pulp and seeds)	Chardonnay
11/22/12			Sultanina
11/19/12	**4 weeks of development**	Fruits (Pulp and seeds)	Chardonnay
11/05/12			Sultanina
12/03/12	**6 weeks of development**	Fruits (Pulp and seeds)	Chardonnay
11/19/12			Sultanina
01/02/13	**Maturation**	Fruits (Pulp and seeds)	Chardonnay
12/17/12			Sultanina

4 CAPÍTULO II

Manipulation of *VviAGL11* expression changes the seed content in grapevine (*Vitis vinifera* L.)

Artigo científico publicado no periódico ‘Plant Science’ (FI: 3,43)

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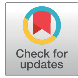
Manipulation of *VviAGL11* expression changes the seed content in grapevine (*Vitis vinifera* L.)



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ABSTRACT

Seedlessness in grapes is a desirable trait, especially for *in natura* consumption. Previously, we showed that *VviAGL11* is the main responsible gene for seed morphogenesis in grapevine. Here we tested the function of this gene in grapevine with the use of plant plasmids. *VviAGL11* was cloned into silencing and overexpression versions of p28iIR plasmid. Reproductive grapevine bunches from different seeded and seedless cultivars were separately treated with *VviAGL11*-harboring plasmids, along with controls. Plasmids were detected in leaves after a month of treatment, and berries, leaves, stems and seeds were analyzed for ectopic gene expression by RT-qPCR after 90 days of plasmid injection. Fruits from the seedless 'Linda' treated with the VviAGL11-overexpression plasmid showed high expression levels of *VviAGL11* and exhibited small seeds that were not found in the untreated control samples. Mature grapes from seeded 'Italia' and 'Ruby' bunches treated with the VviAGL11-silencing plasmid showed decreased *VviAGL11* expression, reduced number of seeds and increased number of seed traces. The present study confirms that *VviAGL11* is a key master regulator of seed morphogenesis in grapevine and corroborates with the applicability of plant plasmids as promising biotechnological tools to functionally test genes in perennial plants in a rapid and confident way.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the major and oldest cultivated plant species worldwide [1], covering an area of 7.5 million hectares [2–4]. Its world production increased from 59.74 million tons in 1990–73.7 million tons in 2014, of which 2.4 tons were table grapes. The large and increasing consumption of table grapes is accompanied by an increasing demand for better quality, not only relative to the visual aspect of fruits, but also focused on taste, flavour and consistency of berries [5,6]. Moreover, the market for table grapes is expanding its preference for seedless grapes [7–9].

The absence of seeds in grapevine, called apyreny, results from two distinct processes named stenospermocarpy and parthenocarpy. In the process of stenospermocarpy, predominantly selected in breeding programs of table grapes, the development of embryo and endosperm starts after fertilization but the endosperm tissue degenerates prematurely, leading to the production of fruits containing seed traces [10]. In parthenocarpy, the ovary develops into a fruit without fertilization, i.e., fruits develop from maternal tissues only, and therefore have no seeds [11,12]. A plant is considered apirenic when it is capable of producing (i) seedless fruits, (ii) fruits with seed traces or (iii) fruits with seeds reduced in number and size [12]. To determine the phenotype of seedlessness, a particular grapevine cultivar is classified by the International Organization of Vine and Wine (OIV) standard descriptor 243, according to which cultivars with a rate of less than 30 mg/seed are considered apirenic. Other useful scale was proposed by Bouquet and Danglot [11] in which the total seed dry matter percentage should be under 40% to be consider as a seedless cultivar.

In silico analysis of the grapevine genome allowed the identification of a candidate gene related to seed morphogenesis called *Vitis vinifera AGAMOUS-LIKE 11* (*VviAGL11*), mapped at the same *Seed development Inhibitor* (*SdI*) locus where the microsatellite molecular marker VMC7F2 was located. VMC7F2 is a highly polymorphic microsatellite marker associated to seedlessness in grapevines with discrimination efficiency

Abbreviations: OIV, Organisation Internationale de la vigne et du Vin; SdI, seed development inhibitor; STK, seedstick; TYLCV, Tomato Yellow Leaf Curl Virus; RNAi, silencing; OX, overexpression; NT, non-treated; GFP, green fluorescent protein; GUS, beta-glucuronidase protein

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Table 1
Experimental overview of the treated cultivars. The cultivars used in this study, their respective phenotype concerning seed presence and the genotype data for *VviAGL11* alleles. In this work, we used three sets of plant plasmids: the experiment control set (pIR + p1470), the *VviAGL11* overexpression set (pIRVviAGL11OX) and the *VviAGL11* silencing set (pIRVviAGL11RNAi). The number of bunches treated for each cultivar and the final number of bunches evaluated were accounted.

Cultivar	Phenotype	Plasmids	Number of treated bunches	Number of evaluated bunches
Prosecco	Seeded	pIR + p1470	6	6
Alvarinho	Seeded	pIRVviAGL11RNAi + p1470	12	12
Chardonnay	Seeded	pIRVviAGL11RNAi + p1470	13	10
Italia	Seeded	pIRVviAGL11RNAi + p1470	10	10
Moscato giallo	Seeded	pIRVviAGL11RNAi + p1470	19	12
Pinot Noir	Seeded	pIRVviAGL11RNAi + p1470	24	15
Ruby	Seeded	pIRVviAGL11RNAi + p1470	9	9
Trebbiano	Seeded	pIRVviAGL11RNAi + p1470	15	14
Clara	Seedless (scar trace)	pIRVviAGL11OX + p1470	9	5
Linda	Seedless (scar trace)	pIRVviAGL11OX + p1470	13	13
Sultanine	Seedless (seed trace)	pIRVviAGL11OX + p1470	3	0

[13–15]. The *VviAGL11* gene (genome access http://genomes.cribi.unipd.it/grape/- Vv18s0041g01880) demonstrated to be an ortholog of the *AtAGL11/STK* gene [12], known to have an important role in seed development in *Arabidopsis* [16,17]. The analysis of the *VviAGL11* transcriptional profile in grapevine showed a large accumulation of transcripts in developing seeds at two, four and six weeks after fruit set, whereas in apirenic varieties, there was an extreme depletion of its expression in all berry tissues evaluated [12,18,19]. *VviAGL11* seems to regulate the differentiation of the endotesta layer of the seed coat. In the absence of *VviAGL11* expression, the endotesta duplication and elongation fail to occur, and the seed development is arrested, forming a seed trace-like structure [12].

Fruits are a major source of fibre, nutrients, and antioxidants which are essential for a healthy diet [3,8,20]. Therefore, fruit crops play a key role in the economy of many countries. Efforts have been made to improve the quality of fruits and crops by using both conventional breeding and genetic transformation approaches [21,22]. Genetic engineering and other biotechnologies to improve plants are mostly dependent on efficient procedures of cell and tissue culture, especially for the obtainment of regenerants via organogenesis or somatic embryogenesis. Tissue culture is laborious and time-consuming. It typically allows the regeneration of only a few lines of genetically modified plants [23]. The grapevine breeding and improvement of commercial cultivars are based on sexual reproduction, vegetative propagation, and somatic mutations [1]. The genetic breeding of grapes is highly dependent on genetic markers and genome association studies which take years in order to obtain segregant generations by crossings. The selection of a specific phenotype, particularly a berry trait, is typically a long process considering the juvenile period (three-to-five years) of grapevine plants. Breeding tools that may be readily applied to cultivated varieties are therefore of great interest, reducing the need for developing new cultivars by crossings that inevitably decharacterizes the original genotype. Grapevine genetic transformation is very inefficient, leading to success rates lower than 10% [22,24,25], and it is highly dependent on the genotype used.

Advances have been made in the plant biotechnology field that may help to circumvent many limitations of the traditional techniques. One of the most interesting and promising new tools, especially for perennial plants, is the expression of genes based on episomal plasmids, such as the IL-60 platform [26]. This platform consists of a universal system of virus-derived vectors for the overexpression or silencing of genes directly in adult plants, opening possibilities for trait delivery in woody species. The IL-60 system is derived from the Tomato Yellow Leaf Curl Virus (TYLCV), a dsDNA that is able to replicate in plant cells and spread to other tissues after inoculation. This so called "plant plasmid" is mechanically introduced into plant tissues and the expression and replication of the episomal DNA occur in a few days. It becomes stable and systemic, eliminating the need for selectable markers, being useful for treating grafting stocks prior to transfer to the field [26–28]. The system is compatible with many species, including woody fruit trees such as grapevine, citrus, and olive [28,29]. Due to the lack of heritability of the episomal DNA, biosafety control might be easier than that advised to conventional genetically modified plants that undergo pollen-mobile genetic transmission.

In the present study, we aimed to functionally characterize the *VviAGL11* gene in grapevine by its overexpression in seedless cultivars and via silencing through RNAi in seeded genotypes, demonstrating its key role as a determinant of the fruit seed/seedless trait. We describe the successful use of virus-derived plant plasmids as a powerful tool for gene functional characterization and as a fast delivery method of valuable traits in grapevine. With this strategy, we were able to detect the systemic presence of the plant plasmids in grapevine plants and observe changes in *VviAGL11* expression pattern on treated plants. More interestingly, we showed that the overexpression of the *VviAGL11* in apirenic grapevine cultivars was able to partially restore seed development and morphogenesis whereas inhibition by *VviAGL11* silencing in pirenic cultivars resulted in the opposite effect, i.g., seeds were abnormally formed, reduced both in number and size.

2. Methods

2.1. Plant material

Six-to-eight years old grapevine plants of 11 cultivars were selected in fields from the Serra Gaúcha region, Rio Grande do Sul, Brazil. Three plants of each cultivar were transferred into greenhouse pots, after top and root pruning, during winter time. The greenhouse experimental areas were located at Embrapa Uva e Vinho in Bento Gonçalves, Rio Grande do Sul, Brazil (29°09′48″S, 51°31′42″O and 616 m altitude). The cultivars were grafted on Paulsen 1103 rootstock, planted on pergola trellising system and managed with conventional annual pruning and control of pests and diseases. Cultivars assayed were the pirenic 'Prosecco', 'Alvarinho, "Chardonnay", 'Italia', 'Moscato giallo', 'Pinot Noir', 'Ruby', 'Trebbiano' and apirenic 'Clara, "Linda" and 'Sultanine' (Table 1). Grapevine bunches chosen for treatment with plant plasmids were at the phenological scales D, G and H1 (Supplementary Fig. 1), according to Baggiolini [30]. Plants from the same cultivars were also kept under the same conditions and used as controls plants, in which no treatment was performed.

2.2. Vector construction and preparation

VviAGL11 DNA fragments were amplified via PCR from a previous cloned 'Chardonnay' seed cDNA [12]. Primers *VviAGL11CDSF* 5′CACC ATGGGGAGAGGAAAGATCG3′ and *VviAGL11CDSR* 5′TTACCCGAGAT GGAGGACCTTCTTATC3′ were defined, allowing the amplification of a 676 bp fragment that was subsequently cloned into the overexpression plasmid pH7WG2D ([31]; Ghent University, Ghent). Primers

VviAGL11F 5′CACCATTGTTCATCTGGGCATTTCG3′, and *VviAGL11R* 5′ GGAGATGAAGTTGGCGGATA-3′ were designed to amplify the initial portion of *VviAGL11* gene. with. The resulting 110 bp amplicon was cloned into the silencing plasmid pH7WIWG2D ([31]; Ghent University, Ghent). All PCR amplifications reported in this study were performed with 10–25 ng of grapevine genomic DNA using Platinum® Pfx DNA polymerase (Applied Biosystems, Foster City) and reaction conditions recommended by the manufacturer. The 35S CaMV-*VviAGL11* cassette and the *VviAGL11* hairpin were respectively transferred from pH7WG2D and pH7WIWG2D to the plant plasmid pIR (Morflora) using the restriction enzymes combinations HindIII/XbaI and NsiI/BglII. Final constructs were confirmed by sequencing in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using primers *pIR*F1 5′TTGCTCACATG TAATG3′ and *pIR*R1 5′ AGTTTTTTGTCTTGCA3′). All primers were synthesized by Integrated DNA Technologies (IDT). The final piIR-derived vectors were named piIR-*VviAGL11*-OX (overexpression) and piIR-*VviAGL11*-RNAi (silencing). Both plasmids were prepared in large amounts and purified using the PureLink® HiPure Plasmid Filter Maxiprep Kit (Thermo Fischer Scientific) following manufacturer's instructions. Solutions combining the p1470 helper plasmid with either piIR-*VviAGL11*-OX or piIR-*VviAGL11*-RNAi were prepared to a final concentration of 20 ng/μL of each plasmid

2.3. Grapevines bunches treatment with plant plasmids

Plant plasmids administration was performed at the base of the grapevine bunch peduncle with a 1 mL hypodermic syringe armed with a needle. A 1:1 proportion solution with 2 μg of the helper plasmid (p1470 [27]) plus 2 μg of the *VviAGL11* overexpression vector (pIRVviAGL11OX) or of the *VviAGL11* silencing vector (pIRVviAGL11RNAi) in was injected in each bunch. The treatment in the peduncle was performed at early D or G stages of development as shown in Supplementary video 1 and Supplementary Fig. 1. The treatments with the plant plasmids were repeated after three days, when bunch development was at the H1 stage, to guarantee maximum plasmid distribution in the growing bunches. Grapevine cultivars, combination of plasmids, number of bunches injected and analyzed are presented in Table 1. A total number of 133 bunches were treated with plasmids solutions, and more than 9.000 berries were analyzed molecular and phenotypically. At six weeks of fruit development, three bunches of each cultivar were sampled. Thereafter, all berries from the middle section of the bunch were picked and mixed. Thirty randomly sampled berries were pooled to constitute three representative samples (10 berries each). The replicates were stored into 50 mL Falcon tubes in −80 °C for later RNA extraction and RT-qPCR reactions. At berry maturation, all bunches available for each cultivar were harvested. Posteriorly, all the berries from the bunches were collected and mixed. Three replicates of 100 berries each were used for phenotypic analysis (300 berries per cultivar/per treatment, divided in three replicates). For anatomical purposes, seeds representing distinct phenotype variants were used as described later on this section.

2.4. Reporter genes analysis

GFP: Leaves from grapevine plants (1 m high) were treated with a pIRGFP + p1470 combination in a concentration of 200 ng/μL. The visualization of the GFP fluorescence was obtained in a stereo microscope equipped with a GPF filter (Leica 165FC).

GUS: Bunches from adult grapevines were treated with a pIRGUS + p1470 combination in a concentration of 1 μg/uL. GUS histochemical assays were performed in different organs of the grapevine plants (berry, seed, leaf, stem, pollen). The tissues were incubated in 1 mM X-Gluc, 100 mM phosphate buffer (pH 7.0), 2 mM KH2Fe, and 0.5% Triton X-100. The samples were incubated for 16 h at 37 °C. Thereafter, the tissues were incubated in 70% ethanol for chlorophyll removal.

2.5. Plant DNA/RNA purification

Genomic DNA was extracted from leaves and fruits at six weeks of development after fruit establishment (fruit set), and from seeds after dissection from fruit pulp following the protocol of Lefort and Douglas [32]. RNA extraction from the same organs was performed by LiCl precipitation using the Zeng and Yang [33] protocol scaled to 2 mL micro centrifuge tubes. Each sample extraction was performed in triplicate and final volumes were pooled before the LiCl precipitation step. Genomic DNA was removed using the TURBO DNA-free Kit (Ambion, Foster City) according to the manufacturer's protocol. RNA integrity and quantity were monitored by agarose gel electrophoresis and spectrophotometric quantitation, respectively.

2.6. RT-qPCR analysis

For *VviAGL11* expression analysis complementary DNAs were synthesized using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City) according to manufacturer's instructions. We used biological for each cultivar, and also technical quadruplicates for the RT-qPCR evaluation. The gene-specific primers *VviAGL11F* 5′CACTTAAT GGGTGATTCCTTGGC3′ and *VviAGL11R* 5′AGCAACTCATGCTTCTTCG ACC3′ were designed with the Oligo Analyzer 3.1 tool (IDT, http://www.idtdna.com) and synthesized by IDT, with the standards settings of 0.2 μM of oligo concentration, 1.5 mM of $MgCl_2$ and 0.2 mM of dNTP. The evaluated tissues were leaves and fruits at six weeks of development after fruit establishment (fruit set). Seeds were dissected from the pulp in seeded cultivars samples and the whole berry was evaluated in seedless cultivars. For the plasmid quantification on berries and seeds, we used a serial standard curve produced by pIRVviAGL1OX (at initial concentration of $1,2.10^{-9}$g), diluted in five magnitude orders: p1 (10^{-9}), p2 (10^{-10}), p3 (10^{-11}), p4 (10^{-12}) and p5 (10^{-13}). 5 ng of total DNA from each sample tested were used for the quantitation assay. All RT-qPCR were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City). SYBR Green (Invitrogen, Carlsbad city) was used to monitor dsDNA synthesis. Each biological sample was analyzed in technical quadruplicates. Cycling consisted of one step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and finished by a dissociation curve between 60 °C and 95 °C. The specificity of PCR amplifications was assessed by the presence of a single peak in melting curves, visualization of single amplification products of expected size in 1% ethidium bromide-stained agarose gel electrophoresis and sequencing of the amplicons. Primer efficiency was calculated by LinRegPCR (version 11.0 [34]). Mean relative gene expression was calculated by the Pfaffl [35] method employing *ACTIN* (GenBank EC969944), *EF1-α* (GenBank EC959059), *GAPDH* (GenBank CB973647) and *α-Tubulin* (GenBank EC930869) as reference gene [36]. Statistical analysis was performed using Prism 6.1 with a Parametric paired T test.

2.7. Characterization of VviAGL11 among grapevine cultivars

Genomic DNA was extracted from leaves following the protocol from Lefort & Douglas [32]. Grapevines cultivars were genotyped by PCR and sequencing with the employment of primers *VviAGL11 F10* 5′GCAAAGCAGTTATTGAAAGC3′ and *VviAGL11 R15* 5′GATGGGAGAA AATATCCGCC3′ for PCR. Amplification products were purified and sequenced with primers *VviAGL11 F14 5′CGTATATCTCCGAACCAAG3′* and *VviAGL11 R14 5′GCTTAATGAGACATTTCAAGCC3′*. Primer design and synthesis were described previously. This strategy enabled us to observe *VviAGL11* SNPs in the CDS region for the determination of *VviAGL11* allele's composition. The products were sequenced in an ABI Prism® 310 Genetic Analyser (Applied Biosystems, Foster City) using standard sequencing protocols described in Falavigna et al. [37]. Sequence analysis was carried with DNA Sequencing Analysis Software v5 (Applied Biosystems, Foster City) and MEGA7 software (http://www.

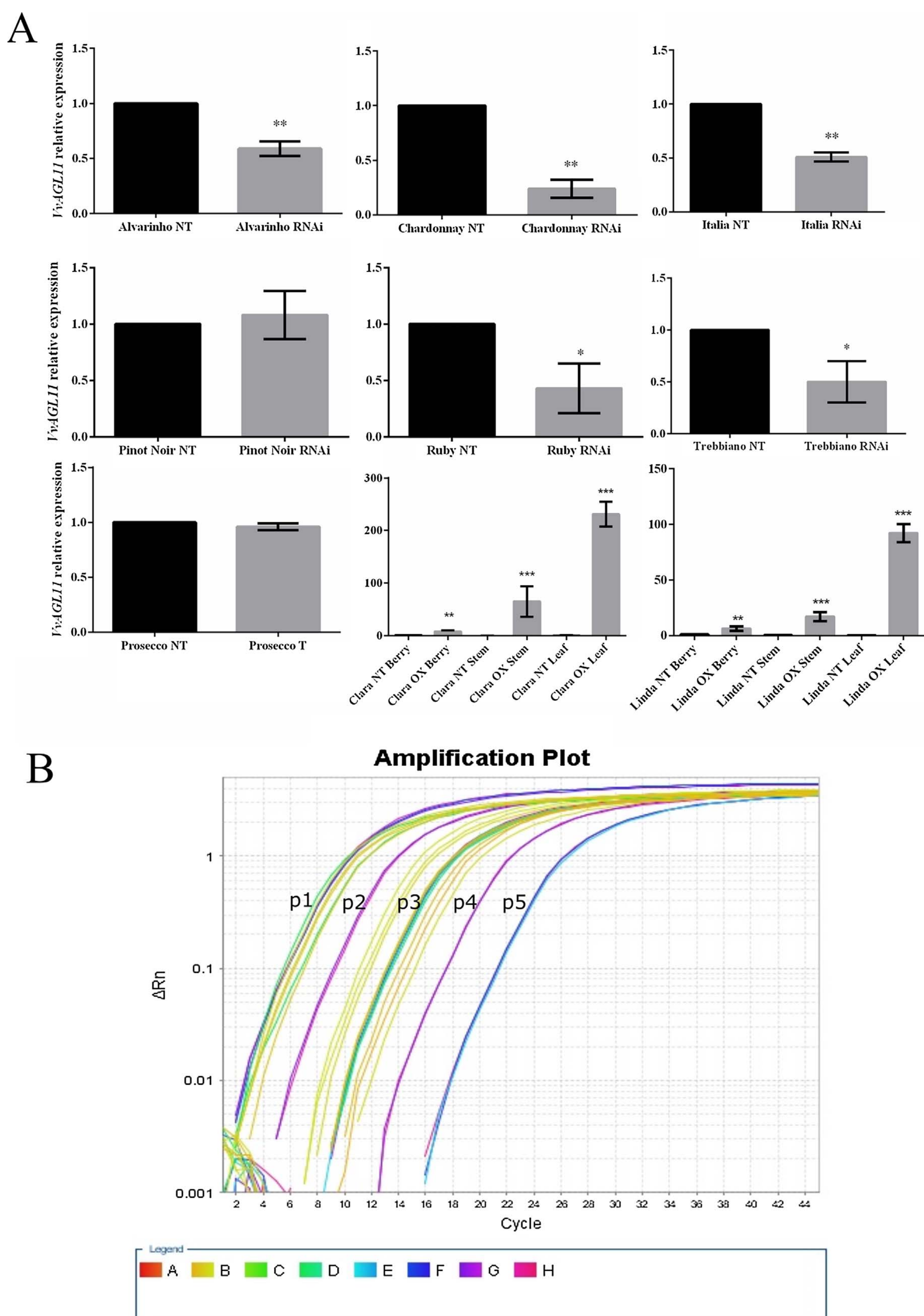

Fig. 1. *VviAGL11* relative expression and vector detection after treatment with plant plasmids. (A) The silenced cultivars, 'Italia' and 'Ruby', demonstrated a decrease in *VviAGL11* expression in seeds of six weeks of development. Linda cultivar demonstrated an accumulation of *VviAGL11* transcripts in berries, stem and leaves after six weeks of fruit development. Prosecco was used as a control. Bars show standard deviation. *α-Tubulin*, *GAPDH* and *EF1-α* were used as reference genes. OX = Overexpression, RNAi = RNA interference, NT = non-treated and T = treated. Parametric paired T test = * p < ,05; ** p < ,001; *** p < ,00001. (B) Quantitative evaluation of pIRVviAGL11 vectors by RT-qPCR. The serial standard curve presents five points: p1 (10^{-9}), p2 (10^{-10}), p3 (10^{-11}), p4 (10^{-12}) and p5 (10^{-13}). The curves from berries and seeds from the treated grapevines shows the high amount of plant plasmids in the samples.

megasoftware.net/home). Sequences were compared with the grapevine reference ('Pinot Noir' PN40024) genome.

2.8. Seed morphological analysis

After grapevine bunches reaches maturity, quantitative and qualitative evaluations of seeds were carried out. The maturation stage of the bunches was determined by Brix degree (°Brix) evaluation in a digital refractometer (Abbe) model 1421 with ten berries from each sampled bunch. The measurements represent an estimate of the total soluble solids on the liquid (mostly sugar), which indicates the sugar content and the phenological maturation of the berries [38]. The total number of seeds and seed traces was estimated from an average of 300 berries from each treated and non-treated cultivar. After determining seed fresh weight, seeds were dried at 50 °C for three days and dry weight was measured, both in a Shimadzu AUX22 analytical balance. We followed the OIV standard descriptor 243 in which the fruit seedlessness classification refers to the seed/seed trace dry weight content, for the classification of resulting fruits of pirenic or apirenic. Statistical analysis was performed using the Prism 6.1 with a Parametric paired T test and $\chi 2$ test.

2.9. Seed morpho-anatomical assay

Fresh seeds and seed traces were transferred to a fixation solution under vacuum [39]. Thereafter, samples were dehydrated in an increasing gradient of ethanol [40] and embedded in 2-hydroxyethyl methacrylate resin [41]. The 8 μm sections were obtained in a Leica RM 2255 microtome. The metachromatic reagent Toluidine Blue O [42] was used to determine seed structure. Slides with sections were submerged in the reagent for 1 min, washed with water and dried on plate at 45 °C. The photographs were obtained with the support of ZEN microscope software from ZEISS.

3. Results

3.1. Reporter gene experiments (GFP and GUS)

Prior to the experiments with adult grapevines, we evaluated the capacity of these vectors to multiply themselves in grapevine leaves and express reporter genes. Our pilot test showed that the leaves were expressing the Green Fluorescent Protein (GFP) after one month of the treatment with pIRGFP + p1470 (Supplementary Fig. 2). Furthermore, we tested the expression of the Beta-Glucuronidase protein (GUS) by injecting a combination of pIR/GUS + p1470 vectors on bunches. After a month we were able to observe the blue staining in seed tissues (Supplementary Fig. 2). Nevertheless, we could not see a GUS activity in other organs evaluated, such as leaves, stem and pollen. We believe that these results are due to the place of injection since the treatments were made in the bases of the bunch peduncle and not in leaves. The absence of GUS activity in pollen samples is in agreement with the plant plasmids technology that states that its vector was not reported in the progeny of treated plants [28].

3.2. Plasmid DNA is systemically translocated and retained in grapevine

Seeded grapevine cultivars Alvarinho, Chardonnay, Italia, Moscato giallo, Pinot Noir, Ruby and Trebbiano, were treated with the *VviAGL11* silencing construction (pIRVviAGL11RNAi + p1470) whereas seedless cultivars Clara, Linda and Sultanine, were treated with *VviAGL11* overexpression construction (pIRVviAGL11OX + p1470). After a month of plant plasmid inoculation, leaves proximal to injected bunch peduncles were sampled for plasmid DNA detection. All treated cultivars resulted PCR-positive. 'Prosecco', randomly selected as the control cultivar and injected with the empty plasmid, was also PCR-positive for vectors presence after treatment with pIR + p1470 (Supplementary Fig. 3). We were able to confirm that all treatments combining different versions of pIR and the helper p1470 plasmid were fully efficient, demonstrating that plasmid DNA was translocated through plant tissues, demonstrating a sectorized branch systemic translocation in treated plants.

3.3. The introduced genes remained stable and functional in grapevine reproductive and vegetative tissues

The expression of *VviAGL11* in Clara and Linda apirenic cultivars was evaluated by RT-qPCR in leaves, stems, and berries. Compared to field plants of the same cultivars, treated 'Clara' and 'Linda' overexpressed *VviAGL11* in all evaluated organs. *VviAGL11* overexpression in 'Linda' ('Linda' OX) berries presented six times more *VviAGL11* transcripts compared to non-treated 'Linda' ('Linda' NT) berries. It was also possible to observe a 17-fold increase in *VviAGL11* relative expression in stems and, in leaves, the expression was up to 90-fold higher than non-treated samples (Fig. 1A). 'Clara' OX also showed a significant increase in *VviAGL11* relative expression. Compared to non-treated plants, the levels of *VviAGL11* mRNA in berries was eight times higher, whereas stems presented a 65-fold increase and leaves demonstrated a remarkable mean of a 231-fold increase in *VviAGL11* relative expression (Fig. 1A). These results show that the plant plasmid harboring the *VviAGL11* overexpression construct was effective in conferring high levels of *VviAGL11* expression in apirenic grapevines. We were able to observe that the introduced gene remained stable and functional after three months of plant treatments.

It was previously demonstrated that *VviAGL11* transcripts accumulate at higher levels in seeds of two, four and six weeks after fruit set [12,19]. We, therefore, evaluated the expression levels of *VviAGL11* in seeds of pirenic cultivars Alvarinho, Chardonnay, Italia, Pinot Noir, Ruby, and Trebbiano treated with the silencing *VviAGL11* construct version (pIRVviAGL11RNAi + p1470) by RT-qPCR. The level of transcripts in plants silenced for *VviAGL11* was statistically lower than those observed in control plants, most of them presenting half or even lower levels of the *VviAGL11* normal expression (Fig. 1A). 'Chardonnay' showed the higher differences between the treated and the control plants. Moreover, 'Pinot Noir' was the only genotype that presented similar levels of *VviAGL11* expression relative to the control plants. The Prosecco plants, treated with an empty vector as control, showed similar levels of *VviAGL11* expression between seeds from treated and non-treated plants (Fig. 1A).

Furthermore, we also quantified the amount of plant plasmids in berries and seeds of the treated cultivar (Fig. 2B). When we compare the amount of plant plasmids in the total DNA extracted from the samples, it is clear that there is a correlation between vector quantity and gene expression. The samples from the cultivars Linda, Clara, Italia and Ruby presents the highest amounts of plant plasmids, almost 1/80 of the total DNA (Supplementary Table 1). These are some of the cultivars that had the most drastic phenotype modifications on their seed content: Linda presenting miniseeds after treatment; and Italia and Ruby presenting significate amounts of seed traces and less total seed dry weight. For the others cultivars, including Prosecco, the rate of plant plasmids versus total DNA varied between 1/600 −1/800.

3.4. Manipulation of VviAGL11 expression affects seed morphogenesis in several grape cultivars

Our study aimed to assess the phenotypic effects of altered *VviAGL11* expression on seed characteristics of each grapevine cultivar. Therefore, we analyzed the mature bunches of treated cultivars and their respective non-treated controls. The phenological maturation stage of berries was estimated by the determination of the °Brix from each bunch sample. Because berries from all cultivars exhibited °Brix degrees above 13, they were all characterized as belonging to the mature stage for final seed size evaluation (Supplementary Fig. 4).

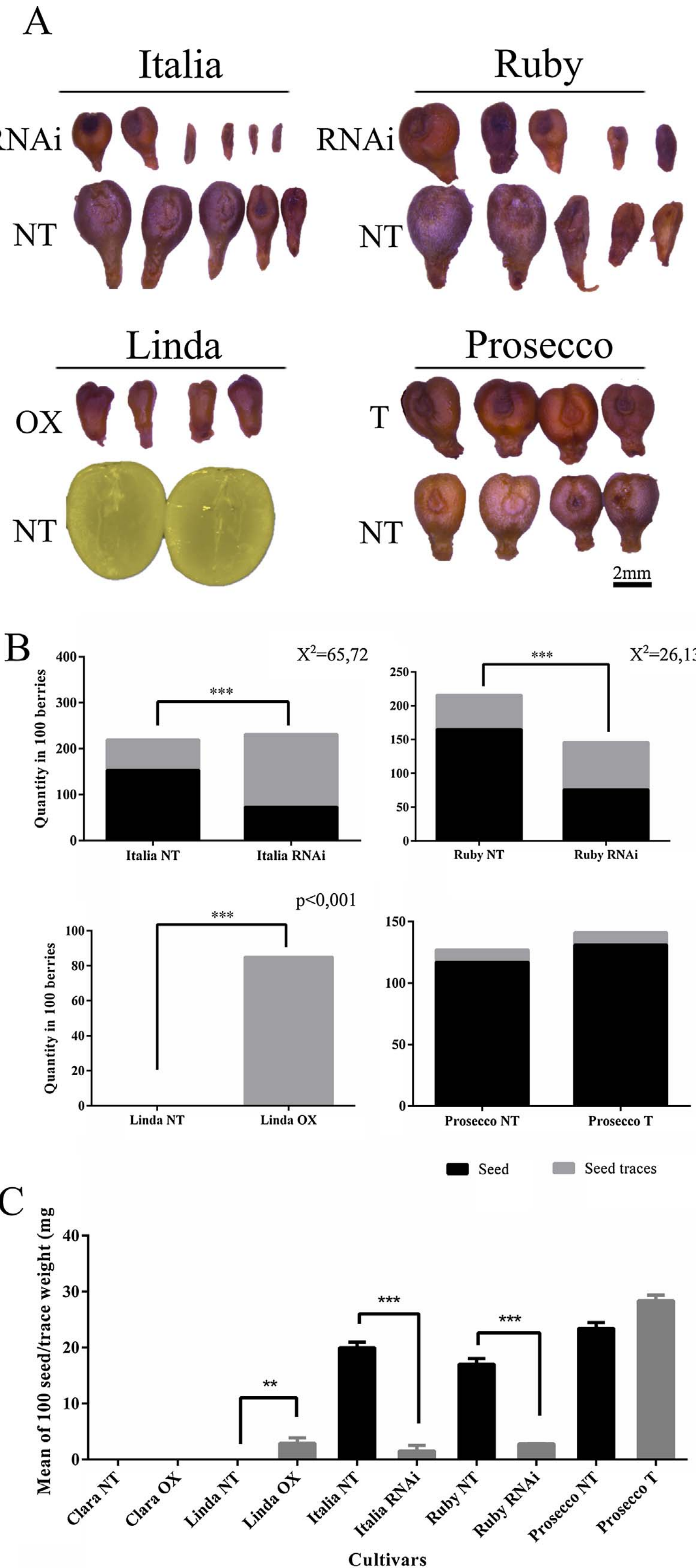

Fig. 2. Seeds and seed traces quantitative evaluation. (A) Seeds and seed traces morphological analysis. 'Linda' OX presents miniseeds that do not exist in 'Linda' NT. (B) The number of seed and seed traces was counted of 100 berries per cultivar. 'Italia' RNAi and 'Ruby' RNAi present an increased number of seed traces and fewer seeds, while 'Linda' OX presents small seeds (counted as seed traces) that 'Linda' NT does not present. 'Italia', 'Ruby' and 'Prosecco' $\chi2$ test * $p < ,05$; ** $p < ,001$; *** $p < ,00001$. (C) Dry seed/traces weight evaluation. Italia RNAi and Ruby RNAi had a significant decrease in dry seed weight. 'Linda' OX presents a dry seed weight that was nonexistent in 'Linda' NT. Prosecco was used as a control. OX = Overexpression, RNAi = RNA interference, NT = non-treated and T = treated. Linda Parametric paired T test = * $p < ,05$; ** $p < ,001$; *** $p < ,00001$.

Subsequently, mature bunches were evaluated for quantitative measurements including berry weight, seed/seed-trace number, fresh seed/seed-trace weight, and dry seed/seed-trace weight. The quantitative evaluation of seeds and seed traces showed that cultivars Alvarinho, Chardonnay, Italia, Linda, Pinot Noir, Moscato giallo and Ruby treated with the silencing version of the *VviAGL11*-harboring plasmid presented a significant reduction in all parameters analyzed between treated and non-treated plants (Fig. 2B and Supplementary Fig. 5).

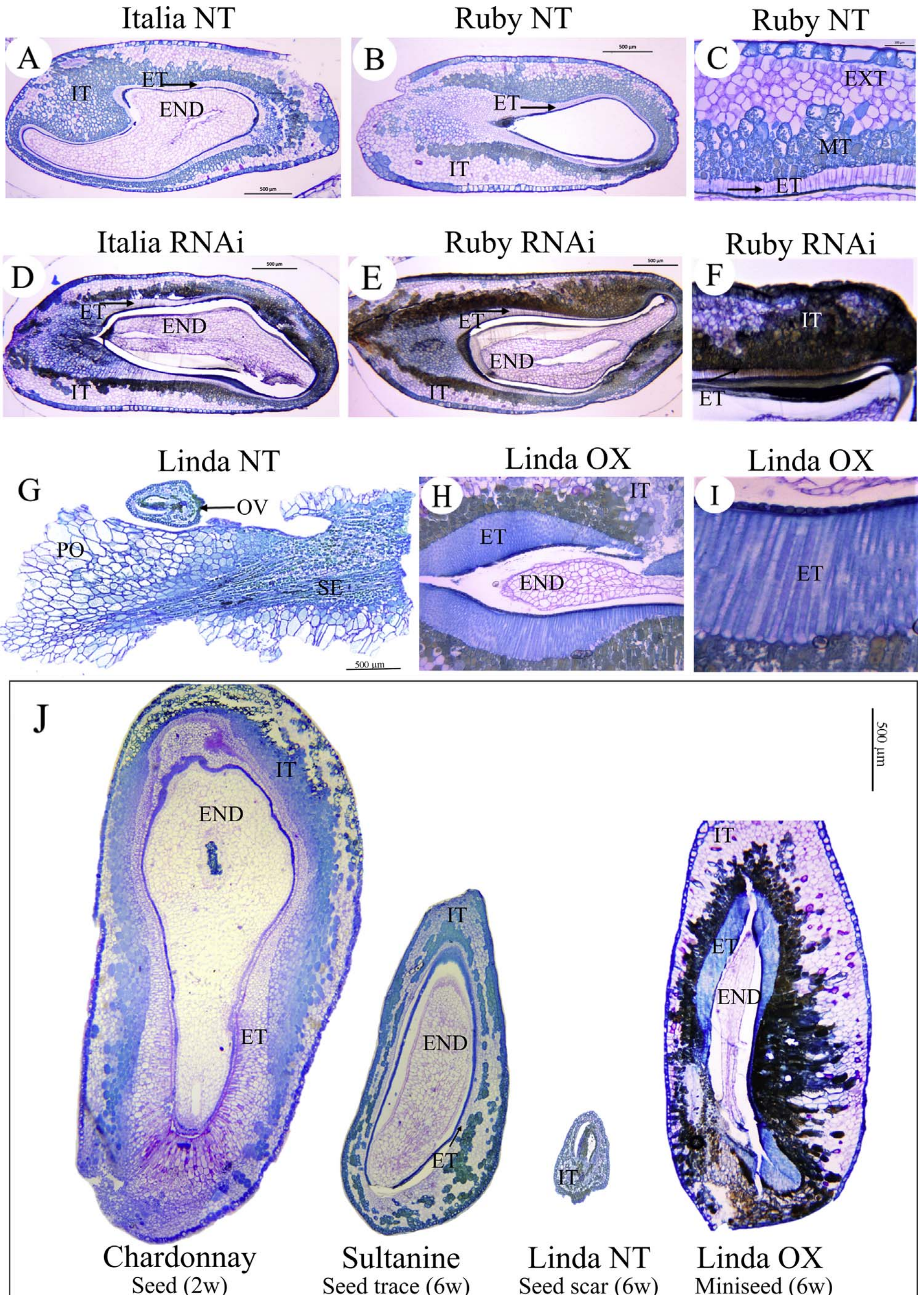

Fig. 3. Comparison of grapevine seed and seed trace morpho-anatomical structure. Seeds were sampled six weeks after fruit set. Light micrographs of grape seed cross sections were stained with toluidine blue O. (A) 'Italia' NT; (B) 'Ruby' NT; (C) 'Italia' RNAi; (D) 'Italia' RNAi; (E) 'Ruby "RNAi"; (F) 'Ruby' RNAi (G-I) 'Linda' OX. (J) Comparison between a normal seed of 'Chardonnay' with two weeks of development, a 'Sultanine' seed trace of six weeks after fruit set, the 'Linda' NT seed trace scar and the 'Linda' OX that presents a miniseed. Endosperm (END), endotesta (ET), mesotesta (MT), exotesta (EXT), teguments (IT), ovule (OV), polp (PO), septum (SE). Scale bar: 500 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Silencing of *VviAGL11* in the seeded cultivars Italia and Ruby rendered a visible and drastic decrease in their total seed dry weight with a reduced number of seeds and a concomitant increase in the number of seed traces (Fig. 2A). Consequently, the reduction in seed size and in number may allow 'Italia' RNAi and 'Ruby' RNAi to change their classification for the OIV-243 description, being considered seedless (apirenic) cultivars (Fig. 2A–C).

Overexpression of *VviAGL11* in the seedless Linda cultivar (Linda OX) resulted in the formation of miniseeds that were not present in the 'Linda' non-treated bunches. Its control, Linda NT, typically presents only seed traces that are almost unobservable to the naked eye (Fig. 2A). The diminutive seeds from 'Linda' OX berries were bigger in size than a seed trace, but they did not achieve a normal mature seed size. Despite the miniseed development in the 'Linda' OX berry samples, its total dry weight was still classified as seedless following the OIV-243 descriptor (Fig. 2C). This indicates that *VviAGL11* overexpression by plant plasmid delivery did affect seed size, increasing it, but it was not sufficient to restore the complete seed development in the Linda genotype background although it can partially restore the formation of the seed coat (Fig. 3G–J).

Finally, the quantitative evaluation of seeds and of seed traces in berries from cultivars Clara (OX), Trebbiano (RNAi) and Prosecco (control) showed no statistically significant differences in the number of seeds or traits comparing treated and untreated plants (Supplementary Fig. 5).

3.5. Seed traces from VviAGL11RNAi-treated plants are anatomically different from non-treated control plants

The seeds from 'Pinot Noir' and 'Chardonnay' were characterized in

previous studies with a particular pear triangular shape and composed of a cuticle, an epidermis, three layers of outer integument (exotesta, mesotesta and endotesta), two inner integument layers, endosperm and embryo [12,43]. The structure of the seed traces from 'Sultanine' was characterized by the undifferentiation of the seed coat inner layer – endotesta (ET), as a consequence of the absence of *VviAGL11* expression, that leads to endosperm and embryo abortion, forming a seed trace [12]. We conducted the morpho-anatomical characterization of seed traces resulting from the treatments of pirenic cultivars Italia and Ruby (VviAGL11RNAi treatment). These two cultivars showed the most significant differences in final seed size and dry weight after plasmid treatments for *VviAGL11* silencing.

In 'Italia' RNAi (Fig. 3D) and 'Ruby' RNAi (Fig. 3E), seed traces presented the same characteristics when compared to 'Italia' NT (Fig. 3A) and 'Ruby' NT (Fig. 3B). However, the internal layer of the seed coat in seeds from *VviAGL11*RNAi-treated plants exhibited an accumulation of phenolic compounds that were probably involved in the beginning of the lignification process (maximization in Fig. 3F). These cells are stained brown because of their polyphenols content. They are also present in the outer integument, both in the exotesta and in the endotesta layers (maximization in Fig. 3F). This concentration of phenolic compounds did not occur in non-treated seed traces from 'Italia' and 'Ruby' (Fig. 3C). Also, the endosperm and the embryo were not developed properly, as expected for a normal seed trace. We were able to observe that the ET layer neither elongated nor duplicated in most of the seed traces from 'Italia' RNAi and 'Ruby' RNAi (Fig. 3D and E). This suggests that *VviAGL11* induces modifications in the seed coat, initiating the lignification of the ET layer and, therefore, producing a high effect on seed development and maturation even without the normal formation of endosperm and embryo.

3.6. *VviAGL11 overexpression induces the development of the endotesta layer in 'Linda' miniseeds*

In order to evaluate the effect of *VviAGL11* overexpression in the 'Linda' seedless cultivar, we first performed an anatomical evaluation of the diminutive seed traces scars present in non-treated plants. These structures present minimal conducting vessels that were identified as septum cells surrounded by parenchyma cells derived from the pulp (Fig. 3G). Additionally, a structure composed of maternal tissues was identified as being the ovule. Within this structure, we were able to observe inner and outer integuments that never developed (Fig. 3G), and that could only be identified after microscopic examination.

'Linda' OX miniseeds presented the most interesting morpho-anatomical results when compared to the seed traces scars from 'Linda' NT. 'Linda' OX miniseeds presented the typical characteristics of seed trace concerning size and death of reproductive tissues, such as endosperm and embryo. However, the seed coat layers were found to be completely different from standard seed traces observed in 'Sultanine' [12]. The ET layer was duplicated and elongated (Fig. 3H and maximization on Fig. 3I). It was possible to observe the accumulation of phenolic compounds on the exotesta layer as well (Fig. 3H and I). The miniseeds presented a vulvar form due to ET layer overdevelopment (Fig. 3J). As a result, OX treated seedless plants showed a seed trace that was intermediary in terms of seed size and lignification profile, being bigger than a typical stenospermocarpic seed trace but, nevertheless, not achieving the size nor the shape of a wild-type like seed.

4. Discussion

Since the beginning of grapevine domestication, first by artificial selection and later by intercrossing and vegetative propagation, the main desirable traits selected were the ones involving fruit taste, size, and reduced seed size and number [44]. Unluckily, this was not the easiest trait combination to be achieved due to the seed effects on fruit size in grapevine [45]. Consequently, its improvement was and still is a laborious and time-consuming work. Furthermore, the molecular mechanisms underlying seed development in grapevine were not fully understood. Previous reports merely stated that the anatomical and morphological changes during normal seed development, from fertilization to maturity, involve three phases (i) rapid cell division; (ii) reserve accumulation and cell expansion due to water uptake; and (iii) slowing down growth and arrest of reserve accumulation [46].

By the time *VviAGL11* was confirmed as a master controlling gene of the seed development in grapevine [12], we then envisaged to use its sequence in a biotechnological approach. In the present study, we constructed vectors for *VviAGL11* ectopic overexpression and silencing through RNA interference. Overall, we treated more than 130 bunches and evaluated more than 9.000 berries from 11 grapevine cultivars. By detecting the plasmid DNA in different plant tissues and organs after months of the original injection, it was possible to confirm that 100% of the plants were multiplying the IL-60 plasmids systemically. Through the evaluation of the *VviAGL11* relative expression, 90% of the cultivars showed a direct correlation between gene expression and plasmid presence. 'Pinot Noir' plants treated with the overexpression construction was the exception, in which no differences in the expression of *VviAGL11* were observed comparing treated and non-treated seeds. This might be due to the phenological stage of the 'Pinot Noir' plants when treatments were performed. 'Pinot Noir' plants presented an early bud break and, at the time of treatment, bunches were at the H1 stage instead of D or G stages like the other cultivars. Therefore, the bunches were more developed. There are no studies suggesting the best combination of time and place of plasmid injection in order to optimize its movement to other tissues in grapevine. For the purpose of our study, plasmids had to reach the ovule integuments in time to allow the overexpression or the silencing of the *VviAGL11* gene during seed formation. It seemed to us that the best stage of bunch development for treatment was the first week after bunch appearance, during grapevine floral development and before ovule fertilization. This stage is typically achieved in a couple of weeks after bud break [30]. Nevertheless, there is room for optimizing the protocol in the DNA delivery strategy, assuring that plasmid constructs will reach the target tissues at the appropriate time.

Treatments with the *VviAGL11*-harboring plasmids led to quantitative and qualitative changes in seeds and/or traits in 78% of the cultivars. Three cultivars showed more evident modifications in the number and characteristics of seeds and/or seed traits. The seeded cultivars 'Italia' and 'Ruby' are two of the main commercialized table grapes in South America, contributing to the economic success of table grape varieties, but still lacking the desirable seedless trait. In the present work, 'Italia' and 'Ruby' were treated for *VviAGL11* silencing. The suppression of *VviAGL11* expression caused a reduction in their seed quantity in a way that their total dry weight decreased to a level acceptable as seedless. To access this information, the standard dry matter data of 100 seeds randomly sampled is used, classified in the following order: class 1 – very low (≤ 10 mg/seed); class 3 – low (21–29 mg/seed); class 5 – average (36–44 mg/seed); class 7 – high (51–59 mg/seed) and class 9 – very high (> 65 mg/seed) [47]. The varieties that fit in classes 1 and 3 are the most desired for table grape seedless selection. Both cultivars, after treatment, turned out to be considered as elite seedless because their dry weight from 100 seeds is less than 10 milligrams. Nevertheless, the small seeds could be sensed by chewing during berry consumption because of their unique seed coat development. These results show that the decrease of *VviAGL11* expression on the integuments was sufficient to interrupt normal seed development, and many seeds became seed traces. Furthermore, the analysis of control plants from 'Prosecco' allowed us to demonstrate that the empty pIR +1470 plasmids alone did not promote changes in treated plants, confirming that the modifications were caused directly by the overexpression or silencing of *VviAGL11*.

'Linda' (BRS Linda) is a seedless grape developed by the Embrapa Uva e Vinho breeding program. Its berry is elliptical, greenish, with a

tiny seed trace, practically invisible. Exploring the extreme seedless phenotype from this cultivar, it was possible to observe that ectopic *VviAGL11* overexpression during berry development partially restored seed formation, determining elongation and duplication of the ET layer in 'Linda' OX berry samples, leading to the development of miniseeds. Notwithstanding, the novel seeds from *VviAGL11*-overexpressed 'Linda' samples did not achieve a normal final size. Nevertheless, the morpho-anatomical examination of the miniseeds samples allowed to show the critical role of this gene in seed formation and growth, being able to 'open space' for endosperm growth and, consequently, embryo development. These results are in agreement with the findings reported by Garcia et al. [48], showing that integuments are able to control and determine the final seed size, due to the elongation of their layers, producing a strong maternal effect on seed growth and development.

Even though we detected the pIR DNA and also *VviAGL11* transcripts level modifications in seeds, only the ET layer was influenced by *VviAGL11* overexpression; and, as a maternal tissue, this layer does not remain in the progeny. This provides an important biosafety barrier for future field applications in grapevines and other plants. Peretz et al. [28] showed that when the progenies (up to F3) of treated tomato plants were evaluated, the pIR plasmid and p1470 were not present. As it was observed for the ASLV vectors [49], the TYLCV-derived plasmids are not heritable, and the progeny will be devoid of plasmid DNA.

VviAGL11, as a MADS-box protein class D, is able to form the so-called floral quartets protein complexes. In this case, MADS-box proteins will bind other proteins from the same family but different classes (A, B, C and E), which afterwards can bind to two *cis*-elements called CArG-boxes and activate gene transcription [50,51]. The formation of transcription factor dimers and multimeric complexes offers a mechanism to increase the diversity of possible DNA-binding proteins enormously [52]. The fact that only the ET layer of grapevine seeds and seed traces presented a contrasting development after the treatments and that *VviAGL11* is the single class D gene in grapevine [12,18] brings into light how important is the arrangement of the MADS-box proteins complexes in a certain temporal and spatial site. Nevertheless, another point to focus is the amount of VviAGL11 protein in the ET layer needed for its proper elongation and duplication. For normal seed formation, the expression of the *VviAGL11* gene should be around 15–25-fold higher relatively comparing to seedless cultivars in which this gene is expressed at a very low rate [12,19]. Regrettably, there is still no clear clue about the reasons for the low expression of *VviAGL11* in seedless cultivars. However, in our study, it was possible to see that the ET layer is actively responsive to the expression levels of this gene. With its overexpression provided by plant plasmids, a higher level of *VviAGL11* transcripts was present in 'Linda' OX samples, leading to the formation of miniseeds in a higher number and more developed than seed traces. This probably induced the overdevelopment of 'Linda' OX seed structure causing the duplication and elongation of the ET layer in a faster way and, consequently, initiating the lignification process. Because the timing of the expression of this key gene was not regulated by our approach, the lignification process was rapid and, consequently, stalled seed growth. These unusual processes formed a final structure that is no longer a seed trace and not even a normal seed, and therefore was named 'miniseed' (Fig. 3J).

The significant differences observed in *VviAGL11* transcript levels combined with the phenotypic seed modifications observed both in 'Italia' RNAi and 'Rubi' RNAi samples, and also the restored seed structure in 'Linda' OX, confirm the previous study of *VviAGL11* complementation of *stk* (*AtAGL11* mutant) that was also able to restore seed number and seed size in the Arabidopsis heterologous background [12]. In addition, it seems clear that the *VviAGL11* transcript levels can be directly correlated to the rate of plant plasmids versus total DNA. The present work contributes with key functional data about *VviAGL11* in grapevine itself, showing, *in situ,* that this gene is a major regulator of seed morphogenesis in grapevine. Furthermore, these results demonstrated the scientific applicability of the IL-60-derived plasmids to manipulate the *VviAGL11* gene expression. However, from the results observed, it was possible to verify that the efficiency of this tool is dependent on the context of its application. Nevertheless, this work represents the follow up characterization step of the *VviAGL11* locus as a target for manipulation of seed number and size in grapevine.

Authors contribution

L.F.R and J.M. conceived original screenings, research plans, designed experiments and analyzed resulting data; L.F.R, V.B., F.S.M, M.M.P, G.P. and J.E.A.M. supervised experiments and writing; J.M. and V.B. performed most experiments; F.S.M and J.E.A.M. provided technical assistance. J.M. wrote the article with contributions from all authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2018.01.013.

References

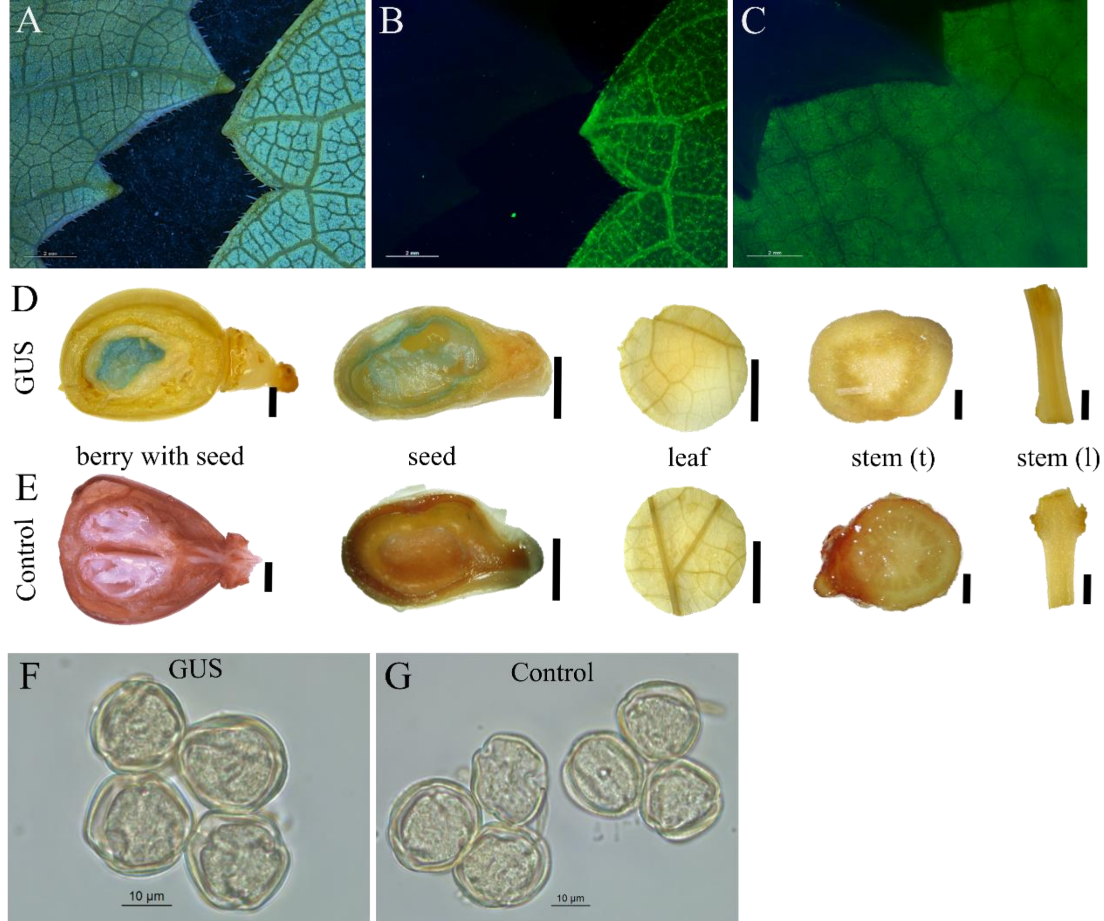
[1] P. This, T. Lacombe, M.R. Thomas, Historical origins and genetic diversity of wine grapes, Trends Genet. 22 (2006) 511–519, http://dx.doi.org/10.1016/j.tig.2006.07.008.
[2] M. Vitivin, Comunicado157 Técnico, (2014), pp. 0–2.
[3] E. Mundial, Comércio Exterior Mundial e Brasileiro de Uva de Mesa: Análise de Indicadores de Competitividade, Tendências e Sazonalidades, (2012).
[4] M. Falcão, G. Bnb, J. Maria, P. Souza, J. Amauri, B. Embrapa, F. Passos, L. Jany, F. Ximenes, AVALIAÇÃO DO FNE RURAL Série Avaliação de Políticas e Programas do BNB Fortaleza Banco do Nordeste do Brasil 12 (2012).
[5] F. Lahogue, P. This, A. Bouquet, Identification of a codominant scar marker linked to the seedlessness character in grapevine, Theor. Appl. Genet. 97 (1998) 950–959.
[6] G. Fanizza, F. Lamaj, L. Costantini, R. Chaabane, M.S. Grando, QTL analysis for fruit yield components in table grapes (Vitis vinifera), Theor. Appl. Genet. 111 (2005) 658–664 doi101007/s00122-005-2016-6.
[7] G.W.B. de Melo, M. Botton, L. da R. Garrido, Produção Orgânica de Uva para Mesa, Doc. Embrapa, 92 –Bento Gonçalves, Embrapa Uva E Vinho 92 (2015) 40 ISSN 1808-4648 http://ainfo.cnptia.embrapa.br/digital/bitstream/item/131061/1/Doc92.pdf.
[8] J.C. Nachtigal, Uvas sem sementes, Revevista Brasileira de Fruticultura 27 (2005), http://dx.doi.org/10.1590/S0100-29452005000100001 0–0.
[9] C. Lacirignola, M. Digiaro, Grapevine industry in the world: state of the art and future trends, CIHEAM 46 (1999) 33–46.
[10] U.A. Camargo, F. Mandelli, M.A.F. Conceição, J. Tonietto, Grapevine Performance and Production Strategies in Tropical Climates 5 (2012), pp. 257–269.
[11] A. Bouquet, Y. Danglot, Inheritance of seedlessness in grapevine (Vitis vinifera L.), Vitis 35 (1996) 35–42.
[12] J. Malabarba, V. Buffon, J.E.A. Mariath, M.L. Gaeta, M.C. Dornelas, M. Margis-Pinheiro, G. Pasquali, L.F. Revers, The MADS-box gene Agamous-like 11 is essential for seed morphogenesis in grapevine, J. Exp. Bot. 68 (2017) 1493–1506, http://dx.doi.org/10.1093/jxb/erx025.
[13] L. Costantini, M.S. Grando, S. Feingold, S. Ulanovsky, N. Mejia, P. Hinrichsen, A. Doligez, P. This, J.A. Cabezas, J.M. Martinez-Zapater, Generation of a common set of mapping markers to assist table grape breeding, Am. J. Enol. Vitic. 58 (2007) 102 http://www.ajevonline.org/cgi/content/abstract/58/1/102.
[14] L.F. Revers, L.J. Welter, P.B. Irala, D.C. Silva, V.S. Lampe, Co-localization of QTLs for Seedlessness and Downy Mildew Resistance in Grapevine, (2014), pp. 449–456.
[15] M. Mejia, N. Gebauer, Identification of QTLs for Seedlessness, Berry Size, and Ripening Date in a Seedless X Seedles Table Grape Progeny, (2007).
[16] R. Favaro, A. Pinyopich, R. Battaglia, M. Kooiker, L. Borghi, G. Ditta, M.F. Yanofsky, M.M. Kater, L. Colombo, MADS-box protein complexes control carpel and ovule

development in arabidopsis, Society 15 (2003) 2603–2611, http://dx.doi.org/10.1105/tpc.015123.2.
[17] A. Pinyopich, G.S. Ditta, B. Savidge, S.J. Liljegren, E. Baumann, E. Wisman, M.F. Yanofsky, Assessing the redundancy of MADS-box genes during carpel and ovule development, Nature (2003) 85–88.
[18] J. Díaz-Riquelme, J. Grimplet, J.M. Martínez-Zapater, M.J. Carmona, Transcriptome variation along bud development in grapevine (Vitis vinifera L.), BMC Plant Biol. 12 (2012) 181, http://dx.doi.org/10.1186/1471-2229-12-181.
[19] N. Mejia, B. Soto, M. Guerrero, X. Casanueva, C. Houel, M.D.L. Angeles Miccono, R. Ramos, L. Le Cunff, J.-M. Boursiquot, P. Hinrichsen, A.-F. Adam-Blondon, Molecular, genetic and transcriptional evidence for a role of VvAGL11 in stenospermocarpic seedlessness in grapevine, BMC Plant Biol. 11 (2011) 57, http://dx.doi.org/10.1186/1471-2229-11-57.
[20] S. Myles, A.R. Boyko, C.L. Owens, P.J. Brown, F. Grassi, M.K. Aradhya, B. Prins, A. Reynolds, J.-M. Chia, D. Ware, C.D. Bustamante, E.S. Buckler, Genetic structure and domestication history of the grape, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 3530–3535, http://dx.doi.org/10.1073/pnas.1009363108.
[21] R. Scorza, Progress in tree fruit improvement through molecular genetics, HortScience 36 (2001) 855–858.
[22] M.A. Gomez-Lim, R.E. Litz, Genetic transformation of perennial tropical fruits, Vitr. Cell. Dev. Biol. 40 (2004) 442–449, http://dx.doi.org/10.1079/IVP2004547.
[23] P. Crouzet, Transgenic plants, Life Sci. 847 (2002) 1–7, http://dx.doi.org/10.1007/978-1-61779-558-9.
[24] C. Fan, N. Pu, X. Wang, Y. Wang, L. Fang, W. Xu, J. Zhang, Agrobacterium-mediated genetic transformation of grapevine (Vitis vinifera L.) with a novel stilbene synthase gene from Chinese wild Vitis pseudoreticulata, Plant Cell. Tissue Organ Cult. 92 (2008) 197–206, http://dx.doi.org/10.1007/s11240-007-9324-2.
[25] L. Sanjurjo, J.R. Vidal, A. Segura, F. De la Torre, Genetic transformation of grapevine cells using the minimal cassette technology: the need of 3'-end protection, J. Biotechnol. 163 (2013) 386–390, http://dx.doi.org/10.1016/j.jbiotec.2012.11.014.
[26] R. Mozes-Koch, O. Gover, E. Tanne, Y. Peretz, E. Maori, L. Chernin, I. Sela, Expression of an entire bacterial operon in plants, Plant Physiol. 158 (2012) 1883–1892, http://dx.doi.org/10.1104/pp.111.186197.
[27] O. Gover, Y. Peretz, R. Mozes-Koch, E. Maori, H.D. Rabinowitch, I. Sela, Only minimal regions of tomato yellow leaf curl virus (TYLCV) are required for replication, expression and movement, Arch. Virol. 159 (2014) 2263–2274, http://dx.doi.org/10.1007/s00705-014-2066-7.
[28] Y. Peretz, R. Mozes-koch, F. Akad, E. Tanne, H. Czosnek, I. Sela, A universal expression/silencing vector in plants [C][OA], Plant Physiol. 145 (2007) 1251–1263, http://dx.doi.org/10.1104/pp.107.108217.
[29] C. Li, M. Gu, N. Shi, H. Zhang, X. Yang, T. Osman, Y. Liu, H. Wang, M. Vatish, S. Jackson, Y. Hong, Mobile FT mRNA contributes to the systemic florigen signalling in floral induction, Sci. Rep. 1 (2011) 73, http://dx.doi.org/10.1038/srep00073.
[30] M. Baggiolini, Stades repères de la vigne, Rev. Rom. d'Agriculture, Vitic. d'Arboriculture 8 (1952) 4–6.
[31] A. Karimi, M. Inzé, D. Depicker, Gateway vectors for Agrobacterium-mediated plant transformation, Trends Plant Sci. 7 (2002) 193–195.
[32] F. Lefort, G.C. Douglas, An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species Acer, Fraxinus, Prunus and Quercus, Ann. For. Sci. 56 (1999) 259–263, http://dx.doi.org/10.1051/forest:19990308.
[33] Y. Zeng, T. Yang, RNA isolation from highly viscous samples rich in polyphenols and polysaccharides, Plant Mol. Biol. Report. 20 (2002) 417, http://dx.doi.org/10.1007/BF02772130.
[34] J.M. Ruijter, C. Ramakers, W.M.H. Hoogaars, Y. Karlen, O. Bakker, M.J.B. van den hoff, A.F.M. Moorman, Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009), http://dx.doi.org/10.1093/nar/gkp045.
[35] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45, http://dx.doi.org/10.1093/nar/29.9.e45.
[36] K.E. Reid, N. Olsson, J. Schlosser, F. Peng, S.T. Lund, An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development, BMC Plant Biol. 6 (2006) 27, http://dx.doi.org/10.1186/1471-2229-6-27.
[37] V. da, S. Falavigna, D.D. Porto, V. Buffon, M. Margis-Pinheiro, G. Pasquali, L.F. Revers, Differential transcriptional profiles of dormancy-related genes in apple buds, Plant Mol. Biol. Report. 32 (2014) 796–813, http://dx.doi.org/10.1007/s11105-013-0690-0.
[38] S. De Defesa, A. Sda, S.S. Brix, (n.d.) C.O.M. Refratômetro P. De, Coordenação Geral de Apoio Laboratorial − CGAL Manual de Métodos de Análises de Bebidas e Vinagres Não alcoólicos Para amostras que não apresentam álcool e sólidos em suspensão, o oBrix poderá ser Coordenação Geral de Apoio Laboratorial − CGAL Manual de (1–7).
[39] E.M. Mcdowell, B.R. Trump, Histological fixatives for diagnostic light and electron microscopy, Arch. Pathol. Lab. Med. 100 (8) (1976) 405–414.
[40] B. Gabriel, Biological Electron Microscopy, (1982), p. 264.
[41] P.O. Gerrits, L. Smid, A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections, J. Microsc. 132 (1983) 81–85, http://dx.doi.org/10.1111/j.1365-2818.1983.tb04711.x.
[42] N. Feder, T.P. O'brien, Plant microtechnique, some principles and new methods, Am. J. Bot. 55 (1968) 123–142.
[43] Y. Cadot, M.T. Miñana-Castelló, M. Chevalier, Anatomical, histological, and histochemical changes in grape seeds from Vitis vinifera L. cv cabernet franc during fruit development, J. Agric. Food Chem. (2006) 9206–9215.
[44] F. Varoquaux, R. Blanvillain, M. Delseny, P. Gallois, Less is better: new approaches for seedless fruit production, Trends Biotechnol. 18 (2000) 233–242, http://dx.doi.org/10.1016/S0167-7799(00)01448-7.
[45] L. Costantini, J. Battilana, F. Lamaj, G. Fanizza, M.S. Grando, Berry and phenology-related traits in grapevine (Vitis vinifera L.): from quantitative trait loci to underlying genes, BMC Plant Biol. 8 (2008) 38 http://www.ncbi.nlm.nih.gov/pubmed/18419811.
[46] M. Cadot, Y. Minãna-Castelló, M.T. Chevalier, Anatomical histological, and histochemical changes in grape seeds from Vitis vinifera L. cv cabernet franc during fruit development, Agric. Food Chem. 54 (2006) 9206–9215.
[47] OIV, Statistique Du Secteur Vitivinicole Mondiale. (2015). http://www.oiv.int/fr/lorganisation-internationale-de-la-vigne-et-du-vin.
[48] D. Garcia, J.N.F. Gerald, I. Lyon, Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis, Society 17 (2005) 52–60, http://dx.doi.org/10.1105/tpc.104.027136.1.
[49] N. Yamagishi, S. Sasaki, K. Yamagata, S. Komori, M. Nagase, M. Wada, T. Yamamoto, N. Yoshikawa, Promotion of flowering and reduction of a generation time in apple seedlings by ectopical expression of the Arabidopsis thaliana FT gene using the Apple latent spherical virus vector, Plant Mol. Biol. 75 (2011) 193–204, http://dx.doi.org/10.1007/s11103-010-9718-0.
[50] M.C. Dornelas, O. Dornelas, From leaf to flower: revisiting Goethe's concepts on the metamorphosis of plants, Brazilian J. Plant Physiol. 17 (2005) 335–343, http://dx.doi.org/10.1590/S1677-04202005000400001.
[51] G. Theißen, A. Becker, A. Di Rosa, A. Kanno, J.T. Kim, T. Münster, K.-U. Winter, H. Saedler, A short history of MADS-box genes in plants, Plant Mol. Biol. 42 (2000) 115–149, http://dx.doi.org/10.1023/A:1006332105728.
[52] S.D. Folter, G.C. Angenent, trans meets cis in MADS science, Trends Plant Sci. 11 (2006) 224–231, http://dx.doi.org/10.1016/j.tplants.2006.03.008.

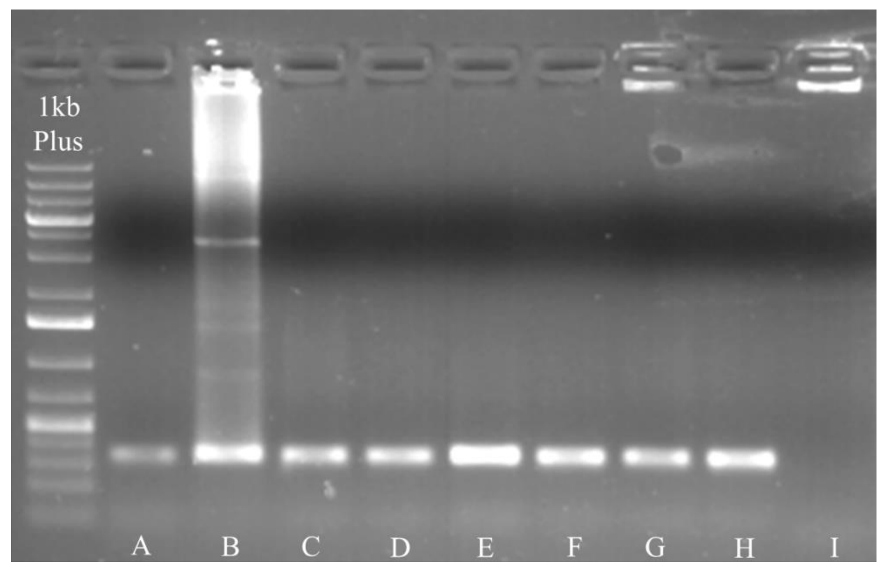
Supplementary material

Supplementary video 1. The treatment procedure. The DNA solution was injected into the base of the bunch vascular system with a hypodermic syringe. A 1:1 proportion with 2 µg of the helper plasmid (p1470, [27]) and 2 µg of the *VviAGL11* overexpression vector (pIRVviAGL11OX) or the *VviAGL11* silencing vector (pIRVviAGL11RNAi) was injected in a 100 µL water based solution.

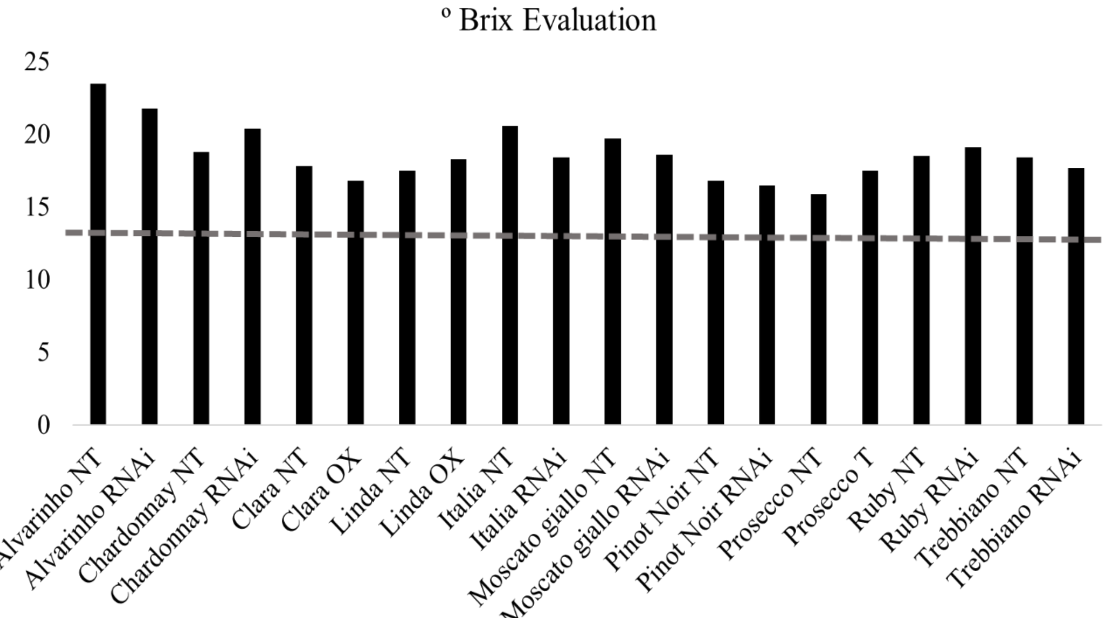
Supplementary figure 1. Stages of grapevine bunch development chosen for treatment with plant plasmids. Initial bunch development stages D and G were mostly used in this study for the first injection of plant plasmids. The H1 stage was used in the second injection. Stage identification was based on Baggiolini [30] phenological scale.



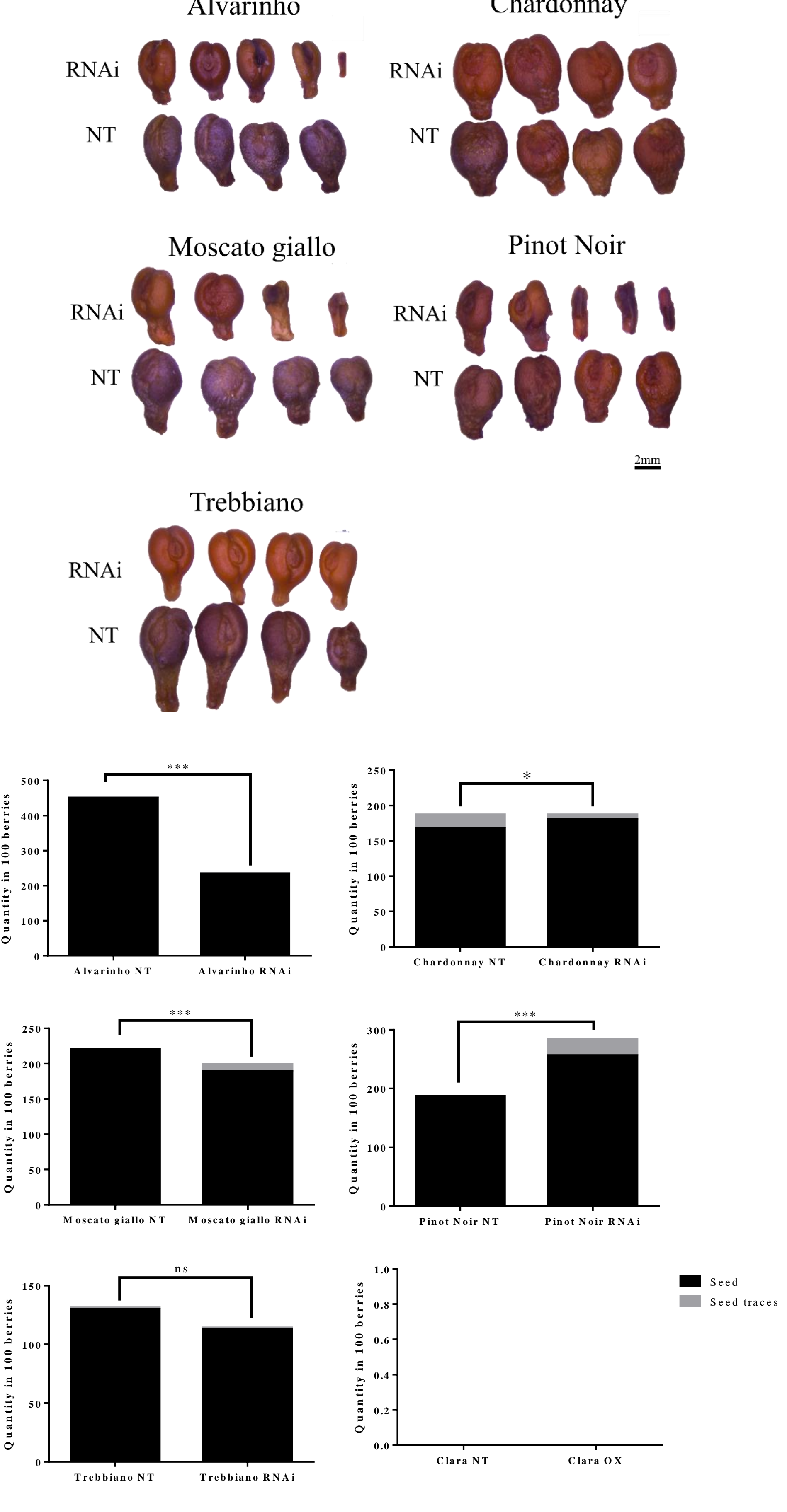
Supplementary figure 2. Reporter genes evaluation on treated tissues with plant plasmids. Grapevine leaves were treated with 1:1 solution of pIR:GFP + p1470 plasmids and after a month it was possible to see GFP fluorescence on the treated leaf (right) (A-B). (A) bright field, (B) GFP filter. (C) Leaf above the treated leaf, demonstrating a systemic presence of the plant plasmids. (D) Tissues from grapevine bunches treated with pIRGUS+p1470. (E) Control samples. Scale bar: (A-C) 2 mm; (D-E) 0,5 cm; (F-G) 10um.



Supplementary figure 3. Gel image of the pIR PCR of the treated cultivars. PCR detection of pIR in treated grapevines. All the cultivars are positive for the pIR presence in their leaves after a month of treatment with the plant plasmids (*pIR* F1 5' – TTGCTCACATGTAATG - 3' e *pIR* R1 5' - AGTTTTTTGTCTTGCA - 3'). First lane: molecular weight marker 1kb plus (Invitrogen, Carlsbad city); lane A: Alvarinho, B: Chardonnay, C: Clara, D: Linda, E: Pinot Noir, F: Ruby, G: Italia, H: Prosecco, I: negative control of the PCR reaction. 1% agarose with ethidium bromide**.**



Supplementary figure 4. °Brix Values for the tested cultivars. The bunches were sampled after complete fruit and berry development. The dashed line on the 13 °Brix represents the establishment of the maturation stage. OX= Overexpression, RNAi= silencing, NT= non-treated and T= treated.



Alvarinho
Chardonnay
RNAi
NT
Moscato giallo
Pinot Noir
2mm
Trebbiano
***
*
***
***
ns
Quantity in 100 berries
Alvarinho NT
Alvarinho RNAi
Chardonnay NT
Chardonnay RNAi
Moscato giallo NT
Moscato giallo RNAi
Pinot Noir NT
Pinot Noir RNAi
Trebbiano NT
Trebbiano RNAi
Clara NT
Clara OX
Seed
Seed traces

Supplementary figure 5. Seeds and seed traces quantitative evaluation. The upper panel shows images from seeds and seed traces morphological analysis. The lower panel shows a graphic comparison between treated and not treated cultivars showing the number of seed and seed traces. This data was achieved by counting this structures in 100 berries per cultivar. OX= Overexpression, RNAi= silencing, NT= non-treated and T= treated. ns: not significant. (Alvarinho $\chi 2$ = 68,21; Chardonnay $\chi 2$ = 5,67; Moscato giallo $\chi 2$ = 19,64; Pinot Noir $\chi 2$ = 11,05)

5 CAPÍTULO III

Desenvolvimento de marcadores moleculares com potencial para a seleção assistida de videiras apirênicas

Desenvolvimento de marcadores moleculares com potencial para a seleção assistida de videiras apirênicas

Resumo

O desenvolvimento de novas cultivares de videiras apirênicas é uma prioridades dos programas de melhoramento de uvas de mesa, e o método baseado na seleção assistida por marcadores moleculares (MAS) é de grande importância agronômica. Estudos sobre os mecanismos moleculares que controlam a morfogênese de sementes demonstraram evidências que sustentam o papel essencial do gene *VviAGL11* no desenvolvimento da semente de videira. O objetivo deste trabalho foi desenvolver marcadores moleculares do tipo *SNPs* e *INDELs* baseados nos alelos do gene *VviAGL11* e avaliar o potencial uso desses marcadores na seleção assistida de apirenia em indivíduos e populações de videiras. Inicialmente, a avaliação de uma população segregante para apirenia com cinco marcadores SSR foi realizada e demonstrou que a porção distal do cromossomo 18, onde está localizado o gene *VviAGL11* possui alta associação com a ausência de sementes, sendo que três marcadores microssatélites, VMC7F2, P3_VVAGL11 e VVIN16, foram significativamente associados à apirenia e poderiam ser usados na forma de haplótipos para seleção assistida. Baseados em mutações do tipo *SNPs* e *INDELs* alelo-específicas de *VviAGL11,* nove marcadores foram desenvolvidos e testados por meio do método da PCR competitiva alelo-específica (KASP®) em indivíduos apirênicos e em uma população de videiras. Para os marcadores VvAGL11_KASP_2, VvAGL11_KASP_3, VvAGL11_KASP_8 e VvAGL11_KASP_9 foram observados polimorfismos segregando nos indivíduos apirênicos genotipados (A_T / T_C / G_T / G_A), respectivamente. Os outros marcadores testados (VvAGL11_KASP_1, 4, 5, 6 e 7) foram homozigotos nesses indivíduos. Os resultados de associação de marcadores com a apirenia em videira (VvAGL11_KASP_ 2, 3, 8 e 9), sugerem a potencialidade do uso dessas marcas em uma estratégia de seleção assistida rápida e eficaz para videiras sem sementes.

Palavras-chave: Apirenia, seleção assistida por marcadores, KASP, *V. vinifera*.

Introdução

A viticultura é uma atividade tradicional em países de clima temperado e nas últimas décadas ganhou grande importância em várias regiões de clima tropical e subtropical. Esta característica abre perspectivas para uma grande expansão na viticultura tropical, principalmente para a produção de uvas de mesa para consumo *in natura* (AMARAL *et al*., 1999). A produção mundial de uva de mesa atingiu 2,1 milhões de toneladas em 2010 (FAO, 2010) e a ausência de sementes, denominada apirenia, é uma das característica mais apreciadas pelos consumidores (BERGAMINI *et al*., 2013). Independentemente do crescente mercado mundial de uvas *in natura*, com até 26% de aumento ao ano nas últimas três décadas, há apenas um crescimento anual de 13% na produção de uvas apirênicas (FAO, 2012; OIV, 2012; LAZZAROTTO & FIORAVANÇO, 2012). Portanto, faz-se necessário uma adaptação do setor produtivo de uvas de mesa para suprir a demanda por esse produto. Por essa razão, muitos programas de melhoramento concentraram esforços na geração de uvas de mesa combinando a apirenia com outras características de interesse agronômico, como o aumento no tamanho das bagas e o sabor de Moscatel (VAROQUAUX *et al*. 2000).

Uma planta é considerada apirênica quando é capaz de produzir frutos sem sementes, com traços de sementes ou com reduzido número e tamanho de sementes (VAROQUAUX *et al*., 2000). Uma cultivar de videira apirênica pode apresentar uma ou mais das características citadas. Além disso, existem duas formas de apirenia: partenocarpia e estenoespermocarpia (STOUT, 1936). Na partenocarpia, o ovário é capaz de se desenvolver em fruto sem que ocorra a fecundação dos óvulos, apenas a partir do desenvolvimento de tecidos maternos, caracterizando seus frutos pela ausência total de sementes (BOUQUET & DANGLOT, 1996). Em contraponto, na estenoespermocarpia a fecundação ocorre normalmente e o desenvolvimento da semente se inicia. No entanto, após algumas semanas, o embrião imaturo morre devido à malformação do endosperma. Esse fato caracteriza a estenoespermocarpia pela produção de frutos com apenas traços de sementes o que faz desta forma de apirenia a mais selecionada em programas de melhoramento de uva de mesa pela possibilidade da aplicação de técnicas de cultura *in vitro* baseadas no resgate do embrião (VAROQUAUX *et al*, 2003; MALABARBA *et al*, 2017).

Para auxiliar na seleção de cultivares elite, melhoristas de videiras podem utilizar a seleção assistida por marcadores moleculares (do inglês, **M**arker **A**ssisted **S**election, **MAS**), que é baseada em marcadores de DNA derivados de pesquisas em biologia molecular e genômica

(BERGAMINI *et al*., 2013). Os marcadores microssatélites se tornaram um tipo popular de marcador molecular para estudar genomas de plantas e ainda são o sistema escolhido para várias aplicações, como estudos de mapeamento, diversidade genética, estrutura genética, impressão digital de indivíduos e estudos de parentesco (JAKSE *et al*., 2013). Os microssatélites de repetições de sequências simples (do inglês, ***S****ingle* ***S****equence* ***R****epeat*, **SSR**) são os marcadores mais eficazes para a genotipagem de videira devido a sua elevada taxa de polimorfismo, o que se reflete em padrões multialélicos em um *locus* específico (THOMAS & SCOTT, 1993; CIPRIANI, 1994; SEFC *et al*., 2000; JAKSE *et al*., 2013).

Contudo, surgiram recentemente novas técnicas que forneceram vários avanços na metodologia de genotipagem, permitindo automatizar parcialmente o processo de seleção, preencher dados em tempo real e comparar e armazenar os dados de genotipagem de uma maneira fácil e eficiente (JAKSE *et al*., 2013). Este é o caso da tecnologia KASP® baseada na reação da polimerase em cadeira (PCR) de forma competitiva e alelo específica. A técnica de KASP combina um sistema homogêneo de informação baseado em fluorescência para a identificação e medição de variações genéticas para detectar polimorfismos de nucleotídeo único (*SNPs*) ou inserções e deleções (*INDELs*) (HE *et al*., 2014).

Recentemente, a compreensão dos mecanismos genéticos e moleculares que controlam a morfogênese de semente em videira foi parcialmente elucidada pela caracterização funcional do gene MADS-box classe D de *Vitis vinifera*, *VviAGL11*. A expressão do gene *VviAGL11* foi comprovada como essencial para o desenvolvimento da camada de endotesta da casca da semente, sendo que a diminuição extrema de transcritos desse gene durante a morfogênese da semente ocasiona má formação das camadas tegumentares dessa estrutura e a geração de traços de sementes ao invés de sementes de tamanho normal (MALABARBA *et al*., 2017). Este gene está localizado na porção distal do cromossomo 18, no *locus SdI* (Inibidor do Desenvolvimento de Sementes), que é o principal *locus* de características quantitativas (*QTL*) relacionado à ausência de sementes (MEJÍA *et al*., 2011, MALABARBA *et al*., 2017).

Com o isolamento e sequenciamento alelo-específico de *VviAGL11* em cultivares apirênicas e pirênicas, diferentes mutações em alelos foram identificadas. Um dos alelo de "Sultanine", a cultivar apirênica, apresentou 99% de similaridade com a sequência do genoma, PN40024. No entanto, o outro alelo de *VviAGL11* de 'Sultanine' apresentou um grupo de polimorfismos que o diferencia dos demais, sendo denominado alelo mutante (SU mut). Este conjunto de polimorfismos consistiu em 28 *INDELs* e 105 *SNPs* (MALABARBA *et al*., 2017).

Mutações como *SNPs* e *INDELs* são a base genética da maioria das variações alélicas e podem ser tratadas como marcadores bialélicos, tendo amplas aplicações em mapeamento genético de alta resolução e em testes diagnósticos (GUIMARÃES *et al*., 2009). Portanto, surgiu a possibilidade da avaliação dessas mutações em uma abordagem de MAS para a apirenia de videiras.

O objetivo que norteou o presente trabalho foi desenvolver novos marcadores moleculares baseados na sequência *VviAGL11*. Assim, avaliamos o potencial biotecnológico de nove marcadores candidatos do tipo *SNP*/*INDELs* pela técnica KASP para a seleção da indivíduos de videira apirênicos.

Material e Métodos

Material vegetal

Noventa e quatro genótipos resultantes da população CNPUV692 ('Villard Blanc' X 'Crimson Seedless') e com segregação para a ausência de sementes foram utilizados para avaliação de marcadores SSR.

Para a avaliação dos marcadores KASP, foram utilizados cem indivíduos da população autofecundada VB ('Villard Blanc X' Villard Blanc '), além de genótipos de Chardonnay, Pinot Noir e Villard Blanc (pirênicos), Sultanine, Concord Seedless e Crimson Seedless (apirênicos). A população VB e os indivíduos utilizados para esse estudo estão localizados na área experimental da Embrapa Uva e Vinho, em Bento Gonçalves, no Rio Grande do Sul, Brasil (29 ° 09 '48 "S, 51 ° 31 '42" W e 616 m de altitude).

O critério de classificação de apirenia seguiu o método descrito por Bouquet e Danglot, 1996, no qual o peso seco de sementes foi utilizado. Neste método são utilizados dados de matéria seca de 100 sementes amostradas aleatoriamente, classificadas na seguinte ordem: classe 1 - muito baixa (40-48 mg / semente); classe 3 - baixa (49-56 mg / semente); classe 5 - média (56-64 mg / semente); classe 7 - alta (64-72 mg / semente) e classe 9 - muito alta (72-80 mg / semente). As variedades que se enquadram nas classes 1 e 3 são as mais desejadas na seleção de uvas de mesa apirênicas, porém as classe 1, 3 e 5 são consideradas igualmente apirênicas.

Avaliação de marcadores SSR

Os indivíduos que compõem a população CNPUV692 foram utilizados neste ensaio. O DNA de cada indivíduo foi purificado conforme descrito por LEFORT & DOUGLAS (1999) e usado em reações de PCR para cada *locus*, conforme descrito por Revers *et al*. (2010). Os marcadores SSR P2_VVAGL11 (MEJÍA *et al*., 2011); P3_VVAGL11 (MEJÍA *et al*., 2011); VMC7F2 (GenBank BV005171); VVIN16 (GenBank BV140662) e UDV108 (GenBank BV097037) foram utilizados (Tabela 1). Os amplicons foram resolvidos em gel de poliacrilamida a 6% e corados com prata, como descrito por CRESTE *et al*. (2001). Os desvios entre as segregações genotípicas observadas e esperadas e as possíveis associações entre os fenótipos e os alelos avaliados foram testados por concordância e independência de qui-quadrado (χ 2).

Tabela 1: Sequência de iniciadores utilizada na avaliação dos marcadores microssatélites.

Marcador molecular SSR	Sequência dos iniciadores forward/reverse	Temperatura de anelamento (ºC)
P2_VVAGL11	TGTACACCAATACGGGTTTCAT/ GTTTGCTGGATTTCCGATGT	61 °C
P3_VVAGL11	CTCCCTTTCCCTCTCCCTCT/ AAACGCGTATCCCAATGAAG	61 °C
VMC7F2	AAGAAAGTTTGCAGTTTATGGTG/ AAGATGACAATAGCGAGAGAGAA	56 °C
VVIN16	ACCTCTATAAGATCCTAACCTG/ AAGGGAGTGTGACTGATATTTC	56 °C
UDV108	TGTAGGGTTCCAAAGTTCAGG/ CCTTTTTATATGTGGTGGAGC	56 °C

Avaliação de marcadores SNP/INDELs

Os nove marcadores do tipo *SNP/INDELs* testados neste trabalho foram escolhidos com base nas mutações de *VviAGL11* identificadas no alelo mutante de Sultanine (SU mut) e denominadas de VvAGL11_Kasp_1 ao 9. Discos foliares dos indivíduos e da população VB avaliada foram coletados e após liofilizados por 24 horas a 4ºC e enviados a empresa LGC Genomics (http://www.lgcgroup.com), Inglaterra. Para realizar a técnica de KASP, o DNA foi

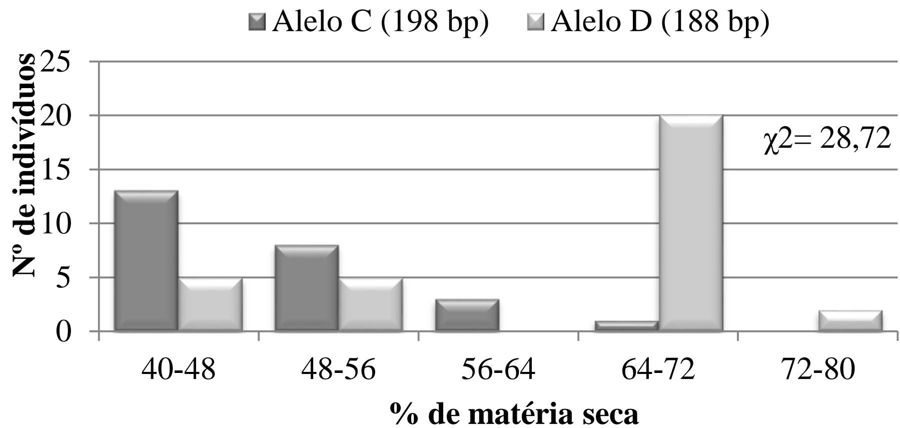
extraído das amostras e três primers foram adicionados na reação de PCR, dois primers forward específicos para cada alelo, cada um com uma sequência única e marcados com fluorescência, e um primer reverso complementar a ambos, permitindo a leitura-ponto final de fluorescência. A fluorescência resultante foi medida e os dados brutos foram interpretados para permitir a atribuição de genótipos a cada amostra de DNA. O sinal fluorescente individual das amostras de DNA é representado como um ponto de dados independente para a identificação da homozigosidade ou heterozigosidade da amostra. Com a obtenção dos resultados foi possível realizar a análise das marcas genotipadas através dos programas *SNP*viewer e Klustercaller, disponibilizados pela empresa (LGC Genomics), conforme visualizado no painel esquerdo da figura 3.

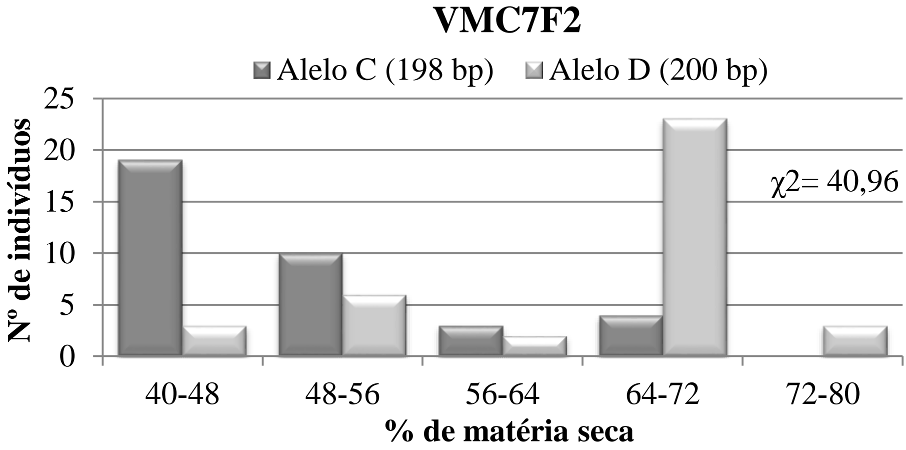
Resultados

Avaliação de marcadores SSR para a ausência de sementes em videira

Os marcadores moleculares SSR selecionados para este estudo (UDV108, VMC7F2, VVIN16, P2_VVAGL11 e P3_VVAGL11) foram previamente descritos como eficientes para a seleção de videiras apirênicas (DOLIGEZ *et al*. 2002, CABEZAS *et al*., 2006, MEJÍA *et al*., 2011). Nossa avaliação da utilização dos marcadores na população CNPUV692, observou que os marcadores UDV108 e P2_VVIAGL11 não apresentaram a segregação esperada e estes foram excluídos da análise. Para os *loci* VMC7F2, VVIN16 e P3_VVIAGL11 foram observados quatro alelos segregantes na população genotipada. A análise da distribuição fenotípica das características avaliadas *versus* a frequência dos alelos dos marcadores SSR genotipados mostra claramente ($p < 0{,}0001$) a associação entre os alelos P3_VVIAGL11 de 198 bp ($\chi 2$ calc = 28,72), VVIN16 de 153 pb ($\chi 2$ calc = 26,64) e VMC7F2 de 198 bp ($\chi 2$ calc = 40,96) com a ausência de sementes em videira (Figura 1).

Por meio da avaliação desses marcadores, foram identificados haplótipos que podem ser utilizados na estratégia de MAS. Ao formarmos o haplótipo VVP, que combina os três marcadores SSR VMC7F2, VVIN16 e P3_VVIAGL11, a seleção de indivíduos apirênicos torna-se 100% precisa, uma vez que elimina a presença de falsos positivos. O mesmo é observado com o uso do haplótipo VP, composto por VVIN16 e P3_VVAGL11, que pode selecionar os indivíduos que apresentem ausência de sementes com 100% de eficiência (Tabela 2).





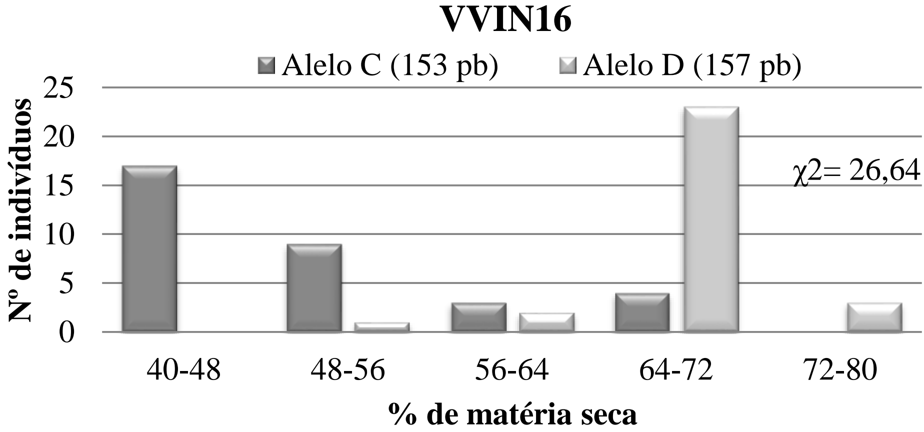


Figura 1: Avaliação dos marcadores moleculares SSR P3_VVAGL11, VMC7F2 e VVIN16. Número de indivíduos e seus alelos associados à apirenia foi calculado por qui-quadrado ($\chi 2$).

Segundo Bouquet e Danglot (1996), os indivíduos que possuem porcentagem de matéria seca baixa (até 64) são consideradas apirênicos. Os indivíduos que se classificam nas classes 7 e 9 (>64) apresentam porcentagem de matéria seca mais elevada e são cultivares consideradas pirênicas.

Tabela 2: Haplótipos de marcadores SSR para seleção assistida de apirenia em videiras. Haplótipo **VVP**= VMC7F2, VVIN16 e P3_VVAGL11; Haplótipo **VP**= VVIN16 e P3_VVAGL11.

	VVP	**VP**	**VMC7F2**
	Tamanho do alelo (pb)		
P3_VVAGL11	**198**	**198**	
VMC7F2	**198**		**198**
VVIN16	**153**	**153**	
Número de indivíduos selecionados	12	23	36
% de indivíduos (sem falso positivo)	100%	100%	91,7%

Desenvolvimento e avaliação de marcadores SNP/INDEL para seleção assistida da apirenia

Visando a melhor exploração do *locus SdI* para a geração de novos e mais eficientes marcadores moleculares para a seleção assistida de apirenia, marcadores do tipo *SNPs* e *INDELs* foram desenvolvidos. Nove sequências gênicas foram selecionados baseadas no sequenciamento completo do *VviAGL11* e na identificação de mutações exclusivas de *SNPs* e *INDELs* para o alelo do cultivar apirênica Sultanine, descrito por Malabarba *et al*., (2017). As nove mutações utilizadas para a genotipagem pela técnica de KASP estão localizadas em diferentes regiões do gene *VviAGL11* (Tabela 3), sendo nomeadas em ordem crescente em relação a sua localização na sequência *VviAGL11*, no sentindo 5' - 3' do gene: VvAGL11_KASP_1; VvAGL11_KASP_2; VvAGL11_KASP_3; VvAGL11_KASP_4; VvAGL11_KASP_5; VvAGL11_KASP_6; VvAGL11_KASP_7; VvAGL11_KASP_8; VvAGL11_KASP_9 (Figura 2 e Figura Suplementar 1). VvAGL11_KASP_ 1 e VvAGL11_KASP_5 são baseados em mutações *INDELs* enquanto os outros marcadores são baseados em *SNPs*. As sequências dos marcadores estão

localizadas em íntrons do gene *VviAGL11*, como VvAGL11_KASP_1, 2, 3, 4, 5 e 7, em éxons, como os marcadores VvAGL11_KASP_6 e 8 e na região 3' UTR, na qual se encontra o marcador VvAGL11_KASP_9 (Figura 2 e Figura Complementar 1).

Tabela 3: Sequências dos marcadores KASP desenvolvidos com base nas mutações do gene *VviAGL11* presentes no alelo de 'Sultanine' (SU mut). Mutação destacada em negrito.

Marcador	Sequência
VvAGL11_ KASP_1	TGATTTGCCTTTTTAATTGAAGACCCTTTTCTGTTGGTAACTTTGTGAAGGCATGAACGAA GTTGATTACTGAARATATTAGCAACATTACTAGGGTTTT**[GGC/-** **]**GGAGATGAGACTGTAAAGTTGGCTAAAACCCTAGAGGCATGTTGNTTTTTAATAATTGG AGATACCATATATGTATGTTGCGAGTGCAG
VvAGL11_ KASP_2	TCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACANATTTTTTCGGTAGATCTTCTCTC ACAAATTACATGCATTATTACCCTTCATNTGTCACTCATACTTCA**[T/A]**TTTNNNNNTTTTT TNTTTTTAAAGCTTGANCAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAGCAGTT TCATAAAGAGATGCTTTGGTAAWTTTTTCTAATATTTTCAGTTGGTGATATAATA
VvAGL11_ KASP_3	AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTCACTG CAGATTATGATTTTTTA**[C/T]**GAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGGATT ACATGGGTACCTGCTTTGATATTTATGATNATTTTCTTTNATCAAAATTTA
VvAGL11_ KASP_4	GGGGAGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATT**[** **T/A]**TATTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAAT CGTATTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAA
VvAGL11_ KASP_5	GATGAAACANACATTATTTGTATTATGTAATCTATTTTANGAACAAAATTTTAAAAATGCT TTAATAANTAGTGAGGATGACTATGATATTTCA[AAAAAAATTATTAATT[/]AAAAAANTT TACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTANATGNCCTTAAAATCACTT ATTTAAATAATAGAAATTAAG
VvAGL11_ KASP_6	TGTTAAAATCATTATNCTACTTTCTTTTTTCTTTTTGGATTATCCCATTGAACTTTCTCGAA TTCTGTNGTTGAACATAGTA**[T/C]**TACCAGCAAGAATCAGCAAAGCTGCGCCAGCAAATA CAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCATTTTTCTCACATG
VvAGL11_ KASP_7	AGACAAGTGATCACTAGATCAGCAAAGCCTATCTTTTTTCCTTTGTTCTAATGTNGTGCTT GTTCTNATGCAATAGCATGAGTTGCTGTTGGCTGAGATTGA**[A/G]**TACTTGCAGAAAAGG GTAATCTCTTGTTCTAAACATCAATTTCCATGTKGGTTCCCTTCTTTTATGATTTTNCCAGA ATTCTTTATCGAGCA
VvAGL11_ KASP_8	TTCATTGAGCTCTGCTGCAYTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGC AAACATGGTRTCAACACATGAGTTYAATGCCATCCAGGCATTAGTTTCTC**[T/G]**CAATTTC TTTCAGCCCAATATGATTGARGGTGGATCCRCAGGCTACCCACTTCCTGATAAGAAGGTC CTCCATCTCGGGTACACTC
VvAGL11_ KASP_9	GTTTTCTTTTGGCTTACAATAAGATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTT GTTTTATGTAGGTAAATGATGG**[A/G]**GGAAAATATCCGCCAACTTCATCTCCTTATGATGT TTAAATAAATCTTTTCCCATGCTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGT TGT

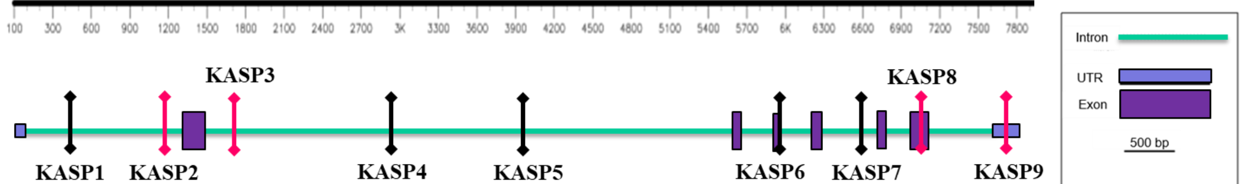


Figura 2. Representação do gene *VviAGL11* gene com a localização dos nove marcadores do tipo KASP. As setas rosas representam os marcadores que demonstraram associação com apirenia (VvAGL11_KASP2; VvAGL11_KASP3; VvAGL11_KASP8 and VvAGL11_KASP9). As setas pretas representam os marcadores KASP que não possuem associação com a ausência de sementes (VvAGL11_KASP1; VvAGL11_KASP4; VvAGL11_KASP5; VvAGL11_KASP6 and VvAGL11_KASP7).

Após avaliação pela técnica KASP, os marcadores VvAGL11_KASP_2; VvAGL11_KASP_3; VvAGL11_KASP_8 e VvAGL11_KASP_9 apresentaram segregação com genótipo heterozigótico mostrando uma correlação com o fenótipo de apirenia (Figura 3). Esses quatro marcadores foram heterozigotos (A_T / T_C / G_T / G_A, respectivamente) para as cultivares apirênicas Sultanine, Concord Seedless e Crimson Seedless e se o genótipo relacionado as cultivares pirênicas Chardonnay, Pinot Noir e a população Villard Blanc autofecundada foi homozigoto (A_A/ T_T/ G_G/ G_G, respectivamente). Interessantemente, o marcador VvAGL11_KASP_8 seleciona os indivíduos pelo *SNP* do alelo *VviAGL11* mutante de 'Sultanine' que ocasiona uma troca de aminoácidos na proteína VviAGL11 no momento da tradução, de Arginina para Leucina (Figura Suplementar 1).

Com a utilização destes marcadores na técnica MAS é possível identificar o genótipo de cada indivíduo, que pode ser homozigoto para o alelo normal sendo assim pirênico, ou pode ser heterozigoto, sendo selecionado como apirênico. Os demais marcadores KASP não apresentaram segregação dos indivíduos analisados, mostrando um genótipo homozigoto em todos os indivíduos exceto na cultivar Sultanine, na qual as mutações foram selecionadas. A cultivar Concord Seedless é descendente da cultivar Concord (Catawba X *Vitis labrusca* L.) e Crimson Seedless é derivada do cruzamento entre Emperor X Fresno C33-199, ambas contendo mutação(ões) ainda não identificada(s) que confere(m) a apirênia ao genótipo Concord Seedless e Crimson Seedless e que aparentemente não está(ão) relacionada(s) aos *SNPs* e *INDELs* utilizados nesse trabalho.

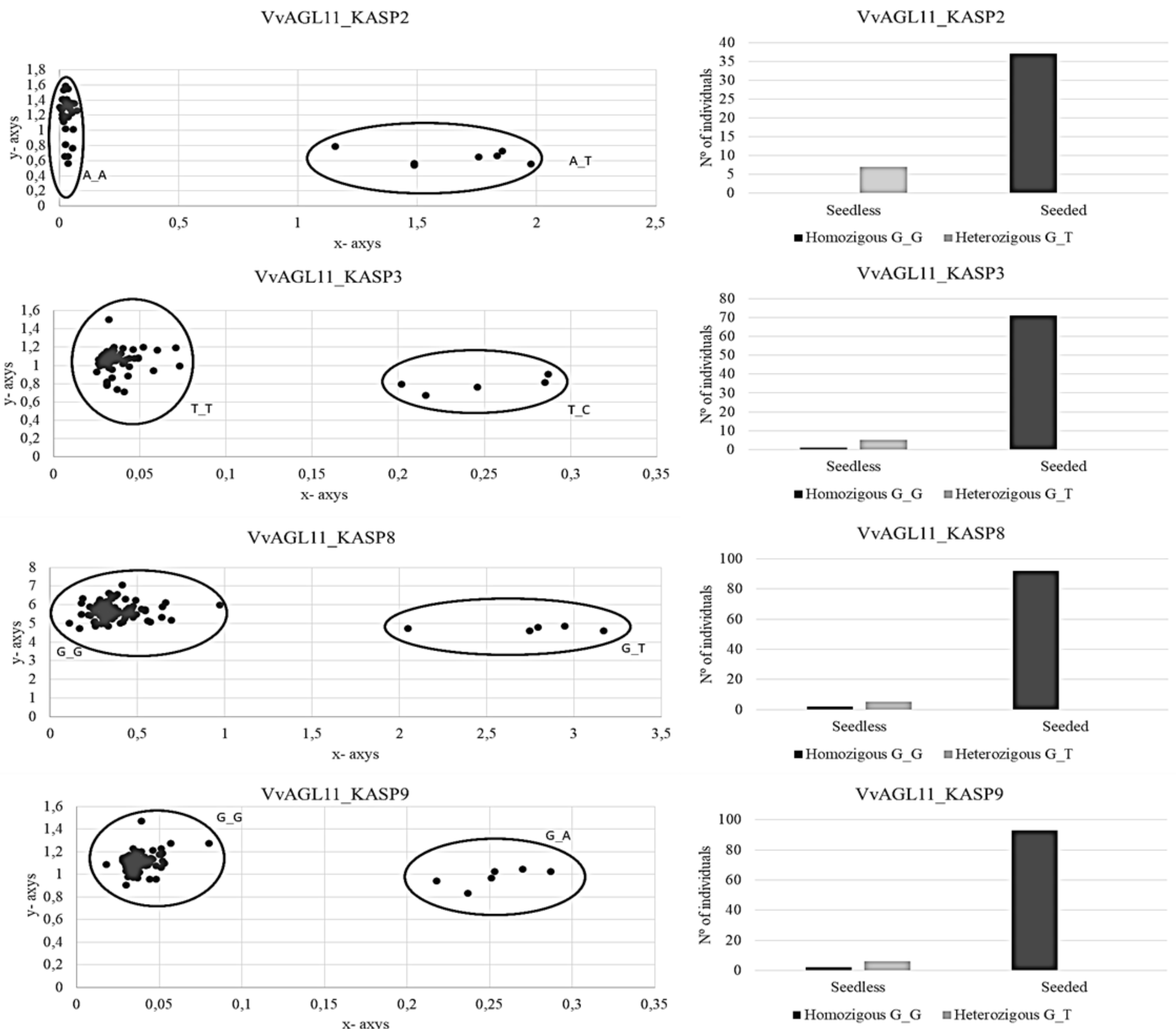


Figura 3. Resultados de marcadores KASP heterozigotos em cultivares apirênicas e na população autofecundada e pirênica VBxVB. O painel esquerdo demonstra os valores da fluorescência de FAM e HEX nos eixos x e y, respectivamente. O painel da direita mostra a relação entre o número de indivíduos versus a característica. Dentro dos círculos estão os indivíduos que foram classificados como pirênicos (grupos densos) e apirênicos (grupos esparsos). Nos grupos esparsos se encontram as cultivares apirênicas, sendo que mais de uma amostra de cada cultivar for analizada.

Discussão

O uso de *QTLs* proporciona uma vantagem para grupos de pesquisa que visam compreender as bases genéticas e moleculares de diferentes processos metabólicos e/ou

estruturais, como é o caso da apirenia. No entanto, além de entender quais são os genes envolvidos na morfogênese das sementes, é imperativo desenvolver métodos para a seleção de indivíduos apirênicos. As aplicações da MAS em programas de melhoramento de plantas são amplas, sendo uma das mais importantes a seleção de características de interesse nas primeiras gerações e em plântulas.

A abordagem de MAS é de grande impacto em programas de melhoramento de espécies perenes, como a videira, macieira, pereira, etc., devido ao longo período de juvenilidade dessas espécies, uma vez que plantas perenes de interesse agronômico requerem diversos anos de acompanhamento a campo para avaliações fenotípicas precisas, especialmente para fenótipos que só podem ser rastreados em plantas que já atingiram a maturação, como os fenótipos desejados em bagas de videira (MEJÍA *et al*., 2011). A partir da seleção de plantas jovens por marcadores moleculares, melhoristas podem descartar indivíduos que apresentam uma combinação genética indesejável ou desfavorável, podendo dedicar seus esforços para a obtenção de outros objetivos.

Neste estudo, a avaliação da apirenia realizada inicialmente por meio dos marcadores microssatélites P2_VVAGL11, P3_VVAGL11, UDV108, VVIN16 e VMC7F2, demonstrou ser uma técnica confiável. Para três dos cinco marcadores SSR a segregação esperada foi obtida e os alelos demonstraram correlação com a presença ou ausência de sementes. Estudos comprovam que marcadores de repetições de sequência simples estão entre os mais confiáveis que podem ser utilizados para MAS, para mapeamento de genoma de videira e para detectar loci de características quantitativas (DOLIGEZ *et al*. 2002; ADAM-BLONDON *et al*. 2004; FANIZZA *et al*., 2005).

Os resultados mostram que o melhor marcador entre os SSRs testados é o marcador VMC7F2. Este marcador está localizado no *locus SdI*, mais precisamente a 463 pb a montante do códon de início da tradução *VviAGL11*, sendo altamente polimórfico e capaz de identificar diferenças entre os alelos do gene *VviAGL11*. A eficácia deste marcador neste estudo foi de 91%, o que está próximo do trabalho de Costantini *et al.* (2008) que descreveu VMC7F2 sendo capaz de selecionar indivíduos de videira apirênicos com 95% de eficácia. A diferença entre a eficácia da seleção pode ser explicada pelo fato de os microssatélites possuírem diferentes taxas de mutação entre *locus* e diversos fatores potenciais podem contribuir para a dinâmica das sequências de microssatélites como o número de repetições, a sequência da repetição, o comprimento de unidades de repetição, interrupções em microssatélites, regiões flanqueadoras e taxa de recombinação (DI RIENZO, *et al*., 1998; JAKSE *et al*., 2013).

Estudos prévios mostraram que o marcador P3_VVAGL11 desenvolvido por Mejía e colaboradores foi validado com 100% de efetividade, tornando-se o melhor para seleção precoce negativa de estenoespermocarpia em populações de *V. vinifera* derivadas de cruzamentos entre indivíduos apirênicos e pirênicos (MIKLOS & JOHN, 1979; AKAGI *et al*., 1996; BERGAMINI *et al*., 2013). No entanto, o marcador P3_VVAGL11 não é capaz de discriminar entre as diferentes classes sem sementes, selecionando apenas os indivíduos que certamente não são apirênicos (BERGAMINI *et al*., 2013). Levando esse fato em consideração, sugerimos a aplicação de haplótipos para seleção inequívoca de videiras sem sementes. Esses haplótipos poderiam ser compostos pelos marcadores P3_VVAGL11, VVIN16 e VMC7F2, ou ainda por P3_VVAGL11 e VVIN16 (Tabela 2), para criar uma alternativa a ser explorada em programas de melhoramento.

Atualmente, técnicas mais eficientes para MAS, do que a análise de SSRs em gel poliacrilamida, têm surgido. Uma delas é o ensaio de genotipagem KASP, que apresenta diversas vantagens, como menor erro de genotipagem em comparação a outras técnicas, geração de dados em grande escala de forma automatizada e genotipagem de menor custo para seleção assistida por marcadores (7.9 - 46.1 % mais barato) (GUIMARÃES *et al*., 2009; SEMAGN *et al*., 2014). A PCR competitiva alelo-específica foi recentemente aplicada na seleção de características de interesse em trigo (CHHETRI *et al*., 2017; TAN *et al*., 2017), soja (SHI *et al*., 2015), milho (SEMAGN *et al*., 2014), arroz (THOMSON *et al*., 2014), e amendoim (KHERA *et al*., 2013) utilizando marcadores do tipo *SNP* em estudos de MAS visando melhorias nessas culturas.

No presente estudo, os marcadores *SNP/INDELs* foram selecionados com base na sequência do gene *VviAGL11* devido ao seu envolvimento no desenvolvimento de sementes e em sua localização no *locus SdI*, o qual explica 50-70% da variância fenotípica da apirênica (BOUQUET & DANGLOT, 1996; DOLIGEZ *et al*., 2002; CABEZAS *et al*., MEJÍA *et al*., 2011). Com a avaliação dos nove marcadores moleculares por meio de KASP foi possível identificar quatro marcadores com segregação diferenciada entre cultivares apirênicas e pirênicas. A forma heterozigótica dos marcadores VvAGL11_KASP_2, 3, 8 e 9 está fortemente associada aos indivíduos apirênicos.

Os resultados apresentados neste estudo contribuem com dados-chave sobre os marcadores relacionados à apirenia e ao gene *VviAGL11,* demonstrando sua potencial aplicabilidade biotecnológica em uma estratégia de seleção assistida por marcadores moleculares. No entanto, outros ensaios devem ser realizados em populações maiores e mais

diversificadas para garantir o uso desses marcadores em uma abordagem confiante de MAS. Com esse objetivo futuro, acessos apirênicos e populações segregantes para apirenia provenientes do Banco de Ativo de Germoplasma (BAG) da Embrapa Uva e Vinho serão genotipadas. Em adição, uma população com ~200 indivíduos, gerada por meio do cruzamento entre as cultivares Villard Blanc X Sultanine nas safras de 2012/2013 e 2013/2014, está em desenvolvimento em campos experimentais da Embrapa Uva e Vinho. Os indivíduos desta população poderão ser fenotipados em relação à presença ou à ausência de sementes nos próximos anos e concomitantemente avaliados quanto ao seus genótipos para os marcadores VvAGL11_KASP_2, 3, 8 e 9.

Referências Bibliográficas

ADAM-BLONDON, A. F.; LAHOGUE-ESNAULT, F.; BOUQUET, A.; BOURSIQUOT, J. M.; THIS, P. Usefulness of two SCAR markers for marker-assisted selection of seedless grapevine cultivars. Vitis, 40: 147–155. 2001.

AMARAL, A. L.; CAMARGO, U. A.; OLIVEIRA, P. R. D. Uva sem sementes: uso da biotecnologia na busca de novas cultivares apirênicas. Biotecnologia Ciência e Desenvolvimento, 2 (10): 108-112. 1999.

ANONYMOUS. Descriptor list for grapevine varieties and Vitis species 243. Organization Internacional de la Vigne et du Vin (OIV), Paris. 1983.

BERGAMINI, C.; CARDONE, M. F.; ANACLERIO, A.; PERNIOLA, R.; PICHIERRI, A.; GENGHI, R., ALBA, V.; FORLEO, R.; CAPUTO, A. R.; MONTEMURRO, C.; BLANCO, A.; ANTONACCI, D. Validation Assay of p3_VvAGL11 Marker in a Wide Range of Genetic Background for Early Selection of Stenospermocarpy in Vitis vinifera L. Molecular Biotechnology 54:1021–1030. 2013.

BOUQUET, A. AND DANGLOT, Y. Inheritance of seedlessness in grapevine (Vitis vinifera L.). Vitis 35: 35-42. 1996.

CABEZAS, J. A.; CERVERA, M. T.; RUIZ-GARCIA, L.; CARRENO, J.; MARTINEZ-ZAPATER, J. M. A genetic analysis of seed and berry weight in grapevine. Genome, 49 (12):1572–1585, 2006.

CHHETRI, M.; BARIANA, H.; WONG, D.; SOHAIL, Y.; HAYDEN, M.; BANSAL, U.; Development of robust molecular markers for marker-assisted selection of leaf rust resistance gene Lr23 in common and durum wheat breeding programs. Molecular Breeding 37: 21. 2017.

CHRISTENSEN, L. P. Raisin Grape Varieties. Pages 38-47 in: Raisin Production Manual. University of California, Agricultural and Natural Resources Publication 3393, Oakland, CA. 2000.

CIPRIANI, G.; MARRAZZO, M. T.; MARCONI, R.; CIMATO, A.; TESTOLIN, R. Microsatellite markers isolated in olive (Olea europaea L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. Theoretical and Applied Genetics, 104 223–228. 2002.

COSTANTINI L., BATTILANA J., LAMAJ F., FANIZZA G., GRANDO M.S. Berry and phenology-related traits in grapevine (Vitis vinifera L.): from quantitative trait loci to underlying genes. BMC Plant Biology 8:38, 2008.

CRESTE, S., TULMANN NETO, A. AND FIGUEIRA, A. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reports 19:299-306. 2001.

DI GENOVA, A.; ALMEIDA, AM.; MUÑOZ-ESPINOZA, C.; VIZOSO, P.; TRAVISANY, D.; MORAGA, C.; PINTO, M.; HINRICHSEN, P.; ORELLANA, A.; MAASS, A. Whole genome comparison between table and wine grapes reveals a comprehensive catalog of structural variants. BMC Plant Biology 7 (14), 1471-2229. 2014.

DOLIGEZ, A., BOUQUET, A., DANGLOT, Y., LAHOGUE, F., RIAZ, S., MEREDITH, C.P., EDWARDS, K.J., THIS, P. Genetic mapping of grapevine (Vitis vinifera L.) applied to the detection of *QTL*s for seedlessness and berry weight. Theoretical and Applied Genetics 105(5):780–795, 2002.

FANIZZA, G.; LAMAJ, F.; COSTANTINI, L.; CHAABANE, R.; GRANDO, M. S. *QTL* analysis for fruit yield components in table grapes (Vitis vinifera). Theoretical and Applied Genetics, 111: 658–664. 2005.

FAO, Food and Agriculture Organization of the United Nations. Disponível em <http://www.faostats.org/>. Acesso em 15 de março de 2018.

GUIMARÃES, C. T.; MAGALHÃES, J. V.; LANZA, M. A.; SCHUSTER, I. Marcadores Moleculares e suas Aplicações no Melhoramento Genético. Informe Agropecuário, Belo Horizonte, v.30, n.253. 2009.

HE, C.; HOLME, J.; ANTHONY, J. *SNP* genotyping: the KASP assay. Volume 1145, 2014, pp 75-86.

JAKŠE, J.; ŠTAJNER, N.; TOMIĆ, L. AND JAVORNIK, B. Application of Microsatellite Markers in Grapevine and Olives. Chapter 2 in The Mediterranean Genetic Code - Grapevine and Olive. http://dx.doi.org/10.5772/3442. 2013.

KHERA, P.; UPADHYAYA, H. D.; PANDEY, M. K.; ROORKIWAL, M.; SRISWATHI, M.; JANILA, P.; GUO, Y.; MCKAIN, M. R.; NAGY, E. D.; KNAPP, S. J. Single nucleotide polymorphism–based genetic diversity in the reference set of peanut (Arachis spp.) by developing and applying cost-effective competitive allele-specific polymerase chain reaction genotyping assays. Plant Genome 6. 2013.

REVERS, L. F.; WELTER, L. J.; IRALA, P. B.; SILVA, D. C.; LAMPE, V. S.; OLIVEIRA, P. R. D.; GARRIDO, L. R. Co-Localization of *QTL*s for seedlessness and downy mildew resistance in grapevine. In: International Conference on Grapevine Breeding and Genetics. v. 10: 58. 2010.

RIBEREAU-GAYON, J. AND PEYNAUD, E. Sciences et techniques de la Vigne. Tome I. Biologie de la Vigne. Sols de Vignobles, Dunod: Paris, Vol. 1, 327. 1980.

ROUNSLEY, S. D.; DITTA, G. S.; YANOFSKY M. F. Diverse roles for MADS box genes in Arabidopsis development. The Plant Cell, 7: 1259–1269. 1995.

RUIJTER, J. M.; RAMAKERS, C.; HOOGAARS, W. M. H.; KARLEN, Y.; BAKKER, O.; VAN DEN HOFF, M. J. B.; MOORMAN, A. F. M. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research 37. 2009.

SEMAGN, K.; BABU, R.; HEARNE S.; OLSEN, M. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Molecular Breeding, v, 33, pp 1-14. 2014.

SEFC, K. M.; LOPES, M. S.; MENDONCA, D.; MACHADO, M.; DA CAMARA MACHADO, A. Identification of microsatellite loci in olive (Olea europaea) and their characterization in Italian and Iberian olive trees. Molecular Ecology; 9, 1171–1173. 2000.

SHI, Z.; LIU, S.; NOE, J.; ARELLI, P.; MEKSEM, K.; LI, Z. *SNP* identification and marker assay development for high-throughput selection of soybean cyst nematode resistance. BMC Genomics 16: 314. 2015.

STOUT, A. B. Seedlessness in grapes. N.Y. State Agric. Expt. Stat. Tech. Bull. 238. 1936.

TAN, C-T.; ASSANGA, S.; ZHANG, G.; RUDD, J. C.; HALEY, S. D.; XUE, Q.; IBRAHIM, A.; BAI, G.; ZHANG, X.; BYRNE, P.; FUENTEALBA, M. P.; LIU, S. Development and validation of KASP markers for wheat streak mosaic virus resistance gene Wsm2. Crop Science 57: 340-349. 2017.

THOMSON, M. J. (2014). High-throughput *SNP* genotyping to accelerate crop improvement. Plant Breed Biotechnology 2: 195-212.

THOMAS, M. R., & SCOTT, N. S. (1993). Microsatellite repeats in grapevine reveal DNA polymorphisms when analyzed as sequence-tagged sites (STSs). Theoretical and Applied Genetics, 86(8), 985-990.

VAROQUAUX, F.; BLANVILLAIN, R.; DELSENY, M.; GALLOIS, P. Less is better: new approaches for seedless fruit production. Trends in Biotechnology, 18: 233–242. 2000.

Material Suplementar

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)

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ch2                  CCACTGATATGGATTGATTTGCCTTTTTAATTGAAGACCCTTTTCTGTTGGTAACTTTGT
VIT_218S0041G01880.2 CCACTGATATGGATTGATTTGCCTTTTTAATTGAAGACCCTTTTCTGTTGGTAACTTTGT
su_wt                CCACTGATATGGATTGATTTGCCTTTTTAATTGAAGACCCTTTTCTGTTGGTAACTTTGT
ch1                  CCACTGATATGGATTGATTTGCCTTTTTAATTGAAGACCCTTTTCTGTTGGTAACTTTGT
                     ************************************************************

su_mut               GAAGGCATGAACGAAGTTGATTACTGAAAATATTAGCAACATTACTAGGGTTTTGGCGGA
su_Di Genova         GAAGGCATGAACGAAGTTGATTACTGAARATATTAGCAACATTACTAGGGTTTTGGCGGA
ch2                  GAAGGCATGAACGAAGTTGATTACTGAAGATATTAGCAACATTACTAGGGTTTT---GGA
VIT_218S0041G01880.2 GAAGGCATGAACGAAGTTGATTACTGAAGATATTAGCAACATTACTAGGGTTTT---GGA
su_wt                GAAGGCATGAACGAAGTTGATTACTGAAGATATTAGCAACATTACTAGGGTTTT---GGA
ch1                  GAAGGCATGAACGAAGTTGATTACTGAAGATATTAGCAACATTACTAGGGTTTT---GGA
                     **************************** *************************   ***
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VvAG11_KASP1

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su_Di Genova           GATGAGACTGTAAAGTTGGCTAAAACCCTAGAGGCATGTTGTTTTTTAATAATTGGAGAT
ch2                    GATGAGACTGTAAAGTTGGCTAAAACCCTAGAGGCATGTTGTTTTTTAATAATTGGAGAT
VIT_218S0041G01880.2   GATGAGACTGTAAAGTTGGCTAAAACCCTAGAGGCATGTTG-TTTTTAATAATTGGAGAT
su_wt                  GATGAGACTGTAAAGTTGGCTAAAACCCTAGAGGCATGTTG-TTTTTAATAATTGGAGAT
ch1                    GATGAGACTGTAAAGTTGGCTAAAACCCTAGAGGCATGTTG-TTTTTAATAATTGGAGAT
                       **************************************** *******************

su_mut                 ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
su_Di Genova           ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
ch2                    ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
VIT_218S0041G01880.2   ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
su_wt                  ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
ch1                    ACCATATATGTATGTTGCGAGTGCAGATATACGTGGAAAGCACAAAAATCCTAACCTGTT
                       ************************************************************

su_mut                 AAACTGTAATTTGGGAAGATATTGTGTTTCTTTTACGTGTTGGATACAACATTGGGTTGT
su_Di Genova           AAACTGTAATTTGGGAAGATATTGTGTTTCTTTTACGTGTTGGATACAACATTGGGTTGT
ch2                    AAACTGTAATTTGGGAAGATATTGTGTTTCTTTTACGTGTTGGATACAACATTGGGTTGT
VIT_218S0041G01880.2   AAACTGTAATTTGGGAAGATATTGTGTTTCTTTTACGTGTTGGATACAACATTGGGTTGT
su_wt                  AAACTGTAATTTGGGAAGATATTGTGTTTCTTTTACGTGTTGGATACAACATTGGGTTGT
ch1                    AAACTGTAATTTGGGAAGATATTGTGTTTCTTTTACGTGTTGGATACAACATTGGGTTGT
                       ************************************************************

su_mut                 GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTATGGTGGGAATTTGGAACT
su_Di Genova           GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTRTGGTGGGAATTTGGAACT
ch2                    GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTATGGTGGGAATTTGGAACT
VIT_218S0041G01880.2   GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTGTGGTGGGAATTTGGAACT
su_wt                  GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTGTGGTGGGAATTTGGAACT
ch1                    GGCCTTGAAGAAAATAAAGAAGAGAAGAAAGTTTGCAGTTTGTGGTGGGAATTTGGAACT
                       **************************************** *******************

su_mut                 GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
su_Di Genova           GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
ch2                    GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
VIT_218S0041G01880.2   GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
su_wt                  GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
ch1                    GCAAAGCTGTTTACATTATAAAACTACTATGTCTTGTATCCGACTTTGGGTAATAAGCCA
                       ************************************************************

su_mut                 TTCGGAAGTCTTTTTCTTCCTCAACCC------CTCTCTCTCTCTCTCTCTTTGAAGAAG
su_Di Genova           TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
ch2                    TTCGGAAGTCTTTTTCTTCCTCAACCCCTCTCTCTCTCTCTCTCTCTCTCTTTGAAGAAG
VIT_218S0041G01880.2   TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
su_wt                  TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
ch1                    TTCGGAAGTCTTTTTCTTCCTCAACCC----CTCTCTCTCTCTCTCTCTCTTTGAAGAAG
                       ***************************      ***************************

su_mut                 TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--CTC
su_Di Genova           TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTCCTCTC
ch2                    TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC-----
VIT_218S0041G01880.2   TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--TTC
su_wt                  TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--TTC
ch1                    TCATTTTCTTCATCTGCACCGCCCATTCTCTCTCGCTATTGTCATCTTTCTCCTC--TTC
                       *******************************************************

su_mut                 TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
su_Di Genova           TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
ch2                    -----TTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCA
VIT_218S0041G01880.2   TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
su_wt                  TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
ch1                    TCTCTCTCTCTCTCTCTCTCTCTCTCTATTGTACTATCTCTCTTCTCTCTCTATCTCTCT
                             *****************************************************

su_mut                 TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCCCTCTCCCTCCCTCTCTCTCTCTCT
su_Di Genova           TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCTCT
ch2                    TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--------CTCCCTCTCCCTCTCT
VIT_218S0041G01880.2   TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCTCT
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su_wt              TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCT
ch1                TTATTGCTCTCTCTCTCCCTTTCCCTCTCCCTCTCC--CTCCCTCTCTCTCTCTCTCT
                   ************************************        * * ***** ******

su_mut             CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTCTCTCTCTTTCA
su_Di Genova       CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
ch2                CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTC------------TCA
VIT_218S0041G01880.2 CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
su_wt              CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
ch1                CAAAGTCACTTTCTACATCTGCACCACCCACTCTCTCTCTCTCTCTC--------TCA
                   *********************************************            ***

su_mut             TATCCGTCTCACATCTCTCCATTGTGGTATATATATATAAGGACTAAACACAAGTCTTAA
su_Di Genova       TATCCGTCTCACATCTCTCCATTGTGGTATATATATATAAGGACTAAACACAAGTCTTAW
ch2                TATCCGTCTCACATCTCTCCATTGTGGTATATATATATAAGGACTAAACACAAGTCTTAA
VIT_218S0041G01880.2 TATCCGTCTCACATCTCTCCATTGTGGTATATATATATAAGGACTAAACACAAGTCTTAA
su_wt              TATCCGTCTCACATCTCTCCATTGTGGTATATATATATAAGGACTAAACACAAGTCTTAA
ch1                TATCCGTCTCACATCTCTCCATTGTGGTATATATATATAAGGACTAAACACAAGTCTTAA
                   ***********************************************************

su_mut             GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
su_Di Genova       GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
ch2                GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
VIT_218S0041G01880.2 GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
su_wt              GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
ch1                GGCACTTTGGCTTCATTGGGATACGCGTTTTTGCACAGCCCGAAATTTTCCGAAAGCTGA
                   ************************************************************

su_mut             AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
su_Di Genova       AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
ch2                AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
VIT_218S0041G01880.2 AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
su_wt              AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
ch1                AGTCGCCGGATTTTGGAAACAAGGTGTGTAGGTGATCTTTTAACAGAAACGTTTCACCAC
                   ************************************************************

su_mut             CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
su_Di Genova       CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
ch2                CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
VIT_218S0041G01880.2 CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
su_wt              CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
ch1                CAAAATTTCCACAATCAACCATTCTCTCTCTGTGAAAACGTTTCGTGCATAACTGGGTAA
                   ************************************************************

su_mut             TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
su_Di Genova       TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
ch2                TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
VIT_218S0041G01880.2 TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
su_wt              TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
ch1                TCTTAGATCTGCTCCCCTCCACACCACAGAATCTACTTTTGCCTACATATGAACATCTGC
                   ************************************************************

su_mut             TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
su_Di Genova       TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
ch2                TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
VIT_218S0041G01880.2 TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
su_wt              TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
ch1                TTTCCATTTCTTCCTCTTTCTTTTGTCAGTGCCCATCTCTCCAAATTTACTTCCACCTCT
                   ************************************************************

su_mut             TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
su_Di Genova       TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
ch2                TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
VIT_218S0041G01880.2 TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
su_wt              TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
ch1                TACATTTTCTTACCATTCTTTTTAGATTTCTTGGCTTGATTTACTCTCTTCTTCCTGCAA
                   ************************************************************

su_mut             CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
su_Di Genova       CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
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ch2                  CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
VIT_218S0041G01880.2 CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
su_wt                CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
ch1                  CACTTTACTCTTCAGTTCTTGATTCTCTTTTGCCTTCTTCATGCATTTGTTTCCATTCTT
                     ************************************************************

su_mut               TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
su_Di Genova         TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
ch2                  TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
VIT_218S0041G01880.2 TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
su_wt                TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
ch1                  TAATTAGTCATTTTCTTATTCTAAACTTTCTTTTCCCTTTTTTCGTTATTTTCAGAATGC
                     ************************************************************

su_mut               AGTTTGATTATTTTGCCTTTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTCTCAG
su_Di Genova         AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTCTCAG
ch2                  AGTTTGATTATTTTGCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
VIT_218S0041G01880.2 AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
su_wt                AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
ch1                  AGTTTGATTATTTTTCC-TTTTTTTCATGTTAAAACAGTATTTGTTTTGGTAGTTTTCAG
                     ************** ** ************************************* ****

su_mut               TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAAGGGAAAAAGCCAGAATTTT
su_Di Genova         TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGARGGGAAAAAGCCAGAATTTT
ch2                  TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAAGGGAAAAAGCCAGAATTTT
VIT_218S0041G01880.2 TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAGGGGAAAAAGCCAGAATTTT
su_wt                TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAGGGGAAAAAGCCAGAATTTT
ch1                  TTTATTTTTTCCATAAATTTCTGTTTCAAAAACCTCTTGAGGGGAAAAAGCCAGAATTTT
                     **************************************** *******************

su_mut               TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
su_Di Genova         TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
ch2                  TTTTCCTGCGTTCTGGTCTTAAGCTTCTTATCCATATCCTAGGTTTATGGGAAATGTGTT
VIT_218S0041G01880.2 TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
su_wt                TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
ch1                  TTTTCCTGCGTTCTGGTCTTAAGCTTCTCATCCATATCCTGGGTTTATGGGAAATGTGTT
                     **************************** *********** *******************

su_mut               AAGGGTCATGGGGTTTTTGAGAAATGTG----GAGAGAGAGAGAGAGAGAGAGAGAGAGA
su_Di Genova         AAGGGTTATGGGGTTTTTGAGAAATGTG----GAGAGAGAGAGAGAGAGAGAGAGAGAGA
ch2                  AAGGGTCATGGGGTTTTTGAGAAATGTGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
VIT_218S0041G01880.2 AAGGGTTATGGGGTTTTTGAGAAATGTG----------------------GAGAGAGAGA
su_wt                AAGGGTTATGGGGTTTTTGAGAAATGTG----------------------GAGAGAGAGA
ch1                  AAGGGTTATGGGGTTTTTGAGAAATGTG----------------------GAGAGAGAGA
                     ****** *********************                      **********

su_mut               GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
su_Di Genova         GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
ch2                  GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
VIT_218S0041G01880.2 GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
su_wt                GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
ch1                  GAGAGAGATGTGTAGACATGCACCCACTTGTAGTAGTATTAGTAGTGGAGATAGATCCTG
                     ************************************************************

su_mut               GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
su_Di Genova         GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
ch2                  GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
VIT_218S0041G01880.2 GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
su_wt                GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
ch1                  GGTTGATTTTTCAAATGGTAGATCATGTTCTCTTCTCTTTGTTCTCATCTCTTCATGTTC
                     ************************************************************

su_mut               TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
su_Di Genova         TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
ch2                  TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
VIT_218S0041G01880.2 TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
su_wt                TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
ch1                  TTTTTTCCTCAGTGGCTTACAGATTTTCTGTCTCCCAATCTTTTGGTTTCCATCTCTTTT
                     ************************************************************
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su_mut                 CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
su_Di Genova           CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
ch2                    CCAAGGTCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
VIT_218S0041G01880.2   CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
su_wt                  CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
ch1                    CCAAGATCTCCTTATTTTCCTACATTATCATCATTATTTTATTATATACACAAGCCTCAC
                       ***** ******************************************************

su_mut                 CCTATTTTTCAAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGA-----
su_Di Genova           CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
ch2                    CCTATTTTTCAAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
VIT_218S0041G01880.2   CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
su_wt                  CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
ch1                    CCTATTTTTCGAACAACATCTAACTTTCTCATTTCTCAGTCTATCATCATTCAGATCTCT
                       ********** ********************************************

su_mut                 --TCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
su_Di Genova           CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
ch2                    CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACAAATTTTTTCGGTAGATCTTC
VIT_218S0041G01880.2   CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
su_wt                  CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
ch1                    CCTCTCTCCTCTCTCCTCTTCACCCTCTACCAAAAACACACATTTTTTCGGTAGATCTTC
                         ************************************** *******************

su_mut                 TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCATTTT-----TT
su_Di Genova           TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAWTTT-----TT
ch2                    TCTCACAAATTACATGCATTATTACCCTTCATGTGTCACTCATACTTCAATTTTTTTATT
VIT_218S0041G01880.2   TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAATTTT----TT
su_wt                  TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAATTTT----TT
ch1                    TCTCACAAATTACATGCATTATTACCCTTCATATGTCACTCATACTTCAATTTT----TT
                       ******************************** **************** ***     **
                                                                  VvAG11_KASP2

su_mut                 TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
su_Di Genova           TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
ch2                    TTTTATTTTTAAAGCTTGATCAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
VIT_218S0041G01880.2   TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
su_wt                  TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
ch1                    TTTTTTTTTTAAAGCTTGAACAGATCTCCAACTCTTTCTCTAGTATTTTGTAATCTTTAG
                       **** ************** ****************************************

su_mut                 CAGTTTCATAAAGAGATGCTTTGGTAATTTTTTCTAATATTTTCAGTTGGTGATATAATA
su_Di Genova           CAGTTTCATAAAGAGATGCTTTGGTAAWTTTTTCTAATATTTTCAGTTGGTGATATAATA
ch2                    CAGTTTCATAAAGAGATGCTTTGGTAATTTTTTCTAATATTTTCAGTTGGTGATATAATA
VIT_218S0041G01880.2   CAGTTTCATAAAGAGATGCTTTGGTAAATTTTTCTAATATTTTCAGTTGGTGATATAATA
su_wt                  CAGTTTCATAAAGAGATGCTTTGGTAAATTTTTCTAATATTTTCAGTTGGTGATATAATA
ch1                    CAGTTTCATAAAGAGATGCTTTGGTAAATTTTTCTAATATTTTCAGTTGGTGATATAATA
                       *************************** ********************************

su_mut                 AAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
su_Di Genova           WAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
ch2                    TAACAATGTATAGTAGTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
VIT_218S0041G01880.2   TAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
su_wt                  TAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
ch1                    TAACAATGTATAGTACTACAAAAATTTTGTTCTTATATACCTAAGCCATTTTTTCTTTTG
                       ************** ********************************************

su_mut                 GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
su_Di Genova           GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
ch2                    GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
VIT_218S0041G01880.2   GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
su_wt                  GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
ch1                    GTGCAATAAACCTAACAGTGTAGTGAACATGGGGAGAGGAAAGATCGAGATCAAGAGGAT
                       ************************************************************

su_mut                 CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
su_Di Genova           CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
ch2                    CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
VIT_218S0041G01880.2   CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
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su_wt                CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
ch1                  CGAAAACACGACCAACCGTCAGGTCACATTCTGCAAGCGAAGGAATGGGCTTTTGAAGAA
                     ************************************************************

su_mut               GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
su_Di Genova         GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
ch2                  GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
VIT_218S0041G01880.2 GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
su_wt                GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
ch1                  GGCTTATGAATTATCAGTGCTATGTGATGCAGAAGTTGCCCTCATCGTCTTCTCCAGCCG
                     ************************************************************

su_mut               CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
su_Di Genova         CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
ch2                  CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
VIT_218S0041G01880.2 CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
su_wt                CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
ch1                  CGGTCGAGTCTATGAGTACTCAAACAACAAGTAATAATTTTCTCCACCATTTCTTCAACC
                     ************************************************************

su_mut               ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
su_Di Genova         ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
ch2                  ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACTGGCAATTAAT
VIT_218S0041G01880.2 ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
su_wt                ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
ch1                  ATCTGCTGAATTTTCTATGTTCATCTTTCTCCATTTTGGTGCATCATACGGGCAATTAAT
                     ************************************************* **********

su_mut               GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
su_Di Genova         GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
ch2                  GGTTTTATATTGATGAGATTAATATCATTAGAAGTTGCAGAACCCTAATGTTGTTAAAGC
VIT_218S0041G01880.2 GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
su_wt                GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
ch1                  GGTTTTATATTGATGAGATTAATATCATCAGAAGTTGCAGAACCCTAATGTTATTAAAGC
                     **************************** *********************** *******

su_mut               AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
su_Di Genova         AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
ch2                  AAAGAAAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
VIT_218S0041G01880.2 AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
su_wt                AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
ch1                  AAAG-AAAAAAAAAAAGAACAGTGGAAGGAGGAGATGCAATAAATTTGAAAATCTAACTC
                     **** *******************************************************

su_mut               ACTGCAGATTATGATTTTTTACGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
su_Di Genova         ACTGCAGATTATGATTTTTTAYGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
ch2                  ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
VIT_218S0041G01880.2 ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
su_wt                ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
ch1                  ACTGCAGATTATGATTTTTTATGAGGGGAGTCAGATTCCTTTTGCTTTATTTAGGAAGGG
                     ********************* **************************************
                                    VvAG11_KASP3
su_mut               ATTACATGGGTACCTGCTTTGATATTTATGAT-ATTTTCTTTCATCAAAATTTAATTAAT
su_Di Genova         ATTACATGGGTACCTGCTTTGATATTTATGAT-ATTTTCTTTCATCAAAATTTAATTAAT
ch2                  ATTACATGGGTACCTGCTTTGATATTTATGAT-ATTTTCTTTCATCAAAATTTAATTAAT
VIT_218S0041G01880.2 ATTACATGGGTACCTGCTTTGATATTTATGATAATTTTCTTTTATCAAAATTTA---AAT
su_wt                ATTACATGGGTACCTGCTTTGATATTTATGATAATTTTCTTTTATCAAAATTTA---AAT
ch1                  ATTACATGGGTACCTGCTTTGATATTTATGATAATTTTCTTTTATCAAAATTTA---AAT
                     ******************************** ********* ***********   ***

su_mut               GAAGGAAAATGCAGATAATATTCTGACATTTCATCAGGCATCTGCTGATGAATCTGAGAA
su_Di Genova         GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
ch2                  GAAGGAAAATGCAGATAATATTCTGACATTTCATCAGGCATCTGCTGATGAATCTGAGAA
VIT_218S0041G01880.2 GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
su_wt                GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
ch1                  GAAGGAAAATGCAGATAATATTCTGACATTTCATCTGGCATCTGCTGATGAATCTGAGAA
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su_mut               AACAATGTCTTCTTCAAGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
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su_Di Genova         AACAATGTCTTCTTCAAGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
ch2                  AACAATGTCTTCTTCATGATTTTTCACTAATTGCATCTTTGACTGATATAAGGAAAATAT
VIT_218S0041G01880.2 AACAATTTCTTCTTCATGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
su_wt                AACAATTTCTTCTTCATGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
ch1                  AACAATTTCTTCTTCATGATTTTTCACTAATTACATCTTTGACTGATATAAGGAAAATAT
                     ****** ********* *************** ***************************

su_mut               GAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
su_Di Genova         SAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
ch2                  CAGAAAGAACACTTTACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
VIT_218S0041G01880.2 CAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
su_wt                CAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
ch1                  CAGAAAGAACACTATACCTATAGTTAGAAACTTCTTCAAAGATTAACTATCAGAGGGTTC
                      ************ **********************************************

su_mut               TTTTTATTGTATGTTTAGTGCATTAAAA-----AAAAATTATAATGAAAAAATAAAAATA
su_Di Genova         TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
ch2                  TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
VIT_218S0041G01880.2 TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
su_wt                TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
ch1                  TTTTTATTGTATGTTTAGTGCATTAAAAAAAATAAAAATTATAATGAAAAAATAAAAATA
                     ****************************     ***************************

su_mut               AAATA-GCAAAAAAACCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTACTGA
su_Di Genova         AAATAGGAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
ch2                  AAATAGGAAAAAAAACCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTACTGA
VIT_218S0041G01880.2 AAATAGGAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
su_wt                AAATAGGAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
ch1                  AAATAGGAAAAAAAATCGAAAAAGTTGAAATCCAACTATCAGTTACACTGGTAGTGCTGA
                     ***** * ******* *************************************** ****

su_mut               TATGCATGCAAGGGAAGACAATCTAGGCAACAGCCAGTTAGGGTTTCTTGTCCTAGCTAG
su_Di Genova         TATGCATGCAAGGGAAGACAATCTAGGCAACARCCAGTTAGGGTTTCTTGTCCTAGCTAG
ch2                  TATGCATGCAAGGGAAGACAATCTAGGCAACAGCCAGTTAGGGTTTCTTGTCCTAGCTAG
VIT_218S0041G01880.2 TATGCATGCAAGGGAAGACAATCTAGGCAACAACCAGTTAGGGTTTCTTGTCCTAGCTAG
su_wt                TATGCATGCAAGGGAAGACAATCTAGGCAACAACCAGTTAGGGTTTCTTGTCCTAGCTAG
ch1                  TATGCATGCAAGGGAAGACAATCTAGGCAACAACCAGTTAGGGTTTCTTGTCCTAGCTAG
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su_mut               TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
su_Di Genova         TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
ch2                  TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
VIT_218S0041G01880.2 TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
su_wt                TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
ch1                  TTCTTCCCATCTCTCCTTACTGTTCCTTTCCAATAATATTTAACATTGTAATCTGTGAGA
                     ************************************************************

su_mut               ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
su_Di Genova         ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
ch2                  ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
VIT_218S0041G01880.2 ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
su_wt                ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
ch1                  ATCTGTATTCCACTGTCCACAGTACAGTATTTAAGAAAAGGGTTATGGCCCTTTTCCTCT
                     ************************************************************
su_mut               TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
su_Di Genova         TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
ch2                  TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
VIT_218S0041G01880.2 TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
su_wt                TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
ch1                  TCCACTCTTTGTTCAAGTCTCAGTTTCTCTGCTCTTCTCTTTCCATTTCCAGCTTTGGGA
                     ************************************************************

su_mut               GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
su_Di Genova         GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
ch2                  GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
VIT_218S0041G01880.2 GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
su_wt                GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
ch1                  GGCTTGGAGCCCATGTATCATAAATCCTTCCCTTGTTTTTCTCCATCTTTTTGTTTTGGG
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su_mut              TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
su_Di Genova        TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
ch2                 TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
VIT_218S0041G01880.2 TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
su_wt               TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
ch1                 TTTCTTCAGCCTAAAAGCTGTGAACCTTTCAATGGAGTCTTTTGGTCCTCTCTTCTCTTT
                     ************************************************************

su_mut              CCCATTCTCATTTAAAGTGCAGTAAAGTGATCACCTTTCACTGCTCTTTGTTTTTTTCAC
su_Di Genova        CCCATTCTCATTTAAAGTGCAGTAAAGTGATCASCTTTCACTGCTCTTTGTTTTTTTCAC
ch2                 CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
VIT_218S0041G01880.2 CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
su_wt               CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
ch1                 CCCATTCTCATTTAAAGTGCAGTAAAGTGATCAGCTTTCACTGCTCTTTGTTTTTTTCAC
                     ********************************* **************************

su_mut              TTGGTGTGGGCAATTTGTGGATATCAAA--TCAGAAATGGGACGGTGTACATCAGCACCC
su_Di Genova        TTGGTGTGGGCAATTTGTGGATATCAAA--TCAGAAATGGGACGGTGTACATCAGCACCC
ch2                 TTGGTGTGGGCAATTTGTGGATATCAAA--TCAGAAATGGGACGGTGTACATCAGCACCC
VIT_218S0041G01880.2 TTGGTGTGGGCAATTTGTGGATATCAAATCTCAGAAATGGGACGGTGTACATCAGCACCC
su_wt               TTGGTGTGGGCAATTTGTGGATATCAAATCTCAGAAATGGGACGGTGTACATCAGCACCC
ch1                 TTGGTGTGGGCAATTTGTGGATATCAAATCTCAGAAATGGGACGGTGTACATCAGCACCC
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su_mut              ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
su_Di Genova        ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGMTCAA
ch2                 ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
VIT_218S0041G01880.2 ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
su_wt               ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
ch1                 ATTACATCTTTGTGTGGGTTTGAATAAATATTTCATATCAATCATTTCATGTTTGCTCAA
                     ******************************************************* ****

su_mut              TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCCCGATTTCTCCCTT
su_Di Genova        TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCCCGATTTCTCCCTT
ch2                 TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
VIT_218S0041G01880.2 TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
su_wt               TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
ch1                 TGCCATCCAAGATTTTTTTTCTTCACTTCATGTCTCATTTTCCTCCTTGATTTCTCCCTT
                     **********************************************  ************

su_mut              GTTCAAGGAAAATTGAAGATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
su_Di Genova        GTTCAAGGAAAATTGAAGATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
ch2                 GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
VIT_218S0041G01880.2 GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATCGAT
su_wt               GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
ch1                 GTTCAAGGAAAATTGAAAATCTTCTCATAGTTGAGATAGTATTTAAAATAGGACATTGAT
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su_mut              CTTACATGAGATTTTACTTTTTTGTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
su_Di Genova        CTTACATGAGATTTTACTTTTTTGTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
ch2                 CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
VIT_218S0041G01880.2 CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
su_wt               CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
ch1                 CTTACATGAGATTTTACCTTTTTTTTTTCAAAATTTTAAATTATTTTTTATACTTTTTAA
                     ***************** ***** ************************************

su_mut              AAATACTTGTTTTTACCCTTAATCAATTAACAAACAAAAAAATAAAATGAAAAAAAAGAC
su_Di Genova        AAATACTTGTTTTTACCCTTAATCAATTAACAAACAAAAAAATAAAATGAAAAAAAAGAC
ch2                 AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAATGA-AAAATG-AAAAAAAGAC
VIT_218S0041G01880.2 AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAATGA-AAAATG-AAAAAAAGAC
su_wt               AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAATGA-AAAATG-AAAAAAAGAC
ch1                 AAATACTTGTTTTTACCCTTAATCAATTAACAAAAAAAATGA-AAAATG-AAAAAAAGAC
                     ********************************** ****  * ****** **********

su_mut              CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA-------------TGGGGGTGGGGGG
su_Di Genova        CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA-------------TGGGGGTGGGGGG
ch2                 CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------TGGGGGTGGGGGTGGAGGGG
VIT_218S0041G01880.2 CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCATGGGGGTGGGGGTGGGGGTGGAGGGG
su_wt               CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------TGGGGGTGGGGGTGGAGGGG
ch1                 CAATTTGAATTTTTTTTTTAATGTTGAGGGTGCA------TGGGGGTGGGGGTGGAGGGG
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su_mut                AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTTTA
su_Di Genova          AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTWTA
ch2                   AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
VIT_218S0041G01880.2  AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
su_wt                 AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
ch1                   AGAAGTCTTCCTTATTATTTATTAATTTTAATGCATTATTTTTAGTCAAATCCAATTATA
                      ********************************************************* **
                                                                         VvAG11_KASP4

su_mut                TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
su_Di Genova          TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
ch2                   TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
VIT_218S0041G01880.2  TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
su_wt                 TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
ch1                   TTGCATAAAATTAATTAACATCATCTTTGATCATCTTTAAATCATATGGATTAAATCGTA
                      ************************************************************

su_mut                TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
su_Di Genova          TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
ch2                   TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
VIT_218S0041G01880.2  TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
su_wt                 TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
ch1                   TTGTTGCTTATTTTCCTTATTCAAATAGAATAAAAATTATTTTAAGATTCTTTATTCTTT
                      ************************************************************

su_mut                ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
su_Di Genova          ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
ch2                   ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
VIT_218S0041G01880.2  ATATATGAGAGAAAAATAGAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
su_wt                 ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
ch1                   ATATATGAGAGAAAAATAAAAGAAATAAATTTGATAAAATATGTGAAAAAATTATTAAGT
                      ***************** ******************************************

su_mut                TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCATTTTTTTTTTGTTTCTTTTTTTTC
su_Di Genova          TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCATTTTTTTTTTGTTTCTTTTTTTTC
ch2                   TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
VIT_218S0041G01880.2  TCAAGCTTTTTTTATTTATTTTTTA-CTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
su_wt                 TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
ch1                   TCAAGCTTTTTTTATTTATTTTTTATTTTTTCATCA-TTTTTTTTTGTTTCTTTTTTTTC
                      *************************  ********* ***********************

su_mut                TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
su_Di Genova          TCTCTCTTAAATTTTYGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
ch2                   TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
VIT_218S0041G01880.2  TCTCTCTTAAATTTTCGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
su_wt                 TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
ch1                   TCTCTCTTAAATTTTTGAAGATTTAAATGCAACCCCTACACTTTACACCTAAGAGAGTTT
                      *************** ********************************************

su_mut                GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
su_Di Genova          GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
ch2                   GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
VIT_218S0041G01880.2  GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
su_wt                 GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
ch1                   GAGGAAAAGTAGGAGAAAAAGAAAACAAAGAAGAAAAATGAAAGAAAGAAAAAGAAAAAT
                      ************************************************************

su_mut                AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
su_Di Genova          AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
ch2                   AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
VIT_218S0041G01880.2  AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
su_wt                 AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
ch1                   AAAGAAATATGTTTAAATTCAATAAATTGTTATTACTTATTTTTCTAAAATTATTTTTAT
                      ************************************************************

su_mut                TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
su_Di Genova          TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
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ch2                    TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
VIT_218S0041G01880.2   TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
su_wt                  TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
ch1                    TCTTTTCTCTCATATACAACCAAACAAAATTTCAAAAATGTAAAATTTTTGATATAGTTT
                       ************************************************************

su_mut                 TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
su_Di Genova           TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACY
ch2                    TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
VIT_218S0041G01880.2   TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACT
su_wt                  TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
ch1                    TCATTAGATTTGATTTTTCATACTTTCCATAATAATCCAAACAAGATCAATTAATAAACC
                       *********************************************************** 

su_mut                 GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
su_Di Genova           GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
ch2                    GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
VIT_218S0041G01880.2   GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
su_wt                  GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
ch1                    GAAACAAAACCTACAATGACTTCATAATAACGGAAGTTTAAAGGAACTAAGGATGAATTG
                       ************************************************************

su_mut                 AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
su_Di Genova           AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
ch2                    AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
VIT_218S0041G01880.2   AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
su_wt                  AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
ch1                    AGATAGATTCGAGGATGGTGTCATAATGATAGGAGTCCAAATTTGGAGGTAAGAATAAAC
                       ************************************************************

su_mut                 TTAAATAAACTCTAAGATGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
su_Di Genova           TTAAATAAACTCTAAGATGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
ch2                    TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
VIT_218S0041G01880.2   TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
su_wt                  TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
ch1                    TTAAATAAACTCTAAGACGATGCTCCAATGATGAATTTCTTTTTTAAAATATTTGAGTAG
                       ***************** ******************************************

su_mut                 ACATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATTATCCCT
su_Di Genova           GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
ch2                    GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
VIT_218S0041G01880.2   GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
su_wt                  GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
ch1                    GCATTTGTCATTAATTTTTCTAACTACAAATAGATTACCAATAAAATGATCATAATCCCT
                        ***************************************************** ******

su_mut                 TTCTACAGAACTTGTCGATTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
su_Di Genova           TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
ch2                    TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
VIT_218S0041G01880.2   TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
su_wt                  TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
ch1                    TTCAACCGAACTTGTCGGTTGTTGTATTGTTTTTTATGATATTTTTATGTAACATGTTAA
                       *** ** ********** ******************************************

su_mut                 ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
su_Di Genova           ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
ch2                    ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
VIT_218S0041G01880.2   ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
su_wt                  ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
ch1                    ATTATAAAATAAATAAATAAGTTATAAGAAGAAAGAAAAAACCAATTCAATTCCAATAAA
                       ************************************************************

su_mut                 AACCTTTGAAATTAATAAATGGTGGGATATGTG--AGAGGTGCAAAGTCTTGGGTTTGAT
su_Di Genova           AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTTGAT
ch2                    AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
VIT_218S0041G01880.2   AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
su_wt                  AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
ch1                    AACCTTTGAAATTAATAAATGGTGGGATATGTGATAGAGGTGCAAAGTCTTGGGTTCAAT
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su_mut               TACCATCATTAAGAATCCCTGGATTACTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
su_Di Genova         TACCATCATTAAGAATCCCTGGATTACTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
ch2                  TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
VIT_218S0041G01880.2 TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
su_wt                TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
ch1                  TACCATCATTAAGAATCCCTGGATTATTCGGAGTTGATTCTAATGGGTGTCGTTGGAATC
                     ************************** *********************************

su_mut               CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGGT
su_Di Genova         CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGGT
ch2                  CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGGT
VIT_218S0041G01880.2 CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGTT
su_wt                CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGTT
ch1                  CCTAAGGTGTCGAAGCCATGGGTGGATTTGAAAGGCCCTATCACAGTGGGGTTTCCGGTT
                     ********************************************************** *

su_mut               TATCAAAAAAAAAAACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAATTGAG
su_Di Genova         TATCAAAAAAAAAAACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAKTGAG
ch2                  TAT----AAAAAACACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
VIT_218S0041G01880.2 TAT--AAAAAACACACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
su_wt                TAT------AAAAAACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
ch1                  TAT--AAAAAACACACACACACACACACAATTTTCATAATAGAAGGTAGAAAAAAGTGAG
                     ***      ** * ***************************************** ****

su_mut               AATAAGGTAGAGAAGATGTTTTTTCC------TAGGTACTCTAGGGTTTTGTGATAATAG
su_Di Genova         AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
ch2                  AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
VIT_218S0041G01880.2 AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
su_wt                AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
ch1                  AATAAGGTAGAGAAGATGTTTTTTCCTAGCTATAGGTACTCTAGGGTTTTGTGATAATAG
                     **************************      ****************************

su_mut               ATTTGGATTTTTTTCCCCCAATAATTCAAATTATTAATTCAATTATAATAAATATAAAAA
su_Di Genova         ATTTGGATTTTTTTCCCCCAATAATTCAAATTATTAATTCAATTATAATAAATATAAAAA
ch2                  ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
VIT_218S0041G01880.2 ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
su_wt                ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
ch1                  ATTTGGATTTTTTT-CCCCAATAATTAAAATTATTAATTCAATTATAATAAATATAAAAA
                     ************** *********** *********************************

su_mut               TCAATGAGAAATTCCATGGAAAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
su_Di Genova         TCAATGAGAAATTCCATGGAAAACCCAAATCAAATGTTGCAATATAGWTCAAGATTAGCA
ch2                  TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
VIT_218S0041G01880.2 TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
su_wt                TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGTTCAAGATTAGCA
ch1                  TCAATGAGAAATTCCATGG-AAACCCAAATCAAATGTTGCAATATAGATCAAGATTAGCA
                     ******************* *************************** ************

su_mut               TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
su_Di Genova         TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
ch2                  TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
VIT_218S0041G01880.2 TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
su_wt                TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
ch1                  TTTACTTATATGCATGCATGTTAATTAGTTTGACCTTAAAGATCTTGTAGTACTGGTTCC
                     ************************************************************

su_mut               CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACTCCTGCATCTAGTAATTAGA
su_Di Genova         CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACWCCTGCATCTAGTAATTAGA
ch2                  CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
VIT_218S0041G01880.2 CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
su_wt                CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
ch1                  CAATATAAAAATGAGAGCCTATTTGGGATAACTTTTTAACACCTGCATCTAGTAATTAGA
                     **************************************** *******************

su_mut               GATGAAACAGACATTATTTGTATTATGTAATCTATTTTACGAACAAAATTTTAAAAATGC
su_Di Genova         GATGAAACAGACATTATTTGTATTATGTAATCTATTTTACGAACAAAATTTTAAAAATGC
ch2                  GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
VIT_218S0041G01880.2 GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
```

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su_wt                  GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
ch1                    GATGAAACATACATTATTTGTATTATGTAATCTATTTTATGAACAAAATTTTAAAAATGC
                       ********* ***************************** *******************

su_mut                 TTTAATAAGTAGTGAGGATGACTATGATATTTCAAAAAAAATTATTAATTAAAAAAATTT
su_Di Genova           TTTAATAAATAGTGAGGATGACTATGATATTTCAAAAAAAATTATTAATTAAAAAAATTT
ch2                    TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
VIT_218S0041G01880.2   TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
su_wt                  TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
ch1                    TTTAATAAATAGTGAGGATGACTATGATATTTCA----------------AAAAAATTTT
                       ******** *************************                ****** ***
                                                      VvAG11_KASP5

su_mut                 ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTACATGTCCTTAAAATCACTT
su_Di Genova           ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
ch2                    ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
VIT_218S0041G01880.2   ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
su_wt                  ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
ch1                    ACTAACAAAAATGTCGATAAATCATTTATAAGTGTTATCTAAATGCCCTTAAAATCACTT
                       ***************************************** *** **************

su_mut                 ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATTAAAAGAAAA-----AAAAATT
su_Di Genova           ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAAATAAAATTT
ch2                    ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAAATAAAATTT
VIT_218S0041G01880.2   ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAAATAAAATTT
su_wt                  ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAAATAAAATTT
ch1                    ATTTAAATAATAGAAATTAAGCTAGATTAATAATGAATGAAAAGAAAAAAAAATAAAATTT
                       ************************************** *********     **** **

su_mut                 GTACAAACCATCCAGTTTAGTACTGGTCCGAACCCCCTTCTAGCTCAAAAGGTCAATAGG
su_Di Genova           GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
ch2                    GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
VIT_218S0041G01880.2   GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
su_wt                  GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
ch1                    GTACAAATCATCCAGTTTAGTACTGGTTCGAACCCCCTTCAAGCTCAAAAGGTCAATCGG
                       ******* ******************* ************ **************** **

su_mut                 ATTGGACTAGGATCCGGTCAATAGTTCGATTGATTAAATCGATCAATT-GGTCCGATGTT
su_Di Genova           ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
ch2                    ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
VIT_218S0041G01880.2   ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
su_wt                  ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
ch1                    ATTGGACTAGGATCTGGTCAACAGTTCGATTGATT-----GATCAATTCGGTCCGATCTT
                       ************** ****** *************     ******** ******** **

su_mut                 TAAAACATTACAAATAACTTATCTTATCTGAGTTTTGATGCATCTCATACATGCAAAGTA
su_Di Genova           TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATACATGCAAAGCA
ch2                    TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATTCATGCAAAGCA
VIT_218S0041G01880.2   TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATACATGCAAAGCA
su_wt                  TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATACATGCAAAGCA
ch1                    TAAAACATTACAAATAACTTATCTTATGTGAGTTTTGGTGCATCTCATTCATGCAAAGCA
                       *************************** ********* ********** ********* *

su_mut                 GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
su_Di Genova           GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
ch2                    GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
VIT_218S0041G01880.2   GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
su_wt                  GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
ch1                    GTTATTGAAAGCCAATACATATTTCATACAAAGTCCACACAAAGAAACTCAAATAAAAAA
                       ************************************************************

su_mut                 ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
su_Di Genova           ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
ch2                    ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
VIT_218S0041G01880.2   ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
su_wt                  ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
ch1                    ATAAAAAAATCAGAGAATAAAATCCTCATGGTAAAGTTGCACCAATAAGACCTATACTGC
                       ************************************************************

su_mut                 ATACAGTTGCAGAGTGGCAGTGCTCTCCATTGTTCTTTCTCTTGCTTTTCAGTGGTGCAA
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su_Di Genova        ATACAGTTGCAGAGTGGCAGTGCTCTYCATTGTTCTTTCTCTTGCTTTTCAGTGGTGCAA
ch2                 ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
VIT_218S0041G01880.2 ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
su_wt               ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
ch1                 ATACAGTTGCAGAGTGGCAGTGCTCTTCATTGTTCTTTCTCTTGCTTTTCAGTGGTGTAA
                    ************************* ******************************* **

su_mut              TCCATGAGAATCTGAACCCTCTGGCAGTGTCTGAAAAAGGGTGGTCATAGCAGTACAACC
su_Di Genova        TCCATGAGAATCTGAACCCTCTGGCAGTGTCTGAAAAAGGGTGGTCATAGCAGTACAACC
ch2                 TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
VIT_218S0041G01880.2 TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
su_wt               TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
ch1                 TCCATGAGAATCTGAACCATCTGGCAGTGTCTGAAAAAGGGAGGTCATAGCAGTACAACC
                    ****************** ********************** ******************

su_mut              AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
su_Di Genova        AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
ch2                 AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
VIT_218S0041G01880.2 AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
su_wt               AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
ch1                 AACCACTCATTTTTCATCTTTCCTTGTATCTTCCCTTGTTTGCAAAATCTCAGTTTTTCT
                    ************************************************************

su_mut              GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
su_Di Genova        GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
ch2                 GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
VIT_218S0041G01880.2 GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
su_wt               GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
ch1                 GGTTGTTTCTTTCCACTTTTGGCTTTCCGCAAACTTTGAATGGAGTCTTGGGTCTTTTCT
                    ************************************************************

su_mut              CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGTAGAAACCAAGA
su_Di Genova        CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGTAGAAACCAAGA
ch2                 CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
VIT_218S0041G01880.2 CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
su_wt               CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
ch1                 CTTTGCCTCTCAAAGCTGCAATCACTGCTCTTTTTTAGTGCTGGAGAGGAGAAACCAGGA
                    ************************************************ ******** **

su_mut              CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
su_Di Genova        CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
ch2                 CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
VIT_218S0041G01880.2 CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
su_wt               CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
ch1                 CAAGACTTTTCAACTGCTATTCTCCATACAAACTCATGAAACTGATGAACAATTGAGCAG
                    ************************************************************

su_mut              TAGGGTCACTGTGCATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
su_Di Genova        TAGGGTCACTGTGYATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
ch2                 TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
VIT_218S0041G01880.2 TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
su_wt               TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
ch1                 TAGGGTCACTGTGTATACTGTATAGTGATTTTAATCATTTTTATGTCTCATAACTTGTGG
                    ************* **********************************************

su_mut              GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
su_Di Genova        GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
ch2                 GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
VIT_218S0041G01880.2 GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
su_wt               GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
ch1                 GTGTTTGTACTGTTACTTCATCTTTCTCATATCAAGTCATAGTCCTTTCACAAACTTGTG
                    ************************************************************

su_mut              CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
su_Di Genova        CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
ch2                 CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
VIT_218S0041G01880.2 CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
su_wt               CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
ch1                 CTGTCATCTCCCATCATATATGGTTTCCTCCTTTAGGGTTTTGCTTTCCGCCTTCACTTT
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su_mut               TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATCATATGTAATATTTCTATCCAT
su_Di Genova         TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATCATATGTAATATTTCTATCCAT
ch2                  TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
VIT_218S0041G01880.2 TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
su_wt                TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
ch1                  TGACTACTTTTGGAGCAATTTTCTCTCTTGAATTCTATGGTATGTAATATTTCAATCCAT
                     **************************************  ************* ******

su_mut               TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
su_Di Genova         TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
ch2                  TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
VIT_218S0041G01880.2 TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
su_wt                TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
ch1                  TTTGCAACAATTTTTTTAATTCATTCTATTTTTATATATTGGAAAACAGCATAAAATCAA
                     ************************************************************

su_mut               CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
su_Di Genova         CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
ch2                  CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
VIT_218S0041G01880.2 CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
su_wt                CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
ch1                  CCATAGATAGGTACAAGAAGGCCAGCTCAGATAGTACAAATGGAGGCTCTACCATGGAGA
                     ************************************************************

su_mut               TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
su_Di Genova         TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
ch2                  TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
VIT_218S0041G01880.2 TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
su_wt                TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
ch1                  TCAATGCCCAAGTAAGAAAACTTCCATTTTATGATAGTTAATGAATCAATCCAAATCCTA
                     ************************************************************

su_mut               TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
su_Di Genova         TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
ch2                  TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
VIT_218S0041G01880.2 TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
su_wt                TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
ch1                  TATGGTTTACTGATCAGATGTCTTATCCACCATGGCTGTTAGAACATCTCAATCCATTCT
                     ************************************************************

su_mut               GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
su_Di Genova         GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
ch2                  GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
VIT_218S0041G01880.2 GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
su_wt                GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
ch1                  GTGGATTGAAAAGTGCACATTTTTCATTAGGCTGGCCACAAATTAAAACCCTCATCATGC
                     ************************************************************

su_mut               TTAGCCATAAACAAATTTGTTAAAATCATTATTCTACTTTCTTTTTTCTTTTTGGATTAT
su_Di Genova         TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
ch2                  TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
VIT_218S0041G01880.2 TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
su_wt                TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
ch1                  TTAGCCATAAACAAATATGTTAAAATCATTATCCTACTTTCTTTTTTCTTTTTGGATTAT
                     **************** *************** ***************************

su_mut               CCCATTGAACTTTCTCGAATTCTGTCGTTGAACATAGTATTACCAGCAAGAATCAGCAAA
su_Di Genova         CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
ch2                  CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
VIT_218S0041G01880.2 CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
su_wt                CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
ch1                  CCCATTGAACTTTCTCGAATTCTGTTGTTGAACATAGTACTACCAGCAAGAATCAGCAAA
                     ************************* ************* ********************
                                                        VvAG11_KASP6

su_mut               GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
su_Di Genova         GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
ch2                  GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
VIT_218S0041G01880.2 GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
```

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su_wt                GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
ch1                  GCTGCGCCAGCAAATACAGATGCTGCAGAATTCTAACAGGTACCCTTGATTCATATTTCA
                     ************************************************************

su_mut               TTTTTCTCACATGTAATTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
su_Di Genova         TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
ch2                  TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
VIT_218S0041G01880.2 TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
su_wt                TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
ch1                  TTTTTCTCACATGCAGTTGAGTGTATATATATATGTCAATTTGCATTTTCCTTCTGCTTG
                     ************* * ********************************************

su_mut               TGTCTTTAACTAGGTTTTTGGCATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
su_Di Genova         TGTCTTTAACTAGGTTTTTGRCATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
ch2                  TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACCTAACG
VIT_218S0041G01880.2 TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
su_wt                TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
ch1                  TGTCTTTAACTAGGTTTTTGACATCAGTTCCCATGCAAAAATTTCAAATATTACTTAACG
                     ******************** ********************************* *****

su_mut               ATCTTAATTAGGTATGTTAAGACTTAAGAGCAGGTTATACTAAGACATTTTGTCTGTGTT
su_Di Genova         ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
ch2                  ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
VIT_218S0041G01880.2 ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
su_wt                ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
ch1                  ATCTTAATTAGATATGTTAAGACTTAAGATCAGGTTGTACTAAGACATTTTGTCTGTGTT
                     *********** ***************** ****** ***********************

su_mut               TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
su_Di Genova         TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
ch2                  TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
VIT_218S0041G01880.2 TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
su_wt                TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
ch1                  TATAGGCACTTAATGGGTGATTCCTTGGCTTCCTTGACTGTGAAGGAGCTAAAGCAGCTC
                     ************************************************************

su_mut               GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACAC--ATA
su_Di Genova         GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACAC--ATA
ch2                  GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
VIT_218S0041G01880.2 GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
su_wt                GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
ch1                  GAGAACAGGCTTGAACGAGGCATCACAAGAATCAGGTCGAAGAAGGTAACGACACCTAGA
                     *******************************************************  * *

su_mut               CACTAATTCTAATCTGGCTGGGCATTTGACTTTGTGACTTAAATGAAATTGAAAAAAAAA
su_Di Genova         CACTAATTCTAATCTGGCTGGGCATTTGACTTTGTGACTTAAATGAAATTGAAAAAAAAA
ch2                  CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
VIT_218S0041G01880.2 CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
su_wt                CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
ch1                  CACTAAATCTAATCTGGCTGAGCATTTGATTTTGAGACTTAAATGAAA-TGGAAAAAAAA
                     ****** ************* ******** **** ************* ** ********

su_mut               ATGGTATCATTCACTGATTA---AATGATACATGAAGATTGAATTAATTTACGATACTGC
su_Di Genova         ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
ch2                  ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
VIT_218S0041G01880.2 ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
su_wt                ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
ch1                  ATGGTATCATTCACTGATTAAATAATAATACTTGAAGGTTGAATTAATTTAAGATACCGC
                     ********************   *** **** ***** ************* ***** **

su_mut               TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
su_Di Genova         TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
ch2                  TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
VIT_218S0041G01880.2 TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
su_wt                TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
ch1                  TTTCACTTGATAACTAAAGCCTGATGGTAGGAACTTCCACCCATCTAGCTTAGTTGGTGA
                     ************************************************************

su_mut               TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
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su_Di Genova         TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
ch2                  TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
VIT_218S0041G01880.2 TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
su_wt                TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
ch1                  TTGAGCTGAATTTTGTCTTGGAAACTTTGTGAAAAATAGACAAGTGATCACTAGATCAGC
                     ************************************************************

su_mut               AAAGCCTATCTTTTTTTCCTTTGTTCTAATGTTGTGCTTGTTCTAATGCAATAGCATGAGT
su_Di Genova         AAAGCCTATCTTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
ch2                  AAAGCCTATCTTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
VIT_218S0041G01880.2 AAAGCCTATCTTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
su_wt                AAAGCCTATCTTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
ch1                  AAAGCCTATCTTTTTTTCCTTTGTTCTAATGTCGTGCTTGTTCTTATGCAATAGCATGAGT
                     ******************************** *********** ***************

su_mut               TGCTGTTGGCTGAGATTGAATACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
su_Di Genova         TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
ch2                  TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
VIT_218S0041G01880.2 TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
su_wt                TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
ch1                  TGCTGTTGGCTGAGATTGAGTACTTGCAGAAAAGGGTAATCTCTTGTTCTAAACATCAAT
                     ******************* ****************************************
                                   VvAG11_KASP7
su_mut               TTCCATGTTGGTTCCCTTCTTTTATGATTTTACCAGAATTCTTTATCGAGCATGTGTTAA
su_Di Genova         TTCCATGTKGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
ch2                  TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
VIT_218S0041G01880.2 TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
su_wt                TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
ch1                  TTCCATGTGGGTTCCCTTCTTTTATGATTTTTCCAGAATTCTTTATCGAGCACCTTTTAA
                     ******** ********************** ********************  * ****

su_mut               TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
su_Di Genova         TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
ch2                  TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
VIT_218S0041G01880.2 TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
su_wt                TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
ch1                  TTTTCTTAGGAAATTGAGCTGGAAAATGAAAGCGTATATCTCCGAACCAAGGTATGAATT
                     ************************************************************

su_mut               AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
su_Di Genova         AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
ch2                  AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
VIT_218S0041G01880.2 AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
su_wt                AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
ch1                  AAATTAGTATAGGAATTGAATGTGATAAAATTGAAGGCACAACAGTTGATACCGATCTAG
                     ************************************************************

su_mut               CAGTTCAGTTGAACTTGTTAGTGTATATATTCCACAATTGATTGAGTTATCATTACTTAA
su_Di Genova         CAGTTCAGTTGAACTTGTTAGTGTATATATTCCACAATTGATTGAGTTATCATTACTTAA
ch2                  CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
VIT_218S0041G01880.2 CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
su_wt                CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
ch1                  CAGTTCAGTTGAACTTGTTAGTGTATATATTTAACAATTGATTGAGCTATCATTACTTAA
                     *******************************  ************* *************

su_mut               TTTTTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
su_Di Genova         TTTTTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
ch2                  TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
VIT_218S0041G01880.2 TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
su_wt                TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
ch1                  TTATTTTGATGTATGAAAACTATCCATATCCATACTACTACTCTTCATTGAGCTCTGCTG
                     ** *********************************************************

su_mut               CATTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTGTCAACAC
su_Di Genova         CAYTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
ch2                  CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
VIT_218S0041G01880.2 CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
su_wt                CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
ch1                  CACTAAACTTTCAGATTGCAGAAGTGGAGAGGCTTCAGCAAGCAAACATGGTATCAACAC
                     ** ************************************************* *******
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```
su_mut               ATGAGTTTAATGCCATCCAGGCATTAGTTTCTCTCAATTTCTTTCAGCCCAATATGATTG
su_Di Genova         ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCTCAATTTCTTTCAGCCCAATATGATTG
ch2                  ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
VIT_218S0041G01880.2 ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
su_wt                ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
ch1                  ATGAGTTCAATGCCATCCAGGCATTAGTTTCTCGCAATTTCTTTCAGCCCAATATGATTG
                     ******* ************************* **************************
                                         VvAGL11_KASP8

su_mut               AAGGTGGATCCGCAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
su_Di Genova         AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
ch2                  AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
VIT_218S0041G01880.2 AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
su_wt                AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
ch1                  AGGGTGGATCCACAGGCTACCCACTTCCTGATAAGAAGGTCCTCCATCTCGGGTACACTC
                     * ********* ************************************************

su_mut               ATCTTCCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
su_Di Genova         ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
ch2                  ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
VIT_218S0041G01880.2 ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
su_wt                ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
ch1                  ATCTTTCGCCATGTTTATTCATTTGCATTCTTTCTCGGTCTAATATTGAAGATTAAAAGA
                     ***** ******************************************************

su_mut               GCACATAAATCTCCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
su_Di Genova         GCACATAAATCWCCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
ch2                  GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
VIT_218S0041G01880.2 GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
su_wt                GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
ch1                  GCACATAAATCACCAAGTAGCCTTAGGTCTGCTAAATTCATCACTACAAATGGTATATGC
                     *********** ************************************************

su_mut               ATGAATAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
su_Di Genova         ATGAAYAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
ch2                  ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
VIT_218S0041G01880.2 ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
su_wt                ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
ch1                  ATGAACAGCTTAATGAGACATTTCAAGCCATGCATTGGGTAGAAAAAAGTTGTGAGATTT
                     ***** ******************************************************

su_mut               CTTCGTCTTTTAGAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
su_Di Genova         CTTCGTCTTTTARAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
ch2                  CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
VIT_218S0041G01880.2 CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
su_wt                CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
ch1                  CTTCGTCTTTTAAAATACTGCATAGTCATTGACTACCCTATATAATCTTGCTGTGAATTA
                     ************ ***********************************************

su_mut               TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTAT
su_Di Genova         TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTAT
ch2                  TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
VIT_218S0041G01880.2 TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
su_wt                TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
ch1                  TATACTAATTATGATGGGCAAAACAAAAATTTGTTCTCCAAATCCTCCTCAAGTTCCTGT
                     ********************************************************** *

su_mut               GATGTGTATGACGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
su_Di Genova         GATGTGTATGACGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
ch2                  GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
VIT_218S0041G01880.2 GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
su_wt                GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
ch1                  GATGTGTATGAGGGCCTAAAGATGATTGTTCATCTGGGCATTTCGATTTTTGCTCATCCT
                     *********** ************************************************

su_mut               TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
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su_Di Genova         TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
ch2                  TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
VIT_218S0041G01880.2 TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
su_wt                TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
ch1                  TAATAATCAGTACTTCTCCAAAGTAAGTAGATTTCCATGTTTTCTTTTGGCTTACAATAA
                     ************************************************************

su_mut               GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
su_Di Genova         GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
ch2                  GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
VIT_218S0041G01880.2 GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
su_wt                GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
ch1                  GATTCAAGTTCGACCTAATTCCTGGGGTTTTTTGCTTTTTGTTTTATGTAGGTAAATGAT
                     ************************************************************

su_mut               GGAGGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
su_Di Genova         GGRAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
ch2                  GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
VIT_218S0041G01880.2 GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
su_wt                GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
ch1                  GGGAGAAAATATCCGCCAACTTCATCTCCTTATGATGTTTAAATAAATCTTTTCCCATG
                     ** ********************************************************
              VvAGL11_KASP9
su_mut               CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
su_Di Genova         CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
ch2                  CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
VIT_218S0041G01880.2 CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
su_wt                CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
ch1                  CTATCCAAACTGTTATGTAACGGAGAATTAATGTCATTGTTGTATTTGGGGTTTCTATGA
                     ************************************************************

su_mut               CTTGGTATTTATCAGCTAGCAACCAGTACTGTTTTGTATAACATTATGACATATAATATA
su_Di Genova         CTTGGTATTTATCMGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
ch2                  CTTGATATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
VIT_218S0041G01880.2 CTTGGTATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
su_wt                CTTGGTATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
ch1                  CTTGGTATTTATCCGCTAGCAACCAGTACTGTTTTGTATAACATTATGATATATAATATA
                     **** ******** *********************************** **********

su_mut               GCCTATTAAAAT
su_Di Genova         GCCTATTAAAAT
ch2                  GCCTATTAAAAT
VIT_218S0041G01880.2 GCCTATTAAAATATACAATATTCCTAATCT
su_wt                GCCTATTAAAAT
ch1                  GCCTATTAAAATATACAATATTCCTAATC
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Figura Suplementar 1. Mutações selecionadas usadas para o desenvolvimento de marcadores KASP. As sequências apresentadas no alinhamento são referentes ao gene *VviAGL11* de PN40024 (VIT_218S0041G01880.2), *VviAGL11* 'alelo Chardonnay 1' (CH1), *VviAGL11* 'alelo Chardonnay 2' (CH2), alelo mutante *VviAGL11* de 'Sultanine' (SU mut), *VviAGL11* 'alelo wt de Sultanine' (SU wt) e a sequência disponível de 'Sultanine' por Di Genova (2014). A sequência sublinhada representa o marcador SSR VMC7F2, as sequências cinzas claras representam UTRs.

6 DISCUSSÃO

A busca e a seleção de características de interesse em videiras como sabor, tamanho do fruto e tamanho de sementes teve início a partir de sua domesticação por seleção artificial e, posteriormente, por cruzamento e propagação vegetativa (VAROQUAUX *et al.,* 2000). Com o advento do descobrimento e do sequenciamento de DNA, diversos fenótipos vegetais de interesse comercial tiveram seus mecanismos moleculares e genéticos melhor compreendidos (HUANG *et al.,* 2013). Apesar do interesse em variedades de videira apirênicas, os mecanismos moleculares subjacentes ao desenvolvimento da semente na videira não foram totalmente elucidados. Estudos anteriores apenas demonstraram as mudanças anatômicas e morfológicas durante o desenvolvimento normal da semente, desde a fertilização até a sua maturação, que pode ser caracterizada por fases como (i) divisão celular rápida; (ii) acumulação de reserva e expansão celular devido à absorção de água; e (iii) diminuição do crescimento e retenção do acúmulo de reserva (CADOT *et al.,* 2006).

Com o intuito de avaliar a função do gene candidato *VviAGL11* durante a morfogênese da semente de videira, foram realizados estudos de genética reversa utilizando plantas de *A. thaliana* homozigotas para a mutação do gene *SEEDSTICK* (*stk*). A técnica de transformação genética "*Floral Dip*" (ZHANG *et al.,* 2006) mediada por *A. tumefaciens*, foi empregada para a geração de plantas transgênicas de *A. thaliana* com expressão ectópica de *VviAGL11*, como apresentado no Capítulo I. A introdução de genes exógenos utilizando *Agrobacterium* é o método mais popular de transformação e se tornou uma ferramenta essencial para os estudos de transferência de genes por meio de engenharia genética, podendo ser utilizado em plantas-modelo para a comprovação funcional de genes candidatos bem como em plantas de interesse agronômico (ZIEMIENOWICZ, 2014). Esse método possui diversas vantagens em relação as metodologias de introdução direta de DNA como, por exemplo, o baixo número de cópias inseridos no genoma, a estabilidade e a herdabilidade da integração do transgene ao DNA endógeno, além de ser um método reprodutível e que apresenta protocolos eficientes em plantas como *A. thaliana* que, por sua vez, são vantajosos modelo de estudo gênico por gerarem grande quantidade de sementes em ciclos de vida relativamente curtos (RIVERA *et al.,* 2012).

Setenta e duas plantas mutantes *stk* de *A. thaliana* foram utilizadas para a transformação com construções vetoriais que induzissem a superexpressão de formas alélicas de *VviAGL11*. Umas das construções foi constituída da sequência codificadora da

proteína VviAGL11 da cultivar com semente Chardonnay (CH). A outra construção possuía a sequência codificadora de *VviAGL11* proveniente do alelo mutante da cultivar apirênica Sultanine (SU mut) com sete *SNP*s, dois deles causadores de modificações de aminoácidos no momento da tradução. Plantas T1, previamente selecionadas por meio da resistência ao antibiótico higromicina e pela expressão de gene repórter GFP (do inglês *Green Fluorescent Protein*), foram testadas por PCR para detecção da presença da construção de complementação e a presença dos alelos de *VviAGL11,* o que resultou em 15 linhagens independentes transformadas com o alelo *VviAGL11* CH e 21 linhagens independentes com o alelo *VviAGL11* SU.

Para a avaliação da complementação do fenótipo e da ortologia entre os genes *VviAGL11* e *AtAGL11,* a caracterização fenotípica foi realizada nas linhagens T1 em comparação com os fenótipos *stk* e WT. O mutante *stk* de *A. thaliana* apresenta cinco fenótipos distintos do tipo selvagem: i) o número de sementes é reduzido pela metade devido ao aborto de sementes ainda imaturas; ii) o tamanho das sementes é diminuído; iii) o tamanho da síliqua também é afetado, reduzindo o seu comprimento; iv) o funículo é mais longo e; v) mais espesso (PINYOPICH *et al.,* 2003, MATIAS-HERNANDEZ *et al.,* 2010). Com a análise quantitativa das síliquas das linhagens T1, foi possível observar um número reduzido de sementes abortadas, aumento do tamanho das sementes, aumento do comprimento das síliquas e modificações nos funículos que se assemelharam ao fenótipo de tipo selvagem.

Dentre as plantas transformadas com o alelo *VviAGL11* CH, seis apresentaram síliquas bem desenvolvidas e aumento do número de sementes, oito demonstraram sementes do tamanho semelhante ao tipo selvagem, sete plantas apresentaram redução da espessura dos funículos e oito mostraram funículos mais curtos. Entre as plantas transformadas com o alelo mutante *VviAGL11* SU, nove apresentaram síliquas como as do tipo selvagem, seis plantas apresentaram aumento do número de sementes, dez demonstraram sementes de tamanho selvagem, dez plantas apresentaram redução da espessura dos funículos e nove apresentaram funículos menores.

A expressão gênica relativa de *VviAGL11* foi avaliada nas plantas transformadas e se identificou um aumento significativo da expressão do respectivo gene, que é ausente em WT e *stk*, correlacionado com as plantas T1 que obtiveram melhor complementação fenotípica. As duas formas alélicas tiveram a capacidade de restaurar o fenótipo no modelo heterólogo, o que não descarta a possibilidade de que as mutações presentes em um dos alelos de ‘Sultanine’ sejam importantes na formação da proteína VviAGL11 e sua posterior atividade

celular como fator de transcrição. Para a comprovação do efeito dessas mutações, experimentos de expressão gênica como a transativação em protoplastos poderão ser realizados futuramente.

Os resultados compilados no Capítulo I permitiram revelar que a expressão ectópica de *VviAGL11* é capaz de controlar a morfogênese de sementes em *A. thaliana* na ausência de expressão de *AtAGL11*, demonstrando que estes dois genes muito semelhantes têm a mesma função *in planta*, sendo verdadeiros ortólogos. Entretanto, em *A. thaliana*, a morfogênese da semente é dependente da expressão de três genes principais, *AGL11, SHP1 e SHP2*, cuja redundância é considerada essencial para manter a robustez da atividade dos genes *MADS-box* no desenvolvimento de órgãos florais (RIJPKEMA *et al.,* 2007). Todavia, em videira, não foram descritos genes candidatos ortólogos aos genes *SHATTERPROOF*. Logo, o gene *VviAGL11* pode ser o único gene *MIKC*^{c} da classe D atuando ativamente na determinação da identidade do óvulo e da semente.

Após a comprovação do papel de *VviAGL11* no desenvolvimento de sementes em modelo heterólogo, o objetivo principal deste estudo tornou-se a avaliação funcional desse gene em videiras. Para tal, fez-se necessário a geração de videiras transformadas e a posterior avaliação de seus frutos com o intuito de fenotipá-los de acordo com características relacionadas às sementes como número, tamanho e forma. Dentre os diversos métodos de transformação de plantas, a transformação indireta por *A. tumefaciens* em embriões somáticos é amplamente empregada (DALE & IRWIN, 1995; DE ANDRADE, 2003). Essa técnica foi utilizada para diversos fins no melhoramento vegetal e, dentre eles, estudos de desenvolvimento e crescimento vegetal, metabolismo, regulação e expressão de genes, extração de produtos medicinais, vacinas transgênicas e produção de anticorpos (BRASILEIRO & DUSI, 1999). Entretanto, esse método é baseado no cultivo celular *in vitro* para a obtenção de plantas completas a partir dos tecidos transgênicos, sendo extremamente laborioso e delongado, ainda mais quando aplicado em plantas perenes com fases juvenis longas como é o caso da macieira e da videira (YAMAMOTO *et al.,* 2004; VIDAL *et al.,* 2010). A transformação genética de videiras via *A. tumefaciens* apresenta ainda baixa eficiência em comparação a culturas não-perenes como milho, soja, arroz, devido a problemas na regeneração das plantas transgênicas pois suas células embriogênicas são altamente recalcitrantes, apresentando alta atividade nucleosídica que pode degradar o DNA no momento da realização de experimentos de transferência gênica (BARANDIARAN *et al.,* 1998).

Felizmente, muitos avanços atingidos no campo da biotecnologia vegetal permitiram auxiliar a contornar as limitações das técnicas tradicionais. Uma das ferramentas mais interessantes e promissoras, especialmente para plantas perenes, é a expressão de genes baseados em plasmídeos episômicos, como a plataforma IL-60 (MOZES-KOCH *et al.,* 2012). Essa plataforma consiste em um sistema universal de vetores derivados do vírus TYLCV (do inglês *Tomato Yellow Leaf Curl Virus*) em sua forma assintomática, devido à inativação de proteínas causadoras de danos celulares por meio da deleção de 60 aminoácidos da proteína do capsídeo viral. Neste método de transformação genética, os vetores são compostos por DNA dupla fita sendo capazes de se replicar em células vegetais e de se espalhar para outros tecidos após a inoculação, não havendo a inserção de DNA exógeno no genoma das plantas transformadas (PERETZ *et al.,* 2007). Dois vetores, chamados de "plasmídeos vegetais", um deles com o gene de interesse e o outro com sequências virais de expressão de proteínas auxiliadoras da mobilidade dos vetores, são introduzidos mecanicamente em tecidos vegetais e a expressão e a replicação do DNA episomal ocorre em alguns dias, tornando a expressão do gene de interesse estável e sistêmica (PERETZ *et al.,* 2007; MOZES-KOCH *et al.,* 2012). Devido à falta de herdabilidade do DNA episômico, aspectos de biossegurança podem ser mais fáceis do que o recomendado para plantas convencionalmente modificadas que sofrem transmissão genética pelo pólen e pelas sementes (MOZES-KOCH *et al.,* 2012). Estes vetores são usados para a superexpressão ou silenciamento de genes diretamente em plantas adultas, abrindo possibilidades de modificação de características em espécies lenhosas (GOVER *et al.,* 2014). O sistema é compatível com muitas espécies, incluindo espécies frutíferas como videiras, cítricos e oliveiras (PERETZ *et al.,* 2007; LI *et al.,* 2011).

Em relação à denominação das plantas transfectadas com os vetores da plataforma IL-60, sua definição é variável e dependente de leis de biosseguranças regentes em cada país. De maneira generalizada, a transformação genética consiste na transferência controlada de genes para o genoma de um organismo vivo por via não sexual, o que pode gerar variabilidade genética não disponível via métodos de melhoramento convencionais (JONES & CASSELLS, 1995; TORRES *et al.,* 2000). Deste modo, define-se planta transgênica como aquelas que apresentam genes diferentes do genoma original obtidos por técnicas de engenharia genética (GANDER & MARCELLINO, 1997; TORRES *et al.,* 2000). No Brasil, porém, a Lei n. 11.105 de 24 de março de 2005, que regulamentou os incisos II, IV e V do § 1º do art. 225 da Constituição Federal do Brasil e estabelece normas para o uso das técnicas

de engenharia genética e liberação no meio ambiente de organismos geneticamente modificados (OGMs), definiu alimentos transgênicos da seguinte forma: Art. 3º Para os efeitos desta Lei, considera-se: “[...] V – organismo geneticamente modificado – OGM: organismo cujo material genético – DNA/RNA tenha sido modificado por qualquer técnica de engenharia genética” (BRASIL, 2005). Portanto, pela regulamentação brasileira, as plantas derivadas do método de expressão de genes de interesse via plasmídeos vegetais possuem seu RNA modificado, mesmo sem haver a inserção do transgene no genoma endógeno, sendo, assim, consideradas transgênicas.

No Capítulo II, são apresentados os resultados da caracterização funcional de *VviAGL11* em videira com a utilização de plasmídeos vegetais, promovendo sua superexpressão em cultivares sem sementes e via silenciamento por meio de RNAi (RNA de interferência) em genótipos com semente. Após um mês da inoculação dos plasmídeos vegetais, foi possível confirmar que todos os tratamentos foram totalmente eficientes, demonstrando que o DNA dos plasmídeos vegetais foi translocado através de tecidos vegetais, atingindo uma translocação sistêmica do ramo setorizado em plantas tratadas. Essa observação também se fez possível com a avaliação da expressão do gene repórter GFP em folhas jovens transfectadas após trinta dias, e da atividade da proteína GUS (β-glicuronidase) em sementes de videira de quatro semanas de desenvolvimento, tendo sido transfectadas seis semanas antes, ainda quando em estádio de formação do cacho.

A avaliação da expressão gênica relativa de *VviAGL11* permitiu mostrar que o plasmídeo vegetal que abriga a construção de superexpressão desse gene foi eficaz na conferência de níveis elevados de expressão, como foi possível observar em bagas de 'Linda' ('Linda' OX), que apresentou seis vezes mais transcritos de *VviAGL11* em comparação com as bagas de 'Linda' não tratadas ('Linda' NT). Também foi possível observar um aumento de 17 vezes na expressão relativa de *VviAGL11* em ramos e, nas folhas, a expressão foi até 90 vezes maior que as amostras não tratadas. Os níveis de transcrito em plantas silenciadas para *VviAGL11* foi estatisticamente inferior ao observado nas plantas-controle, a maioria apresentando metade ou, mesmo, níveis mais baixos da expressão normal de *VviAGL11.* As plantas da cultivar Prosecco, tratadas com um vetor vazio como controle, apresentaram níveis semelhantes de expressão de *VviAGL11* entre as sementes de plantas tratadas e não tratadas.

Pelo presente estudo também teve-se por objetivo avaliar os efeitos fenotípicos da expressão alterada de *VviAGL11* nas características de semente de cada cultivar de videira.

Portanto, cachos maduros das cultivares tratadas e seus respectivos controles não tratados foram avaliados. A avaliação dos cachos maduros deu-se de forma quantitativa com a obtenção de dados incluindo peso de baga, número de semente/traços de semente, peso fresco e seco de sementes/traço de semente. O silenciamento de *VviAGL11* nas cultivares pirênicas Italia e Ruby provocou uma diminuição visível e drástica do peso seco total das sementes, com número reduzido de sementes e aumento concomitante no número de traços de sementes. De maneira inversa, a superexpressão de *VviAGL11* na cultivar apirênica Linda (Linda OX) resultou na formação de minisementes que não estavam presentes nos cachos não tratados da Linda. Linda NT geralmente apresenta apenas vestígios de sementes que são praticamente inobserváveis a olho nu. As sementes diminutas das bagas de "Linda" OX exibiram-se maiores em tamanho do que traços de sementes, mas não atingiram um tamanho normal de semente madura. Apesar do desenvolvimento das minissementes nas amostras de 'Linda' OX, seu peso seco total ainda foi classificado como apirênica. Esses resultados permitiram indicar que a superexpressão de *VviAGL11* por meio de plasmídeos vegetais afetou o tamanho das sementes, aumentando-as, mas não foi suficiente para restaurar o desenvolvimento completo da semente no genótipo Linda.

Quando avaliados por meio de ensaios histológicos, os traços de semente resultantes das cultivares pirênicas Italia e Ruby tratadas com os vetores de silenciamento de *VviAGL11* apresentaram um acúmulo de compostos fenólicos nas camadas da casca, provavelmente envolvidos com o início do processo de lignificação. Foi possível identificar a presença de células preenchidas com polifenóis no tegumento externo, tanto na exotesta como na camada da endotesta (ET), local específico da expressão de *VviAGL11*. Essa concentração de compostos fenólicos não ocorreu em traços de sementes não tratados de 'Italia' e 'Ruby'. Adicionalmente, a camada ET não se alongou nem se duplicou na maioria dos traços de sementes de 'Italia' RNAi e 'Ruby' RNAi. Já as minissementes de 'Linda' OX apresentaram os resultados morfoanatômicos mais interessantes quando comparadas às cicatrizes de traços de sementes de 'Linda' NT. As minissementes de ‘Linda’ OX apresentaram as características típicas de traços de sementes quanto ao tamanho e à morte dos tecidos reprodutivos como endosperma e embrião. No entanto, as camadas do revestimento das sementes foram completamente diferentes dos padrões de traços de sementes observados em 'Sultanine' (MALABARBA *et al.,* 2017). A camada ET apresentou duplicação e alongamento. Também foi possível observar o acúmulo de compostos fenólicos na camada exotesta. Esses dados permitiram sugerir que a expressão de *VviAGL11* induz modificações na casca da semente,

iniciando a lignificação da camada ET e, portanto, produzindo um alto efeito no desenvolvimento e na maturação das sementes, mesmo sem a formação normal de endosperma e embrião.

As diferenças significativas nos níveis de transcrição de *VviAGL11* combinadas com as modificações fenotípicas observadas tanto em cultivares com semente quanto em cultivares apirênicas confirmaram o estudo prévio da complementação de *VviAGL11* em mutantes *stk,* apresentado no Capítulo I, que foi capaz de restaurar o número e o tamanho de sementes no modelo heterólogo *Arabidopsis* (MALABARBA *et al.,* 2017). Os dados apresentados no Capítulo II contribuem com dados funcionais fundamentais sobre *VviAGL11* na própria videira demostrando, *in loco*, que esse gene é um importante regulador da morfogênese das sementes. Ademais, os resultados obtidos com a plataforma IL-60 demonstraram a aplicabilidade científica e biotecnológica dos plasmídeos vegetais em plantas perenes.

Apesar do contínuo avanço no descobrimento dos mecanismos moleculares vegetais que regem processos biológicos importantes, o desenvolvimento e a seleção de variedades de uva de mesa são considerados investimentos de longo prazo e de alto custo. Visando a inovação deste sistema, a geração de plantas que apresentem caracteres de interesse por técnicas de engenharia genética é vantajosa para os grandes mercados produtores de frutíferas perenes, além da utilização de ferramentas biotecnológicas como marcadores moleculares, que auxiliem na aceleração da geração e seleção de novas cultivares (CABEZAS *et al.,* 2006). Como ferramenta-suporte na seleção indireta, são utilizados dados de marcadores moleculares juntamente com dados fenotípicos para a identificação de marcadores ligados a *QTL*s, que possam ser testados e utilizados posteriormente na seleção assistida por MAS (DE MORAIS JÚNIOR, 2013).

Interessantemente, *QTL*s relacionados com a ausência de sementes (locus *SdI*) e com a resistência ao fungo *Plasmopara viticola* (locus *Rpv3*), causador do míldio, foram detectados na porção distal do cromossomo 18 de *V. vinifera* (REVERS *et al.,* 2010). No Capítulo III, estão demonstrados os resultados do desenvolvimento e utilização de marcadores moleculares para a seleção da apirenia em videira, enquanto os estudos realizados com marcadores moleculares para resistência ao míldio, além da avaliação de genes localizados no locus *Rpv3*, estão apresentados junto ao Anexo I desta tese. Inicialmente, uma população segregante (POP692) para a resistência a doenças e para a ausência de sementes foi genotipada com cinco marcadores microssatélites associados aos

QTLs colocalizados no cromossomo 18. Após a realização dos testes de ajustamento $\chi2$, dois dos cinco marcadores, UDV108 e P2_VVIAGL11, não apresentaram a segregação esperada e foram excluídos das análises. Para os loci VMC7F2, VVIN16 e P3_VVAGL11 foram observados quatro alelos segregando na população genotipada. A análise da distribuição fenotípica dos caracteres avaliados *versus* a frequência de alelos dos marcadores SSR genotipados, mostraram claramente ($p<0,0001$) a associação entre os alelos P3_VVAGL11-198 pb ($\chi2 = 28,72.$), VVIN16-157 pb ($\chi2 = 26,64$) e VMC7F2-198 pb ($\chi2 = 40,96$) com os indivíduos apirênicos. Além disso, os mesmos marcadores demonstraram possuir forte correlação com a resistência ao míldio: P3_VVAGL11-185 pb ($\chi2 = 28,9$), VVIN16-154 pb ($\chi2 = 26,81$) e VMC7F2-210 pb ($\chi2 = 36,93$).

Com a avaliação destes marcadores, foram identificados haplótipos para a resistência ao míldio e para o caractere apirênico. Com a utilização do haplótipo VVP, composto pelos três marcadores ou, ainda, o haplótipo VP (VVIN16 e P3_VVAGL11) na MAS, todos os indivíduos selecionados foram verdadeiramente apirênicos, eliminando falsos positivos. A utilização do haplótipo RM de resistência ao míldio (do inglês *Mildew Resistance*), que é composto pelos três marcadores, apresentou 100% de exatidão na seleção de indivíduos resistentes, também eliminando falsos positivos.

Apesar da eficácia da seleção de indivíduos com a utilização dos marcadores microssatélites citados acima, atualmente surgiram técnicas de MAS mais eficientes do que a análise de SSRs por gel de poliacrilamida. Uma delas é o ensaio de genotipagem KASP®. Esse ensaio utiliza a PCR competitiva alelo-específica combinada com um sistema de informação homogênea à base de fluorescência para a identificação e a medição da variação genética que ocorre ao nível de nucleotídeos para detectar *SNPs* ou inserções e deleções (*INDELs*) (HE *et al.,* 2014). Essa técnica apresenta várias vantagens como menor erro de genotipagem em amostras de DNA de controle positivo quando comparada com outras técnicas e menor custo de genotipagem (7,9- 46,1% mais barato) (SEMAGN *et al.,* 2014). Assim, tais marcadores têm gerado dados em larga escala de maneira automatizada e a custos reduzidos, sendo úteis para a construção de mapas genéticos de alta densidade para estudos de associação genética e para a seleção assistida de caracteres de interesse (GUIMARÃES *et al.,* 2009).

A partir da confirmação da função de *VviAGL11* como um gene essencial para o desenvolvimento das sementes na videira, foi iniciada uma abordagem biotecnológica com a utilização de sua sequência para a geração de marcadores moleculares de alta eficiência.

Com o sequenciamento completo do gene *VviAGL11* e a identificação de mutações do tipo *SNP*s e *INDEL*s únicos para o alelo da cultivar apirênica, foram desenvolvidas nove marcas para a genotipagem de populações segregantes para apirenia. Além disso, 41 marcas foram desenvolvidas para a seleção de indivíduos resistentes ao míldio, uma vez que, conforme informado anteriormente, um conjunto de genes de resistência está localizado na porção distal do cromossomo 18. Essas marcas foram baseadas no painel GrapeReSeq_Illumina_20K (https://urgi.versailles.inra.fr/Species/Vitis/GrapeReSeq_Illumina_20K).

As marcas foram desenvolvidas tendo em vista a versatilidade de utilização da técnica de PCR competitiva alelo específica (KASP®) para serem testadas em populações segregantes para apirenia e/ou resistentes ao míldio. Os marcadores VvAGL11_KASP 1 ao 9 foram avaliados na população 'Villard Blanc' X 'Villard Blanc' e em genótipos apirênicos. Após avaliação pela técnica de KASP, os marcadores VvAGL11_KASP2, VvAGL11_KASP3, VvAGL11_KASP8 e VvAGL11_KASP9 demonstraram correlação com o fenótipo de apirenia. Em relação aos marcadores desenvolvidos para a seleção de indivíduos resistentes ao míldio, dois marcadores, Rpv3_15 e Rpv3_33, demonstraram segregação heterozigótica com forte associação à resistência ao *P. viticola*. Em ambos os casos, na seleção de indivíduos apirênicos e/ou resistentes ao míldio, faz-se necessária a avaliação das marcas selecionadas em diferentes populações segregantes para as características de interesse e com parentais distintos dos utilizados até o momento. Esses experimentos já estão em andamento e poderão acrescentar dados ao estudo descrito no Capítulo III. Somente assim os marcadores desenvolvidos neste estudo poderão ser comprovados como boas alternativas para programas de melhoramento de videira auxiliados por MAS.

Muitas questões sobre o funcionamento de *VviAGL11* ainda não foram respondidas. Os estudos mais recentes permitiram comprovar que o gene *AGL11* é atuante na casca das sementes em videira (MALABARBA *et al.,* 2017; MALABARBA *et al.,* 2018), à semelhança de seu ortólogo em *A. thaliana* (*AtAGL11*) (PINYOPICH *et al.,* 2003) e, também, como seu ortólogo em tomate (*TAGL11*) que apresenta 85% de similaridade com *AtAGL11* e possui expressão preferencial nas camadas de tegumento interno da casca (OCAREZ & MEJÍA, 2016). Além de sua função na morfogênese de sementes, com a indução do alongamento e a divisão de células do tegumento, o gene *AGL11* pode ter papel fundamental na produção de metabólitos secundários, como na produção de

proantocianidinas (MIZZOTTI *et al.,* 2014). Esses compostos fenólicos, antes classificados como taninos condensados, são encontrados nas cascas de sementes e conferem rigidez e proteção para essa estrutura (MICHAUD *et al.,* 1971). A colocalização de transcritos de *AGL11* e de compostos fenólicos relacionados à maturação das sementes, como observado em ensaios histológicos apresentados nos Capítulos I e II, pode ser estudada em videiras de uma maneira mais aprofundada para a maior compreensão da atuação deste gene nas rotas metabólicas do desenvolvimento das sementes de uva.

Complementarmente, estudos relacionados com a proteína VviAGL11 podem identificar seus genes-alvo e avaliar sua preferência por elementos *cis* (CArG-box) críticos às interações proteicas da família MADS-box. Ao mesmo tempo, pode-se realizar a caracterização das proteínas MADS-box de videira que interagem com a proteína VviAGL11 para a formação dos complexos quaternários de ativação da transcrição de genes-alvo. Durante o desenvolvimento da presente tese de doutorado, foram realizados ensaios laboratoriais visando a expressão da proteína VviAGL11 em procariotos, porém não houve sucesso na purificação dessa proteína até o momento. A geração de anticorpos para o reconhecimento de uma sequência peptídica única para o gene *AGL11* de videira foi realizada por uma empresa contratada. Infelizmente, os anticorpos aparentam apenas reconhecer o peptídeo quando em sua forma pura, não sendo capazes de identificar a proteína VviAGL11 no extrato proteico total (resultados não apresentados).

Outro ponto importante é que, por meio do presente estudo, houve a comprovação da função de *VviAGL11*, demonstrando que a expressão deste gene na camada ET da semente é necessária para o desenvolvimento normal da estrutura. No entanto, ainda não há a compreensão da causa da baixa expressão de *VviAGL11* em cultivares apirênicas. Com o sequenciamento alelo-específico completo deste gene foi possível observar que a causa não está baseada em grandes inserções provenientes de elementos genéticos móveis, como transposons ou retrotransposons. Apenas pequenas mutações foram encontradas nos alelos da cultivar apirênica Sultanine, juntas formando um conjunto de polimorfismos únicos, com alguns *SNP*s modificando sequências consenso CArG-box ou com *INDEL*s aumentando a distância entre os elementos *cis* encontrados na maior região intrônica de *VviAGL11* (MALABARBA *et al.,* 2017). Ademais, para desenvolvimento futuro, sugerem-se estudos com as proteínas MADS-box de videira visando responder se *VviAGL11* possuiu um *feedback* de regulação e se suas regiões intrônicas são importantes para o desencadeamento e para a manutenção de sua expressão durante o desenvolvimento das sementes.

7 REFERÊNCIAS BIBLIOGRÁFICAS

ADAM-BLONDON, A. F.; LAHOGUE-ESNAULT, F.; BOUQUET, A.; BOURSIQUOT, J. M.; THIS, P. Usefulness of two SCAR markers for marker-assisted selection of seedless grapevine cultivars. *Vitis*, 40: 147–155. 2001.

ADAM-BLONDON, A. F.; ROUX, C.; CLAUX, D.; BUTTERLIN, G.; MERDINOGLU, D.; THIS, P. Mapping 245 SSR markers on the *Vitis vinifera* genome: a tool for grape genetics. *Theoretical and Applied Genetics*, 109 (5): 1017-1027. 2004.

ADAMCZYK, B. J. & FERNANDEZ, D. E. *MIKC** MADS-domain heterodimers are required for pollen maturation and tube growth in *Arabidopsis*. *Plant Physiology,* 149: 1713–1723. 2009.

ANGENENT, G. C. & COLOMBO, L. Molecular control of ovule development. *Trends in Plant Science*, 1 (7): 228–232. 1996.

ANGENENT, G. C.; FRANKEN, J.; BUSSCHER, M.; VAN DIJKEN, A.; VAN WENT, J. L.; DONS, H. J. M.; VAN TUNEN, A. J. A novel class of MADS-box genes is involved in ovule development in petunia. *The Plant Cell*, 7: 1569–1582. 1995.

BARANDIARAN, X.; DI PIETRO, A.; MARTÍN, J. Biolistic transfer and expression of a uidA reporter gene in different tissues of *Allium sativum* L. *Plant Cell Reports,* 7: 737–741. 1998.

BECKER, A. & THEIßEN, G. The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Molecular Phylogenetics and Evolution,* 29: 464–489. 2003.

BERGER, F.; GRINI, P. E.; SCHNITTGER, A. Endosperm: an integrator of seed growth and development. *Current Opinion in Plant Biology*, 9: 664–670. 2006.

BOSS, P. K.; SENSI, E.; HUA, C.; DAVIES, C.; THOMAS, M. R. Cloning and characterization of grapevine (*Vitis vinifera* L.) MADS-box genes expressed during inflorescence and berry development. *Plant Science,* 162: 887–895. 2002.

BOSS, P. K.; VIVIER, M.; MATSUMOTO, S.; DRY, I. B.; THOMAS, M. R. A cDNA from grapevine (*Vitis vinifera* L.), which shows homology to AGAMOUS and SHATTERPROOF, is not only expressed in flowers but also throughout berry development. *Plant Molecular Biology,* 45, 541–553. 2001.

BOUQUET, A. & DANGLOT, Y. Inheritance of seedlessness in grapevine (*Vitis vinifera* L.). *Vitis,* 35: 35-42. 1996.

BRAMBILLA, V.; BATTAGLIA, R.; COLOMBO, M.; MASIERO, S.; BENCIVENGA, S.; KATER, M.; COLOMBO, L. Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in *Arabidopsis. The Plant Cell,* 19 (8): 2544–2556. 2007.

BRASIL. Lei Nº 11.105, 24/03/05 - Regulamenta os incisos II, IV e V do § 1o do art. 225 da Constituição Federal, estabelece normas de segurança e mecanismos de fiscalização de atividades que envolvam organismos geneticamente modificados – OGM e seus derivados, cria o Conselho Nacional de Biossegurança – CNBS, reestrutura a Comissão Técnica Nacional de Biossegurança – CTNBio, dispõe sobre a Política Nacional de Biossegurança – PNB revoga a Lei no 8.974, de 5 de janeiro de 1995, e a Medida Provisória no 2.191-9, de 23 de agosto de 2001, e os arts. 5º, 6º, 7º, 8º, 9º, 10º e 16º da Lei no10. 814, de 15 de dezembro de 2003, e dá outras providências. Disponível em: <http://www.planalto.gov.br/ccivil_03/_Ato2004- 2006/2005/lei/L11105.htm.>. 2005.

BRASILEIRO, A. C. M. & DUSI, D. M. A. Transformação genética de plantas. V.2, In: TORRES, A. C.; CALDAS, L.S.; BUSO, J. A. Cultura de tecidos e transformação genética de plantas. *Brasília: EMBRAPA–SPI/EMBRAPA–CNPH:* 679–735. 1999.

BUSI, M. V.; BUSTAMANTE, C.; D'ANGELO, C.; HIDALGO-CUEVAS, M.; BOGGIO, S B.; VALLE, E. M.; ZABALETA, E. MADS-box genes expressed during tomato seed and fruit development. *Plant Molecular Biology,* 52: 801–815. 2003.

CABEZAS, J. A.; CERVERA, M. T.; RUIZ-GARCIA, L.; CARRENO, J.; MARTINEZ-ZAPATER, J. M. A genetic analysis of seed and berry weight in grapevine. *Genome,* 49 (12):1572–1585, 2006.

CADOT, Y.; MINÃNA-CASTELLÓ, M. T.; CHEVALIER, M. Anatomical, Histological, and Histochemical Changes in Grape Seeds from Vitis vinifera L. cv Cabernet franc during Fruit Development. *Agriculture and Food Chemistry,* 54: 9206-9215. 2006.

CAMARGO, U. A. Uvas sem sementes cultivares BRS Morena, BRS Clara e BRS Linda. (Sistema de Produção, 8 ISSN 1678-8761, Versão Eletrônica). Disponível em: <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Uva/UvasSemSementes/cultivares.htm>. 2005.

CAUSIER, B.; SCHWARZ-SOMMER, Z.; DAVIES, B. Floral organ identity: 20 years of ABCs. *Seminars in Cell and Developmental Biology,* 21: 73–79. 2010.

CHRISTENSEN, L. P. Raisin Grape Varieties. Pages 38-47 in: *Raisin Production Manual*. University of California, Agricultural and Natural Resources Publication 3393, Oakland, CA. 2000.

COLOMBO, L.; FRANKEN, J.; KOETJE, E.; VAN WENT, J.; DONS, H. J.; ANGENENT, G. C.; VAN TUNEN, A. J. The petunia MADS box gene FBP11 determines ovule identity. *The Plant Cell,* 7 (11):1859-1868. 1995.

COLOMBO, L.; FRANKEN, J.; VANDERKROL, A. R.; WITTICHY, P. E.; DONSY, H. J. M.; ANGENENT, G. C. Downregulation of ovule-specific MADS box genes from petunia results in maternally controlled defects in seed development. *The Plant Cell,* 9: 703–715, 1997.

COSTANTINI, L.; BATTILANA, J.; LAMAJ, F.; FANIZZA, G.; GRANDO, M.S. Berry and phenology-related traits in grapevine (*Vitis vinifera* L.): from quantitative trait loci to underlying genes. *BioMedCentral Plant Biology*, 8 (38): 1471-2229. 2008.

DALBO, M. A.; YE, G. N.; WEEDEN, N. F.; STEINKELLNER, H.; SEFC, K. M.; REISCH, B. I. A gene controlling sex in grapevines placed on a molecular marker-based genetic map. *Genome,* 43: 333-340. 2000.

DALE, P. J. & IRWIN, J. A. The release of transgenic plants from containment, and the move towards their widespread use in agriculture. *Developments In Plant Breeding*, 3: 425-431, 1995.

DEYHOLOS, M. K. & SIEBURTH, L. E. Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *The Plant Cell,* 12: 1799–1810. 2000.

DE ANDRADE, S. R. M. Transformação de Plantas. Documentos 102 – EMBRAPA CERRADOS. Planaltina (DF), p. 1-28, 2003. Disponível em: <www.cpac.embrapa.br/baixar/326/t>.

DE MELLO, L. M. R. Panorama da Vitivinicultura brasileira 2014. In: *Hortifrúti,* 2015. Disponível em: <http://www.uvibra.com.br/pdf/PanoramadaVitiviniculturabrasileira2014abr2015.pdf>.

DÍAZ-RIQUELME, J.; LIJAVETZKY, D.; MARTÍNEZ-ZAPATER, J. M.; CARMONA, M. J. Genome wide analysis of *MIKC*^{C}-type MADS-box genes in grapevine. *Plant Physiology,* 149: 354–369. 2009.

DI GENOVA, A.; ALMEIDA, A. M.; MUÑOZ-ESPINOZA, C.; VIZOSO, P.; TRAVISANY, D.; MORAGA, C.; PINTO, M.; HINRICHSEN, P.; ORELLANA, A.; MAASS, A. Whole genome comparison between table and wine grapes reveals a comprehensive catalog of structural variants. *BMC Plant Biology*, 7 (14): 1471-2229. 2014.

DOLIGEZ, A.; BOUQUET, A.; DANGLOT, Y.; LAHOGUE, F.; RIAZ, S.; MEREDITH, C. P.; EDWARDS, K. J.; THIS, P. Genetic mapping of grapevine (*Vitis vinifera* L.) applied to the detection of *QTL*s for seedlessness and berry weight. *Theoretical and Applied Genetics,* 105 (5):780–795, 2002.

DORNELAS, M. C. & DORNELAS, O. From leaf to flower: revisiting Goethe's concepts on the ¨metamorphosis¨ of plants. *Brazilian Journal of Plant Physiology*, 17 (4): 335-343. 2005.

EGLI, D. B. In: Seed biology and the yield of grain crops. *CAB International*. ISBN 0852992412. 178. 1998.

EMERSHAD, R. L.; RAMMING, D. W.; SERPE, M. D. In: Ovulo embryo development and plant formation from stenospermic genotypes of *Vitis vinifera*. *American Journal of Botany*. 1989.

FANIZZA, G.; LAMAJ, F.; COSTANTINI, L.; CHAABANE, R.; GRANDO, M. S. *QTL* analysis for fruit yield components in table grapes (*Vitis vinifera*). *Theoretical and Applied Genetics,* 111: 658–664. 2005.

FAO, Food and Agriculture Organization of the United Nations. Disponível em <http://www.faostats.org/>. Acesso em 12 de abril de 2018.

FASOLI, M.; DAL SANTO, S.; ZENONI, S. The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. *The Plant Cell,* 24: 3489-3505. 2012.

FAVARO, R.; PINYOPICH, A.; BATTAGLIA, R.; KOOIKER, M.; BORGHI, L.; DITTA, G.; YANOFSKY, M. F.; KATER, M.; COLOMBO, L. MADS-Box protein complexes control carpel and ovule development in *Arabidopsis*. *The Plant Cell,* 15: 2603-2611. 2003.

FISCHER, B. M.; SALAKHUTDINOV, I.; AKKURT, M.; EIBACH, R.; EDWARDS, K. J.; TÖPFER, R.; ZYPRIAN, E. M. Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine. *Theoretical and Applied Genetics*, 108: 501-515. 2004.

GANDER, E. S. & MARCELLINO, L. H. Plantas transgênicas. *Biotecnologia Ciência & Desenvolvimento*, 1: 34-37, 1997.

GARCIA, D.; FITZ-GERALD, J. N.; BERGER, F. Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in *Arabidopsis. The Plant Cell,* 17: 52–60. 2005.

GOVER, O.; PERETZ, Y.; MOZES-KOCH, R.; MAORI, E.; RABINOWITCH, H. D.; SELA, I. Only minimal regions of tomato yellow leaf curl virus (TYLCV) are required for replication, expression and movement. *Archives of virology,* 159: 2263–2274. 2014.

GRAMZOW, L. & THEIßEN, G. Phylogenomics of MADS-box genes in plants—two opposing life styles in one gene family. *Biology,* 2: 1150–1164. 2013.

GRAMZOW, L. & THEIßEN, G. Phylogenomics reveals surprising sets of essential and dispensable clades of MIKCc-group MADS-box genes in flowering plants. *Journal of Experimental Zoology*. Part B, *Molecular and Developmental Evolution,* 324: 353–362. 2015.

GRIMPLET, J.; MARTÍNEZ-ZAPATER, J. M.; CARMONA, M. J. Structural and functional annotation of the MADS-box transcription factor family in grapevine. *BMC Genomics,* 17: 80. 2016.

GUIMARÃES, C. T.; MAGALHÃES, J. V.; LANZA, M. A.; SCHUSTER, I. Marcadores Moleculares e suas Aplicações no Melhoramento Genético. *Informe Agropecuário*, Belo Horizonte, 30: 253. 2009.

HE, C.; HOLME, J.; ANTHONY, J. *SNP* genotyping: the KASP assay. *Methods in Molecular Biology*, 1145: 75-86. 2014.

HAUGHN, G. & CHAUDHURY, A. Genetic analysis of seed coat development in *Arabidopsis*. *Trends Plant Science*, 10: 472-477. 2005.

HONG, R. L.; HAMAGUCHI, L.; BUSCH, M. A.; WEIGEL, D. Regulatory elements of the floral homeotic gene AGAMOUS identified by phylogenetic footprinting and shadowing. *The Plant Cell*, 16: 1296-1309. 2003.

HUANG, K.; LOUIS, J. M.; DONALDSON, L.; LIM, F. L.; SHARROCKS, A. D.; CLORE, G. M. Solution structure of the MEF2A-DNA complex: structural basis for the modulation of DNA bending and specificity by MADS-box transcription factors. *EMBO Journal,* 19: 2615-2628. 2000.

JAILLON, O.; AURY, J; M.; NOEL, B. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature,* 449: 463–467. 2007.

JENSEN, W. A. Observations on the fusion of nuclei in plants. *Journal of Cellular Biology*, 23: 669–672. 1964

JENSEN, W. A. & FISHER, D. B. Cotton embryogenesis: double fertilization, *Phytomorphology,* 17: 261–269. 1968.

JONES, P. W. & CASSELLS, A. C. Criteria for decision making in crop improvement programmes — Technical considerations. *Developments in Plant Breeding*, 3: 465-476. 1995.

DE MORAIS JÚNIOR, O. P. Seleção genômica ampla (GWS) aplicada ao melhoramento populacional. Programa de Pós-graduação em Genética e Melhoramento de Plantas, *Universidade Federal de Goiás (UFG)*, Goiás, 2013.

JURGENS, G. & MAYER, U. *Arabidopsis*. In: Embryos: Colour Atlas of Development, J. B. L. Bard, ed (*London: Wolfe Publishing*): 7–21. 1994.

KAUFMANN, K.; MELZER, R.; THEIßEN, G. *MIKC*-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Gene,* 347: 183-198. 2005.

KOOIKER, M.; AIROLDI, C. A.; LOSA, A.; MANZOTTI P. S.; FINZI, L.; KATER M. M.; COLOMBO, L. BASIC PENTACYSTEINE 1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic *Arabidopsis* gene SEEDSTICK. *The Plant Cell,* 17: 722–729. 2005.

KRAMER, E. M.; JARAMILLO, M. A.; DI STILIO, V. S. Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. *Genetics*, 166 (2): 1011-1023. 2004.

LACIRIGNOLA, C. & DIGIARO, M. Grapevine industry in the world: state of the art and future trends. In: Proceedings of the Mediterranean network on grapevine clostero viruses. *Ciheam,* 29: 33-46. 1999.

LAHOGUE, F.; THIS, P.; BOUQUET, A. Identification of a codominant marker linked to the seedlessness character in grapevine. *Theoretical and Applied Genetics,* 97: 1638-1649. 1998.

LAZZAROTTO, J. J. & FIORAVANÇO, J. C. Comércio exterior mundial e brasileiro de uva de mesa: análise de indicadores de competitividade, tendência e sazonalidades. *Documentos Embrapa Uva e Vinho*: 44-54. 2012.

LI, C.; GU, M.; SHI, N.; ZHANG, H.; YANG, X.; OSMAN, T.; LIU, Y.; WANG, H.; VATISH, M.; JACKSON, S.; HONG, Y. Mobile FT mRNA contributes to the systemic florigen signalling in floral induction. *Scientific Reports,* 1: 73. 2011.

LIU, C.; CHEN, H.; ER, H. L.; SOO, H. M.; KUMAR, P. P.; HAN, J. H.; LIOU, Y. C.; YU, H. Direct interaction of AGL24 and SOC1 integrates flowering signals in *Arabidopsis*. *Development,* 135: 1481-1491. 2008.

LODHI, M. A.; DALY, M. J. Y.; WEEDEN, N. F.; REISCH, B. I. A molecular marker based linkage map of *Vitis*. *Genome*, 38: 786–794. 1995.

LOHMANN, J. U. & WEIGEL, D. Building beauty: the genetic control of floral patterning. *Development Cell*, 2: 135–142. 2002.

LOPEZ-DEE, Z. P.; WITTICH, P.; ENRICO, P. E. M.; RIGOLA, D.; DEL BUONO, I.; GORLA, M. S.; KATER, M. M.; COLOMBO, L. OsMADS13, a novel rice MADS-box gene expressed during ovule development. *Developmental Genetics*, 25: 237 – 244. 1999.

LUSCOMBE, M. N.; AUSTIN, S. E.; BERMAN, H. M.; THORNTON, J. M. An overview of the structures of protein-DNA complexes. *Genome Biology Reviews*, 1: 1-37. 2000.

MALABARBA, J. Estrutura do gene *VvAGL11* e análise da expressão durante a morfogênese da semente de videira. Dissertação de Mestrado. - Curso de Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, *UFRGS*, Porto Alegre (RS). 2014.

MALABARBA, J.; BUFFON, V.; MARIATH, J. E. A.; GAETA, M. L.; DORNELAS, M. C.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L, F. The MADS-box gene Agamous-like 11 is essential for seed morphogenesis in grapevine. *Journal of Experimental Botany,* 68: 1493–1506. 2017.

MALABARBA, J.; BUFFON, V.; MARIATH, J. E. A.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L. F. Manipulation of *VviAGL11* Expression Changes the Seed Content in Grapevine (*Vitis vinifera* L.). *Plant Science,* 269: 126-135. 2018.

MARINVAL, P. Vigne sauvage et Vigne cultive´e dans le Bassin me´diterrane´en: Emergence de la viticulture - Contribution archeobotanique. In L'Histoire du Vin, une Histoire de Rites (Office International de la Vigne et du Vin, eds): 137–172. 1997.

MATIAS-HERNANDEZ, L.; BATTAGLIA, R.; GALBIATI, F.; RUBES, M.; EICHENBERGER, C.; GROSSNIKLAUS, U.; KATER, M. M.; COLOMBO, L. VERDANDI is a direct target of the MADS domain ovule identity complex and affects embryo sac differentiation in *Arabidopsis*. *The Plant Cell,* 22: 1702–1715. 2010.

MEJÍA, N.; GEBAUER, M.; MUNOZ, L.; HEWSTONE, N.; MUNOZ, C.; HINRICHSEN, P. Identification of *QTL*s for seedlessness, berry size, and ripening date in a seedless x seedless table grape progeny. *American Journal of Enology and Viticulture,* 58 (4): 499–507. 2007.

MEJÍA, N.; SOTO, B.; GUERRERO, G.; CASANUEVA, X.; HOUEL, C.; MICCONO, M. L. A.; RAMOS, R.; LE CUNFF, L.; BOURSIQUO, J. M.; HINRICHSEN, P. ADAM-BLONDO, A. F. Molecular, genetic and transcriptional evidence for a role of *VvAGL11* in stenospermocarpic seedlessness in grapevine. *BMC Plant Biology*, 11 (57): 1471-2229. 2011.

MELZER, R. & THEIßEN, G. Reconstitution of 'floral quartets' in vitro involving class B and class E floral homeotic proteins. *Nucleic Acids Research,* 37: 2723–2736. 2009.

MENDES, M. A.; GUERRA, R. F.; BERNS, M. C.; MANZO, C.; MASIERO, S.; FINZI, L. MADS domain transcription factor mediate short-range looping that is essential for target gene expression in *Arabidopsis*. *The Plant Cell,* 25 (7): 2560-2572. 2013.

MICHAUD, J.; LACAZE, P.; MASQUELIER, J. Fractionnement des oligomeres flavanoliques du raisin. *Bulletin de la Société de Pharmacie de Bordeaux*, 110: 111-116. 1971.

MIZZOTTI, C.; EZQUER, I.; PAOLO, D.; RUEDA-ROMERO, P.; GUERRA, R. F.; BATTAGLIA, R.; ROGACHEV, I.; AHARONI, A.; KATER, M. M.; CAPORALI, E.; COLOMBO, L. SEEDSTICK is a Master Regulator of Development and Metabolism in the *Arabidopsis* Seed Coat. *PLoS Genetics,* 10 (12): e1004856. 2014.

MOZES-KOCH, R.; GOVER, O.; TANNE, E.; PERETZ, Y.; MAORI, E.; CHERNIN, L.; SELA, I. Expression of an Entire Bacterial Operon in Plants. *Plant Physiology,* 158, 1883–1892. 2012.

NACHTIGAL, J.C. Avanços tecnológicos na produção de uvas de mesa. In: Congresso Brasileiro de Viticultura e Enologia, 10, Bento Gonçalves. Anais. Bento Gonçalves: Embrapa Uva e Vinho, 2003.

OCAREZ, N. & MEJÍA, N. Suppression of the D-class MADS-box AGL11 gene triggers seedlessness in fleshy fruits. *Plant Cell Reports,* 35: 239–254. 2016.

OIV. Descriptor list for grapevine varieties and Vitis species 243. Organization Internacional de la Vigne et du Vin (OIV), Paris. 2016.

OIV. Organization Internacional de la Vigne et du Vin (OIV), Paris. Statiques du secteur vitivinicole mondial. Disponível em <http://www.oiv.int/oiv/cms/index>. Acesso em 26 de junho de 2018.

PARENICOVÁ, L.; DE FOLTER, S.; KIEFFER, M.; HORNER, D. S.; FAVALLI, C.; BUSSCHER, J.; COOK, H. E.; INGRAM, R. M.; KATER, M. M.; DAVIES, B. Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in *Arabidopsis*: new openings to the MADS world. *The Plant Cell*, 15 (7): 1538-1551. 2003.

PELLEGRINI, L.; TAN, S.; RICHMOND, T. J. Structure of serum response factor core bound to DNA. *Nature,* 376: 490-498. 1995.

PERETZ, Y.; MOZES-KOCH, R.; AKAD, F.; TANNE, E.; CZOSNEK, H.; SELA, I. A Universal Expression / Silencing Vector in Plants [C][OA]. *Plant physiology,* 145: 1251–1263. 2007.

PINYOPICH, A.; DITTA, G., SAVIDGE, B.; LILJEGREN, SJ.; BAUMANN, E.; WISMAN, E.; YANOFSKY, M. Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature,* 424: 85–88. 2003.

PRATT, C. Reproductive anatomy in cultivated grapes. A review. *American Journal of Enology and Viticulture,* 22 (2): 92-109. 1971.

PROTAS, J. F. S. Uvas sem sementes cultivares BRS Morena, BRS Clara e BRS Linda. (Sistema de Produção, 8 ISSN 1678-8761, Versão Eletrônica). Disponível em: <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Uva/UvasSemSementes/apresentacao.htm>. *Embrapa Uva e Vinho*. 2005.

RAVEN, P. H.; EVERT, R. F; EICHHORN, S. E. Biologia Vegetal. 7ª ed. *Editora Guanabara Koogan* S.A., Rio de Janeiro. 2007.

REVERS, L. F.; WELTER, L. J.; IRALA, P. B.; SILVA, D. C.; LAMPE, V. S.; OLIVEIRA, P. R. D.; GARRIDO, L. R. Co-Localization of *QTLs* for seedlessness and downy mildew

resistance in grapevine. In: *International Conference on Grapevine Breeding and Genetics,* 10: 449-456. 2010.

RIBEREAU-GAYON, J. & PEYNAUD, E. Sciences et techniques de la Vigne. Tome I. Biologie de la Vigne. *Sols de Vignobles*, Dunod: Paris, l. 327. 1980.

RIAZ, S.; DANGL, G. S.; EDWARDS, K. J.; MEREDITH, C. P. A microsatellite marker based framework linkage map of *Vitis vinifera* L. *Theoretical and Applied Genetics,* 108: 864–872. 2003.

RIJPKEMA, A. S.; GERATS, T.; VANDENBUSSCHE, M. Evolutionary complexity of MADS complexes. *Current Opinion in Plant Biology*, 10: 32-38. 2007.

RIVERA, A. L.; LIM, M. G.; FERNANDEZ, F.; LOSKE, A. M. Physical methods for genetic plant transformation. *Physics of Life Reviews,* 9: 308–345. 2012.

ROUNSLEY, S. D.; DITTA, G. S.; YANOFSKY M. F. Diverse roles for MADS box genes in *Arabidopsis* development. *The Plant Cell,* 7: 1259–1269. 1995.

SAMACH, A.; ONOUCHI, H.; GOLD, S. E.; DITTA, G. S.; SCHWARZ-SOMMER, Z.; YANOFSKY, M. F.; COUPLAND, G. Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science* 288: 1613–1616. 2000.

SCHILLING, S.; PAN, S.; KENNEDY, A.; MELZER, R. MADS-box genes and crop domestication: the jack of all traits. *Journal of Experimental Botany,* 69 (7): 1447–1469. 2018.

SCHMIDT, R. J.; VEIT, B.; MANDEL, M. A.; MENA, M.; HAKE, S.; YANOFSKY, M. F. Identification and molecular characterization of ZAG1 the maize homolog of the *Arabidopsis* floral homeotic gene AGAMOUS. *The Plant Cell,* 5 (7): 729–737. 1993.

SCHWARZ-SOMMER, Z.; HUIJSER, P.; NACKEN, W.; SAEDLER, H.; SOMMER, H. Genetic control of flower development by homeotic genes in *Antirrhinum majus*. *Science,* 250: 931–936. 1990.

SEMAGN, K.; BABU, R.; HEARNE S.; OLSEN, M. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. *Molecular Breeding*, 33: 1-14. 2014.

SMACZNIAK, C.; IMMINK, R. G. H.; ANGENENT, G. C.; KAUFMANN, K. Developmental and evolutionary diversity of plant MADS-domain factors: Insights from recent studies. *Development,* 139: 3081–3098. 2012.

SONG, I. J.; NAKAMURA, T.; FUKUDA, T.; YOKOYAMA, J.; ITO, T.; ICHIKAWA, H. Spatiotemporal expression of duplicate agamous orthologues during floral development in Phalaenopsis. *Development Genes and Evolution,* 216: 301–313. 2006.

STADLER, R.; LAUTERBACH, C.; SAUER, N. Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in *Arabidopsis* seeds and embryos. *Plant Physiology,* 139: 701–712. 2005.

STOUT, A. B. Seedlessness in grapes. New York State Agricultural Experiment Station. 238. 1936.

THEIßEN, G.; SAEDLER, H. MADS-box genes in plant ontogeny and phylogeny. Haeckel's biogenetic law revisited. *Current Opinion in Genetic Development*, 5 (5): 628–639. 1995.

THEIßEN, G. & SAEDLER, H. Plant biology. Floral quartets. *Nature,* 409: 469-471. 2001.

THEIßEN, G. Development of floral organ identity: stories from the MADS house. *Current Opinion in Plant Biology,* 4: 75-85. 2001.

THEIßEN, G.; MELZER, R.; RÜMPLER, F. MADS-domain transcription factors and the floral quartet model of flower development: linking plant development and evolution. *Development* 143: 3259–3271. 2016.

THIS, P; LACOMBE, T; THOMAS, M. Historical origins and genetic diversity of wine grapes. *Trends in Genetics,* 22 (9): 511-519. 2006.

TORRES, A. C.; FERREIRA, A.T.; SÀ, F. G. de; BUSO, J. A.; CALDAS, L. S.; NASCIMENTO, A. S.; BRIGIDO, M. de M.; ROMANO, E. Glossário de biotecnologia vegetal. Brasília (DF), Embrapa –CNPH: 128. 2000.

TZENG, T. Y.; CHEN, H. Y.; YANG, C. H. Ectopic expression of carpel-specific MADS box genes from lily and lisianthus causes similar homeotic conversion of sepal and petal in *Arabidopsis*. *Plant Physiology,* 130: 1827–1836. 2002.

UNGRU, A. H. Analysis of communication pathways during seed development in *Arabidopsis thaliana*. Dissertação de mestrado. 2010.

VARDI, A.; LEVIN, I.; CARMI, N. Induction of seedlessness in citrus, from classical techniques to emerging biotechnological approaches. *Journal of the American Society for Horticultural Science,* 133: 117-126. 2008.

VAROQUAUX, F.; BLANVILLAIN, R.; DELSENY, M.; GALLOIS, P. Less is better: new approaches for seedless fruit production. *Trends in Biotechnology,* 18: 233–242. 2000.

VELASCO, R.; ZHARKIKH, A.; TROGGIO, M. A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE,* 2: 1326. 2007.

VIDAL, J. R.; GOMEZ, C.; CUTANDA-PEREZ, M. C.; SHRESTHA, B. R.; THOMAS, M. R.; TORREGROSA, L. Use of gene transfer technology for functional studies in grapevine. *Australian Journal of Grape and Wine Research,* 16: 138–151. 2010.

WANG, X.D.; QIN, C.; SU, J.; WANG, J.; CAO, L.; GUO, Q. G. Advances in in citrus seedless mechanism. *South China Fruits*, 41: 60-63. 2012.

YAMAMOTO, T.; KIMURA, T.; SOEJIMA, J.; SANADA, T.; BAN, Y.; HAYASHI, T. Identification of quince varieties using SSR markers developed from pear and apple. *Breed Science,* 54: 239–244. 2004.

YUN, P. Y.; KIM, S. Y.; OCHIAI, T.; FUKUDA, T.; ITO, T.; KANNO, A. AVAG2 is a putative D-class gene from an ornamental asparagus. *Sexual Plant Reproduction,* 17: 107–116. 2004.

ZHANG, X.; HENRIQUES, R.; LIN, S.; NIU, Q.; CHUA, N. Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nature Protocols*, 1: 1-6. 2006.

ZIEMIENOWICZ, A. Agrobacterium-mediated plant transformation: Factors, applications and recent advances. *Biocatalysis and Agricultural Biotechnology,* 3: 95–102. 2014.

8 ANEXO I

Structure and molecular characterization of the *Rpv3* locus and its relation to downy mildew resistance in grapevine (*Vitis* spp.)

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Title

Structure and Molecular Characterization of the *Rpv3* Locus and its Relation to Downy Mildew Resistance in Grapevine (*Vitis* spp.)

Short-title

P. viticola resistance genes from the *Rpv3* locus in *Vitis vinifera*

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Abbreviations: DPI: Days Post Inoculation; HAI: Hours After Inoculation; HR: Hypersensitivity reaction; OIV: Organisation Internationale de la Vigne et du Vin; LG: Linkage Group; RG: Resistance genes.

Abstract

Plasmopara viticola is the oomycete that causes downy mildew in grapevine. This disease implies in large economic and environmental impacts, due to its pathogenicity causing partial or total crop losses and demanding repeated fungicide sprays as a control measure. Varying levels of resistance to *P. viticola* allowed quantitative trait loci and major resistance genes to be mapped. The *Rpv3* locus is located on chromosome 18, on a region enriched in TIR-NBS-LRR genes, and the phenotype associated is a high hypersensitive response to downy mildew inoculation on resistant genotypes. In this work, we aimed to identify candidate genes associated with resistance to downy mildew located inside the *Rpv3* interval and to evaluate their transcriptional profiles in a susceptible (Cabernet Sauvignon) and a resistant (Villard Blanc) grapevine cultivar after *P. viticola* inoculation. Candidate genes were identified by representational differential analysis and also by *in silico* functional enrichment tests. Many functional genes were found at the *Rpv3* locus that are putatively associated with resistance to diseases. In total, seventeen genes were evaluated by RT-qPCR. Differences in the steady-state expression of these genes were observed between the two grapevine cultivars. Moreover, four genes were found to be expressed only for Villard Blanc, which can indicate their association to the hypersensitivity reaction. Based on *Rpv3*-located polymorphic SNPs we identified forty-one markers for for potential assisted selection. After genotypic and phenotypic evaluations on segregant populations, two markers, Rpv3_15 and Rpv3 33, were classified as efficient for downy mildew resistance identification. The present study confirms the involvement of the *Rpv3* locus in conferring resistance to *P. viticola* infection and presents a promising biotechnological tool for the selection of young resistant individuals.

Keywords: Downy mildew resistance, *Plasmopara viticola,* RT-qPCR, *Rpv3* locus, *V. vinifera.*

69 **Introduction**

70

71 Fungal diseases are one of the main constraints to grape production, both
72 quantitatively and qualitatively (GARRIDO, 2008). In humid subtropical areas the
73 major diseases causing pathogens in the grapevines aerial parts and limiting cultivation
74 are the fungi *Elsinoe ampelina* and *Uncinula necator,* causing the anthracnose, and
75 powdery mildew, respectively, and the oomycota *Plasmopara viticola,* responsible for
76 the downy mildew disease (IBRAVIN, 2012). *Plasmopara viticola* (Berk. Et Curt.)
77 Berl. et de Toni. is a major biotrophic oomycete belonging to the Pernosporaceae
78 (Chromalveolata), known to be a large family of phytopathogenic microorganisms
79 (GRENVILLE-BRIGGS & VAN WEST, 2005). Downy mildew infection can cause
80 total production loss by the destruction of inflorescences and/or fruits and by early plant
81 defoliation (ERWIN & RIBEIRO, 1996). In addition, even after controlled, *P. viticola*
82 infection may still trigger future crop damages, due to the poor formation of the
83 branches and consequent plant weakened development (TESSMANN & VIDA, 2005;
84 GARRIDO & SÔNEGO, 2007). When in the grapevine tissues, mainly leaves and
85 bunches, *P. viticola* develop by obtaining nutrients from the infected plant cells with the
86 help of specialized structures, the haustories. These structures also allow the exchange
87 of signals involved in the compatibility infection establishment (POLESANI *et al.*,
88 2010). *Vitis vinifera* cultivars are completely susceptible to the attack of this pathogen,
89 therefore to reduce losses, the intense application of multiple fungicides becomes
90 necessary during each new season (CADLE-DAVIDSON, 1998; BELLIN *et al.,* 2009).
91 Nevertheless, these palliative actions increased production costs, pose a risk factor for
92 human health and generate environmental impacts in the application areas, such as
93 contamination of soil with chemical residues (BLASI *et al.*, 2011). Besides, there are
94 some strains with resistance to certain fungicides, which leads to a decrease in the
95 efficiency of disease control (WANG *et al.*, 2013; BLUM *et al.*, 2010).

96 Plant defense responses to pathogens are triggered by the recognition of
97 pathogen exogenous chemical signatures by the host, followed by the immune response
98 signal transduction mechanisms resulting in a significant reprogramming of the plant
99 cell metabolism, which involves changes in gene expression activity (DÍEZ-NAVAJAS
100 *et al.*, 2008; PINTO *et al.* 2012). This response triggered by plants does not inhibit
101 colonization of the pathogen, but it limits the extent of propagation since the action of
102 resistance proteins (R) accelerates and amplifies the innate basal response process

103 (BELKHADIR *et al*., 2004). The downy mildew resistance mechanism includes cell
104 wall strengthening, pathogenesis-related (PR) protein synthesis, production of
105 antimicrobial compounds, such as phytoalexins, and the assembly of complex responses
106 such as the hypersensitivity reaction (HR), which consists of the programmed cell death
107 of the cells around the infected region, blocking the pathogen progression (POLESANI
108 *et al*., 2010). North American grapevine species (*V. riparia, V. cinera, V. labrusca, V.*
109 *rupestris, V. berlandieri, V. lincecumii* and *Muscadinia rotundifolia*) present variable
110 resistance levels to *P. viticola* attack (KORTEKAMP & ZYPRIAN, 2003; UNGER *et*
111 *al*., 2007; DÍEZ-NAVAJAS *et al*., 2008). More than 200 resistance genes (R) were
112 identified by the construction of systematic genome maps (VELASCO *et al*., 2007) and
113 the genomic DNA sequencing of *V. amurensis* and *V. riparia* (DI GASPERO &
114 CIPRIANI, 2003). Many of these genes are located in genomic regions associated with
115 resistance to *P. viticola* in wild *Vitis* species and many have orthologous genes in
116 *Arabidopsis thaliana* that regulate pathways of defense against pathogens (POLESANI
117 *et al*., 2010). Furthermore, the variable levels of resistance to *P. viticola* presented by
118 North American species allowed the mapping of resistance genes and quantitative
119 character loci (QTLs) as already identified by Merdinoglu *et al*. (2003); Fischer *et al*.
120 (2004); Welter *et al*. (2007), Revers *et al*. (2010), Blasi *et al*. (2011), Moreira *et al*.
121 (2011) and Schwander *et al*. (2012). Eight major loci were detected with great effect on
122 downy mildew resistance in grapevines: *Rpv1* (MERDINOGLU *et al*., 2003), *Rpv2* e
123 *Rpv3* (BELLIN *et al*., 2009), *Rpv5* (MARGUERIT *et al*., 2009), *Rpv8* (BLASI *et al.,*
124 2011), *Rpv10* (SCHWANDER *et al*., 2012), *Rpv11* (SALMASO *et al*., 2008;
125 SCHWANDER *et al*., 2012) e *Rpv12* (VENUTI *et al*., 2013).

126 The *Rpv3* locus is located in the LG 18 and was identified by different research
127 groups as being a QTL associated with a strong HR against downy mildew, in resistant
128 individuals (FISCHER *et al*., 2004; WELTER *et al*., 2007; BELLIN *et al*., 2009;
129 REVERS *et al*., 2010; SCHWANDER *et al*., 2012). This locus accounts for up to 75%
130 of the phenotypic variation, and more than 30 R genes encoding TIR-NBS-LRR
131 proteins and receptor proteins of the LRR-kinase type are located within the *Rpv3*
132 region (VELASCO *et al*., 2007; BELLIN *et al*., 2009). The TIR-NBS-LRR proteins are
133 related to the so-called gene-to-gene resistance response. This mechanism is based on
134 the presence of the proteins encoded by the R genes that act in the recognition of
135 specific race effectors, the Avr proteins (TAKKEN *et al*., 2006; DÍEZ-NAVAJAS *et al*.,
136 2008; DRY *et al*., 2010). TIR-NBS-LRR proteins are responsible for monitoring the

137 integrity of components of the basal immune system, based on the recognition pathogen
138 Avr proteins. Any perturbation of the components by the effector action/Avr proteins
139 can trigger a greater expression of the TIR-NBS-LRR proteins (TAKKEN *et al*., 2006).
140 These proteins are composed of three major domains: 1) an amino-terminal variable
141 domain (coiled-coil (CC) or a homologous domain Toll/Interleukin -1- Receptor (TIR),
142 2) a central nucleotide binding site (NBS), and 3) leucine-rich repeats (LRR), which is
143 responsible for the recognition of pathogen effectors (TAKKEN *et al*., 2006). With the
144 increase in TIR-NBS-LRR levels, the induction of a rapid defense response initiates,
145 characterized by calcium and ion fluxes, extracellular oxidative burst, and
146 transcriptional reprogramming in the infected sites and in the peripheral regions,
147 culminating with the HR, ceasing the growth of the pathogen (BELKHADIR *et al*.,
148 2004; JONES & TAKEMOTO, 2004; GREENBERG & VINATZER, 2003). Therefore,
149 these proteins are determinants of the specificity of the immune response (TAKKEN *et*
150 *al*., 2006; DÍEZ-NAVAJAS *et al*., 2008; DRY *et al*., 2010).

151 In this work we aimed to identify candidate genes associated with resistance to
152 downy mildew at the *Rpv3* locus in grapevine and evaluate the transcriptional profiles
153 of these genes in a susceptible cultivar, Cabernet Sauvignon, and in a resistant cultivar,
154 Villard Blanc, after challenging with *P. viticola*. Moreover, we evaluated the
155 biotechnological applicability of 41 SNP–type candidate markers, located within the
156 *Rpv3* locus and associated to the resistance phenotype.

157

158 **Materials and methods**

159

160 *P. viticola challenge assay*

161 Leaves from the cultivar Isabel (*V. labrusca*) infected with downy mildew were
162 harvested from a vineyard in the district of Faria Lemos (29º06'13,09 " South and
163 51º36'26,10 " West - 362 meters altitude) in Bento Gonçalves – RS, Brazil.. The leaves
164 were humidified and stored in a humid chamber at 25 ºC and in the absence of light,
165 until sporangia formation. For *P. viticola* challenge assay, 20 young branches of
166 'Cabernet Sauvignon', a *V. vinifera* cultivar, susceptible to mildew, and of 'Villard
167 Blanc', a complex hybrid resistant to mildew, were harvested at Embrapa Uva e Vinho
168 (29º09'48" South and 51º31'53.95" Weast – 640 meters altitude) and rooted on a
169 Styrofoam box. A suspension containing $3x10^5$ sporangia / mL of *P. viticola* was
170 sprayed onto the abaxial surface of leaves from these two cultivars. This experiment

171 was carried out in biological triplicates, under controlled environmental conditions
172 (temperature of 25 ± 2 ºC and 100% relative humidity). Ten leaves per biological
173 replicate were sampled at 0 (time which was used as a control) 6, 12, 24, 48 and 72
174 hours after inoculation (HAI). These leaves were frozen in liquid nitrogen and stored at
175 -80 ºC until processing.
176

177 *Representational difference analysis (RDA)*

178 Young branches of 'Villard Blanc' and 'Cabernet Sauvignon' were challenged
179 with *P. viticola* sporangia as described above. Leaf samples were obtained at 0, 6, 12,
180 24, 48 and 72 hours after inoculation (HAI). Total RNA was isolated (as described in
181 *Plant RNA extraction* section) and equimolar aliquots from each 'Villard Blanc' sample
182 points were assembled and used for the synthesis of a cDNA pool. This pool was
183 assayed to obtain a collection of differentially expressed genes using representational
184 difference analysis (RDA). Two steps of subtractive hybridization were performed. At
185 first, an RNA pool from mock-inoculated 'Villard Blanc' was used as driver and an
186 RNA pool from downy mildew-inoculated 'Villard Blanc' was used as tester. In the
187 second subtraction, the driver was the same and tester was the differential product of the
188 first subtraction. Amplicons from the second subtraction were cloned into pGEM-T
189 Easy vectors (Promega, Madison). A total of 2,229 transformants were obtained, of
190 which 384 were randomly chosen for sequencing. The resulting sequences were then
191 analyzed using the SisGen Automatized System of Sequence Analysis
192 (http://genoma.embrapa.br/genoma/), a unified tool that dynamically integrates data
193 from various databases.
194

195 *P. viticola resistance-related candidate genes selection*

196 For candidate genes selection, Fisher's test was performed using the online software
197 Blast2Go (B2G) (GÖTZ *et al.*, 2008). All genes located at the distal portion of
198 chromosome 18, corresponding to the *Rpv3* locus region, were evaluated for enrichment
199 in functional annotation terms (GO) related to resistance, taking the entire grapevine
200 gene set as a reference. Direct and reverse primers specific for each candidate gene were
201 designed manually, limiting the size of amplicons to the maximum of 200 base pairs.
202 The candidate genes were named according to their position at the *Rpv3* locus in the
203 12X version of the genome (Jaillon et al., 2007).

204 *Plant RNA extraction*

205 Total RNA was isolated from grapevine leaves by LiCl precipitation using the
206 Zeng and Yang (2002) protocol scaled to 2 mL volumes. Each sample extraction was
207 performed in triplicate and final volumes were pooled before the LiCl precipitation step.
208 Genomic DNA was removed using the TURBO DNA-free Kit (Ambion, Foster City)
209 according to the manufacturer's protocol. RNA integrity and quantity were monitored
210 by agarose gel electrophoresis and spectrophotometric quantitation, respectively.

211

212 *RT-qPCR analysis*

213 Complementary DNAs were synthesized using the GeneAmp RNA PCR Core
214 Kit (Applied Biosystems, Foster City) according to manufacturer's instructions. The
215 gene-specific primers were designed using the Oligo Analyzer 3.1 tool (IDT,
216 http://www.idtdna.com) and synthesized by IDT, with the standard settings of 0.2 µM
217 of oligo concentration, 1.5 mM of $MgCl_2$ and 0.2 mM of dNTP (Supplementary Table
218 1). RT-qPCR was performed on a StepOnePlus Real-Time PCR System (Applied
219 Biosystems, Foster City). SYBR Green (Invitrogen, Carlsbad) was used to monitor
220 dsDNA synthesis. Each biological sample was analyzed in technical quadruplicates.
221 Cycling consisted of one step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15
222 s, 60 °C for 1 min, and finished by a dissociation curve between 60 °C and 95 °C. The
223 specificity of PCR amplification was assessed by the presence of a single peak in
224 melting curves, visualization of single amplification products of expected size in 3%
225 ethidium bromide-stained agarose gel electrophoresis and sequencing of the amplicons.
226 Primer efficiency was calculated using LinRegPCR software (version 11.0). Mean
227 relative gene expression was calculated by the Pfaffl (2001) method employing *ACTIN*
228 (GenBank EC969944) as a reference gene (REID *et al.*, 2006). All data are represented
229 as averages of four replicates techniques and three biological replicates. Statistical
230 analysis of the significant differences between the genes and the samples was performed
231 using One-way ANOVA followed by Dunett's test ($p < 0.05$), in which the 0h time-point
232 of each cultivar was used as a control for comparison to the following time-points of the
233 same cultivar samples.

234

235 *Evaluation of P. viticola resistant individuals*

236 Ninety-four grapevine genotypes resulting from the CNPUV692 population
237 ('Villard Blanc' X 'Crimson Seedless') and segregating for downy mildew resistance

238 were used for SSR markers evaluation. For the KASP markers, two hundred individuals
239 from the self-fertilized population VB (‘Villard Blanc’ X ‘Villard Blanc’) were used.
240 Moreover, ‘Villard Blanc’ leaves were used for resistance control evaluation and leaves
241 from ‘Cabernet Sauvignon’ were used as a positive control of the infection. For
242 phenotyping their resistance to *P. viticola*, sixteen leaf discs of 1 cm in diameter were
243 detached from each individual and a suspension containing $3x10^5$ sporangia/mL of *P.*
244 *viticola* was sprayed onto the abaxial surface of the discs. Next, they were incubated in
245 Petri dishes under humid conditions, at 25ºC and medium light (~150 $\mu molm^{-2}s^{-1}$). The
246 phenotypic classification of downy mildew resistance was determined according to the
247 descriptor OIV - 452 (ANONYMOUS, 1983), in which the most resistant individuals
248 are classified as 1 and the most susceptible as 9. Individuals are considered resistant if
249 scored 1 to 3. Disease progression was monitored for nine days. Five days post
250 inoculation (DPI), photographs from the dishes were taken until the ninth day and a
251 score was given for each individual by the end of the experiment (9 DPI) (Figure 4).
252 These experiments were done in two distinct years.
253
254 *SSR markers evaluation*
255 Individuals from the CNPUV692 population were used in this assay. Each
256 individual DNA was purified as described by LEFORT & DOUGLAS (1999) and used
257 in PCR amplifications for each locus as described by Revers *et al*. (2010). The SSR
258 markers P2_VVAGL11 (MEJÍA *et al*., 2011; F- 5’ TGTACACCAATACGGGTTTCAT
259 3’ and R- 5’ GTTTGCTGGATTTCCGATGT 3’), P3_VVAGL11 (MEJÍA *et al*., 2011;
260 F- 5’ CTCCCTTTCCCTCTCCCTCT3’ and R- 5’ AAACGCGTATCCCAATGAAG
261 3’), VMC7F2 (GenBank BV005171; F- 5’ AAGAAAGTTTGCAGTTTATGGTG 3’
262 and R- 5’ AGATGACAATAGCGAGAGAGAA 3’) and VVIN16 (GenBank
263 BV140662; F- 5’ ACCTCTATAAGATCCTAACCTG 3’ and R- 5’
264 AAGGGAGTGTGACTGATATTTC 3’) UDV108 (F- 5’
265 TGTAGGGTTCCAAAGTTCAGG and R 5’CTTTTTATATGTGGTGGAGC 3’) were
266 used. The amplicons were resolved on 6% polyacrylamide gel and stained with silver
267 nitrate as described by CRESTE *et al*. (2001). Deviations between the observed and
268 expected genotype segregations and the possible associations between the phenotypes
269 and the alleles evaluated were tested by concordance and chi-square independence (χ^2).
270
271

272 *SNP markers evaluation*

273 The forty-one SNP-type markers tested in this work were chosen based on the
274 GrapeReSeq_Illumina_20K chip
275 (https://urgi.versailles.inra.fr/Species/Vitis/GrapeReSeq_Illumina_20K). Leaf discs
276 from the two hundred individuals from the self-fertilized population VB ('Villard
277 Blanc' X 'Villard Blanc') were shipped to LGC Genomics (http://www.lgcgroup.com,
278 England). To perform the KASP technique, DNA was extracted from the samples and
279 three primers were added in the PCR, two allele-specific forward primers, each with a
280 single tail sequence, labelled with fluorescence, and a reverse primer complementary to
281 both, allowing end-point fluorescence reading. The resulting fluorescence was measured
282 and the raw data was interpreted to allow the assignment of genotypes to each DNA
283 samples. The fluorescent signal of each individual DNA sample is represented as an
284 independent data point, whereby the identification of the allele is homozygous or
285 heterozygous for a particular sample.

286

287 *DNA amplicons sequencing*

288 All the DNA amplicons sequenced for this work were analyzed in an ABI
289 Prism® 310 Genetic Analyser (Applied Biosystems, Foster City) using standard
290 sequencing protocols described in Falavigna *et al.* (2014). Sequence analysis was
291 carried with DNA Sequencing Analysis Software v5 (Applied Biosystems, Foster City)
292 and MEGA7 software (http://www.megasoftware.net/home). Sequences were compared
293 with the grapevine reference ('Pinot Noir' PN40024) genome.

294

295 **Results**

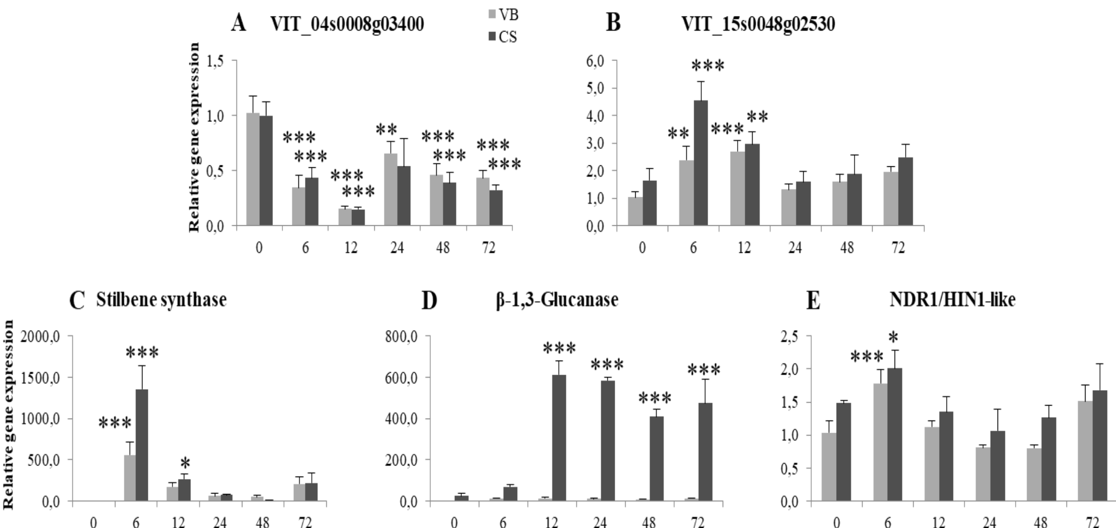
296

297 *Identification and characterization of associated resistance genes for downy mildew*
298 *disease*

299 In this study we researched differentially expressed genes after a challenge with
300 *P. viticola* in leaves of 'Villard Blanc' and 'Cabernet Sauvignon'. Amongst all specific
301 cDNAs from the RDA approach, two candidate genes were identified,
302 VIT_04s0008g03400 and VIT_15s0048g02530. The gene VIT_04s0008g03400 is an
303 ethylene-responsive transcription factor from the DREB sub-A-4 family of ERF/AP2
304 transcription factors (AGUDELO-ROMERO *et al.*, 2015). When compared to 0 HAI,
305 this gene had a high decrease in relative expression at 6 and 12 HAI, and kept a low

expression in all time-points for both cultivars (Figure 1A). The other candidate, VIT_15s0048g02530 is predicted to be a hairpin-induced 1 gene and a possible Arabidopsis ortholog of an L-TYPE LECTIN RECEPTOR KINASE S4 (AT3G55550.1), described to be involved with defense response to bacterium and oomycetes and with protein phosphorylation (Bouwmeester & Govers, 2009; Eggermont *et al*., 2017). After challenge with *P. viticola,* VIT_15s0048g02530 presented a significant increase in relative expression at 6 and 12 HAI. Moreover, the expression was induced in both cultivars in the following time-points, 24, 48 and 72, showing a pattern of increase in transcript levels (Figure 1B).

Next, we characterized the transcriptional profile of genes described in the literature to be associated with defense response against fungi: Stilbene Synthase (VIT_16s0100g00990), β-1,3-glucanase (PR3, VIT_08s0007g06060) and NDR1/HIN1-like (VIT_16s0098g00890). Relative gene expression profiles using RT-qPCR were obtained for these genes from each sample point of the two cultivars challenged with downy mildew. The expression profile of Stilbene Synthase gene was similar for both cultivars, with an increase in relative expression at 6hs, and with significantly higher levels at 12hs for 'Cabernet Sauvignon' (Figure 1C). For the β-1,3-glucanase gene, the induction of gene expression in 'Cabernet Sauvignon" was delayed, starting at 12hs after infection with *P. viticola*. Nevertheless, this induction was significant in all posterior time-points when compared to 'Villard Blanc' samples (Figure 1D). For NDR1/HIN1-like the effect of inoculation time was similar between the resistant and susceptible cultivars and the expression peak occurred at 6 hours, with no significant differences after this time-point (Figure 1E). According to bioinformatics analysis, NDR1/HIN1-like is a probable ortholog of the Arabidopsis NDR1 gene which belongs to the large family of NHL (for NDR1/HIN1 like) (DÖRMANN *et al*., 2000). AtNDR1 is involved in the induction of systemic acquired resistance salicylic acid-dependent, after pathogen infection and many CC-NBS-LRR proteins require NDR1 to trigger multiple signalling defense pathways in Arabidopsis (CENTURY *et al*., 1997).



334

335 **Figure 1. Transcriptional profile of candidate genes after *P. viticola***
336 **inoculation.** Candidate genes selected from the RDA (A and B). Candidate genes from
337 the literature (C-E). Actin gene expression was used as a reference. Axys *x* shows hours
338 after *P. viticola* inoculation (HAI). VB= Villard Blanc; CS= Cabernet Sauvignon. One-
339 way ANOVA followed by Dunett's test, in which the 0h time-point of each cultivar was
340 used as a control for comparison to the following time-points, * p< 0,05; ** p< 0,001;
341 *** p<0,0001.

342

343 *Identification of Rpv3 associated resistance genes for downy mildew disease*

344 We also aimed to test a set of genes that are located in the *Rpv3* locus, LG18.
345 According to Fisher's test, the genomic region of the *Rpv3* locus is enriched with genes
346 associated with defense responses, programmed cell death, signal transduction, immune
347 system processes and responses to stress. About 70 genes were found at the *Rpv3* locus,
348 of which 40 had at least one of the TIR-NBS-LRR domains, while eight genes had all
349 three domains. From this set of 40 predicted candidate genes, 24 were selected for RT-
350 qPCR evaluation (Table 1), 8 of them for having the 3 TIR-NBS-LRR domains and 16
351 for having more GO terms associated with programmed cell death and defense
352 responses, besides being located close to the apyreny selection molecular marker
353 VMC7F2 (CABEZAS *et al.,* 2006) and having at least one of the TIR-NBS-LRR
354 domains. Fisher's test results for each of the 70 *Rpv3* gens are described in detail in
355 supplementary table 2. The location of the 24 genes on the *Rpv3* locus selected for the
356 accomplishment of the present work is demonstrated in figure 2.

357

358 *Rpv3 candidate genes characterization*

359 To characterize the candidate genes, conventional PCR reactions based on
360 genomic DNA were performed to confirm their presence in the genome of both
361 cultivars, Villard Blanc and Cabernet Sauvignon, as well as to confirm the size of the
362 amplification product of each gene, based on the grapevine genome cultivar. Of the 24
363 candidate selected genes, 22 presented amplification product. 'Cabernet Sauvignon'
364 DNA presented the 22 genes, dissimilar to 'Villard Blanc' that presented only 17
365 candidate genes, under the conditions tested. This allowed us to confirm the presence of
366 the selected candidate genes in the genomes of the two cultivars (Supplementary Figure
367 1). A few genes presented differences in the amplification product sizes, between
368 cultivars. The gene locus, specific primers and expected amplification products are
369 described in Supplementary Table 1.

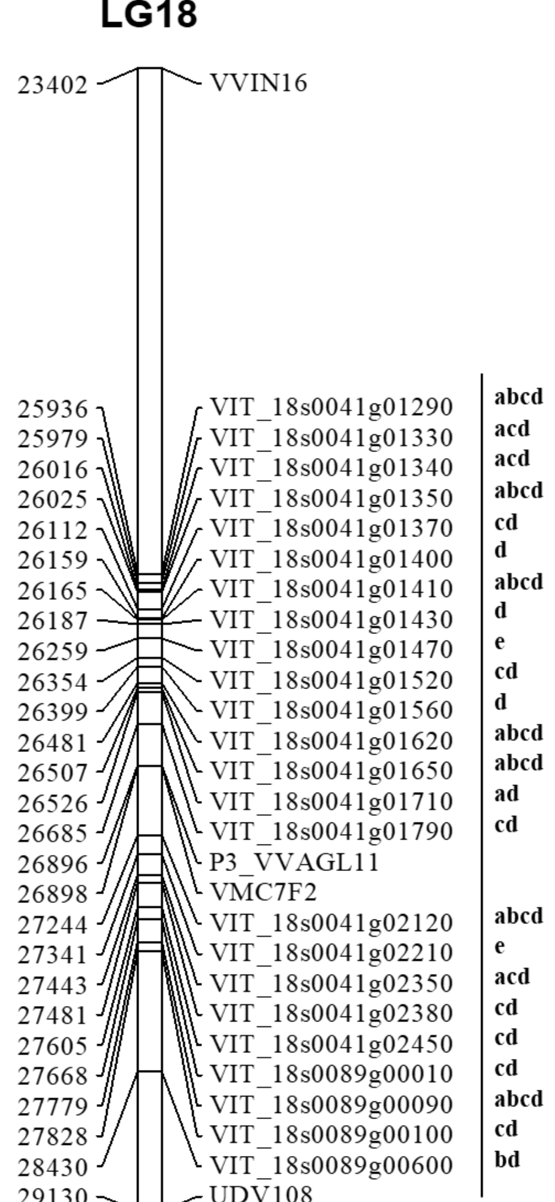
370

371 *Rpv3 candidate genes expression in response to P. viticola infection*

372 The transcriptional profile of the candidate genes was obtained by RT-qPCR
373 after spraying *P. viticola* sporangia on the susceptible cultivar Cabernet Sauvignon and
374 the resistant cultivar Villard Blanc. *Actin* gene expression was used to normalize the
375 data from all genes and zero hours after inoculation (0 HAI) was used as a calibrator. Of
376 the twenty-two pairs of specific primers used to evaluate the candidate genes, nine did
377 not show RT-qPCR amplification. For twelve genes it was observed amplification
378 products on expected sizes (Figure 2 and Figure 3). The genetic specificity of the twelve
379 sequenced PCR products were all confirmed by sequencing.

380 The twelve genes that presented expression were divided into three groups,
381 according to their expression pattern: 1) the ones that presented expression in both
382 cultivars samples (seven genes); 2) the ones that only had expression in the Villard
383 Blanc cultivar samples (four genes) and the 3) one gene that was only expressed in
384 'Cabernet Sauvignon' samples (Figure 3).

385 From the first group, the transcriptional profile of the genes
386 VIT_18s0041g01350, VIT_18s0041g01620 and VIT_18s0089g00090 demonstrated an
387 increase in relative gene expression after challenge with *P. viticola* in both cultivars.
388 The gene VIT_18s0041g01350 presented a transcriptional profile with high levels for
389 'Villard Blanc' samples with statistical differences after 6-12 HAI (Figure 3B).



390

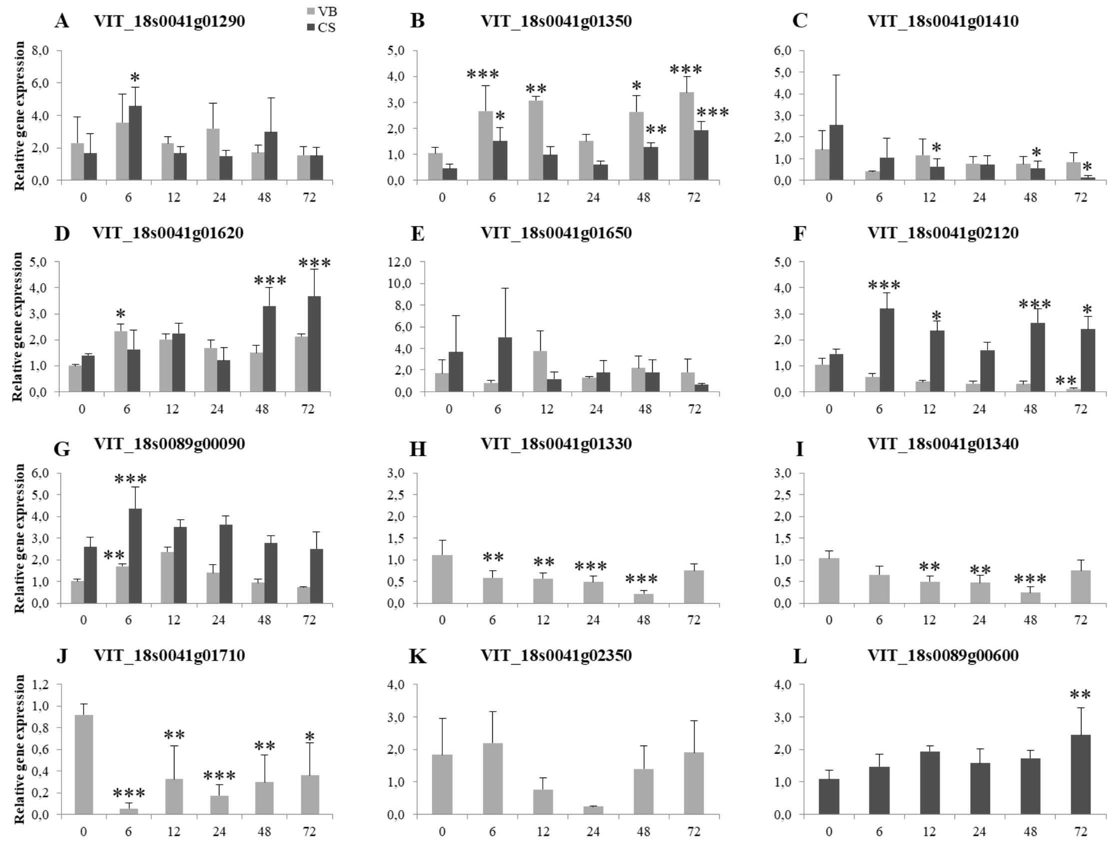
391

392 **Figure 2. The position of the candidate genes at *Rpv3* locus on the distal portion of**
393 **chromosome 18.** Letters represent the presence of amplification products for each
394 cultivar, Villard Blanc (VB) and Cabernet Sauvignon (CS), by PCR and by RT-qPCR
395 (a= RNA VB; b= RNA CS; c= DNA VB and d= DNA CS).

396

397 Furthermore, for the Cabernet Sauvignon cultivar, the gene
398 VIT_18s0089g000090 increased in expression showing a significant difference of
399 approximately 2-4X more expression after 6 HAI (Figure 3G). Gene
400 VIT_18s0041g01620 presented a significant difference in 48 and 72 HAI when
401 compared to 0 HAI showing an approximately 2.5X increase in expression in ‘Cabernet

402 Sauvignon' (Figure 3D). Gene VIT_18s0041g02120 presented a higher level of
403 transcripts for Cabernet Sauvignon samples in most of the time-points while for Villard
404 Blanc cultivar its expression decreased after *P. viticola* inoculation (Figure 3F). This
405 tendency of differential expression, with a decrease curve happened for other genes,
406 such as VIT_18s0041g01290, VIT_18s0041g01410 and VIT_18s0041g01650 (Figure
407 3A, 3C and 3E, respectively).



409

410 **Figure 3. Transcriptional profile of candidate genes at the *Rpv3* locus after *P.*
411 *viticola* inoculation.** Actin gene expression was used as a reference. Axis *x* shows hours
412 after *P. viticola* inoculation (HAI). VB= Villard Blanc; CS= Cabernet Sauvignon. One-
413 way ANOVA followed by Dunett's test, in which the 0h time-point of each cultivar was
414 used as a control for comparison to the following time-points, * p< 0,05; ** p< 0,001;
415 *** p<0,0001.

416

417 Interestingly, four genes presented expression only for the resistant cultivar
418 Villard Blanc after *P. viticola* challenge, representing the second group. Genes
419 VIT_18s0041g01330 and VIT_18s0041g01340 showed a gradual decrease in relative
420 gene expression in the period between 0 and 48hs after challenge with *P. viticola*

421 (Figure 3H and Figure 3I). Moreover, gene VIT_18s0041g01710 presented a statistical
422 difference in relation to 0 HAI with significant decrease at all time-points for resistant
423 cultivar Villard Blanc (Figure 3J).

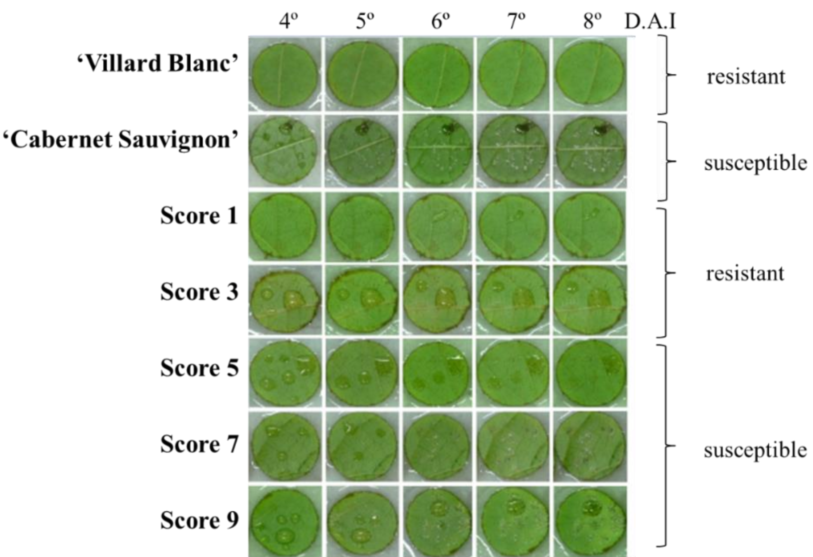
424 The VIT_18s0089g00600 gene, that composes the third group, was the only one
425 that was expressed uniquely in the susceptible cultivar samples. This gene showed a
426 slight increase in expression after challenge with *P. viticola*, demonstrating 2.5X more
427 expression at 72 HAI when compared to 0 HAI (Figure 3L).

428

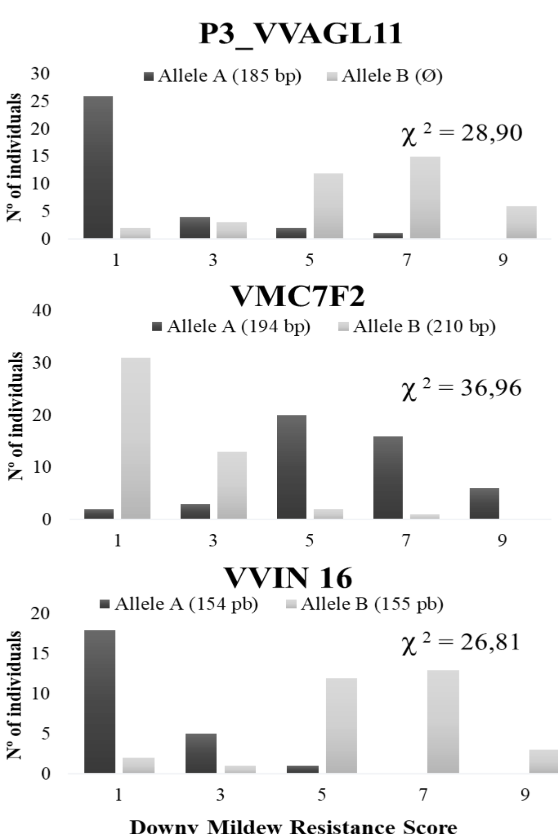
429 *Evaluation of SSR markers for downy mildew resistance*

430 The co-localization of the *Rpv3* locus and the *SdI* locus (Seed development
431 Inhibitor) gave us the opportunity to test molecular markers that were already stablished
432 for seedlessness assisted selection. These markers (UDV108, VMC7F2, VVIN16,
433 P2_VVAGL11 and P3_VVAGL11) were efficiently described for their >80% selection
434 of apirenic grapevines (DOLIGEZ *et al*. 2002, CABEZAS *et al*., 2006, MEJIA *et al*.,
435 2011). For this analysis, we performed the phenotyping of the resistance individuals by
436 a *P. viticola* challenge assay which is described in Material and Methods section in
437 figure 4. After performing the $\chi2$ adjustment tests, UDV108 and P2_VVAGL11 did not
438 present the expected segregation and were excluded from the analysis. For the loci
439 VMC7F2, VVIN16 and P3_VVAGL11 four segregating alleles were observed in the
440 genotyped population. Analysis of the phenotypic distribution of the evaluated
441 characters versus the allele frequency of the genotyped SSR markers shows the
442 association between the alleles P3_VVAGL11-185 bp ($\chi2$ calc = 28.9), VVIN16-154 bp
443 ($\chi2$ calc = 26.81) and VMC7F2-210 bp ($\chi2$ calc = 36.93) with downy mildew resistance
444 (Figure 5). By these markers evaluation, haplotypes were identified for resistance to
445 downy mildew. The use of the haplotype, that combines the three SSR markers,
446 VMC7F2, VVIN16 and P3_VVAGL11, presents 100% accuracy in the selection of
447 resistant individuals, also eliminating false positives.

448



449 **Figure 4. Downy Mildew infection progression on leaf discs.** Leaf discs were
450 monitored for 8 days after *P. viticola* inoculation. Leaf discs from the parental 'Villard
451 Blanc' were used as the resistance control and 'Cabernet Sauvignon' was used as
452 positive control for the infection.



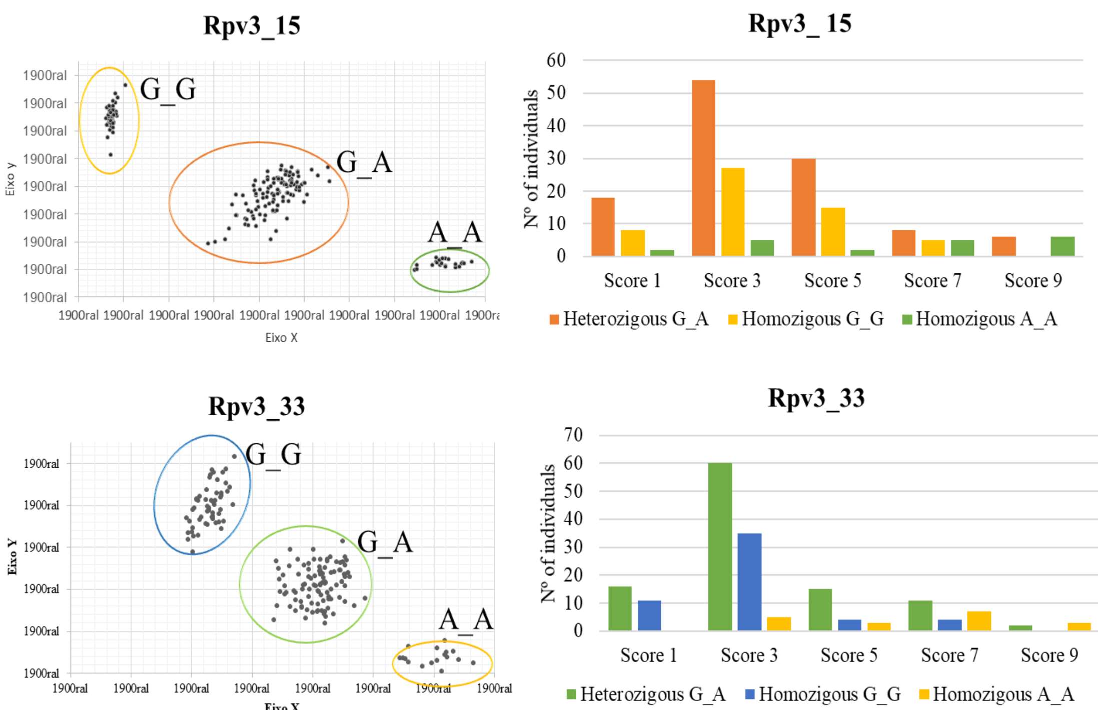
453

454 **Figure 5. SSR markers evaluated for downy mildew resistance.** Number of
455 individuals and their genotypes versus the score for *P. viticola* resistance (OIV - 452).

456 *Development and evaluation of SNPs markers for P. viticola resistance assisted*
457 *selection*

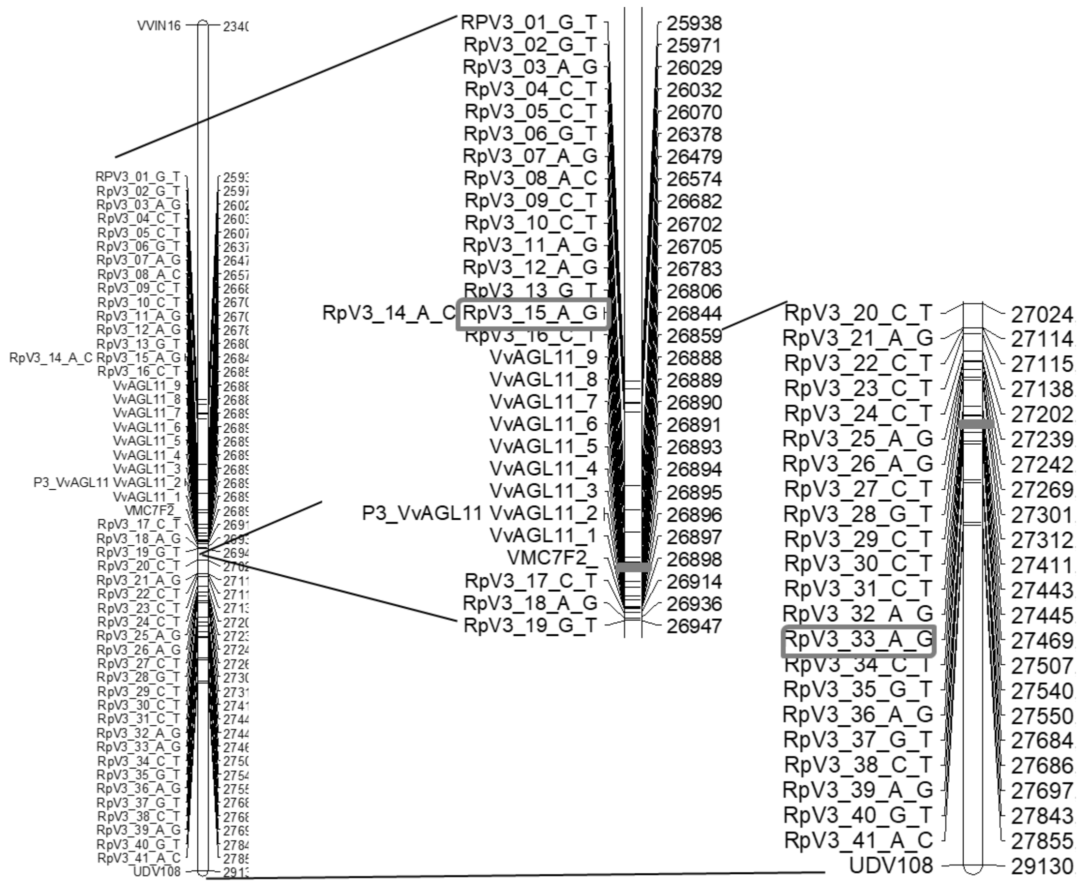
458 In order to develop new and efficient molecular markers for assisted selection of
459 downy mildew resistance, we choose SNPs that are located in the *Rpv3* locus. These
460 selected mutations were based on a high throughput genotyping of different grapevine
461 backgrounds (Supplementary Table 3). We developed forty-one markers to be tested by
462 allele competitive PCR. Of those, Rpv3_15 and Rpv3_33 loci presented segregating
463 polymorphisms in the genotyped population. The other markers tested were
464 homozygous in this population. Analysis of the phenotypic distribution versus allele
465 frequency clearly showed ($p < 0.0001$) the association between the Rpv3_15 (χ^2 calc=
466 59.81, position chr18_26844557) and the Rpv3_33 (χ^2 calc= 119.9, position
467 chr18_27469511) with mildew resistance (Figure 6). All the candidate markers tested
468 are represented at chromosome 18 in figure 7.

469



470

471 **Figure 6. Evaluation of SNP-type markers in downy mildew resistant segregating**
472 **population.** The left panel shows the fluorescence results for FAM and HEX in the axis
473 *x* and *y*, respectively. Right panel demonstrates the relation between the number of
474 individuals and their genotype versus the score for downy mildew resistance (OIV -
475 452).

476 

477 **Figure 7. SNP-type candidate marker's position at the *Rpv3* locus.** The markers
478 were developed for competitive allele-specific PCR (KASP) for the evaluation of
479 downy mildew resistant segregation. The SSR markers on the distal portion of
480 chromosome 18 are the ones tested in this work (VVIN16, P3_VVAGL11, VMC7F2
481 and UDV108).

482

483 **Discussion**

484 In this work we aimed to establish favourable conditions for *P. viticola*
485 development for a precise evaluation of its effect on gene expression of two cultivars
486 that presents contestants responses to pathogen attack. It was imperative that the
487 experiments would present high humidity conditions due to the necessity of free water
488 for the production and release of sporangia, formation of zoospores, and subsequent
489 infection of healthy tissues (TESSMANN & VIDA, 2005). Furthermore, to guarantee
490 the assay reproducibility the temperature for fungal growth was adjusted at 25ºC
491 because this pathogen has broad optimal growth temperature ranges, from 6ºC to 28ºC.
492 Nevertheless, the optimum temperature for the development and fruiting of the
493 pathogen are between 18ºC and 25ºC, where the sporangia can form in up to 8 hours

(RIBEIRO, 2001). With these standards, during our phytoassays, we were able to identify reddish-brown tissue of necrotic spots, corresponding to the oil stain, in the upper region of the infected leaves in all the performed assays. These stains are due to the pathogen recognition system which triggers localized resistance reactions such as the hypersensitivity reaction (DURRANT & DONG, 2004). When in nature, over time, these stains increase in size and unite occupying much of the leaf blade with the tissue in the spot's area becoming dry, causing the affected leaves to fall prematurely and depriving the plant to generate photoassimilates (RIBEIRO, 2001; GARRIDO & SÔNEGO, 2007).

We chose to work with the cultivar Cabernet Sauvignon, which is one of the most prestigious *V. vinifera* varieties in the world, being cultivated in all the producing regions (BATALHA, 2013). It is used mainly for the elaboration of wines by its capacity to maintain characteristics like aromas and flavours independently of the region where it is cultivated (FIGUEIRA, 2013). However, this variety is totally sensitive to a series of biotic stresses, for example, to the attack of grapevine pathogens such as *P. viticola* and *U. necator* (FIGUEIRA, 2013). Contrasting to 'Cabernet Sauvignon', the downy mildew resistant cultivar selected for this study is Villard Blanc, also known as Seyve Villard 12,375 is a member of the Seyve Villard family. During the 60's, in France, this cultivar was one of the most cultivated varieties (INRA, IFV, Montpellier SupAgro, 2013). 'Villar Blanc' is a complex hybrid composed by accesses of six Vitis species: *V. aestivalis, V. berlandieri, V. cinera, V. lincecummi, V. rupestris* and *V. vinifera* (INRA, IFV, Montpellier SupAgro, 2013). This cultivar presents a strong HR that does not allow the spread of infection beyond the infected cell, which may delay the growth of the pathogen in many interactions, particularly those involving haustoria parasites (JONES & DANGL, 2006).

Our candidate gene expression analysis results demonstrate the intricacy of the downy mildew resistance mechanism. Studies show that the time and intensity with which the infected organism develops the defense process presents a major genotypic component, thus the process of resistance induction is functional, spatially and temporally complex (BELLIN *et al.*, 2009; PINTO *et al.*, 2012). From the two genes selected by the RDA approach, VIT_15s0048g02530 shows that 'Cabernet Sauvignon' and 'Villard Blanc' had a fast but transient response against *P. viticola*. In addition, the expression analysis of genes selected from the literature demonstrates the triggering of 'Cabernet Sauvignon' defense response. Especially for β-1,3-glucanase transcriptional

528 profile, ‘Cabernet Sauvignon’ presented extremely high levels when compared to
529 ‘Villard Blanc’ showing that some defense pathways are being activated, but their
530 products are not sufficient to prevent mildew infection.

531 In this work we also tested candidate genes located at the *Rpv3* locus region that
532 were annotated as related to fungi resistance. All genes tested are predicted to be
533 resistance genes (R) against fungi, which are determinants of plant immune response
534 specificity. Some of the PCR performed did not show amplification for one of the
535 cultivars (Supplementary Figure 1). The absence of amplification products for the
536 Villard Blanc cultivar may be related to the fact that this cultivar is a complex hybrid
537 and, as a consequence, has when compared with the Pinot Noir cultivar, from which the
538 full genome was sequenced.

539 The results comprising genes that were expressed in both cultivars shows
540 interesting data for genes VIT_18s0041g01350, VIT_18s0041g01620,
541 VIT_18s0089g00090 genes. These genes were expressed in both cultivars with
542 significant differences between time-points. According to Blast2GO, the
543 VIT_18s0041g01350 and VIT_18s0041g01620 are related to processes of apoptosis,
544 ATP binding, innate immune response, transmembrane activity receptor and signal
545 transduction. The gene VIT_18s0089g00090 presents GO terms related to apoptosis
546 processes, transmembrane activity receptors, binding nucleotides and signal
547 transduction. In addition, the two genes, VIT_18s0041g01620 and
548 VIT_18s0089g00090. present the three TIR-NBS-LRR domains. The results suggest
549 that these three genes may be related to the effector-triggered immune response process
550 that results in the assembly of the hypersensitivity (HR) reaction in resistant individuals.
551 A transcriptional profile for the genes VIT_18s0041g01330 and VIT_18s0041g01340
552 were only visible in the resistant cultivar. Both genes present the three domains, TIR,
553 NBS and LRR and according to Blas2GO, both have GO terms related to processes of
554 apoptosis, binding proteins, innate immune response and stress-causing factors, among
555 others. Their transcriptional profiles suggest that these genes may be related to the
556 process of immune response triggered by the effector that results in HR. The decrease
557 gene expression levels, as compared to 0 HAI, presented by these two genes might be
558 related to part of the infection response, as already reported in a study by Soanes &
559 Talbot (2008). Moreover, genes VIT_18s0041g02350 and VIT_18s0041g01710
560 presented a transcriptional profile only in the mildew resistant 'Villard Blanc' cultivar
561 after challenge with the pathogen. Both genes present GO terms associated with the

562 process of apoptosis, ATP binding, defense responses and stress, among others. Their
563 transcriptional profile suggests that they may be associated with the defense response
564 against *P. viticola* as seen for this cultivar. This set of genes that only present transcripts
565 for the Villard Blanc resistant cultivar have two possible explanations: on one hand a
566 different set of primers might be needed to amplify these genes in 'Cabernet
567 Sauvignon', maybe due to differences in their mRNA on this cultivar. Nevertheless,
568 'Cabernet Sauvignon' is closely related to the genome cultivar, Pinot Noir. On the other
569 hand, these four genes could be the main responsible genes for the 'Villard Blanc'
570 effective defense response upon downy mildew infection related to *Rpv3* locus.

571 Our results deviate from the work of Polesani and collaborators (2010) in which
572 they observed that the resistant cultivar, in their case cultivar Gloire (*V. riparia*), starts
573 assembling the defense response mechanism against downy mildew in the first 24 hours
574 after contact with the pathogen. This is the period in which the establishment of the
575 compatible plant-pathogen interaction is taking place (POLESANI *et al*., 2010). In our
576 evaluation of the candidate genes transcriptional profiles, it was possible to observe that
577 the Villard Blanc cultivar presented almost a constant expression of the genes tested,
578 saved the induction of gene VIT_18s0041g01350. This shows that the resistant cultivar
579 is, in a way, always producing the necessary defense response compounds against the
580 pathogen. In the case of *V. vinifera* cultivars, it is known that they are susceptible to the
581 attack of *P. viticola*, although they present defense responses against other pathogens,
582 thus indicating that the defense components are not activated in response to this specific
583 pathogen (KORTEKAMP, 2006). This fact can be confirmed by the presence of the
584 selected candidate genes in the genome of both cultivars, demonstrating the absence of
585 one or more components in *V. vinifera* that would be necessary to trigger *P. viticola*
586 defense response, like the four genes expressed only in Villard Blanc cultivar. This
587 probably happens because the resistance mechanism is dependent on the activation of R
588 genes, which encode cellular receptors that detect the presence of a particular pathogen,
589 leading to the activation of the signal transduction pathways (WANG *et al*., 2013). In
590 addition, the susceptibility of this and other species may occur due to failure to mount
591 the effective defense response, which leads to defective recognition of the pathogen
592 (VELASCO *et al*., 2007). Fung *et al*. (2008) evaluated the interaction between the *V.*
593 *vinifera* and *V. aestivalis* species and the *E. necator* pathogen, which is the agent that
594 causes powdery mildew. These authors observed that for the *V. aestivalis* species only
595 three genes presented modulation as a consequence of the infection, whereas for the *V.*

596 *vinifera* species, reprogramming occurred in a high number of genes before the
597 pathogen infection, which justifies the increase in the level of expression presented by
598 the Cabernet Sauvignon cultivar for some genes.
599 Our results upon SSR markers confirmed that the markers P3_VVAGL11,
600 VVIN16 and VMC7F2 appear to be sufficiently close to the *Rpv3* locus to allow their
601 use in a molecular marker-assisted selection strategy for downy mildew. These three
602 molecular markers have now a dual purpose; as they can be used in the previously
603 known diagnosis of the character of seedlessness, also known as apyreny, and also in
604 the evaluation of downy mildew resistance in grapevine. Even though we achieved good
605 results with the SSR, nowadays there are more efficient marker-assisted selection
606 techniques than SSR analysis by polyacrylamide gel. One of these is the KASP®
607 genotyping assay. This assay uses a unique form of PCR (competitive allele-specific
608 PCR) combined with a homogeneous fluorescence-based information system for the
609 identification and measurement of genetic variation occurring at the nucleotide level to
610 detect single nucleotide polymorphisms (SNPs) or insertions and deletions (INDELs)
611 (HE *et al*., 2014). With the evaluation of our forty-one SNP based molecular markers,
612 we were able to identify two that are heterozygous and segregate in the 'Villard Blanc'
613 self-fertilized population. Markers Rpv3_15 and Rpv3_33 allow the selection of
614 resistant individuals by a technique that has several advantages such as a lower
615 genotyping error in positive control DNA samples compared to other techniques and
616 lower cost of KASP genotyping for marker-assisted selection (7.9-46.1% cheaper)
617 (SEMAGN *et al*. al., 2014). Thus, these markers can generate data on a large scale, in
618 an automated way and at reduced costs (GUIMARÃES *et al*., 2009). Moreover, our data
619 on KASP markers presenting the Rpv3_15 and Rpv3_33 markers as strongly associated
620 with downy mildew resistance could be combined with other markers, such as the
621 VMC7F2 microsatellite marker, to create an alternative to be explored in breeding
622 programs. Taken together, the results presented by our work contribute with key
623 functional data about the *Rpv3* locus and its associated-resistant genes, demonstrating
624 the biotechnological applicability of molecular markers in an assisted selection strategy.
625
626 **Authors contribution statement**
627
628 L.F.R, J.M., A.W. and V.B. conceived original screenings, research plans,
629 designed experiments and analyzed resulting data; L.F.R and V.B. supervised

630 experiments and writing; J.M., A.W and V.B. performed most experiments; L.F.R and
631 D.D.P provided technical assistance. J.M. and A.W wrote the article with contributions
632 from all authors.
633

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635

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638

639 **References**
640

641 AGUDELO-ROMERO, P.; ERBAN, A.; REGO C.; CARBONELL-BEJERANO P.;
642 NASCIMENTO, T.; SOUSA, L.; MARTÍNEZ-ZAPATER, J. M.; KOPKA, J.;
643 FORTES, A. M. Transcriptome and metabolome reprogramming in *Vitis vinifera* cv.
644 Trincadeira berries upon infection with *Botrytis cinerea*. Journal of Experimental
645 Botany, 66, 1769–1785. 2015.

646 ANONYMOUS. Descriptor list for grapevine varieties and Vitis species 452.
647 Organization Internacional de la Vigne et du Vin (OIV), Paris. 1983.

648 BATALHA, Vinhas e Vinhos. http://www.vinhosbatalha.com.br/vinhas.aspx.

649 BELKHADIR, Y.; SUBRAMANIAM, R.; DANGL, J. L. Plant disease resistance
650 protein signaling: NBS-LRR proteins and their partners. Current Opinion in Plant
651 Biology. 7, 391-399, 2004.

652 BELLIN, D.; PERESSOTTI, E.; MERDINOGLU, D.; WIEDEMANN-
653 MERDINOGLU, S.; ADAM-BLONDON, A.-F.; CIPRIANI, G.; MORGANTE, M.;
654 TESTOLIN, R. and DI GASPERO, G. Resistance to *Plasmopara viticola* in grapevine
655 'Bianca' is controlled by a major dominant gene causing localized necrosis at the
656 infection site. Theoretical and Applied Genetics. 163-176, 2009.

657 BLASI, P.; BLANC, S.; WIEDEMANN-MERDINOGLUE, S.; PRADO, M.; RÜHL E.
658 H.; PERE, M.; MERDINOGLU, D. Construction of a reference linkage map of *Vitis*
659 *amurensis* and genetic mapping of *Rpv8*, a locus conferring resistance to grapevine
660 downy mildew. Theoretical and Applied Genetics 123. 43-53. 2011.

661 BLUM, M.; WALDNER, M.; GISI, U. A single point mutation in the novel *PvCesA3*
662 gene confers resistance to the carboxylic acid amide fungicide mandipropamid in
663 *Plasmopara viticola*. Fungal Genetics and Biology 47. 499-510. 2010.

664 CABEZAS, J. A.; CERVERA, M. T.; RUIZ-GARCIA, L.; CARRENO, J.;
665 MARTINEZ-ZAPATER, J. M. A genetic analysis of seed and berry weight in
666 grapevine. Genome, 49 (12):1572–1585, 2006.

667 CADLE-DAVIDSON, L. Variation within and between *Vitis* spp. for foliar resistance to
668 the downy mildew pathogen *Plasmopara viticola*. Plant Disease. v.92, p. 1577-1584,
669 2008.

670 CENTURY, K. S.; SHAPIRO, A. D.; REPETTI, P. P.; DAHLBECK, D.; HOLUB, E.;
671 and STASKAWICZ, B. J. NDR1, a pathogen-induced component required for
672 Arabidopsis disease resistance. Science 278:1963-1965. 1997.

673 CRESTE, S.; TULMANN NETO, A.; and FIGUEIRA, A. Detection of single sequence
674 repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining.
675 Plant Molecular Biology Reports 19:299-306. 2001.

676 DI GASPERO G. & CIPRIANI, G. Nucleotide binding site/leucine-rich repeats, Pto-
677 like and receptor-like kinases related to disease resistance in grapevine. Molecular
678 Genetics Genomics. v. 269, p. 612-623, 2003.

679 DÍEZ-NAVAJAS, A.M.; WIEDEMANN-MERDINOGLU S.; GREIF, C.;
680 MERDINOGLU, D. Nonhost versus host resistance to the grapevine downy mildew,
681 *Plasmopara viticola*, studied at the tissue level. Phytopathology, v. 98, p. 776-780,
682 2008.

683 DOLIGEZ, A.; BOUQUET, A.; DANGLOT, Y.; LAHOGUE, F.; RIAZ, S.;
684 MEREDITH, C.P.; EDWARDS, K.J.; THIS, P. Genetic mapping of grapevine (Vitis
685 vinifera L.) applied to the detection of QTLs for seedlessness and berry weight.
686 Theoretical and Applied Genetics 105(5):780–795, 2002.

687 DÖRMANN, P.; GOPALAN, S.; HE, S. Y.; AND BENNING, C. A gene family in
688 *Arabidopsis thaliana* with sequence similarity to NDR1 and HIN1. Plant Physiology
689 and Biochemistry. 38:789-796. 2000.

690 DRY, I. B.; FEECHAN, A.; ANDERSON, C.; JERMAKOW, A. M.; BOUQUET, A.;
691 ADAM-BLONDON, A. F. and THOMAS, M.R. Molecular strategies to enhance the
692 genetic resistance of grapevines to powdery mildew. Australian Journal of Grape and
693 Wine Research, v. 16, p. 94-105, 2010.

694 DURRANT W. E.; DONG X. Systemic acquired resistance. Annual Review of
695 Phytopathology v. 42, p. 185-209, 2004.

696 ERWIN, D.C.; RIBEIRO, O.K. Phytophthora Diseases Worldwide. American
697 phytopathological Society. St. Paul, MN, p. 562, 1996.

698 FALAVIGNA, V. S.; PORTO, D. D.; BUFFON, V.; PINHEIRO-MARGIS, M.;
699 PASQUALI, G.; REVERS, L. F. Differential Transcriptional Profiles of Dormancy-
700 Related Genes in Apple Buds. Plant Molecular Biology Reporter 32 (4), 796-813. 2014.

701 FAO, Food and Agriculture Organization of the United Nations.
702 <http://www.faostats.org/>.

703 FIGUEIRA, D. Cabernet Sauvignon a casta sagrada. Prazeres do vinho.
704 http://www.dfwines.com.br/public/pdf/midia/002.pdf.

705 FISCHER, B.M.; SALAKHUTDINOV, I.; AKKURT, M.; EIBACH, R.; EDWARDS,
706 K. J.; TOPFER, R.; and ZYPRIAN, E. M. Quantitative trait locus analysis of fungal
707 disease resistance factors on a molecular map of grapevine. Theoretical and Applied
708 Genetics, v. 108, p. 501-515, 2004.

709 FLOR H. H. Current status of the gene-for-gene concept. Annual Review of
710 Phytopathology. v. 9, p. 275-296, 1971.

711 FUNG, R. W. M.; GONZALO, M.; FEKETE, C.; KOVACS, L. G.; HE, Y.; MARSH,
712 E.; MCINTYRE, L. M.; SCHACHTMAN, D. P; QIU, W. Powdery mildew induces
713 defense–oriented reprogramming of the transcriptome in a susceptible but not in a
714 resistant grapevine. Plant Physiology. v.146, p. 234-249, 2008.

715 GARRIDO, L. R.; BOTTON, M.; MELO, G. W. B.; FAJARDO, T. V. M.; NAVES, R.
716 de L. Manual de identificação e controle de doenças, pragas e deficiências nutricionais
717 da videira. Bento Gonçalves: Embrapa Uva e Vinho, 2008.

718 GARRIDO, L. R. & SÔNEGO, O. R. Manejo de doenças da videira. *In:* FERREIRA,
719 Ana Carolina Naves. Manejo integrado de doenças de fruteiras. Brasília: Sociedade
720 Brasileira de Fitopatologia, cap. 4, 2007.

721 GÖTZ, S.; GARCÍA-GÓMEZ, J. M.; TEROL, J.; WILLIAMS, T. D.; NAGARAJ, S.
722 H.; NUEDA, M. J.; ROBLES, M.; TALÓN, M.; DOPAZO, J.; CONESA, A. High-
723 throughput functional annotation and data mining with the Blast2GO suite. Nucleic
724 Acids Research, v. 36, p. 3420-3435, 2008.

725 GREENBERG, J. T.; VINATZER, B. A. Identifying type III effectors of plant
726 pathogens and analyzing their interaction with plant cells. Current Opinion
727 Microbiology v. 6, p. 20-28, 2003.

728 GRENVILLE-BRIGGS, L. J.; VAN WEST, P. The biotrophic stages of oomycete-plant
729 interactions. Advances in Applied. Microbiology. v. 57, p.217–243, 2005.

730 GUIMARÃES, C. T.; MAGALHÃES, J. V.; LANZA, M. A.; SCHUSTER, I.
731 Marcadores Moleculares e suas Aplicações no Melhoramento Genético. Informe
732 Agropecuário, Belo Horizonte, v.30, n.253. 2009.

733 HE, C.; HOLME, J.; ANTHONY, J. SNP genotyping: the KASP assay. Methods in
734 Molecular Biology, 1145, 75-86. 2014.

735 IBRAVIN. A VITIVINICULTURA BRASILEIRA. Bento Gonçalves, 2012.
736 http://www.ibravin.org.br/brasilvitivinicola.php.

737 INRA; IFV; Montpellier SupAgro.Fiche Variétale Villard Blanc B. Plant Grape.
738 http://www.montpellier.inra.fr/

739 JONES, D. A.; TAKEMOTO, D. Plant innate immunity – direct and indirect
740 recognition of general and specific pathogen-associated molecules. Current Opinion in
741 Immunology. v. 16, p. 48-62, 2004.

742 JONES, J. D. G. & DANGL, J. L. The plant immune system. Nature Publishing Group,
743 v. 444, p. 323-329, 2006.

744 KORTEKAMP, A. Expression analysis of defence-related genes in grapevine leaves
745 after inoculation with a host and a non-host pathogen. Plant Physiology and
746 Biochemistry. v. 44, p.58-67, 2006.

747 KORTEKAMP, A. & ZYPRIAN, E. Characterization of *Plasmopara* resistance in
748 grapevine using in vitro plants. Journal of Plant Physiology. v. 160, p. 1393-1400, 2003.

749 LEFORT, F. & DOUGLAS, G. C. An efficient micro-method of DNA isolation from
750 mature leaves of four hardwood tree species Acer, Fraxinus, Prunus, and Quercus.
751 Annals of Forest Science. v.56, p. 259-263, 1999.

752 MARGUERIT, E.; BOURY, C.; MANICKI, A.; DONNART, M.; BUTTERLIN, G.;
753 NÉMORIN, A.; WIEDEMANN-MERDINOGLU, S.; MERDINOGLU, D.; OLLAT,
754 N.; DECROOCQ, S. Genetic dissection of sex determinism, inflorescence morphology
755 and downy mildew resistance in grapevine. Theoretical and Applied Genetics, v. 118, p.
756 1261-1278, 2009.

757 MEJÍA, N.; SOTO, B.; GUERRERO, G.; CASANUEVA, X.; HOUEL, C.; MICCONO,
758 M. L. A.; RAMOS, R.; LE CUNFF L.; BOURSIQUO, J. M.; HINRICHSEN, P.;
759 ADAM-BLONDO, A. F. Molecular, genetic and transcriptional evidence for a role of
760 VvAGL11 in stenospermocarpic seedlessness in grapevine. BMC Plant Biology 11
761 (57), 1471-2229. 2011.

762 MERDINOGLU, D.; WIEDEMAN-MERDINOGLU, S.; COSTE, P.; DUMAS, V.;
763 HAETTY, S.; BUTTERLIN, G.; GREIF, C. Genetic analysis of downy mildew
764 resistance derived from 'Muscadinia rotundifolia'. In: Acta Horticulturae. v. 603, p.
765 451-456, 2003.

766 MOREIRA, F. M.; MADINI, A.; MARINO, R.; ZULINI, L.; STEFANINI, M.;
767 VELASCO, R.; KOZMAM, P.; GRANDO, S. Genetic linkage maps of two
768 interspecific grape crosses (*Vitis* spp.) used to localize quantitative trait loci for downy
769 mildew resistance. Tree Genetics & Genomes. v. 7, p. 153-167, 2011.

770 PFAFFL, M.W. A new mathematical model for relative quantification in real-time RT-
771 PCR. Nucleic Acids Research, v. 29, p. e45, 2001.

772 PINTO, K. M. S.; CORDEIRO DO NASCIMENTO, L.; GOMES, E. C. S.; DA SILVA,
773 H. F.; MIRANDA, J. R. Efficiency of resistance elicitors in the management of
774 grapevine downy mildew *Plasmopara viticola*: epidemiological, biochemical and
775 economic aspects. European Journal of Plant Pathology. v. 134, p. 745-754, 2012

776 POLESANI, M.; BORTESI, L.; FERRARINI, A.; ZAMBONI, A.; FASOLI, M.;
777 ZADRA, C.; LOVATO, A.; PEZZOTTI, M.; DELLEDONNE, M.; POLVERARI, A.
778 General and species-specific transcriptional responses to downy mildew infection in a
779 susceptible (*Vitis vinifera*) and a resistant (*V. riparia*) grapevine species. BMC
780 Genomics, v.11, p. 117-132, 2010.

781 REID, K. E.; OLSSON, N.; SCHLOSSER, J.; PENG, F.; & LUND, S. T. An optimized
782 grapevine RNA isolation procedure and statistical determination of reference genes for
783 real-time RT-PCR during berry development. BMC Plant Biology, v. 6, p. 27-37, 2006.

784 REVERS, L. F.; WELTER, L. J.; IRALA, P. B.; SILVA, D. C.; LAMPE, V. S.;
785 OLIVEIRA, P. R. D.; GARRIDO, L. R. Co-Localization of QTLs for seedlessness and
786 downy mildew resistance in grapevine. In: International Conference on Grapevine
787 Breeding and Genetics. v. 10: 58. 2010.

788 RIBEIRO, I. J. A. Doenças causadas por fungos e bactérias na cultura da videira. *In:*
789 BOLIANI, Aparecida Conceição; CORRÊA, Luiz de Souza. Cultura de uvas de mesa:
790 do plantio à comercialização. Ilha Solteira: ALGRAF. p. 237-239, 2001.

791 SALMASO, M.; MALACARNE, G.; TROGGIO, M.; FAES, G.; STEFANINI, M.;
792 GRANDO, M. S.; VELASCO, R. A grapevine (Vitis vinifera L.) genetic map
793 integrating the position of 139 expressed genes. Theoretical and Applied Genetics. v.
794 116, p.1129-1143, 2008.

795 SCHWANDER, F.; EIBACH, R.; FECHTER, I.; HAUSMANN, L.; ZYPRIAN, E.;
796 TÖPFER, R. *Rpv10:* a new *locus* from the Asian *Vitis* gene pool for pyramiding downy
797 mildew resistance *loci* in grapevine. Theoretical and Applied Genetics. 163- 176, 2012.

798 SEMAGN, K.; BABU, R.; HEARNE S.; OLSEN, M. Single nucleotide polymorphism
799 genotyping using Kompetitive Allele Specific PCR (KASP): overview of the
800 technology and its application in crop improvement. Molecular Breeding, v, 33, pp
801 1-14. 2014.

802 SOANES, M. D. & TALBOT, N. J. Moving targets: rapid evolution of oomycete
803 effectors. Trends in Microbiology 16. 508-510. 2008.

804 TAKKEN, F. L. W.; ALBRECHT, M.; TAMELING, W. I. L.; Resistance proteins:
805 molecular switches of plant defence. Current Opinions in Plant Biology 9, 383-390.
806 2006.

807 TESSMANN, D. J. & VIDA, J. B. Principais doenças fúngicas da videira. Atualidades
808 agrícolas, São Bernando do Campo, 14-15. 2005.

809 UNGER, S., BÜCHE, C.; BOSO, S.; KASSEMEYER, H. H. The course of colonization
810 of two different *Vitis* genotypes by *Plasmopara viticola* indicates compatible and
811 incompatible host-pathogen interaction. Phytopathology. v. 97, p. 780-786, 2007.

812 VELASCO, R.; ZHARKIKH, A.; TROGGIO, M.; CARTWRIGHT, D. A.; CESTARO,
813 A.; PRUSS, D. A high quality draft consensus sequence of the genome of a
814 heterozygous grapevine variety. Plos One. v. 12, e1326, 2007.

815 VENUTI, S.; COPETTI, D.; FORIA, S.; FALGINELLA, L.; HOFFMANN, S.;
816 BELLIN, D.; CINDRIĆ, P.; KOZMA, P.; SCALABRIN, S.; MORGANTE, M.;
817 TESTOLIN, R.; DI GASPERO, G. Historical introgression of the downy mildew
818 resistance gene *Rpv*12 from the Asian species *Vitis amurensis* into grapevine varieties.
819 Plos One. 8, 61228. 2013.

820 WANG, P.; LIU, C.; WANG, D.; LIANG, C.; ZHAO, K.; FAN, J. Isolation of
821 resistance gene analogs from grapevine resistant to downy mildew. Scientia
822 Horticulturae. v. 150, p. 326-333, 2013.

823 WELTER, L. J.; GOKTURK-BAYDAR, N.; AKKURT, M.; MAUL, E.; EIBACH, R.;
824 TOPFER, R.; and ZYPRIAN, E. M. Genetic mapping and localization of quantitative
825 trait loci affecting fungal disease resistance and leaf morphology in grapevine (*Vitis*
826 *vinifera* L). Molecular Breeding, v. 20, p. 354-379. 2007.

827 ZENG, YING; YANG, TAO. RNA isolation from highly viscous samples rich in
828 polyphenols and polysaccharides. Plant Molecular Biology Reporter. v. 20, p. 417a-
829 417e, 2002.

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Supplementary Material

Supplementary Figure 1. Amplification products of the candidate genes located in *Rpv3* locus. PCR from genomic DNA of the cultivar Villard Blanc (VB) and Cabernet Sauvignon (CS). On the left the molecular ladder marker (GeneRuler^{TM} Low Range DNA Ladder and 1 Kb Plus DNA Ladder). C = negative control.

848 **Supplementary Table 1 – Specific primers for candidate genes.** The first column
849 contains the candidate genes names, followed by their specific primers sequence and the
850 expected product size. The Vv04s0044g00580 is the reference gene Actin.

Locus	Primers forward/reverse	Amplification product size (expected)
VIT_18s0089g00090	CCA GAG GAA GGA GGA ACC C GGC AAT GAG ATC CCA ATT GAG	166 bp
VIT_18s0089g00100	CCA CCA AAC TTT CAT CCA AAT G GCT GTT GTG AAT CAT TGA GAT TG	127 bp
VIT_18s0089g00600	CCA CTT TCT CTC GCA AGT C ATC AAG ATT CTG GAG GTC C	138 bp
VIT_18s0041g01340	CCA CAG AAA CGC TTG AAA TG TCT CAG TTA TTT TAT TCG TTC GTC	178 bp
VIT_18s0041g01620	GGT GAG CAT GGA TCA GTA AGA GA GAT GCA AGT GTG GTT GAG CC	152 bp
VIT_18s0041g02120	ACT TTA TTG AAT TTA AGA GGG TGC C GAT GTA TTG AGG AGG GCA ATT CG	127 bp
VIT_18s0041g02450	ATC GGC AGG TGA TGC TAA G TCT TCC CCA ACA TTT TCA ATG G	156 bp
VIT_18s0041g01400	GCT AGT CAT TTA TTA TCT GGC AAA C CGA TTG GAG TGT GGA AGT GAT	154 bp
VIT_18soo41g01370	GTA TTC CAC AAT GTT TAT TCC ACA ATG TAT GAT AAG CCA CTA ACC CAA TAT C	123 bp
VIT_18s0041g01410	GGA GAG GCA TTT TCC ATT CAT G CAA ATT AAG GAA GTT GAA GTT ACC C	133 bp
VIT_18s0041g01430	GGC TCT TTA CCC TTT TCG TG CTC CAA TCC ATG AAA CTG CTA	189 bp
VIT_18s0041g01470	GCA CTA CCA AGT AGC ATT GTG ATC CCA CTC CTG TTG AAA TAA AG	137 bp
VIT_18s0041g01520	CTC TTC TTC TAC CTC AGT CCG GAT TCA AAC TTC ATG CTG GAT G	102 bp
VIT_18s0041g01560	CGC CCA AGA TCA GAA GCA G CCA CCC TTC CAA TTC CAA AAC	175 bp

VIT_18s0041g01650	CAT GCC AAC ATT GAA ACC TTC A GAC GAG TTC ATC CAA GCA CC	156 bp
VIT_18s0041g01710	GCT TTA CTT CTC ATC TCT ACT CGG GAC AAG AAT GGC TAT CCT TGA TTC	140 bp
VIT_18s0041g01790	CGA CTG TAT CAT CTG ACA TCA CT TCA CAA TAC AAA TTC CGT TGC C	196 bp
VIT_18s0041g02210	CAC TGA AGT TGC TAA TGT ATC TGG CAC AAT ACT CGG TGA AGT AGG AAT A	187 bp
VIT_18s0041g02350	GGT GAA CTC AGG GAA CTT AGA T CGC TGG CAA AGA TGG TAG G	106 bp
VIT_18s0041g02380	GGG GTT GGT CCA CCT ACT G ATT GTC GTA TCT TCA AGT TGG C	127 bp
VIT_18s0041g01350	GAC ATT GGG ACT GCC AGC GGG ATT GAT CCT GTG AGC G	212 bp
VIT_18s0041g01290	CCT CCA GAA GTG CGG AAC GTC TGT CGT TGG CTT AGA CC	209 bp
VIT_18s0089g00010	CTT GTT CGG TAC AAT CTC GCC CGA TAT TTC GGG GGA TCA GG	176 bp
VIT_04s0044g000580	CTT GCA TCC CTC AGC ACC TT TCC TGT GGA CAA TGG ATG GA	82 bp

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852 **Supplementary Table 2 – Fisher's test results.** The first column contains the
853 candidate genes names, followed by their GO terms from the Fisher's test and by their
854 domain.

Gene – Locus	Fisher's test	Domains
VIT_18s0041g01370	Defense response, Programmed cell death Apoptosis, Cell death, Death, Immune system process, Immune response, Innate immune response, Activity receptor transmembrane, Ligation de nucleotide, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside trifosfatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, Signal transduction activity, Transmission signal, Signaling process	LRR
VIT_18s0041g01400	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system	No TIR-NBS-LRR domain

	process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	
VIT_18s0041g01410	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pirofosfatase activity Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	TIR
VIT_18s0041g01430	Defense responses, Nucleotide-binding, Stress response, Ligation	LRR
VIT_18s0041g01460	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	No TIR-NBS-LRR domain
VIT_18s0041g01470	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction	LRR

	activity, signal transducer activity, Transmission signal, Signaling process	
VIT_18s0041g01520	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor	LRR
VIT_18s0041g01560	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, Signal transducer activity, Transmission signal, Signaling process	No TIR-NBS-LRR domain
VIT_18s0041g01570	Defense response, Apoptose, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, defense response, Apoptose, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, Signal transducer activity, Transmission signal, Signaling process	TIR
VIT_18s0041g01650	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	TIR
VIT_18s0041g01710	Defense response, Apoptosis, Programmed	TIR

	cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	
VIT_18s0041g01790	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	LRR
VIT_18s0041g02120	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	TIR LRR Disease resistance protein NB-ARC
VIT_18s0041g02210	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity,	TIR

	Transmission signal, Signaling process	
VIT_18s0041g02310	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	No protein
VIT_18s0041g02350	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	LRR
VIT_18s0041g02370	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation	No data
VIT_18s0041g02380	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	LRR
VIT_18s0041g02450	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune	TIR, NB-ARC, C-terminal leucine-rich

	response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation	repeats, N-terminal AAA+ (ATPases)
VIT_18s0089g00010	Defense response, Cytoplasmatic Portion, Organelles, Organelles Intracellulares, Intracellular Portion, Organelle de Ligation de Membrane Intracellular, Stress response, Ligation, Transmission signal, Sinal de tradução, Signaling process	TIR, NB-ARC, C-terminal leucine-rich repeats, N-terminal AAA+ (ATPases)
VIT_18s0089g00060	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	TIR
VIT_18s0089g00090	Defense response, Apoptosis, Programmed cell death, Cell death, Death, Immune system process, Immune response, Innate immune response, Transmembrane activity receptor, Nucleotide-binding, Stress response, Activity receptor, Ligation, Pyrofosfatase and activity, Nucleoside-triphosphatase activity, Hydrolase activity, acting on acid anhydrides, Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, Transduction signal, Molecular transduction activity, signal transducer activity, Transmission signal, Signaling process	TIR LRR NB-ARC
VIT_18s0041g02030	No data	
VIT_18s0041g01300	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion, Organelle ligada a Membrane Intracellular, Nucleotide binding, Ligation	No TIR-NBS-LRR domain
VIT_18s0041g01350	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion, Organelle associated to Membrane Intracellular, Nucleotide-binding, Activity receptor, Ligation, Transduction signal, Activity of molecular transduction, Signal transduction activity, Signaling process	LRR Kinase-like protein
VIT_18s0041g01550	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion, Organelle linked to Intracellular membrane, Nucleotide-	Tubulin family

	binding, Stress response, Ligation, Activity de pirofosfatase, Activity Nucleoside trifosfatase , Hydrolase activity	
VIT_18s0041g01780	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Stress response,	Proteases
VIT_18s0041g01920	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion, Nucleotide binding, Ligation, Activity pirofosfatase, Activity Nucleoside trifosfatase, Hydrolase activity	Kinase protein
VIT_18s0041g01930	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion	PPR Family protein; Aldolase
VIT_18s0041g02150	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion	Lipases, Esterases
VIT_18s0041g02220	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion	No data
VIT_18s0041g02270	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion, Nucleotide binding, Ligation, Ligation FMN	Reduction or oxidation process and aldolases
VIT_18s0041g02300	Cytoplasmatic Portion, Organelle, Organelle Intracellular, Intracellular Portion, Nucleotide binding, Ligation, Activity de pirofosfatase, Activity Nucleoside trifosfatase, Hydrolase activity	No data
VIT_18s0041g01420	Organelle, Organelle Intracellular, Intracellular Portion, Nucleotide binding, Ligation, Activity pirofosfatase, Activity Nucleoside trifosfatase, Hydrolase activity	No data
VIT_18s0041g01880	Organelle, Organelle Intracellular, Intracellular Portion, Ligation	MADS domains, K-box domain
VIT_18s0041g02150	Organelle, Organelle Intracellular, Intracellular Portion	Lipase, Esterase
VIT_18s0041g01780	Intracellular Portion, Stress response	Acid proteases
VIT_18s0041g02050	Intracellular Portion, Nucleotide-binding, Stress response, Ligation, Ligation FMN	FMN oxidation-reduction; TIM-Aldolase;
VIT_18s0041g02070	Intracellular Portion, Nucleotide-binding, Stress response, Ligation, FMN Ligation	TIM Aldolase; FMN – oxidoreduction
VIT_18s0041g01290	Nucleotide binding, Ligation	Kinase protein
VIT_18s0041g01860	Nucleotide binding, Ligation	AIG1 (Bacteria resistance protein)
VIT_18s0041g02010	Nucleotide binding, Stress response, Ligation, Ligation FMN	FMN (oxidation-reduction) TIM (aldolase)
VIT_18s0041g02020	Nucleotide binding, Ligation, Ligation FMN	FMN, TIM
VIT_18s0041g02040	Nucleotide binding, Stress response, Ligation, Ligation FMN	FMN, TIM

VIT_18s0041g02060	Nucleotide binding, Ligation, Ligation FMN	FMN, TIM
VIT_18s0041g02080	Nucleotide binding, Ligation, Ligation FMN	FMN, TIM
VIT_18s0041g02090	Nucleotide binding, Ligation, Ligation FMN	FMN, TIM
VIT_18s0041g02260	Nucleotide binding, Ligation, Ligation FMN	FMN, TIM
VIT_18s0041g02400	Nucleotide binding, Ligation	FMN, TIM
VIT_18s0041g01380	Ligation, DNA integration	TIR
VIT_18s0041g01510	Ligation	Cytochrome P450
VIT_18s0041g01530	Ligation	Cytochrome P450 – site conserved
VIT_18s0041g01580	Ligation	Cytochrome P450 – site conserved
VIT_18s0041g01590	Ligation	Cytochrome P450 – site conserved
VIT_18s0041g01620	Ligation	Domain TIR, Disease resistance protein, NB-ARC domain, LRR
VIT_18s0041g01720	Ligation	LRR
VIT_18s0041g01800	Ligation	Cytochrome P450 – site conserved
VIT_18s0041g01810	Ligation	Cytochrome P-450 grup E class I
VIT_18s0041g01820	Ligation, DNA integration	No data
VIT_18s0041g02190	Ligation	Disease resistance protein, NB-ARC
VIT_18s0041g02320	Ligation	No data
VIT_18s0041g02360	Ligation, DNA integration	No data
VIT_18s0041g02440	Ligation	LRR
VIT_18s0041g02470	Ligation	LRR
VIT_18s0089g00040	Ligation	LRR
VIT_18s0089g00050	No data	LRR
VIT_18s0089g00080	Ligation	LRR
VIT_18s0089g00110	Ligation	unintegrated

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860 **Supplementary Table 3:** Sequences for KASP markers for downy mildew resistance.

Marker	Sequence
chr18_25931148 _G_T	AAAACTATGATAAGAAAAGCATAAGTAATCTTCATCTAG CTTTTATAAAA[T/G]TACTCTTCCTTCCATCATTGTATTGC AGCTTGTTATGAATTGTGATACAC
chr18_25938392 _G_T	TACGAAACCCTCTCCCACGGCAATACCCGAAAAGTCTGA CTGTACGCTGG[T/G]ATTGAAACCTCGCCATTGCCAGCAG TGAAGGATATTAGTACCATTGACAA
chr18_25971906 _A_G	ATTATACGCCATACCCTAAAATAGTTTGAAGATGATGAA GACCTTGACTC[A/G]AGAACCTTGACAATTACATGAAGAT AATTAAGGTCTTAAGCATCCCTTAA
chr18_26029420 _A_G	AGGGACAGAGAAACCAAACCAGTCAAATTTGTCACCCAA GACGGGAATGG[A/G]CCAGAAAAGTAGTTTATCGACAAA TCCAGAGTCCGTAAGTTGCTCAACTC
chr18_26032150 _C_T	TTTGAAATATCAAACAACGGTTGAAACTGTCAATTCATA ATTTGGATATA[T/C]GCATAATGTCACAGACACTAGTCAG CACAGAGTTTTTAGAAAGCATGACA
chr18_26070570 _C_T	AGGAGTACATTCATCAGTACATTCCCTTATGTTCGCTTTA TTTTGGATGG[T/C]TGAATACCTACACTTTACATTTCTCTT CAACATTTTTTTTTTGGGTTTAT
chr18_26378128 _G_T	ATATATAATATTAAGGGTCAGTAATCAACTGGCATGCAA GAATCAGGCTC[T/G]TCAAGAAACTGAGAAATAGAGGGT TGTATATTTTAAACATTTTCTAATAC
chr18_26479543 _A_G	GAAAAAGAATATGAATGGTAGTTGTAAGAAAATTAAAAA ATAAAATCATT[A/G]ACTCTTCACTCTACCAAACTAGGGA TGACAACGAGGCGAGTTTGGGATGG
chr18_26574837 _A_C	AGTCTTCTTTCAACAACTAAGCTCTGTGCCTTAATGACTG AATTTTCTTT[A/C]ACAGACCTTCTCTAGCAACCAGCCATC TCTGAGCCTTCTGCTTCCATCGC
chr18_26682879 _C_T	TTTAGTCAGTTCAAAAAAACCTATAACTCTATTTTGCTGT GAGGCCCTGG[T/C]GATTGAATGATGTCTATGAATTTCTT GCTCACCTATTGCAAAGATTCTCA
chr18_26702868 _C_T	GAGGGAACTTGCATTCTAATAGAAATAAGGAAACAAATC CGATTACGGGT[T/C]AACTTCCCCCTGGCAATGTCCTGTA GAATATAAATTAAGGAAGCTTCCGT
chr18_26705069 _A_G	ATGCCAAGATGGAAAAAAGTGTAGGACATTCAAGACAA GGTATCTTGTGT[A/G]GAGTTTATACCATCAATACAATTG ACTTATGCATGTTATACAAATGCGGA
chr18_26783449 _A_G	ATAACCTGATGCCAAATGGCACAACCTTCCATACCTCCA AAAAAAAAAAA[A/G]AGGTGGTAATTATTGTGAAAAACA CTTGAATGAAAAACCTAGGTGCACAG
chr18_26806875 _G_T	GGTCTTCTATTTTATGATGCGAATGAACTTTGGGGCTAAT ATGAAGGGAA[T/G]AATTGGCAGAGCAGGAATTAAGAAG TTGGTTTTGCAGAAAGGGAGTGAAA
chr18_26844263 _A_C	AAGATGAGTGATGGTAGTAGTAGTGTGTATTACCATATTT ATGTCTGTGT[A/C]CCATGATGGTGTGTAGAGTTAAGAAA ATTGTATCTTGGGATGCATGGTTT

chr18_26844557_A_G	TTTAAATCAAGGGCCAGCTCTTCTGGCGTTACAAGCACTTGTGAATTACA[A/G]AGAACTATATTGAGAGATGGCCAAATTGTTAATGTCATTGACACTCCTGG
chr18_26859467_C_T	GCATAAATGTGGTGTTTGTAACTATTTCCCTATGAACTCCAACCTAGCCC[T/C]GATAAACTTTGGATGTTATTGCATATTGGCTCAACAACTGTTGCATGCAT
chr18_26914334_C_T	CGCATCAACTAAATTACAGCCCCTATATGAACAAAGGAAGAACCTCAATC[T/C]CGAATGCCAAGTTCGACTTCAAGCTCATCATCATCTTCAAAAAGCTTTAG
chr18_26936099_A_G	ATCCATTGCTCAATATGAGCCTGCCAAAACCAATTATACAAGGTAGCATC[A/G]TAAGAAACTCTATTTTGGTGGAAAACATATCAAAATTTTTCTTGAGGATA
chr18_26947500_G_T	TCTAAAAGACAACAACAAAAATCCCAGATAGGATACAGTCTGCAAAATCT[T/G]TTGGCTCTGATTTTGATCATCTACATTGTCCCTTAAAAATCCCTAGGAGT
chr18_27024319_C_T	CAGAAGAAATATCCCAAAACAAATCACTTATTGTTGAATCAATATCTGAA[T/C]CTTACGAGTTAAATGAGTTGGAGCGAGTTCACGAGAGTCGAAGAAAGACT
chr18_27114046_A_G	TGTTGGATAGCATAAATCTCCACAAGGAGACAATAATAACTTAAATGAAG[A/G]AGTTAGCTAAATAAGCTTGTGACTATTCTGATATTCACCACATTGCCCTT
chr18_27115054_C_T	CCACTAGGAAAGTAAGTTAAAAGATTAAAAGATTAACAATGTCAAACCCA[T/C]CATTTTCATCCCAAAAGAGCATGTTTGGTGAGTAGGAAACAAATAAGGGC
chr18_27138071_C_T	TGTACAAGCACTCAAGCGCTATCTCTGACCGATGTGGGATTCAGATACCA[T/C]TTCTCATATTTAAAAAATTAATTGAACTAAACGGTCCACCCGCACAAGAG
chr18_27202895_C_T	TTGATTATTGATAGGTAAACATCAAATGTTCTTTGGCTTTCACCATGCCT[T/C]AAAAATCTTATCAACTCAATTTAAAAGATATTATGAACATATGGAAAGAG
chr18_27239329_A_G	ACCACTCTGGAGTAGTTATGGTACACAAGTGGTTGATTCATACATCCTTC[A/G]CCAAAACATGGAAATGTTCTCTTTGTAACTATTTTACTAACTTTCTTGCC
chr18_27242809_A_G	TTCTCAGAAGCATTTGCCACATAAATCACACTCCAAACCCATATTAGAGC[A/G]ACACGAATATCCCAAGAACAAGCAGAACATAAAAAGCCCATTGTTTCTGC
chr18_27269431_C_T	GGTATGTTACCTTGCCACATCCTTACTAGATCAAACATCATCTCAGTTCT[T/C]TCTCTATGCAAAGCTTTAAGGTGTGGAAGGAATCTTATTTTATTCAAAAA
chr18_27301869_G_T	GCACACAAACATATTTATGAGACTCAGCTGCCCATCCTTCGTCACTGTGG[T/G]GCATCCAAGCGAGCTTCTGTTGAAAGTTTTCTTTCTTTTGAATTGACTCA
chr18_27312437_C_T	ATCAGTTAGCCTTTGAGTGAATAAATTAGGGAGATAAAATGAGTATTACT[T/C]CTTGGATCATTGGAGTTGAGACAATCCAATGGAGCAGAGCTTTTGGCTTT
chr18_27411386_C_T	TCGCCTTTTCGGTTTGTGAACTTCTAGTGGGTTAATGGAAAAATAGAACT[T/C]ACAATTGGTAGATGAAGATGAGAGTGAGCTGAGAAGACGGAGGAGCGAAG
chr18_27443784	ATCATTTTCCTTTTATATGGGTAATGACAGAAAAATGATC

_C_T	AAATACAAAG[T/C]CTGTCTGCGTAATGATTACGCCTCAT CTAAGAAGATGATAAAAATTTCTT
chr18_27445707 _A_G	ATGATCAAAAGTAAAATGCTTGAAGTTCAAATATTTTTA TGAAAAATAA[A/G]TTCTTGTATTAGTCCTCACCGTATGCT TTTCTAATACATGATAGATGTCC
chr18_27469511 _A_G	CAAAACACAAGAGTGATTCCAATTCCTTTATGCAGGAGT CAACTCCCACT[A/G]GGTTCTGAGCATCATTTGAGGATGC ACAATTTAAATATTTTGAAATATCT
chr18_27507736 _C_T	AATCGAAGAACTATTAAGAAAGAGAGAGTGATATGACTG CAGCTCATGTG[T/C]GTATATATAGAAAATAAACAACACA ATTAAAAACGAGCAATAAAGCTTTA
chr18_27540333 _G_T	ACGACTCGACCGATTTGAACCAGACCCTGCTGATACTTTG AACCCTGACT[T/G]CTTCAAAATGGATGCTTCTTCTTCTTC TCAACATTGGAGGTATGATGCCT
chr18_27550063 _A_G	GTAGGTGTCCATCCATCATAAAACCATCTCCTAATTCAAG AAACTAAATA[A/G]GTACTTACACGTAAATTCATCCACAA ATGAAGTAAATGAAGATAAAAAAA
chr18_27684016 _G_T	ATGAATTGGTAATCCGTTTGTTGACTCAAAGATTTAGTTT GTGCCCATTT[T/G]TTTGAATTAAATTAGAAGAATGGCCA TTTGATGTGCATTGTCTTTTGTGT
chr18_27686573 _C_T	ATCATATCCTTGCACTAGAGATTTCTTTATTATTGTGTTAG AGAATGTTA[T/C]ATCCATGAATTGTCTGGCTATTATTTCC TGATCAATTTGGCCTGTGCAGG
chr18_27697956 _A_G	CTCTAAACAAAAACCCTGCTGAACTAATTTTATATTTTAT GGTGAGAAAG[A/G]GACCCTCAAAGATGGCCACAAAAAC ATGGCATCCTTACATCAGCAAAAGG
chr18_27843004 _G_T	TTTTCTCTAGCTCTTAGGCCCTATGATCAACTAAGGCTTTT AGGTTCTCC[T/G]TAACCCAGATTTTCTTTCCAATGGGCGA TTTAAAACCTATTCAAGAATTG
chr18_27855218 _A_C	TGGATGCTAAGGGAGGCCATGGTTGAACAGAAATCGGAG GAAAAATAAAA[A/C]ATATAAAATTATCAAGTCCATGGG GTATTTAATTAGGCCATAAGAGATCG

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9 ANEXO II

Tendril coiling in grapevine: Jasmonates and a new role for GABA?

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Tendril Coiling in Grapevine: Jasmonates and a New Role for GABA?

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Abstract
Grapevine (*Vitis vinifera* L., Vitaceae) belongs to the genus *Vitis*, and is characterized as a vine due to the presence of tendrils, which are located opposite to leaves. Tendrils are thigmo-responsive organs, able to carry out delicate mechanosensory responses upon touch and related stimuli. These organs are an adaptation of the plant to climb with the help of support to higher places and finally remain at a position with favorable light quality. In previous studies on *Bryonia dioica* (Cucurbitaceae), phytohormones of the jasmonate class were identified as the endogenous hormone signals to initiate coiling of the tendrils. Strikingly, this is still the only example for jasmonate-induced tendril coiling. In grapevine, three compounds (12-oxo-phytodienoic acid, jasmonic acid (JA), and JA isoleucine conjugate) of the jasmonate class were found at higher concentrations in non-coiled tendrils when compared with coiled ones. Upon treatment with phytohormones, we could confirm the activity of jasmonates on tendril coiling in grapevine. However, not jasmonates but a non-proteinogenic amino acid, γ-aminobutyric acid (GABA), was detected to accumulate in grapevine tendrils at significantly higher levels than in all other tissues, independent of their coiling status. For GABA we detected a significant, transient positive effect on tendril coiling. Use of a GABA synthesis blocker, 3-mercaptopropionic acid, caused reduced GABA- but not JA-induced coiling scores. No additive effect of JA plus GABA was detectable on the tendrils' coiling score. Thus, for grapevine, our data demonstrate a strong activity of jasmonates in tendril coiling induction even without mechanical stimuli and, furthermore, the data also suggest that GABA can independently promote tendril coiling.

Keywords γ-Aminobutyric acid · Jasmonic acid · *Vitis* · Tendril · Coiling

Introduction

From ancient times until today, grapevines have had a significant and intricate relation with mankind (This et al. 2006). These species, mainly from the *Vitis* genus, have a high agronomical importance and are consumed worldwide *in natura* and are appreciated as a divine drink: wine (Lacirignola and Digiaro 1999). The grapevine family, Vitaceae, is one of the plant families described as vines due to a peculiar plant organ known as the tendril. Grapevines were classified by Darwin as tendril-bearers-type plants because their tendrils are long, slender, and filiform structures (Darwin 1865). These specific organs are adapted for climbing on a support object or host plant (Bowling and Vaughn 2009). This allows the plant to use stable structural supports, finally being able to grow in various ecological niches and also to reach sunlight (Isnard and Silk 2009).

Tendrils might have different evolutionary backgrounds depending on the plant family. In *Vitis*, meristematic protuberance primordia, which are formed by the terminal and axillary bud meristems—give rise to both grapevine tendrils and inflorescences (Pratt 1971; Srinivasan and Mullins 1979). For that reason, grapevine tendrils are morphologically homologous to inflorescences. This was elegantly demonstrated by treating grapevine tendrils with cytokinins, which converted them into inflorescences and bunches, showing its evolutionary

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background and the potential of tendrils to become reproductive organs (Srinivasan and Mullins 1979).

Tendrils are highly sensitive and can quickly coil around objects in response to mechanical stimuli (Braam 2004; Fukano and Yamawo 2015). This is due to their thigmoresponsive capability, promoting a directional (thigmotropism, for example, grapevine) or non-directional (thigmonasty, for example, *Bryonia dioica*) growth movement, which occurs as a mechanosensory response to a touch stimulus (Jaffe et al. 2002). The coiling mechanism of the tendril is classified into three main movements: (1) circumnutation, a movement increasing the probability to contact supports; (2) contact coiling, in which the stimulated tendril coils around a support; and (3) free coiling, in which the tendril develops helical coils along its axis. This last step allows the plant to get closer to the support (Jaffe and Galston 1968).

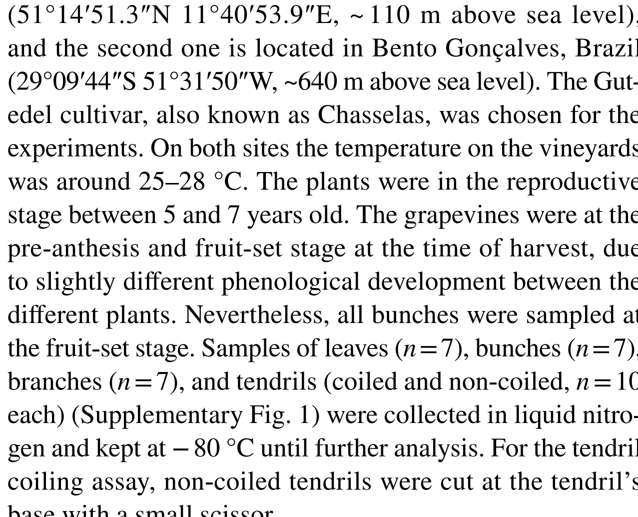
The coiling response appears to be mediated by phytohormones. Many studies characterized the importance of phytohormones in tendril coiling based on the Cucurbitaceae plant *B. dioica* (for example, Falkenstein et al. 1991; Weiler et al. 1993). The phytohormones mainly described in tendril coiling include jasmonates and auxin (Blechert et al. 1999; Stelmach et al. 1999; Engelberth 2003). Applying methyl jasmonate (MJ) or jasmonic acid (JA) was sufficient to induce tendril coiling in *B. dioica* even in the absence of mechanical stimuli (Falkenstein et al. 1991). The JA-induced response in tendril coiling is not rapid, starting after a lag-phase of several hours (Weiler et al. 1993). Additional studies suggested that the JA-precursor, 12-oxo-phytodienoic acid (OPDA), might be the endogenous signal transducer, not JA (Weiler 1997; Stelmach et al. 1999). However, JA-Ile as the most active jasmonate was not known at that time. All those studies showed the importance of jasmonates for tendril coiling—at least in *B. dioica;* interestingly, tendrils of *Pisum sativum* do not respond (Engelberth 2003). To our knowledge, *B. dioica* is still the only plant in which jasmonates were shown to induce tendril coiling.

Here, we aimed to study whether grapevine tendril coiling is also influenced by phytohormones, in particular jasmonates. We further analyze a role for GABA in the coiling process of this organ. Therefore, we determined the levels of various stress-related phytohormones and GABA in different grapevine organs with a focus on coiled and non-coiled tendrils. In addition, we performed experiments with grapevine tendrils to analyze the role of JA and GABA in the tendril coiling process.

Materials and Methods

Plant Material

We performed the experiments in two vineyards. The first one is located close to the city of Freyburg, Germany (51°14′51.3″N 11°40′53.9″E, ~110 m above sea level), and the second one is located in Bento Gonçalves, Brazil (29°09′44″S 51°31′50″W, ~640 m above sea level). The Gutedel cultivar, also known as Chasselas, was chosen for the experiments. On both sites the temperature on the vineyards was around 25–28 °C. The plants were in the reproductive stage between 5 and 7 years old. The grapevines were at the pre-anthesis and fruit-set stage at the time of harvest, due to slightly different phenological development between the different plants. Nevertheless, all bunches were sampled at the fruit-set stage. Samples of leaves ($n=7$), bunches ($n=7$), branches ($n=7$), and tendrils (coiled and non-coiled, $n=10$ each) (Supplementary Fig. 1) were collected in liquid nitrogen and kept at −80 °C until further analysis. For the tendril coiling assay, non-coiled tendrils were cut at the tendril's base with a small scissor.



Extraction and Quantification of Phytohormones

We analyzed the following compounds: JA; *cis*-(+)-OPDA; JA-L-isoleucine conjugate (JA-Ile), 12/11-hydroxy-JA (OH-JA), 12/11-hydroxyl-JA-L-isoleucine (OH-JA-Ile), and 12-carboxy-JA-L-isoleucine (COOH-JA-Ile). Around 250 mg of fresh tissue from either leaves, bunches, branches, and tendrils (coiled and non-coiled) were frozen in liquid nitrogen, ground, and weighed for phytohormones analysis. The extraction procedure and determination of JA and JA-Ile were carried out as described before (Vadassery et al. 2012; Scholz et al. 2017) with small changes. In this study, a different mixture of labeled jasmonates was used as the internal standard. Instead of 15 ng of JA-[^{13}C6]-Ile conjugate used in the previous study, 60 ng of D_6-JA-Ile (HPC Standards GmbH, Cunnersdorf, Germany) was used. Additionally, the 60 ng of 9,10-D_2-9,10-dihydro JA was replaced by 60 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany). Phytohormones were quantified on an API 5000 LC–MS/MS system (Applied Biosystems, Darmstadt, Germany). Because it was observed that both the D_6-labeled JA and D_6-labeled JA-Ile contained 40% of the corresponding D_5-labeled compounds, the sum of the peak areas of the D_5- and D_6-compound was used for quantification.

Extraction and Quantification of GABA

Similar to phytohormones quantification, 250 mg of fresh tissue from either leaves, bunches, branches, and tendrils (coiled and non-coiled) were frozen in liquid nitrogen and weighed for the determination of GABA content per g fresh weight. The material was homogenized by maceration. Later on, the amino acids (including GABA) were extracted twice with a total of 2 ml of methanol. Supernatants were combined and dried using a Concentrator plus (Eppendorf, Hamburg, Germany) and re-suspended in 500 µl of methanol.

The extract was diluted 1:20 (v/v) with water containing the internal standard. The algal amino acid mix ^{13}C, ^{15}N (Isotec, Miamisburg, USA) was used as a standard at a concentration of 10 µg of the algal amino acid mix ml^{-1}. The content of GABA was analyzed by LC–MS/MS according to previous studies (Scholz et al. 2017). An API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) was operated in positive ionization mode with multiple reaction monitoring (MRM) to monitor analyte parent ion→product ion: GABA (*m/z* 104.1→87.1; DP 51, CE 17) an MRM specific for GABA. GABA in the sample was quantified using U-^{13}C, ^{15}N-Ala applying a response factor of 1.0.

Tendril Coiling Assay

We used tendrils from 20 different plants. The whole experiment was independently performed twice to ensure maximal reproducibility. Each time we incubated ten non-coiled tendrils, five being selected as short ones (5–6 cm) and five as long ones (8–10 cm), in water or different solutions containing either GABA (Sigma-Aldrich), 3-mercaptopropionic acid (3-MPA) (Sigma-Aldrich), JA (Sigma-Aldrich), or combinations thereof: (1) deionized water; (2) GABA (50 µM); (3) JA (50 µM); (4) GABA and JA (50 µM each); GABA and 3-MPA (50 µM each); JA and 3-MPA (50 µM each); 3-MPA (50 µM each). After harvesting, the tendrils were directly transferred into the respective solutions. Five Petri dishes, with 20 ml of each solution, were used for incubation of two tendrils per plate, in a total of forty plates in each experimental replicate. The plates were placed in a phytochamber during the time of tendril coiling evaluation. The growth chamber was adjusted to 50–60% humidity and 21 °C with a 10-h-light/14-h-dark photoperiod with a light intensity of 100 mmol m^2 s^{-1}. Tendrils were evaluated at 0, 5, 20, 30, and 48 h; the 0 h time point was taken immediately after transfer into the solutions. The coiling scale (CS, for clarification see Fig. 1a) was set according to the work of Weiler et al. (1993) and calculated as follows: multiply the number of tendrils in each scale by the percentage of the scale and sum it up, then divide the sum by five sum of scales (0, 25, 50, 75, and 100%), in each time point for each treatment. For example, time point: 0 h, treatment: water, tendrils at 0%: 6, tendrils at 25%: 4; coiling score = (0 × 6) + (25 × 4)/5 = 20.

Statistics

The statistical analyses are indicated in the respective figure legends. Two-way ANOVA, the Student's test, or a Chi-square test was used. All experiments were repeated two times to ensure reproducibility and 10–20 plants were used in each treatment for each experimental time point. Different letters indicate significant differences between treatments.

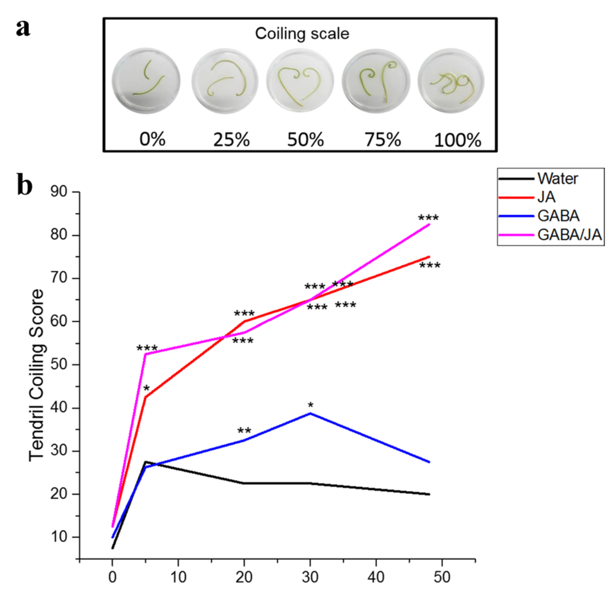


Fig. 1 Tendril coiling abilities of exogenously applied JA and GABA on non-coiled grapevine tendrils at different time points. **a** Coiling scale for grapevine tendrils. 0% = no observable effect; 25% = coiling only at apical, but not basal (cut) end; 50% = coiling at apical as well as basal (cut) end, but median part straight; 75% = fully coiled, loose spiral; and 100% = tendril fully coiled, tight spiral on both ends. Photos were taken at 48 h after starting the incubation. **b** Quantitative score on tendril coiling induction upon treatments with water; GABA (50 µM); JA (50 µM); GABA/JA (50 µM each). Statistically significant differences between treatments and water control were analyzed by Chi-square with four degrees of freedom (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$) and found for GABA at 20 h $\chi^2 = 10.00$; JA at 20 h $\chi^2 = 62.5$; at 30 h $\chi^2 = 80.27$; at 48 h $\chi^2 = 151.25$; GABA/JA at 5 h $\chi^2 = 22.7$; at 20 h $\chi^2 = 54.44$; at 30 h $\chi^2 = 80.27$; at 48 h $\chi^2 = 195.31$

We used GraphPad Prism 6 and Origin Pro for data analysis and graph composition.

Results and Discussion

Phytohormone Analysis in Various Grapevine Tissues

Jasmonates are known to be involved in plant defense against herbivore attack and mechanical wounding but these signaling molecules can also work as regulatory compounds in growth and development of a non-stressed plant (Wasternack 2007). Before specifically addressing the question of whether or not jasmonates might play a role in grapevine tendril coiling, we first determined the general levels of jasmonate-related compounds in different grapevine organs, that is, leaves, bunches, branches, and tendrils. As expected, for leaves, bunches, and branches, we observed some differences in the phytohormones levels, very likely representing tissue-specific characteristics

(Table 1 and Supplementary Table 1). We next investigated whether the conformation of the tendril-influenced jasmonate levels. For that reason, we sampled two types of grapevine tendrils: (1) tendrils that were already coiled and (2) tendrils that were not coiled, but fully expanded (Supplementary Fig. 1D). For all jasmonates including OPDA, the non-coiled tendrils showed higher (between 2.5- and 7.2-fold) levels than coiled ones (Table 1). The degradation metabolites (OH-JA, OH-JA-Ile and COOH-JA-Ile) of jasmonates followed the same trend (Supplementary Table 1). These observations can be explained by a certain capacity of non-coiled tendrils to sense the environment and maybe to defend itself from a herbivore attack, whereas a coiled tendril has already fulfilled its main objective of being attached to a support.

We also tested other stress-related hormones, SA and ABA, on the same samples used for jasmonate evaluation. SA is known to be mainly involved in stress responses against biotrophic and hemibiotrophic pathogens (Grant and Lamb 2006; Bari and Jones 2009). The roles of ABA are vast, from drought stress resistance to regulation of plant development processes (Wasilewska et al. 2008; Bari and Jones 2009). For both phytohormones we observed a kind of tissue-specific distribution for the particular hormones (Table 1). The levels of SA were highest in branches and elevated in tendrils, independently of their coiling conformation. This suggests that stem-related tissues might have a generally high level of SA compared with other tissues. Furthermore, ABA showed smaller differences in the accumulation levels between all tissues evaluated. Bunches and branches presented the highest levels, whereas tendrils and leaves had almost the same ABA concentrations (Table 1). Nevertheless, uncoiled tendrils possess higher concentrations of ABA than coiled ones (Table 1). These results are similar to those observed for jasmonates, in which non-coiled tendrils seem to have a higher amount of defense molecules, which could mean that these structures can be more resilient to biotic and/or abiotic stress. However, Bangerth (1974) stated that ABA beside gibberellins and kinetin did not induce tendril coiling in cucumber (*Cucumis sativus*).

Tendrils Show Higher GABA Levels

Another stress-related molecule we analyzed is GABA (γ-aminobutyric acid). This four-carbon non-proteinogenic amino acid is present in microorganisms, animals, and plants. It acts as an inhibitory neurotransmitter in the mammalian central nervous system by regulating ion channels (Shelp 2012). However, in plants, GABA was reported to be involved in diverse processes, such as influencing cell respiration by regulating the Krebs cycle and acting as a compound for signaling development and growth (Bouché and Fromm 2004; Bown and Shelp 2016). In *Arabidopsis* and tobacco, optimal pollen, pistil, and root growth were described to be dependent on GABA levels (Mirabella et al. 2008; Renault et al. 2011; Yu et al. 2014). GABA can accumulate in plants upon abiotic stress such as salt and cold (Wallace et al. 1984; Ramputh and Bown 1996). It also was proven to be involved in defense responses against herbivore attack and mechanical wounding in a jasmonate-independent manner (Ramputh and Bown 1996; Scholz et al. 2015; Bown and Shelp 2016). In addition, there is evidence for a GABA-induced GABA accumulation (Scholz et al. 2017).

Thus, we also included GABA in our analyses of grapevine tissues. Our results show that GABA is present in leaves, bunches, and branches in equivalent amounts (Fig. 2). Strikingly, GABA levels were up to fivefold higher in tendrils, with apparently no difference between coiled and non-coiled tendrils. These data suggest that GABA could have a role in tendril development or growth.

Impact of JA and GABA on Tendril Coiling in Grapevine

To examine the effects of JA and GABA on tendril coiling in grapevine, we performed experiments with non-coiled tendrils that were treated with different solutions of these particular compounds. Thereafter, we applied a coiling scale for the classification of the tendril coiling process (Fig. 1a). Our treatments demonstrate that JA can induce coiling in grapevine tendrils without touching. This effect is visible already after 5 h (Fig. 1b). We furthermore observed that

Table 1 Phytohormone levels (ng g^{-1} FW) in different grapevine tissues

	cis-OPDA	JA	JA-Ile	SA	ABA
Leaf	133 ± 75.4	9.0 ± 4.5	0.7 ± 0.9	63.5 ± 19.4	354 ± 105
Bunch	158 ± 90.2	8.5 ± 5.0	2.3 ± 1.9	129 ± 30.8	913 ± 200
Branch	459 ± 163	32.9 ± 6.5	0.9 ± 0.4	1012 ± 440	894 ± 150
Tendril non-coiled	203 ± 86**	30.2 ± 7.2***	2.9 ± 1.7***	328.4 ± 72.85 ns	559.6 ± 90.6***
Tendril coiled	80.3 ± 49.7**	7.0 ± 2.0***	0.4 ± 0.1***	294.4 ± 79.73 ns	393.7 ± 69.98***

Shown are average levels ± SE ($n = 7$ for leaf, branch, bunch; $n = 10$ for tendrils, coiled and non-coiled). Statistically significant differences for the same phytohormones between the tendrils were analyzed by Student's *t* test: *ns* non-significant; ** $p < 0.001$; *** $p < 0.0001$

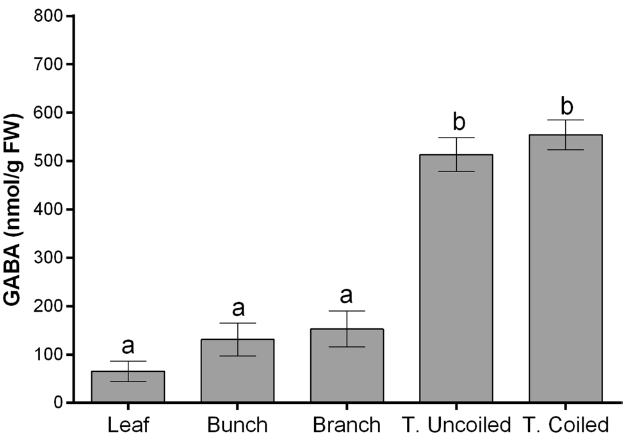


Fig. 2 GABA levels in different grapevine tissues. Fully expanded leaves, bunches in fruit-set stage, non-lignified branches, tendrils (coiled and non-coiled) were analyzed. Shown are average levels ± SE, $n = 7$ for leaf, branch, bunch; $n = 10$ for tendrils. Significant differences between the tissues for the samples were analyzed by two-way ANOVA ($p < 0.05$, Mann–Whitney-U test) and indicated by different letters. Each individual tendril was harvested from individual plants. Samples were harvested around noon and immediately frozen

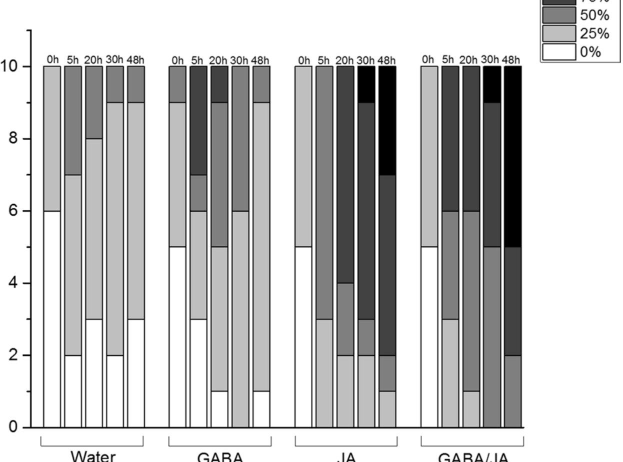


Fig. 3 Distribution of coiled tendrils between the coiling scales (see Fig. 1a) at different time points. Tendril coiling induction upon treatments with water; GABA (50 µM); JA (50 µM); GABA/JA (50 µM each) with scores based on the tendril coiling scale for grapevine tendrils (0% = no observable effect; 25% = coiling only at apical, but not basal (cut) end; 50% = coiling at apical as well as basal (cut) end, but median part straight; 75% = fully coiled, loose spiral; and 100% = tendril fully coiled, tight spiral on both ends)

after the first 5 h the JA-induced effect on tendril coiling increased a bit slower but continuously (Fig. 1b). The fast initial coiling phase (between 0 and 5 h) that can be seen in all approaches is very likely a result of rapid water uptake and the predetermined tendril anatomy; obviously, in the presence of JA a combined effect of water uptake and JA-mediated coiling seems to take place (Fig. 1b). GABA also induced coiling of tendrils without any touch although in the early phase not to the same extent as JA (Fig. 1b). However, between 5 and 30 h of incubation, the increase of the GABA-induced coiling score is similar to that of JA-induced coiling. Later on, the GABA effect on tendrils faded suggesting that this process is transient and reversible. To get an impression about the distribution of tendrils with respect to the particular CS, we did a more detailed analysis as shown in Fig. 3. In the water control, the majority of the tendrils did belong to 0 and 25% CS and only a few were found having 50% CS. Upon GABA treatment, a few tendrils with a 75% coiling score appeared rapid and transiently while the majority of the tendrils belonged to the 25 and 50% coiling CS. The latter also disappeared but at later time. JA treatment caused a majority of tendrils with a CS of higher than 50% already after 20 h. Here, after 30 h, for the first time tendrils with 100% of CS were detected but never a transient effect (Fig. 3). To find out whether GABA and JA have additive or synergistic effects on tendril coiling, we treated them with a combination of both compounds. Neither additive nor synergistic effects were found. Instead, we observed that only after 5 h the coiling score was slightly more pronounced due to a higher number of tendrils belonging to 75% CS. Later on, there were no more clear differences to the treatment with JA alone (Figs. 1b, 3). However, still an open question is whether cutting of the tendrils might induce a higher jasmonate level, which in turn could prime the tendril tissue towards GABA sensitivity and GABA-induced coiling response. This possibility will be tested in further experiments using GABA and/or JA on intact tendrils.

To further evaluate the effects of GABA on grapevine tendrils, we tested if its absence would affect tendril coiling by applying 3-MPA. This compound was described first to inhibit the GABA-generating enzyme glutamate decarboxylase (GAD) in vitro and in vivo, causing a decrease in GABA content in rat brains (Lamar 1970; Karlsson et al. 1974). In plants, the application of 50 µM 3-MPA was shown to affect GABA synthesis, causing an abnormal formation of pollen tubes due to the disruption of actin organization in these organs. With higher amounts (2 mM), 3-MPA completely inhibited pollen germination (Yu et al. 2014). Using 50 µM 3-MPA, our results show that the GABA-induced tendril coiling score was significantly reduced by the inhibitor after 20 and 30 h (Fig. 4). This seems to conflict with the inhibitory effect of 3-MPA on GABA synthesis. However, the work of Scholz et al. (2017) demonstrated a GABA-induced endogenous GABA accumulation in *Arabidopsis* leaves. Thus, applying GABA from outside would also increase GABA synthesis in tendrils, which will be inhibited in the presence of 3-MPA. Moreover, treatment with 3-MPA alone reduced the coiling score at the same time points compared with the water

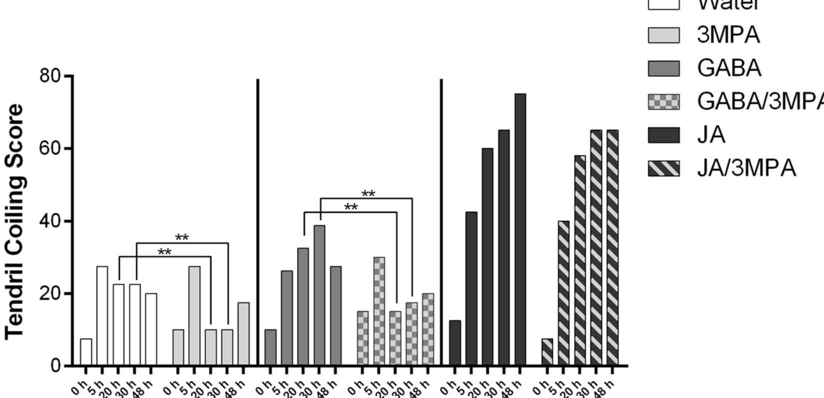


Fig. 4 Effect of 3-MPA on GABA-mediated coiling induction on grapevine tendrils at different time points. Coiling score was determined upon treatments with water; 3-MPA (100 µM); GABA (50 µM); GABA/3-MPA (50 µM/100 µM); JA (50 µM); and JA/3-MPA (50 µM/100 µM). Statistically significant differences between treatments were analyzed by Chi-square with four degrees of freedom (**$p < 0.001$). Water *X* 3-MPA at 20 h $\chi^2 = 15.625$ and at 30 h $\chi^2 = 15.625$; GABA X GABA/3-MPA at 20 h $\chi^2 = 9.423$ and at 30 h $\chi^2 = 11.653$

control (Fig. 4). Both results suggest that a continuous synthesis of GABA seems necessary for tendril coiling. Interestingly, when 3-MPA was applied along with JA it reduced the effect of JA only slightly after 48 h (Fig. 4) suggesting that 3-MPA indeed affects the GABA pathway and does not have a non-specific effect on tendril coiling. Because of the selective effect of 3-MPA together with the finding that there is no combined effect of JA and GABA (Fig. 1b), it is tempting to speculate that both compounds act independently. A similar observation was made before in *Arabidopsis* plants, which were challenged with herbivores (Scholz et al. 2015).

Taken together, our results show that JA has a strong impact on grapevine tendril coiling, similar to what has been described for *B. dioica* tendrils (Blechert et al. 1999; Stelmach et al. 1999). Moreover, also for GABA tendril coiling-inducing activity could be shown. Although the JA effect was progressive and more pronounced, the GABA effect was weaker and transient. Whether and how both signaling compounds may act together or independently from each other remains an open question and will be addressed in further studies.

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Author Contributions JM and AM conceived and designed the research; JM, MR, GP, and AM performed the experiments; JM and AM wrote the manuscript. All authors contributed to the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Bangerth F (1974) Interaktionen von Auxin und Äthylen bei der thigmotropen Bewegung der Ranken von *Cucumis sativus*. Planta 117:329–338. https://doi.org/10.1007/BF00388027

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–488. https://doi.org/10.1007/s11103-008-9435-0

Blechert S, Bockelmann C, Füßlein M et al (1999) Structure-activity analyses reveal the existence of two separate groups of active octadecanoids in elicitation of the tendril-coiling response of *Bryonia dioica* Jacq. Planta 207:470–479. https://doi.org/10.1007/s004250050506

Bouché N, Fromm H (2004) GABA in plants: Just a metabolite? Trends Plant Sci 9:110–115. https://doi.org/10.1016/j.tplants.2004.01.006

Bowling AJ, Vaughn KC (2009) Gelatinous fibers are widespread in coiling tendrils and twining vines. Am J Bot 96:719–727. https://doi.org/10.3732/ajb.0800373

Bown AW, Shelp BJ (2016) Plant GABA: not just a metabolite. Trends Plant Sci 21:811–813. https://doi.org/10.1016/j.tplants.2016.08.001

Braam J (2004) In touch—plant responses to mechanical stimuli. New Phytol 165:17. https://doi.org/10.1111/j.1469-8137.2004.01263.x

Darwin C (1865) On the movements and habits of climbing plants. Bot J Linn Soc 9: 1–118

Engelberth J (2003) Mechanosensing and signal transduction in tendrils. Adv Space Res 32:1611–1619

Falkenstein E, Groth B, Mithöfer A, Weiler EW (1991) Methyljasmonate and α-linolenic acid are potent inducers of tendril coiling. Planta 185:316–322. https://doi.org/10.1007/BF00201050

Fukano Y, Yamawo A (2015) Self-discrimination in the tendrils of the vine *Cayratia japonica* is mediated by physiological connection. Proc R Soc Lond 282:20151379. https://doi.org/10.1098/rspb.2015.1379

Grant M, Lamb C (2006) Systemic immunity. Curr Opin Plant Biol 9:414–420. https://doi.org/10.1016/j.pbi.2006.05.013

Isnard S, Silk WK (2009) Moving with climbing plants from Charles Darwin's time into the 21st century. Am J Bot 96:1205–1221. https://doi.org/10.3732/ajb.0900045

Jaffe MJ, Galston AW (1968) The physiology of tendrils. Annu Rev Plant Physiol 19(1):417–434

Jaffe MJ, Leopold AC, Staples RC (2002) Responses in plants and fungi. Am J Bot 89:375–382. https://doi.org/10.3732/ajb.89.3.375

Karlsson A, Fonnum F, Malthe-Sørenssen D, Storm-Mathisen J (1974) Effect of the convulsive agent 3-mercaptopropionic acid on the levels of GABA, other amino acids and glutamate decarboxylase in different regions of the rat brain. Biochem Pharmacol 23:3053–3061

Lacirignola C, Digiaro M (1999) Grapevine industry in the world: state of the art and future trends. In: Martelli G.P. (ed.), Digiaro M. (ed.). Proceedings of the Mediterranean network on grapevine closteroviruses 1992–1997 and the viroses and virus-like diseases of the grapevine a bibliographic report, 1985–1997. Bari: CIHEAM, 1999. p. 33–46. (Options Méditerranéennes : Série B. Etudes et Recherches; n. 29). Mediterranean Network on Grapevine Closteroviruses (1992–1997) 46:33–46

Lamar C (1970) Mercaptopropionic acid: a convulsant that inhibits glutamate decarboxylase. J Neurochem 17(2):165–170

Mirabella R, Rauwerda H, Struys EA et al (2008) The Arabidopsis her1 mutant implicates GABA in *E*-2-hexenal responsiveness. Plant J 53:197–213. https://doi.org/10.1111/j.1365-313X.2007.03323.x

Pratt C (1971) Reproductive anatomy in cultivated grapes. A review. Am J Enol Vitic 22:91–109

Ramputh A, Bown A (1996) Rapid γ-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae. Plant Physiol 8:6–9

Renault H, El Amrani A, Palanivelu R et al (2011) GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in *Arabidopsis thaliana*. Plant Cell Physiol 52:894–908. https://doi.org/10.1093/pcp/pcr041

Scholz SS, Reichelt M, Mekonnen DW et al (2015) Insect herbivory-elicited GABA accumulation in plants is a wound-induced, direct, systemic, and jasmonate-independent defense response. Front Plant Sci 6:1128. https://doi.org/10.3389/fpls.2015.01128

Scholz SS, Malabarba J, Reichelt M et al (2017) Evidence for GABA-induced systemic GABA accumulation in Arabidopsis upon wounding. Front Plant Sci 8:1–9. https://doi.org/10.3389/fpls.2017.00388

Shelp BJ (2012) Does long-distance GABA signaling via the phloem really occur? Botany 90:897–900. https://doi.org/10.1139/b2012-066

Srinivasan C, Mullins MG (1979) Flowering in *Vitis*: conversion of tendrils into inflorescences and bunches of grapes. Planta 145:187–192. https://doi.org/10.1007/BF00388716

Stelmach BA, Müller A, Weiler EW (1999) 12-Oxo-phytodienoic acid and indole-3-acetic acid in jasmonic acid-treated tendrils of *Bryonia dioica*. Phytochemistry 51:187–192. https://doi.org/10.1016/S0031-9422(99)00017-5

This P, Lacombe T, Thomas MR (2006) Historical origins and genetic diversity of wine grapes. Trends Genet 22:511–519. https://doi.org/10.1016/j.tig.2006.07.008

Vadassery J, Reichelt M, Hause B et al (2012) CML42-mediated calcium signaling coordinates responses to Spodoptera herbivory and abiotic stresses in Arabidopsis. Plant Physiol 159:1159–1175. https://doi.org/10.1104/pp.112.198150

Wallace W, Secor J, Schrader LE (1984) Rapid accumulation of gamma-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol 75:170–175. https://doi.org/10.1104/pp.75.1.170

Wasilewska A, Vlad F, Sirichandra C et al (2008) An update on abscisic acid signaling in plants and more. Mol Plant 1:198–217. https://doi.org/10.1093/mp/ssm022

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100:681–697. https://doi.org/10.1093/aob/mcm079

Weiler EW (1997) Octadecanoid-mediated signal transduction in higher plants. Naturwissenschaften 84(8):340–349

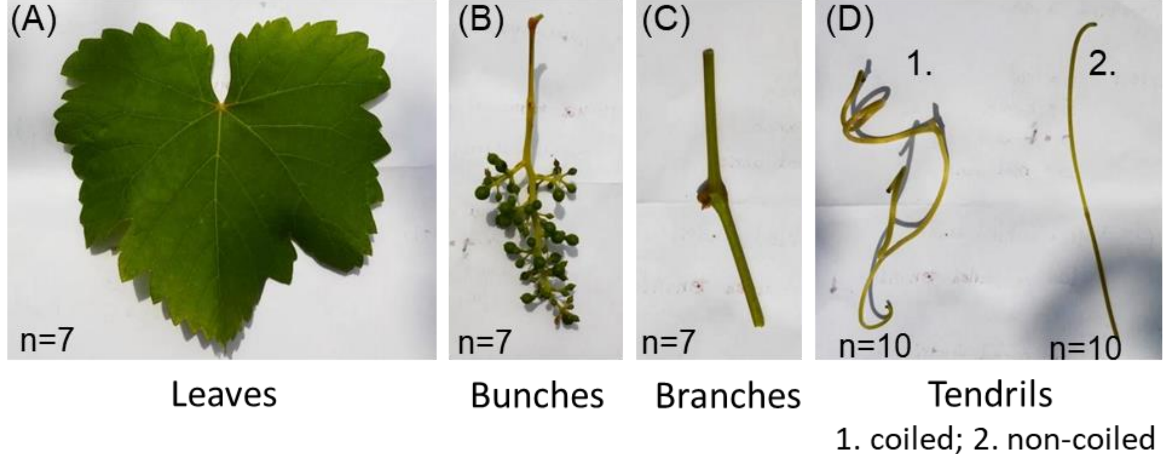
Weiler EW, Albrecht T, Groth B et al (1993) Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendril coiling response of *Bryonia dioica*. Phytochemistry 32:591–600. https://doi.org/10.1016/S0031-9422(00)95142-2

Yu GH, Zou J, Feng J et al (2014) Exogenous γ-aminobutyric acid (GABA) affects pollen tube growth via modulating putative Ca^{2+}-permeable membrane channels and is coupled to negative regulation on glutamate decarboxylase. J Exp Bot 65:3235–3248. https://doi.org/10.1093/jxb/eru171

Tendril coiling in grapevine: Jasmonates - and a new role for GABA?

Jaiana Malabarba, Michael Reichelt, Giancarlo Pasquali and Axel Mithöfer

Supplementary Figures and Tables



Supplementary Fig. 1 Grapevine organs samples for phytohormones evaluation. (A) Fully expanded leaves, (B) Bunches in the fruit-set stage, (C) Unlignified branches (D) Tendril coiled and non-coiled were sampled for phytohormones measurements

Supplementary Table 1. Evaluation of jasmonate metabolites (ng g^{-1} FW) in different grapevine tissues.

	OH-JA	OH-JA-Ile	COOH-JA-Ile
Leaf	3.7± 2.0	0.8 ± 0.2	0.1 ± 0.1
Bunch	34.6 ± 15.5	9.6 ± 4.2	0.7 ± 0.5
Branch	11.7 ± 6.0	1.7 ± 1.4	0.2 ± 0.1
Tendril non-coiled	73,4 ± 42,9 ns	34,3 ± 14,7 *	2,02 ± 0,8 ***
Tendril coiled	42,4 ± 23,76 ns	19,7 ± 12,3 *	0,51 ± 0,4 ***

Shown are average levels ± SE (*n* = 7 for leaf, branch, bunch; *n* = 10 for tendrils, coiled and non-coiled). Statistically significant differences for the same phytohormones between the tendrils were analyzed by Student's t-test: ns, non-significant; * = $p<0.01$; *** = $p<0.0001$

10 ANEXO III

Evidence for GABA-Induced Systemic GABA Accumulation in *Arabidopsis* upon Wounding

Artigo científico publicado no periódico ‘Frontiers in Plant Science’ (FI: 4,29)

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Evidence for GABA-Induced Systemic GABA Accumulation in *Arabidopsis* upon Wounding

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The non-proteinogenic amino acid γ-aminobutyric acid (GABA) is present in all plant species analyzed so far. Its synthesis is stimulated by either acidic conditions occurring after tissue disruption or higher cytosolic calcium level. In mammals, GABA acts as inhibitory neurotransmitter but its function in plants is still not well understood. Besides its involvement in abiotic stress resistance, GABA has a role in the jasmonate-independent defense against invertebrate pests. While the biochemical basis for GABA accumulation in wounded leaves is obvious, the underlying mechanisms for wounding-induced GABA accumulation in systemic leaves remained unclear. Here, the *Arabidopsis thaliana* knock-out mutant lines *pop2-5,* unable to degrade GABA, and *tpc1-2*, lacking a wounding-induced systemic cytosolic calcium elevation, were employed for a comprehensive investigation of systemic GABA accumulation. A wounding-induced systemic GABA accumulation was detected in *tpc1-2* plants demonstrating that an increased calcium level was not involved. Similarly, after both mechanical wounding and *Spodoptera littoralis* feeding, GABA accumulation in *pop2-5* plants was significantly higher in local and systemic leaves, compared to wild-type plants. Consequently, larvae feeding on these GABA-enriched mutant plants grew significantly less. Upon exogenous application of a D_2-labeled GABA to wounded leaves of *pop2-5* plants, its uptake but no translocation to unwounded leaves was detected. In contrast, an accumulation of endogenous GABA was observed in vascular connected systemic leaves. These results suggest that the systemic accumulation of GABA upon wounding does not depend on the translocation of GABA or on an increase in cytosolic calcium.

Keywords: γ-aminobutyric acid, herbivory, *Spodoptera littoralis*, MecWorm, calcium, plant defense, systemic signaling

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INTRODUCTION

The four carbon non-proteinogenic amino acid γ-aminobutyric acid (GABA) is present in a multitude of organisms and was also detected in all plant species analyzed so far (Shelp et al., 2009). In contrast to mammals – where GABA acts as inhibitory neurotransmitter by regulating ion channels – its function in plants is still not completely understood. It seems that the role of GABA

in plants is quite diverse. Several studies indicate that GABA is not only involved in regulating metabolic pathways like the Krebs cycle, it additionally acts as a signaling molecule in plant growth and development (Bouche and Fromm, 2004; Fait et al., 2008). For example, it was reported that a tightly regulated GABA level is required for optimal root and pollen tube growth (Palanivelu et al., 2003; Mirabella et al., 2008; Renault et al., 2011). Additionally, it was shown that various abiotic as well as biotic stress stimuli induce an elevation of the GABA level in plant tissue (Bown and Shelp, 1997; Kinnersley and Turano, 2000). For example, it was observed that salt and cold stress or tissue damage of soybean (*Glycine max*) leaves leads to rapid accumulation of GABA up to 25-fold (Wallace et al., 1984; Ramputh and Bown, 1996). *Arabidopsis thaliana* plants producing a lower constitutive level of GABA showed a higher susceptibility to drought stress due to a stomata closure defect which could be rescued by increasing the internal GABA level (Mekonnen et al., 2016). Also, external application of GABA to *Oryza sativa* seedlings and *Piper nigrum* plants could enhance the individuals' performance under heat and drought stress conditions, respectively (Nayyar et al., 2014; Vijayakumari and Puthur, 2016).

Previous studies suggest that GABA acts as a player in plant defense against insect herbivores, although the exact impact of an elevated GABA concentration on the invertebrates is not clear (Ramputh and Bown, 1996; Bown et al., 2006; Scholz et al., 2015; Bown and Shelp, 2016). On the one hand, insects feeding on high concentration of GABA show a reduced performance. Rearing lepidopteran *Choristoneura rosaceana* cv Harris larvae on artificial diet supplemented with 1.6–2.6 μmol GABA (g fresh weight)$^{-1}$ reduced growth and survival of the insects and delayed their development (Ramputh and Bown, 1996). Similar, *Spodoptera littoralis* larvae fed with a GABA concentration up to 1 μmol (g food)$^{-1}$ gained less weight compared with the control group (Scholz et al., 2015). On the other hand, attacked plants had an elevated level of GABA in plant tissue in response to herbivore contact. The movement of crawling insects on the leaf surfaces of *Nicotiana tabacum* and *Glycine max* plants was sufficient to stimulate the local GABA level by five-fold after 5–10 min (Bown et al., 2002). In *Arabidopsis thaliana* Col-0 plants, feeding of *S. littoralis* larvae for 3 h increased the local GABA concentration by two-fold (Scholz et al., 2015). Interestingly, continuous mechanical wounding with the robotic caterpillar MecWorm (Mithöfer et al., 2005) resulted in an up to 10-fold elevation of the local endogenous GABA level (Scholz et al., 2015).

In *Arabidopsis*, GABA is mainly produced from L-glutamate; the decarboxylation reaction is catalyzed by glutamate decarboxylases (GADs), which are encoded by five genes. GAD1 and GAD2 are the most abundantly expressed members of this family (Shelp et al., 2012; Scholz et al., 2015). It was shown that GAD activity is induced by two different mechanisms: (i) in intact plant tissue and neutral pH GAD activity is stimulated in a Ca^{2+}-dependent manner by binding of calmodulin to the CAM-binding site (Snedden et al., 1995); (ii) after wounding of plant cells the vacuolar content is released and the cytosol is acidified which leads to a Ca^{2+}-independent activation of GADs (Carroll et al., 1994). The degradation of GABA is carried out in the mitochondrial matrix by a GABA-transaminase (GABA-T/POP2). In *Arabidopsis*, GABA-T is encoded by only one gene whose knock-out results in constitutive elevated level of GABA in the *pop2-5* plant (Palanivelu et al., 2003).

In recent studies, the focus in plant wounding-induced stress signaling shifted from only analyzing the locally treated leaf to considering the whole plant response including the systemic leaves (Farmer et al., 2013; Mousavi et al., 2013; Kiep et al., 2015). When the plant was wounded or attacked by a herbivore on one leaf, a spike in cytosolic calcium, $[Ca^{2+}]_{cyt}$, was generated locally as well as in adjacent leaves with vascular connections to the treated leaf. In *Arabidopsis thaliana* plants, wounding leaf 8 including its midrib, caused a $[Ca^{2+}]_{cyt}$ elevation in vascular connected leaves 5, 8, and 13 (Dengler, 2006; Kiep et al., 2015). The response in the local leaf was immediate whereas the response in the systemic leaves showed a delay of 1–2 min. A similar response pattern was observed for plants fed by *S. littoralis* larvae (Kiep et al., 2015). Strikingly, the $[Ca^{2+}]_{cyt}$ elevation in adjacent leaves after wounding is dependent on the presence of the Ca^{2+}-permeable vacuolar channel TWO PORE CHANNEL 1 (TPC1-2) (Kiep et al., 2015). The systemic signaling is quite complex and the elevation of $[Ca^{2+}]_{cyt}$ was accompanied by e.g., an increase in ROS and electrical signals moving to systemic tissues of the plant (Gilroy et al., 2016). Additionally, it was observed that a precursor of the defense-related phytohormone jasmonic acid (JA) was able to travel from the wounded local tissue to unwounded systemic leaves when applied exogenously (Jimenez-Aleman et al., 2015). Similarly, a systemic accumulation of GABA in unwounded adjacent leaves was detected after mechanical wounding of one single defined leaf in *Arabidopsis thaliana* (Scholz et al., 2015).

Thus, the open question is how insect feeding or wounding initiates the systemic accumulation of GABA in the plant. Two different mechanisms are conceivable. First, the observed systemic cytosolic calcium elevation could trigger the production of GABA in the systemic leaves by interaction of GADs with calmodulin. Second, no signaling is involved but GABA itself can travel from the treated leaf through the vascular system to the systemic leaves. Here, we addressed and challenged these hypotheses. Therefore, we used an *Arabidopsis tpc1-2* mutant lacking a systemic cytosolic calcium signal after wounding to study the impact of calcium. In addition, we characterized the *pop2-5* mutant upon wounding and herbivore attack and, due to its inability to degrade GABA, employed this mutant line in transport studies with D_2-labeled GABA.

MATERIALS AND METHODS

Plant Growth and Treatment

Four to 5-week-old plants grown in 10 cm round pots were used for all experiments. *Arabidopsis thaliana* Columbia-0 wild-type and mutant plants (*tpc1-2* and *pop2-5*) were kept at short day conditions after stratification for 2 days at 4°C. The growth chamber was adjusted to 50–60% humidity and 21°C with a

10-h-light/14-h-dark photoperiod using FLUORA® L 36W/77 bulbs (OSRAM, Garching, Germany) with a light intensity of 100 μmol m^2 s^{-1}. Seeds of *tpc1-2* were kindly provided by Prof. Edgar Peiter (University Halle) and *pop2-5* seeds (GK_157D10) were purchased from GABI-Kat directly (Kleinboelting et al., 2012). To ensure the same starting conditions, all plants used for one assay were sown and germinated on the same day and were kept in the same growth chamber. For experiments investigating the systemic response and translocation of metabolites the leaves of each plant were counted according to their age (Dengler, 2006; Farmer et al., 2013; Kiep et al., 2015).

MecWorm treatment was used for mechanical wounding of the plant with punches every 5 s, totaling 12 punches per minute on treated leaf 8 (Mithöfer et al., 2005; Scholz et al., 2015). To investigate the systemic response upon treatment of leaf 8, the local and systemic leaves 5, 8, 9, 11, and/or 13 were analyzed. Untreated plants, kept exactly as the treated plants, were used as controls. The duration of the MecWorm treatment used is indicated in the respective figures. To study the wounding-induced systemic translocation of GABA in the plant, a double-deuterated GABA (Sigma–Aldrich, Munich, Germany) was used and applied to *pop2-5* mutant plants. The *pop2-5* mutant was used for this assay since it cannot degrade GABA due to the knock-out of the *GABA-T* gene (Palanivelu et al., 2003). According to (Jimenez-Aleman et al., 2015), 20 μl of 50 μM D_2-GABA or water was applied to local wounds on leaf 8, which were generated with a pattern wheel (PRYM_610940, Prym, Stolberg, Germany) without wounding the midrib. On each side of the midrib, the leaf was wounded with six vertical motions. D_2-GABA and water-treated plants were kept for 1.5 and 3 h with a cover to prevent evaporation. Samples of leaf 8 and chosen systemic leaves were harvested after the indicated time points. To avoid additional accumulation of GABA due to the cutting and harvesting process, all samples were harvested in less than 1 min and directly stored in liquid nitrogen. For all plants the leaves were harvested from oldest to youngest leaf (starting with leaf 5). All samples were kept at −80°C till further analysis.

Insect Material and Feeding Assays

Larvae of the generalist herbivore *Spodoptera littoralis* were hatched and reared on artificial diet (Bergomaz and Boppre, 1986) at 23–25°C with 10-h-light/14-h-dark cycles. For the 1 week long-term feeding assay, three larvae of first instar were placed on every wild-type and mutant plant. To achieve similar starting conditions, all larvae determined for one plant genotype were pooled and weighed prior to the experiment. The minimal starting weight of 30 larvae was set to 60 mg to ensure a survival of all larvae. After 1 week the weight of every larva was recorded separately. For the short-term feeding assay third instar larvae were used after they were kept overnight without food. This treatment ensures an immediate start of feeding after placement on the plant. The locally fed leaves were collected after the indicated time points and kept at −80°C till further analysis.

Extraction and Quantification of GABA

Two hundred and fifty milligram of fresh leaves were frozen in liquid nitrogen and weighed to enable a GABA determination per g fresh weight. If multiple leaves of a plant were collected, then leaves were cut according to their age starting with the oldest (leaf 5). The leaf material was homogenized in a Geno/Grinder® 2010 (Spex Sample Prep, Stanmore, UK) equipped with aluminum racks. The racks were cooled in liquid nitrogen prior to usage to prevent a thawing of leaf material during the whole homogenization process. The amino acids (including GABA) were extracted twice with a total of 2 ml of methanol on ice. Supernatants were combined and dried using a Concentrator plus (Eppendorf, Hamburg, Germany) and re-suspended in 500 μl of methanol. The extract was diluted 1:20 (v/v) with water containing the internal standard. The algal amino acid mix ^{13}C, ^{15}N (Isotec, Miamisburg, OH, USA) was used as internal standard at a concentration of 10 μg ml^{-1} in all samples. The concentration of unlabeled and D_2-GABA (M + 2) was analyzed by LC-MS/MS according to previous studies (Scholz et al., 2015). An API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) was operated in positive ionization mode with multiple reaction monitoring (MRM) to monitor analyte parent ion → product ion: GABA (*m/z* 104.1 →87.1; DP 51, CE 17); this MRM is specific for GABA and does not detect any α-aminobutyric acid, D_2-GABA (*m/z* 106.1 →89.1+ *m/z* 106.1 →88.1; DP 51, CE 17).

Extraction and Quantification of Phytohormones

Like the quantification of GABA, 250 mg of leaf material was used for phytohormone analysis. The extraction procedure and determination of JA and JA-Ile was carried out on ice as described before (Vadassery et al., 2012) with small changes. In this study, a different mixture of labeled jasmonates was used as internal standard. Instead of 15 ng of JA-[^{13}C6]-Ile conjugate used in the previous study, 60 ng of D_6-JA-Ile (HPC Standards GmbH, Cunnersdorf, Germany) was used. Additionally, the 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid was replaced by 60 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany). Since it was observed that both the D_6-labeled JA and JA-Ile contained 40 % of the corresponding D_5-labeled compounds (which were not included in the analysis method), the obtained results were divided by 1.7 to exclude this inaccuracy.

Statistics

All plants of different mutant lines used for one experiment were grown in the same growth chamber. Independent experiments were treated as a completely randomized design. Experiments were repeated three times to ensure reproducibility and 5–10 plants were used in each treatment for each experiment time point. Data of all independent experiments were pooled and analyzed. For comparison of two groups, the Student's *t*-test or Mann Whitney *U*-test was applied. For statistical analyses of multiple groups, one-way analysis of variance (one-way ANOVA) or two-way analysis of variance (two-way ANOVA) were used as indicated in the figure legends. Different letters indicate significant differences between treatments. GraphPad

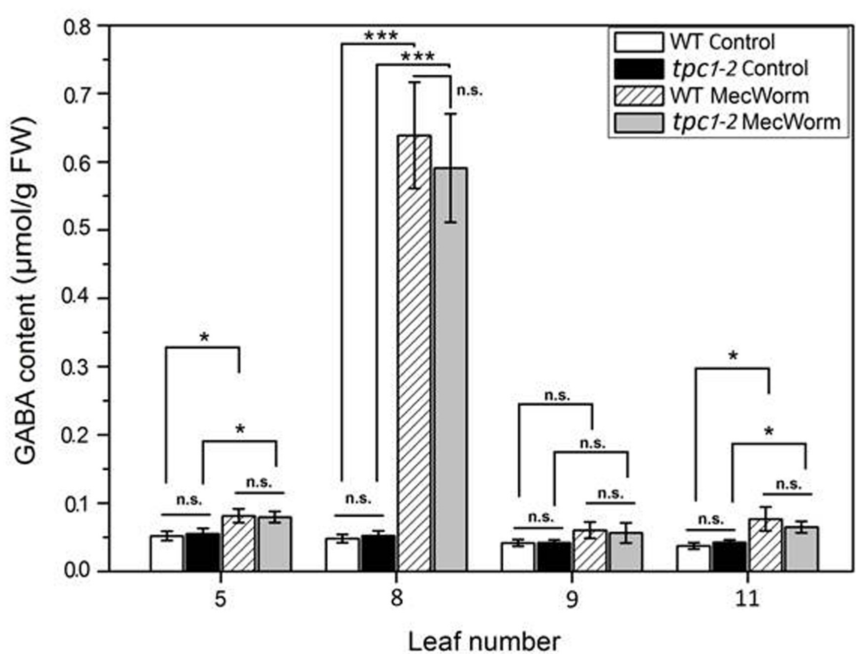

FIGURE 1 | Accumulation of GABA in individual *Arabidopsis* leaves of wild-type (WT) and *tpc1-2* plants after MecWorm treatment. Mean [±SE, $n = 6$ (control), 8 (MecWorm)] levels of GABA were determined in individual leaves of untreated control plants and plants after 1.5 h treatment with MecWorm. In treated plants, leaf 8 was subjected to mechanical damage and systemic leaves 5, 9, and 11, and treated leaf 8 were analyzed for GABA level. Significant differences between the GABA level in the same leaf of the control and treated plant were analyzed by *t*-test (for each leaf separately, $p < 0.05$, Mann–Whitney *U*-test), $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

Prism 6 and Origin Pro were used for data analysis and graph composition.

RESULTS AND DISCUSSION

GABA Accumulates in Systemic Untreated Leaves in the Calcium-Channel Mutant *tpc1-2*

To examine if the production of GABA in untreated systemic leaves is induced in a Ca^{2+}/calmodulin-dependent manner (Snedden et al., 1995), the systemic GABA accumulation was analyzed after MecWorm treatment in the *tpc1-2* mutant. Recently, it was observed, that the *tpc1-2* mutant did not show a systemic $[Ca^{2+}]_{cyt}$ elevation after mechanical wounding (Kiep et al., 2015). Thus, *tpc1-2* is a good model to study whether or not the systemic GABA accumulation depends on the systemic calcium signal. As expected, after 1.5 h of MecWorm treatment on WT leaf 8, this leaf showed the highest GABA accumulation. However, a significant increase in GABA concentration was also observed in systemic leaves 5 and 11 (**Figure 1**). With 0.05 μmol g FW^{-1} the *tpc1-2* plants showed the same basic GABA level in control plants as the WT and also a comparable increase in systemic leaves 5 and 11 (**Figure 1**). This indicates that the insect-like wounding-induced systemic $[Ca^{2+}]_{cyt}$ elevation has no or limited influence on the amount of GABA produced in systemic leaves. For soybean GAD it was observed that an increase in $[Ca^{2+}]_{cyt}$ up to 7–11 μM is necessary to decrease the enzyme's Km value about 55% (Snedden et al., 1995). The determined stress-induced $[Ca^{2+}]_{cyt}$ elevations in *Arabidopsis* showed an increase to a maximum of 0.6–2 μM (Knight et al., 1996, 1997; Whalley and Knight, 2013). Hence, the wounding-induced systemic $[Ca^{2+}]_{cyt}$ elevation appears to be insufficient to account for the increasing GAD activity observed in the present study.

The GABA Accumulating *pop2-5* Mutant Responds Efficiently to Mechanical Wounding and *Spodoptera littoralis* Feeding

Due to the results obtained with *tpc1-2* plants, the possibility of direct GABA transport to systemic leaves was investigated. Therefore, we employed the GABA-transaminase knock-out mutant line, *pop2*-5. This mutant has not been used and characterized in the context of herbivory. Previous studies observed that increased concentrations of GABA can affect insect's development (Ramputh and Bown, 1996; MacGregor et al., 2003; Bown et al., 2006; Scholz et al., 2015). We used the *pop2-5* mutant to further evaluate the effect of high endogenous GABA concentration *in planta* on the herbivorous larvae of *Spodoptera littoralis* in a feeding assay (**Figure 2**). Our results demonstrate that the larvae, feeding 1 week on *pop2-5* plants, gained significantly less weight than the larvae

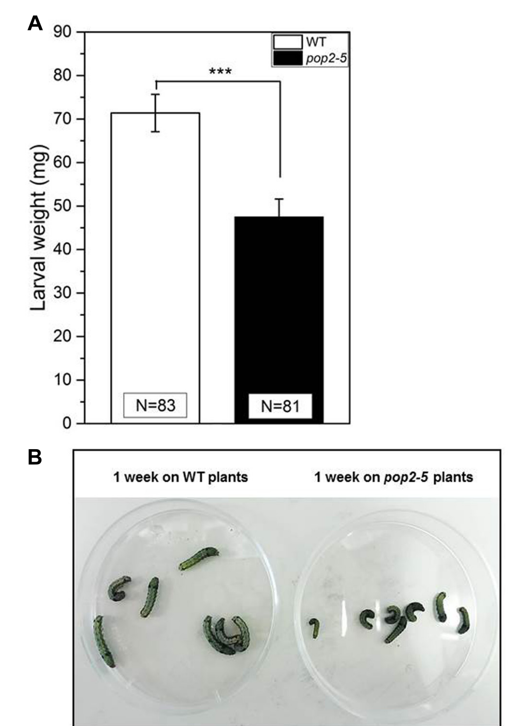

FIGURE 2 | Feeding assay of *Spodoptera littoralis* larvae on *Arabidopsis* WT and *pop2-5* plants. *S. littoralis* first instar larvae were pre-weighed and three larvae were placed on each plant. The larval weight (mean ± SE) was measured after 7 days of feeding. **(A)** Final weight of larvae after feeding for 1 week on WT (white) and *pop2-5* (black) plants. The total number of larvae weighed (N) is indicated in the bars. **(B)** Larval macroscopic view after 1 week of feeding on the indicated mutants. Experiments were repeated three times independently. Significant differences between WT and GABA mutant plants after feeding were analyzed by Student's *t*-test (***$P \leq 0.001$).

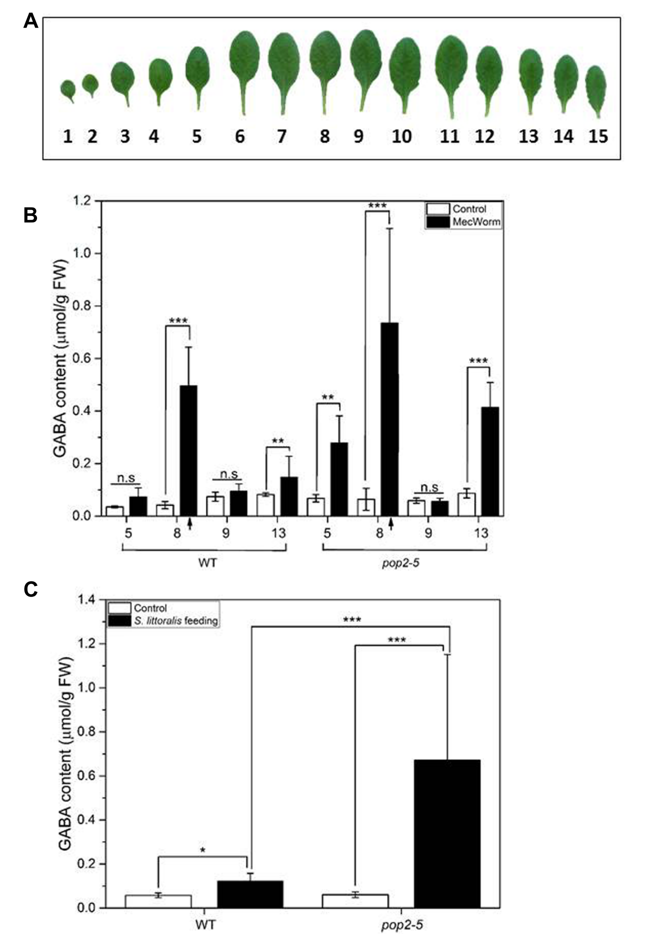

FIGURE 3 | Accumulation of GABA in individual *Arabidopsis* leaves after MecWorm treatment and *Spodoptera littoralis* short-term feeding assay. (A) Leaves of 4-week old *Arabidopsis thaliana* plants in growing order, from the oldest to the youngest. **(B)** Mean (±SE, $n = 5$) levels of GABA were determined in individual leaves of untreated control plants (white) and plants after treatment with MecWorm for 1.5 h (black). In treated plants, leaf 8 was subjected to mechanical damage (indicated by arrow) and systemic leaves 5, 9, and 11, and treated leaf 8 were analyzed for GABA level. **(C)** Mean (±SE, $n = 5$) levels of GABA were determined in individual leaves of untreated control plants (white) and plants after feeding of third instar *S. littoralis* larvae for 1.5 h (black). Significant differences between the GABA level were analyzed by *t*-test (for each leaf separately, $p < 0.05$, Mann–Whitney *U*-test), *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. No significant difference in GABA concentration was found between untreated genotypes.

feeding on WT plants (**Figure 2A**). The difference could also be observed by macroscopic inspection (**Figure 2B**). In previous experiments with GABA-supplemented artificial diet it was shown, that larvae of *C. rosaceana* and *S. littoralis* feeding on high levels of GABA showed decreased survival, delayed development and decreased gain of weight, respectively (Ramputh and Bown, 1996; Scholz et al., 2015). This indicates that the constitutive GABA accumulation in *pop2*-5 plants seems to contribute to the enhanced resistance against *S. littoralis* larvae attack while the exact effect of GABA on the insects is not known. Enhanced GABA levels in the insect might lead to permanent activation of GABA-activated Cl^--channels resulting in hypertension or paralysis (Sattelle, 1990; Bown et al., 2006).

We further investigated the GABA response upon MecWorm wounding in well-developed leaves of *pop2-5* plants (**Figure 3A**). With MecWorm we are able to investigate the impact of the isolated wounding process without the contribution of insect-derived compounds in oral secretions (Mithöfer et al., 2005). As shown in **Figure 3B**, continuous mechanical wounding of leaf 8 significantly elevated the amount of GABA in the treated leaf in both WT and *pop2-5* plants. For WT plants, apart from the local GABA accumulation, we observed a significant increase of GABA in leaf 13, which is directly connected to leaf 8 and a non-significant increase in the indirectly connected leaf 5 (Dengler, 2006). For *pop2-5* plants the local GABA accumulation of leaf 8 was followed by a significant increase of GABA in leaves 5 and 13. In preliminary experiments we observed that the GABA elevation in the adjacent leaves is a time-dependent response, and after 90 min of feeding-like wounding of leaf 8 a significant increase of GABA was detectable in leaf 13. As expected, there was no significant increase of GABA on leaf 9,

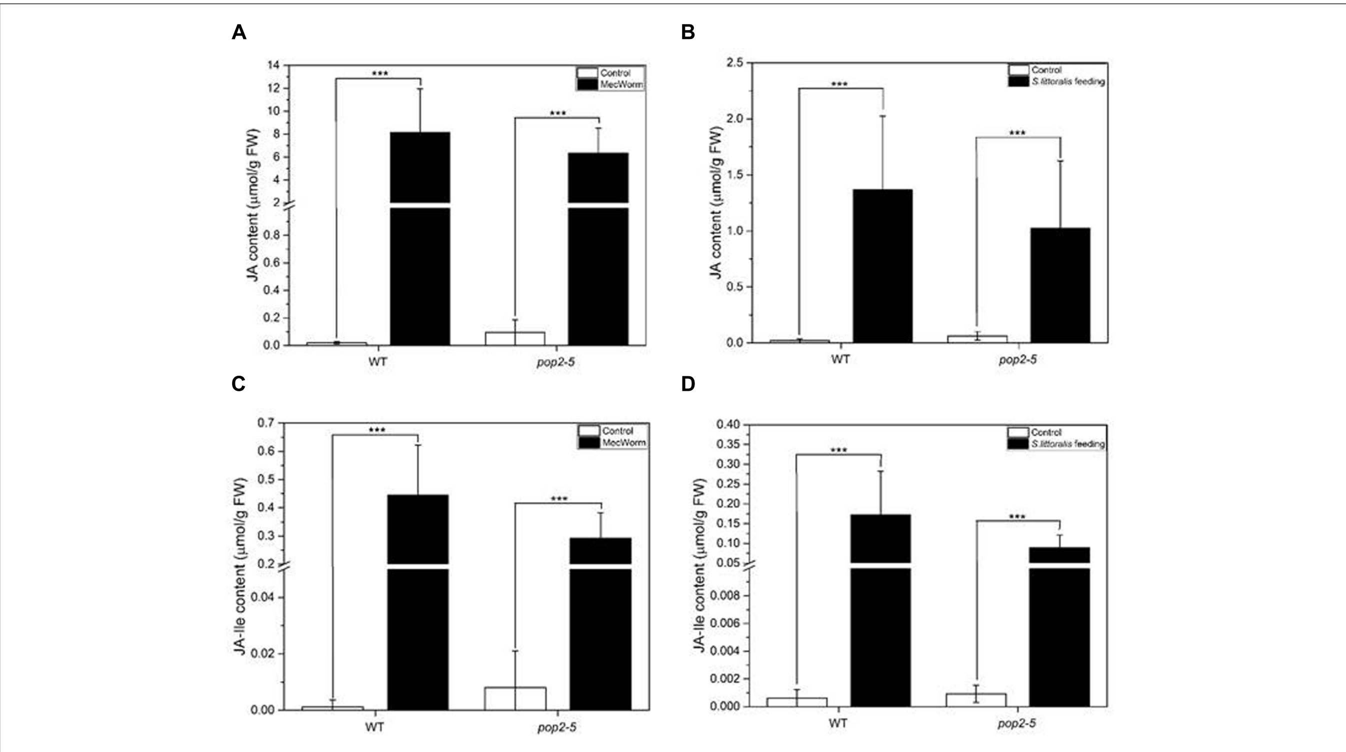

FIGURE 4 | Accumulation of jasmonates in *Arabidopsis* leaves after *Spodoptera littoralis* short time feeding assay and MecWorm treatment. JA levels were analyzed after mechanical wounding for 1.5 h **(A)** and after *S. littoralis* feeding for 3 h **(B).** JA-Ile levels were analyzed after mechanical wounding for 1.5 h **(C)** and after *S. littoralis* feeding for 3 h **(D).** For MecWorm treated plants, leaf 8 was subjected to mechanical damage and analyzed for jasmonate level. For quick feeding assay hormone level was analyzed from local fed leaves of third instar *S. littoralis* feeding for 1.5 h. Untreated leaves from untreated plants were used as controls. Statistically significant differences between the JA and JA-Ile levels of the control and treated plant were analyzed by Student's *t*-test (***$P \leq 0.001$). No significant differences in JA and JA-Ile levels were found between the analyzed genotypes.

as it is not connected to treated leaf 8. Analysis of the GABA concentration after 1.5 h of *S. littoralis* feeding revealed that the larvae caused a 6-fold higher GABA accumulation in *pop2-5* compared to WT plants, in the fed leaves (**Figure 3C**). The synthesis of GABA in *pop2-5* plants during treatments should be the same but in WT there is more GABA degradation by the transaminase during the assay, so the accumulation at the end was lower in the WT (**Figure 3C**). Here, it should be mentioned that in this set of experiments no clear difference in GABA concentrations between untreated WT and *pop2*-5 plants was found. This contradicts earlier findings including our own study (Palanivelu et al., 2003; Miyashita and Good, 2008; Clark et al., 2009; Scholz et al., 2015) where in *pop2-5* plants a higher GABA level was detected. However, this might be explained by different growth conditions, i.e., long vs. short day. Nevertheless, taken together, the results indicate that also in *pop2-5* mutant plants GABA is an efficient inducible systemic defense factor against insect feeding.

Jasmonate Levels Are Not Affected in the *pop2-5* Mutant

The accumulation of jasmonates was not influenced by different GABA levels in *gad1/2* and *gad1/2 x pop2-5* mutants. Similarly the accumulation of GABA was not changed in *jar1*, a JA-Ile jasmonate signaling mutant (Scholz et al., 2015). We decided to further test the involvement of jasmonates on GABA accumulation also in the non-GABA-degrading *pop2-5* mutant. Therefore we measured the levels of JA and its bioactive derivative, (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), in *Arabidopsis* WT and in *pop2-5* plant*s* both upon mechanical wounding and herbivore treatment. The concentration of JA and JA-Ile significantly increased after feeding assay with *S. littoralis* and after MecWorm treatment in both tested genotypes (**Figure 4**). In all cases the MecWorm treatment caused higher jasmonate accumulation but the non-treated controls showed no differences between WT and *pop2-5* plants. These data support earlier results suggesting that GABA accumulation is not jasmonate-dependent (Scholz et al., 2015) and *vice versa*. However, we cannot completely rule out that an octadecanoic dependent, but JA/JA-Ile independent, signaling pathway might still be involved in GABA accumulation.

D_2-GABA Treatment Increases GABA Concentration in Systemic Adjacent Leaves

To further investigate if GABA is transported to or synthetized in the adjacent leaves, we treated *Arabidopsis pop2-5* plants

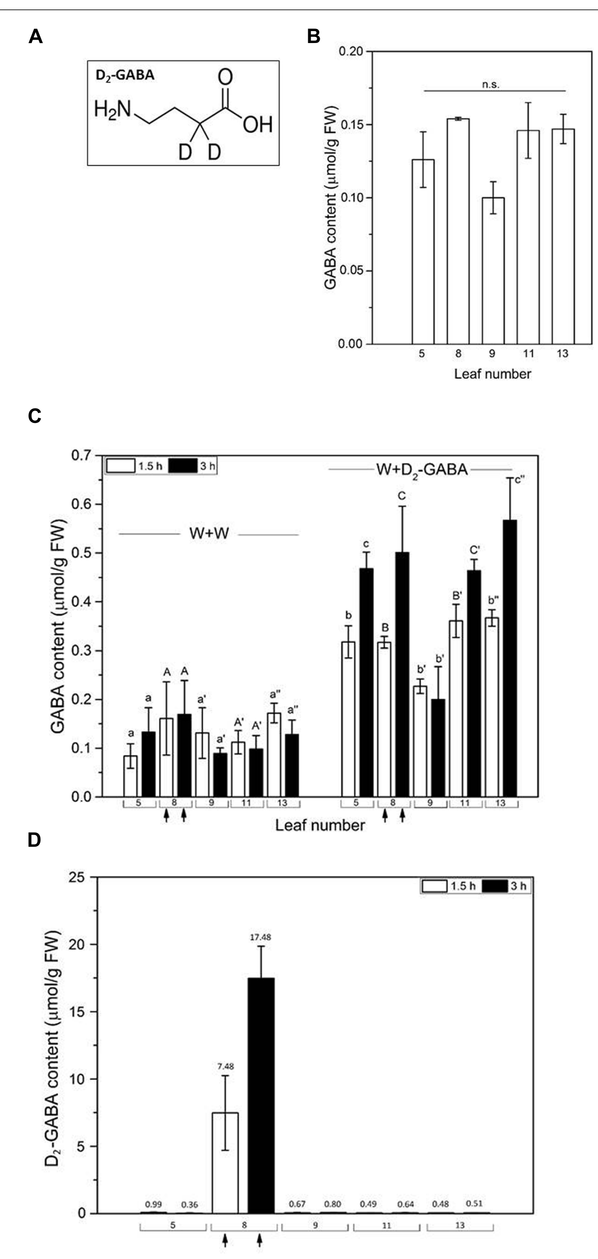

FIGURE 5 | Accumulation of GABA after isotopically labeled GABA (D_2-GABA) treatment in *pop2-5* plants. (A) Chemical structure of D_2-GABA used. **(B)** Mean (±SE, $n = 7$) level of unlabeled GABA in untreated leaves from control plants. **(C)** Mean (±SE, $n = 7$) level of unlabeled GABA in *pop2-5* plants was determined after wounding leaf 8 with a pattern wheel (indicated by arrow), applying water (wounding + water, W + W) or D_2-GABA (wounding + D_2-GABA, W + D_2-GABA) on the wounds and incubating for 1.5 h (white) and 3 h (black). No statistically significant difference was found between the controls (in B) and the wounding plus water treatment. **(D)** Mean (±SE, $n = 7$) levels of D_2-GABA were determined in different leaves after wounding leaf 8 with a pattern wheel (indicated by arrow), applying D_2-GABA on the wounds and incubating for 1.5 h (white) and 3 h (black). Values below 1 µmol (g FW)$^{-1}$ do not represent D_2-GABA but an unknown endogenous compound and should be considered as noise. Significant differences between the treatments were analyzed by One-way ANOVA and Two-way ANOVA ($p < 0.05$, SNK-test) and are indicated by different letters.

with the double-deuterated D_2-GABA (**Figure 5A**) on a leaf that was wounded with a pattern wheel. This approach would enable us to observe any translocation of GABA in the plant leaves, because no D_2-GABA degradation takes place in the *pop2-5* mutant. The basic GABA levels in different leaves of control *pop2-5* plants were comparable (**Figure 5B**). Leaves of the *pop2-5* plants that were slightly pattern wheel-wounded and incubated with water, showed a GABA concentration similar to control plants (**Figures 5B,C**), indicating that the weak single mechanical wounding had no effect compared to continuous mechanical wounding achieved with MecWorm treatment. Interestingly, when GABA was measured in leaf 8-wounded *pop2-5* plants that were treated with D_2-GABA, an increase of non-labeled GABA was detected, with the accumulation of GABA in directly connected (leaf 13) and indirectly connected (leaves 5 and 11) leaves. GABA accumulation was significantly higher after 1.5 h and increased more after 3 h (**Figure 5C**). Even leaf 9 showed, compared to wounding and water treatment (W + W), a slight elevation of unlabeled GABA after the application of D_2-GABA (W + D_2-GABA). These results suggest that GABA synthesis was stimulated by the combination of the pattern wheel wounding and D_2-GABA incubation in both the treated leaf and systemic leaves (**Figure 5C**). When we further analyzed the concentration of D_2-GABA on *pop2-5* treated plants it was only possible to detect higher D_2-GABA level in the locally treated leaf 8 indicating that D_2-GABA was taken up. However, the fact that in the adjacent leaves D_2-GABA was detected not even nearly as in leaf 8 (**Figure 5D**) suggests that it is not transported from the local wounded leaf to the systemic leaves. This result, combined with the observation that also the systemic cytosolic calcium elevation after wounding is not responsible for activation of GABA synthesis in systemic unwounded leaves, demonstrates that there has to be a different signal initiating GABA synthesis and accumulation in systemic leaves. Previous studies in animals presented data concerning GABA-mediated positive feedback loop (Kamermans and Werblin, 1992; Fenalti et al., 2007) and also in plants a several-fold endogenous accumulation of GABA after external application of GABA has been found for different tissues (Shi et al., 2010; Shang et al., 2011; Malekzadeh et al., 2014; Vijayakumari and Puthur, 2016). Further studies are required to establish a role for phloem B-type cells in long-distance signaling in plants (Shelp, 2012). In a study concerning drought stress tolerance with a Poaceae species, *Agrostis stolonifera*, GABA was shown to enhance the accumulation of GABA and of other amino acids (glycine, valine, proline, 5-oxoproline, serine, threonine, aspartic acid and glutamic acid) (Li et al., 2017). Interestingly, in barley plants it was shown that external application of GABA was able to induce action potentials (APs) accompanied by cytoplasmatic acidification short of the position of the stimulus (Felle and Zimmermann, 2007). APs and other electrophysiological reactions represent conceivable signaling candidates for systemic leaves because they have been shown to be involved in wound-induced leaf-to-leaf signaling (Zimmermann et al., 2009, 2016; Mousavi et al., 2013).

CONCLUSION

Wounding of plant tissue and cell disruption caused by feeding insects demonstrated that GABA synthesis and accumulation can be a rapid defense response against invertebrate pests. Other studies proposed that GABA might have a role as signaling molecule, activated upon abiotic stresses. Our results demonstrate that under stress-conditions GABA cannot only act as a defense metabolite, but also as a signaling molecule. The approach with isotopically labeled GABA demonstrates in addition that a high local concentration of GABA in the challenged leaf can trigger a *de novo* synthesis of GABA in systemic untreated leaves. This systemic accumulation of GABA is neither dependent on the systemic cytosolic calcium elevation induced by the wounding nor on the direct transport of GABA from wounded to systemic leaves. The signal responsible for this observation remains still unclear but electrophysiological reactions might represent conceivable signaling candidates.

AUTHOR CONTRIBUTIONS

SS, JM, and AM conceived and designed the research; SS, JM, MR, and MH performed the experiments; SS, JM, FL, and AM wrote the manuscript. All authors contributed to the manuscript.

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REFERENCES

Bergomaz, R., and Boppre, M. (1986). A simple instant diet for rearing arctiidae and other moths. *J. Lepid. Soc.* 40, 131–137.

Bouche, N., and Fromm, H. (2004). GABA in plants: just a metabolite? *Trends Plant Sci.* 9, 110–115. doi: 10.1016/j.tplants.2004.01.006

Bown, A. W., Hall, D. E., and MacGregor, K. B. (2002). Insect footsteps on leaves stimulate the accumulation of 4-aminobutyrate and can be visualized through increased chlorophyll fluorescence and superoxide production. *Plant Physiol.* 129, 1430–1434. doi: 10.1104/pp.006114

Bown, A. W., Macgregor, K. B., and Shelp, B. J. (2006). Gamma-aminobutyrate: defense against invertebrate pests? *Trends Plant Sci.* 11, 424–427. doi: 10.1016/j.tplants.2006.07.002

Bown, A. W., and Shelp, B. J. (1997). The metabolism and functions of [gamma]-aminobutyric acid. *Plant Physiol.* 115, 1–5. doi: 10.1104/pp.115.1.1

Bown, A. W., and Shelp, B. J. (2016). Plant GABA: not just a metabolite. *Trends Plant Sci.* 21, 811–813. doi: 10.1016/j.tplants.2016.08.001

Carroll, A. D., Fox, G. G., Laurie, S., Phillips, R., Ratcliffe, R. G., and Stewart, G. R. (1994). Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell suspensions. *Plant Physiol.* 106, 513–520. doi: 10.2307/4276085

Clark, S. M., Di Leo, R., Van Cauwenberghe, O. R., Mullen, R. T., and Shelp, B. J. (2009). Subcellular localization and expression of multiple tomato gamma-aminobutyrate transaminases that utilize both pyruvate and glyoxylate. *J. Exp. Bot.* 60, 3255–3267. doi: 10.1093/jxb/erp161

Dengler, N. G. (2006). The shoot apical meristem and development of vascular architecture. *Can. J. Bot.* 84, 1660–1671. doi: 10.1139/b06-126

Fait, A., Fromm, H., Walter, D., Galili, G., and Fernie, A. R. (2008). Highway or byway: the metabolic role of the GABA shunt in plants. *Trends Plant Sci.* 13, 14–19. doi: 10.1016/j.tplants.2007.10.005

Farmer, E., Mousavi, S., and Lenglet, A. (2013). Leaf numbering for experiments on long distance signalling in *Arabidopsis*. *Protoc. Exch.* doi: 10.1038/protex.2013.071

Felle, H. H., and Zimmermann, M. R. (2007). Systemic signalling in barley through action potentials. *Planta* 226, 203–214. doi: 10.1007/s00425-006-0458-y

Fenalti, G., Law, R. H., Buckle, A. M., Langendorf, C., Tuck, K., Rosado, C. J., et al. (2007). GABA production by glutamic acid decarboxylase is regulated by a dynamic catalytic loop. *Nat. Struct. Mol. Biol.* 14, 280–286. doi: 10.1038/nsmb1228

Gilroy, S., Białasek, M., Suzuki, N., Górecka, M., Devireddy, A. R., Karpiński, S., et al. (2016). ROS. Calcium, and electric signals: key mediators of rapid systemic signaling in plants. *Plant Physiol.* 171, 1606–1615. doi: 10.1104/pp.16.00434

Jimenez-Aleman, G. H., Scholz, S. S., Heyer, M., Reichelt, M., Mithöfer, A., and Boland, W. (2015). Synthesis, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0. *Biochim. Biophys. Acta* 12, 1545–1553. doi: 10.1016/j.bbalip.2015.09.002

Kamermans, M., and Werblin, F. (1992). GABA-mediated positive autofeedback loop controls horizontal cell kinetics in tiger salamander retina. *J. Neurosci.* 12, 2451–2463.

Kiep, V., Vadassery, J., Lattke, J., Maaß, J.-P., Boland, W., Peiter, E., et al. (2015). Systemic cytosolic Ca2+ elevation is activated upon wounding and herbivory in Arabidopsis. *New Phytol.* 207, 996–1004. doi: 10.1111/nph.13493

Kinnersley, A. M., and Turano, F. J. (2000). Gamma aminobutyric acid (GABA) and plant responses to stress. *Crit. Rev. Plant Sci.* 19, 479–509. doi: 10.1080/07352680091139277

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P., and Weisshaar, B. (2012). GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Res.* 40, D1211–D1215. doi: 10.1093/nar/gkr1047

Knight, H., Trewavas, A. J., and Knight, M. R. (1996). Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* 8, 489–503. doi: 10.1105/tpc.8.3.489

Knight, H., Trewavas, A. J., and Knight, M. R. (1997). Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.* 12, 1067–1078. doi: 10.1046/j.1365-313X.1997.12051067.x

Li, Z., Yu, J., Peng, Y., and Huang, B. (2017). Metabolic pathways regulated by abscisic acid, salicylic acid and gamma-aminobutyric acid in association with improved drought tolerance in creeping bentgrass (*Agrostis stolonifera*). *Physiol. Plant.* 159, 42–58. doi: 10.1111/ppl.12483

MacGregor, K. B., Shelp, B. J., Peiris, S., and Bown, A. W. (2003). Overexpression of glutamate decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. *J. Chem. Ecol.* 29, 2177–2182. doi: 10.1023/A:1025650914947

Malekzadeh, P., Khara, J., and Heydari, R. (2014). Alleviating effects of exogenous Gamma-aminobutiric acid on tomato seedling under chilling stress. *Physiol. Mol. Biol. Plants* 20, 133–137. doi: 10.1007/s12298-013-0203-5

Mekonnen, D. W., Flügge, U. I., and Ludewig, F. (2016). Gamma-aminobutyric acid depletion affects stomata closure and drought tolerance of *Arabidopsis thaliana*. *Plant Sci.* 245, 25–34. doi: 10.1016/j.plantsci.2016.01.005

Mirabella, R., Rauwerda, H., Struys, E. A., Jakobs, C., Triantaphylides, C., Haring, M. A., et al. (2008). The Arabidopsis *her1* mutant implicates GABA in E-2-hexenal responsiveness. *Plant J.* 53, 197–213. doi: 10.1111/j.1365-313X.2007.03323.x

Mithöfer, A., Wanner, G., and Boland, W. (2005). Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling

insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137, 1160–1168. doi: 10.1104/pp.104.054460

Miyashita, Y., and Good, A. G. (2008). Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. *Plant Cell Physiol.* 49, 92–102. doi: 10.1093/pcp/pcm171

Mousavi, S. A. R., Chauvin, A., Pascaud, F., Kellenberger, S., and Farmer, E. E. (2013). GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling. *Nature* 500, 422–426. doi: 10.1038/nature12478

Nayyar, H., Kaur, R., Kaur, S., and Singh, R. (2014). γ-Aminobutyric acid (GABA) imparts partial protection from heat stress injury to rice seedlings by improving leaf turgor and upregulating osmoprotectants and antioxidants. *J. Plant Growth Regul.* 33, 408–419. doi: 10.1007/s00344-013-9389-6

Palanivelu, R., Brass, L., Edlund, A. F., and Preuss, D. (2003). Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. *Cell* 114, 47–59. doi: 10.1016/S0092-8674(03)00479-3

Ramputh, A. I., and Bown, A. W. (1996). Rapid γ-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae. *Plant Physiol.* 111, 1349–1352. doi: 10.1104/pp.111.4.1349

Renault, H., El Amrani, A., Palanivelu, R., Updegraff, E. P., Yu, A., Renou, J. P., et al. (2011). GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in *Arabidopsis thaliana*. *Plant Cell Physiol.* 52, 894–908. doi: 10.1093/pcp/pcr041

Sattelle, D. B. (1990). "GABA receptors of insects," in *Advances in Insect Physiology*, eds P. D. Evans and V. B. Wigglesworth (Cambridge, MA: Academic Press), 22.

Scholz, S. S., Reichelt, M., Mekonnen, D. W., Ludewig, F., and Mithöfer, A. (2015). Insect herbivory-elicited GABA accumulation in plants is a wound-induced. direct, systemic, and jasmonate-independent defense response. *Front. Plant Sci.* 6:1128. doi: 10.3389/fpls.2015.01128

Shang, H., Cao, S., Yang, Z., Cai, Y., and Zheng, Y. (2011). Effect of exogenous gamma-aminobutyric acid treatment on proline accumulation and chilling injury in peach fruit after long-term cold storage. *J. Agric. Food Chem.* 59, 1264–1268. doi: 10.1021/jf104424z

Shelp, B. J. (2012). Does long-distance GABA signaling via the phloem really occur? *Botany* 90, 897–900. doi: 10.1139/b2012-066

Shelp, B. J., Allan, W. L., and Faure, D. (2009). "Role of γ -aminobutyrate and γ-hydroxybutyrate in plant communication," in *Plant-Environment Interactions*, ed. F. Baluška (Berlin: Springer-Verlag), 73–84. doi: 10.1007/978-3-540-89230-4_4

Shelp, B. J., Bozzo, G. G., Zarei, A., Simpson, J. P., Trobacher, C. P., and Allan, W. L. (2012). Strategies and tools for studying the metabolism and function of γ-aminobutyrate in plants. II. Integrated analysis. *Botany* 90, 781–793. doi: 10.1139/b2012-041

Shi, S. Q., Shi, Z., Jiang, Z. P., Qi, L. W., Sun, X. M., Li, C. X., et al. (2010). Effects of exogenous GABA on gene expression of *Caragana intermedia* roots under NaCl stress: regulatory roles for H_2O_2 and ethylene production. *Plant Cell Environ.* 33, 149–162. doi: 10.1111/j.1365-3040.2009.02065.x

Snedden, W. A., Arazi, T., Fromm, H., and Shelp, B. J. (1995). Calcium/calmodulin activation of soybean glutamate decarboxylase. *Plant Physiol.* 108, 543–549. doi: 10.1111/j.1365-3040.2009.02065.x

Vadassery, J., Reichelt, M., Hause, B., Gershenzon, J., Boland, W., and Mithöfer, A. (2012). CML42-mediated calcium signaling coordinates responses to *Spodoptera* herbivory and abiotic stresses in Arabidopsis. *Plant Physiol.* 159, 1159–1175. doi: 10.1104/pp.112.198150

Vijayakumari, K., and Puthur, J. T. (2016). γ-Aminobutyric acid (GABA) priming enhances the osmotic stress tolerance in *Piper nigrum* Linn. plants subjected to PEG-induced stress. *Plant Growth Regul.* 78, 57–67. doi: 10.1007/s10725-015-0074-6

Wallace, W., Secor, J., and Schrader, L. E. (1984). Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiol.* 75, 170–175. doi: 10.1007/s10725-015-0074-6

Whalley, H. J., and Knight, M. R. (2013). Calcium signatures are decoded by plants to give specific gene responses. *New Phytol.* 197, 690–693. doi: 10.1111/nph.12087

Zimmermann, M. R., Maischak, H., Mithöfer, A., Boland, W., and Felle, H. H. (2009). System potentials, a novel electrical long-distance apoplastic signal in plants. induced by wounding. *Plant Physiol.* 149, 1593–1600. doi: 10.1104/pp.108.133884

Zimmermann, M. R., Mithöfer, A., Will, T., Felle, H. H., and Furch, A. C. (2016). Herbivore-triggered electrophysiological reactions: candidates for systemic signals in higher plants and the challenge of their identification. *Plant Physiol.* 170, 2407–2419. doi: 10.1104/pp.15.01736

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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11 *CURRICULUM VITAE*

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- SCHOLZ, SANDRA S[1].; MALABARBA, JAIANA[1]; REICHELT, MICHAEL; HEYER, MONIKA; LUDEWIG, FRANK; MITHÖFER, AXEL. Evidence for GABA-Induced Systemic GABA Accumulation in *Arabidopsis* upon Wounding. *Frontiers in Plant Science*, 8: 1-9. 2017.

- MALABARBA, JAIANA[1]; BUFFON, VANESSA; MARASCHIN, FELIPE S.; MARIATH, JORGE E.A; MARGIS-PINHEIRO, MÁRCIA; PASQUALI, GIANCARLO; REVERS, LUÍS F. Manipulation of *VviAGL11* Expression Changes the Seed Content in Grapevine (*Vitis vinifera* L.) *Plant Science*, 269: 126-135. 2018.

- MALABARBA, JAIANA[1]; REICHELT, MICHAEL; PASQUALI, GIANCARLO; MITHÖFER, AXEL. Tendril Coiling in Grapevine: Jasmonates and a New Role for GABA? *Journal of Plant Growth Regulation*, 1-7.2018.

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RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

- MALABARBA, J.; SCHOLZ, S. S.; REICHELT, M; Fuch, A; MITHÖFER, A. Is there a role of Annexin1 in *A. thaliana* defense response against herbivore attack? Max Planck for Chemical Ecology Retreat, Frohberg, Germany, 2017.

- MALABARBA, J.; SCHOLZ, S. S.; REICHELT, M., MEYER, M; LUDEWIG, F.; MITHÖFER, A. Evidence for GABA-Induced Systemic GABA Accumulation in *Arabidopsis* upon Wounding. IMPR Symposium. Jena, Germany, 2017.

- MALABARBA, J.; Buffon V.; FRANCIO, L.; MARGIS-PINHEIRO, M.; MARASCHIN, F. S.; REVERS, L. F.; PASQUALI, G. Plant Plasmids as a Biotechnological Tool for Seed Morphogenesis Characterization in Grapevine. Annual Congress if The Strategy Process Biotechnology 2020, Germany, 2016.

- MALABARBA, J.; BUFFON, V.; FRÂNCIO, L.; MARASCHIN, F. S.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS L. F. Plant Plasmids as a Biotechnological Tool for Seed Morphogenesis Characterization in Grapevine. I Biotecsul, Congress of Biotechnology of the South Region. FEEVALE, 2016.

- FRÂNCIO, L.; MALABARBA, J.; BUFFON, V.; REVERS, L. F.; WAIRICH, A. Development of Markers for Assisted Selection of Seedlessness and Downy Mildew Resistance In Grapevine. I Biotecsul, Congress of Biotechnology of the South Region. FEEVALE, 2016.

- MALABARBA, J.; BUFFON, V.; PASQUALI, G.; REVERS, LF structural and functional studies *VvAGL11* gene and its role in morphogenesis of *Vitis vinifera* seeds. In: 12th Meeting of Scientific Initiation and Post-Graduates and 8th Meeting of Embrapa Grape and Wine, Bento Gonçalves, 2014.

- MALABARBA, J.; BUFFON, V.; WAIRICH, A.; MARIATH, J.; GAETA, M.; DORNELAS, M. C.; REVERS, LF Gene expression analysis of *VvAGL11* in grapevine reproductive tissues by in situ hybridization. In: IV Brazilian Symposium on Molecular Genetics of Plants, Bento Gonçalves, 2013.

- WAIRICH, A.; MALABARBA, J.; BUFFON, V.; REVERS, LF Evaluation of resistance to *Plasmopara viticola* in the self-fertilized segregating population from Villard Blanc (Vitis spp.). In: IV Brazilian Symposium on Molecular Genetics of Plants, Bento Gonçalves, 2013.

- MALABARBA, J.; BUFFON, V.; WAIRICH, A.; MARIATH., J.; GAETA, M.; DORNELAS, M. C.; PASQUALI, G.; REVERS, LF. Spatio-temporal expression pattern of the gene *VvAGL11* in grapevine reproductive tissues by in situ hybridization. In: 11th Meeting of Scientific Initiation and Post-Graduates 7th Meeting of Embrapa Grape and Wine, Bento Gonçalves. 2013.

- WAIRICH, A.; MALABARBA, J.; BUFFON, V.; REVERS, L. F.; Welter LJ. Resistance evaluation for *Plasmopara viticola* in a population segregating for resistance self-fertilized 'Villard Blanc' (*Vitis* spp.). In: 11th Meeting of Scientific Initiation and Post-Graduates 5th Meeting of Embrapa Grape and Wine, 2012, Bento Gonçalves, 2013.

- MALABARBA, J.; WAIRICH, A..; BUFFON, V.; CZERMAINSKI, A..; REVERS, LF. SSR markers Assessment with the potential for assisted selection of seedless and mildew resistant grapevines. In: XXII Brazilian Congress of Fruits, Bento Gonçalves, 2012.

- WAIRICH, A.; MALABARBA, J.; BUFFON, V.; CZERMAINSKI, A.; REVERS, LF. Transcriptional profile analysis of candidate genes associated with resistance to downy mildew in grapes, present in Rpv3 locus. In: XXII Brazilian Congress of Fruits, Bento Gonçalves, 2012.

- MALABARBA, J.; BUFFON, V.; CZERMAINSKI, A.; REVERS, LF. Transcriptional profile characterization of a gene associated with estenoespermocarpia in grapevine (*Vitis vinifera* L.). III Biodiversity Symposium, UFSM, Santa Maria, RS. 2011

HISTÓRICO CURSO BIOLOGIA CELULAR E MOLECULAR - Doutorado – início em 01/08/2014					
Período Letivo	Código	Disciplina	Créditos	Conceito	Situação
2017/01	BCM10201	Redação Científica - Manuscrito	1	A	Aprovado
2016/02	BCM11001	Orientação de Estagiários de Iniciação Científica	1	A	Aprovado
2016/02	BCM10301	Seminário do Centro de Biotecnologia	1	A	Aprovado
2016/01	BCM10101	Atividade Didática	2	A	Aprovado
2016/01	BCM079	Curso de Bioestatística	3	A	Aprovado
2016/01	BCM10301	Seminário do Centro de Biotecnologia	1	A	Aprovado
2016/01	BCM10401	Seminários de Dados Experimentais - Apresentação	1	A	Aprovado
2016/01	BCM10401	Seminários de Dados Experimentais - Frequência	1	A	Aprovado
2016/01	BCM10501	Tópicos Avançados	2	A	Aprovado
2015/02	BCM13040	Biossegurança e Boas Práticas de Laboratório	2	A	Aprovado
2015/01		Plant Development and hormones: a crash course	1	A	Aprov. Ext.
2015/01	BCM10301	Seminário do Centro de Biotecnologia	2	A	Aprovado
2015/01	BCM10501	Tópicos Avançados	2	A	Aprovado
2014/02	BCM13001	Purificação de Proteínas	4	A	Aprovado
2014/01	BCM15000	Aproveitamento de Créditos do Mestrado	12	A	Aprovado
2015/02		Exame de Qualificação	-	-	Aprovado
2013/01		Proficiência em Inglês	-	-	Aprovado
2015/01		Proficiência em Francês	-	-	Aprovado
		Créditos Cursados com Aprovação neste curso:		35	
		Créditos por Aproveitamento Externo:		1	
		Total:		36	