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Amanda Malvessi Cattani

Compreendendo a dormência de gemas de macieira (*Malus × domestica* Borkh.) por meio da integração de estímulos externos, sinalização hormonal e mecanismos moleculares

Tese de Doutorado

Porto Alegre

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Orientadores: Dr. Luís Fernando Revers e Dr. Giancarlo Pasquali

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BANCA EXAMINADORA

Dr. Antonio Vargas de Oliveira Figueira
Centro de Energia Nuclear na Agricultura
Universidade de São Paulo
São Paulo, SP

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Universidade Federal do Rio Grande do Sul
Porto Alegre, RS

Dra. Janette Palma Fett
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Universidade Federal do Rio Grande do Sul
Porto Alegre, RS

Dr. Arnaldo Zaha – Relator/Membro Suplente
Programa de Pós-Graduação em Biologia Celular e Molecular
Universidade Federal do Rio Grande do Sul
Porto Alegre, RS

INSTITUIÇÕES E FONTES FINANCIADORAS

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

%	Porcentagem
°C	Grau Celsius
ABA	Ácido abscísico
ABI1	Proteína insensível à ácido abscísico 1 (do inglês, *ABSCISIC ACID INSENSITIVE 1 protein*)
ABPM	Associação Brasileira de Produtores de Maçã
ABRE	Elemento responsivo à ABA (do inglês, *ABA-responsive element*)
AGL24	Gene codificador do fator de transcrição AGAMOUS-LIKE 24
AP2	Domínio de ligação ao DNA da família APETALA2
ARR10	Proteína reguladora de resposta do tipo-B de Arabidopsis 10 (do inglês, *ARABIDOPSIS RESPONSE REGULATOR 10 protein*)
BAP	6-benzilaminopurina
BRR	Gene codificador do fator de trancrição regulador de respota do tipo-B (do inglês, *type-B response regulator gene*)
CALS1	Gene codificador da enzima de síntese de calose 1 (do inglês, *callose synthase 1 gene*)
CAMTA3	Gene codificador do ativador transcricional de ligação à calmodulina 3 (do inglês, *calmodulin binding transcription activator* 3 *gene*)
CaMV	Vírus-do-mosaico-da-couve-flor (do inglês, *cauliflower mosaic virus*)
CBF	Gene codificador do fator de transcrição C-REPEAT BINDING FACTOR
cDNA	DNA complementar (do inglês, *complementary DNA*)
CDS	Região codificante (do inglês, *coding region*)
CEASAS	Centrais de abastecimento de hortifrutigranjeiros
CK	Citocinina
CKX1	Gene codificador da enzima citocinina oxidase/desigrogenase 1 (do inglês, *ck oxidase/dehydrogenase 1 gene*)
Col-0	*Arabidopsis thaliana* acesso Columbia-0
COR	Genes de resposta ao frio (do inglês, *cold responsive genes*)

CRLK	Gene codificador de proteína quinase relacionada ao receptor regulado por cálcio/calmodulina (do inglês, *calcium/calmodulin-regulated receptor-like kinase gene*)
DAM	Gene codificador do fator de transcrição MADS-box associado à dormência (do inglês, *dormancy-associated MADS-box gene*)
DHZ	Di-hidrozeatina
DNA	Ácido desoxirribonucleico (do inglês, *deoxyribonucleic acid*)
DNase	Desoxirribonuclease
EBB	Gene codificador do fator de transcrição EARLY BUD-BREAK
ETR	Receptor de etileno (do inglês, *ethylene receptor*)
evg	Em constante cresimento (do inglês, *evergrowing)*
FT	Gene codificador do florígeno FLOWERING LOCUS T
GA	Giberelina
gDNA	DNA genômico
GID1	Receptor de giberelina 1 (do inglês, *GA insensitive dwarf 1 receptor*)
gusA	Gene codificador da enzima β-glicuronidase
HF	Horas de frio
HK	Proteína Histidina quinase (do inglês, *HISTIDINA KINASE protein*)
HOS1	Gene codificante para a enzima E3 ligase de alta expressão de genes osmoticamente responsivos 1 (do inglês, *high expression of osmotically responsive 1 gene*)
HP	Enzima Histidina fosfotransferase (do inglês, *Histidina phosphotransferase enzyme*)
ICE	Gene indutor da expressão de *CBF* (do inglês, *inducer of CBF expression gene*)
IPPCC	Painel Intergovernamental de Mudanças Climáticas (do inglês, *Intergovernmental Panel on Climate Change*)
IPT	Enzima fosfato-isopenteniltransferase adenosina (do inglês, *adenosine phosphate-isopentenyltransferase enzyme*)
MEKK	Proteína MKK quinase (do inglês, *MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE*)

MKK	Proteína MPK quinase (do inglês, *MITOGEN-ACTIVATED PROTEIN KINASE KINASE*)
MPK	Proteína quinase ativada por mitógeno (do inglês, *MITOGEN-ACTIVATED PROTEIN KINASE*)
NCED	Gene codificador da enzima dioxigenase de 9-*cis*-epoxycarotenoid (do inglês, *9-cis-epoxycarotenoid dioxygenase gene*)
NPTII	Gene codificador da enzima neomicina fosfotransferase II (do inglês, *neomycin phosphotransferase II gene)*
OST1	Proteína quinase OPEN STOMATA 1
pb	Par(es) de bases
PCR	Reação em cadeia da DNA polimerase (do inglês, *polymerase chain reaction*)
PKKPAGR	Domínio PKKP/RAGRxKFxETRHP presente em proteínas do tipo CBF
PKL	Remodelador de cromatina PICKLE
PP2C	Proteína fosfatase 2C (do inglês, *PROTEIN PHOSPHATE 2C*)
PYL	Proteína realacionada à pirabactina (do inglês, *PYRABACTIN-like protein*)
PYR	Proteína de resistência à pirabactina (do inglês, *PYRABACTIN RESISTANCE protein*)
QTL	*locus* de característica quantitativa (do inglês, *quantitatve trait locus*)
RCAR	Proteínas denominadas de componentes de receptores de ABA (do inglês, *COMPONENTS OF ABA RECEPTORS proteins*)
REC	Domínio receptor dos genes Reguladores de Reposta do tipo-A e tipo-B
RNA	Ácido ribonucleico (do inglês, *ribonucleic acid*)
ROS	Espécies reativas de oxigênio (do inglês, *reactive oxygen species*)
RT-qPCR	PCR quantitativa precedida de transcrição reversa (do inglês, *reverse transcription-quantitative PCR*)
SNP	Polimorfismo de nucleotídeo único (do inglês, *single nucleotide polymorphism*)
SnRK2	Proteína quinase 2s relacionada à SNF1 (do inglês, *SNF1-related protein kinase 2s*)
SVL	Gene realcionado à *SVP* (do inglês, *SVP-like*)
SVP	Gene codificador do fator de transcrição SHORT VEGETATIVE PHASE
T1	Primeira geração transgênica (do inglês, *first filial generation of transgenic*)

T-DNA	DNA de transferência (do inglês, *transfer DNA*)
TDZ	Tidiazuron
WT	Tipo-selvagem (do inglês, *wild-type*)
WUS	Gene codificador do fator de transcrição WUSCHEL
Z	Zeatina
ZEP	Gene codificador da zeaxantina epoxidase (do inglês, *zeaxanthin epoxidase*)

RESUMO

Plantas perenes cultivadas em climas temperados adquiriram ao longo do processo evolutivo um mecanismo adaptativo chamado de dormência o qual permite a sua sobrevivência em condições adversas de crescimento. Em macieira (*Malus* × *domestica* Borkh), a indução e a liberação da dormência são finamente reguladas e abrangem diversos aspectos que vão desde a percepção da célula ao frio até as respostas moleculares que desencadeiam os eventos fisiológicos. Sabendo-se da complexa rede regulatória por trás desse controle, o presente estudo visou contribuir com o seu entendimento por meio da caracterização dos genes *MdoCBFs* e *MdoICE1*, envolvidos nas vias de aclimatação ao frio, bem como dos genes *MdoBRRs*, fatores de transcrição responsáveis por modular a resposta dependente de citocinina (CK), um importante hormônio vegetal promotor do crescimento. O estudo contou com a avaliação de um conjunto de dados contendo perfis transcricionais, quantificações hormonais e ensaios de transativação *in vivo* usando protoplastos de Arabidopsis. Os dados revelaram que, ao longo do ciclo da dormência, os genes *MdoCBF2* e *MdoCBF4* são expressos somente durante a fase inicial da endodormência enquanto que os genes *MdoBRR1* e *MdoBRR8* tem sua expressão acentuada em uma fase posterior, que engloba a transição da endo- para a ecodormência. Além disso, o aumento de transcritos dos genes *MdoBRRs* é concomitante com a regulação negativa de *MdoDAM1*, importante gene envolvido na manutenção da dormência. Nesta mesma fase, há um incremento nos níveis de CK, seguido de um aumento na quantidade de giberelina (GA) e redução drástica da expressão dos genes *MdoCBFs*. O antagonismo entre os perfis trancricionais de *MdoBRRs e MdoDAM1* também foi revelado em gemas ecodormentes tratadas com CK, onde houve aumento nos níveis de transcritos de *MdoBRR9* e *MdoBRR10* e diminuição da expressão de *MdoDAM1*. De maneira similar, ensaios de transativação *in vivo* permitiram mostrar que ambos os estímulos de CK e as expressões transientes de *MdoBRR1*, *MdoBRR8* ou *MdoBRR10* levaram à modulação negativa de *MdoDAM1*. Por meio da mesma técnica, foi possível observar que os genes *MdoCBF4* e *MdoCBF5* são modulados de forma dependente de frio mas independente do ativador transcricional codificado por *MdoICE1*. Por fim, os dados foram integrados em um modelo hipotético que indica uma via de regulação bem orquestrada mediada tanto por *MdoCBFs* e GA quanto por *MdoBRRs* e CK que, respectivamente, levam à ativação e à repressão de *MdoDAM1*, um regulador-chave da dormência de gemas.

ABSTRACT

Throughout the evolutionary process, temperate perennial plants developed an adaptive mechanism called dormancy that allows their survival in adverse growth conditions. In apple trees (*Malus* × *domestica* Borkh), the induction and release of dormancy is finely regulated and covers several aspects, ranging from cold perception by cells to molecular responses that trigger physiological events. Knowing that dormancy control is dependent of complex network, the present study aimed to contribute to its understanding by the characterization of the *MdoCBFs* and *MdoICE1* genes involved in cold acclimation pathways, as well as the *MdoBRRs* genes encoding transcription factors responsible for modulating cytokinin (CK)-dependent genes. The study inferences were based on transcriptional profiles, hormonal quantifications and *in vivo* transactivation assays using Arabidopsis protoplasts. The data revealed that, throughout the dormancy cycle, the *MdoCBF2* and *MdoCBF4* genes were expressed only during the initial phase of endodormancy while the *MdoBRR1* and *MdoBRR8* genes have their transcription enhanced in a later stage, along the transition from endo- to ecodormancy. Moreover, the increase in *MdoBRR* transcript levels was concomitant with the negative regulation of *MdoDAM1*, an important gene involved in dormancy maintenance. At this same stage, high levels of CK, followed by increased amounts of gibberellins (GA) and drastic decrease in *MdoCBFs* expression were also observed. The antagonism between the transcriptional profiles of *MdoBRRs* and *MdoDAM1* was also revealed in ecodormant buds treated with CK, which presented higher transcript levels of *MdoBRR9* and *MdoBRR10* genes along with a decrease in *MdoDAM1* expression. Similarly, *in vivo* transactivation assays showed that both CK stimulus and transient expression of *MdoBRR1*, *MdoBRR8* or *MdoBRR10* led to the negative modulation of *MdoDAM1*. With the same technique, it was possible to observe that *MdoCBF4* and *MdoCBF5* genes were modulated in a cold-dependent manner but independently of the transcriptional activator encoded by *MdoICE1*. Finally, the present data were integrated into a hypothetical model that indicates a well-orchestrated pathway mediated by *MdoCBFs* and GA as well as by *MdoBRRs* and CK, which respectively lead to activation and repression of *MdoDAM1*, a key regulator of bud dormancy.

1 INTRODUÇÃO

1.1 O MECANISMO ADAPTATIVO CHAMADO DORMÊNCIA

A macieira (*Malus × domestica* Borkh.) é uma frutífera de clima temperado que ao longo do processo evolutivo desenvolveu mecanismos adaptativos que possibilitaram sua sobrevivência em condições extremas de baixa temperatura e luz. Esse mecanismo é definido como dormência e se caracteriza pela baixa atividade metabólica das plantas e o cessar do crescimento vegetativo até que condições favoráveis de desenvolvimento sejam restabelecidas (ROHDE & BHALERAO, 2007). Dentre os principais aspectos fisiológicos envolvidos nesse processo, destacam-se a formação de gemas, a inibição do crescimento apical, a aquisição de tolerância ao frio/dessecação e a senescência foliar (COOKE *et al.,* 2012). As gemas são estruturas vegetativas resultantes de modificações dos primórdios foliares em escamas rígidas que protegem os tecidos vegetais do congelamento e da dessecação. Neste contexto, elas são definidas como órgãos meristemáticos primários na formação de brotos em plantas dicotiledôneas e são responsáveis por garantir um novo ciclo reprodutivo em espécies perenes (HORVATH, 2010).

Devido à sua importância, a dormência de gemas foi dividida em três fases por LANG *et al.* (1987), conhecidas como para-, endo- e ecodormência. De forma simplificada, a paradormência, também chamada de dominância apical, é induzida por sinais provenientes de outras partes da planta, levando a inibição do crescimento. A endodormência é determinada por sinais internos que não permitem a brotação das gemas mesmo em condições favoráveis de crescimento. Por fim, a ecodormência é mediada por estímulos externos como frio e estresse hídrico que, quando superados, permitem que o crescimento vegetativo seja reassumido (LANG *et al.,* 1987).

Os mecanismos que envolvem o processo de regulação da dormência são complexos e muito bem orquestrados, envolvendo diversos aspectos como estímulos ambientais, vias moleculares, sinalização hormonal e modificações epigenéticas. O conjunto organizado de todos esses fatores leva tanto à indução da dormência quanto à retomada do crescimento.

1.1.1 Sinais Ambientais Envolvidos na Regulação da Dormência

Em geral, os principais sinais ambientais que estimulam o estabelecimento da dormência são dias consecutivos com fotoperíodo curto e baixas temperaturas, embora seu grau de relevância dependa de cada espécie (COOKE *et al.,* 2012). Em espécies de *Betula* (bétula), *Populus* (álamo) e *Salix* (salgueiro), o fotoperíodo é o principal estímulo (HOWE *et al.*, 1996; JUNTTILA, 1976), enquanto que para espécies de *Malus* (maçã), *Pyrus* (pera) e *Sorbus* (sorveira), temperaturas baixas são mais determinantes (HEIDE & PRESTRUD, 2005; HEIDE, 2011). Em macieira, a exposição às baixas temperaturas durante o outono induz os eventos fisiológicos da entrada em dormência, enquanto que a posterior contínua exposição ao frio durante o inverno leva à sua superação e consequente floração na primavera (HEIDE & PRESTRUD, 2005).

Com a percepção dos primeiros sinais de frio, há o desencadeamento de eventos bioquímicos e fisiológicos no interior das gemas que resulta no estabelecimento da fase de endodormência. Nesta fase, o crescimento vegetativo é inibido até que haja a completa satisfação do requerimento de frio necessário para que ocorra a transição para o estádio de ecodormência. Atingida a ecodormência, as gemas tornam-se aptas a brotar após um adequado período de exposição à luz e a temperaturas mais elevadas (~25 °C; LANG *et al.*, 1987).

O requerimento de frio, ou seja, o somatório das horas de exposição a baixas temperaturas necessário para o início da brotação, é genótipo-dependente e varia conforme a cultivar de macieira. As variedades que necessitam de um somatório de horas de frio (HF) inferior a 450 HF como 'Eva' e 'Castel Gala' são classificadas como de baixo requerimento. As cultivares como 'Monalisa' e 'Fred Hough', que necessitam de um acúmulo de 450 a 700 HF, são agrupadas como de médio requerimento. As demais variedades que brotam com mais de 700 HF, como é o caso de 'Gala' e 'Fuji', amplamente conhecidas e comercializadas, são consideradas de alto requerimento de frio (CAETANO *et al.*, 2013).

A dificuldade em se estabelecer uma temperatura padrão para a estimativa do acúmulo de frio levou à criação de vários modelos bioclimáticos que permitiram propor uma análise qualitativa e quantitativa do efeito da temperatura sobre a fenologia das plantas. Os modelos de 'Horas de frio ponderadas' (EREZ & LAVEE, 1971), 'Utah' (RICHARDSON

et al., 1974) e 'Carolina do Norte' (SHALTOUT *et al.,* 1983) designam diferentes pesos às ações do frio dependendo de cada temperatura. O modelo 'Carolina do Norte' é uma adaptação do modelo 'Utah' para a cultura de macieira e considera a faixa funcional entre 1,6 °C e 16,4 °C, com pico máximo em 7,2 °C. Temperaturas inferiores a 1,6 °C e superiores a 16,5 °C não são consideradas efetivas para a superação da dormência (SHALTOUTUNRATH & AKADEMIYA, 1983). Considerando esse modelo, o método mais utilizado para mensurar a quantidade acumulada de frio é pela soma diária das horas com temperaturas iguais ou inferiores a 7,2 ºC.

1.1.2 O Balanço Hormonal ao longo do Ciclo da Dormência de Gemas

As plantas, como organismos sésseis e complexos, dependem de uma cadeia coordenada de eventos que determinam todas as fases de suas vidas. O balanço hormonal é fundamental em todos os estádios, inclusive ao longo da dormência. Hormônios vegetais, ou fitormônios, são pequenas moléculas sinalizadoras que ocorrem naturalmente e afetam o metabolismo fisiológico das plantas (DAVIES, 2010). Além de regular processos de desenvolvimento e crescimento ao longo do ciclo de vida vegetal, os fitormônios podem desencadear respostas adaptativas induzidas por estímulos externos como mudanças ambientais e estresses bióticos ou abióticos. Baseado nesse contexto, a seguir serão explorados em maiores detalhes os principais hormônios envolvidos nas diferentes etapas do ciclo da dormência de gemas.

1.1.2.1 Ácido abscísico (ABA)

O ácido abscísico (ABA) é um importante hormônio vegetal associado à inibição do crescimento e que desempenha papel significativo na regulação da dormência de gemas. Em várias espécies de árvores, os níveis endógenos de ABA aumentam no estabelecimento da dormência e diminuem durante a sua liberação (CHMIELEWSKI *et al.*, 2018; TUAN *et al.*, 2017; WANG *et al.*, 2016). Baseado em diversos estudos, um modelo proposto de regulação mediada por ABA para o estabelecimento da endodormência é apresentado na recente

revisão realizada por LIU & SHERIF (2019) e simplificado na Figura 1. Neste modelo, os autores propõem que, ao final do ciclo vegetativo, as plantas iniciam a percepção das primeiras temperaturas baixas juntamente com a redução do fotoperíodo e ativam as vias de síntese e sinalização de ABA. Essa ativação se dá por meio de múltiplas etapas de fosforilação que acabam por regular positivamente enzimas como ZEAXANTHIN EPOXIDASE (ZEP) e 9-CIS-EPOXYCAROTENOID DIOXYGENASE (NCED), essenciais na síntese de ABA. Além delas, os fatores envolvidos no complexo receptor de ABA, que contém as proteínas PYRABACTIN RESISTANCE (PYR)/PYRABACTIN-*like* (PYL)/COMPONENTS OF ABA RECEPTORS (RCAR), também são ativados. Após ABA ligar o complexo PYR-PYL-RCAR, as proteínas PROTEIN PHOSPHATE 2Cs (PP2C) associam-se a ele e são desativadas. Assim, há a fosforilação de SNF1-RELATED PROTEIN KINASE 2s (SnRK2), anteriormente suprimida pela PP2C por meio da reação oposta. Com a SnRK2 fosforilada, há o estímulo da expressão de genes contendo, em seus promotores, os elementos *cis* denominados ABA-RESPONSIVE ELEMENT (ABRE), que em geral são responsáveis pela modulação das respostas dependentes de ABA (LIU & SHERIF, 2019; Figura 1).
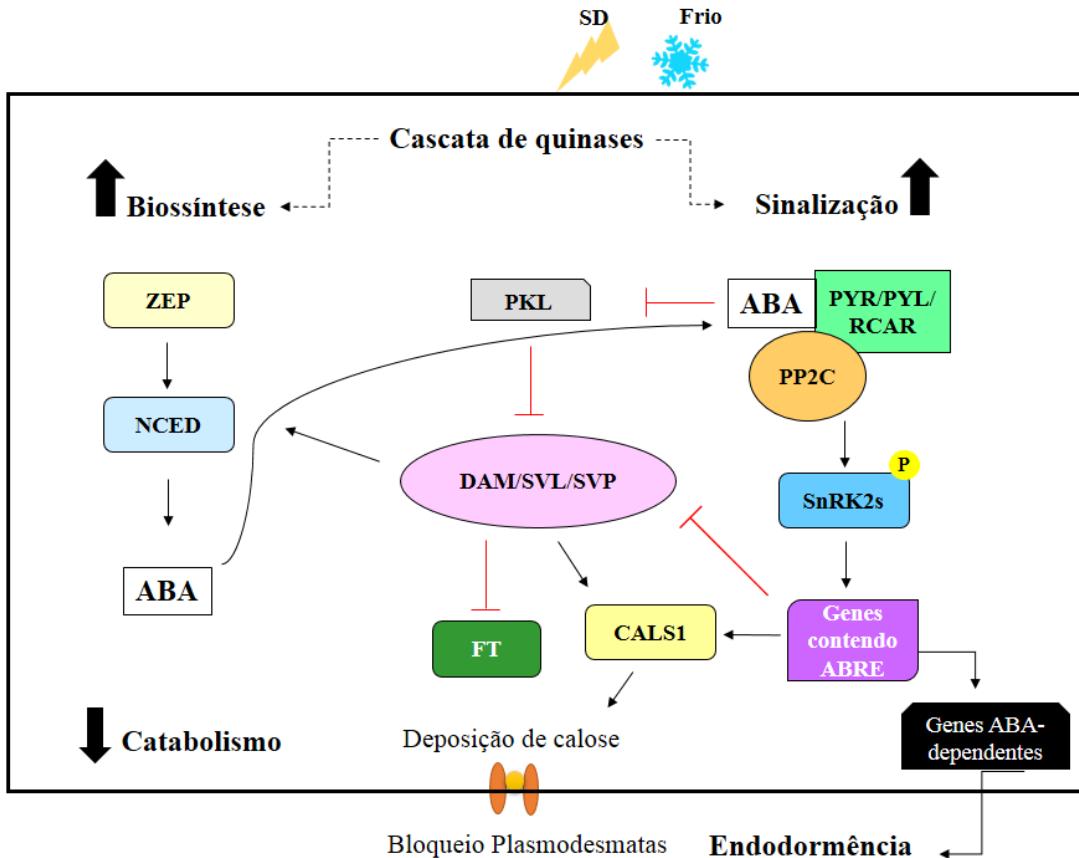
Figura 1. Regulação da endodormência via ABA.

Um modelo hipotético baseado em dados disponíveis na literatura mostra que o estímulo mediado por frio e fotoperíodo curto (SD) ativa cascatas de quinases que por meios ainda a serem entendidos leva a indução das vias de biossíntese e sinalização de ABA, ao mesmo passo que a dimuição das suas rotas de catabolismo. O aumento da síntese de ABA mediada pelas enzimas ZEP e NCED induz a via de sinalização mediada pelo complexo PYR/PYL/RCAR e PP2C que leva a fosforilação da proteína quinase SnRK2s e consequente ativação de genes contendo o *cis* elemento responsivo à ABA chamado ABRE. Dentre os genes contendo ABRE, destaca-se o *CALS1*, responsável pela síntese de calose que se deposita nos plasmodesmatas e interrompe a comunicação intercelular, fator chave no estabelecimento da endormência. Os altos níveis de ABA também levam a ativação de genes *DAM*/*SVL*/*SVP* por meio da repessão do remodelador de cromatina PKL. Assim, o florígeno FT é reprimido, auxiliando no cessar do crescimento. Em uma via de *feedback* negativo, os genes contendo os *cis* elementos ABRE podem ainda reprimir *DAM*. Adaptada de LIU & SHERIF (2019).

Enquanto as rotas de síntese e sinalização por ABA são ativadas durante a endormência, as vias de catabolismo são reprimidas. Com isso, a célula assegura altos níveis de ABA que podem, assim, modular os importantes fatores envolvidos na regulação da dormência como os genes *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*), *SHORT VEGETATIVE PHASE* (*SVP*) e *SPV-like* (*SVL*). A presença de ABA inativa o remodelador de cromatina PICKLE (PKL) que mantém *DAM* desligado, levando à ativação do gene. Além disso, os genes *DAM* podem promover a síntese de ABA através da ativação do gene *NCED* (TUAN *et al.,* 2017). De maneira a manter a homeostase da célula, os genes contendo os *cis* elementos ABRE são ainda capazes de reprimir *DAM* (TUAN *et al.,* 2017; Figura 1).

ABA pode ainda regular o estabelecimento da dormência por meio do bloqueio da comunicação intercelular. Nas plantas, o transporte célula-célula depende da conectividade de canais especializados entre células adjacentes chamados plasmodesmatas. O fluxo simplástico através dos plasmodesmas é controlado pela deposição e pela degradação de calose (WU *et al.*, 2018). A síntese de calose mediada pela enzima CALLOSE SYNTHASE 1 (CALS1) é regulada positivamente por ABA, o que leva ao seu acúmulo nos canais e bloqueio da passagem de água, nutrientes, moléculas sinalizadoras e fatores de crescimento como o FLOWERING LOCUS T (FT), contribuindo para o estabelecimento da endodormência (TYLEWICZ *et al.,* 2018).

1.1.2.2 Giberelinas (GA)

As giberelinas (GAs) são um grande grupo de compostos diterpenoides tetracíclicos que exercem efeitos significativos em um amplo espectro de processos biológicos em plantas. De todas as GAs numericamente codificadas, apenas em GA1, GA3, GA4 e GA7 encontrou-se bioatividade. Em relação ao seu papel na dormência, um efeito antagônico ao ABA é observado. Em muitas espécies lenhosas, os níveis de GA estão diminuídos na indução da dormência e aumentados durante a sua liberação (RINNE *et al.*, 2011; ZHENG *et al.*, 2018; ZHUANG *et al.*, 2015).

Diferentemente de ABA, especula-se que a conectividade do canal de plasmodesmata pode ser restaurada por GA4, o qual pode induzir a expressão da β-1,3-glucanase de forma a promover a hidrólise da calose e a liberação do transporte simplástico

entre células adjacentes (RINNE *et al.*, 2011). Outro modelo recente propõe que a indução da enzima de síntese de calose CALS1 por ABA e SVL é capaz de reprimir o metabolismo de GA e garantir o estabelecimento da dormência (SINGH *et al.*, 2019).

Além do envolvimento na restauração da comunicação celular, GA pode ainda aumentar a produção de ROS, importantes na quebra de dormência (BEAUVIEUXWENDEN & DIRLEWANGER, 2018; ZHUANG *et al.*, 2013), e ativar vias metabólicas associadas ao reestabelecimento do crescimento. Em *Prunus mume*, por exemplo, o tratamento com GA4 levou à ativação de muitas vias do metabolismo energético, incluindo aquelas associadas ao metabolismo de açúcar (ZHUANG *et al.*, 2015). Os açúcares solúveis são considerados importantes fontes de energia na sustentação do crescimento. Além disso, a sacarose é um elemento potencial de sinalização que pode indiretamente regular positivamente a expressão de genes relacionados ao ciclo celular (RUAN *et al.*, 2010).

A avaliação do balanço hormonal ao longo da progressão da dormência revela um interessante *crosstalk* entre ABA e GA, onde seus níveis podem ser inversamente correlacionados (WEN *et al.*, 2016). As proteínas DELLA, importantes reguladores negativos da via de transdução de sinal de GA, estão também envolvidas na mediação desse *crosstalk*. Estudos utilizando plantas de Arabidopsis mutantes para o do receptor de GA *insensitive dwarf 1* (*gid1*) permitiram demonstrar um acúmulo de proteínas DELLAs e consequente aumento da expressão de XERICO, uma proteína conhecida por induzir a síntese de ABA (ARIIZUMI *et al.*, 2013; ECKARDT, 2007). Do mesmo modo, a aplicação exógena de ABA leva à repressão da expressão do mesmo receptor de GA GID1 e induz a expressão dos genes DELLA, inativando as respostas dependentes de GA que levam ao favorecimento do crescimento. No entanto, no que parece ser um mecanismo de *feedback* negativo, longas horas de tratamento de ABA reprimem a expressão de *DELLA* (YUE *et al.*, 2018). Além disso, o acúmulo de ABA em reposta a estresses estabiliza as proteínas DELLA indicando que a elevação dos níveis de ABA no início da dormência pode levar à inibição das respostas mediadas por GA e o estabelecimentos dos eventos fisiológicos associados à dormência (ARIIZUMI *et al.*, 2013; LIU & SHERIF, 2019).

1.1.2.3 Citocininas (CK)

As citocininas (CKs) naturalmente encontradas nas células vegetais são moléculas derivadas da adenina, possuindo cadeias laterais isoprenóides ou aromáticas ancoradas à posição N^6 do anel de adenina. As CKs isoprenóides são muito mais comuns em plantas e sua abundância relativa é muito superior à das CKs aromáticas (SAKAKIBARA, 2006). As adeninas N^6-(Δ^2-isopentenyl) (iP) e suas formas hidroxiladas chamadas de zeatina (Z) e di-hidrozeatina (DHZ) são representantes típicos de CKs isoprenóides. A zeatina ocorre em dois isômeros, *cis* (*c*) e *trans* (*t*), referindo-se à posição do grupo hidroxila na molécula. A maior parte da atividade fisiológica é atribuída à *t*-zeatina, enquanto que, embora presente nas células, a *c*-zeatina é considerada uma forma inativa ou fracamente ativa de CK (GAJDOŠOVÁ *et al.,* 2011).

As CKs estão envolvidas em diversos aspectos do crescimento e do desenvolvimento de plantas, sendo associadas à promoção da divisão e diferenciação celular, bem como no auxílio da indução da atividade meristemática (ARGUESO *et al.*, 2012; MOK & MOK, 2001). Quando relacionadas a processos regulatórios envolvidos na dormência, seu papel é melhor compreendido durante a paradormência ou dominância apical, embora estudos já venham elucidando sua importância durante a liberação da endodormência (revisado em LIU & SHERIF, 2019)

Dentre os vários modelos propostos para os mecanismos envolvidos no controle da paradormência, a interação entre auxinas e CK é bem recorrente. De maneira geral, quando todas as gemas do ramo estão intactas, as auxinas derivadas das gemas apicais reprimem as enzimas ADENOSINE PHOSPHATE-ISOPENTENYLTRANSFERASES (IPTs) que catalisam a primeira etapa da síntese de CK. Após a retirada da gema apical, o fluxo de auxina cessa, os genes *IPTs* são expressos e a síntese de CK é reestabelecida, levando à brotação das gemas laterais (SHIMIZU-SATO & MORI, 2001).

De forma similar ao que ocorre na paradormência, plantas expressando ectopicamente o gene *IPT* mostraram maiores níveis de CK e um período de liberação da dormência antecipado. Além disso, plantas superexpressando o gene de catabolismo *CK OXIDASE/DEHYDROGENASE 1* (*CKX1*) apresentaram níveis reduzidos do hormônio, inibição do metabolismo e proliferação celular, ausência da resposta à GA3 e um período de

dormência ampliado (HARTMANN *et al.,* 2011). Estudos ainda permitiram mostrar um importante *crosstalk* entre ABA e CK, onde receptores envolvidos na via de sinalização de CK atuam como reguladores negativos de ABA (TRAN *et al.*, 2007; SHINOZAKI & YAMAGUCHI-SHINOZAKI, 2010).

Embora alguns dados envolvendo CK na liberação da dormência já tenham sido revelados, muitas lacunas ainda permanecem abertas. Atualmente, tem se proposto que CK possa atuar de um modo anterior às vias de resposta envolvendo ABA e GA por meio do estímulo da atividade meristemática, embora evidências mais concretas precisam ser adicionadas ao modelo proposto (LIU & SHERIF, 2019).

1.1.2.4 Outros hormônios

O etileno foi o primeiro hormônio associado à via de regulação da endodormência e induz uma cadeia fisiológica de eventos que culminam no cessar do crescimento por meio do seu efeito negativo nas vias de síntese e sinalização de GA (DUBOIS *et al.,* 2013). Curiosamente, o etileno pode ainda apresentar papel na liberação da dormência, uma vez que a utilização de um inibidor da sua sinalização levou ao aumento dos níveis de ABA (OPHIR *et al.,* 2009). No processo de liberação da dormência, hormônios como o ácido salicílico (WEN & LIANG, 1994) e auxinas podem ser ainda associados. Estas últimas auxiliam na degradação da calose e no restabelecimento do fluxo simplástico nas células, bem como na ativação de enzimas envolvidas na via de síntese de GA (ALONI & PETERSON, 1997; NORIEGA & PÉREZ, 2017).

1.1.3 A Complexa Rede Molecular Abrangendo a Dormência de Gemas

Os processos de aclimatação ao frio e estabelecimento da dormência sobrepõem-se em espécies lenhosas, sendo governados por mecanismos orquestrados que envolvem a modulação da expressão de diversos genes (CHINNUSAMYZHU & SUNKAR, 2010; ROHDE & BHALERAO, 2007; THOMASHOW *et al.*, 2001). Dentre eles, importantes famílias gênicas como C-*REPEAT BINDING FACTORS* (*CBF)*, *INDUCER OF CBF*

EXPRESSION (*ICE*), *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*) e *TYPE-B RESPONSE REGULATOR* (*BRR)* destacam-se. As vias de regulação desses importantes genes serão exploradas detalhadamente a seguir, bem como a contextualização do seu potencial papel na via de regulação do ciclo da dormência de gemas.

1.1.3.1 A rota de sinalização controlada por *CBF e ICE*

Uma das rotas de resposta ao frio mais bem estudadas em Arabidopsis refere-se aos fatores de transcrição denominados DEHYDRATION RESPONSIVE ELEMENT BINDING (DREB)/C-REPEAT BINDING FACTORS, doravante chamados de CBFs. Eles pertencem à família de fatores de transcrição APETALA2 (AP2) e ligam os *cis*-elementos DRE/CRT dos genes de resposta ao frio (*COR*), modulando sua expressão de maneira estresse-dependente (SAKUMA *et al.*, 2002; STOCKINGERGILMOUR & THOMASCHOW, 1997). Além disso, as proteínas CBFs tem outros dois domínios característicos, PKKP/RAGRxKFxETRHP (abreviado para PKKPAGR) e DSAWR, que parecem ser importantes na ligação ao DNA, mesmo que análises *in silico* tenham permitido reconhecer o domínio PKKPAGR como um sinal de localização nuclear (CANELLA *et al.,* 2010; WISNIEWSKI *et al.,* 2014). Em Arabidopsis, 6 genes *CBFs* foram descritos, sendo 3 deles (*CBF1-3*) regulados pelo relógio circadiano e induzidos rapidamente em reposta a baixas temperaturas (FOWLER; COOK & THOMASHOW, 2005). Os genes *AtCBF1* e *AtCBF3* são os primeiros a serem ativados e modulam o mesmo conjunto de genes *COR*. Em seguida, há a regulação positiva de *AtCBF2* que, por sua vez, regula negativamente a expressão de *AtCBF1* e *AtCBF3*, o que garante que a via de aclimatação ao frio dependente de CBF seja acionada somente de uma maneira estresse-dependente (NOVILLOMEDINA & SALINAS, 2007). Além disso, sabe-se que os três *AtCBFs* são ativados por três fatores de transcrição distintos. *AtCBF1* é modulado pelo INDUCER OF CBF EXPRESSION 2 (ICE2; FURSOVA *et al*., 2009) e, aparentemente, também pelo ativador transcricional CALMODULIN BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3; DOHERTY *et al*., 2009). O gene *AtCBF2* é ativado por CAMTA3 (DOHERTY; VAN BUSKIRK; MYERS; et al., 2009), enquanto que *AtCBF3* tem como regulador o gene *AtICE1* (CHINNUSAMY *et al.,* 2003).

A via de aclimatação dependente de CBF é acionada por estímulos mediados por baixas temperaturas que levam a um aumento nos níveis endógenos de cálcio presentes no citoplasma das células (KNIGHT, 1999; THOMASHOW, 1999). Um dos mais recentes modelos propostos para Arabidopsis, simplificado na Figura 2, infere que o influxo de cálcio ativa enzimas quinases como a CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASES 1 e 2 (CRLK1/2), as quais iniciam uma cascata de sinalização mediada por proteínas quinases ativadas por mitógenos que, tipicamente, atuam em série, englobando as proteínas MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE (MEKK), MITOGEN-ACTIVATED PROTEIN KINASE KINASE (MKK) e MITOGEN-ACTIVATED PROTEIN KINASE (MPK). As CRLK1 e CRLK2 iniciam a cascata MEKK1–MKK1/2–MPK4 que antagoniza a via MEKK-MKK4/5–MPK3/6, responsável por promover a inibição de ICE1, o ativador essencial da expressão de *CBF3*. Com o desligamento da rota de inativação de ICE1, o mesmo é acionado e, consequentemente, ativa a resposta mediada por CBF (YANG *et al.,* 2010; LI *et al*., 2017a; ZHAO *et al*., 2017; LIU *et al*., 2019; Figura 2).
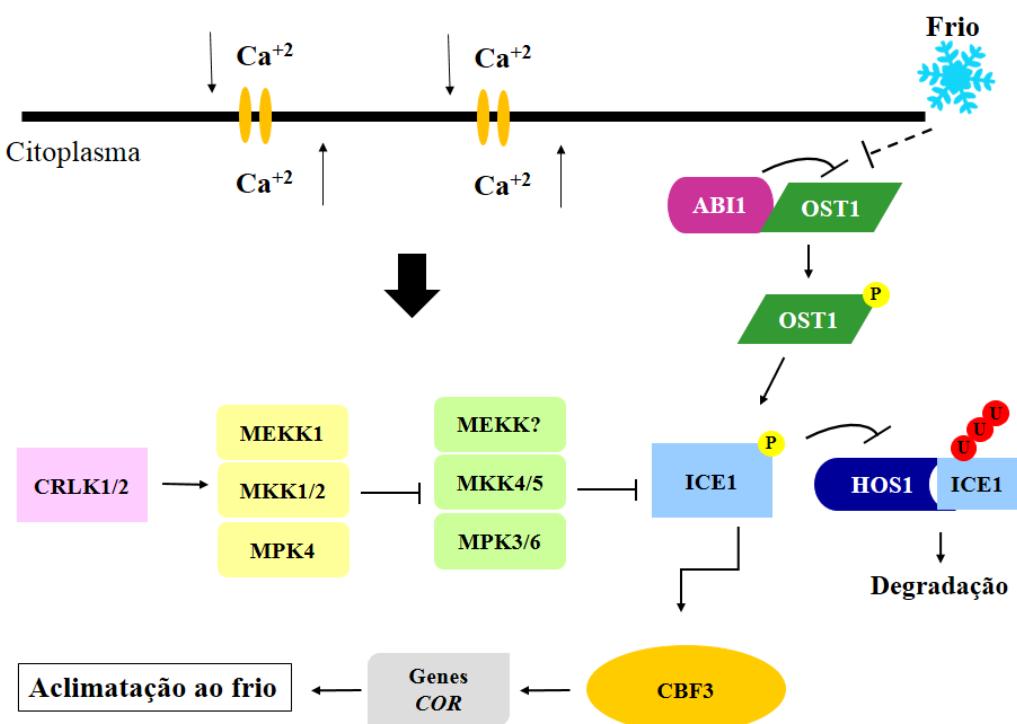
Figura 2. Modelo proposto para a regulação de *CBF3* via ICE1 em Arabidopsis.
O frio aciona o influxo de cálcio (Ca^{+2}) na célula, levando à modulação do receptor CRLK e indução de um conjunto de quinases ativadas por mitógenos (MEKK1-MKK1/2-MPK4). Esse grupo de quinases inativa o complexo inibidor de ICE1 composto por MEKK?-MKK4/5-MPK3/6. Em uma via paralela, as temperaturas baixas levam à disrupção do complexo ABI1/OST1, possibilitando que OST1 seja fosforilado. Em sequência, há a ativação de ICE1 por meio de fosforilação, inibindo assim a sua via de degradação por ubiquitinação mediada pela enzima HOS1. Por fim, ICE1 é capaz de ativar *CBF3* e consequentemente modular a expressão dos genes *COR*, levando a via de resposta à aclimatação ao frio mediada por CBF. P: Fosfato, U: Ubiquitina. Adaptado de LIU *et al*. (2019) e DING *et al*. (2015).

Em uma via paralela, o estresse induzido por frio, por meios ainda a serem entendidos, inativa o complexo OPEN STOMATA 1 (OST1)/ ABSCISIC ACID INSENSITIVE 1 (ABI1), que mantém OST1 defosforilado. Com a ativação de OST1, há a fosforilação de ICE1, inibindo, asssim, a sua degradação por meio de ubiquitinação pela enzima E3 ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1). Enquanto isso, a proteína OST1 também concorre com HOS1 pela ligação, liberando-a do complexo HOS1-ICE1. Assim, a dupla função do OST1 contribui para a estabilidade de ICE1 de forma a aumentar a expressão de *CBF3* e acionar o conjunto de genes envolvidos na aclimatação ao frio (Figura 2; DING *et al.,* 2015).

O genes *ICE* codificam fatores de transcrição do tipo hélice-volta-hélice pertencentes à família MYC, sendo capazes de ligar os respectivos *cis*-elementos encontrados no promotor dos genes *CBF* (CHINNUSAMY *et al.,* 2003). Basicamente, a ativação da função de *ICE* está mais associada a eventos de modificação pós-traducional do que a direta regulação da sua expressão gênica (FURSOVAPOGORELKO & TARASOV, 2009; NAKAMURA *et al.*, 2011). Mecanismos como fosforilação, SUMOilação e interações proteína-proteína vêm sendo relacionados com a sua ativação e/ou repressão (AGARWAL *et al.,* 2006; MIURA *et al.,* 2007; ZHAO *et al.,* 2017). Em Arabidopsis, dois genes codificantes de proteínas do tipo ICE foram descritos (CHINNUSAMY *et al.*, 2003;

FURSOVAPOGORELKO & TARASOV, 2009) e a completa caracterização do mutante de perda de função *ice1* revelou menores indíces de transcritos do gene *AtCBF3*, e consequente diminuição da expressão dos genes regulados por ele, o que levou as plantas a uma disfunção na tolerância ao frio e ao congelamento (CHINNUSAMY *et al.,* 2003).

Dentre as diversas proteínas codificadas pelos genes *COR* destacam-se aquelas envolvidas no metabolismo de carboidratos e lipídios, em modificações da parede celular, na detoxificação de espécies reativas de oxigênio (ROS), no metabolismo e na sinalização hormonal, além da ativação de proteínas anticongelantes (CHINNUSAMYZHU & SUNKAR, 2010; SHI *et al.*, 2017; ZHAO *et al.*, 2016). Além dos estímulos de frio, alterações no relógio circadiano, mudanças no fotoperíodo, sinais hormonais, estresse salino e hídrico também podem ser responsáveis pela modulação da atividade de *CBFs* (DONGFARRÉ & THOMASHOW, 2011; JIANG *et al.*, 2017; KANG *et al.*, 2011; MAGOME *et al.*, 2008).

Embora a superexpressão de *CBFs* leve a um aumento na tolerância a estresses abióticos, uma redução drástica no crescimento das plantas é observada, sugerindo que estes genes atuem também em uma ampla gama de aspectos do desenvolvimento vegetal (GILMOURARTUS & THOMASHOW, 1992; KURBIDAEVA & NOVOKRESHCHENOVA, 2011; THOMASHOW, 1999). Características como atrasos na floração e antecipação da senescência das folhas também são relatadas em plantas expressando ectopicamente genes *CBF*s (SHARABI-SCHWAGER *et al.,* 2010; SUO *et al.,* 2016). Da mesma maneira, a superexpressão de um *CBF* de pêssego (*PpCBF1*) em maçã resultou em plantas com maior tolerância ao congelamento, redução do crescimento e atraso na brotação (WISNIEWSKI *et al.*, 2011). Ainda nessas plantas, uma modulação em fatores-chave do estabelecimento da dormência, como os genes *DAM* e *EARLY BUD-BREAK* (*EBB*) foi observada, além de genes envolvidos na homeostase de hormônios, como giberelinas (GAs), citocininas (CKs) e auxinas (ARTLIP *et al.*, 2019; WISNIEWSKINORELLI & ARTLIP, 2015)**.** Vias de aclimatação independentes de CBF também foram descritas, embora a maioria dos mecanismos envolvidos seja ainda pouco compreendida (revisado em LIU *et al.*, 2019).

1.1.3.2 Genes *DAM*, reguladores-chave na endodormência

Os genes *DORMANCY-ASSOCIATED MADS-BOX* (*DAMs*) foram descritos inicialmente em um mutante espontâneo de pêssego, chamado de "*evergrowing*" (*evg*), e caracterizado pela sua incapacidade em adentrar o estado de dormência (RODRIGUEZ *et al.*, 1994). A análise do mutante *evg* resultou na observação de diferenças em um lócus contendo uma repetição em *tandem* de seis genes do tipo MIKC^c MADS-box. Dentre os seis genes associados à perda do fenótipo, quatro deles (*PpeDAM1-4)* foram fisicamente deletados enquanto que dois (*PpeDAM5-6)* apresentaram quantidades baixas de transcritos (BIELENBERG *et al.,* 2008).

Desde a descoberta em pêssego, os genes *DAM* vêm sendo extensivamente carcaterizados em outras espécies (revisado em FALAVIGNA *et al*., 2019). Na planta modelo Arabidopsis, eles estão filogeneticamente associados a importantes reguladores da floração como *SHORT VEGETATIVE PHASE* (*SVP*) e *AGAMOUS-LIKE* 24 (*AGL24).* Nesse mesmo contexto, vários trabalhos diferentes apontam para uma expressão sazonal dos genes *DAM* ao longo de um ciclo anual de cultivo, apresentando acúmulo de transcritos durante a endodormência e posterior repressão após a completa satisfação do requerimento de frio (BIELENBERG *et al.*, 2008; KUMAR, G. *et al.*, 2017; MIMIDA *et al.*, 2015; PORTO *et al.*, 2016; SASAKI *et al.*, 2011b; YAMANE *et al.*, 2011; ZHAO *et al.*, 2018; ZHU *et al.*, 2015). Além disso, eles podem integrar sinais ambientais distintos (como fotoperíodo e/ou temperatura), a fim de permitir uma regulação fina e precisa das diferentes fases da dormência de gemas (YAMANE *et al.,* 2011).

Concordando com os dados transcricionais, a expressão constitutiva de *DAM6* de damasco em álamo, por exemplo, induziu a interrupção do crescimento, a formação de gemas e o estabelecimento da endormência (SASAKI *et al.,* 2011a). Da mesma forma, em maçã, plantas transgênicas superexpressando *MdoDAMb* e *MdoSVPa* apresentaram brotação atrasada e modificações na arquitetura da planta (WU *et al.*, 2017).

Embora os dados diponíveis permitam provar que os genes *DAM* são fundamentais no estabelecimento e na manutenção da dormência, ainda pouco se conhece sobre os fatores envolvivos na regulação da sua expressão gênica. Como previamente mencionado na seção anterior, alguns estudos apontam que os genes *CBFs* podem estar diretamente ligados à

ativação de genes *DAM* por meio da resposta dependente de frio (NIU *et al.,* 2016; WISNIEWSKI *et al.*, 2015; ZHAO *et al.*, 2018). Ao passo que pouco se sabe sobre os reguladores envolvidos na ativação/repressão de genes *DAM*, pouco se conhece também sobre seus alvos. Acredita-se que eles podem antagonizar a função do gene *FLOWERING LOCUS T* (FT), fundamental na floração de diversas espécies (NIU *et al.*, 2016). A regulação de genes *DAM* é ainda mediada por fatores epigenéticos e hormonais, que serão melhor abordados nas seções futuras.

1.1.3.3 BRRs na mediação das respostas dependentes de CK

Os REGULADORES DE RESPOSTA DO TIPO-B (BRRs) são fatores de transcrição da família MYB-*like* (HOSODA e*t al.,* 2002a; IMAMURA; HANAKI *et al.,* 1999) que participam das etapas finais da rota de sinalização de CK, uma importante classe de hormônios vegetais envolvidos na promoção do crescimento (KAKIMOTO, 2003; TO & KIEBER, 2008; WERNER & SCHMÜLLING, 2009; HILL *et al.*, 2013). Além do domínio de ligação ao DNA, eles possuem um domínio receptor (REC) característico que conta com um resíduo de Asp conservado, fundamental para a sua ativação (IMAMURA *et al*., 1999).

A via de sinalização de CK pode ser relacionada a um 'sistema de dois componentes' responsável pela percepção/resposta a estímulos ambientais (TO & KIEBER, 2008). O sistema consiste basicamente de uma proteína histidina quinase (primeiro componente), capaz de reconhecer o estímulo ambiental e fosforilar o regulador de resposta (segundo componente). Inicialmente, acreditava-se que esse tipo de via estivesse presente somente em procariotos (CHEUNG & HENDRICKSON, 2010). Entretanto, estudos com o receptor de etileno, ETHYLENE RECEPTOR 1 (ETR1 *et al.*, 1993) e fotorreceptores de fitocromos de Arabidopsis (SCHNEIDER-POETSCH, 1992) começaram a extrapolar esses conceitos a sistemas eucarióticos (LOHRMANN *et al.,* 2002).

A rota de sinalização de CK consiste de múltiplas etapas de fosforilação e inicia com a percepção de CK pela célula por meio de receptores encontrados na membrana plasmática e/ou no retículo endoplasmático. Uma vez que as moléculas de CK são percebidas, ocorre a indução da autofosforilação de um resíduo de His no domínio quinase da proteína HISTIDINA KINASE (HK). O fosfato é posteriormente transferido para um resíduo de Asp

no domínio receptor da mesma HK. A via segue com a transferência do fosfato da HK para uma HISTIDINA PHOSPHOTRANSFERASE (HP) que, fosforilada, consegue permear o núcleo. Consequentemente, o resíduo de Asp do domínio REC de um regulador de resposta é fosforilado, culminando, assim, na sua ativação (Figura 3). Os reguladores de resposta são divididos em tipo-A e tipo-B, dependendo de sua função. Os do tipo-A possuem apenas o domínio REC e são normalmente apontados como reguladores negativos da via (TO *et al.*, 2004), enquanto que os do tipo-B são responsáveis pela modulação dos genes responsivos a CK. Dentre os possíveis genes alvos, pode-se destacar aqueles relacionados ao desenvolvimento vegetal, à resposta a estresses, à transdução de sinal e aos estímulos bióticos e abióticos (WERNER & SCHMÜLLING, 2009).
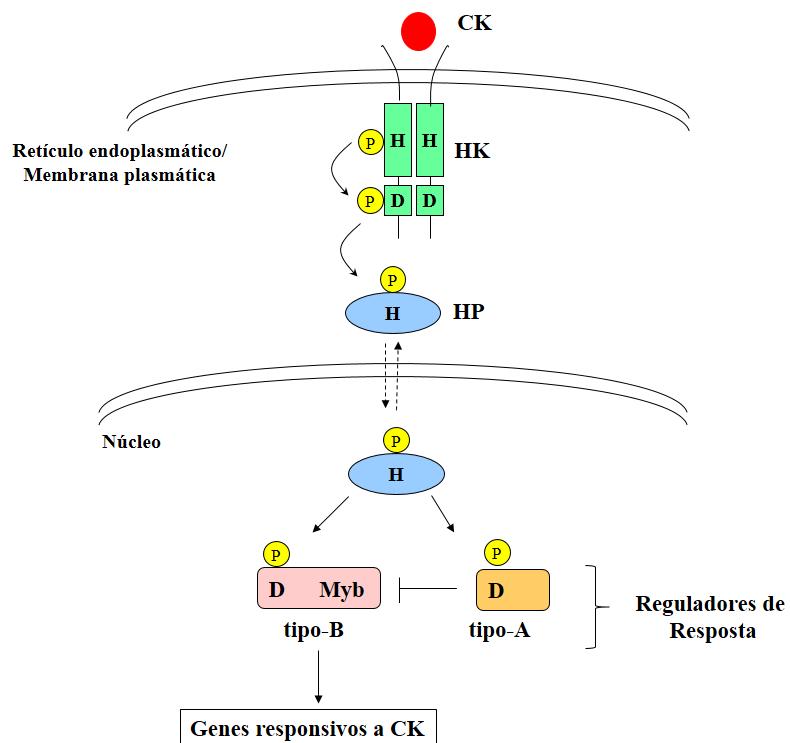
Figura 3. Via de sinalização de citocininas (CK).
Os receptores HK presentes no retículo endoplasmático e /ou membrana plasmática das células vegetais precebem o estímulo mediado por CK e acionam a via de transdução de sinal, que se inicia com a autofosforilação do domínio quinase de HK. O fosfato (P) é então transferido para o domínio receptor da mesma HK e em seguida transferido para a enzima

HP, que fosforilada, consegue permear o núcleo. No núcleo, o fosfato é transferido para os reguladores de resposta que são divididos em tipo-A e tipo-B. Os reguladores de resposta do tipo-B modulam a expressão de genes responsivos a CK, enquanto que os do tipo-A são reguladores negativos da via. Figura adaptada de EL-SHOWK *et al.* (2013).

Embora alguns membros da família *BRR* tenham funções mais específicas, muitos deles são redundantes (ISHIDA *et al.,* 2008). Essa característica foi evidenciada em estudos avaliando diferentes mutantes de perda de função para esses genes em Arabidopsis. Comparações entre plantas possuindo mutações simples (*arr1-3*), (*arr10-5*) ou (*arr12-1*) e plantas selvagens revelaram poucas alterações no fenótipo. As diferenças mais expressivas foram encontradas no mutante triplo (*arr1-3 arr10-5 arr12-1*) que, de maneira geral, apresentou diminuição na divisão celular em órgãos meristemáticos, aumento no tamanho das sementes, aumento na sensibilidade à luz, raízes primárias abortadas e perda da sensibilidade à aplicação exógena de CK (ARGYROS; MATHEWS; CHIANG; et al., 2008).

De forma semelhante, plantas expressando ectopicamente *AtARR10* ativaram vias de regulação a estresses abióticos mediados por baixas temperaturas, luz e disponibilidade de água (ZUBO *et al.*, 2017). Nessas plantas, a aplicação de CK levou à modulação de genes associados à biossíntese, à degradação, ao transporte e à sinalização da própria CK, além de auxinas e ABA, evidenciando um *crosstalk* hormonal interessante. No mesmo estudo, o gene *WUSCHEL* (*WUS*), um fator de transcrição importante no estabelecimento e na manutenção do meristema apical, foi identificado como alvo direto da regulação por *AtARR10* (ZUBO *et al.,* 2017).

Embora a família gênica de *BRRs* já tenha sido relatada em macieira (LI *et al.*, 2017), *Arabidopsis* (D'AGOSTINODERUÈRE & KIEBER, 2000), álamo (RAMÍREZ-CARVAJAL; MORSE & DAVIS, 2008), arroz (SCHALLER *et al.,* 2006), pêssego (IMMANEN *et al.,* 2013) e pera (NI *et al.,* 2017), ainda não há evidências claras sobre o papel destes fatores ao longo da regulação do ciclo da dormência.

1.1.4 A Importância da Regulação Epigenética desde a Dormência até a Floração

A regulação de características complexas como a dormência também passa por eventos epigenéticos que envolvem modificação de histonas, metilação do DNA e síntese de pequenos RNAs não-codificadores (RICHARDS *et al.,* 2017; RÍOS *et al.,* 2014). Essas modificações são herdáveis e modulam a expressão de genes sem alterar sua sequência nativa. Além disso, fornecem um mecanismo regulatório adicional em resposta a sinais internos ou externos (BOND & FINNEGAN, 2007).

A modificação de histonas e a metilação do DNA é fortemente influenciada por estímulos de frio, desempenhando um papel chave na aclimatação das plantas em resposta a condições extremas (BANERJEEWANI & ROYCHOUDHURY, 2017). Ao longo da dormência, diferentes padrões de marcas epigenéticas são observadas. Em geral, uma maior metilação do DNA e uma menor acetilação de histonas são observadas em gemas dormentes se comparadas aos estádios ativos de desenvolvimento (KUMAR & SINGH, 2016; SANTAMARÍA *et al.*, 2009). Nesse contexto, uma completa e atualizada revisão dos principais mecanismos epigenéticos envolvendo os processos de regulação da dormência e florescimento de plantas está descrita no **Capítulo I** da presente tese. Esta seção integra o volume 88 do livro "*Advances in Botanical Research*" que evidencia o potencial uso dos estudos epigenéticos em programas de melhoramento e o desenvolvimento de novas cultivares.

1.2 A IMPORTÂNCIA DA MALEICULTURA PARA O BRASIL

A macieira é uma frutífera de clima temperado, tendo como origem a Ásia Central. No Brasil, o início da cultura ocorreu, provavelmente, no município de Valinhos, SP, em 1926. Em meados da década de 1970, já se estabeleciam os primeiros pomares comerciais no país. Em 1988, o Brasil passou a exportar maçãs, atingindo a autossuficiência em 1998 quando as exportações ultrapassaram as importações, o que vislumbra a rápida evolução do setor (PETRI *et al*., 2011).

Atualmente, a maleicultura destaca-se no cenário nacional, principalmente na região Sul, com uma produção de 1,1 milhão de toneladas em 2018 (KIST, 2018). É um mercado em constante atividade e que movimenta cifras significativas tanto internamente, como por meio de exportação. Em 2018, por exemplo, segundo a Associação Brasileira de Produtores de Maça (ABPM), a maçã foi o terceiro produto mais vendido dentre os produtos de fruticultura nas centrais de abastecimentos (CEASAS) dos estados, superada apenas pela banana e pela melancia. O comércio total da fruta atingiu 448,7 milhões de quilos, terceiro maior volume alcançado nos registros existentes desde 2009. Do total da produção vendida no sistema atacadista brasileiro em 2018, a maior parcela, de 44,2%, teve origem em Santa Catarina e 33,2% no Rio Grande do Sul, sendo os principais municípios fornecedores São Joaquim (SC) e Vacaria (RS), seguidos da catarinense Fraiburgo e da gaúcha Caxias do Sul. O montante acumulado resultante das vendas girou em torno de 1,8 bilhão de reais. Levantamentos feitos pela Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Epagri) mostraram que, nos últimos cinco anos, a média de participação de cultivares na produção brasileira de maçã foi de 56% para o grupo Gala; 39% para Fuji; e 6% para outras variedades (KIST, 2019).

No mercado internacional, em 2018, houve o crescimento das exportações se comparado ao período de 2017, com um aumento de 28,1% do volume e 25,3% em termos de valores, resultando em 71 mil toneladas exportadas que movimentaram US$ 52 milhões. O produto foi direcionado a 25 países, sendo os maiores compradores Bangladesh e Rússia (KIST, 2019).

No aspecto socioeconômico, o setor proporciona um dos maiores índices de demanda de mão-de-obra, atingindo cerca de 1,5 emprego por hectare, conforme cálculo da ABPM. Considerando que a área de pomares atinge a ordem de 33 mil hectares, o mercado de trabalho no segmento oferece oportunidades a mais de 49 mil pessoas, e outras 98 mil colocações indiretas, perfazendo 148 mil empregos gerados. Para efeitos comparativos, na produção da maçã são empregadas 150 pessoas em 100 hectares cultivados enquanto que, nos grãos, a média atinge, no máximo, uma pessoa na mesma área (KIST, 2019).

1.3 OS DESAFIOS DO ATUAL CENÁRIO PRODUTIVO DA MAÇÃ

As boas práticas culturais como fertilização, condução das plantas, poda, quebra da dormência, raleio e manejo de pragas e doenças são fundamentais para a produtividade e a viabilidade dos pomares de maçã. Nesse âmbito, vale ressaltar que as principais regiões produtoras do país não são capazes de acumular o frio suficiente para a quebra de dormência das gemas sendo, assim, necessário um tratamento químico para uniformizar a brotação e a floração. Essas regiões tem um acúmulo médio de 450 HF, o que é praticamente metade do requerido para as cultivares mais amplamente plantadas e aceitas no mercado como Gala e Fuji (~800 HF).

Nesse contexto, o indutor químico mais utilizado é a cianamida hidrogenada, comercialmente conhecida como Dormex®. No entanto, este composto é classificado como altamente tóxico ao ser humano (categoria I), podendo provocar ulcerações nos olhos, pele e trato respiratório, além de inibir a aldeído desidrogenase, levando à síndrome de acetaldeído. Esta síndrome é caracterizada por vômito, hiperatividade parassimpática, dispneia, hipotensão e desorientação. Em 2002, a Itália determinou a suspensão temporária das vendas do produto comercial Dormex® devido aos vários casos registrados de intoxicação por esse produto. Em 2008, a União Europeia proibiu a sua comercialização (SETTIMI *et al.,* 2005).

Embora altamente tóxico, a não utilização desses indutores químicos nas gemas levaria a uma florada desuniforme que se estenderia além do normal, resultando em uma queda de mais ou menos 40% a 50% da produção. A insuficiência no acúmulo de frio é ainda agravada com a presença de invernos com temperaturas cada vez mais altas, o que também altera os níveis de precipitação de chuvas, essenciais nos períodos de floração e de crescimento dos frutos. Consequências desses fenômenos puderam ser vistas em 2018 com a diminuição no tamanho dos frutos e queda na produtividade de 0,2 milhão de quilos de frutos em relação à 2017 (KIST, 2018).

Em 2019, um relatório especial do Painel Intergovernamental de Mudanças Climáticas (IPCC) revelou que, desde o período pré-industrial (1850-1900), a temperatura média do ar observada na superfície da terra aumentou 1,53 °C (variando de 1,38 °C a 1,68 °C). Além disso, o estudo mostrou que as mudanças climáticas já afetaram a produção de alimentos devido ao aquecimento, mudanças nos padrões de precipitação e maior frequência de alguns eventos extremos. Em regiões de baixas latitudes, por exemplo, houve diminuição

nos rendimentos de algumas culturas como milho e trigo, enquanto que regiões produtoras com maior latitude foram afetadas positivamente (IPCC, 2019). Isso demonstra que as constantes mudanças ambientais podem levar a uma modificação do atual cenário agrícola mundial, tornando terras antes viáveis para determinados cultivos em inviáveis e vice-versa.

O quinto e mais atual relatório geral mundial de mudanças climáticas baseado nas mais recentes pesquisas científicas, técnicas e socioeconômicas divulgado pelo IPCC em 2014, tendo o sexto com previsão de lançamento em 2021, prevê que o aumento médio da temperatura global da superfície terrestre no ano de 2100 será de 3,7 °C a 4,8 °C (em comparação ao período pré-industrial) se as emissões de gases do efeito estufa permanecerem como estão (IPCC, 2014). Modelando-se o cenário atual em relação às condições de acúmulo de frio nas regiões do sul do Brasil, haveria uma potencial redução de 85% a 98% das áreas de plantio com acúmulo médio de 400 HF até 2070 (MONTEIRO & FARIAS, 2013). Tratando-se do cultivo de espécies perenes de clima tropical, o aumento das temperaturas resultaria na alteração de eventos fenológicos além da brotação, como anormalidades na fertilização devido à sincronização incorreta da floração de cultivares auto-incompatíveis, mudanças no tamanho da flor e no tempo da antese e modificações nos períodos de colheita dos frutos bem como na sua logística de comercialização (CANNELL & SMITH, 1986; VITASSE *et al.*, 2014; ENSMINGER *et al., 2007*; ABBOTT et al., 2015; CELTON *et al*., 2011; DIRLEWANGER *et al*., 2012; EREZ, 2000, SCAVEN & RAFFERTY, 2013).

Considerando a importância econômica da maleicultura no atual cenário brasileiro e visando enfrentar os principais desafios encontrados no setor, o uso de novas tecnologias se faz cada vez mais fundamental. Para tal, o entendimento de como ocorrem os processos biológicos ao longo do desenvolvimento da planta é essencial. No âmbito da genômica, desvendar os integrantes da complexa rede molecular que regula todas as fases do ciclo da dormência possibilitaria o desenvolvimento de novas ferramentas biotecnológicas que poderiam auxiliar nos atuais programas de melhoramento convencional de macieiras como o uso de marcadores moleculares, por exemplo. Além disso, potenciais genes reguladores poderiam ser silenciados, integrados ou editados no genoma, gerando plantas geneticamente modificadas mais adaptadas aos atuais e futuros cenários produtivos. Por fim, os avanços tecnológicos possibilitariam a manutenção da cadeia produtiva, mantendo-a competitiva no mercado externo.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Contribuir para o entendimento dos mecanismos moleculares envolvendo o processo de regulação da dormência de gemas de macieira por meio da integração das respostas mediadas por baixas temperaturas, sinalização hormonal e caracterização dos genes *MdoCBFs*, *MdoICE1* e *MdoBRRs* durante a transição da endo- para a ecodormência.

2.2 OBJETIVOS ESPECÍFICOS

a. Identificar e caracterizar o potencial papel regulatório dos genes *MdoBRRs* por meio de perfis transcricionais ao longo do ciclo da dormência de gemas de macieira;

b. Investigar se os genes *MdoBRRs*, bem como o estímulo mediado por CK, podem modular a expressão de *MdoDAM1*;

c. Caracterizar o padrão de expressão dos genes *MdoCBFs* e *MdoICE1* ao longo do ciclo da dormência de gemas de macieira;

d. Compreender como o ciclo da dormência se sobrepõe à via de aclimatação ao frio mediada por ICE1-CBF.

Os resultados dos estudos relativos aos objetivos específicos **a** e **b** foram relatados em formato de artigo científico submetido à publicação e estão apresentados no **Capítulo II**. O conjunto de resultados dos objetivos **c** e **d** estão apresentados no **Capítulo III** sob forma de artigo científico a ser encaminhado à publicação.

Na sessão de anexos da presente tese encontra-se o pedido de patenteabilidade referente aos potenciais inventos gerados no estudo relatado no **Capítulo II** (Anexo I). Além disso, o Anexo II traz um artigo publicado referente à análise genética do lócus responsável pelo tempo de brotação em gemas de macieira. Esta parte do trabalho foi realizada adjacentemente aos estudos apresentados nos capítulos que compõem a presente tese.

3 CAPÍTULO I

The control of bud break and flowering time in plants: contribution of epigenetic mechanisms and consequences in agriculture and breeding

Capítulo publicado no livro "Advances in Botanical Research - Plant Epigenetics Coming of Age for Breeding Applications"

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The Control of Bud Break and Flowering Time in Plants: Contribution of Epigenetic Mechanisms and Consequences in Agriculture and Breeding

Amanda Malvessi Cattani[*,a], **Tiago Sartor**[*,a],
Vítor da Silveira Falavigna[§], **Diogo Denardi Porto**[¶],
Carolina Pereira Silveira[‖], **Paulo Ricardo Dias de Oliveira**[‖] **and**
Luís Fernando Revers[*,‖,1]

[*]Graduate Program in Cell and Molecular Biology, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[§]AGAP, Univ. Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier, France
[¶]Centro de Pesquisa Agropecuária do Trópico Semiárido, Empresa Brasileira de Pesquisa Agropecuária, Petrolina, Brazil
[‖]Centro Nacional de Pesquisa de Uva e Vinho, Empresa Brasileira de Pesquisa Agropecuária, Bento Gonçalves, Brazil
[1]Corresponding author: E-mail: luis.revers@embrapa.br

Contents

[a] These authors contributed equally to this work.

Abstract

In perennial plants, the release of bud dormancy, with subsequent flowering, resembles the vernalization process of *Arabidopsis thaliana* and cereals. Especially for perennial crops from temperate regions, dormancy is an important adaptive trait for both survival and growth. Exposure to sufficient chilling during winter dormancy ensures the normal phenological traits in subsequent growing periods. Here, we compile research data on mechanisms controlling the overlapping developmental processes that define dormancy induction, maintenance and release, bud burst and flowering. Recent findings highlight the relevance of genome-wide epigenetic modifications related to dormancy events, and more specifically the epigenetic regulation of *DORMANCY-ASSO-CIATED MADS-box*, *FLOWERING LOCUS C* and *FLOWERING LOCUS T* genes, key integrators of vernalization effectors on flowering. The roles of plant growth regulators in controlling bud break and flowering are discussed in relation to epigenetic mechanisms. A growing body of knowledge demonstrates that epigenetic regulation plays a key role in these processes in perennial horticultural and forestry plants. We discuss the most relevant molecular and genomics research that contribute to better understanding of the dormancy process and pave the way to precise manipulation of dormancy-related horticultural traits, such as flowering time. Finally, some of the challenges for further research in bud dormancy and consequences in agriculture are discussed within the context of global climate change.
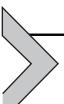
1. INTRODUCTION

Most of perennial tree species in temperate areas are cultivated in regions with well–differentiated seasons. In addition, perennials differ from annual and biennial lifestyles in their ability to turn on/off growth in response to environmental and seasonal changes. As an adaptation to long periods of unfavorable environmental conditions, including cold winters and low light availability, many perennial plants have developed a mecha–nism of protection called dormancy. The first definition of dormancy was proposed by Lang, Early, Martin, and Darnell (1987) as "the absence of

visible growth in any plant structure containing a meristem". Later, Rohde and Bhalerao (2007) proposed another definition aiming to accurate the expression "visible growth" and the theory that dormancy is not only absence of growth, but also include the inability to resume it. Dormancy was therefore redefined as "the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favorable conditions" (Rohde & Bhalerao, 2007). This definition mainly describes the meristem states including axillary dormant buds with correlative inhibition or apical dominance, but does not include physical dormancy or ecodormancy (Rohde & Bhalerao, 2007), one of the three physiological stages of bud dormancy defined by Lang et al. (1987). Indeed, considering ecodormancy, growth inhibition is determined by adverse external stimuli and once environmental conditions become favorable, the meristematic activity restarts. The two other dormancy stages defined by Lang et al. (1987) are paradormancy and endodormancy. In paradormancy, distal organs signalize growth suppression (apical dominance), whereas in endodormancy, plant internal signals are determinant for growth arrest. Endodormant buds are not capable of resuming growth even when exposed to growth–promoting conditions. Visible changes can be observed in plants during dormancy establishment, maintenance and release. These include the cessation of apical growth, bud development, acquisition of cold/desiccation tolerance, and leaf senescence, as observed in deciduous species (Cooke, Eriksson, & Junttila, 2012). Initially, leaf primordia are modified to form hard scales during bud set (Horvath, 2010). When plants start resuming growth, bud burst can be observed as a swelling (green tip), followed by emission of young leaves and could be related to B and C stages of Fleckinger phenological scale (EPPO, 1984).

Within this context, buds are defined as "the primary shoot-producing meristematic organs for dicotyledonous plants" (Horvath, 2010) and are largely responsible for ensuring a new reproductive cycle in perennial species. Complex and orchestrated mechanisms that involve environmental stimulus, molecular pathways, hormonal signaling, and epigenetic modifications are responsible for both bud set and bud break. As a rule of thumb, photoperiod and temperature are the major signals that stimulate bud set, although their relative importance depends on the species (Cooke et al., 2012). In species of *Betula* (birch), *Populus* (aspen) and *Salix* (willow tree), photoperiod is the major stimulus (Howe, Gardner, Hackett, & Furnier, 1996; Junttila, 1976), whereas for *Malus* (apple), *Pyrus* (pear) and *Sorbus* (mountain ash) species, low temperatures are more determinant (Heide, 2011;

Heide & Prestrud, 2005). Dormancy is a cyclic and dynamic process and in some cases, the same stimuli that induce bud set are essential for dormancy release and bud break. In *Malus* species, for example, exposure to low temperatures during the autumn induces bud set, and continuous chilling exposure over the winter triggers the release from dormancy, indicating that the meristem responds to temperature changes during its endogenous development. The molecular mechanisms that control dormancy establishment and release are still far from a complete elucidation, although different models have been proposed (Campoy, Ruiz, & Egea, 2011; Horvath, 2009; Rinne et al., 2011).

Epigenetic modifications play a crucial role during dormancy regulation, from bud set to bud break, and are generally divided into active or repressive modifications. Active modifications include trimethylation of histone H3 lysine 4 (H3K4me3), di- or trimethylation of histone H3 lysine 36 (H3K36me2/me3), and acetylation of histone H3 lysine 9 (H3K9ac). Repressive modifications include histone deacetylation, trimethylation of histone H3 lysine 27 (H3K27me3), methylation of histone H3 lysine 9, and depending on its position relative to a gene, DNA methylation (see chapter 2 of this book). Nucleosome remodeling may also act to activate or repress gene expression (Guo et al., 2015; He, 2012).

In this chapter, we will discuss the already known regulatory mechanisms that regulate dormancy, bud break and flowering, focusing on the epigenetics events that could directly or indirectly influence these pathways. Potential biotechnological applications for breeding purposes are also presented.

2. CONTROL OF DORMANCY AND FLOWERING TIME

2.1 Chilling Requirement

Although the molecular mechanisms responsible for cold perception and rest completion in dormant buds are far from being fully understood, empirical knowledge about the effects of cold exposure on bud phenology is being employed successfully since a long time in dormancy research as well as for fruit production. Early conceptual models that are still in use today postulate that individual buds somehow record the amount of time of exposure to temperatures typically in the range from 0 to 7.2°C (Chandler, Kimball, Philp, Tufts, & Weldon, 1937; Lamb, 1948). These first models already provided a good correlation with bud phenology and proved to

be useful for researchers and growers. Temperature records from meteorological stations, often in hourly intervals, could be used to estimate dormancy status in tree orchards, and the concept of chilling hours (hours of exposure to temperatures that contribute to dormancy progression) became an important tool for bud dormancy research in trees.

Further analysis of the interaction between climate and bud phenology showed that under slightly warmer temperatures, bud dormancy still progresses, although not in the same pace as with lower temperatures. Towards warmer environments (nearly 15°C), bud dormancy remain unchanged, and when temperature is close to 20°C, the effect of cold is negated: the record of cold exposure is progressively erased and further exposure to chilling is necessary to compensate the period of time under warm temperatures (Erez, Couvillon, & Hendershott, 1979; Young, 1992). These discoveries were implemented in new bud dormancy completion models in which, instead of chilling hours, the exposure to cold temperatures is counted as chilling units, whereas the duration under warmer temperature is either considered of no effect or subtracted from the chilling unit count (Richardson, Seeley, & Walker, 1974; Shaltout, Unrath, & Akademiya, 1983). The exact ranges of temperatures are specific to each model.

All the aforementioned models based on ambient temperature data in temperate regions provide a good prediction of bud phenology. However, when applied to sites with warm winters, the predictive power of these models is not accurate enough. With that in mind, Erez, Fishman, Linsley-Noakes, and Allan (1990) developed a more elaborate conceptual model based on a two-step process for the memory of cold exposure. First, chilling (higher efficiencies occurring between 6°C and 8°C) induces the accumulation of a precursor, which is thermally labile. Therefore, this first reaction is reversible after sufficient time of exposure to higher temperatures. Second, when the precursor accumulates to a threshold value, a second reaction takes place that is not reversible. It produces, in conceptual terms, a stable factor that signals a fixed amount of cold exposure. This process is repeated several times during winter, until the number of dormancy-releasing factors (chilling units) reaches a critical level. The performance of this so called Dynamic Model has been shown to be superior to previous models in warm winter regions (Dennis, 2003; Erez et al., 1990).

The total amount of chilling hours (or chilling units) required for bud endodormancy release is referred to as chilling requirement (CR), and is genetically controlled (Hauagge & Cummins, 1991; Labuschagné, Louw, Schmidt, & Sadie, 2002; Ruiz, Campoy, & Egea, 2007). CR is

variable across cultivars of commercial tree species, which is highly valuable for breeding, allowing crops to achieve high yields in diverse climates.

2.2 QTLs for Bud Dormancy

The heritable nature of CR has been extensively used to map loci significantly contributing to this trait (Allard et al., 2016; Celton et al., 2011; Frewen et al., 2000; Graham et al., 2009; Urrestarazu et al., 2017; van Dyk, Soeker, Labuschagne, & Rees, 2010). Bud dormancy, at first glance, appears as a set of discrete phenotypes (*e.g.* endodormant or ecodormant buds) although in genetic research, it is considered as a quantitative trait. The phenotypic data used as input to quantitative trait loci (QTL) mapping is typically the time period, measured in days, from a reference date until half of all buds of each individual burst during the growing season. The reference date used in most cases is the time of bud flush of the early blooming parental genotype, and is directly related to the CR of each sibling.

One of the best characterized loci controlling bud dormancy in trees, however, was not mapped as a quantitative trait, but rather as a categorical trait with two discrete phenotypes: setting or not setting buds before winter. The peach genotype named "evergrowing" is unable to set cold-hardened buds, and the analysis of a population derived from a cross between *evergrowing* and a high CR peach cultivar allowed the identification of the *EVG* locus located in peach chromosome 1 as responsible for the trait (Wang, Georgi, Reighard, Scorza, & Abbott, 2002). Sequencing and annotation of the *EVG* locus revealed the presence of six MADS-box genes, whose sequences are similar to the *A. thaliana SHORT VEGETATIVE PHASE* (*SVP*) gene, which acts as a transcriptional repressor in distinct flowering and hormonal-signaling pathways (see Section 4, Gregis et al., 2013). These genes were named *DORMANCY-ASSOCIATED MADS-box* (*DAM*) genes 1 to 6 (Bielenberg et al., 2008) and their expression patterns were later shown to correlate with several phases of the dormancy-cycle, strongly suggesting them as important players for dormancy regulation in peach (Li, Reighard, Abbott, & Bielenberg, 2009). *DAM* genes were further identified in other perennials such as leafy spurge (Horvath, Sung, Kim, Chao, & Anderson, 2010), apricot (Sasaki et al., 2011; Yamane, Kashiwa, Ooka, Tao, & Yonemori, 2008), pear (Ubi et al., 2010) and apple (Falavigna et al., 2014; Mimida et al., 2015; Porto et al., 2016).

QTL mapping of CR in peach also pointed to the *EVG* locus as contributing to bud dormancy variation (Fan et al., 2010). Fine mapping of the

EVG locus, combined to DNA sequencing of extreme phenotypes from segregating populations, is in agreement with previous data showing that two peach *DAM* genes, *PpeDAM5* and *PpeDAM6*, are playing major roles during bud dormancy (Zhebentyayeva et al., 2014). Furthermore, sequence data showed a tight association between large insertions in *PpeDAM5* and *PpeDAM6* first introns and low CR (Zhebentyayeva et al., 2014).

Besides peach, QTLs for bud dormancy in regions syntenic to the *EVG* locus were found in other *Prunus* species (Castède et al., 2015; Olukolu et al., 2009; Sánchez-Pérez, Dicenta, & Martínez-Gómez, 2012). However, no *DAM* homologs could be found in major QTLs for CR in apple (Celton et al., 2011; Urrestarazu et al., 2017; van Dyk et al., 2010), pear (Gabay et al., 2017) or poplar (Rohde et al., 2011), although minor QTLs were identified coinciding with the position of *DAM* genes in apple and pear (Allard et al., 2016; Gabay et al., 2017). Possibly, *DAM* genes are more conserved in these species, or the role played by the *DAM* homologs in apple, pear and poplar is not the same as in *Prunus* species (Romeu et al., 2014).

2.3 Epigenetic Modifications of *FLC* x Dynamic Model of CR

Similarly to the variation in CR of tree crop cultivars, the model plant *A. thaliana* show natural variation in flowering time (Bloomer & Dean, 2017; Coustham et al., 2012). Interestingly, up to 70% of this variation is due to polymorphisms of only two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Bloomer & Dean, 2017). The role of FLC in the regulation of flowering time in *A. thaliana* is very well demonstrated in the literature. FLC is a floral repressor and is highly expressed in most tissues during the vegetative phase (Choi, Hyun, & Kang, 2009). During winter, if the plant is exposed to sufficient cold, *FLC* expression is permanently silenced, unlocking downstream pathways of floral induction.

In an elegant approach, Angel, Song, Dean, and Howard (2011) proposed that the dynamics of *FLC* silencing by epigenetic modifications is the main mechanism underlying the memory of cold exposure. During winter, the cold-induced plant-homeodomain (PHD) protein VERNALIZATION INSENSITIVE 3 (VIN3) accumulates in a region spanning the first exon and a portion of the first intron of the *FLC* gene, named nucleation region. VIN3 recruits POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) proteins, which modify the chromatin in the nucleation region by adding the H3K27me3 mark (trimethylation of Lysine 27 of Histone H3), a repressive mark stable over cell cycles (Feng, Jacobsen, & Reik, 2010). After returning to warmer temperatures, the remainder of

the *FLC* chromatin is silenced by the same mechanism under a positive feed-back process (Angel et al., 2011). The model predicts that *FLC* gene expression in each individual cell is bistable, that is, can either be activated or silenced. The quantitative signal transduction is achieved when in a population of cells, each cell respond autonomously to cold exposure. Later on, cold-induced *FLC* antisense transcripts (*COOLAIR*) were demonstrated to participate in the silencing process during the first weeks of chilling, but the overall model has been shown to be accurate (Fig. 1; Bloomer & Dean, 2017).
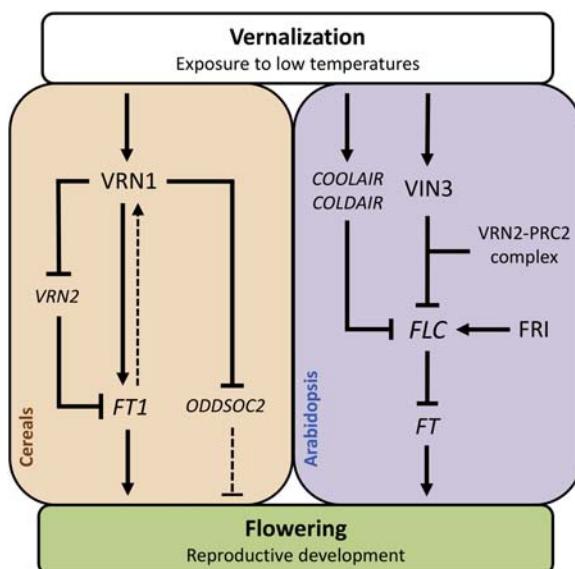
Figure 1 *Vernalization pathways in Arabidopsis and cereals.* Vernalization is the process that renders a plant competent to flower after a period of exposure to chilling temperatures. In cereals, such as wheat and barley, cold exposure triggers the accumulation of VRN1 protein, which directly binds to the flower-inducing *FT1* promoter and induces its expression. FT1 might promote the expression of *VRN1* in a feedback loop regulation. In parallel, VRN1 also binds to the promoter and represses two flowering repressors, *VRN2* (a repressor of *FT1* in cereals) and *ODDSOC2* (Andrés & Coupland, 2012; Deng et al., 2015). In *Arabidopsis*, the flower inhibitor *FLC* gene is initially repressed via cold-induced transcription of the *FLC* antisense transcript *COOLAIR*. After 3 weeks of continuous chilling, a *FLC* sense transcript from intron 1 (*COLDAIR*) is also induced. VIN3, a plant homeodomain (PHD) protein, accumulates during continuous cold exposure and associates with the VRN2-containing PRC2 complex to form a PHD-PRC2 complex. This complex promotes trimethylation of histone H3 lysine 27 (H3K27me3) at the *FLC* chromatin to epigenetically repress the *FLC* gene, allowing *FT* to be expressed and induce flowering (Ietswaart, Wu, & Dean, 2012; Li & Cui, 2016; Song et al., 2012a).

A. thaliana accessions differing in flowering time were screened for variation in *FLC* sequence (Li et al., 2014). Interestingly, most variation occurred in noncoding regions of the *FLC* alleles and the predicted FLC protein was identical over all tested genes. Moreover, polymorphism in the nucleation region showed the strongest association with *FLC* silencing phenotypes (Li et al., 2014). One of the early flowering accessions, *Lov-1*, revealed a single nucleotide polymorphism (SNP) that disrupts one of two conserved binding sites for B3 transcriptional regulators (Coustham et al., 2012). This site was later found to be targeted by the transcriptional repressor VIVIPAROUS1/ABI3-LIKE (VAL1), which is necessary for the nucleation of PHD-PRC2 proteins (Qüesta, Song, Geraldo, An, & Dean, 2016).

Epigenetic regulation is regarded as a strong candidate molecular mechanism for bud dormancy progression (Ríos, Leida, Conejero, & Badenes, 2014). Conceptually, the similarities between the Dynamic model and the *FLC*-VIN3-PRC2 regulon are worth noting. The intermediate thermolabile precursor and the stable factor preconized by the model show high resemblance to the VIN3 concentration and histone modifications in the *FLC* locus, respectively. *VIN3* expression is cold-induced, but its protein concentration rapidly declines in the warm (Angel et al., 2011). After the trimethylation of histones at the *FLC* locus, the cold signal is stably and irreversibly transduced in each cell. Individual cells autonomously and stochastically responding to chilling resembles the Dynamic Model of CR, where the independant accumulation of still unknown dormancy-releasing factors is necessary in order to lead to endodormancy release.

From a conceptual point of view, the characterization of epigenetic modifications seems a very promising approach to better understand bud dormancy regulation and flowering at the molecular level. The literature on this subject, especially in recent years, has grown significantly (see next section). *DAM* genes are the main candidates for the role of *FLC* in dormant buds, mainly from studies on *Prunus* species. Although recent findings were able to identify *FLC*-like genes in fruit tree species (Kumar et al., 2017, 2016; Niu et al., 2016; Porto et al., 2016; Takeuchi et al., 2018) to this date, well established homologs of *FLC* remain to be found. On the other hand, homologs of the flowering integrator gene *FLOWERING LOCUS T* (*FT*) were already identified in several tree genomes (Kotoda et al., 2010). An overview of the *FT* role in flowering-time regulation is present on Section 4 of this chapter. Interestingly in poplar, two *FT* paralogs, *FT1* and *FT2*, control dormancy transitions (Böhlenius et al., 2006; Hsu et al., 2011). The first is induced by chilling and signals dormancy release, while

the second one promotes vegetative growth and inhibition of bud set during summer (Hsu et al., 2011). The identification of upstream regulators of each *FT* ortholog can provide valuable information over the control of bud dormancy.

3. EPIGENETIC AS A KEY MECHANISM FOR DORMANCY REGULATION

3.1 Chromatin Modifications during Dormancy and Bud Break

Although bud dormancy is a well-characterized process at the physiological level, the molecular mechanisms controlling this process are only recently starting to be unveiled. Indeed, as demonstrated in the previous section, it is exactly the similarities to other better established phenomena in model plant species such as seed dormancy and flowering that guided the studies over bud dormancy control. As expected, recent studies have provided evidences that bud dormancy is under complex regulatory pathways including, hormonal control, the overlap of cold and photoperiod pathways, miRNAs regulation, and several epigenetic mechanisms. At present, a huge focus is being given to the contribution of epigenetics during plant developmental processes (reviewed in Banerjee, Wani, & Roychoudhury, 2017; Gallusci et al., 2017; Richards et al., 2017), with a special attention to bud dormancy (Ríos et al., 2014). The first clue suggesting that dormancy is epigenetically regulated comes from the study of the major role of cold in this process. Both induction and overcoming of the trait are directly linked to cold exposure, in a way similar to the "vernalization memory" described in *A. thaliana* that involves epigenetic mechanisms (Banerjee et al., 2017). In this context, histone modifications, changes in DNA methylation patterns, and the regulatory role of small non-coding RNAs (sncRNAs) have already been observed during dormancy in several perennial species.

For example, early studies in chestnut using suppression subtractive hybridization were able to identify genes involved in phosphorylation of histone H3 (H3 kinase *CsaAUR3*) during bud burst, and in H2B monoubiquitination (histone mono–ubiquitinase *CsaHUB2*) and acetylation of histone H3 serine (histone acetyltransferase *CsaGCN5L*) in dormant apical buds (Santamaría, Rodríguez, Cañal, & Toorop, 2011). The same group already reported that DNA methylation levels were higher in dormant apical chestnut buds, while H4 histone acetylation levels were higher in

non-dormant ones. Interestingly, the same methylation pattern was not observed in axillary buds, confirming that the DNA methylation levels observed in apical buds is a seasonal trend (Santamaría et al., 2009). In a study using full-genome microarray analysis, the regulation of genes encoding components of the DNA methylation and chromatin remodeling machineries were investigated at distinct stages of the active—dormancy cycle in hybrid aspen (Karlberg et al., 2010). It was demonstrated that short-day and low temperature treatments induced the expression of several histone deacetylases (*HDA14*, *HDA08*, *HDA9*, *SIN3*), a histone lysine methyltransferase (*SUVR3*) and histone ubiquitination (*HUB2*) genes. Simultaneously, a *DEMETER*-like gene, which encodes a DNA glycosylase, was downregulated by short-day treatment, possibly leading to a further increase in DNA methylation and chromatin compaction. On the other hand, long-term low temperature exposure induced a *DML* (*DEMETER*-related) gene, probably leading to demethylation and activation of genes that contribute to dormancy release.

The first evidence demonstrating that changes in cytosine methylation patterns may play a regulatory role in the control of dormancy release was obtained from MSAP (Methylation Sensitive Amplified Polymorphism) and RNA-seq analyses in apple. This study also showed that chilling availability appears to modulate the expression of genes involved in active DNA methylation and demethylation, with a progressive decrease in DNA methylation levels being observed during dormancy release. Using the same approach, high expression levels of the histone acetyltransferase *HCA1* and the histone deacetylases *HDA14* and *HDA19* where identified towards the initiation of active growth, while downregulation of other histone deacetylases like *HDA06* and *HDA08* was observed in the same time points (Kumar et al., 2017). Another study showed that dormancy release triggered the induction of two types of histones, the canonical H2A and an H2A.Z histone variant (Falavigna et al., 2014). In *A. thaliana*, H2A.Z-containing nucleosomes coordinate one of the thermosensory responses (Kumar & Wigge, 2010), being usually more expressed during the S phase of the cell cycle (March-Díaz & Reyes, 2009). The expression of these genes during dormancy release suggests that the dynamic balance in their opposing activities is necessary to regulate the transcriptional levels of target genes.

In poplar, epigenetic changes seem to take place during dormancy in order to control differential gene expression. The balance of epigenetic marks related to low transcriptional activity during winter (i.e. DNA

methylation and histone H4 hypoacetylation) with chromatin modifications compatible with increased gene expression during growth resumption (i.e. DNA hypomethylation and histone H4 acetylation) were observed (Conde, González-Melendi, & Allona, 2013). Further studies demonstrated that growth reactivation is preceded by DNA demethylation mediated by *DML*, thus promoting the necessary changes to apical bud physiology in order to start the bud break process (Conde et al., 2017b, 2017a). The DNA methylation in poplar is a widespread process in non-condensed chromatin and is associated to tissue-specific gene expression. Moreover, in the shoot apical meristem (SAM), this process responds to variations of water availability and affects the expression of genes involved in hormonal pathways (Lafon-Placette et al., 2013, 2018). Taken together, these findings indicate that the dynamics of genomic DNA methylation levels could be involved in the regulation of dormancy—growth cycle.

Recently, the ABA-mediated regulation of dormancy in response to photoperiod has been proposed for growth cessation (Tylewicz et al., 2018). In this model, ABA accumulation in the buds triggers plasmodesmata closure, ensuring bud growth arrest until sufficient chilling has been accumulated. The role of ABA in the induction of several epigenetic marks such as histone modifications, DNA methylation and short interfering RNA pathways was already documented for several development processes and stress responses in plants (Chinnusamy, Gong, & Zhu, 2008). In strawberry, dormancy establishment induced global DNA methylation in young leaves, and these changes were synchronized with endogenous ABA levels (Zhang et al., 2012c). However, how ABA is able to modulate epigenetic changes during the bud dormancy process is yet to be discovered.

3.2 Epigenetic Regulations of *DAM* and *EBB* Genes

Some of the most emblematic genes associated to bud dormancy regulation, the *DAM* genes, are also under strong epigenetic control. Both H3K4me3 and H3K27me3, two epigenetic marks related to activation and repression of transcription, respectively, are recurrently found over *DAM* genes in the different species that have been analyzed. The leafy spurge *EesDAM1* and peach *PpeDAM6* genes showed both an increase of the H3K27me3 and a decrease of the H3K4me3 levels in promoter regions when comparing eco-to endodormancy (Horvath et al., 2010; Leida, Conesa, Llácer, Badenes, & Ríos, 2012). In peach, the same epigenetic changes were also identified at the transcriptional start site (TSS) and in the largest intron of *PpeDAM6* (Leida et al., 2012). Interestingly, these two *DAM* genes

displayed a seasonal expression profile during dormancy, with a peak of transcription during endodormancy that correlates to the levels of the aforementioned epigenetic marks. Additionally, the abundance of acetylation of histone H3 (H3ac), another epigenetic mark associated with gene expression, in the promoter of *PpeDAM6* decreased during dormancy release (Leida et al., 2012). A sequential chain of events affecting *PpeDAM6* chromatin status was identified: loss of H3K4me3 and H3ac modifications during dormancy release would contribute to gene repression, while increase of H3K27me3 at the same time point would enable long-term gene inactivation (Leida et al., 2012). These two pioneering studies have provided the first evidence that epigenetic regulation of *DAM* genes may be operating during dormancy in a manner similar to the regulation of the *Arabidopsis FLC* gene during vernalization.

Further studies in peach were aimed to identify putative regulations of the *DAM* locus, which is responsible for the *EVG* phenotype (Bielenberg et al., 2008). Genes involved in chromatin remodeling were identified in QTLs for nine traits related to bud dormancy, flowering and fruit harvest, emphasizing a prominent role of chromatin modification pathways in this process (Romeu et al., 2014). Thereafter, de la Fuente, Conesa, Lloret, Badenes, and Ríos (2015) identified a significant enrichment of H3K27me3 during dormancy release concomitantly with reduced expression at different regions of the *DAM* locus, and this may be one of the controlling mechanisms responsible for the differential transcriptional profiles observed for *PpeDAM* genes during bud dormancy (Li et al., 2009). In pear, the *DAM* gene *PpyMADS13-1* displayed a seasonal expression pattern that resembles the one identified for *EesDAM1* and *PpeDAM6* (Saito et al., 2013). Besides a reduction of H3K4me3 prior to endodormancy release, the authors identified a tendency for *PpyMADS13-1* promoter to lose the histone variant H2A.Z during dormancy progression (Saito et al., 2015). Within this context, the decrease of *PpyMADS13-1* expression during dormancy progression, along with the removal of this histone variant, add a new layer of complexity to the epigenetic regulation on dormancy–related genes. Finally, recent studies demonstrated that DNA methylation and small interference RNAs (siRNAs) may also participate to the regulation of the sweet cherry *DAM* genes *PavMADS1* and *PavMADS2*. Near dormancy release, *PavMADS1* promoter presented an increase in DNA methylation as well as in the abundance of matching siRNAs (Rothkegel et al., 2017). These findings suggest that both DNA methylation and siRNAs are one of the mechanisms responsible for the seasonal expression profile of these

genes during the dormancy process. Taken together, these results indicate that a complex regulatory network involving epigenetic marks is acting over the *DAM* genes during dormancy.

Another important dormancy regulator gene is called *EARLY BUD-BREAK 1* (*EBB1*), which controls the timing of bud break in poplar by playing an integrative role in the reactivation of the SAM after dormancy (Yordanov, Ma, Strauss, & Busov, 2014). To date, the molecular regulation of bud break and its epigenetic control are poorly understood. Interestingly, transcripts of *DML* gene were localized in poplar apices, similar to that reported for *EBB1* (Conde et al., 2017b; Yordanov et al., 2014). However, the direct target genes of *DML*-dependent DNA demethylation and *EBB1* are not related, indicating that they may act by separate pathways to control bud break. In pear, the orthologous *EBB1* gene *PpyEBB* was highly induced prior to bud break in flower buds, and the analysis of its chromatin status revealed high levels of active histone modifications (H3K4me3) during ecodormancy in relation to endodormancy (Anh Tuan et al., 2016). However, no changes were detected in the levels of H3K27me3 in the same time points. Interestingly, two of the most important dormancy regulators discovered so far, *DAM* and *EBB* genes, are under strong epigenetic control. Taken together, these evidences show that epigenetic mechanisms play an important role in the regulation of bud dormancy, and a better understanding of these processes may help developing a valuable resource to genetically manipulate this trait.

4. *FT*: A MASTER SWITCH CONTROLLING FLOWERING IN ANGIOSPERMS

4.1 The Florigen *FT* Integrates Signals from Different Floral Pathways

The decision to flower is one of the most important steps for plant reproductive success and survival and represents a major life cycle transition in which meristem cells must commit with reproductive development. The flowering pathways have been extensively studied in the model plant *A. thaliana* (Amasino, 2005, 2010; Andrés et al., 2014; Lee et al., 2006; Mateos et al., 2015; Michaels & Amasino, 1999; Moon, Lee, Kim, & Lee, 2005). Five major floral pathways were characterized so far and they finely tune the time of flowering via endogenous (gibberellins, autonomous, and aging), as well as environmental factors (photoperiod and vernalization)

(Blümel, Dally, & Jung, 2015; Khan, Ai, & Zhang, 2014; Lucas-Reina et al., 2016; Song, Ito, & Imaizumi, 2013).

The vernalization and the autonomous pathways control flowering via repression of the *FLC* gene. The photoperiodic pathway involves the perception of the day length by the circadian clock that induces the expression of *CONSTANS* (*CO*). In *Arabidopsis*, flowering is anticipated during long days and delayed during short days, making it a facultative long day plant. The gibberellins (GA) pathway is a hormonal control of flowering and studies have reported that GA levels increase in the leaves and meristems of *Arabidopsis* plants, inducing the expression of the *FT* gene (Andrés et al., 2014; Song et al., 2013). The aging pathway is regulated by the balance of two microRNAs, *miR156* and *miR172*, that regulate transition from juvenile to adult and reproductive phase. All of the flower signaling pathways in *Arabidopsis* are integrated by the *FT* gene, which ultimately regulates the expression of the floral meristem identity genes *LEAFY* (*LFY*) and *APETALA 1* (*AP1*). These major floral pathways are summarized in Fig. 2. Other recently discovered floral pathways include regulation of flowering via ambient temperature, stress, nutrients and sugar balance (for detailed reviews on the flowering pathways see Andrés & Coupland, 2012; Blümel et al., 2015; Cho, Yoon, & An, 2017; Khan et al., 2014; Lucas-Reina et al., 2016; Song et al., 2013).

Different plant species may present different signaling mechanisms for the same endogenous and environmental factors that trigger vegetative-to-reproductive meristem transition, meaning that there is a functional diversification in floral pathways among angiosperms (Blümel et al., 2015; Lucas-Reina et al., 2016). For example, wheat, barley, and *Arabidopsis* all have a cold (vernalization) requirement to induce flowering. However, while in *Arabidopsis* vernalization causes the repression of the flowering-inhibitor *FLC* gene, in wheat and barley vernalization induces the expression of the *VERNALIZATION 1* (*VRN1*) gene that directly binds to *FT* and promotes inflorescence formation (Fig. 1; Andrés & Coupland, 2012; Deng et al., 2015).

The hormone GA seems to affect flower formation differently in *Arabidopsis* compared to several perennial fruit trees including apple, mango, cherry, peach, apricot, almond, and lemon (Khan et al., 2014; Turnbull, Anderson, & Winston, 1996; Upreti et al., 2013; Zhang et al., 2016). While GA is a potent inducer of flowering in *Arabidopsis*, it has been reported that exogenous application of GA usually leads to a reduction of flower formation in fruit trees (Khan et al., 2014; Turnbull et al., 1996; Zhang et al., 2016).
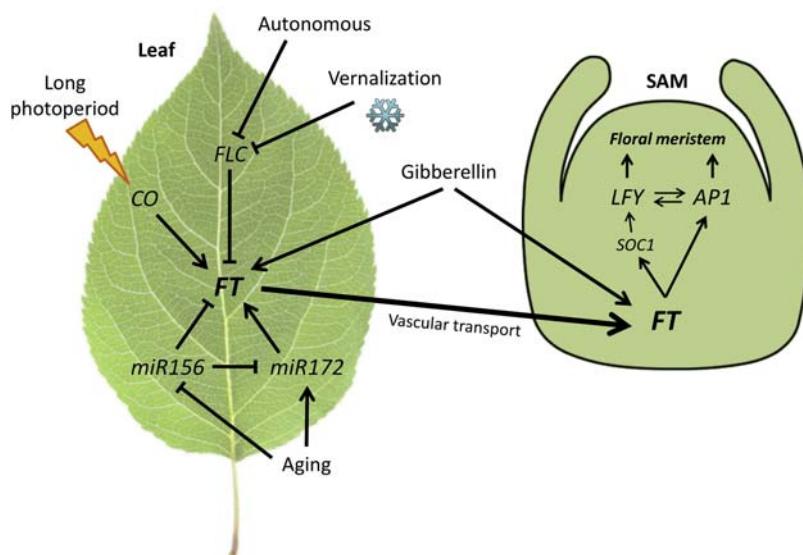
Figure 2 *Major floral pathways in Arabidopsis.* Different signaling pathways converge to the *FT* gene, whose protein and mRNA are translocated to the shoot apical meristem (SAM) via vascular transport (Andrés & Coupland, 2012; Jackson & Hong, 2012). The FT protein in the SAM induces the expression of *SOC1*, an activator of *LFY*. At the same time, FT protein also induces the expression of *AP1*, another floral meristem identity gene. The signal initiated by *FT* is amplified via feedforward regulation: *FT* induces *LFY* and *AP1*, and *LFY* and *AP1* continue to induce each other. This mechanism ensures that after *FT* activation, the meristem becomes committed with reproductive development and the process can no longer be reversed.

Moreover, application of paclobutrazol, an inhibitor of GA biosynthesis, was able to even increase the flowering rate of apple and promote early-flowering in mango (Upreti et al., 2013; Zhang et al., 2016). Zhang et al. (2016) have proposed a model in which GA acts by reducing cytokinin (CK) levels in apple shoots, and that a high CK/GA ratio is necessary to promote flower formation. Notwithstanding, the pathways by which GA affects flowering differently in *Arabidopsis* and in most perennials, are not fully understood. This highlights the difficulty of describing a universal model for floral signaling pathways in angiosperms and has led many review papers to focus on the flowering pathways of specific groups of plants (Deng et al., 2015; Guo et al., 2015; Hanke, Flachowsky, Peil, & Hättasch, 2007; Ramírez & Davenport, 2010; Sun et al., 2014; Weller & Ortega, 2015).

Despite all of the efforts aiming at understanding how different species perceive environmental cues and endogenous signals to promote flower

formation, only scarce information concerning the molecular signaling of reproductive meristem formation is available for perennials with a winter dormancy period. This may be due to the fact that differentiation of vegetative meristems into flower organs usually occurs concomitantly with the formation of buds for the next growing cycle (known as flower buds) (Foster, Johnston, & Seleznyova, 2003; Hanke et al., 2007) and also because plants tend to resume flowering soon or immediately after bud break, which makes dormancy the main limiting factor for the cultivation of temperate plants in warmer climates.

Noteworthy, regardless of the pathway that a given plant may utilize to promote floral development, the integration of the flowering pathways seems to converge to the *FT* gene and this appears to be highly conserved among higher plants, between dicots and monocots, and between annual and perennial species (Blümel et al., 2015; Khan et al., 2014; Lucas-Reina et al., 2016; Putterill & Varkonyi-Gasic, 2016). For instance, in both rice and *Arabidopsis*, the *FT* gene is expressed in leaves and the protein and mRNA are translocated to the apical meristem, acting as a strong promoter of flowering (Amasino & Michaels, 2010; Andrés & Coupland, 2012; Jackson & Hong, 2012; Sun et al., 2014). Similarly, studies involving woody perennial dicots and the annual herbaceous plant *Arabidopsis* revealed the conserved role of *FT* genes on flowering (Kotoda et al., 2010; Tränkner et al., 2010). More specifically, the constitutive expression of the apple *MdoFT1* gene was able to induce flowering of *in vitro* shoot cultures of apple and *Populus* (Kotoda et al., 2010; Tränkner et al., 2010). Ectopic expression of the apple *MdoFT1* or *MdoFT2* genes in the annual plant *Arabidopsis* was also able to induce an early-flowering phenotype under long days in wild-type background plants (Kotoda et al., 2010; Tränkner et al., 2010). Besides, several other reports involving the homologous and heterologous expression of *FT* and *FT*-like genes from different plants show that *FT* is able to promote flowering regardless of the species (Blümel et al., 2015; Putterill & Varkonyi-Gasic, 2016; Wickland & Hanzawa, 2015). Thus, the *FT* signaling network appears to constitute a highly conserved convergence hub for the flowering pathways in higher plants (Fig. 2).

4.2 Epigenetic Control of *FT* Expression

The expression of a given gene is affected by different factors, including the genetic information that is contained in the DNA sequence (*e.g. cis*-elements in a promoter), and by epigenetic modifications in the chromatin that can, in some cases, be transmitted to progeny (*e.g.* histone modifications and DNA

methylation; see chapters 1 and 2). Like many other genes, *FT* chromatin is also subjected to epigenetic modifications that participate to the control of gene expression, thereby determining flowering time, as illustrated below.

As part of the ambient temperature flowering pathway, *Arabidopsis* plants growing under long days in temperatures of 27°C show accelerated flowering compared to plants growing at 23°C. This has been attributed to PHYTOCHROME INTERACTING FACTOR 4 (PIF4) binding to the *FT* promoter under high temperatures (27°C). At 23°C, binding of PIF4 to the *FT* promoter is hampered by the occupancy of histone H2A variant H2A.Z at the *FT* locus. Exposure of plants to high temperatures of 27°C triggers the eviction of H2A.Z nucleosomes occupying the *FT* locus. The rate of histone H2A.Z occupancy in the chromatin is one of the factors that mediate temperature responses in *Arabidopsis* and its presence on the *FT* locus decreases accordingly under high temperatures, allowing the binding of PIF4 to the *FT* promoter and flowering (Fig. 3; Lucas–Reina et al., 2016; Song et al., 2013).

The protein AT-HOOK MOTIF NUCLEAR LOCALIZED 22 (AHL22) was described as a repressor of flowering. Overexpression of *AHL22* causes a late–flowering phenotype by reducing the levels of *FT* mRNA. AHL22 binds to AT-rich DNA sequences within the *FT* locus where it recruits histone deacetylases (HDACs), HDA1/HDA19, HDA6, and HDA9. These HDACs remove acetyl groups from the N-terminal tail of histones H3, thus promoting the silencing of the *FT* gene. Evidence also suggests that AHL22 mediates dimethylation of histone H3K9 by recruiting specific histone methyltransferases, as suggested by the increase of H3K9me2 mark abundance in lines overexpressing *AHL22* (Fig. 3; Yun, Kim, Jung, Seo, & Park, 2012).

In *Arabidopsis*, it has been shown that activation of the *FT* gene by CO in the photoperiodic pathway is dependent on the direct interaction between MORF RELATED GENE (MRG) group proteins, MRG1 and MRG2, with the CO protein. The proteins MRG1 and MRG2 recognize H3K4me3 and H3K36me3 histone marks on the *FT* chromatin and allow the binding of CO to the *FT* promoter (Fig. 3). The *mrg1 mrg2* double mutant has reduced *FT* mRNA levels and a late–flowering phenotype associated with impaired binding of CO to the *FT* promoter under long photoperiods (Bu et al., 2014).

Presence of H3K4me3 close by the TSS is generally associated with active transcription (Guo et al., 2015; He, 2012). Absence of two redundant H3K4me3 demethylases, *Jumonji 4* (*AtJmj4*) and *EARLY FLOWERING 6*
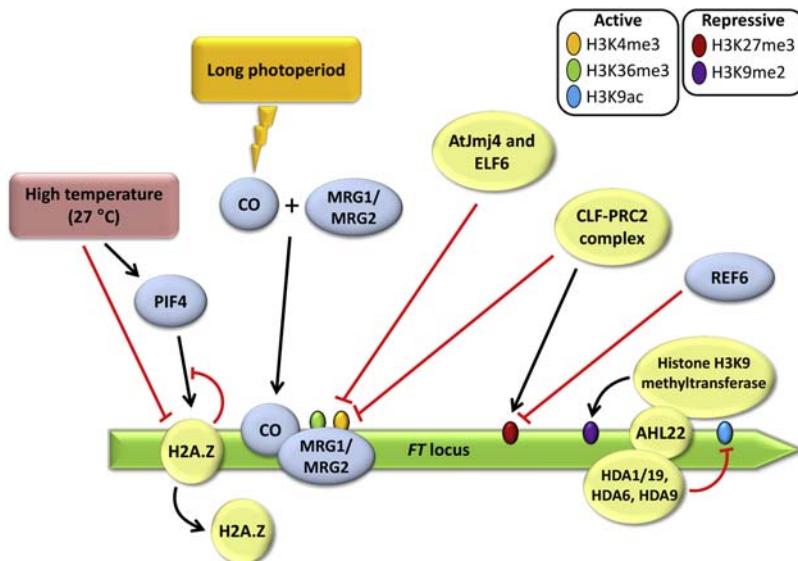
Figure 3 *Epigenetic mechanisms controlling* FT *expression in Arabidopsis.* The binding site of PIF4 to the *FT* promoter is occupied by the histone variant H2A.Z. Exposure to high temperatures causes the eviction of H2A.Z from the *FT* locus, allowing the binding of PIF4. MRG1 and MRG2 recognize methylated H3K4 and H3K36 histone marks, allowing the binding of CO to the *FT* promoter. The binding of CO can be counteracted by the activity of H3K4me3 demethylases (AtJmj4 and ELF6). The CLF-PRC2 complex silences *FT* via trimethylation of histone H3K27 and via demethylation of H3K4me3 marks. REF6 removes H3K27me3 marks, acting against the CLF-PRC2 complex to allow *FT* transcription. AHL22 represses *FT* by recruiting histone deacetylases (HDA1/19, HDA6, and HDA9) and histone H3K9 methyltransferases. Active and repressive histone modifications are indicated in the upper right, respectively. Proteins acting to activate *FT* transcription are depicted in blue, whereas proteins whose function is to silence *FT* expression are pictured in yellow.

(*ELF6*), causes an early–flowering phenotype in *Arabidopsis* under both short and long days (Fig. 3; Jeong et al., 2009). In another study, CURLY LEAF (CLF), an Enhancer of zeste protein constitutive of PRC2, was shown to be a strong repressor of *FT* by mediating the trimethylation of H3K27 and by partially suppressing H3K4me3 marks into the *FT* chromatin (Jiang, Wang, Wang, & He, 2008). In contrast, RELATIVE OF EARLY FLOWERING 6 (REF6) was shown to be an important histone H3K27 demethylase promoting *FT* expression, acting dynamically against the CLF-PRC2 complex (Fig. 3; He, 2012).

Interestingly, it has been demonstrated that deposition of active and repressive histone modifications occur independently of each other

and are mutually antagonistic (Jiang et al., 2008; Shafiq, Berr, & Shen, 2014). In other words, activation or repression of *FT* expression in *Arabidopsis* relies on the relative abundance of active or repressive epigenetic marks. The implications of this finding is that, in order to fully understand how plants control the time of flowering, it may be necessary to look further into the mechanisms that regulate the balance of active and repressive epigenetic marks on the *FT* chromatin.

In rice, chromatin methylation also appears to play an important role in the expression pattern of two *FT* homologs, *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*). Knockdown plants with reduced synthesis of S-Adenosyl-l-methionine, a universal methyl group donor, show low levels of histone H3K4me3 and DNA methylation at the *Hd3a* and *RFT1* loci, which was associated with reduced expression of these genes and late–flowering phenotype (Sun et al., 2014). Two genes that are responsible for histone H3K36 di– and tri-methylation, *SET DOMAIN GENE 724* (*SDG724*) and *SDG725*, were also shown to be important for flowering time regulation in rice. A loss–of–function mutant for *SDG724* and a knockdown plant for *SDG725* were associated with a reduction of H3K36 methylation and transcription of *RFT1* and *Hd3a*, and of other flower–related genes (Sun et al., 2014). The presence of H3K9ac around the TSS of *RFT1* was also correlated with active gene transcription and flowering in rice (Sun et al., 2014).

As mentioned above, little information regarding the flowering pathways of perennial plants is available, and even less or no information at all on the epigenetic regulation of *FT* genes of perennials might exist. Nevertheless, a study with sexual dimorphism in poplar has shown that DNA methylation is an important regulatory factor for flower development in *Populus tomentosa* (Song et al., 2012b). In another work using the early–flowering trifoliate orange *Poncirus trifoliate*, the authors treated plants with 5-azacytidine, a DNA methyltransferase inhibitor, and observed several abnormalities related to vegetative development, without noticeable changes in the flowering time of the already precocious trifoliate orange (Zhang, Mei, Liu, Khan, & Hu, 2014). Notwithstanding, it was observed an increase in *CiFT* transcript accumulation with increasing concentrations of 5-azacytidine. The expression level of other flowering genes *CiLFY*, *CiAP1*, *TERMINAL FLOWER 1* (*CiTFL1*), and *CiFLC* was highest at 250 μM of 5-azacytidine and then decreased at higher concentrations (Zhang et al., 2014). Because a decrease in the overall DNA methylation pattern led to an increase in the expression of five major floral genes, the

authors concluded that DNA methylation plays an important role in the regulation of the early-flowering trait of trifoliate orange (Zhang et al., 2014).

The knowledge regarding how plants flower is of utmost importance for breeding programs, especially for perennial fruit trees, in which a long juvenile phase represents a significant hindrance. Activation of *FT* and *FT*-like genes through homologous or heterologous expression in juvenile perennials is sufficient to accelerate entrance into the reproductive phase. *FT* is regarded as the master switch that must be turned on by angiosperm plants to initiate flower formation and complete their reproductive cycle. Epigenetics is a rapidly growing field and we address here that a direct link between epigenetics and the *FT* gene is essential to flowering. As a future perspective, we believe that understanding the genetic mechanisms underlying the epigenetic control of *FT* expression (and how this is linked to repression of *FT* expression in juvenile perennials) could help breeding programs seek important characteristics to shorten the juvenile phase in perennial fruit trees.
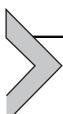
5. CHROMATIN REMODELING AND HORMONAL STIMULUS: AN INHERENT PART OF THE FLOWERING PROCESS

The regulation of flowering time depends on many factors including environmental, genetic, and epigenetic mechanisms as detailed in the previous sections. Hormonal regulation plays a central role in this process, integrating internal and external stimulus. In this section, we will explore the importance of well-known phytohormones in the flowering process, connecting them with epigenetic modifications that occur in the chromatin during floral transition (Campos-Rivero et al., 2017; Yamamuro, Zhu, & Yang, 2016).

5.1 Gibberellins

In the model plant *A. thaliana*, the timing of flowering mediated by GA signaling has been extensively studied, leading to the elucidation of some of the underlying molecular mechanisms. The induction of flowering starts with the perception of bioactive GAs and is mediated, among other factors, by nuclear proteins called DELLA. Briefly, DELLA are negative regulators of GA signaling that sequestrate transcriptional activators, inhibiting flowering (Achard & Genschik, 2009; Harberd, 2003). In leaves, the transcriptional activators of *FT*, like CO and PIF4 are sequestered by

DELLA proteins, preventing them from binding to DNA and activating *FT* transcription (Conti, 2017; de Lucas et al., 2008; Tiwari et al., 2010; Wang et al., 2016; Xu et al., 2016). In the SAM, DELLAs bind to SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcriptional regulators and prevent their function as transactivators on target genes (Conti, 2017; Hyun et al., 2016; Yu et al., 2012). When GA is present, the GID1 receptor undergoes conformational changes, increasing its affinity for DELLA proteins (Griffiths et al., 2006; Willige et al., 2007). This interaction (DELLA–GID1) favors the binding of the E3 Ubiquitin ligase SLEEPY1 (SLY1) that marks DELLA for degradation by proteasome 26 (Dill, Thomas, Hu, Camille, & Steber, 2004). Without DELLA proteins, the activators CO and PIF4 are free to promote *FT* expression. In the SAM, SPLs are able to activate *LFY, FRUITFUL (FUL), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* and *AP1* by directly binding to their promoter regions. Expression of these genes contribute to the amplification of the FT-FD signal and causes the activation of the floral meristem identity genes (Abe et al., 2005; Conti, 2017; Melzer et al., 2008; Moon et al., 2003). In contrast to *Arabidopsis*, application of exogenous GAs were shown to inhibit flower formation in some perennial crops like apple (Marcelle & Sironval, 1963; Zhang et al., 2016), peach (Southwick & Glozer, 2000) and citrus (Goldberg-Moeller et al., 2013; Guardiola, Monerri, & Agusti, 1982; Tan & Swain, 2006), as previously described in this chapter. Some works reporting the molecular link between chromatin modifications and GA responses have been already published and will be now more thoroughly discussed.

PKL genes code for an CHD3-family ATP-dependent chromatin remodeling factor, that promote the trimethylation of H3K27, contributing for tissue-specific gene repression in plants (Ogas, Kaufmann, Henderson, & Somerville, 1999; Zhang et al., 2008, 2012b). Interestingly, analyses of *pkl* mutant plants showed a defective ability to respond to GA, demonstrating the important role of methylation in the induction of GA transcriptional network (Rider et al., 2003; Zhang et al., 2008). A recent study showed that PKL is required to promote flowering in Arabidopsis, probably due to its close interaction with DELLA proteins (Park et al., 2017). Another interesting point is that histone modifications are able to regulate genes involved in GA biosynthesis. Some examples as *GA REQUIRING1 (GA1), GA2, GA3, ENT-KAURENOIC ACID HYDROXYLASE1 (KAO1)* and *KAO2* have already been reported to undergo H3K27ac modification (Charron, He, Elling, & Deng, 2009).

Dioxygenase genes (*GA2ox7* and *GA3ox2*) are the major targets for light regulation in the GA metabolism (Kamiya & García-Martínez, 1999) and showed to be downregulated by H3K27me3. The higher transcript accumulation of *GA2ox7* and *GA3ox2* correlates with the reduction of H3K27me3 abundance over the promoter regions and first exons of dioxygenase genes, removing the repressive effect of this modification (Charron et al., 2009). On the other hand, genes that are not affected by the GA signaling have no differences in H3K27me3 levels (Charron et al., 2009). These results suggest an import role of epigenetics modifications in GA biosynthesis, signaling and response, influencing flowering time.

5.2 Jasmonic Acid

Jasmonic acid (JA) is a fatty acid–derived molecule that orchestrates different responses to abiotic and biotic stress in plants (Browse, 2009; Stintzi & Browse, 2000) and plays important functions in the regulation of floral development in *Arabidopsis* (Park et al., 2002; Stintzi & Browse, 2000), rice (Cai et al., 2014; Xiao et al., 2014), maize (Acosta et al., 2009; Yan et al., 2012), tomato (Li et al., 2004) and tobacco (Stitz, Hartl, Baldwin, & Gaquerel, 2014). The currently accepted model proposes that, in the absence of JA, repressors of JASMONATE-ZIM domain (JAZ) family interact with the transcriptional factors MYB21 and MYB24, preventing them to activate responsive JA genes. In contrast, the JA signal makes JAZ proteins a potential target for the F-box protein CORONATINE INSENSITIVE PROTEIN 1 (COI1) to ubiquitination and subsequent degradation (Chini et al., 2007). As soon as JAZ is degraded, these factors are released and can activate genes involved in anther development and filament elongation (Song et al., 2011).

Studies developed in *Arabidopsis* have now revealed the importance of HDA6, a RPD3-type histone deacetylase, in JA response, senescence and flowering (Wu, Zhang, Zhou, Yu, & Chaikam, 2008). The interaction of HDA6 with COI1, has been demonstrated (Devoto et al., 2002), suggesting an important connection between JA response and histone modifications. The analysis of *axe1-5*, a splice site mutant of *AtHDA6* (Murfett, Wang, Hagen, & Guilfoyle, 2001) and HDA6-RNAi plants have showed that the JA responsive genes, *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* were downregulated in these plants, reinforcing the idea that HDA6 is necessary to the JA-responsive pathway (Wu et al., 2008). Furthermore, *axe1-5* and HDA6-RNAi plants displayed increased leaf longevity and late flowering compared to wild type plants. These plants also showed an hyperacetylation of histones

at the *FLC* gene and an increase in its expression, suggesting that HDA6 is required for *FLC* deacetylation and repression (Wu et al., 2008). Little is known about epigenetics modification and JA response in perennial plants, revealing a huge gap that remains to be investigated.

5.3 Brassinosteroids

Brassinosteroids (BR) are a class of polyhydroxylated steroidal phytohormones that are involved in many aspects of plant biology, including cell elongation, cell division, root growth, photo-morphogenesis, stomatal and vascular differentiation and seed germination (Gudesblat & Russinova, 2011; Wei & Li, 2016). Phenotype analyses of BRs defective mutants revealed a delayed flowering time, indicating a positive role of BRs in the establishment of flowers (Domagalska et al., 2007; Li, Li, Chen, & An, 2010). A higher level of *FLC* transcripts and increased levels of histone H3 acetylation normally associated with active chromatin at the *FLC* locus were observed in these mutants, suggesting that BRs could contribute to a silenced epigenetic state at the *FLC* promoter, helping to downregulated it and promote flowering (Domagalska et al., 2007).

The transcriptional factor BRI1-EMS suppressor 1 (BES1), one of the major regulators of BR signaling that coordinates several genes in response to BR (Yin et al., 2005), directly interacts with REF6 and its homolog, EARLY FLOWERING 6 (EFL6), both jumonji N/C (JmjN/C) domain-containing proteins essential to catalytic demethylation of histones (Agger, Christensen, Cloos, & Helin, 2008). REF6 and EFL6 (recruited by BES1) have been shown to be involved in BR signaling by affecting histone methylation in the promoters of BR-responsive genes (Noh et al., 2004; Yu et al., 2008). BES1 can also recruit a histone lysine methyltransferase called SET DOMAIN GROUP 8 (SDG8), which is implicated in H3K36 di- and trimethylation (Wang et al., 2014; Xu et al., 2008).

Analysis of the *rice plasticity 1* (*rpl1*) mutant that is impaired in a gene involved in plastic response of rice plants to environmental changes, have also demonstrated an interesting connection between BRs and histone modification (Zhang et al., 2012a). The *rpl1* mutant showed an increased in DNA methylation level and a decrease in overall histone acetylation. Responses to various plant phytohormones including BRs were also negatively affected. Noteworthy, the putative rice BR receptor *OsaBRI1*, a key hormone signaling gene, was extremely downregulated in the *rlp1*

mutant concomitantly to changes in histone marks in the *OsaBRI1* gene locus (Zhang et al., 2012a), reinforcing the importance of epigenetic regulations in BR perception and signaling.

5.4 Ethylene

Ethylene (ET) is a volatile organic chemical compound involved in fruit ripening, senescence of leaves, response to stress and a floral repressor in *Arabidopsis* (Achard et al., 2006). The transcription factor ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-like (EIL) mediate ET transcriptional responses (Guo & Ecker, 2003). EIN3 accumulation delays flowering by activating the *ETHYLENE RESPONSE 1* (*ERF1*) related genes. The negative role of ET in flowering through the EIN3-ERF1 is attributed to reduced bioactive GA levels, causing enhanced accumulation of DELLA proteins (Achard et al., 2007). In the work reported by Zhou, Zhang, Duan, Miki, and Wu (2005), the importance of HDA19, a RPD3-type histone deacetylase, in integrating hormonal stimulus and epigenetic regulations was analyzed in *Arabidopsis*. By overexpressing *HDA19*, an increased expression of *ERF1* was detected, showing that histone deacetylation positively regulates *ERF1*, contributing to an increased delay in flowering (Zhou et al., 2005). These observations contributed to elucidate the roles of histone modification and ET response. However much more efforts need to be done to completely understand this hormonal-epigenetics regulation.

5.5 Salicylic Acid

Salicylic acid (SA) is an important phytohormone that belongs to the group of phenolic compounds and plays distinct roles in many aspects of plant growth and development, as well as in disease resistance (Vicente & Plasencia, 2011). The importance of SA in the flowering process is already described (Cleland & Ajami, 1974; Lee & Skoog, 1965) but the knowledge about its role in the floral regulatory network is still very limited. Arabidopsis SA-deficient plants show a late-flowering phenotype and an increased expression of *FLC*. Low levels of *FT* transcripts were also observed in these mutants compared to wild type, under short day or long day conditions (Martínez, Pons, Prats, & León, 2004).

On the other hand, the *CO* and *SOC1* genes showed a different profile under the same daylength conditions in SA-deficient plants (Martínez et al., 2004). *CO* and *SOC1* expression decreased in long day conditions, while in short day-grown plants, amounts of *CO* transcripts increased and *SOC1* remained stable (Martínez et al., 2004). Given that, *CO* and *SOC1* are

strictly regulated by light changes (Suárez-López et al., 2001), suggesting that SA regulates flowering by interacting with the photoperiod dependent pathway (Martínez et al., 2004; Vicente & Plasencia, 2011).

Sumoylation is a post-translational modification that conjugates small ubiquitin modifier peptides (SUMO) to protein substrates (Mahajan, Delphin, Guan, Gerace, & Melchior, 1997; Matunis, Coutavas, & Blobel, 1996). In mammals and yeasts sumoylation has been included in the list of histone modifications associated to transcriptional repression (Garcia-Dominguez & Reyes, 2009; Nathan et al., 2006; Shiio & Eisenman, 2003). In plants, it is related to biotic and abiotic stress responses, flowering and development (Chosed, Mukherjee, Lois, & Orth, 2006; Jin et al., 2008; Novatchkova, Budhiraja, Coupland, Eisenhaber, & Bachmair, 2004). Analysis of plant mutants in the *SUMO E3 ligase* (*SIZ1*) gene, which play crucial role during SUMO steps, revealed an early flowering time phenotype under short day (Jin et al., 2008). It has also showed elevated SA levels, suggesting an important connection between SUMO modification and SA response (Jin et al., 2008). The authors concluded that under low levels of SA an increased *SIZ1* expression is observed, contributing to sumoylation of *FLOWERING LOCUS D* (*FLD*) and its consequent low expression. The *FLD* gene encodes a plant homolog to an important protein in mammals histone deacetylase complexes (He, Michaels, & Amasino, 2003). Mutations in *FLD* and its consequent low expression promote hyperacetylation of histones in *FLC* chromatin, leading to its transcriptional activation and delayed flowering time (He et al., 2003). Nevertheless, when high levels of SA are perceived, *SIZ1* is repressed, which increases FLD levels and results in lower *FLC* transcript amounts, allowing floral transition (Campos-Rivero et al., 2017; Jin et al., 2008).

5.6 Cytokinins

CK are plant hormones involved, in cell proliferation, differentiation and many other related processes (Werner et al., 2008; Werner & Schmülling, 2009). Their role in the progress of dormancy to flowering is still unclear, although studies showed that cell division is higher in SAM during floral transition (Jacqmard, Gadisseur, & Bernier, 2003). The literature about epigenetic regulation and the role of CK in flowering induction is still sparse, but some insights have been described (Godge, Kumar, & Kumar, 2008; Li et al., 2008; Meijón, Jesús Cañal, Valledor, Rodríguez, & Feito, 2011; Tanaka et al., 1997). The study of Li et al. (2008) analyzed *A. thaliana* plants mutated for the *S-adenosyl-L-homocysteine hydrolase* (*SAHH*) gene, which

codes for a key enzyme in the stabilization of methylation potential in cells (Palmer & Abeles, 1979; Tanaka et al., 1997). They also investigated RNAi lines with reduced *AtSAHH2* gene expression and observed a global reduction of DNA methylation levels and high levels of endogenous CK, even when compared to CK overproducers (Catterou et al., 2002; Chang, Jones, Banowetz, & Clark, 2003).

Similarly, increased levels of CK were observed in *sahh1-1* tobacco mutants (Tanaka et al., 1997). In tobacco plants, it was also observed that CK upregulate genes encoding Cytosine DNA methyltransferases (Li et al., 2008). The overexpression of *SAHH1* and *Adenosine kinase 1* (*ADK1*) in response to CK was similarly detected (Li et al., 2008; Pereira et al., 2007). Taken together, these results indicate a 'feedback–regulatory loop between cytokinin and DNA methylation: reduced DNA methylation potential in plant cells seems to lead to an increased level of CK, which in turn stimulates the genes encoding enzymes of the DNA methylation machinery' (Li et al., 2008). Moreover, the delayed flowering time found in Arabidopsis mutants knockdown for *SAHH* could be related to the accumulation of CK in response to reduction of global DNA methylation (Campos–Rivero et al., 2017; Li et al., 2008; Rocha et al., 2005). In the same way, the treatment with 9-(S)-(2,3-dihydroxypropyl)–adenine (DHPA), an inhibitor of SAHH in tobacco plants resulted in global DNA hypomethylation, flower morphology alterations, reduced fertility and upregulation of floral organ identity genes (Fulneček et al., 2011). The analysis of a gene coding for a CK binding protein, *PETUNIA CYTOKININ BINDING PROTEIN* (*PETCBP*) from *Petunia hybrida* cv. Mitchell, revealed a highly similar sequence (85%−90%) to the *SAHH* gene from several plant species (Godge et al., 2008). Authors suggest that PETCBP could be a positive regulator of CK response, mainly by modulating CK signal transduction. They also report that antisense expression of this gene resulted in plants with delayed flowering time (Godge et al., 2008).

Another work reported by Meijón et al. (2011) investigated if the treatment with GA inhibitors applied to improve flower production in ornamental azaleas plants could also influence DNA methylation and other phytohormones (as CK) during floral transition. They found that before flower set, low DNA methylation levels were seen, but when floral organs formation was achieved, genomic DNA became hypermethylated. They also observed low levels of GAs (as expected) and high endogenous CK amounts during floral transition. The authors suggested that CK could

induce DNA demethylation, thereby contributing to the induction of gene expression during flowering transition (Meijón et al., 2011).

5.7 Abscisic Acid

ABA is a phytohormone associated mostly with drought stress and important to coordinate an adaptive response during water deprivation (Shinozaki & Yamaguchi-Shinozaki, 2007). Moreover, ABA plays important roles in plant development, even without stress stimuli, like induction of seed and bud dormancy (Barrero et al., 2005). During the transition of flowering, both positive and negative effects of ABA have been reported (Riboni, Test, Galbiati, Tonelli, & Conti, 2016, 2013; Shu et al., 2016; Wang et al., 2013; and reviewed by; Shu, Luo, Meng, & Yang, 2018). Briefly, the delay of *A. thaliana* floral transition is strictly related to *ABSCISIC ACID-INSENSITIVE* (*ABI*) genes. Studies analyzing the *Arabidopsis abi3*, *abi4* and *abi5* mutants revealed an early flowering phenotype; consistently to that, transgenic plants overexpressing those genes (*ABI3*, *ABI4* and *ABI5*) showed delayed floral transition (Shu et al., 2016; Wang et al., 2013). *ABI4* and *ABI5* directly induces *FLC* transcription, while FLC further represses expression of *FT* in Arabidopsis, regulating the flowering process in a negative way (Shu et al., 2018; Wang et al., 2013).

Recently, Shu et al. (2016) proposed that ABI4 negatively controls GA biogenesis, suggesting that it may repress flower formation through the GA flowering pathway. Positive regulation of flowering mediated by ABA response occurs especially under environmental stress conditions, such as drought (Riboni, Galbiati, Tonelli, & Conti, 2013, 2016). It is already described that drought stress causes early flowering in various plant species and high level of ABA biosynthesis (Budak, Hussain, Khan, Ozturk, & Ullah, 2015; Munemasa et al., 2015; Riboni, Test, Galbiati, Tonelli, & Conti, 2014). Molecular mechanisms required for ABA response in order to promote flowering and overpass the drought stress is still under evaluation, but some key factors were already described. Riboni et al. (2016) proposed that in Arabidopsis, ABA induces the drought escape response by promoting *FT* expression, for which *CO* and flower–promoting gene *GIGANTEA* (*GI*) genes are required in a photoperiod dependent manner. In citrus species, water deficit promotes flowering, and the higher level of ABA was also detected during the floral inductive stage (Li et al., 2017). Furthermore, in litchi species, drought stress combined with low temperature remarkably promoted flowering; however, the detailed relationship

between ABA biosynthesis and/or signaling and flowering in litchi needs further investigation (Shen, Xiao, Qiu, Chen, & Chen, 2016).

In the epigenomic context, the nuclear interaction of SWI/SNF (chromatin remodeling complex) with *ABA HYPERSENSITIVE* (*HAB1*) gene, involved in negative regulation of ABA signaling and flower induction in Arabidopsis, was reported as necessary for ABA-responsive gene regulation (Rodriguez, Leube, & Grill, 1998; Saez, Rodrigues, Santiago, Rubio, & Rodriguez, 2008).

Another work showed that Arabidopsis mutants impaired in the *ABA OVERLY-SENSITIVE* (*ABO4*) gene that encodes an important regulator of ABA plant responses (Liu et al., 2010), showed an early flowering phenotype. *abo4-1* plants showed lower expression of *FLC* and high amounts of *FT* transcripts. An altered histone modification pattern in these two loci were also observed (Yin et al., 2009). Thirunavukkarasu et al., (2014) reported that stress-responsive genes involved in the ABA signaling promote floral transition and are regulated by epigenetic events under water stress condition in maize. Altogether, these findings suggest an important crosstalk between epigenetics, ABA responses, drought stress and flowering.

5.8 Auxins

Auxins are phytohormones involved in many aspects of plant biology such as vascular tissue formation, adventitious root initiation, tropistic responses, apical dominance and fruit development (Goldfarb, Lanz-Garcia, Lian, & Whetten, 2003; Luo, Zhou, & Zhang, 2018; Sundberg & Østergaard, 2009). The first link between auxin and floral transition was reported when the auxin transport mutant *pin1* (*PIN-FORMED1*) was characterized (Gälweiler et al., 1998). The *PIN1* gene encodes for a member of the putative auxin efflux regulator proteins, important in polar auxin transport (Gälweiler et al., 1998) and have been related to plant organ formation by regulating auxin distribution (Benková et al., 2003; Furutani et al., 2004). The most intriguing characteristic of the *pin1* mutant was the formation of inflorescence without flowers. Two other mutants showed very similar phenotypes: *pinoid* (Bennett, Alvarez, Bossinger, & Smyth, 1995) and *monopteros* (*mp*) (Przemeck, Mattsson, Hardtke, Sung, & Berleth, 1996). PINOID is a serine/threonine protein kinase and have been reported to be involved in signaling and polar auxin transport (Benjamins, Quint, Weijers, Hooykaas, & Offringa, 2001). MP/ARF5 is a member of a family of transcription factors called auxin response factors (ARFs), and are essential to auxin signaling and response (Hardtke &

Berleth, 1998; Quint & Gray, 2006). Interestingly, these mutants could progress from vegetative to reproductive phase while the inflorescence is formed, however no flower is observed (Cheng & Zhao, 2007). These findings demonstrate the important role of auxin in flower formation, even if the complete pathways connecting auxins and formation of floral primordial remain unclear (Cheng & Zhao, 2007). The link between epigenetic, auxin response and floral initiation was reported in a recent study which identified an auxin hormone-regulated chromatin state switch in order to promote floral primordial development in *Arabidopsis* (Wu et al., 2015). Based on these findings a model for cell auxin sensing and recruitment of chromatin remodeling factors was proposed. The presence of low auxin levels triggers a repressor system for auxin responsive genes that is orchestrated by monopteros (MP) and auxin sensitive Aux/IAA proteins (ASP). In this way, ASP binds to the MP factor associated with its target loci. ASP directly recruits the TOPLESS (TPL) repressor and the histone deacetylase HDA19, preventing gene expression (Guilfoyle & Hagen, 2012; Szemenyei, Hannon, & Long, 2008; Wu et al., 2015; Yamaguchi et al., 2013). The system also prevent recruitment of SWI/ SNF ATPase subgroup BRAHMA (BRM) and SPLAYED (SYD) chromatin remodeling complexes, creating an inactive chromatin state (Wu et al., 2015). However, when high levels of auxin are present, ASPs are degraded and prevent the action of TPL and HD19. The recruiting of BRM or SYD complexes is possible and an open chromatin state is achieved, enabling the activation of the general transcriptional machinery (Wu et al. 2015). In loss of function studies, the authors also showed that the SWI/SNF ATPase activity is essential for flower primordium initiation, revealing a 'simple and elegant mechanism for small-signaling-molecule-regulated chromatin state switch' that respond to auxin signal and is essential for flower development (Wu et al., 2015).
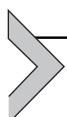
6. BUD BREAK AND FLOWERING: CONSEQUENCES IN AGRICULTURE AND BREEDING

Throughout the evolution, temperate fruit trees have developed bud dormancy as an adaption to seasonality (Campoy et al., 2011; Ionescu, Møller, & Sánchez-Pérez, 2017). Bud break and subsequent flowering happen after chilling and heating requirements have been fulfilled during winter and spring, respectively. Within the context of global climate change, there are a number of evidences that increasing temperatures results in

shifting the timing of phenological events, mainly in springtime, when plants resume growth (Hänninen & Tanino, 2011; Legave, Guédon, Malagi, El Yaacoubi, & Bonhomme, 2015; El Yaacoubi, Malagi, Oukabli, Hafidi, & Legave, 2014). Early bud break and flowering have many implications such as an increased risk of frost damage (Cannell & Smith, 1986; Vitasse, Lenz, & Karner, 2014) affecting the photosynthetic capacity of the trees (Ensminger, Schmidt, & Lloyd, 2007), bud burst delay combined with low burst and poor fruit set (Abbott, Zhebentyayeva, Barakat, & Liu, 2015; Celton et al., 2011; Dirlewanger et al., 2012; Erez, 2000), fertility abnormalities due to mis-synchronization of flowering of self-incompatible cultivars (Dirlewanger et al., 2012), changes in flower size and timing of anthesis (Scaven & Rafferty, 2013) and modifications of fruit harvesting periods and fruit marketing logistics (Dirlewanger et al., 2012). The production of floral scent, nectar and pollen can also be affected by temperature. Altered floral scent emission at higher temperatures affect the detectability of flowers by pollinating insects, such as moths, that rely on long-distance cues to locate floral resources (Kevan & Baker, 1983; Yuan, Himanen, Holopainen, Chen, & Stewart, 2009). Changes in nectar production and composition have immediate effects on pollinators activity and fitness (Burkle & Irwin, 2009), especially for those insects, such as some lepidopterans and wasps, that rely on nectar for amino acids as well as for sugars (Kevan & Baker, 1983). Similarly, decreased pollen production affect the reproductive success of many bees, which may need to collect pollen from a large number of plants to successfully rear their offspring (Müller et al., 2006). Along with the effects of warming on floral traits, elevated temperatures can alter other plant characteristics such as vegetative growth. It has been observed that plants exposed to winter warming of $1.5°C$ via open top chambers were several centimeters taller than plants in control chambers (Liu, Mu, Niklas, Li, & Sun, 2012). In contrast to the negative effects of the warming temperatures over the floral traits, increased vegetative growth may represent a benefit for agriculture in certain circumstances, such as wood and fruit crop production in sub-optimal environments. For example, the Brazilian apple cultivar 'Castel Gala', which is originated from a spontaneous bud sport mutation from a 'Gala Standard' tree, features a low CR when compared to the original cultivar, exhibits a precocious growth cycle that starts 25 days earlier and produces fruits that are anatomically and nutritionally equivalent (Denardi & Seccon, 2005). Due to its earlier bud burst, 'Castel Gala' also presents taller and more vigorous plants, reaching full production capacity earlier and can be planted in warmer

climate sites than its parental type. Examples like this and the abovementioned information help pave the way to better analyze the challenging context of breeding strategies to deal with bud phenology traits in order to generate adapted cultivars to the new climate scenarios for agriculture.

Bud dormancy can still be considered a biological black box, in which the outputs to certain inputs can be predicted with some degree of certainty, but the internal workings remain obscure. Epigenetic regulation seems to play roles in some central steps of the bud dormancy cycles, including gene expression adjustments that remain stable over cell cycles and the quantification of cold exposure. Although the participation of epigenetic modifications in bud dormancy is suggested by many research data in the literature, the genes responsible for transducing epigenetic marks into phenological behaviour remain to be fully characterized. *DAM* and *EBB* genes, at this time, are the most promising candidates; however, further studies on repressors of *FT* transcription in temperate woody plants may uncover new pieces of this puzzle.

Exploiting epigenetic variations (DNA methylation and histone PTMs) for breeding applications depends on the plant propagation strategies (sexual versus clonal, McKey, Elias, Pujol, & Duputié, 2010). Because DNA methylation patterns can be transmitted after mitosis and meiosis, DNA methylation marks could be useful in all crops, irrespective to their propagation mode. By contrast, histone modifications are reset during meiosis (Ingouff et al., 2010; Xiao & Wagner, 2015), therefore, they would be of little benefit for breeding purposes in sexually propagated crops. However, they could be applicable for clonally propagated crops, such as in perennial fruit crops, because new epigenetic patterns could be maintained in meristems for grafting propagation (Bräutigam et al., 2013; Douhovnikoff & Dodd, 2014). As examples of heritable epigenetic states, epimutations, irrespective of their origin (induced or natural epialleles), are generally stable in plant populations and can be selected as classical phenotypes in breeding schemes. An extensive review about the use of epigenetics for breeding purposes is described by Gallusci et al. (2017).

A successful example of the use of epigenetic research and its application for breeding purposes has been demonstrated in the African oil palm *Elaeis guineensis,* where a flower and fruit abnormality known as 'mantled' can develop in oil palm cultivars derived from tissue culture and the resulting mantled palms can become unproductive (Ong-Abdullah et al., 2015). By performing a genome-wide, unbiased, DNA methylation analysis to look for loci epigenetically associated with the mantled

phenotype, it was discovered that hypomethylation of a single *Karma* family retrotransposon embedded in the intron of the homeotic gene *DEFICIENS* is common to all mantled clones and associated with aberrant splicing and termination of the gene transcript. Loss of methylation — dubbed the *Bad Karma* epiallele — predicts a loss of oil palm yield and this property enable screening for higher-performing clones at the plantlet stage. This finding is likely to provide a way to detect unproductive palms much earlier than was previously possible, enabling their timely replacement in plantations, which besides the obvious economic importance, also benefits the environment. The results found for the 'mantled' phenotype in *E. guineensis* have also other key implications. For instance, a well-planned and performed genome-wide methylation mapping can pinpoint precise spots in the genome of a non-model organism that are responsible for a trait of interest.

Unveiling the molecular mechanisms responsible for the regulation of dormancy completion and flowering time in woody perennials is an important front for both basic and applied research. The compatibility between CR and the local climate is one of the main factors determining the success of a temperate fruit production enterprise. Conventional tree breeding, especially for dormancy traits, is costly and highly time-consuming because of protracted generation times. The advances in genomic research in woody perennials has now opened the opportunity to assist the breeders with tools generated through extensive sequencing, genome wide association studies and high density genotyping arrays that lead to the discovery of highly predictive molecular markers for traits of horticultural interest (Laurens et al., 2018). Therefore, markers linked to genes proved to participate in dormancy and flowering can be an invaluable asset for assisted breeding strategies. In combination with a biotechnological approach, master controllers of flowering time and bud dormancy could be used as tools to shorten the juvenile phase of target plant materials in order to accelerate breeding programs. This type of approach is already being suggested with the employment of transgenes that have the ability to induce flowering, such as *FT* (Yamagishi, Kishigami, & Yoshikawa, 2014), *TFL* (Freiman et al., 2012) and *BpMADS4* (Flachowsky et al., 2011; Weigl, Wenzel, Flachowsky, Peil, & Hanke, 2015) genes, but the use of dormancy regulators in similar strategies is still to be proposed. A different and potentially game changer strategy would be to modify the expression of dormancy controlling genes by means of state of the art technologies such as CRISPR/Cas9 (Bortesi & Fischer, 2015). This could potentially be done in already grown plants

employing viral vectors, a plant-breeding technique with no transmission of genetic modification to the next generation (Yamagishi et al., 2014). In fruit production, the application of chemical compounds is already used as a tool to modulate flowering time and increase yields (see review in Ionescu et al., 2017). Because epigenetic mechanisms participate in gene expression control and hormonal signaling, an alternative for plant breeding approaches is to engineer flowering time by combining efforts on modern genetics and chemicals via the external application of flower-inducing compounds. This is an illustration of how the understanding of flowering time and dormancy control can be important to applied research and to agriculture.

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4 CAPÍTULO II

Type-B cytokinin response regulators link hormonal stimuli and molecular responses during dormancy overcome in apple (Malus × domestica Borkh.)

Artigo submetido ao periódico "Plant Molecular Biology"

TITLE

Type-B cytokinin response regulators link hormonal stimuli and molecular responses during dormancy overcome in apple (*Malus × domestica* Borkh.)

Amanda Malvessi Cattani[1,2], Vítor da Silveira Falavigna[3], Carolina Pereira Silveira[2], Vanessa Buffon[2], Felipe dos Santos Maraschin[4], Giancarlo Pasquali[1], Luís Fernando Revers[1,2*]

[1]Graduate Program in Cell and Molecular Biology, Center for Biotechnology, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[2]Laboratory of Plant Molecular Genetics, Embrapa Uva e Vinho, Bento Gonçalves, RS, Brazil.

[3]Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D50829, Köln, Germany

[4]Departament of Botany, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

*To whom correspondence should be addressed. Tel: +5554 3455-8039/3455 8056 Fax: +5554 3451-2792; e-mail: luis.revers@embrapa.br

SUMMARY

Type-B response regulators (BRRs) are essential transcription factors in cytokinin (CK) signaling pathway, being responsible for CK-responsive gene modulation. We performed a genome-wide analysis aiming to identify apple *BRR* family members and understand their involvement in dormancy regulation in apple trees. This investigation resulted in the identification of ten *MdoBRR* protein-coding genes. A higher expression of three *MdoBRR* (*MdoBRR1*, *MdoBRR9* and *MdoBRR10*) was observed in dormant buds in comparison to other developmental stages. Interestingly, these three *MdoBRR* genes were upregulated in a CK-dependent manner in ecodormant buds. Transcription profiles determined during dormancy cycle under field and artificially controlled conditions, revealed that *MdoBRR1* and *MdoBRR8* played important roles in the transition from endo- to ecodormancy, probably mediated by endogenous CK stimuli. The expression of *MdoBRR7*, *MdoBRR9* and *MdoBRR10* was induced in ecodormant buds exposed to warm temperatures, indicating a putative role in growth resumption after chilling requirement fulfillment. Contrasting expression patterns between *MdoBRRs* and *MdoDAM1*, an essential dormancy establishment regulator, were observed during dormancy cycle and in CK-treated buds. Thereafter, *in vivo* transactivation assays showed that CK stimuli combined with transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* resulted in downregulation of the reporter gene *gusA* driven by the *MdoDAM1* promoter. These evidences point to the integration of CK-triggered responses through MdoBRRs that are able to downregulate *MdoDAM1*, thus contributing to dormancy overcome in apple.

SIGNIFICANCE STATEMENT

This work reports experimental evidences that contribute to the understanding of apple dormancy release mediated by CK stimuli and MdoBRRs activation. The results suggest an important role of *MdoBRRs* in the transition from endo- to ecodormancy. *In vivo* transactivation assays showed that CK stimuli and the transient overexpression of *MdoBRR1, MdoBRR8 and MdoBRR10* were able to downregulate *MdoDAM1*, an essential dormancy establishment regulator.

INTRODUCTION

The cytokinin (CK) signal transduction is a multiple step phosphorelay pathway that culminates in the activation of type-B response regulators (BRRs; Kakimoto 2003; To and Kieber 2008; Werner and Schmülling 2009; Hill et al. 2013). This protein family is characterized by the presence of two essential domains: a signal receiver (REC), involved in phosphorylation-mediated switches of response regulators, and a Myb-like DNA binding domain (Imamura et al. 1999; Hosoda et al. 2002). The REC domain core consists of a quintet of highly conserved amino acid residues comprising three adjacent Asp, one Ser/Thr and one Lys residue. In the Asp triplet, the first one is associated with the phosphorylation site and the other two with metal ion binding (Mg^{2+}), important for phosphoryl group changes. The conserved Thr/Ser interacts with the phosphoryl group and together with the conserved Lys enables phosphorylation-mediated conformational changes. The Myb-like output of BRRs shares a B-motif of approximately 60 amino acids and is responsible for DNA binding and transcription factor activity (Sakai et al. 2000; Hosoda et al. 2002). BRRs play a pivotal role in the early CK plant responses, being involved in shoot cell division, seed and root development and light responses (Argyros et al. 2008).

CKs are generally considered positively regulators of shoot apical meristems through stimulating cell division and negatively regulators of root apical meristem through promoting cell differentiation (Mok and Mok 2001; Argueso et al. 2012; Kieber and Schaller 2018). Besides that, studies have suggested that CKs are important regulators of bud dormancy release, acting upstream of the gibberellic and abscisic acid response pathways by stimulating meristematic activity (Cutting et al. 1991; Faust 1997; Liu and Sherif 2019).

Dormancy is defined as a self-arrest state of the shoot apical meristem, which is maintained even under growth-promoting conditions (Paul et al. 2014). Perception of external signals like photoperiodic changes and chilling exposure as well as internal stimuli such as hormonal balance, genetic and epigenetic regulation are key factors modulating the main physiological aspects of bud dormancy (Beauvieux et al. 2018; Lloret et al. 2018; Cattani et al. 2018; Miotto et al. 2019). Bud dormancy cycle is divided into endo-, eco- and paradormancy stages (Lang et al. 1987). Endodormant buds are not capable of resuming growth even when exposed to permissive conditions. This growth arrest is mainly determined by endogenous plant signals. At the ecodormancy stage, growth inhibition is associated to adverse external stimuli, and once environmental conditions become favorable, the vegetative growth restart. Paradormancy is related to growth suppression by distal organs signaling, and is usually referred as apical dominance (Lang et al. 1987).

The dormancy mechanism in *Malus* species is cyclic and the same perception of low temperatures during the autumn that induces bud set is necessary for budburst, as a result of continuous chilling exposure over the winter until spring. The chilling requirement for dormancy release is genotype dependent and guided by a complex molecular network. The first important dormancy regulator genes were identified in the

evergrowing (*evg*) peach mutant, which phenotype is associated to dormancy settlement failure (Bielenberg et al. 2008). The peach *evg* locus is related to six tandemly repeated MIKC^c-type MADS-box genes named *Dormancy-associated MADS-box* (*DAM*; Bielenberg et al. 2008). These genes have been widely associated with dormancy cycle control in several tree species, and are phylogenetic related to the Arabidopsis floral regulator SHORT VEGETATIVE PHASE (SVP; reviewed in Falavigna et al. 2019). In Arabidopsis, SVP plays important roles in the response to ambient temperature changes and in the modulation of hormonal responses, delaying flowering by directly repressing flowering-time genes (Lee et al. 2007; Andrés et al. 2014). Similarly, environmental signals, hormonal pathways and epigenetic changes modulate the transcriptional regulation of *DAM* genes during bud dormancy (Falavigna et al. 2019). However, the transcriptional regulators identified so far do not fully explain the expression dynamics of the *DAM* genes during the dormancy cycle.

In apple (*Malus × domestica* Borkh.), one of the most cultivated perennial species worldwide, ectopic expression of *MdoDAMb* and *MdoSVPa* resulted in delayed bud break and changes in plant architecture due to constrained lateral shoot outgrowth (Wu et al. 2017). Moreover, the promoter analysis of *MdoDAM1*, an important regulator of endodormancy, revealed the presence of BRRs binding sites, suggesting that the CK pathway may act upstream of the *DAM* genes (Porto et al. 2016). However, the functional relevance of these sites has not yet been evaluated. Therefore, considering the importance of CK in plant growth and the lack of knowledge about transcriptional regulators of the *DAM* genes, this study aimed to understand the roles of MdoBRRs factors during dormancy and investigate whether they could be involved in the modulation of *DAM* genes expression, a key regulator of dormancy cycle.

RESULTS

Identification, structural and phylogenetic analysis of apple *BRR* genes

The *in silico* identification of apple *BRR*s was performed based on the new haploid genome GDDH13 v1.1 (Daccord et al. 2017) and resulted in 10 predicted gene models (Table 1). In this study, we propose an update on the nomenclature of the members of apple BRR gene family, based on: the identification of two extra genes (*MdoBRR2* and *MdoBRR3)* and the use of a more curated version of the apple genome compared to the previous study conducted by Li et al (2017). Additionally, the *MdoBRR* gene names were inferred following the guideline developed for Rosaceae family members (Jung et al. 2015).

Table 1: Identification of apple *BRR* genes

Genome accession[a]	Chromosomal localization[a]	Li *et al.* (2017)	Proposed nomenclature	GenBank accession
MD08G1059000	Chr08:4701285..4705253	*MdRRB3*	*MdoBRR1*	MN590295
MD16G1036100	Chr16:2601907..2605099	-	*MdoBRR2*	MN590296
MD01G1047800	Chr01:15007174..15008926	-	*MdoBRR3*	MN590297
MD02G1229600	Chr02:27354515..27358617	*MdRRB1*	*MdoBRR4*	--
MD16G1108400	Chr16:7572032..7576420	*MdRRB10*	*MdoBRR5*	MN590298
MD13G1108300	Chr13:7783159..7786774	*MdRRB7*	*MdoBRR6*	MN590299

MD16G1159400	Chr16:12890367..12894414	*MdRRB11*	*MdoBRR7*	MN590300
MD13G1159700	Chr13:12567112..12571259	*MdRRB9*	*MdoBRR8*	MN590301
MD13G1019800	Chr13:1246553..1250362	*MdRRB6*	*MdoBRR9*	MN590302
MD16G1017900	Chr16:1305300..1309682	*MdRRB4*	*MdoBRR10*	MN590303

^aGenome accession codes and chromosomal localization are provided by the '*Malus × domestica* Genome' (http://rosaceae.org/) based on GDDH13_v1.1 version.

Full-length coding regions of seven identified *MdoBRRs (MdoBRR1; MdoBR5-10)* amplified from cDNA were confirmed by sequencing using specific primers (Table S1). Partial sequences were obtained for two *MdoBRR*s (*MdoBRR2* and *MdoBRR3)*, covering 70% and 40% of the coding regions, respectively. For *MdoBRR4*, just the RT-qPCR amplicon could be retrieved under our PCR conditions. These genes are located in a genomic region rich in repeated sequences, what may explain the difficulty in obtaining amplification products. With the exception of *MdoBRR4*, all sequences (partial and full-length) were deposited in the GenBank database and accession codes are presented in Table 1. In the case of partial sequences, the genome GDDH13 v1.1 predicted gene models for *MdoBRR2*, *MdoBRR3* and *MdoBRR4* were used as input for further analysis.

The functional annotation of MdoBRR deduced-protein sequences was performed with InterPro (Mitchell et al. 2019) and resulted in the identification of the REC domain (IPR001789) and the plant Myb-like family (IPR006447) domain (Figure 1A). The motif related to MdoBRR REC domain shows the quintet of highly conserved amino acid residues essential to REC activity (Figure S1A). The conserved Lys and Ser residues were found in all MdoBRR proteins. The presence of the three-conserved Asp was observed in seven MdoBRRs. MdoBRR2, MdoBRR3 and MdoBRR4 exhibited an amino acid substitution in the second position of the triplet (Figure S1A). The B-motif related to Myb-like family in MdoBRRs shares a group of highly conserved amino acid residues in all protein sequences. Exceptionally, MdoBRR2 presents gaps and a good number of substitutions (Figure S1B).

A)

	REC domain	Myb domain

B)
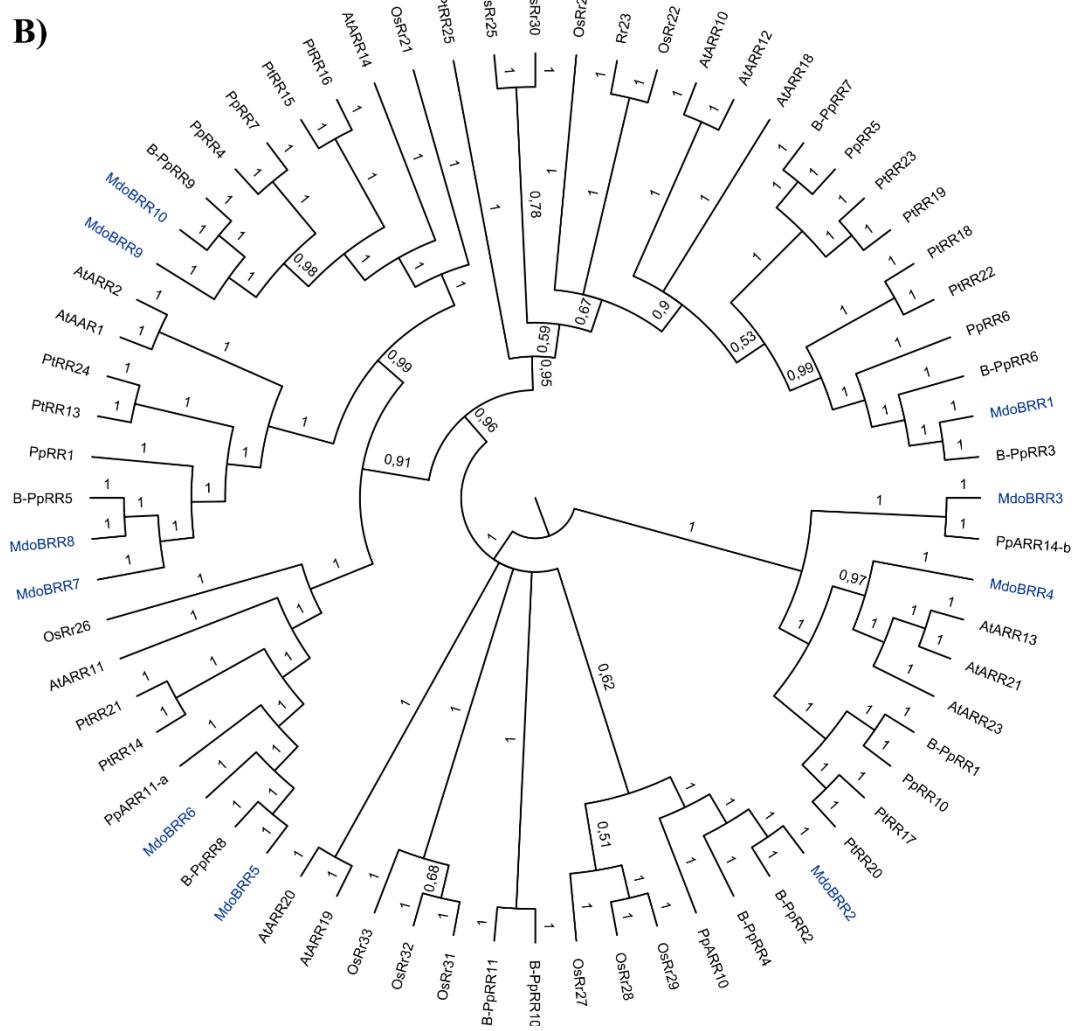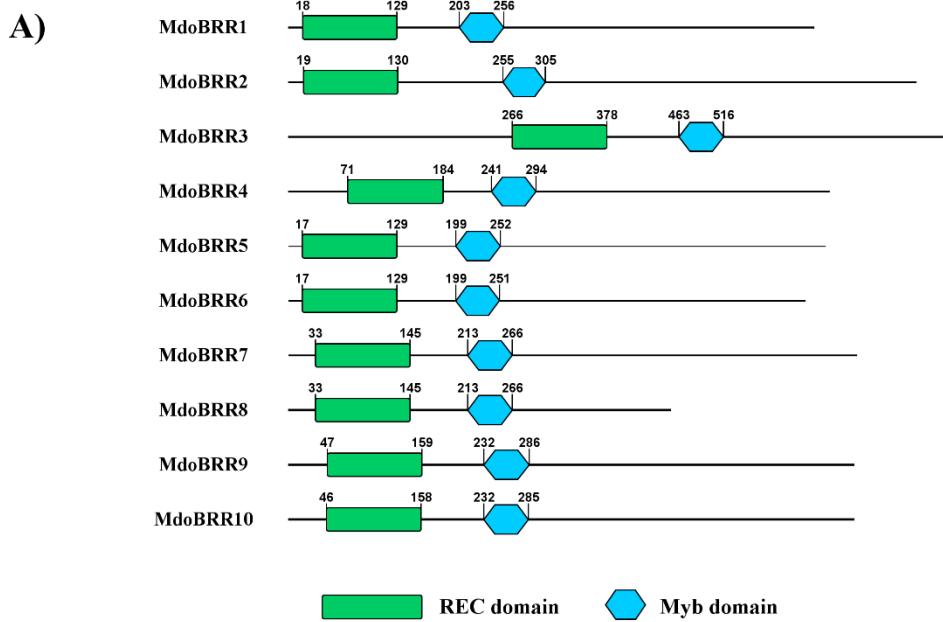
Figure 1. Identification of conserved domains and phylogenetic analysis of MdoBRRs. (A) Graphical illustration representing the position of the two conserved domains found in MdoBRR proteins. The first Met of each protein is considered as position number 1. **(B)** The phylogenetic tree was inferred by the Bayesian method using 68 protein sequences annotated as BRR from six different species (*A. thaliana*, *O. sativa* sub. Japonica, *P. trichocarpa*, *P. persica*, *P. bretschneideri* and *M. domestica*). MdoBRRs are colored in blue.

The plant model Arabidopsis (*Arabidospis thaliana*), the monocot rice (*Oryza sativa* sub. Japonica) and the dicot species poplar (*Populus trichocarpa*), peach (*Prunus persica*) and pear (*Pyrus bretschneideri*) were used in MdoBRR phylogenetic analysis (Table S2). The ten MdoBRRs deduced-protein sequences along with 58 other full-length proteins already described as BRRs were used for phylogenetic inference. The results allowed the identification of six MdoBRRs putative orthologs with pear and peach (Figure 1B). Pear proteins B-PpRR3, B-PpRR2, B-PpRR8, B-PpRR5 and B-PpRR9 are more closely related to MdoBRR1, MdoBRR2, MdoBRR5, MdoBRR8 and MdoBRR10, respectively and the PpARR14-b and MdoBRR3 grouped separately. The remaining proteins MdoBRR6, MdoBRR7, MdoBRR9 grouped in the same cluster of MdoBRR5, MdoBRR8 and MdoBRR10, respectively. The MdoBRR4 did not group with any other apple, pear or peach protein sequences, but with three Arabidopsis BRRs (AtARR13, AtARR21 and AtARR23).

Expression patterns of *MdoBRR* genes in different apple developmental stages

Based on the Fleckinger scale (EPPO 1984) we selected 12 tissues that encompass vegetative, reproductive and dormant cycles of apple trees to analyze *MdoBRR*s expression. Results showed that in general, among all tissues, the highest expression levels were observed for *MdoBRR6-10* genes (Figure 2). *MdoBRR6* was preferentially expressed in seeds of unripe fruit (JS) and in leaves independently of the stage (E2L, IL and JL). *MdoBRR7* transcripts accumulated in apple inflorescences (E2IN). Expression of *MdoBRR8* is higher in flowering (E2IN) and fruit development (J). *MdoBRR9* and *MdoBRR10* together with *MdoBRR1* presented higher expression levels in close terminal buds (A), especially *MdoBRR10* (Figure 2), leading us to better investigate their roles during the bud dormancy cycle. Transcriptional profile of *MdoBRR5* showed higher transcript levels in skin and pulp of unripe 40 mm apple fruit (J). *MdoBRR2*, *MdoBRR3* and *MdoBRR4* exhibited the lowest transcript levels (Figure 2). *MdoBRR4* expression was only barely detected in seeds of unripe fruit (JS). Based on these results, these three *BRRs* (*MdoBRR2-4*) were excluded from further analyses.
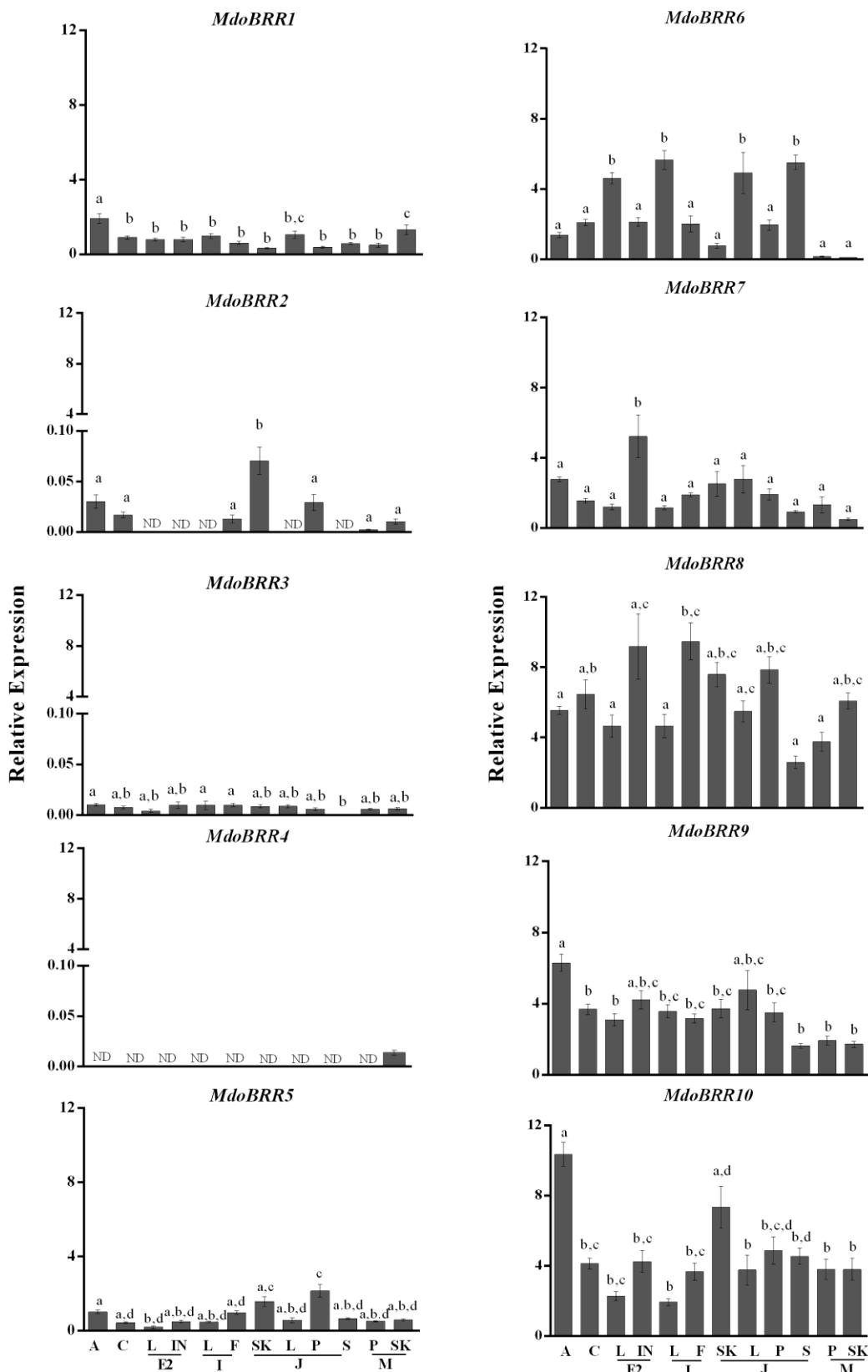
Figure 2. Gene expression of *MdoBRRs* in apple developmental stages. The horizontal axis displays the developmental stages according to Fleckinger's phenological scale (EPPO, 1984). **"A"**, closed dormant buds; **"C"**, buds in late sprouting stage; **"E2 L"**,

young leaves at E2 stage; **"E2 IN"**, flower buds at E2 stage; **"I L"**, leaves at I stage; **"I F"**, whole set fruits at I stage; **"J SK"**, fruit skin at J stage; **"J L"**, mature leaves at J stage; **"J P"**, fruit pulp at J stage; **"J S"**, seeds at J stage; **"M P"**, pulp from mature fruits at M stage; **"M SK"**, skin from mature fruits at M stage. Relative expression was calculated based on the calibrator represented by the levels of mRNA from *MdoBRR5* gene at stage A. Different letters indicate statistically significant differences between means using one-way ANOVA followed by Tukey's test ($p \leq 0.05$). Error bars represent the standard error of the mean. ND: None detected.

Transcriptional profiles of *MdoBRR* genes during the bud dormancy cycle

In apple trees, chilling hours (CH) exposure stimulates removal of the endodormancy physiological blocks that inhibit growth, culminating in ecodormancy. Subsequently, buds are able to resume growth (bud break) after a certain amount of warm temperatures. In order to monitor all stages of dormancy cycle progression, an artificially controlled induction assay was performed to determine the transcriptional profile of *MdoBRRs* and *MdoDAM1* under the perspective of chilling accumulation and growth promotion conditions. Additionally, quantification of the most prevalent CK found in higher plants, zeatin, at the active *trans* form (*t*-zeatin; Schmitz et al. 1972) was performed in these samples. 'Royal Gala' buds were collected in the field in the beginning of endodormancy with 315 CH and conditioned in a dark chamber with the constant temperature of 3 °C. In this condition, ecodormancy was achieved at 1,067 CH. After that, buds were transferred to permissive growth conditions (25±1.5 °C, 16 h photoperiod and 70 % relative humidity) for 360 h in order to favor bud break.

Results revealed that *MdoBRR1* and *MdoBRR8* showed a peak of expression after the transition from endo- to ecodormancy (after 1000 CH). At the same time point, *MdoDAM1* expression started to decrease (Figure 3). The initial hours under growth promoting conditions (25 °C) upregulated *MdoBRR10* transcripts, which remained constant all over the sampled time-points. The expression of *MdoBRR9* has also increased from 24 to 96 h during 25 °C temperature exposition. In a later response, after 168 h at growth permissive conditions, dormant buds started to accumulate *MdoBRR7* transcripts. The importance of *MdoBRR7*, *MdoBRR9* and *MdoBRR10* during bud growth resettlement was further reinforced in the 'Royal Gala' annual cycle transcriptional profile assay, which demonstrated upregulation of these three *MdoBRRs* during summer (Feb/09) when compared to autumn and winter (May-Aug/09; Figure S2). The expression of *MdoBRR5* and *MdoBRR6* did not change along the dormancy cycle (Figure 3). Peaks of *t*-zeatin accumulation were observed before the ecodormancy stage, decreasing after that, and resuming after prolonged warm temperatures exposure, close to bud break (Figure 3).
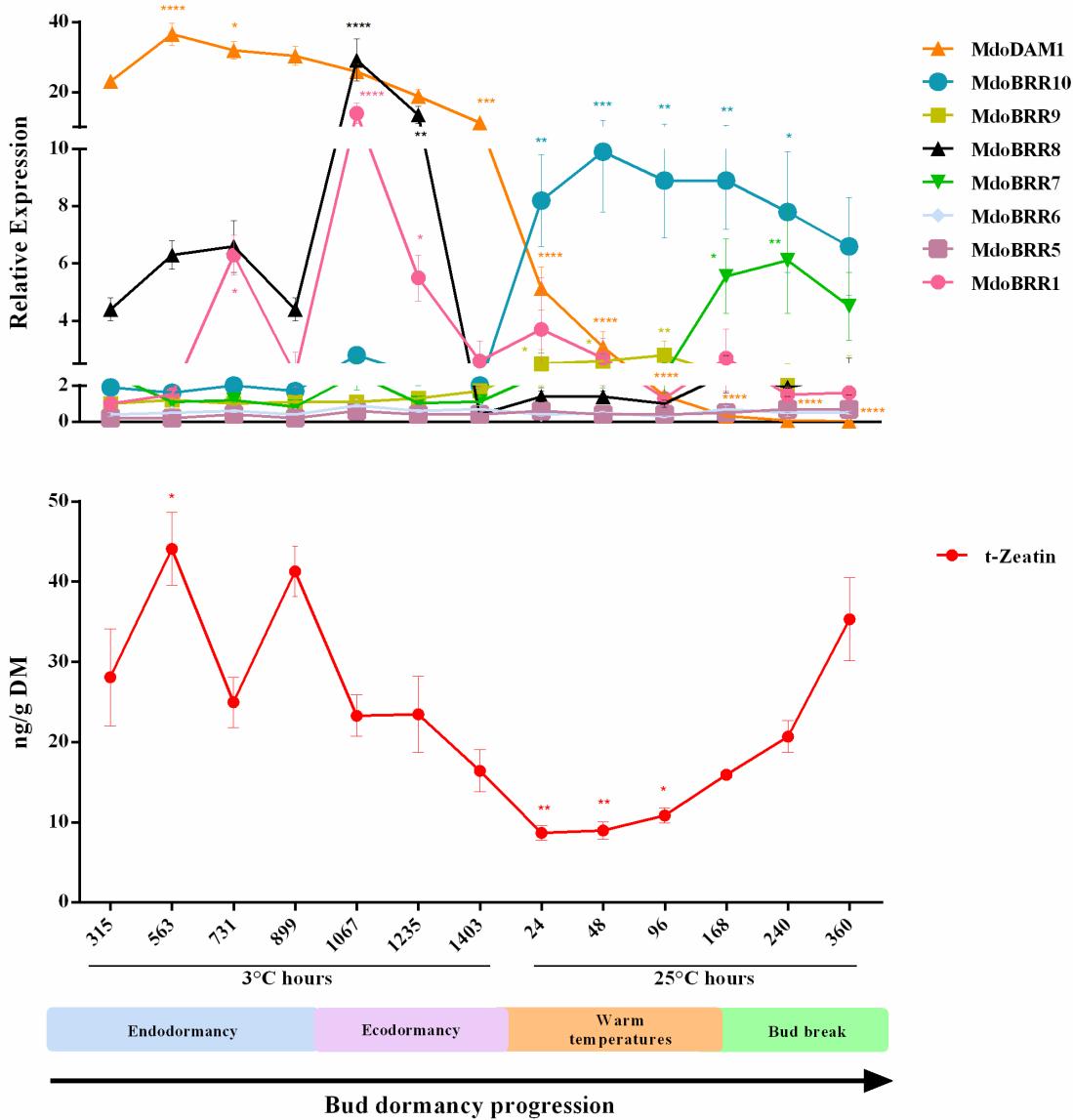
Figure 3. Expression pattern of *MdoDAM1* and *MdoBRR* genes over 'Royal Gala' bud dormancy progression under artificially controlled conditions. RT-qPCR was performed with dormant buds of 'Royal Gala' twigs sampled in the begging of endodormancy with 315 CH and conditioned into dark controlled chambers (3 °C) until the ecodormancy stage was reached (50% of green tip buds). After 1,403 CH, the ecodormant buds were transferred to growth permissive conditions (25±1.5 °C, 16 h photoperiod and 70 % relative humidity) for 24 to 360 h in order to favor bud break. Expression data was calculated in relation to *MdoBRR1* gene at 315 CH point. *t*-zeatin quantification was done using UPLC-ESI-MS/MS. The asterisks indicate statistical differences (*0.01 < p < 0.05, **0.001 < p <0.01***) between means of mRNA levels of each evaluated gene at 315 CH and the distinct time-points sampled. Amounts of *t*-zeatin were compared in the same way. Error bars represent the standard error of the mean. DM: dry mass.

Aiming to study the pattern of *MdoBRRs* expression during the transition from endo- to ecodormancy in the context of field cultivation conditions, 'Castel Gala' buds were sampled at four time-points from 118 to 778 CH (Figure 4). Castel Gala cultivar was chosen because it is a natural mutant of 'Gala' (Kidd´s Orange X Golden Delicious) that requires 50% less CH to bud break (Denardi and Seccon 2005) becoming capable of reach ecodormancy in natural field conditions. The same is not observed in the Royal Gala cultivar, that is also a spontaneous mutation from 'Gala', more commercially attractive but not able to accumulate enough CH to reach ecodormancy without the usage of chemical inducers, such as hydrogen cyanamide.
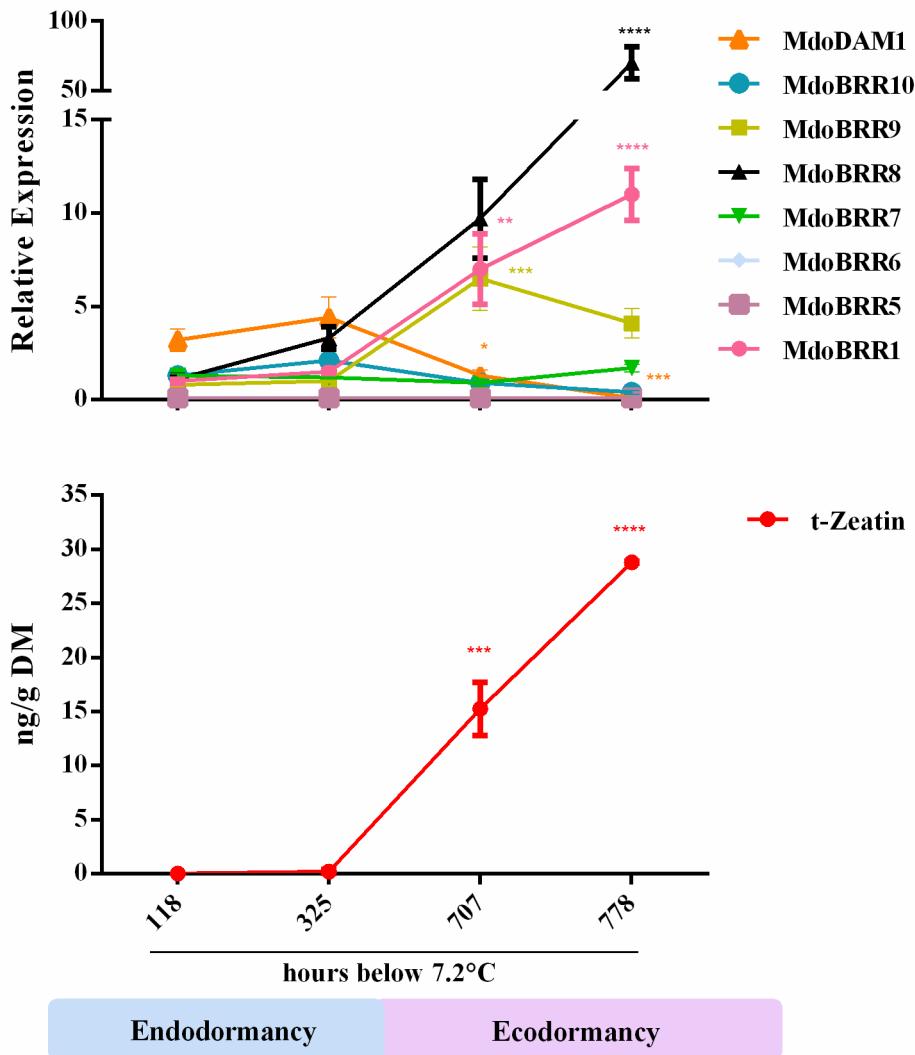
Figure 4. Transcriptional profile of *MdoDAM1* and *MdoBRR* genes under the field transition from endo- to ecodormancy in 'Castel Gala' buds. Relative expression of *MdoDAM1* and seven *MdoBRRs* was evaluated by RT-qPCR in dormant buds of 'Castel Gala' sampled in two different time-points of endo- (118 and 325 CH) and ecodormancy (707 and 778 CH). Calculation of expression data and statistical analysis was performed as described in Figure 3 considering point 118 CH as reference; $*0.01 < p < 0.05$, $**0.001 < p < 0.01***$. Error bars represent the standard error of the mean.

In 'Castel Gala' buds, the transition from endo- to ecodormancy was observed at approximately 700 CH, concomitantly with increased transcript amounts of *MdoBRR1*, *MdoBRR8* and *MdoBRR9* from 325 to 707 CH and *t*-zeatin amounts. From 707 to 778 CH, transcript amounts of *MdoBRR1* and *MdoBRR8* kept increasing in the same way as *t*-zeatin (Figure 4), suggesting the induction of *MdoBRRs* by the endogenous CK during ecodormancy stage. In an opposite way, the highest levels of *MdoDAM1* were observed from 118 to 325 CH, during endodormancy, followed by a significant reduction at 700 and 778 CH. The expression of the other four *MdoBRRs* (*MdoBRR5-7*; *MdoBRR10*) remained constant all over the dormancy cycle.

Taken together, these results indicate that CK accumulation with consequent *MdoBRR* upregulation and activation, followed by *MdoDAM1* downregulation constitute important steps in the transition from endo- to ecodormancy stage, as well as for bud break.

CK triggers *MdoBRR* gene expression in ecodormant buds

In order to determine if the CK stimulus could affect *MdoBRR* expression and investigate if the dormant stage could influence on this modulation, exogenous CK was applied in the same way on both endo- and ecodormant apple buds. The most effective treatment in *MdoBRR* transcriptional activation was achieved in ecodormant buds treated by the TOP method (direct CK application, for more details see Experimental Procedures). In this case, *MdoBRR9* and *MdoBRR10* demonstrated almost four-fold more transcripts when compared to the control after treatment with 4 mM of 6-benzylaminopurine (BAP). In these same samples, downregulation of *MdoDAM1*expression was also observed (Figure 5A). Conversely, thidiazuron (TDZ) treatments showed a clear tendency of *MdoBRR9* and *MdoBRR10* induction with a significant *MdoDAM1* repression. The CK influx through diffusion via xylem into ecodormant buds (BASE method) resulted in lower responses compared do the direct CK application (TOP method). In this case, after 16 h *MdoBRR7* expression was induced with 4 mM TDZ at the same time that *MdoDAM1* was repressed, showing again an opposite modulation of *MdoBRR* and *MdoDAM1* transcriptional patterns by the CK stimulus (Figure 5B). However, the same was not observed with *MdoBRR1* and *MdoBRR7* activation with 1 mM BAP treatment (Figure 5B). The expression of *MdoBRR5*, *MdoBRR6* and *MdoBRR8* did not change in any of the tested conditions (Figure S3). After 48 h of treatment with the BASE method, no significant modulation of *MdoBRR* by CK was observed in ecodormant buds (Figure S3).
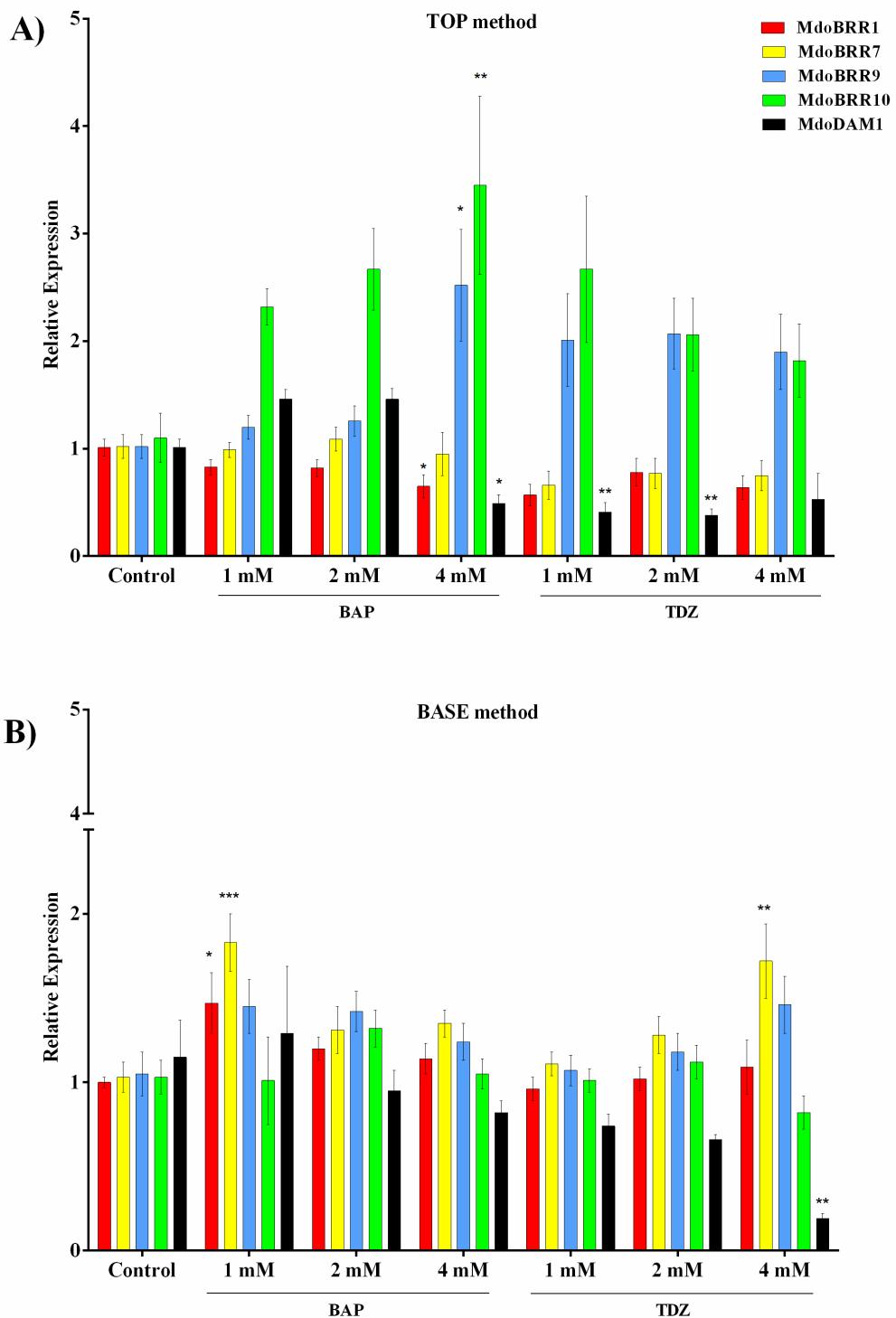
Figure 5. The transcriptional modulation of *MdoDAM1* and *MdoBRRs* in CK-treated ecodormant buds. The relative expression of *MdoBRRs* and *MdoDAM1* genes were measured in ecodormant buds treated for 16 h with 1, 2 or 4 mM BAP or TDZ by the methods of **(A)** direct application (TOP) or **(B)** influx (BASE). The expression level of each gene in the different treatments was calibrated and compared to its respective control. Asterisks indicate statistically significant differences between means using one-way ANOVA followed by Dunnet's test (*$0.01 < p < 0.05$, **$0.001 < p < 0.01$***). Error bars represent the standard error of the mean.

The influence of the dormancy stage in the CK-mediated *MdoBRRs* transcriptional modulation could be clearly seen when exogenous CK was applied to endodormant buds. In this case, the direct application of CK (TOP method) did not induce any *MdoBRR* expression (Figure S4A), resulting in a completely different response to that obtained in ecodormant buds (Figure 5). Additionally, CK transport in endodormant buds for 16 h (BASE method) resulted mainly in downregulation of *MdoBRR6* (Figure S4B) and after 48 h, just a subtle induction of *MdoBRR6* and *MdoBRR10* was achieved in the TDZ treatment (Figure S4C).

In summary, these findings corroborate with those found during the dormancy cycle evaluation (Figures 3 and 4) which showed that *MdoBRR* activation is only achieved during the bud ecodormancy stage. Furthermore, we could also observe that the CK signal was not just able to increase the level of *MdoBRR9* and *MdoBRR10* transcripts, but also to downregulate *MdoDAM1* expression.

CK and transient overexpression of *MdoBRR*s negatively regulate *MdoDAM1* gene expression

Considering the different putative roles of *MdoBRRs* and *MdoDAM1* during the bud dormancy cycle, and taking into account that the CK stimuli induces *MdoBRRs* expression whilst downregulates *MdoDAM1*, we aimed to evaluate whether MdoBRR could modulate *MdoDAM1* expression through an *in vivo* transactivation assay using Arabidopsis protoplasts. For this purpose, a segment of 503 bp from the *MdoDAM1* promoter region containing two putative BRR binding sites (Figure S5A; Porto *et al.*, 2016) was used to control the *gusA* reporter gene (*MdoDAM1prom::gusA)* in the pGUSXX-90 vector (Pasquali et al. 1994). Based on their consistent expression pattern related to a possible role during bud dormancy, *MdoBRR1*, *MdoBRR8* and *MdoBRR10* were chosen to be tested as effectors on the transactivation assays (Figures 3 to 5).

Results revealed that all MdoBRRs tested were able to bind BRR motifs and downregulate *MdoDAM1prom::gusA* activity in wild type protoplasts (Figure 6A). Wondering to know if CK signal is also capable to repress *MdoDAM1* expression as observed in CK-treated ecodormant buds (Figure 5), Arabidopsis wild type protoplasts suspensions were transfected just with *MdoDAM1prom::gusA* construction, without any effector (MdoBRR1, MdoBRR8 or MdoBR10). For this group of samples, TDZ was applied in increasing concentrations (as described in experimental procedures section), leading to a significant reduction in *MdoDAM1prom::gusA* activity even when the lowest concentration was tested, confirming the repressive effect of CK signal on *MdoDAM1* gene regulation (Figure 6B).
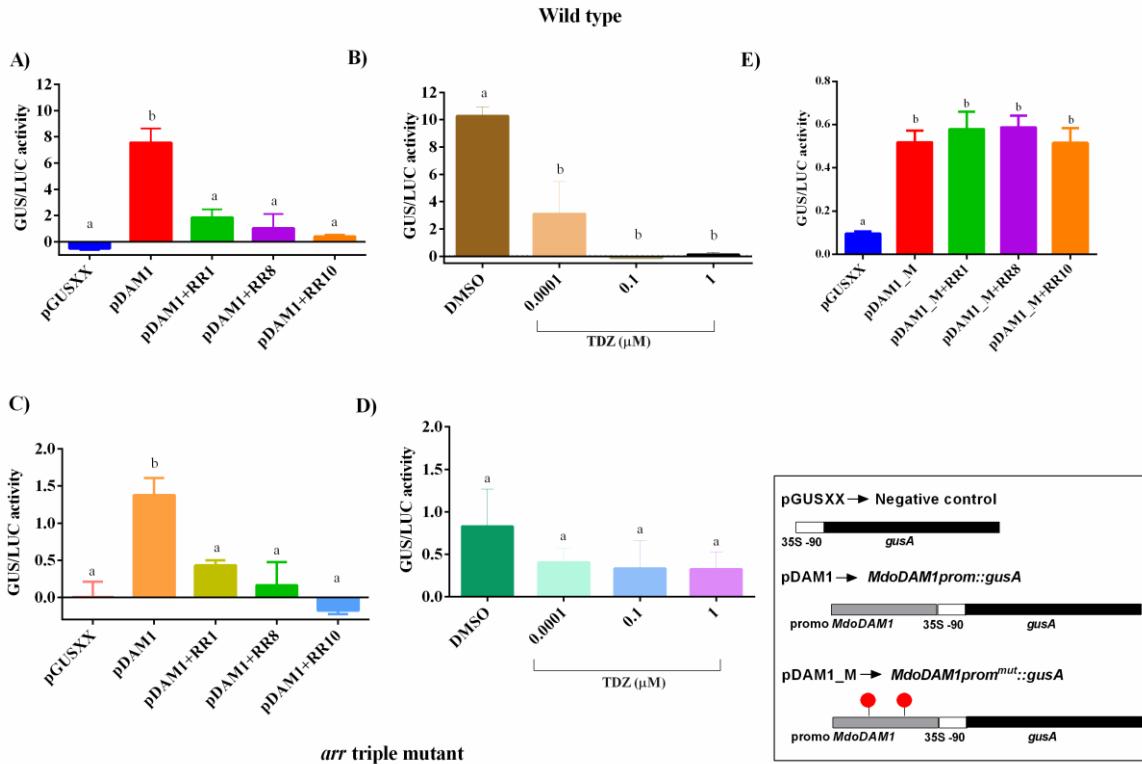
Figure 6. *MdoDAM1* **downregulation mediated by MdoBRRs and CK signal.** The modulation of *gusA* driven by the *MdoDAM1* promoter through *MdoBRR1*, *MdoBRR8* and *MdoBRR10* was evaluated in Arabidopsis wild type **(A)** and *arr* triple loss of function mutant protoplasts **(C)**. The effect of exogenous application of TZD (from 0.0001 to 1.0 μM) and DMSO (control) was also tested for both wild type **(B)** and *arr* backgrounds **(D)**. Site-specific mutations were introduced into the *MdoDAM1* promoter and the biding ability of MdoBRRs effetors was tested in wild type protoplasts **(E).** The GUS/LUC activity was calculated through dividing the enzymatic GUS activity by rLUC luminescence. RR1, RR8 and RR10 represent *MdoBRRs* respective coding regions cloned into the pART7 vector for protoplasts transient overexpression. Different letters indicate statistically significant differences between means using one-way ANOVA followed by Tukey's test ($p \leq 0.05$). Error bars represent the standard error of the mean.

The availability of the Arabidopsis triple loss-of-function mutant (*arr1-3 arr10-5 arr12-1*) allowed us to perform the same assay described above, with the advantage of minimizing the Arabidopsis ARR(s) endogenous effects and possible technical artifacts, since these plants have lost the ability to respond to external CK stimuli (Argyros et al. 2008). Consistent with the previous observations, the assay employing *arr* triple mutant protoplasts resulted in *MdoDAM1prom::gusA* repression when transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* was tested, confirming that *MdoDAM1* downregulation is due to MdoBRRs (Figure 6C). Moreover, the comparison between control (DMSO) and TDZ treatments resulted in no significant differences in *MdoDAM1prom::gusA* activity, reinforcing the hypothesis that *MdoDAM1* downregulation occurs by cellular perception of CK stimuli through ARRs (Figure 6D). In order to confirm the MdoBRRs ability to bind in BRR regulatory motifs, site-specific mutations were introduced into the two BRR biding sites found along the *MdoDAM1* promoter region (Figure S5B). The results showed that the loss of native BRR biding sites

disrupted the ability of MdoBRR1, MdoBRR8 and MdoBRR10 to downregulate *MdoDAM1prom^{mut}::gusA* activity, confirming the hypothesis that the transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* is responsible for the transcriptional repression of *MdoDAM1* (Figure 6E). These findings suggest a redundant mechanism on which *MdoDAM1* downregulation could be mediated by CK signaling (probably mediated by the activation of already existing pools of BRRs in the cell) followed by *de novo* transcriptional induction of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* and CK post-translational activation.

DISCUSSION

BRR genes were firstly reported in Arabidopsis (D'Agostino et al. 2000) followed by rice (Schaller et al. 2006); poplar (Ramírez-Carvajal et al. 2008), peach (Immanen et al. 2013), pear (Ni et al. 2017) and apple (Li et al. 2017). It is already known that activated BRRs are responsible for transcription modulation in a CK-dependent manner of many downstream genes involved in plant development (reviewed by Kieber and Schaller 2018). However, their potential regulatory role during the bud dormancy cycle has not been well explored. Our investigation based on the GDDH13_v1.1 apple genome dataset (Daccord et al. 2017) rendered the identification of ten *MdoBRRs*, including two additional members for the family (*MdoBRR2* and *MdoBRR3*; Table 1) when compared to the previous report (Li et al. 2017) that explored the apple draft genome described by Velasco et al. (2010). It is important to consider that the GDDH13_v1.1genome has a higher accuracy and a better contig assembly with a consequent more precise gene annotation. As example of the problems of the first apple genome draft assembly and annotation, the three predicted gene models (MDP0000607144, MDP0000307383 and MDP0000124301) were considered as true *MdoBRRs* by Li et al. (2017) but, in fact, these sequences are miss-predicted versions of *MdoBRR1*, *MdoBRR6* and *MdoBRR9*, respectively and are not even assigned in GDDH13_v1.1 dataset.

The ten MdoBRR deduced proteins (Table 1) contain the two typical REC and Myb-like domains necessary for activation and DNA binding, respectively (Figure 1A). Detailed analysis of all MdoBRR REC motifs revealed the lack of one of the three highly conserved Asp residues in MdoBRR2, MdoBRR3 and MdoBRR4 deduced-proteins (Figure S1). Another class of response regulators is characterized by atypical receiver domains and is related to the absence of one or more residues in the core region of the REC domain. This class is called pseudo-response regulators and also lacks the invariant phospho-accepting Asp residue, often replaced by a Glu residue (Mizuno and Nakamichi, 2005; Bourret 2010). Although MdoBRR2, MdoBRR3 and MdoBRR4 deduced-proteins do not have the Asp triplet, they present the phospho-accepting Asp residue (Figure S1) and consequently do not fit to this definition, remaining as true members of the apple BRR family. Exactly these three BRR genes showed very low expression levels in all apple tissues tested (Figure 2), which could be associated to a potential pseudogenization process through loss-of-function mutations, once the recent duplication of the apple genome created two copies of most genes, resulting in gene function diversification (Panchy et al. 2016).

The transcripts from nine out of the ten *MdoBRR* genes were detected in all twelve apple tissues evaluated. *MdoBRR4* was the exception, whose transcripts were detected only in seeds of 40 mm developing fruit (Figure 2). This wide distribution of *BRR* expression was already reported in Arabidopsis and rice (Mason et al. 2004; Ito and Kurata 2006). We have also observed higher amounts of *MdoBRR* transcripts during developing stages rather than in ripened fruit (Figure 2). These data are in agreement with

peach *BRR* expression profiles that present higher transcript amounts in rapidly growing tissues like semi-opened flowers, fruits at expansion stage and young leaves (Zeng et al. 2017). Similar results were also found in pear in the early stage of fruit growth (Ni et al. 2017) and in Arabidopsis where *BRR* expression was observed at shoot apical meristematic cells and young developing leaves (Mason et al. 2004). In general, our data could not be related to the results obtained by Li et al. (2017), probably by the distinct tissue sampling strategy used and apple cultivars tested ('Gala Baigent' *versus* 'Nagafu').

Our particular interest in studying *MdoBRRs* was to gather evidences to understand their involvement in bud dormancy regulation. Within this context, we found three *MdoBRRs* (*MdoBRR1*, *MdoBRR9* and *MdoBRR10*) that showed significant levels of expression in bud tissues (Figure 2). The evaluation of *MdoBRR* expression during dormancy cycle demonstrated that the transition from endo- to ecodormancy in 'Castel Gala' buds is associated with *MdoBRR1*, *MdoBRR8* and *MdoBRR9* transcriptional upregulation, *t*-zeatin accumulation and *MdoDAM1* gene repression (Figure 4). In agreement, 'Royal Gala' ecodormancy stage was achieved concomitantly with *MdoBRR1* and *MdoBRR8* transcriptional activation and *MdoDAM1* downregulation. Moreover, when ecodormant buds were transferred to permissive growth conditions, we could observe the negative regulation of *MdoBRR1*, *MdoBRR8 and MdoDAM1* and the activation of three *MdoBRR* in a continuous (*MdoBRR10*), early (*MdoBRR9)* and late (*MdoBRR7*) response to warm temperatures (Figure 3). Accordingly, the evaluation of 'Royal Gala' buds during a full year growing cycle showed higher transcriptional activity of *MdoBRR7*, *MdoBRR9* and *MdoBRR10* during the summer (Feb/10; Figure S2) and a remarkable reduction of *MdoDAM1* expression in the same period (Porto et al. 2016).

The potential regulatory role of *BRR* genes in the transition from endo- to ecodormancy was also reported in pear RNA-Seq analysis that showed increased transcript levels of *B-PpRR9/6* and *B-PpRR5/3* genes in 'Suli', and *B-PpRR7* gene in 'Kosui' after endodormancy release (Ni et al. 2017). Complementing these findings, our phylogenetic studies revealed putative orthology between MdoBRR1 and B-PpRR3; MdoBRR8 and B-PpRR5; MdoBRR10 and B-PpRR9 proteins (Figure 1B), the same *MdoBRRs* involved in apple buds dormancy cycle transition.

Although the expression of *MdoBRR1* and *MdoBRR8* showed similar patterns in the transition from endo- to ecodormancy between field ('Castel Gala') and artificially controlled ('Royal Gala') conditions, the same was not observed for *t*-zeatin (Figures 3 and 4). In this case, it is important to consider that the CK source is different in these two distinct dormancy datasets. The origin of endogenous CKs that control shoot branching is still controversial. Some studies associate it with root-derived CK and others with local production (Faiss et al. 1997; Matsumoto-Kitano et al. 2008; Muller and Leyser 2011). 'Castel Gala' buds were sampled from plants under field conditions, and the two sources of CK (root-derived and locally synthesized) were available. On the other hand, the progression of 'Royal Gala' dormancy was evaluated under controlled artificial conditions where independent twigs containing terminal buds were sampled from apple trees, being in this case, the CK local biosynthesis, the only source.

Little is known about the consequences of exogenous CK treatments in buds at dormant stage. The vast majority of reported studies tested CK just in newly flowered buds (Chen et al. 2014; Fogelman et al. 2015; Li et al. 2016). Therefore, in order to investigate whether *MdoBRRs* transcriptional regulation could be mediated by CK stimuli in endo- and ecodormant buds, we envisaged to test different concentrations, types of synthetic CKs and treatments. The most relevant effect of the treatments was the transcriptional activation observed for *MdoBRR9* and *MdoBRR10* (Figure 5) in ecodormant buds treated with 4 mM BAP. In agreement to our results, the activation of

Arabidopsis *BRR* (*ARR10*) in a CK-dependent manner has been already reported (Hill et al. 2013; Zubo et al. 2017). The transcriptome analysis of rice plants treated with CK showed that the *BRR* gene *OsRR22* was downregulated in roots and upregulated in shoots. In addition, *OsRR26* was downregulated in roots and unaffected in shoots (Raines et al. 2016). The lack of a widespread *BRR* upregulation in response to CK stimulus could be related to its mode of action. BRR proteins are present independently of CK signaling and their activation is primarily related to the Asp phosphorylation in the REC domain rather than protein turnover. This is in contrast with other hormone-regulated transcriptional activators like EIN3, involved in ethylene signaling, which is just stabilized in the presence of ethylene (Guo and Ecker 2003; Kurepa et al. 2014). Even though our results demonstrated that *MdoBRRs* could be activated by the CK stimulus in ecodormant buds (Figure 5), the same response was not observed in endodormant buds (Figure S4). This can be attributed to callose accumulation in the plasmodesmatas that closes the symplastic transport and interrupts intercellular communication during endodormancy (Wu et al. 2018). Throughout the process of dormancy overcoming, callose is degraded and the flow of water, nutrients and signaling molecules is reestablished. In peach bud cells, for example, the fluidity of the plasma membranes increases with chilling accumulation during dormancy release (Portrat et al. 1995). Moreover, studies have demonstrated that TDZ is not effective to induce growth during apple endodormancy and it only exerts an effect when a substantial part of the chilling requirement is reached (Steffens and Stutte 1989; Faust et al. 1991).

Since 2008, with their initial characterization in the *evg* peach mutant (Bielenberg et al. 2008), *DAM* genes have been associated to dormancy establishment and maintenance in many species. The current evaluated dormancy cycles demonstrated higher levels of transcripts of *MdoDAM1* during endodormancy and a drastic reduction towards transition to ecodormancy (Figures 3 and 4). Accordingly, apple *DAM* genes (*MdoDAM1*, *MdoDAM3*,and *MdoDAM4)* are characterized by a seasonal oscillating transcript accumulation pattern, with a remarkable repression at ecodormancy stage (Mimida et al. 2015; Porto et al. 2016). Based on the potential different roles of *MdoDAM1* and *MdoBRR1*, *MdoBRR8* and *MdoBRR10* during the dormancy cycle, *in vivo* transactivation assays were performed in order to evaluate the MdoBRR ability to modulate *MdoDAM1* expression. Our results demonstrated an interesting regulatory mechanism on which transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* showed a repressive effect on the *MdoDAM1* gene promoter (Figure 6A). Additionally, the CK signal led to a downregulation of *MdoDAM1prom::gusA* (Figure 6B), agreeing with results found in CK-treated ecodormant buds, where *MdoDAM1* was also repressed (Figure 5).

The use of Arabidopsis *arr* triple loss-of-function mutant confirmed the consistency of the previously data obtained using wild type plants in two aspects: minimizing the effect of endogenous BRRs (Figure 6C) and discarding possible technique artifacts once the mutant does not respond to exogenous CK stimuli (Figure 6D; Argyros et al. 2008). Moreover, the regulatory region of *MdoDAM1* shares the same DNA motif [AGAT(T/C)] found in already described CK-responsive genes (Hosoda et al. 2002; Zubo et al. 2017) and the introduction of site-specific mutations in native BRR biding sites (Figure S5B) disrupted *MdoDAM1prom^{mut}::gusA* modulation by MdoBRR1, MdoBRR8 and MdoBRR10 (Figure 6E). Therefore, this data strongly suggest that MdoBRR proteins recognize regulatory *cis*-elements acting as negative regulators of *MdoDAM1* expression.

The regulatory mechanism that involves *MdoDAM1* modulation by MdoBRRs might be associated to a physical barrier, created by MdoBRR protein binding. This barrier prevents DNA correct structural conformation necessary to *MdoDAM1* activation

by other MADS-box factors that recognize the CArG-box elements found along the promoter region (Figure S5A; Kaufmann et al. 2005). Thus, in a very simple way, our proposed hypothetical model is based on accumulation of CK in dormant buds towards the transition from endo- to ecodormancy, which triggers MdoBRRs transcriptional activity and post-translational modifications. Once phosphorylated, MdoBRRs bind to *MdoDAM1* promoter, repressing it. Turning off *MdoDAM1* expression, dormancy release is then favored (Figure 7).
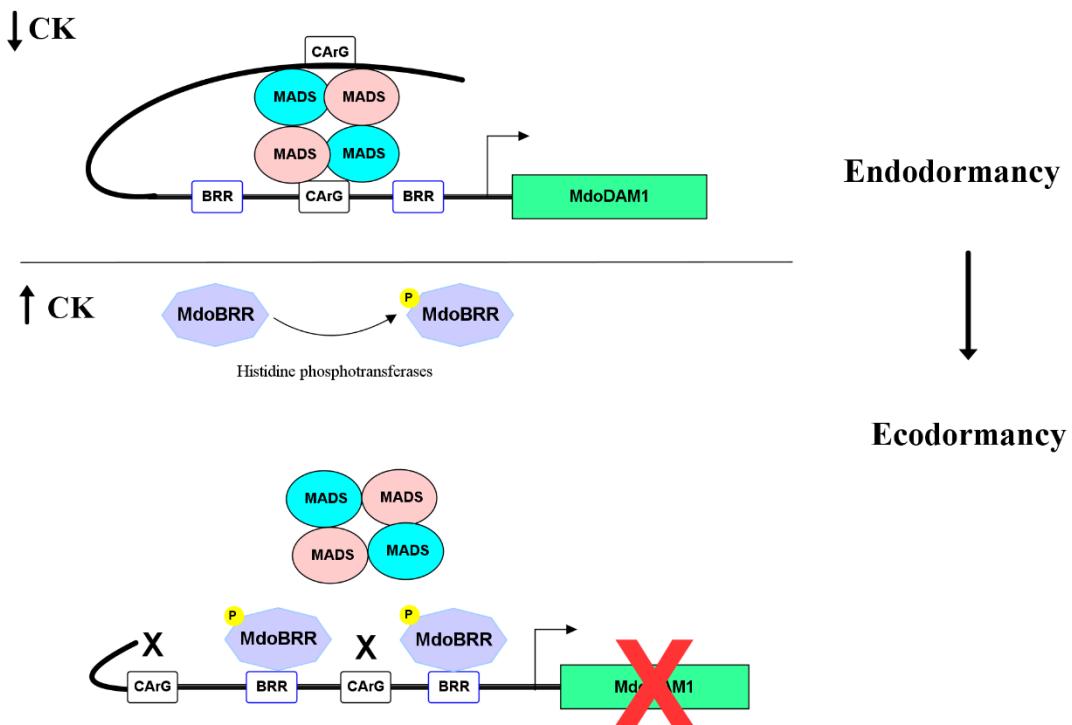
Figure 7. Hypothetical model of *MdoDAM1* modulation through CK signal and consequent *MdoBRRs* transcriptional activation towards the transition from endo- to ecodormancy in apple buds. The model is based on accumulation of CK in dormant buds towards the transition from endo- to ecodormancy, which activate MdoBRRs transcriptional activity and post-translational modifications (highlighted in yellow). Once activated, MdoBRRs bind to *MdoDAM1* promoter on their BRRs motifs, preventing that transcriptional activators, like MADS-box factors, bind to their *cis*-elements (CArG boxes), creating a physical barrier that does not enable DNA correct conformation, culminating in the repression of *MdoDAM1* gene. P: phosphate group. Designed using IBS v.10.3

In conclusion, throughout the dormancy cycle, our results indicate that *MdoBRR1* and *MdoBRR8* apparently exhibit an important regulatory role towards the transition from endo- to ecodormancy, while *MdoBRR7*, *MdoBRR9* and *MdoBBR10* genes were upregulated only after ecodormancy was achieved and favorable environmental conditions were restored, indicating their potential roles in growth resumption. Besides that, in the ecodormancy stage, *MdoBRR1*, *MdoBRR9*, *MdoBBR10* were activated in a CK-dependent manner, leading to *MdoDAM1* downregulation. *In vivo* transactivation

assays showed that the transient overexpression of *MdoBRR1, MdoBRR8* and *MdoBRR10* genes and CK stimuli have negative regulatory effects on *MdoDAM1* expression. Finally, these findings link CK hormonal stimulus with molecular responses through a regulatory mechanism of *MdoDAM1* repression mediated by CK-activated MdoBRRs, that within the complex regulation of dormancy in perennials, represents a contribution to the understanding of dormancy overcome in apple.

EXPERIMENTAL PROCEDURES

Identification, sequencing and phylogenetic analysis of *MdoBRRs*

The identification of predicted gene models coding for *BRRs* was performed by BLASTP (Altschul et al. 1990) using the apple genome version GDDH13_v1.1 (https://www.rosaceae.org/; Daccord et al. 2017). Two conserved domains were used as queries: REC (Imamura et al. 1999) and a Myb-like DNA binding domain (Hosoda et al. 2002). Only predicted gene models that were annotated as response regulators and exhibited both domains were selected for further analysis. To confirm the presence of the conserved domains, the deduced-protein sequences of the selected candidates were used as input in NCBI's CDD (Conserved Domain Database) tool (Marchler-Bauer et al. 2015). Further, protein sequences of MdoBRRs were used as input for the InterPro v.70.0 online search tool (Mitchell et al. 2019) in order to assign domain families. The graphical representation of MdoBRR protein domain localization was generated by IBS v.10.3 (Liu et al. 2015).

Sequence confirmation for coding regions of the ten *MdoBRR* candidates was performed through Sanger sequencing using M13 and walking primers in order to cover full-length sequences (Table S1). *MdoBRR* CDS regions were amplified by PCR using Platinum™ Pfx DNA Polymerase (Invitrogen, USA) with specific primers designed using Vector NTI (Invitrogen, USA; Table S1), followed by amplicon purification (PureLink™ PCR Purification Kit; Invitrogen, USA) and cloning into the pENTR™/D-TOPO™ (Invitrogen, USA) as instructed by manufacturers. *In vitro* sequencing results were aligned to Daccord *et al.* (2017) genome reference using MEGA7 7.0 version software (Kumar *et al.*, 2016).

For phylogenetic analysis, deduced full-length protein sequences of 68 type-B response regulators from *Arabidopsis thaliana*, *Malus × domestica*, *Oryza sativa* sub. japonica, *Populus trichocarpa*, *Prunus persica* and *Pyrus bretschneideri* were aligned using MUSCLE (Edgar 2004). References and accession codes from all sequences are listed in Table S2. The phylogenetic tree was inferred using MRBAYES version 3.1.2 (Huelsenbeck & Ronquist, 2001) employing the mixed amino acid substitution model. Ten million generations were run, sampled every 100 generations and the first 25% of the trees were discarded as burn-in. The remaining ones were summarized in a consensus tree, which was visualized and edited using FIGTREE v.1.4.4 (http://tree.bio.ed.ac.uk/software/Figuretree/). All *in silico* analysis were performed using software's default settings.

RNA extraction and RT-qPCR

RNA extraction was performed as described by Zeng & Yang (2002) and was scaled to micro-centrifuge tubes. The integrity of RNA samples was assessed by 1% agarose gel electrophoresis. DNA contaminants were removed by TURBO DNAse treatment (Applied Biosystems, USA) and cDNA synthesis from 1 µg of RNA was

carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer instructions. The complete removal of DNA contaminants was confirmed by PCR using intron-flanking primers for *WD40* (*WD40-LIKE REPEAT DOMAIN*) gene. The specific primers for Reverse Transcribed quantitative PCR (RT-qPCR) were designed by Vector NTI Advance 10 (Invitrogen, USA; Table S1) employing the default parameters. RT-qPCR was performed as described in Falavigna *et al.* (2014). Biological samples (n=3) were analyzed in three technical replicates. Relative expression of mRNA was calculated by the Pfaffl method (Pfaffl 2001). For the *MdoBRRs* transcriptional profiles in apple developmental stages, the expression of *MdoBRR5* at stage A was chosen as calibrator. For the dormancy cycle experiments, Pfaffl calculation calibrators were *MdoBRR1* transcripts at 118 CH for 'Castel Gala' and *MdoBRR1* expression at 315 CH for 'Royal Gala'. In the CK treatments, gene expression is varying in relation to its respective control. Threshold cycle (CT) values were normalized by reference genes *MDH* (*malate dehydrogenase*), *TMp1* (*type 1 membrane protein-like*) and *WD40* (*transcription factor WD40-like repeat domain*) when samples of tissues were analysed. Specifically for bud samples calculations, *TMp1* was replaced by *ARC5* as described in Perini et al. (2014).

Transcriptional profile of *MdoBRRs* in different apple developmental stages

Sampling strategy was done according to Perini et al. (2014) and different plant tissues were harvested from 3-year-old clones of Gala Baigent® apple trees grafted on Marubakaido rootstock with M.9 as interstem. Apple trees were located in the experimental orchard at the Temperate Fruit Tree Experimental Station of Embrapa Uva e Vinho (TFES), in Vacaria, RS, Brazil (28°30'50''S, 50°54'41''W, 972 m altitude). Tissues were collected through the vegetative and reproductive cycle of 2009/2010. The different developmental stages were classified according to the Fleckinger scale (EPPO 1984) and their complete description is reported in Perini et al. (2014). The chosen tissues were (A) closed terminal buds and (C) bud-set at initial bursting, essential to understand dormancy progression. Furthermore, at blooming stage, we selected (E2IN) flower buds and (E2L) young leaves. Following the cycle, fruit set was also analyzed with (IF) whole 10 mm just-set fruits and (IL) mature leaves. The fruit development was monitored through (JL) leaves, (JP) pulp, (JSK) skin and (JS) seeds of unripe 40 mm fruits. (M) apple mature fruits with approximately 70 mm diameter partitioned into (MP) pulp and (MSK) skin were also investigated (Perini et al. 2014). Three sets of ten clonal trees each were considered as three biological replicates. From each set, equal samples were harvested and frozen in liquid nitrogen in the field and stored at -80 °C until RNA extraction.

Dormancy cycle evaluation

Terminal dormant buds of 'Castel Gala' were harvested at four different time points under field conditions (118, 325, 707 and 778 CH) in the experimental orchard at TFES during the year of 2016 (Table S3a). Immediately thereafter, 45 buds divided into three biological replicates (15 each) were frozen in liquid nitrogen and stored at -80 °C until RNA extraction. In order to evaluate bud dormancy physiological stage (endo- or ecodormancy), a batch of samples (40 twigs) at each sampled time-point was forced to bud break in a growth chamber (25±1.5 °C, 16 h photoperiod and 70 % relative humidity). After 35 days, the percentage of bud break was calculated by dividing the number of green tip buds by all viable buds tested. In this study, we defined that the transition from

endo- to ecodormancy was reached when more than 50% of the buds were at the green tip stage. For the dormancy treatment under artificially controlled conditions, twigs from the Royal Gala cultivar were sampled in the experimental orchard at TFES in June 2016 with 315 CH. The twigs containing terminal buds (20 cm long) were decontaminated and wrapped in black plastic bags as described by Falavigna et al. (2015). Chilling treatments were performed by placing bags inside growth chambers in the dark with terminal buds positioned upwards with a constant temperature of 3 °C. Sampling occurred in six different chilling points (563, 731, 899, 1,067, 1,235 and 1,403)

   After 1,403 CH, twigs were transferred to growth permissive conditions (25±1.5 °C, 16 h photoperiod and 70% relative humidity) and sampled after 24, 48, 96, 168, 240 and 360 h). Buds were frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Three biological replicates, constituted of 15 twigs each were used in the 13 different treatments.

   For 'Royal Gala' annual growing cycle evaluation, buds were harvested during a full year at six different dates to cover all seasons (Table S3b). Sampling was done in an experimental area belonged to a commercial orchard in Papanduva, SC, Brazil (26°26'68"S, 50°05'47"W, at 788 m altitude), during the 2009/2010 cycle.

t-zeatin quantification

   The hormone extraction was performed adding 4.0 mL of extraction solution (methanol:water:formic acid, 75:20:5, v:v:v) in a tube containing 500 mg of plant material. Samples were incubated at -20 °C for 3 h. After that, they were conditioned in an ultrasound bath (40 kHz frequency) for 25 min at 4 °C and supernatants were recovered by centrifugation at 1,750 g at 4 °C for 30 min. Extraction step was repeated twice. The supernatant was transferred into a new 1.5 micro-centrifuge tube and dried in a benchtop vacuum centrifuge (Eppendorf, DE) at 1,400 rpm at 30 °C under 20 mbar vacuum pressure until reaching the mark of 100 µL of residual liquid. To this remaining solution, 1.0 mL of water was added followed by full homogenization in a vortex. The samples were then transferred to Oasis MCX columns (Waters Corporation, USA). The column elution was conducted using a gradient of ammonium hydroxide (Acros Organics cat. no 205840025) from 0.004 to 0.4 M. To the eluted solution, 1.7 % w/v of PVPP (Sigma, cat. no. P6755) was added and the samples were homogenized in a vortex. The sample supernatants were recovered by centrifugation at 10,000 g for 45 min at 4 ºC. The supernatant was then transferred into a new 1.5 micro-centrifuge tube and dried in the vacuum centrifuge as described above. After drying, samples were ressuspended in 75 µL of methanol and filtered through a 0.22 µm polytetrafluoroethylene (PTFE) membrane. The quantification analysis was carried in UPLC-ESI-MS/MS (Waters Corporation, USA). Results are represented in ng of *t*-zeatin / g of dry mass.

CK treatments

   Twigs of 'Royal Gala' apple trees were harvested in the experimental orchard at TFES. Sampled twigs containing terminal buds (10 cm long) were sanitized as described by Falavigna et al. (2015) and CK treatments were performed by two different ways: the TOP method consisted in dipping terminal buds of each twig in 10 mL of the tested CK solution for 10 min. Subsequently, twigs were vertically planted in floral foams inside a growth chamber (25±1.5 °C, 16 h photoperiod and 70% relative humidity) for 16 h. The BASE method consisted in submerging the basal portion of the twigs in 10 mL of the tested CK solution for 16 or 48 h. During this period, the twigs were conditioned in the

growth chamber described above. After each time-point, terminal buds were harvested, frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Two types of synthetic CK were used: BAP (Sigma, cat. no B3408) and TDZ (Sigma, cat. no 45686) in three different concentrations (1, 2 and 4 mM). All CK solutions were diluted in 0.5 % Tween 20 (Sigma, cat. no P7949). Treatments were performed in three biological replicates with 15 twigs each, totalizing 45 twigs in each of the 21 different treatments. A solution of 0.5 % Tween 20 was used as control. Endodormant (254 CH - 9 % bud break) and ecodormant (1,614 CH − 100 % bud break) buds were used in each CK treatment.

Vector constructions for transactivation assays

The promoter region of *MdoDAM1* (-614 to -111 bp) was amplified by PCR from gDNA of 'Royal Gala' buds using Platinum™ Pfx DNA Polymerase (Invitrogen, USA) and specific primers (Table S1), followed by amplicon purification (PureLink™ PCR Purification Kit; Invitrogen, USA) and cloning into the entry vector pGEM®-T Easy (Promega, USA) as instructed by manufactures. The directional cloning of *MdoDAM1* promoter into the multiple cloning site 1 (MCS1) of pGUSXX-90 vector (Pasquali et al. 1994) was done by double-digest with BamHI and HindIII restriction enzymes. The ligation reaction was performed by T4 DNA ligase (New Engand BioLabs, EUA) following manufactures instructions.

The mutated *MdoDAM1* promoter for the two BRR biding sites was synthetized and cloned into pMK-RQ vector by GeneArt® Gene Synthesis (Thermo Fisher Scientific, USA). Four nucleotides substitutions on each BRR biding site were introduced (-518A>C, -517G>C, -516A>T, -513T>G) and (-179A>C, -178G>C, -177A>T, -176T>G). The mutated *MdoDAM1* promoter was digested from pMK_RQ entry vector and cloned into pGUSXX-90 by double-digest with the same restriction enzymes used in the native version. The final construction consists of the promoter region of the respective native or mutated version of *MdoDAM1* promoter fused to CaMV 35S -90 minimal promoter region, that together guide the expression of *gusA* reporter gene into pGUSXX-90 vector, creating the vectors constructions *MdoDAM1promo*::*gusA* (native promoter) and *MdoDAM1promo^mut*::*gusA* (mutated promoter). The cloning of both native and mutated *MdoDAM1* promoter regions as well as the BRR biding sites was confirmed by sequencing.

The transient overexpression of *MdoBRR1*, *MdoBRR8* or *MdoBRR10* was achieved by cloning each CDS region into the pART7 vector (Gleave 1992) using Gateway® technology (Thermo Fisher Scientific, USA). The *Renilla Luciferase (rLuc)* gene cloned into p2rL7 (De Sutter et al. 2005) was used as an internal control of the transfection process.

Transactivation assay

Five-week-old *A. thaliana* plants were used for protoplast isolation by the Tape-Arabidopsis Sandwich method (Wu et al. 2009). Two genotypes of *A. thaliana* were used in the experiments: Col-0 (wild type) and the loss of function triple mutant *arr1-3*/*arr10-5*/*arr12-1*, purchased from the Arabidopsis Biological Resource Center (ABRC; Stock number: CS39992). *A. thaliana* mutant plants were genotyped by PCR using specific primers to T-DNA insertion sites (Table S1). PEG–calcium mediated transfection method was used to deliver plasmid DNA into protoplasts, followed by 16 h incubation to allow gene expression (Yoo et al. 2007). Three independent plasmids were transfected to 1×10^5

protoplast suspension: p2rL7::*rLuc, MdoDAM1promo*::*gusA* (native or mutated version) and the respective pART7::*MdoBRR* tested.

In the assay using CK treatments, protoplasts were transfected just with p2rL7::*rLuc, and MdoDAM1promo*::*gusA* without MdoBRRs effectors. After overnight incubation, three different concentrations of TDZ (1.0, 0.1 and 0.0001 µM; diluted in 1% of DMSO) were added to protoplasts for 4 h. DMSO 1% was also used as control. For each transactivation experiment, four biological and three technical replicates were analyzed. Fluorescence and luminescence were evaluated as described in Yoo et al. (2007) using a SpectraMax® i3 Multi-Mode Detection Platform (Molecular Devices, USA).

Statistical analysis

All datasets were statistically analyzed using GraphPad Prism version 6.01 for Windows, GraphPad Software (La Jolla, California, USA). All datasets were compared using One-way ANOVA followed by a multiple comparison test (Tukey or Dunnet) with statistical significance set at 0.05. For transcriptional profiles in different apple developmental stages and transactivation assays, Tukey multiple comparison test was used in order to compare all against all. In the evaluation of *MdoBRRs* and *MdoDAM1* expression in the dormancy cycle, *t*-zeatin quantification and CK treatments experiments, Dunnet multiple comparison test was the choice, once samples were compared against a specific point. For 'Castel Gala' dormancy cycle evaluation, the relative expression of each *MdoBRR* and *t*-zeatin amounts were compared to 118 CH time-point. In controlled conditions for 'Royal Gala', the time-point chosen was 315 CH. Relative expression of each *MdoBRR* tested in the CK treatments was compared to its respective control.

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SUPPORTING INFORMATION

A)


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           E-value                                              REC domain
MdoBRR1    1.6E-30   MRVLAVDDDPICLELLEALLRRCQYHVTTTSQAITALELLR-NKDKFDLVISDVHMPDM-DGFKLLELVGLEMDLPVIMLSVNGDHKLVMKGITHGACDYLLKPVRIEQLKNIW
MdoBRR2    1.2E-15   LQILVVPFDTLSLMCIASTLEKYSFKVTTTALASVALSMIKEQKYHYDLVMANISMPDK-DKFSLLQVL-HKNEIPVIFMSSEVNIDVAKKALAEGACFFLQKPVSSEDLKNVW
MdoBRR3    1.0E-17   VTVLAVDGDSACLTLLSRMLCKLGYKVLTAKRACDALSIAQKKESELHLVLTEVHLPDM-DKYELLERMRAVSKLPVVIMSDDDDENAMLGGLFKGAVYYIVKPLTMDSLKNLW
MdoBRR4    5.5E-16   LRILVLPVDADFLTFISGLLKLLKHEVVTFMNPIQALSALLLAEHKFHLVITDLYLPDIFSGLQVIRRVRDEFKLPLIVASADNSESTILAALENGAVHYTVKPVCFSDVKNLW
MdoBRR5    6.7E-32   LRVLVVDDDPTWLKILEKMLKKCSYEVTTCGLATEALCLLREKKNGYDIVISDVNMPDM-DGFKLLEHVGLEMDLPVIMMSVDGETSRVMKGVQHGACDYLLKPIRMKELRNIW
MdoBRR6    5.3E-32   LRVLVVDDDPTWLKILEKMLKKCSYEVTTCGLARDALHLLRQKKDGYDIVISDVNMPDM-DGFRLLEHVGLEMDLPVIMMSVDGETSRVMKGVQHGACDYLLKPIRMKELRNIW
MdoBRR7    1.9E-29   LRVLVVDDDPTCLMILERMLRTCLYEVTKCNRAEIALSLLRENKNGFDIVISDVHMPDM-DGFKLLEQVGLEMDLPVIMMSADDGKSVVMKGVTHGACDYLIKPVRIEALKNIW
MdoBRR8    1.6E-30   LRVLVVDDDPTCLMILERMLRTCLYEVTKCNRAESALSLLRENKNGYDIVISDVHMPDM-DGFKLLEQVGLEMDLPVIMMSADDGKSVVMKGVTHGACDYLIKPVRIEALKNIW
MdoBRR9    9.0E-29   LRVLVVDDDTTCLRILELMLLRCLYQVTTCSEATVALNLLRERKDCFDVVLSDVHMPDM-DGFKLLEHVGLEMDLPVIMMSADGRTSVVMRGIRHGACDFLIKPISEAELKNIW
MdoBRR10   5.7E-29   LRVLVVDDDTTCLRILELMLLRCLYQVTTCSEATVALNLLRERKDCFDVVLSDVHMPDM-DGFKLLEHIGLEMDLPVIMMSADGRTSVVMRGIRHGACDFLIKPVREAELKNIW
consensus            ...*..*.*...*  .....*... ..*...  .  **....... ...*......**. ...............*....*... ....... .**.....**........*.*
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B)


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           E-value             Myb-like domain
MdoBRR1    4.1E-25   KPRVVWSVDLHRKFVAAVNQLGIDKAVPKKILDLMNVEKLTRENVASHLQKYRL-
MdoBRR2    8.2E-18   ----VWTTELHLKFTAAISALGDQKARPRRILKWMNVPDITVRQVASHLQKYRKN
MdoBRR3    1.1E-19   KPKMVWTNELHKKFVQAVTLLGVDSAHPKKILQHMNVPGLWKENXSSHLQKYRL-
MdoBRR4    7.2E-25   KHKVEWTSELQDRFLEAINYIGLDKAVPKRILEVMNVEGLTRENVASHLQKYRM-
MdoBRR5    2.4E-21   KARVVWSVDLHQKFVKAVHQIGFDKVGPKKILDLMNVPWLTRENVASHLQKYRL-
MdoBRR6    1.3E-19   KARVVWSVDLHQKFVKAVHQIGFD-IGPKKILDLMNVPWLTRENVASHLQKYRL-
MdoBRR7    1.7E-24   KPRVVWSVELHQQFVGAVNQLGIDKAVPKKILELMNVPGLTRENVASHLQKYRL-
MdoBRR8    1.7E-24   KPRVVWSVELHQQFVGAVNQLGIDKAVPKKILELMNVPGLTRENVASHLQKYRL-
MdoBRR9    3.3E-24   KPRVVWSVELHQQFVTAVNQLGLDKAVPKRILELMNVPGLTRENVASHLQKFRL-
MdoBRR10   4.2E-24   KPRVVWSVELHQQFVTAVNQLGLDKAVPKRILEMMNVPGLTRENVASHLQKFRL-
consensus            .....*...*...*. *....*.....*..**..***..........*****.*.
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Figure S1. Conserved motifs found in apple BRRs. Alignment of protein motifs related to the two conserved domains found using InterPro v.70.0 database. **(A)** The quintet (DDDS/TK) of essential amino acid residues for REC activity is highlighted in black. **(B)** Alignment of the B-motif related to Myb-like DNA biding domain family in MdoBRRs.
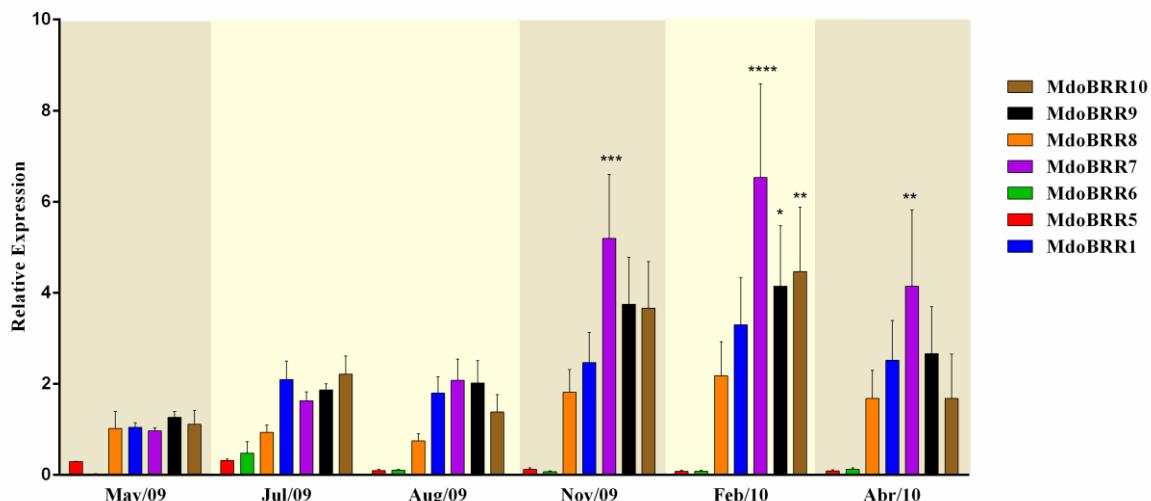
Figure S2. Expression pattern of MdoBRR in 'Royal Gala' buds during an annual growing cycle. The relative expression of each evaluated *MdoBRR* was compared to its respective data found in May/09. The calibrator value used in Pfaffl calculation was *MdoBRR1* at May/09. Statistical analysis was done through One-way ANOVA followed by Dunett test with GraphPad Prism v.6 software (*$0.01 < p < 0.05$, **$0.001 < p < 0.01$***).

A)

B)

C)
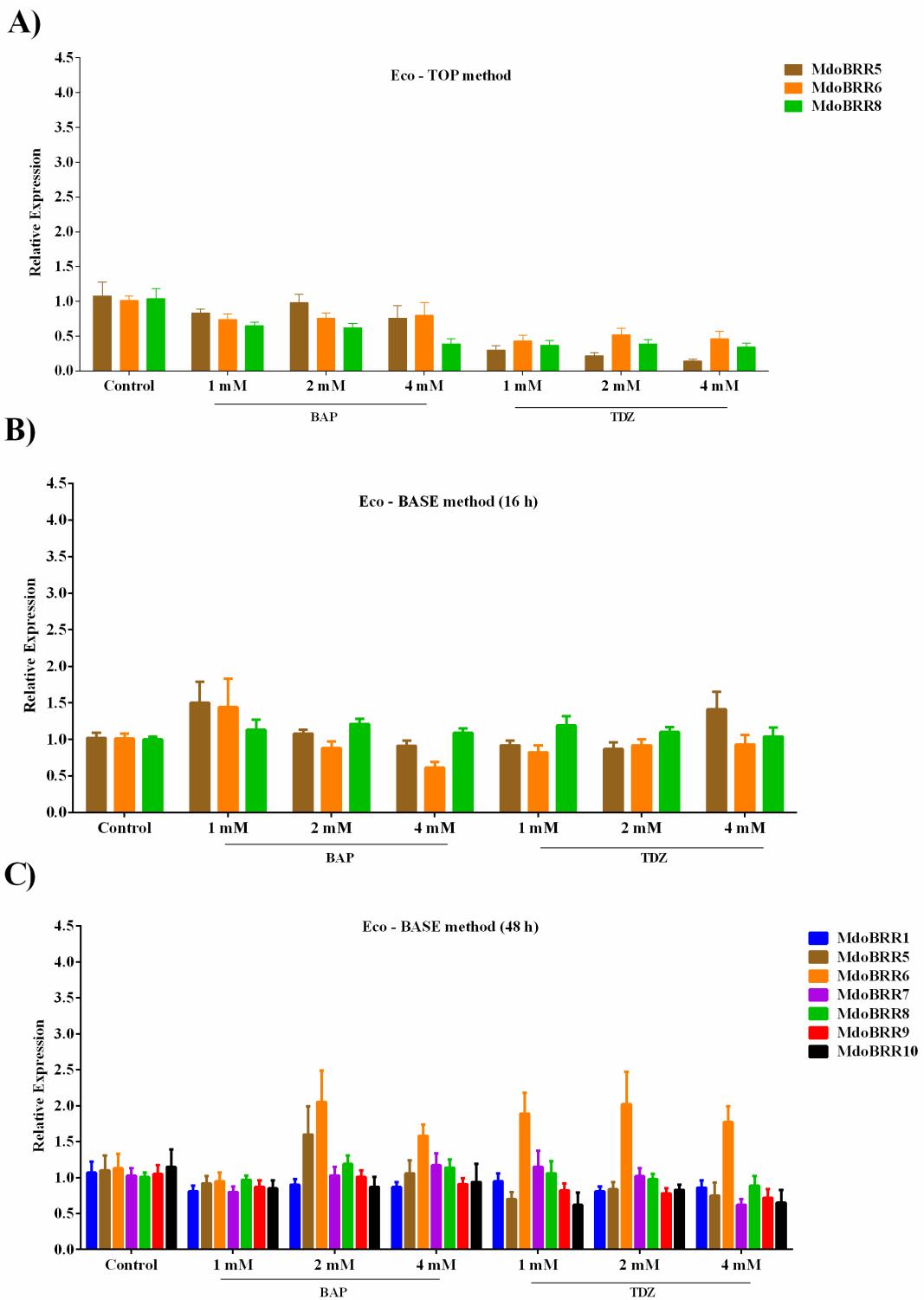
Figure S3. CK treatments in ecodormant apple buds. Relative expression of *MdoBRRs* that did not respond to CK stimulus is shown for (A) TOP, (B) BASE for 16 h or (C) 48 h treatment methods for ecodormant buds. Statistical analysis resulted in non-significant differences (One-way ANOVA followed by Dunett test with GraphPad Prism v.6 software).

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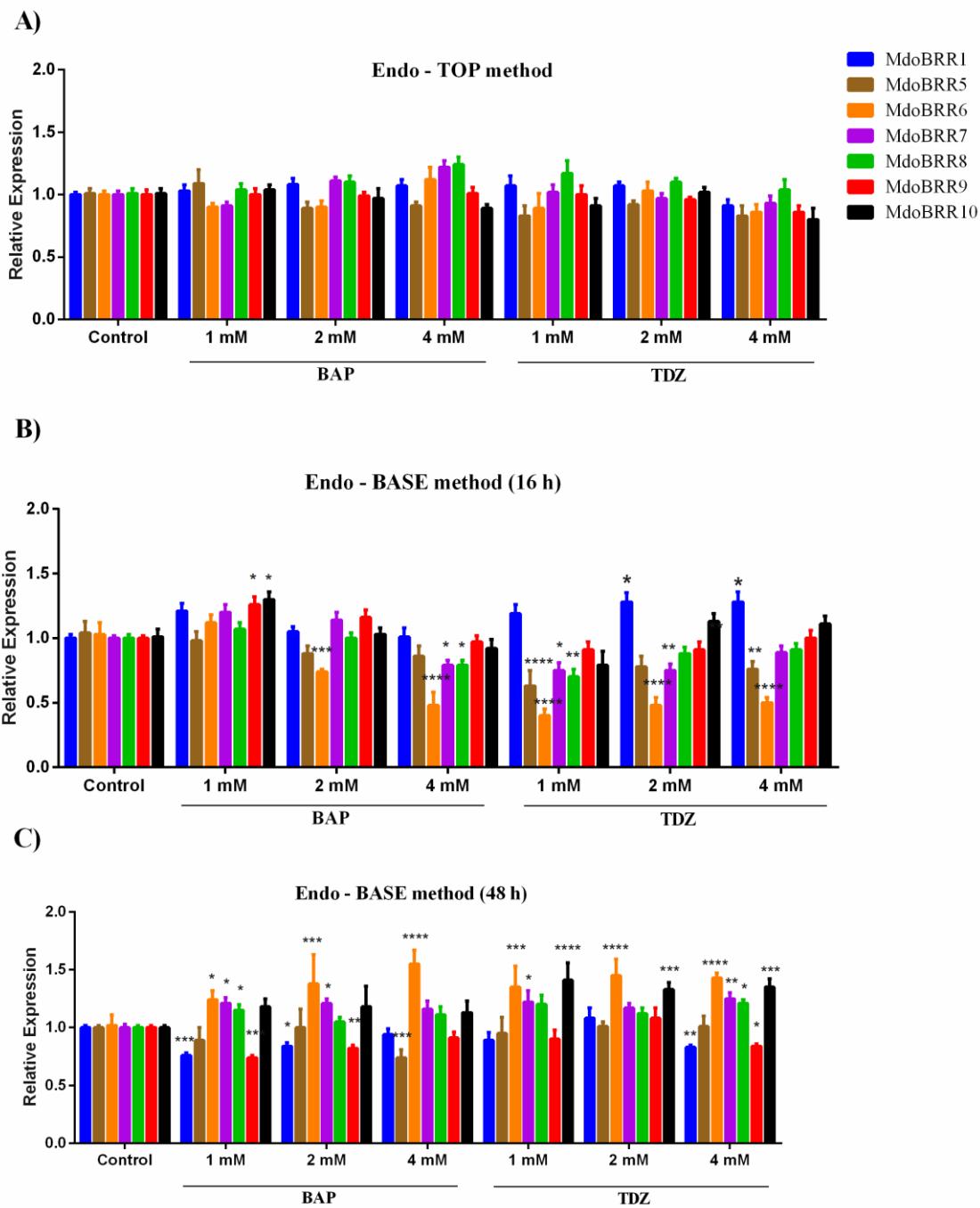
Figure S4. CK treatments in endodormant apple buds. Endodormant buds treated with BAP or TDZ at 1, 2 or 4 mM by the (A) TOP or BASE method for (B) 16 h or (C) 48 h. Statistical analysis was performed comparing the relative expression of each evaluated gene to its corresponding control with One-way ANOVA followed by Dunett test with GraphPad Prism v.6 software (*$0.01 < p < 0.05$, **$0.001 < p < 0.01$***).

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Figure S5. Structure of *MdoDAM1* promoter region. (**A**) The representative image is composed by the promoter region (green box), exons (orange boxes), and introns (black lines). The *cis*-elements are represented by a vertical black line, along with the distance from the start codon; adapted from Porto et al. (2016). (**B**) Alignment among predicted *MdoDAM1* promoter region (Ref) and sequencing results (Seq) of wild type (wt) and mutated (Mt) sequences. The sequence of BRRs motifs are highlighted in red for wild type and in blue for mutated sites.

Table S1. Primers used in this work

Reference sequence[a]	Usage	Sequence (5'-3')	Product (bp)
MdoBRR1; MD08G1059000	CDS amplification	CACCATGACCGTTGAGCG	2,114
		CTATCTCCTTTCCTGTTTGAT	
	RT-qPCR	AGCTTGTCGGGCTTGAGAT	85
		TGGGTGATTCCCTTCATCAC	
	Sequencing	CATTGACTGGACCTGCG	-
		GCTCAGCCATCTGCGT	
		CCACAATGCTGGTTCGTTG	
MdoBRR2; MD16G1036100	CDS amplification	CACCATGGAAGACATGAGCCCTA	2,235
		CTATGGTGAATCCGGGCTCT	
		caccATGTCATTGATTCAAGCTTC	-
	RT-qPCR	GAATGTGCCTGATATAACGGTGCG	93
		CTCGTCGTGCCAACCTCCTG	
MdoBRR3; MD01G1047800	CDS amplification	CACCATGTATTTGCGCAACAAC	2,334
		TCACCAGAAATCATCGAAATTG	
		CACCTGAATGATGAGAAGGCCGT	873
		TTAATCAAGGATTACAGAGC	
	RT-qPCR	CTCGTCCATGCTGCTTTCCC	95
		GGCTTTCCACCAGTCGTTTTTCT	
MdoBBR4; MD02G1229600	CDS amplification	caccATGCTCTCTCCCCCTCTTTC	1,926
		CTACACGTTCGAGAGGAGCTTG	
		caccATGCGAAGGCCTAAGAGAGG	1476
		CTACCATGACAAATATTTATACGA	
	RT-qPCR	ACAAGGCTGTTCCAAAAAGAATCC	87
		GATATTTCTGCAAATGACTTGC	
MdoBRR5; MD16G1108400	CDS amplification	caccATGGAGAGTAGCTTCT	1,914
		CTATGACAGAATATTCTGATGGG	
	RT-qPCR	GAGCAGAAGCCACTTATTCAGTTG	119
		GAAGGTCTTGGACTAATGGAAGG	
	Sequencing	TGGCAGCATGTCTTTAGAAGG	-
MdoBRR6; MD13G1108300	CDS amplification	caccATGGAGAGTAGCTTCTC	1,842
		TCATGATATGAATAGACCTTGACC	
	RT-qPCR	GTCTGTTTGGCAGAAGTTGGTCC	118
		GCAGCCTACTCAAGTAGAGCCG	
MdoBRR7;* MD16G1159400	RT-qPCR	GAACTTCATCAACAGTTTGTGGGC	81
		CAGGAACATTCATCAACTCCAAAAT	
	Sequencing	CAAAACAGCTTGCCAACTTGC	-
MdoBRR8; MD13G1159700	CDS amplification	caccATGCATCTGAGCAAC	2,013
		CTACACAGGAATATTATCCAAGGA	
	RT-qPCR	GCGGGTTGTTTGGTCAGTCG	95
		ACTCCAGAATTTTCTTAGGCACGG	
MdoBRR9; MD13G1019800	CDS amplification	CACCATGGCTGCTTTGCAG	2,013
		CTAATCAGCGAAAGGACTCATG	
	RT-qPCR	GGGACCTCTTAGGAATCTCGGA	109
		GATCCTTCCAGGGTTCAAGTTGC	
	Sequencing	ATGATGATGGTGATACAGAAAACG	-
		CCACAATCCGTTATTTCTGCTG	
MdoBRR10; MD16G1017900	CDS amplification	caccATGGCTGCTTTGCAG	2,217
		CTAATCAGTGAAAGGACTCAT	
	RT-qPCR	GGTGCCCTAGTCAAGCAGGAG	117

		CAAGAGACATCGGCTAACCACAAA	
	Sequencing	CAAGCGTTGTTATGAGAGGA	-
		TCTTGAAATGATGAATGTACCCG	
		ACCTTGCCGTTCGTTTAATGT	
Manson, et al. (2005)[b]	arr triple mutant genotyping	CTTCAAGCACTAGCCGTCACAGGTCAGTT	1,306[d]
		AATGTTATCGATGGAGTATGCGTCAAAGT	
Argyros, et al. (2008)[b]		GCCACCTTCAGGTGAGAGTTAGACTATGAT	1,149[d]
		AGCTGACAAAGAAAAGGGAAAATGGAGTTT	
SIGnAL; Salk[c]		GTGATTTGATCCACATATGGTG	1,151[d]
		GCTGATTAGCCACACCACTGA	
Mdo*DAM1*; MDP0000322567	RT-qPCR	GGAGAAGGGGGATTTTCAAGA	288
		CGTGATGAATCGTGGGTTGA	
	Promoter amplification	GGATCCCCGTTGATTGAAGTTCCATG	515
		TTATCAGAAACCCAGAAAATATATTGAAGCTT	

[a]Primers were designed using GDDH13_v1.1 apple genome as reference and accession codes are available at http://rosaceae.org/.

[b]Primers already described by the authors

[c]Primers designed by "T-DNA Primer Design" tool available at http://signal.salk.edu/tdnaprimers.2.html

[d]Amplicon size when Arabidopsis wild type DNA is used as template

*Primers used for coding region (CDS) amplification of *MdoBRR7* are the same as those employed for Mdo*BRR6* since genes share the same 5' and 3' ends. RT-qPCR primers are specific

Table S2. Type B reponse regulators protein sequences used in phylogenetic analysis

Organism	Gene name	Genome acession code[a]	Reference
Arabidospis thaliana	*ARR1*	At3g16857	TAIR Gene Family avalible in https://www.arabidopsis.org/browse/genefamily/ARR.jsp
	ARR2	At4g16110	
	ARR10	At4g31920	
	ARR11/ARP3	At1g67710	
	ARR12	At2g25180	
	ARR13	At2g27070	
	ARR14	At2g01760	
	ARR18	At5g58080	
	ARR19	At1g49190	
	ARR20	At3g62670	
	ARR21	At5g07210	
	ARR23	At5g62120	
Oryza sativa sub. Japonica	*Rr21*	Os03g12350	Pareek et al. (2006); Ito and Kurata (2006); Doi et al. (2004); Schaller et al. (2006)
	Rr22	Os06g08440	
	Rr23	Os02g55320	
	Rr24	Os02g08500	
	Rr25	Os06g43910	
	Rr26	Os01g67770	
	Rr27	Os05g32880	
	Rr28	Os04g28160	
	Rr29	Os04g28130	
	Rr30	Os10g32600	
	Rr31	Os08g35650	
	Rr32	Os08g17760	
	Rr33	Os08g35670	
	PtRR13	Potri.010G001000.1	Immanen et al. (2013)

	PtRR14	Potri.008G181000.1	
	PtRR15	Potri.008G135500.1	
	PtRR16	Potri.010G105600	
	PtRR17	Potri.012G133800.1	
	PtRR18	Potri.006G262100.1	
Populus trichocarpa	*PtRR19*	Potri.018G111300	
	PtRR20	Potri.015G136000	
	PtRR21	Potri.010G053100	
	PtRR22	Potri.018G021300.1	
	PtRR23	Potri.006G188000	
	PtRR24	Potri.010G001000.1	
	PtRR25	Potri.018G094700.1	
	PpARR10	ppa023099	
	PpARR11-a	ppa002679	
	PpARR14-b	ppa020007m	
	PpRR1	ppa002408	
Prunus persica	*PpRR10*	ppa023185	Immanen et al. (2013); Zeng et al. (2017)
	PpRR4	ppa002900	
	PpRR5	ppa018934	
	PpRR6	ppa024819	
	PpRR7	ppa002493	
	B-PpRR1	Pbr032760.1	
	B-PpRR2	Pbr013363.1	
	B-PpRR3	Pbr19035.2	
	B-PpRR4	Pbr28774.1	
	B-PpRR5	Pbr011993.1	
Pyrus bretschneideri	*B-PpRR6*	Pbr020017	Ni et al. (2017)
	B-PpRR7	Pbr034277.2	
	B-PpRR8	Pbr036239.1	
	B-PpRR9	Pbr012127.1	
	B-PpRR10	Pbr034723.1	
	B-PpRR11	Pbr039458.1	
	MdoBRR1	MD08G1059000	
	MdoBRR2	MD16G1036100	
	MdoBRR3	MD01G1047800	
	MdoBRR4	MD02G1229600	
Malus× domestica	*MdoBRR5*	MD16G1108400	Present data
	MdoBRR6	MD13G1108300	
	MdoBRR7	MD16G1159400	
	MdoBRR8	MD13G1159700	
	MdoBRR9	MD13G1019800	
	MdoBRR10	MD16G1017900	

[a]Protein full-leght sequences were extracted from Plant TFDB (http://planttfdb.cbi.pku.edu.cn/) or PLAZA databases (https://bioinformatics.psb.ugent.be/plaza/)

Table S3a. Dates and chilling accumulation of 'Castel Gala' buds harvested in 2016 in Vacaria, Rio Grande do Sul - Brazil.

Date	Hours bellow 7.2°C
05/04/2016	118
06/09/2016	325
07/28/2016	707
08/18/2016	778

Table S3b. Dates and chilling accumulation of 'Royal Gala' buds harvested in 2009 and 2010. The experimental area belonged to a commercial orchard in Papanduva, Santa Catarina, Brazil.

Season	Date	Hours bellow 7.2°C
Autumn	05/26/2009	53
Winter	07/01/2009	282
Winter	07/30/2009	446
Spring	11/24/2009	0
Summer	02/08/2010	0
Autumn	04/13/2010	0

5 CAPÍTULO III

Characterization of potential regulatory functions of *MdoICE1* and *MdoCBF*s genes during dormancy progression in apple.

Artigo a ser submetido ao periódico "Plant Science"

TITLE

Characterization of potential regulatory functions of *MdoICE1* and *MdoCBF*s genes during dormancy progression in apple.

Amanda Malvessi Cattani[1, 3], Cássia Beatriz de Souza[2, 3], Vítor da Silveira Falavigna[4] Tiago Sartor[3], Vanessa Buffon[3], Giancarlo Pasquali[1], Luís Fernando Revers[1,3*]

[1]Graduate Program in Cell and Molecular Biology, Center for Biotechnology, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[2]Undergraduate Program in Biotechnology and Bioprocess Engineering, Universidade Estadual do Rio Grande do Sul, Bento Gonçalves, RS, Brazil.

[3]Laboratory of Plant Molecular Genetics, Embrapa Uva e Vinho, Bento Gonçalves, RS, Brazil.

[4]Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D50829, Köln, Germany

*To whom correspondence should be addressed. Tel: +5554 3455-8039/3455 8056 Fax: +5554 3451-2792; e-mail: luis.revers@embrapa.br

ABSTRACT

Dormancy is an important adaptive response of plants from temperate regions to increase their protection from low temperatures during winter. As soon as cold is perceived, *CBF* genes are rapidly upregulated, promoting the modulation of downstream responsive genes. The upstream regulation of *CBF*s is mediated, among others factors, by *ICE1* that starts the transcriptional cascade used by plants to cold acclimation. In apple trees, the *ICE1* gene was found in a QTL for bud break time, becoming an interesting candidate in the regulation of this trait. Aiming to understand if ICE1-CBF pathway is involved in apple bud dormancy control, we characterized the transcriptional profile of *MdoCBF*s and *MdoICE1* genes throughout the dormancy cycle and tested if MdoICE1 is capable to modulate them in a cold-dependent manner. The analysis of different plant developmental stages demonstrated that *MdoICE1*, *MdoCBF2*, *MdoCBF4* and *MdoCBF5* were highly expressed in dormant buds. Further, the evaluation of buds dormancy cycle revealed the accumulation of *MdoCBF2* and *MdoCBF4* transcripts during the beginning of endodormancy phase. The expression of *MdoICE1* did not oscillate throughout the transition from endo- to ecodormancy, being repressed only after 96 h of exposure to warm temperatures (25 °C). Additionally, in these samples, the analysis of the growth regulators that may affect CBF-dependent cold acclimation route, like abscisic (ABA), gibberellic (GA) and salicylic (SA) acids reveled peaks of ABA accumulation throughout all cycle. GA amounts increased in the periods of ecodormancy and bud break while SA was exclusively upregulated at the bud burst stage. The *in vivo* transactivation assay showed that cold stimuli increased GUS activity in the reporter constructions driven by the promoters of *MdoCBF4* and *MdoCBF5* genes. Room temperature incubation and the addition of MdoICE1 effector were not able to activate any of the tested *MdoCBF* promoters. Finally, the results showed that *MdoCBF2* and *MdoCBF4* are important genes

in endodormancy establishment and their activation is mediated by cold stimulus, independently of MdoICE1 activation.

Key words: Apple, Bud dormancy, *MdoCBFs*, *MdoICE1,* Cold sensing

1. INTRODUCTION

Dormancy is an important adaptive trait for temperate fruit crops and it overlaps with cold acclimation processes induced by low and non-freezing temperatures in apple trees [1]. The winter low-temperature signals induce bud-set and endodormancy establishment. At this stage, apical meristems are protected inside scales, forming the terminal buds, and cell communication is blocked by callose deposition in plasmodesmata channels avoiding the influx of water, nutrients and signaling molecules [2,3]. Throughout continuous chilling accumulation, endodormancy is released and ecodormancy is achieved. The amount of chilling accumulation to reach ecodormancy is genotype/variety dependent. At the ecodormancy stage, growth is only restored after an appropriate period of exposure to light and warm temperatures [4].

The cold hardiness involves cellular adaptations like biochemical changes, hormonal homeostasis, metabolic balance and gene expression regulation in order to prevent cell dehydration by extracellular ice and freezing temperatures. [5]. In this scenario, the COLD-INDUCIBLE C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTORS (CBF/DREB), henceforth called CBF, are key activators that regulate a number of *COLD-REGULATED* (*COR*) genes [6,7]. CBFs are transcriptional factors that have three particular domains: the AP2 DNA binding, the PKKP/RAGRxKFxETRHP (abbreviated to PKKPAGR) and the DSAWR motif. Moreover, in some cases, they also show a LSWY signature in the C-terminus region [5].

In Arabidopsis, several studies have been correlating *CBF* expression to frost tolerance [8–11]. The cellular pathway is triggered by low temperatures which leads to an increase in cytosolic calcium levels, changing calcium-sensor proteins that activate pre-existing transcription factors to induce *CBF*s expression [12,13]. One of the most important *CBF* activators is the INDUCER OF CBF EXPRESSION 1 (ICE1) that belongs to the family of MYC-like bHLH transcriptional factors. The *ice1* loss of function mutation in Arabidopsis disrupted cold and freezing tolerance and resulted in a drastic reduction in the expression of *CBF3* and its target genes [14]. In the same way, *ICE1* overexpression in wild-type plants improved freezing tolerance and activated the *CBF* regulon in a cold-dependent manner [14]. CBFs are able to modulate *COR* genes that encode a diverse array of proteins such as enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants; molecular chaperones and other antifreeze proteins [15].

In apple, four *ICE*, five *CBF*s and one pseudo-*CBF* genes were already identified [16,17]. The ectopic expression of peach *CBF1* in apple trees resulted in plant growth reduction, increased cold tolerance, anticipation of leaf senescence and delayed bud break [17–19]. Similarly, the ectopic expression of *MbDREB1*, a CBF-type transcription factor from dwarf apple (*Malus baccata*), enhanced plant tolerance to low temperature, drought and salt stress via both abscisic acid (ABA)-dependent and independent pathways [17,20]. Besides ABA, other plant growth regulators like gibberellins (GA) and salicylic acid (SA) might affect the CBF-dependent cold acclimation pathway [21–23].

Among all identified *ICE* genes in apple, *MdoICE1* was already described in a QTL related to bud break time [24]. Another study also demonstrated that *MdoICE1* is

differentially expressed throughout the dormancy cycle in cultivars with contrasting chilling requirement [25], indicating a potential regulatory role in dormancy control. Moreover, the overexpression of an *MdoICE*-like gene, encoded by *MdoCIbHLH*, improved cold tolerance in Arabidopsis and tobacco transgenic plants if compared to wild-type [16].

Based on the idea that dormancy and cold acclimation are overlapping mechanisms in woody species, and little is known about CBF role along the bud dormancy cycle, the aim of this study was to characterize the apple *CBF* gene family in the perspective of bud dormancy regulation. Additionally, we investigated whether the potential regulator *MdoICE1* is able to modulate them.

2. MATERIAL AND METHODS

2.1 Structural analysis of apple *CBF*s and *ICE1* genes

The *in silico* functional annotation of the predicted apple CBFs and ICE1 proteins [16,17] was performed by NCBI's CDD (Conserved Domain Database) for MdoCBFs [26] and by SMART (Simple Modular Architecture Research) for MdoICE1 [27]. The search for the two CBF specific motifs PKKPAGR and DSAWR was done by BLAST Needleman-Wunsch Global Align tool [28] using as query the PKKPAGR motif found in Arabidopsis *CBF1* gene [29] and the DSAWR signature.

The validation of the predicted coding region (CDS) of *MdoCBFs* was performed through Sanger sequencing. CDSs were amplified by PCR using Platinum™ Pfx DNA Polymerase (Invitrogen, USA) and specific primers designed by Vector NTI v.10 software (Invitrogen, USA; Table S1). Amplicons were purified (PureLink™ PCR Purification Kit; Invitrogen, USA) and cloned into pENTR™/D-TOPO™ (Invitrogen, USA) as instructed by the manufacturer. M13 primers were used in the sequencing reactions. Sequencing results were aligned against the GDDH13 reference genome [30] by MEGA7 7.0 version software [31]. The graphical representation of the localization of the protein domains was designed by IBS v.10.3 [32].

2.2 Transcriptional profile in apple developmental stages

Thirteen different apple tissues and organs were sampled from Gala Baigent® trees in the experimental orchard at the Temperate Fruit Tree Experimental Station of Embrapa Uva e Vinho (TFES), in Vacaria, RS, Brazil (28°30'50''S, 50°54'41''W, 972 m altitude). The sampling strategy was based on the Fleckinger scale [33] and encompassed reproductive and vegetative cycles. Closed terminal buds (A), buds at initial bursting (C), flower buds (E2IN), young leaves (E2L), 10 mm just-set fruit (IF) and mature leaves (IL) were harvested. The fruit development was also monitored in unripe 40 mm fruit through leaves (JL), skin (JSK), pulp (JPU) and (JS) seeds. Apple mature fruit with approximately 70 mm diameter partitioned into skin (MSK), pulp (MPU), and (MS) seeds were also investigated. The complete descriptions of the developmental stages evaluated in this study can be found in Perini *et al.* (2014). Three biological replicates containing a pool of samples harvested from ten clonal trees were frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

2.3 RNA extraction and RT-qPCR

RNA extraction was performed as described by Zeng & Yang (2002) with modifications found in Falavigna *et al.* (2014). One μg of total RNA per sample was treated with TURBO DNAse (Applied Biosystems, USA) and reverse-transcribed following manufacturer instructions in a 20 μl reaction volume by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with random primers. The complete removal of DNA contaminants was confirmed using intron-flanking primers for *TRANSCRIPTION FACTOR WD40-LIKE REPEAT DOMAIN* (*WD40*) gene through PCR reaction.

The obtained cDNA was hundred-fold diluted with water and 10μl was used in RT-qPCR. Analyses were carried out in a 20 μl volume using SYBR ® Green (ThermoFisher, USA) in three technical repeats as described in Falavigna *et al.* (2014). RT-qPCR specific primers were designed by Vector NTI Advance v.10 (Invitrogen, USA; Table S1). The relative expression level was calculated using the Pfaffl method [36] and the threshold cycle (CT) values were normalized by the reference genes *MALATE DEHYDROGENASE* (*MDH*), *TYPE 1 MEMBRANE PROTEIN-LIKE* (*TMp1*) and *WD40* when all tissues were analysed. For bud samples calculation, *TMp1* was replaced by *ARC5* as described in Perini *et al.* (2014). Primers efficiencies were obtained from Lin-RegPCR version 2017.1 tool [37]. The calibrator used in Pfaffl calculation when all apple tissues were analysed were the stages A (*MdoCBFs*) or MPU (*MdoICE1*). *MdoCBF* genes were calibrated by *MdoCBF1*. For dormancy cycle evaluation, the calibrator is *MdoCBF1* at 180 CH.

2.4 Dormancy cycle evaluation

The dormancy cycle evaluation was performed using dormant buds from twigs of 'Royal Gala' sampled in the orchard of TFES with 180 chilling hours (CH) in June 2017. Twigs (20 cm long) were decontaminated and wrapped in black plastic bags as described by Falavigna *et al.* (2015). The bags were placed inside growth chambers in the dark with terminal buds positioned upwards in a constant temperature of 3 °C. Sampling occurred every seven days (168 h) of chilling exposition (316, 484, 652, 820, 988 and 1,156, 1,324 and 1,492). After 1,492 CH, twigs were transferred to growth permissive conditions (25±1.5 °C, 16 h photoperiod and 70% relative humidity) and sampled after 24, 48, 96, 144, 240 and 360 h. Buds at the different time-points were frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Three biological replicates, containing 15 buds each, were used in each of the 15 different treatments. In order to evaluate the physiological stage of bud dormancy (endo- or ecodormancy), a batch of 40 twigs at each sampled point was conditioned in a growth chamber (25±1.5 °C, 16 h photoperiod and 70% relative humidity). After 35 days, the percentage of bud break was calculated by dividing the number of green tip buds by all viable buds tested. In this study, we defined that the transition from endo- to ecodormancy was reached when more than 50% of the buds were at the green tip stage.

2.5 Hormonal quantification

The hormone extraction was performed adding 4.0 mL of extraction solution (methanol:water:formic acid, 75:20:5, v:v:v) in a tube containing 500 mg of plant material. Samples were incubated at -20 °C for 3 h. After that, they were conditioned in an ultrasound bath (40 kHz frequency) for 25 min at 4 °C and supernatants were recovered by centrifugation at 1,750 g at 4 °C for 30 min. Extraction step was repeated twice. The supernatant was transferred into a new 1.5 micro-centrifuge tube and dried in

a benchtop vacuum centrifuge (Eppendorf, DE) at 1,400 rpm at 30 °C under 20 mbar vacuum pressure until reaching the mark of 100 µL of residual liquid. To this remaining solution, 1.0 mL of water was added followed by full homogenization in a vortex. The samples were then transferred to Oasis MCX columns (Waters Corporation, USA). The column elution was conducted using a gradient of ammonium hydroxide (Acros Organics cat. no 205840025) from 0.004 to 0.4 M. To the eluted solution, 1.7 % w/v of PVPP (Sigma, cat. no. P6755) was added and the samples were homogenized in a vortex. The sample supernatants were recovered by centrifugation at 10,000 g for 45 min at 4 ℃. The supernatant was then transferred into a new 1.5 micro-centrifuge tube and dried in the vacuum centrifuge as described above. After drying, samples were ressuspended in 75 µL of methanol and filtered through a 0.22 µm polytetrafluoroethylene (PTFE) membrane. The quantification analysis was carried in UPLC-ESI-MS/MS (Waters Corporation, USA). Results are represented in ng per g of dry mass of each phytohormone.

2.6 *MdoICE1* sequencing

The CDS sequence of *MdoICE1* was PCR amplified using iProof™ High-Fidelity DNA Polymerase (Bio-Rad Laboratories, USA) and specific primers (Table S1). cDNA of dormant buds from three different cultivars ('Eva', 'Castel Gala' and 'Gala Standard') were used as templates. Amplicons were purified (PureLink™ PCR Purification Kit - Invitrogen, USA) and cloned into the entry vector pGEM®-T Easy (Promega, USA) as instructed by manufactures. The transformants were confirmed by restriction enzyme digestion and submitted to Sanger sequencing. In order to cover all *MdoICE1* sequence, T7, SP6 and one internal primer were used in the sequencing reactions (Table S1). The results from sequencing were analyzed and aligned using MEGA7 7.0 version software [31].

2.7 Constructions of vectors used in transactivation assays

The promoter region of *MdoCBF1* (from -2,290 to -1 bp), *MdoCBF2* (from -2,166 to -1 bp), *MdoCBF3* (from -2,451 to -1 bp), *MdoCBF4* (from -2,500 to -1 bp) and *MdoCBF5* (from -2,248 to -1 bp) were amplified by PCR from gDNA of 'Royal Gala' buds using Platinum™ Pfx DNA Polymerase (Invitrogen, USA) and specific primers (Table S1). Amplicons were purified using PureLink™ PCR Purification Kit, (Invitrogen, USA) and cloned into the entry vector pGEM®-T Easy (Promega, USA) as instructed by manufactures. The directional cloning of all five *MdoCBFs* promoters into the multiple cloning site 1 (MCS1) of pGUSXX-90 vector [39] was done by double digestion with appropriate restriction enzymes for each case. Ligation reaction was performed by T4 DNA ligase (New England BioLabs, EUA). The final construction consists of the promoter region of the respective *MdoCBF* fused to CaMV 35S -90 minimal promoter region that together guide the expression of *gusA* reporter gene into pGUSXX-90 vector, creating the constructions *MdoCBFxpromo*::*gusA*. The cloning of all *MdoCBFs* promoter regions was confirmed by sequencing. The *MdoICE1* transient overexpression was achieved by cloning its CDS region into the pART7 vector [40] using Gateway® technology (Thermo Fisher Scientific, USA). The internal control of the transfection process is made by *Renilla Luciferase* (*rLuc*) gene cloned into p2rL7 [41].

2.8 Transactivation assays

Five-week-old Arabidopsis plants were used for protoplast isolation by the Tape-Arabidopsis Sandwich method [42]. PEG–calcium mediated transfection was used to deliver plasmid DNA into protoplasts. After transfection, protoplasts were incubated under 25 °C or 0 °C by 16 h [43]. Three independent plasmids were transfected to 1×10^5 protoplast suspension: p2rL7::*rLuc, MdoCBFpromo*::*gusA* and pART7::*MdoICE1*. For each transactivation experiment, four biological and three technical replicates were analyzed. Fluorescence and luminescence were evaluated as described in Yoo *et al.* (2007) using a SpectraMax® i3 Multi-Mode Detection Platform (Molecular Devices, USA). Results are presented as the fraction of GUS activity and *rLUC* luminescence.

2.9 Statistical analysis

One-way ANOVA followed by a multiple comparison test (Tukey or Dunnet) with significance set to 0.05 was used in statistical analysis. The calculation was performed by GraphPad Prism version 6.01 for Windows, GraphPad Software (La Jolla, California, USA). Tukey multiple comparison test was used in transcriptional profiles of different apple developmental stages and transactivation assays, aiming to compare all against all. Dunnet multiple comparison test was the choice in the hormonal quantification and transcriptional profiles of *MdoCBFs* and *MdoICE1* during dormancy cycle in order to compare dataset with 180 CH time-point.

3.   RESULTS AND DISCUSSION

3.1 Structure analysis of *MdoCBFs*

The *MdoCBF* gene family was reported in 2011[17], right after the first apple genome draft became public [44]. The recent availability of the high-quality GDDH13_v1_1 apple genome [30] allowed us to reanalyze and confirm the presence of all the five *MdoCBF* genes previous described (Table S2). The full-length coding regions of *MdoCBF1*, *MdoCBF3* and *MdoCBF5* were confirmed by sequencing using specific primers (Table S1). Additionally, the sequence of *MdoCBF1* submitted in the GenBank (Table S2) also matched to our *in vitro* results. For *MdoCBF2* gene, only partial sequence (60%) of the coding region was obtained. The alignment among *MdoCBF2* prediction in the GDDH13 genome [30] and the *in vitro* sequencing results revealed that the coding region of *MdoCBF2* in GDDH13 genome was annotated in the wrong direction. The correct open reading frame is localized in antisense DNA strand and corresponds to that found in the GenBank (Table S2). For *MdoCBF4*, only the RT-qPCR amplicon could be retrieved under our PCR conditions. The sequencing of *MdoICE1* also confirmed GDDH13 prediction [30].

The confirmed deduced-protein sequences of the three MdoCBFs (MdoCBF1, MdoCBF3 and MdoCBF5) were used as query on NCBI's CDD search. For MdoCBF2 and MdoCBF4, sequences were obtained from GenBank and GDDH13 genome, respectively. The protein functional annotation confirmed the presence of the AP2 domain (cd00018) in all analyzed proteins (Figure 1A). Additionally to AP2, the two important PKKPAGR and DSAWR domains were also found (Figure 1A). The alignment of all motifs revealed a high degree of amino acid residues conservation among family members (Figure 1B).

Figure 1. Conserved domains found in MdoCBFs proteins. (A) The *in silico* functional annotation of all MdoCBF proteins revealed the presence of AP2 domain (pink), PKKPAGR (yellow) and DSAWR (green) motifs. **(B)** The alignment of amino acid residues from MdoCBFs motifs demonstrated a high degree of conservation among family members.

3.2 Dormant apple buds showed high transcripts amounts of *MdoCBF2*, *MdoCBF4* and *MdoCBF5*

Characterization of the transcriptional profile of all *MdoCBFs* and *MdoICE1* among different apple developmental stages revealed that *MdoCBF4* has remarkable expression in dormant buds (A), being practically not detected in other plant tissues (Figure 2). Dormant buds (A) also presented high transcriptional levels of *MdoCBF2* and *MdoCBF5*.

Moreover, *MdoCBF2* and *MdoCBF5* showed similar transcript patterns, with high expression in skin and pulp of ripe and unripe fruits (JSK, JPU, MSK and MPU). *MdoCBF3* transcripts were higher in the skin of J and M stages than other tissues and *MdoCBF1* gene was preferentially expressed in seeds of mature fruits (MS; Figure 2).
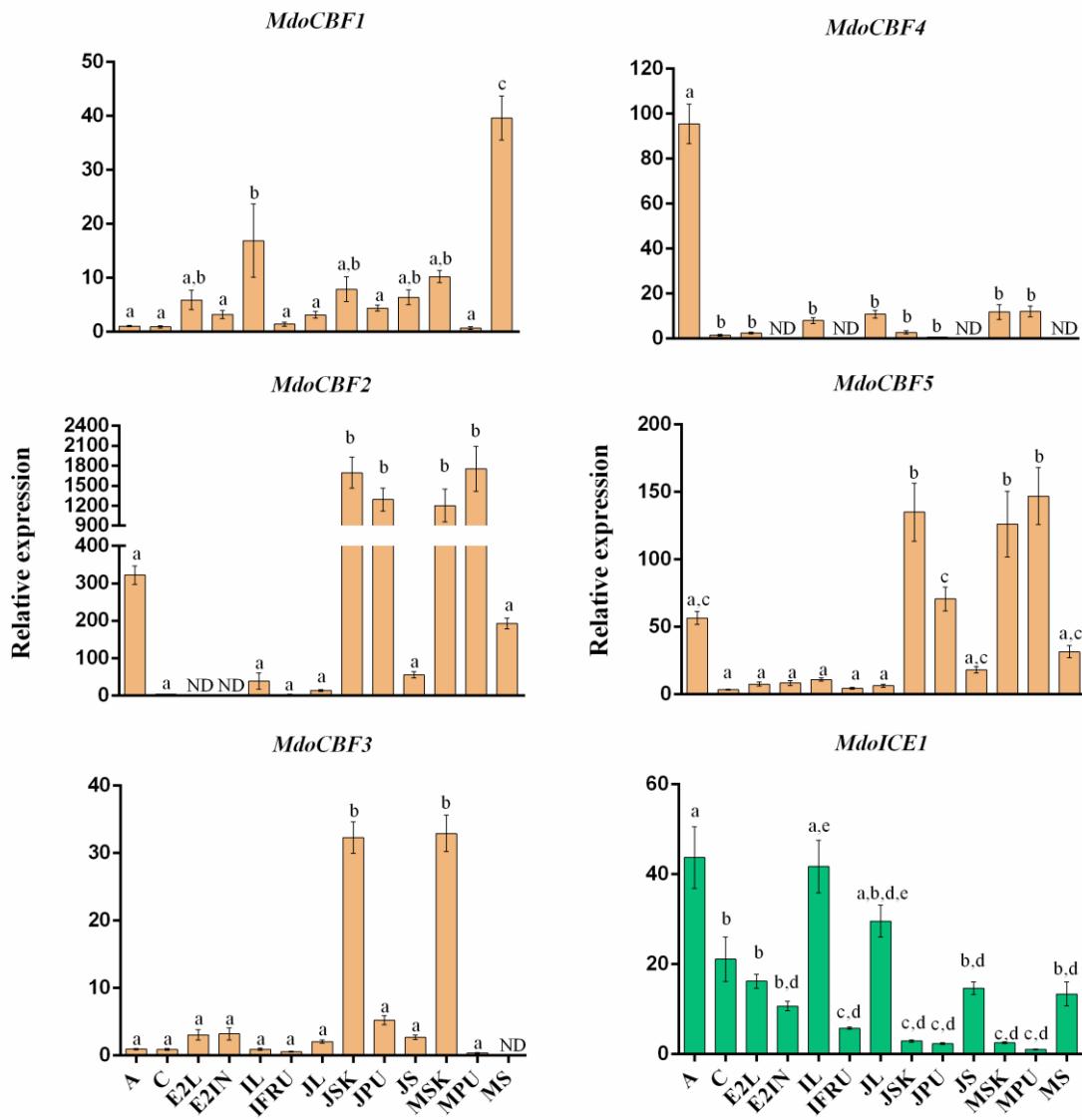
Figure 2. Transcriptional profiles of *MdoCBF*s and *MdoICE1* among different apple developmental stages. The relative expression of five *MdoCBF*s and *MdoICE1* genes were evaluated in dormant buds (A), green tip buds (C), young leaves (E2L), flowers (E2IN), 10 mm developing fruit divided in leaves (IL) and whole fruit (IFRU). Moreover, 40 mm unripe fruit were investigated on leaves (JL), skin (JSK), pulp (JPU) and seeds (JS). Mature apple fruit of 70 mm (M) were portioned in the same way. The relative expression found in the different developmental stages was compared to the mRNA levels of *MdoBRR1* at A stage for *MdoCBF*s gene family. For *MdoICE1* its expression level at MPU stage was used as reference. Different letters indicate statistically significant differences between means using one-way ANOVA followed by Tukey's test ($p \leq 0.05$). Error bars represent the standard error of the mean. ND: None detected.

In general, our data demonstrated that *MdoCBF*s are preferentially expressed in dormant buds and apple fruit (Figure 2). According to that, studies using the Tea plant *Camellia sinensis* showed high levels of *CBF* transcripts during fruit development [45]. The transcriptional activation of *CBF*s genes has also been reported in pulp and skin of grape fruits during postharvest storage at low temperatures [46]. Similarly, the CBF-dependent cold tolerance pathway was triggered in different fruits such as tomato, peach, kiwifruit and mango that were kept under long-term periods of chilling [47–49].

In poplar, the tissue-specific induction of *CBF1* and *CBF3* expression was seen in dormant buds, leaves and stem [50]. In a similar fashion, grape *CBF1-3* were upregulated in dormant buds and young leaves [51]. In agreement, our data also demonstrated high levels of *MdoCBF2*, *MdoCBF4* and *MdoCBF5* transcripts in dormant buds, however the same was not observed in leaves, on which little expression of *MdoCBF*s was detected (Figure 2).

In comparison to *MdoCBF* genes, we noted a more widespread accumulation of *MdoICE1* transcripts among different apple developmental stages. This general expression pattern was also observed in Arabidopsis and wheat [14,52]. Specifically, we could highlight that *MdoICE1* expression is higher in dormant buds (A), young (IL) and mature leaves (JL; Figure 2) [24]. In agreement, previous results showed that the expression of an *ICE*-like gene (*MdCIbHLH1*) was higher in dormant and floral buds, young and matures leaves than in fruit [16]. Interestingly, dormant buds showed high levels of both *MdoCBFs* and *MdoICE1* transcripts, taking us to further investigate if the regulation of dormancy cycle in apple buds is modulated by ICE1-CBF route.

3.3 *MdoCBF2* and *MdoCBF4* genes were upregulated in the endodormant phase.

The accumulation of chilling hours (CH) over time leads to the transition from endo- to ecodormancy. In sequence, ecodormant buds conditioned long enough under favorable growing conditions become able to bud break. The expression of *MdoCBFs* and *MdoICE1* was analyzed during a complete bud dormancy cycle under controlled conditions. The results showed that *MdoCBF2* and *MdoCBF4* were activated in the beginning of endodormancy, between 316 to 484 CH, and drastically decreased at the ecodormant stage (reached after exposure to 1156 CH) and under permissive conditions (Figure 3). In accordance to that, another study using Japanese apricot reported that the transcript amounts of *PmCBF*s were strongly induced under low temperatures and repressed in the flower blooming process [53]. The potential regulatory role of *CBF* genes in bud dormancy control was reported in different studies that analyzed the ectopic expression of peach *CBF1* in apple plants [17–19]. The overexpression of *PpCBF1* resulted in plants exhibiting growth arrest, early bud set and leaf senescence together with delayed bud break [17,19]. Additionally, PpCBF1 has close homology to MdoCBF2. The amino acid identity between PpCBF1 and MdoCBF2 is 67%, higher if compared to the 60% found between MdoCBF2 and MdoCBF1, for example [17]. The transcript levels of the other three *MdoCBFs* (*MdoCBF1*, *MdoCBF3* and *MdoCBF5*) did not change throughout the cycle (Figure 3).
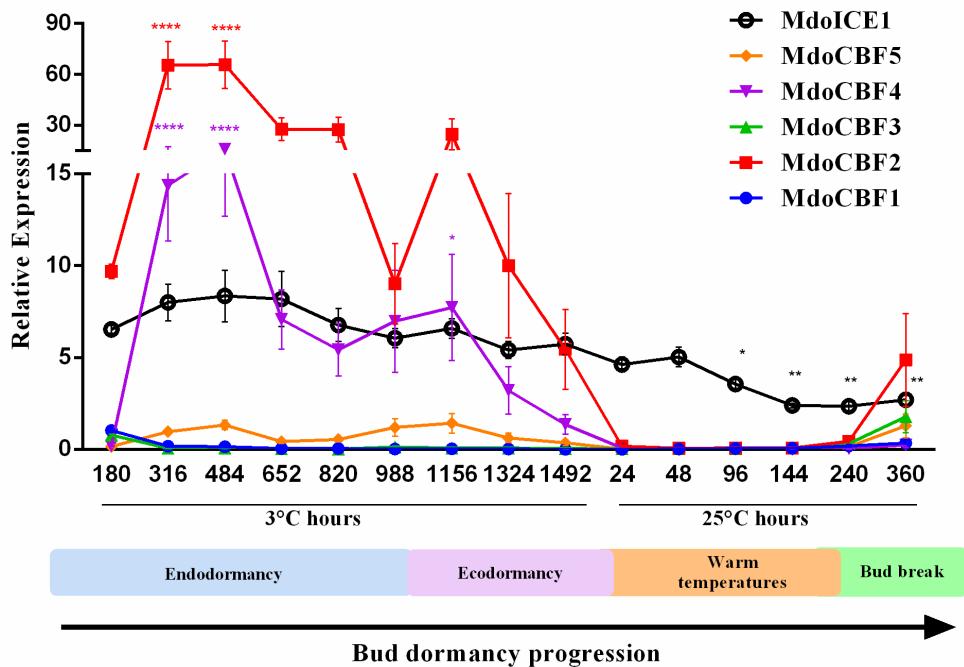
Figure 3. Expression patterns of *MdoCBF* gene family and *MdoICE1* during a complete bud dormancy cycle. Transcriptional activation of *MdoCBF2* and *MdoCBF4* could be observed at 316 and 484 hours of chilling treatment. Relative expression was calibrated by mRNA levels of *MdoCBF1* sampled at 180 CH. Statistical analysis was performed comparing the different temperature treatments to the 180 CH time-point of each tested gene; *$0.01 < p < 0.05$, **$0.001 < p < 0.01$***. Error bars represent the standard error of the mean.

The expression of *MdoICE1* did not oscillate alongside transition from endo- to ecodormancy, being only repressed after 96 h of exposure to warm temperatures. The absence of an oscillatory expression pattern of *MdoICE1* throughout the dormancy cycle does not mean that it plays a minor role in the CBF-dependent cold acclimation pathway. In Arabidopsis, the cold-mediated *CBF* activation is a rapid response which can occur within the first 15 minutes of exposure to low temperatures [8]. As so, it has been proposed that *CBF* transcriptional activators like ICE1 should be already present in the cell to enable this rapid activation [14]. Further, the mode of action of ICE1 is associated with post-translational modification like phosphorylation and sumoylation rather than gene expression activation [54]. Reinforcing this idea, studies have showed that the expression of Arabidopsis *ICE1* and *MdCIbHLH1* genes was only slightly upregulated by cold stimuli [14,16].

In the context of dormancy cycle control, the potential regulatory role of *ICE1* was already reported in pear and apple. In pear, high expression of a putative *ICE1* gene was found during endodormancy [55]. In apple, *ICE1* was differentially expressed in cultivars with contrasting chilling requirements during the dormancy stage [32]. Moreover, a recent study localized the *MdoICE1* gene in a QTL for bud break time in apple trees [24].

3.4 ABA, GA and SA play different roles during the bud dormancy cycle

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Beyond genetic and molecular control, the plant hormonal balance is essential for a proper dormancy cycle regulation. Within this context, responses mediated by CBFs have been related to abscisic acid (ABA), gibberellin (GA3) and salicylic acid (SA) homeostasis [56–60]. The evaluation of bud dormancy cycle progression reveled that ABA presented an oscillatory pattern throughout the cycle, while GA3 has accumulated right after the ecodormancy was reached (Figure 4). SA was present in large concentration during bud break time (Figure 4).
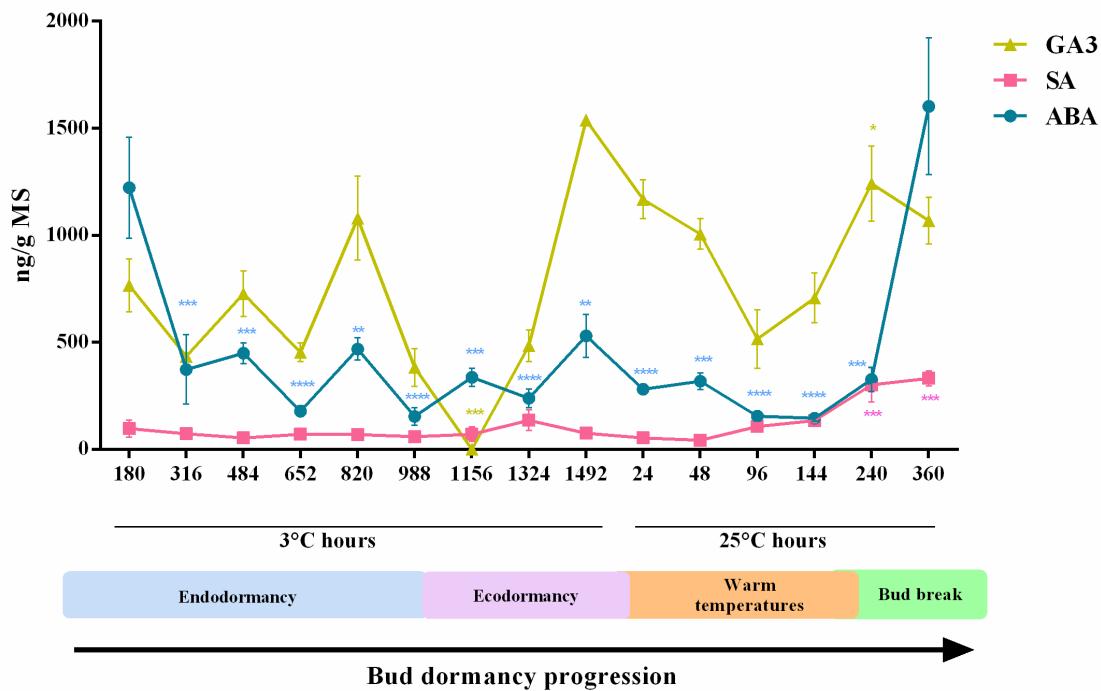
Figure 4. Quantification of ABA, GA and SA during the bud dormancy cycle. Abscisic acid (ABA), gibberellin (GA3) and salicylic acid (SA) were measured through UPLC-ESI-MS/MS in 'Royal Gala' buds throughout chilling accumulation and growing conditions. The data were compared to 180 CH point of each quantified hormone. MS: dry mass. $*0.01 < p < 0.05$, $**0.001 < p < 0.01***$. Error bars represent the standard error of the mean.

Clustering the expression profiles and hormone quantification we observed that *MdoCBF*2 and *MdoCBF4* repression is concomitant with GA increased levels during ecodormancy (Figures 3 and 4). According to that, *CBF*-overexpression in Arabidopsis, soybean and tobacco decreases GA levels [23,56,57]. RNA-seq analysis in apple trees overexpressing peach *CBF1* showed that genes involved in storage and inactivation of GA were generally upregulated [58]. Moreover, in these same plants, the *MdoRGL*s genes, which encode DELLA proteins, were upregulated. DELLAs are negative regulators of GA signaling and act restraining growth [18]. This balance suggests that GA stimuli might repress *CBF* expression, assisting the reestablishment of vegetative growth during dormancy overcome.

SA levels and *MdoICE1* expression also demonstrated distinct profiles. During bud break, *ICE1* is downregulated while higher quantities of SA were observed (Figures 3 and 4). Studies have related SA to cold signaling regulation and growth control [60, 61]. Additionally, the loss of function *ice1* mutant revealed that several SA-inducible genes were upregulated [61], reinforcing the opposite profile between them.

Since decades, ABA has been related to growth inhibition and dormancy induction (reviewed in Liu & Sherif [59]). In grapevine, for example, exogenous ABA applications increased *CBF* expression [60]. In Arabidopsis, ICE1 activity is post-translationally modulated by OST1, a key component of ABA signaling [62]. Unfortunately, under our tested conditions, we were not able to infer any biological association among ABA quantification and *MdoCBF*s or *MdoICE1* expression throughout the dormancy cycle. These results could be related to our sampling strategy, on which buds were harvested from independent twigs rather than apple trees, limiting the availability of ABA sources. Besides that, our data were analyzed only in the scope of chilling accumulation and it is already known that the induction of dormancy by ABA is strictly related to photoperiod changes [63,64].

3.5 Sequencing of *MdoICE1* CDS in apple cultivars with contrasting chilling requirement

Aiming to investigate whether gene mutations in the CBF-dependent cold acclimation pathway could be involved in the modulation of apple chilling requirement, we analyzed the CDS region of *MdoICE1*, one of the first low temperature sensors of the route. As already mentioned, this gene was identified in a QTL for bud break time [24], becoming an interesting candidate for dormancy cycle regulation. The CDS region of *MdoICE1* was sequenced in three apple cultivars with distinct chilling requirements ('Eva', 'Castel Gala' and 'Gala'). 'Castel Gala' is a spontaneous mutation of 'Gala' (Kidd´s Orange X Golden Delicious) that requires 50% less CH to bud break [65]. 'Eva' results from a cross between 'Anna' and 'Gala' [66] and is also a low chilling requirement cultivar.

The comparison among 'Eva', 'Castel Gala' and 'Gala' revealed that the CDS sequence of *MdoICE1* from 'Eva' presented two unique SNPs. These polymorphisms resulted in two amino acid substitutions (Q116R and T234M) in the deduced-protein (Figure 5). Three additional SNPs were found in all tested cultivars if compared to GDDH13 prediction [30]. From them, two resulted in amino acid substitution (N33S and V303L) and one in a synonymous mutation (Figure S1).

Figure 5. Mutations identified in the deduced protein of MdoICE1 from Eva cultivar. The two amino acid substitution found in 'Eva' MdoICE1 are presented in red. The b-HLH domain and S-rich motif are also shown.

The Arabidopsis loss of function *ice1* mutant is the result of a punctual amino acid substitution (R236H). This modification is responsible for significant reduction in plant chilling and freezing tolerance [14]. The R236H mutation found in *ice1* plants is localized between bHLH domain and S-rich motif and does not affect ICE1 DNA-binding ability.

Alternatively, it has been proposed that it may be interfering in homo-oligomer formation, protein stability, nuclear localization and posttranslational modification [14].

Interestingly, the 'Eva' unique mutation (T234M) is also present between the bHLH domain and the S-rich motif (Figure 5). All tested cultivars share three SNPs in the CDS region, resulting in two amino acids substitutions in the deduced-protein sequence if compared to GDDH13 prediction (Figure S1;27). This fact could be associated to allelic variation, considering that GDDH13 is a 'Golden Delicious' doubled-haploid tree. Unfortunately, the identified mutations could not be associated to distinct profiles of chilling requirement in the analyzed cultivars.

3.6 Cold stimuli mediate *MdoCBF4* and *MdoCBF5* activation

The upstream transcriptional regulation of *CBF* by ICE1 is well explored in Arabidopsis, but poorly understood in woody species [5,14]. In order to investigate if the CBF dependent pathway is modulated by ICE1 in apple, *in vivo* transactivation assays in Arabidopsis protoplasts were employed. Results showed a clear activation of *MdoCBF4promo::gusA* and *MdoCBF5promo::gusA* after cold stimuli. The same was not observed for construction carrying *MdoCBF1*, *MdoCBF2* and *MdoCBF3* promoters, although a tendency of *MdoCBF3promo*::*gusA* upregulation could be seen (Figure 6). Independently of temperature, the addition of MdoICE1 effector to the cells, did not affect *gusA* expression driven by any *MdoCBF* promoter (Figure 6).
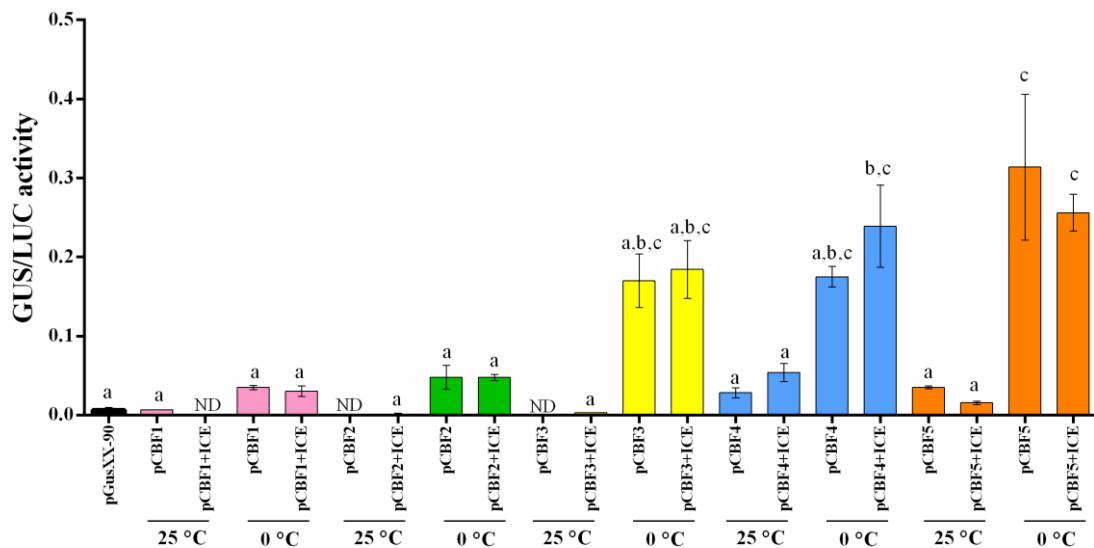
Figure 6. *MdoCBFs* modulation by MdoICE1 under cold stimuli. *MdoCBF4* and *MdoCBF5* promoters activated the reporter gene *gusA* in a cold dependent manner, independently of MdoICE1 effector. pGusXX-90: negative control, pCBF: *MdoCBFpromo*::*gusA*; ICE1: pART7::*MdoICE1*. Different letters indicate statistically significant differences between means using one-way ANOVA followed by Tukey's test ($p \leq 0.05$). Error bars represent the standard error of the mean. ND: None detected.

In agreement to our data, the activation of *CBF* expression in a cold-dependent manner was already reported in several species [17,51,67,68]. In Arabidopsis, for example, RNA blot analysis showed that under normal conditions, *ICE1*-overexpression

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did not transcriptionally activate the *CBF3* gene, being necessary the presence of cold stimuli for its proper modulation [14].

Even though other studies have demonstrated a cold-mediated activation of *MdoCBF1* and *MdoCBF2* [16,18,24], the same was not observed under our tested conditions. The evaluation of apple plants kept at 4 °C revealed that the expression of these two genes was induced after approximately one-half hour, peaking at 2 hours and then slowly diminished over a 24 hours period [17]. In our assay, *gusA* activity was measured only after 16 h under low temperature, indicating that the initial response may have been lost. Therefore, we hypothesize that *MdoCBF1* and *MdoCBF2* have an early cold response, whereas *MdoCBF4* and *MdoCBF5* are late-induced. Additionally, other mechanisms and regulatory regions that are not encompassed in our study might modulate *MdoCBF1* and *MdoCBF2* gene expression.

The investigation based on *MdoICE-like* gene *(MdCIbHLH1)* revealed that MdCIbHLH1 protein could specifically bind to only one of the four MYC recognition sites associated with cold signaling, in the promoter of *AtCBF3*. In contrast, the Arabidopsis ICE1 is able to bind to all sites [14,16]. Further, ChIP-PCR assays showed that MdCIbHLH1 specifically recognized three region of the *MdoCBF2* promoter. However, it failed to bind to the promoters regions tested in the *MdoCBF1*, *MdoCBF3*, *MdoCBF4* and *MdoCBF5* genes [16]. Similarly, we observed that transient overexpression of *MdoICE1* had no effect on the activity of *MdoCBF3promo*::*gusA,* *MdoCBF4promo*::*gusA* and *MdoCBF5promo*::*gusA*. In the case of *MdoCBF1promo*::*gusA* and *MdoCBF2promo*::*gusA,* it is difficult to draw conclusions once we cannot see a clear response of them to the cold stimulus, needing further investigation. Therefore, it is possible that *MdoCBFs* genes may have alternative upstream regulators.

4. CONCLUSIONS

The CBF-dependent cold acclimation pathway is one of the most well explored mechanisms of adaptation in Arabidopsis. Besides the many recent efforts done by the scientific community to extrapolate this data to other species, several gaps remain to be elucidated. In this study, the ICE1-CBF route was investigated in the perspective of dormancy control. The analysis of transcriptional profiles revealed that *MdoCBF2*, *MdoCBF4*, *MdoCBF5* and *MdoICE1* were highly expressed in dormant buds if compared to other tissues. In this scenario, a remarkable expression of *MdoCBF2* and *MdoCBF4* was observed in the beginning of endodormancy followed by a drastic drop at ecodormancy and bud break phases. In contrast, the *MdoICE1* gene presented constant levels of transcripts, being repressed only after 96 h of exposure to growth permissive conditions. During ecodormancy, the downregulation of *MdoCBF*s expression was concomitant to GA accumulation. In a similar fashion, *MdoICE1* repression overlapped with SA increase, indicating a possible crosstalk between ICE1-CBF and hormone homeostasis. *In vivo* transactivation assays demonstrated that *MdoCBF4promo*::*gusA* and *MdoCBF5promo*::*gusA* were activated in a cold-dependent manner, irrespective to MdoICE1. Finally, our data suggest that *MdoCBF2* and *MdoCBF4* are important endodormancy regulators and may be regulated by alternative factors, besides MdoICE1. Thus, the data presented represent a new step towards understanding the orchestrated molecular networks involved in the control of bud dormancy.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

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APPENDIX A. SUPPLEMENTARY DATA

Table S1. Primers used in this work

Reference sequence	Usage	Sequence (5'-3')	Product (bp)
MdoIce1; MD09G1003800	CDS amplification	caccgaattcaaaacaATGCTTCCGATGTCGAGCG	1603
		ggatccTTACATCATCATATCATGAAATCCAG	
	RT-qPCR	GTCTGCTCCAACCCTTTTGA	113
		GCCGTGAAACCCATCAGAAC	
	Sequencing	GTCTGCTCCAACCCTTTTGA	-
MdoCBF1; MD07G1262900	CDS amplification	caccATGGATGGTTGCTCTAATTACTACG	666
		TTAAATGGAAAAACTCCATAAGGGC	
	RT-qPCR	AACGCCAAGGACATTCAGAC	91
		CCACAGGAGATCCACAAACC	
MdoCBF2; MD06G1072200	CDS amplification	caccATGCGCCCGAGCTGCCATCT	393
		TCAACTTGCAACTCCAAAAAGAAGTG	
	RT-qPCR	GCTTGTGTCGTCGGAACTC	102
		GCTCAATTTCTCCGACGCAG	
MdoCBF3; MD01G1196100	CDS amplification	caccATGGATGCTTTCTCTCATTACTACG	663
		TCAAATGGAAAAACTCCATAATGGC	
	RT-qPCR	AGGCTTGTGGTGCTTCTG	84
		CCGCTTCCTCATCCATAAAA	
MdoCBF4; MD04G1067800	CDS amplification	caccATGTGGGCAACATTTCTCCACG	954
		TTACTTTGAGAAGCTCCACAGATTG	
	RT-qPCR	GGGAGAAGGATTGTGGATGT	90
		ACGAGCTGCCACTTTGCTCC	
MdoCBF5; MD06G1072300	CDS amplification	caccATGAATACGATCTTCAGTCAAATCTC	705
		TCAATTTGAGAAGCTCCACAGAC	
	RT-qPCR	GCACCCGATTTACAGAGGAG	111
		GCCGTCTGATAAGTTCCGAG	

Table S2: List of genome accession of *MdoCBFs* and *MdoICE1*

Gene name	Genome accession[a]	Chromosomal localization[a]	GeneBank accession
MdoICE1	MD09G1003800	Chr09:335088..338411	-
MdoCBF1	MD07G1262900	Chr07:33199100..33199765	HM992942
MdoCBF2	MD06G1072200	Chr06:17755189..17755884	HM992941
MdoCBF3	MD01G1196100	Chr01:29427231..29427893	-
MdoCBF4	MD04G1067800	Chr04:9200318..9201271	-
MdoCBF5	MD06G1072300	Chr06:17779619..17780323	-

[a]Genome accession codes and chromosomal localization are provided by the '*Malus* × *domestica* Genome' (http://rosaceae.org/) based on GDDH13_v1.1 version.

Figure S1: CDS and deduced-protein alignment of MdoICE1 from Eva, Castel Gala and Gala cultivars.

MdoICE1 CDS alignment:

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Gala           1  ATGCTTCCGATGTCGAGCGGCGCCGCTTGGATGGGCGGAGACGAAGACGACGCAGCGTCT
Castel_Gala    1  ATGCTTCCGATGTCGAGCGGCGCCGCTTGGATGGGCGGAGACGAAGACGACGCAGCGTCT
Eva            1  ATGCTTCCGATGTCGAGCGGCGCCGCTTGGATGGGCGGAGACGAAGACGACGCAGCGTCT
GDDH13         1  ATGCTTCCGATGTCGAGCGGCGCCGCTTGGATGGGCGGAGACGAAGACGACGCAGCGTCT


Gala          61  TGGACCAGAAACAGCACCACCACCCACAACAACAACAGCAACGAGGCAGAGCCCAGAAGA
Castel_Gala   61  TGGACCAGAAACAGCACCACCACCCACAACAACAACAGCAACGAGGCAGAGCCCAGAAGA
Eva           61  TGGACCAGAAACAGCACCACCACCCACAACAACAACAGCAACGAGGCAGAGCCCAGAAGA
GDDH13        61  TGGACCAGAAACAGCACCACCACCCACAACAACAACAACAACGAGGCAGAGCCCAGAAGA


Gala         121  AACGACCAAGACTCGAGCTTGGGAGCTTCTTTTTCAAACTTCAAATCAATGCTGGAAGGT
Castel_Gala  121  AACGACCAAGACTCGAGCTTGGGAGCTTCTTTTTCAAACTTCAAATCAATGCTGGAAGGT
Eva          121  AACGACCAAGACTCGAGCTTGGGAGCTTCTTTTTCAAACTTCAAATCAATGCTGGAAGGT
GDDH13       121  AACGACCAAGACTCGAGCTTGGGAGCTTCTTTTTCAAACTTCAAATCAATGCTGGAAGGT


Gala         181  GACTGGTACATGAACAACGTTCTCAGCAACCCAGCTCAAGACCTCCATGCCTTCTCTTCC
Castel_Gala  181  GACTGGTACATGAACAACGTTCTCAGCAACCCAGCTCAAGACCTCCATGCCTTCTCTTCC
Eva          181  GACTGGTACATGAACAACGTTCTCAGCAACCCAGCTCAAGACCTCCATGCCTTCTCTTCC
GDDH13       181  GACTGGTACATGAACAACGTTCTCAGCAACCCAGCTCAAGACCTCCATGCCTTCTCTTCC


Gala         241  ACCCAAGCTTCTGAAACTACACTTGCTCCTCTGCAACCAATCGACTCCTCCGCCTCCTGT
Castel_Gala  241  ACCCAAGCTTCTGAAACTACACTTGCTCCTCTGCAACCAATCGACTCCTCCGCCTCCTGT
Eva          241  ACCCAAGCTTCTGAAACTACACTTGCTCCTCTGCAACCAATCGACTCCTCCGCCTCCTGT
GDDH13       241  ACCCAAGCTTCTGAAACTACACTTGCTCCTCTGCAACCAATCGACTCCTCCGCCTCCTGT


Gala         301  TCTCCGTCGCCGGCCTTCTCTCTCGACCCTTCACAGCCGCCACAGCAGTTTTTGCCTCCA
Castel_Gala  301  TCTCCGTCGCCGGCCTTCTCTCTCGACCCTTCACAGCCGCCACAGCAGTTTTTGCCTCCA
Eva          301  TCTCCGTCGCCGGCCTTCTCTCTCGACCCTTCACAGCCGCCACAGCGGTTTTTGCCTCCA
GDDH13       301  TCTCCGTCGCCGGCCTTCTCTCTCGACCCTTCACAGCCGCCACAGCAGTTTTTGCCTCCA


Gala         361  AAATCCTGCTTCTCCTCTCTCCTCAACGTCGTCTGCTCCAACCCTTTTGATAACAGCTTC
Castel_Gala  361  AAATCCTGCTTCTCCTCTCTCCTCAACGTCGTCTGCTCCAACCCTTTTGATAACAGCTTC
Eva          361  AAATCCTGCTTCTCCTCTCTCCTCAACGTCGTCTGCTCCAACCCTTTTGATAACAGCTTC
GDDH13       361  AAATCCTGCTTCTCCTCTCTCCTCAACGTCGTCTGCTCCAACCCTTTTGATAACAGCTTC


Gala         421  GACCTGGGCTGCGACGCTGGGTTTCTGGGCTCCTTTCAGGGAAATCAGCCTTCCAATTCC
Castel_Gala  421  GACCTGGGCTGCGACGCTGGGTTTCTGGGCTCCTTTCAGGGAAATCAGCCTTCCAATTCC
Eva          421  GACCTGGGCTGCGACGCTGGGTTTCTGGGCTCCTTTCAGGGAAATCAGCCTTCCAATTCC
GDDH13       421  GACCTGGGCTGCGACGCTGGGTTTCTGGGCTCCTTTCAGGGAAATCAGCCTTCCAATTCC


Gala         481  TCTGTTCTGATGGGTTTCACGGCCCTCAATTCTCATGCTCAAATGGGTACTCCCGAACTC
Castel_Gala  481  TCTGTTCTGATGGGTTTCACGGCCCTCAATTCTCATGCTCAAATGGGTACTCCCGAACTC
Eva          481  TCTGTTCTGATGGGTTTCACGGCCCTCAATTCTCATGCTCAAATGGGTACTCCCGAACTC
GDDH13       481  TCTGTTCTGATGGGTTTCACGGCCCTCAATTCTCATGCTCAAATGGGTACTCCCGAACTC


Gala         541  AGTTCGAGCGCCGAGTTTCCAGCCAGTCGGTTGCTTCCGGTAACGGACAACGCCAATGTA
Castel_Gala  541  AGTTCGAGCGCCGAGTTTCCAGCCAGTCGGTTGCTTCCGGTAACGGACAACGCCAATGTA
Eva          541  AGTTCGAGCGCCGAGTTTCCAGCCAGTCGGTTGCTTCCGGTAACGGACAACGCCAATGTA
GDDH13       541  AGTTCGAGCGCCGAGTTTCCAGCCAGTCGGTTGCTTCCGGTAACGGACAACGCCAATGTA


Gala         601  CTCGACGGCGACTTTGGCTTCGAAGGCTTCGATGGCTCGGCCGGCGCTCAGCTGCTGAAC
Castel_Gala  601  CTCGACGGCGACTTTGGCTTCGAAGGCTTCGATGGCTCGGCCGGCGCTCAGCTGCTGAAC
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Eva         601  CTCGACGGCGACTTTGGCTTCGAAGGCTTCGATGGCTCGGCCGGCGCTCAGCTGCTGAAC
GDDH13      601  CTCGACGGCGACTTTGGCTTCGAAGGCTTCGATGGCTCGGCCGGCGCTCAGCTGCTGAAC


Gala        661  CGGGCTAAGCTCCTGTTCCCTCCCATGGGTGCTCAGCCCACGCTTTTCCAGAAGCGCCGC
Castel_Gala 661  CGGGCTAAGCTCCTGTTCCCTCCCATGGGTGCTCAGCCCACGCTTTTCCAGAAGCGCCGC
Eva         661  CGGGCTAAGCTCCTGTTCCCTCCCATGGGTGCTCAGCCCATGCTTTTCCAGAAGCGCCGC
GDDH13      661  CGGGCTAAGCTCCTGTTCCCTCCCATGGGTGCTCAGCCCACGCTTTTCCAGAAGCGCCGC

Gala        721  CAAAACTCTGTAGGCGATGGGGGTGATAAATTGGGAAATTTGGAGATTTCGGGTCCCAGG
Castel_Gala 721  CAAAACTCTGTAGGCGATGGGGGTGATAAATTGGGAAATTTGGAGATTTCGGGTCCCAGG
Eva         721  CAAAACTCTGTAGGCGATGGGGGTGATAAATTGGGAAATTTGGAGATTTCGGGTCCCAGG
GDDH13      721  CAAAACTCTGTAGGCGATGGGGGTGATAAATTGGGAAATTTGGAGATTTCGGGTCCCAGG


Gala        781  TACGGAGGACTACTGGAGAGTTTGGAGAAGAAAAGGAAGAGGAACGAGGAGGGTGAGATG
Castel_Gala 781  TACGGAGGACTACTGGAGAGTTTGGAGAAGAAAAGGAAGAGGAACGAGGAGGGTGAGATG
Eva         781  TACGGAGGACTACTGGAGAGTTTGGAGAAGAAAAGGAAGAGGAACGAGGAGGGTGAGATG
GDDH13      781  TACGGAGGACTACTGGAGAGTTTGGAGAAGAAAAGGAAGAGGAACGAGGAGGGTGAGATG


Gala        841  GAGGAGGGGAGTCTGGATGTGTCCGGTTTGAATTATGACTCGGATGACTTCAATGAGTAC
Castel_Gala 841  GAGGAGGGGAGTCTGGATGTGTCCGGTTTGAATTATGACTCGGATGACTTCAATGAGTAC
Eva         841  GAGGAGGGGAGTCTGGATGTGTCCGGTTTGAATTATGACTCGGATGACTTCAATGAGTAC
GDDH13      841  GAGGAGGGGAGTCTGGATGTTTCCGGTTTGAATTATGACTCGGATGACTTCAATGAGTAC


Gala        901  AGTCAATTGGAGGTGGAGGAGAATGCGAAGAATGGTGGAAGCAATTCGAATGCCAACAGC
Castel_Gala 901  AGTCAATTGGAGGTGGAGGAGAATGCGAAGAATGGTGGAAGCAATTCGAATGCCAACAGC
Eva         901  AGTCAATTGGAGGTGGAGGAGAATGCGAAGAATGGTGGAAGCAATTCGAATGCCAACAGC
GDDH13      901  AGTCAAGTGGAGGTGGAGGAGAATGCGAAGAATGGTGGAAGCAATTCGAATGCCAACAGC


Gala        961  ACTGTCACCGGTGTAGAGGGAGGAGACCGGAAGGGCAAGAAGAAGGGTTTGCCGGCCAAA
Castel_Gala 961  ACTGTCACCGGTGTAGAGGGAGGAGACCGGAAGGGCAAGAAGAAGGGTTTGCCGGCCAAA
Eva         961  ACTGTCACCGGTGTAGAGGGAGGAGACCGGAAGGGCAAGAAGAAGGGTTTGCCGGCCAAA
GDDH13      961  ACTGTCACCGGTGTAGAGGGAGGAGACCGGAAGGGCAAGAAGAAGGGTTTGCCGGCCAAA


Gala       1021  AATTTGATGGCGGAGAGGCGGCGGAGGAAGAAGCTCAATGATAGGCTCTACATGCTTAGG
Castel_Gala 1021 AATTTGATGGCGGAGAGGCGGCGGAGGAAGAAGCTCAATGATAGGCTCTACATGCTTAGG
Eva        1021  AATTTGATGGCGGAGAGGCGGCGGAGGAAGAAGCTCAATGATAGGCTCTACATGCTTAGG
GDDH13     1021  AATTTGATGGCGGAGAGGCGGCGGAGGAAGAAGCTCAATGATAGGCTCTACATGCTTAGG


Gala       1081  TCTGTTGTACCCAAGATAAGCAAGATGGATAGGGCGTCCATACTAGGGGATGCAATTGAT
Castel_Gala 1081 TCTGTTGTACCCAAGATAAGCAAGATGGATAGGGCGTCCATACTAGGGGATGCAATTGAT
Eva        1081  TCTGTTGTACCCAAGATAAGCAAGATGGATAGGGCGTCCATACTAGGGGATGCAATTGAT
GDDH13     1081  TCTGTTGTACCCAAGATAAGCAAGATGGATAGGGCGTCCATACTAGGGGATGCAATTGAT


Gala       1141  TATTTGAAAGAGCTTCTACAAAGGATTAATGACCTCCATAACGAGCTGGAGTCAGCTCCA
Castel_Gala 1141 TATTTGAAAGAGCTTCTACAAAGGATTAATGACCTCCATAACGAGCTGGAGTCAGCTCCA
Eva        1141  TATTTGAAAGAGCTTCTACAAAGGATTAATGACCTCCATAACGAGCTGGAGTCAGCTCCA
GDDH13     1141  TATTTGAAAGAGCTTCTACAAAGGATTAATGACCTCCATAACGAGCTGGAGTCAGCTCCA


Gala       1201  CCCGGATCTTTGCTGCCTGCTTCAACAAGTTTTCATCCGTTGACACCCACTCCATCCACC
Castel_Gala 1201 CCCGGATCTTTGCTGCCTGCTTCAACAAGTTTTCATCCGTTGACACCCACTCCATCCACC
Eva        1201  CCCGGATCTTTGCTGCCTGCTTCAACAAGTTTTCATCCGTTGACACCCACTCCATCCACC
GDDH13     1201  CCCGGATCTTTGCTGCCTGCTTCAACAAGTTTTCATCCGTTGACACCCACTCCATCCACC


Gala       1261  CTTCCCTGCCGTGTTAAAGAAGAGCTCTGCCCAAGCTCCTTGCTAAGCCCCAAAACTCAG
Castel_Gala 1261 CTTCCCTGCCGTGTTAAAGAAGAGCTCTGCCCAAGCTCCTTGCTAAGCCCCAAAACTCAG
Eva        1261  CTTCCCTGCCGTGTTAAAGAAGAGCTCTGCCCAAGCTCCTTGCTAAGCCCCAAAACTCAG
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GDDH13       1261 CTTCCCTGCCGTGTTAAAGAAGAGCTCTGCCCAAGCTCCTTGCTAAGCCCCAAAACTCAG


Gala         1321 CCGAAGGTGGAGGTTCGGGTAAGGGAAGGGCGAACTGTTAATATCCACATGTTCTGTTCT
Castel_Gala  1321 CCGAAGGTGGAGGTTCGGGTAAGGGAAGGGCGAACTGTTAATATCCACATGTTCTGTTCT
Eva          1321 CCGAAGGTGGAGGTTCGGGTAAGGGAAGGGCGAACTGTTAATATCCACATGTTCTGTTCT
GDDH13       1321 CCGAAGGTGGAGGTTCGGGTAAGGGAAGGGCGAACTGTTAATATCCACATGTTCTGTTCT


Gala         1381 CGGAGACCAGGTCTCTTGCTCTCTACCATGAGGGCCTTGGATAACCTTGATTTGGACGTC
Castel_Gala  1381 CGGAGACCAGGTCTCTTGCTCTCTACCATGAGGGCCTTGGATAACCTTGATTTGGACGTC
Eva          1381 CGGAGACCAGGTCTCTTGCTCTCTACCATGAGGGCCTTGGATAACCTTGATTTGGACGTC
GDDH13       1381 CGGAGACCAGGTCTCTTGCTCTCTACCATGAGGGCCTTGGATAACCTTGATTTGGACGTC


Gala         1441 CAGCAGGCTGTGATCAGCTGCTTCAATGGGTTTGCTTTAGATGTGTTCCGAGCTGAGCAA
Castel_Gala  1441 CAGCAGGCTGTGATCAGCTGCTTCAATGGGTTTGCTTTAGATGTGTTCCGAGCTGAGCAA
Eva          1441 CAGCAGGCTGTGATCAGCTGCTTCAATGGGTTTGCTTTAGATGTGTTCCGAGCTGAGCAA
GDDH13       1441 CAGCAGGCTGTGATCAGCTGCTTCAATGGGTTTGCTTTAGATGTGTTCCGAGCTGAGCAA


Gala         1501 TGCAGGGAAAACCAGTTCTTGCCAGAGCAAATAAAAGCAGTACTTTTGGATTCAGCTGGA
Castel_Gala  1501 TGCAGGGAAAACCAGTTCTTGCCAGAGCAAATAAAAGCAGTACTTTTGGATTCAGCTGGA
Eva          1501 TGCAGGGAAAACCAGTTCTTGCCAGAGCAAATAAAAGCAGTACTTTTGGATTCAGCTGGA
GDDH13       1501 TGCAGGGAAAACCAGTTCTTGCCAGAGCAAATAAAAGCAGTACTTTTGGATTCAGCTGGA


Gala         1561 TTTCATGATATGATGATGTAA
Castel_Gala  1561 TTTCATGATATGATGATGTAA
Eva          1561 TTTCATGATATGATGATGTAA
GDDH13       1561 TTTCATGATATGATGATGTAA
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MdoICE1 deduced-protein alignment:

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Gala            1 MLPMSSGAAWMGGDEDDAASWTRNSTTTHNNNSNEAEPRRNDQDSSLGASFSNFKSMLEG
Castel_Gala     1 MLPMSSGAAWMGGDEDDAASWTRNSTTTHNNNSNEAEPRRNDQDSSLGASFSNFKSMLEG
Eva             1 MLPMSSGAAWMGGDEDDAASWTRNSTTTHNNNSNEAEPRRNDQDSSLGASFSNFKSMLEG
GDDH13          1 MLPMSSGAAWMGGDEDDAASWTRNSTTTHNNNNEAEPRRNDQDSSLGASFSNFKSMLEG


Gala           61 DWYMNNVLSNPAQDLHAFSSTQASETTLAPLQPIDSSASCSPSPAFSLDPSQPPQQFLPP
Castel_Gala    61 DWYMNNVLSNPAQDLHAFSSTQASETTLAPLQPIDSSASCSPSPAFSLDPSQPPQQFLPP
Eva            61 DWYMNNVLSNPAQDLHAFSSTQASETTLAPLQPIDSSASCSPSPAFSLDPSQPPQRFLPP
GDDH13         61 DWYMNNVLSNPAQDLHAFSSTQASETTLAPLQPIDSSASCSPSPAFSLDPSQPPQQFLPP


Gala          121 KSCFSSLLNVVCSNPFDNSFDLGCDAGFLGSFQGNQPSNSSVLMGFTALNSHAQMGTPEL
Castel_Gala   121 KSCFSSLLNVVCSNPFDNSFDLGCDAGFLGSFQGNQPSNSSVLMGFTALNSHAQMGTPEL
Eva           121 KSCFSSLLNVVCSNPFDNSFDLGCDAGFLGSFQGNQPSNSSVLMGFTALNSHAQMGTPEL
GDDH13        121 KSCFSSLLNVVCSNPFDNSFDLGCDAGFLGSFQGNQPSNSSVLMGFTALNSHAQMGTPEL


Gala          181 SSSAEFPASRLLPVTDNANVLDGDFGFEGFDGSAGAQLLNRAKLLFPPMGAQPTLFQKRR
Castel_Gala   181 SSSAEFPASRLLPVTDNANVLDGDFGFEGFDGSAGAQLLNRAKLLFPPMGAQPTLFQKRR
Eva           181 SSSAEFPASRLLPVTDNANVLDGDFGFEGFDGSAGAQLLNRAKLLFPPMGAQPMLFQKRR
GDDH13        181 SSSAEFPASRLLPVTDNANVLDGDFGFEGFDGSAGAQLLNRAKLLFPPMGAQPTLFQKRR


Gala          241 QNSVGDGGDKLGNLEISGPRYGGLLESLEKKRKRNEEGEMEEGSLDVSGLNYDSDDFNEY
Castel_Gala   241 QNSVGDGGDKLGNLEISGPRYGGLLESLEKKRKRNEEGEMEEGSLDVSGLNYDSDDFNEY
Eva           241 QNSVGDGGDKLGNLEISGPRYGGLLESLEKKRKRNEEGEMEEGSLDVSGLNYDSDDFNEY
GDDH13        241 QNSVGDGGDKLGNLEISGPRYGGLLESLEKKRKRNEEGEMEEGSLDVSGLNYDSDDFNEY
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Gala         301  SQLEVEENAKNGGSNSNANSTVTGVEGGDRKGKKKGLPAKNLMAERRRRKKLNDRLYMLR
Castel_Gala  301  SQLEVEENAKNGGSNSNANSTVTGVEGGDRKGKKKGLPAKNLMAERRRRKKLNDRLYMLR
Eva          301  SQLEVEENAKNGGSNSNANSTVTGVEGGDRKGKKKGLPAKNLMAERRRRKKLNDRLYMLR
GDDH13       301  SQVEVEENAKNGGSNSNANSTVTGVEGGDRKGKKKGLPAKNLMAERRRRKKLNDRLYMLR


Gala         361  SVVPKISKMDRASILGDAIDYLKELLQRINDLHNELESAPPGSLLPASTSFHPLTPTPST
Castel_Gala  361  SVVPKISKMDRASILGDAIDYLKELLQRINDLHNELESAPPGSLLPASTSFHPLTPTPST
Eva          361  SVVPKISKMDRASILGDAIDYLKELLQRINDLHNELESAPPGSLLPASTSFHPLTPTPST
GDDH13       361  SVVPKISKMDRASILGDAIDYLKELLQRINDLHNELESAPPGSLLPASTSFHPLTPTPST


Gala         421  LPCRVKEELCPSSLLSPKTQPKVEVRVREGRTVNIHMFCSRRPGLLLSTMRALDNLDLDV
Castel_Gala  421  LPCRVKEELCPSSLLSPKTQPKVEVRVREGRTVNIHMFCSRRPGLLLSTMRALDNLDLDV
Eva          421  LPCRVKEELCPSSLLSPKTQPKVEVRVREGRTVNIHMFCSRRPGLLLSTMRALDNLDLDV
GDDH13       421  LPCRVKEELCPSSLLSPKTQPKVEVRVREGRTVNIHMFCSRRPGLLLSTMRALDNLDLDV


Gala         481  QQAVISCFNGFALDVFRAEQCRENQFLPEQIKAVLLDSAGFHDMMM*
Castel_Gala  481  QQAVISCFNGFALDVFRAEQCRENQFLPEQIKAVLLDSAGFHDMMM*
Eva          481  QQAVISCFNGFALDVFRAEQCRENQFLPEQIKAVLLDSAGFHDMMM*
GDDH13       481  QQAVISCFNGFALDVFRAEQCRENQFLPEQIKAVLLDSAGFHDMMM*
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6 DISCUSSÃO

O grande impulso inicial ao desenvolvimento comercial da maleicultura no Brasil se deu a partir da criação do Projeto de Fruticultura de Clima Temperado (Profit), pelo Estado de Santa Catarina, por meio da Lei nº 4.263, de 1968, e a Lei Federal nº 5.106 de 1966, também conhecida como Lei dos Incentivos Fiscais. Baseado na nova legislação, em meados da década de 1970, iniciou-se a implementação de novos pomares que alavancaram o setor e o tornaram um dos mais importantes no cenário nacional (PETRI & LEITE, 2008; KIST, 2019).

O cultivo de plantas perenes, como a maciera, em clima temperado requer a adaptabilidade das mesmas às condições adversas encontradas nos períodos de outono/inverno. Após o ciclo vegetativo e a percepção das primeiras temperaturas baixas, há a queda das folhas e a formação de estruturas vegetais chamadas de gemas, caracterizando o período inicial da dormência. As gemas, por sua vez, protegem os tecidos meristemáticos de condições extremas e garantem que o próximo ciclo reprodutivo seja viável (HORVATH, 2010). Além de um importante mecanismo adaptativo, a dormência é uma característica agronômica de grande relevância uma vez que a produtividade dos pomares está estritamente relacionada ao seu adequado processo de superação. Especialmente em regiões produtoras de maçã como o sul do Brasil, que não possui clima típico temperado, capaz de aportar o frio suficiente para que a brotação ocorra espontaneamente em condições normais de cultivo, é imprescindível a busca por soluções que visem diminuir o requerimento de frio hibernal das plantas e, ao mesmo tempo, sejam capazes de manter os padrões exigidos para a comercialização dos frutos.

No âmbito genético, sabe-se que o controle molecular da dormência de gemas é altamente hereditário e está estritamente relacionado a vias de aclimatação ao frio. Na planta modelo Arabidopsis, uma das vias mais bem estudadas em resposta às baixas temperaturas é mediada pelos genes *CBF*s, codificadores de fatores de transcrição do tipo AP2 que modulam a expressão de diversos genes em resposta ao frio (GILMOURARTUS & THOMASHOW, 1992; STOCKINGERGILMOUR & THOMASCHOW, 1997). Em macieira, um estudo pioneiro identificando os genes *CBF*s baseou-se no primeiro "rascunho" publicado do genoma (VELASCO et al., 2010) que mesmo emblemático, dadas as dificuldades na sua obtenção, possui muitos problemas associados à predição e anotação de genes. A recente disponibilização de versões mais curadas do genoma

(DACCORD et al., 2017; ZHANG et al., 2019) nos permitiu reanalisar os dados já reportados na literatura e realizar estudos objetivando investigar se a via de aclimatação ao frio dependende de CBF seria capaz de se sobrepor aos passos da dormência (**Capítulo III**). Nesse cenário, observou-se que gemas dormentes apresentam maior nível de expressão dos genes *MdoCBF2*, *MdoCBF4* e *MdoCBF5*. Curiosamente, os genes *MdoCBF2* e *MdoCBF5* também apresentaram altas quantidades de transcritos ao longo do desenvolvimento dos frutos, o que pode estar vinculado a um mecanismo de proteção, desencadeado por eles afim de minimizar possíveis danos causados pelo armazenamento prolongado sob baixas temperaturas durante etapas de pós-colheita (LIANG et al., 2013; MA et al., 2014; VAZQUEZ-HERNANDEZ et al., 2017).

O acompanhamento completo da progressão dos estádios da dormência de gemas de 'Royal Gala' confirmou a importância de *MdoCBF2* e *MdoCBF4* nesses tecidos, uma vez que foram os únicos dois membros da familia gênica que revelaram um padrão sazonal de expressão ao longo do ciclo. Os dados apresentados no **Capítulo III** mostram ainda que o ínicio da endodormência é marcado pela ativação transcricional de *MdoCBF2* e *MdoCBF4*, com declínio progressivo ao longo do acúmulo de HF (3 °C), queda drástica quando atingida a ecodormência e níveis insignificantes quando as gemas foram submetidas a temperaturas quentes (25 °C).

As primeiras evidências concretas do potencial papel regulatório de CBF no controle da dormência de gemas foram obtidas a partir de um estudo utilizando plantas de macieira superexpressando o gene *CBF1* de pêssego, que apresenta grande homologia ao gene *MdoCBF2* (WISNIEWSKI *et al.,* 2011). Linhagens de macieiras transgênicas apresentaram redução do crescimento, aumento da tolerância ao frio e ao congelamento, antecipação da senescência das folhas e entrada da dormência, bem como atraso no início da brotação (WISNIEWSKI *et al*., 2011). Surpreendentemente, as plantas transformadas foram ainda induzidas a entrar em dormência por meio da exposição à fotoperíodo curto, resposta não convencional, uma vez que espécies de macieiras são induzidas por frio e não pela diminuição da disponibilidade de luz, como espécies de pessegueiro (HEIDE & PRESTRUD, 2005). Além do mais, nessas plantas não foi notada a alteração do perfil transcricional dos genes *MdoCBF*s endógenos, indicando que esse fenômemo incomum está de fato relacionado à superexpressão do gene *CBF1* de pêssego e não aos *CBF*s de macieira (WISNIEWSKI *et al.,* 2011). De forma a confirmar os dados iniciais, a linhagem com maior expressão de *PpCBF1* foi plantada à campo e teve o seu desenvolvimento

acampanhado ao longo de três ciclos vegetativos (ARTLIP *et al.,* 2014). Comprovando os resultados anteriores, a planta apresentou redução no crescimento, antecipação da senescência das folhas, atraso na brotação e aumento da tolerância ao frio (ARTLIP *et al.,* 2014).

A homeostase hormonal é outro ponto chave no controle da dormência que vem sendo amplamente explorado e ligado à modulação da expressão de diversos genes, inclusive àqueles relacionados com as vias de resposta às baixas temperaturas. Neste aspecto, nossos dados permitiram apontar que o aumento nos níveis de GA3 é simultâneo a uma diminuição nos transcritos de *MdoCBF2* e *MdoCBF4* durante a ecodormência. De forma similar, o estudo que avaliou plantas tranformadas com *PpCBF1* revelou que, na primavera, período de brotação das plantas, os genes envolvidos no armazenamento e na inativação de auxinas, GAs e CKs foram geralmente super-regulados, enquanto que os de síntese, captação ou transdução de sinal tiveram seus níveis diminuídos (ARTLIP *et al.,* 2019). Ademais, a expressão ectópica de *PpCBF1* em macieira levou a um aumento na expressão dos genes *DELLA*, reguladores negativos de GA (WISNIEWSKINORELLI & ARTLIP, 2015). Com isso, pode-se hipotetizar que o efeito antagônico de GA sob *CBF* pode auxiliar no reestabelecimento do crescimento.

Em Arabidopsis, um dos principais ativadores transcricionais de *CBF* é o fator de trancrição da família bHLH denominado ICE1 (CHINNUSAMY *et al.,* 2003). Mutantes de perda de função para esse gene perdem a tolerância ao congelamento e apresentam um transcriptoma alterado em relação à resposta a baixas temperaturas (CHINNUSAMY *et al.,* 2003). Em macieira, um QTL (do inglês, *Quantitative Trait Loci*) associado ao tempo de brotação revelou a presença do gene *MdoICE1,* tornando-o um candidato interessante no controle molecular da dormência (MIOTTO et al., 2019). Nesse contexto, a análise de perfis de expressão apresentados no **Capítulo III** da presente tese mostra que embora expresso em quase todos os tecidos, há um acúmulo maior de transcritos de *MdoICE1* em gemas dormentes. A avaliação da sua expressão ao longo do ciclo da dormência se mostrou constante tanto nas fases de endo- como de ecodormência. Entretanto, a ausência de sazonalidade ao longo do ciclo não necessariamente o faz um ator secundário, uma vez que sua ativação é preferencialmente mediada por modificações pós-traducionais e não pela direta modulação da sua expressão gênica (LIU *et al.*, 2019; ZHAO *et al.*, 2017). Esse modelo é embasado por estudos que mostraram que a ativação de *CBF* após a percepção do frio é rápida, insinuando que o seu ativador já deva estar presente na célula,

necessitando apenas de modificações pontuais que levem ao desencadeamento da via (JAGLO-OTTOSEN *et al.,* 1998).

Ensaios *in vivo* de transativação visando entender se a via de regulação mediada por ICE1-CBF também é verdadeira em maciera mostraram que somente as construções contendo o gene reporter *gusA* dirigido pelo promotores dos genes *MdoCBF4* e *MdoCBF5* foram ativadas. Essa regulação positiva foi somente observada quando as células foram condicionadas a uma temperatura de 0 °C. Os ensaios conduzidos a 25 °C e aqueles em que o efetor MdoICE1 foi adicionado não surtiram efeito na modulação de *gusA*. Dados semelhantes foram apresentados em um estudo com maçã utilizando um gene relacionado a *ICE* (*MdCIbHLH*), onde o mesmo não foi capaz de ligar especificamente em 4 dos 5 sítios MYC encontrados no promotor de *AtCBF3*, como reportado para AtICE1 (FENG *et al.,* 2012). Além do que, ensaios *in vitro* mostraram a inabilidade de *MdCIbHLH* em ligar nas diversas regiões regulatórias de *MdoCBF1*, *MdoCBF3*, *MdoCBF4* e *MdoCBF5* (FENG *et al.,* 2012)*,* sugerindo que a via de aclimatação ao frio dependente de CBF possa não estar diretamente vinculada à ativação mediada por MdoICE1. Embora o estudo prévio mostre que *MdCIbHLH* é capaz de ligar no promotor de *MdoCBF2*, o mesmo não foi observado nos nossos resultados. O frio também não foi responsável por ativar as vias regulatórias desse gene. Nesse caso, uma conclusão final é ainda preliminar, uma vez que a atividade enzimática de *gusA* no ensaio reportado foi avaliada após 16 h e outros estudos mostram que a expressão de *MdoCBF*s é aumentada após a exposição ao frio já nas primeiras 2 h e vai diminuindo gradualmente até 24 h (WISNIEWSKI *et al.,* 2011).

A ativação dos genes *CBF* leva à modulação de diversos genes *COR*. Dentre eles, tem se sugerido que os fatores de transcrição denominados DAM podem estar sendo potencialmente regulados por essa via (NIU et al., 2016; SAITO et al., 2015; ZHAO et al., 2018). Desde sua descrição em 2008, os genes *DAM* vêm sendo objeto de estudo em diversas espécies e se caracterizam por apresentarem um perfil consistente e sazonal ao longo do ciclo da dormência (BIELENBERG *et al.*, 2008; KUMAR *et al.*, 2017; MIMIDA *et al.*, 2015; PORTO *et al.*, 2016; SASAKI *et al.*, 2011b; ZHAO *et al.*, 2018; ZHU *et al.*, 2015). Em macieira, por exemplo, os genes *MdoDAM1*, *MdoDAM3* e *MdoDAM4* apresentam transcritos acentuados nos períodos de estabelecimento e manutenção da endodormência e caem ao longo do acúmulo de HF (KUMAR *et al.*, 2017; MIMIDA *et al.*, 2015; PORTO *et al.*, 2016). Entre os processos regulatórios envolvendo

essa familia gênica, destacam-se estímulos hormonais como ABA e eventos epigenéticos como a metilação e a acetilação de histonas (DE LA FUENTE *et al.,* 2015; LEIDA *et al.,* 2012; SAITO *et al.,* 2015). Embora esses mecanismos acima citados sejam melhor explorados, o conhecimento acerca de fatores que regulam a expressão dos genes *DAM*, além da via mediada por CBF, é ainda limitada e carece de investigações mais profundas. Baseado nesse fato, a busca por *cis*-elementos em regiões regulatórias nos seis genes *DAM* descritos em macieira revelou presença de sítios de ligação para os fatores de transcrição denominados reguladores de resposta do tipo-B de Arabidopsis (ARR10; PORTO et al., 2016). Interessantemente, somente 1,73% dos genes de macieira apresentam sítio de ligação para ARR10. Por fim, embora ainda hajam discordâncias sobre a sua direta ligação ao florígeno FT (SAITO *et al.*, 2015; WU *et al.*, 2017), acredita-se que a ativação transcricional de *DAM* leve à repressão do mesmo e consequente inibição do crescimento (HAO *et al.*, 2015; NIU *et al.*, 2016).

Os reguladores de resposta do tipo-B (BRRs), potenciais reguladores de *MdoDAM1* conforme descrito por PORTO *et al.* (2016) são fatores de transcrição envolvidos nas etapas finais da via de sinalização de CK e são responsáveis por modular a expressão de seus genes alvos (EL-SHOWKRUONALA & HELARIUTTA, 2013). A percepção de CK pela célula aciona uma sequência de eventos de fosforilação que culmina na transferência de um fosfato para um resíduo de Asp no domínio receptor (REC) dos BRRs, tornando-os, assim, aptos a acionar as respostas dependentes de CK (TO & KIEBER, 2008). Os BRRs de macieira foram inicialmente descritos em 2017, embora de maneira superficial e com diversos problemas metodológicos (LI *et al.*, 2017). Os resultados apresentados no **Capítulo II** da presente tese permitiram caracterizar de forma extensiva essa família gênica com foco especial no controle da dormência. Entre os principais resultados, destaca-se o aumento da expressão dos genes *MdoBRR1* e *MdoBRR8* no período de transição da endo- para a ecodormência, que ocorre concomitantemente com a regulação negativa de *MdoDAM1*. Esses dados foram confirmados tanto em ensaios de frio controlado como em condições a campo. Nesses dois ciclos, também foram mensurados os níveis de CK que puderam ser fortemente correlacionados com o aumento da transcrição dos genes *MdoBRR*s em 'Castel Gala' (campo), embora o mesmo não tenha ficado tão evidente no ciclo de 'Royal Gala' (controlado). Mesmo que ainda haja divergências sobre a origem da CK (síntese local ou transportada via xilema) disponível no período de brotação (FAISS *et al.,* 1997;

MATSUMOTO-KITANO *et al.,* 2008), é valido considerar que as gemas das estacas de 'Royal Gala' contaram somente com a síntese local enquanto que, em 'Castel Gala', as mesmas puderam usufruir de fontes provenientes de toda a planta.

Da mesma maneira que a ativação de *MdoBRRs* ao longo do ciclo da dormência está vinculada ao aumento endógeno dos níveis de CK, gemas ecodormentes tratadas com 6-benzilaminopurina (BAP) a 4 mM mostraram 4 vezes mais transcritos dos genes *MdoBRR10* e *MdoBRR9* se comparadas ao controle. Interessantemente, o mesmo não foi encontrado para gemas endodormentes, as quais responderam muito pouco aos diferentes tratamentos testados. Sabe-se que na endormência a comunicação intercelular mediada por plasmodesmatas é bloqueada por meio do acúmulo de calose. Isso leva à interrupção do transporte simplástico e a consequente obstrução da passagem de água, nutrientes e moléculas sinalizadoras (WU *et al.*, 2018). Além disso, a adição de CK exógena em gemas ecodormentes levou à repressão do gene *MdoDAM1*, mais uma vez evidenciando a relação antagônica com os *MdoBRR*s.

O efeito negativo de *MdoBRRs* na regulação do gene *MdoDAM1* foi confirmado por meio de um ensaio de transativação *in vivo* utilizando protoplastos de Arabidopsis submetidos a diferentes condições. Primeiramente, células superexpressando de forma transiente o gene *MdoBRR1*, *MdoBRR8* ou *MdoBRR10* mostraram atividade diminuída do gene repórter *gusA* dirigido pelo promotor de *MdoDAM1 (pMdoDAM1::gusA)*. De acordo, a aplicação de CK exógena em células contendo somente a construção *pMdoDAM1::gusA* também levou à diminuição da atividade do gene repórter. Em uma segunda abordagem, objetivando minimizar os efeitos dos *ARRs* endógenos e possíveis artefatos da técnica, foram utilizados protoplastos de Arabidopsis mutados para 3 desse genes: *arr1, arr10 e arr12.* Nesse novo *background* genético, a expressão transiente de *MdoBRR1*, *MdoBRR8* e *MdoBRR10* continuou atuando de forma negativa na atividade de *pMdoDAM1::gusA*, reforçando que os resultados acima relatados não eram frutos da ligação de ARRs endógenos. Quando os protoplastos mutantes foram tratados com CK, não houve diminuição da atividade de *pMdoDAM1::gusA* como observado nos protoplastos selvagens, descartando artefatos da técnica, uma vez que essas células não respondem mais aos estímulos externos de CK (ARGYROS *et al.,* 2008). Os dados ainda concordam com aqueles encontrados em gemas ecodormentes, onde a aplicação de CK levou à repressão de *MdoDAM1*. Um terceiro ensaio, utilizando o promotor de *MdoDAM1* contendo mutações pontuais nos seus dois *cis*-elementos putativos para BRRs, revelou

que a perda dos sítios nativos levou à inabilidade de *MdoBRR1*, *MdoBRR8* e *MdoBRR10* em modular a expressão do gene repórter. Enfim, todos os resultados apresentados permitiram indicar um forte efeito negativo de *MdoBRR* mediado por CK na regulação de *MdoDAM1*. Hipotetiza-se então, que essa regulação negativa seja decorrente de uma barreira física criada pela ligação dos *MdoBRRs* na região promotora, que bloqueia a ligação de ativadores transcricionais do tipo MADS-box nos seus motivos CArG, levando à diminuição nos níveis de expressão de *MdoDAM1*.

Baseado no conjunto de resultados relatados nesse trabalho (**Capítulos II** e **III**), propomos um modelo hipotético do controle da dormência de gemas de macieira que abrange tanto aspectos moleculares quanto fisiológicos. Os eventos iniciam-se com a percepção dos primeiros sinais de frio que, dependente ou independentemente de *MdoICE1*, levam à ativação dos genes *MdoCBF2* e *MdoCBF4* que, então, modulam os genes *MdoDAM1*, desencadeando o início da endodormência (Figura 4).
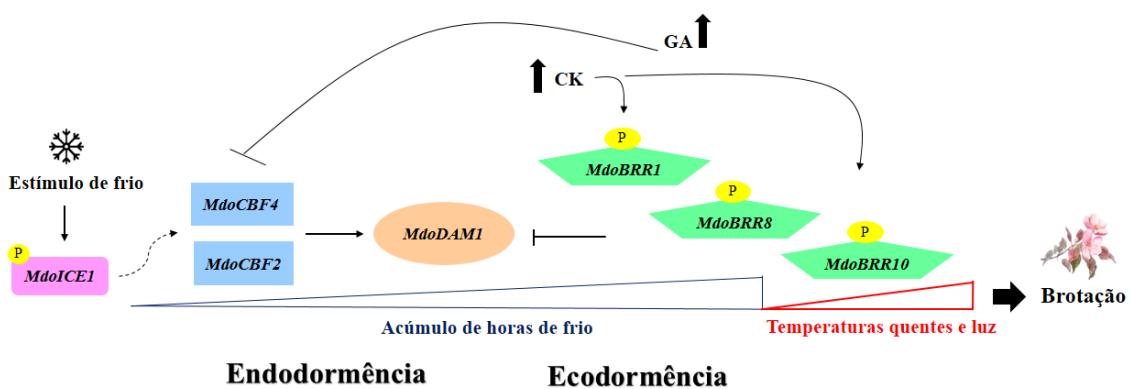
Figura 4. Modelo hipotético da regulação do ciclo da dormência em gemas de macieira. Baseado nos resultados encontrados no presente estudo, simplificamos os achados em um modelo hipotético que integra os mecanismos moleculares desencadeados pela percepção do frio, bem como os eventos fisiológicos ao longo da progressão da dormência de gemas. O estímulo ao frio leva a ativação de ICE1, que por mecanismos ainda não completamente entendidos leva a ativação dos genes *MdoCBF4* e *MdoCBF2* e indução de *MdoDAM1*, auxiliando no estabelecimento da endodôrmencia. Ao longo do acúmulo de horas de frio, há um aumento nos níveis de CK e ativação de *MdoBRR1 e MdoBRR8*, levando a inibição de *MdoDAM1* e transição pra ecodormência.

Paralelamente, o aumento de GA na ecodormência auxilia na repressão de *MdoDAM1,* bem como o acúmulo de transcritos de *MdoBRR10* ao longo da exposição a condições de crescimento.

Ao longo do acúmulo de HF, há um aumento nos níveis de CK que levam à ativação dos genes *MdoBRR1* e *MdoBRR8* e a consequente repressão do gene *MdoDAM1*. Paralelamente, ocorre ainda o aumento das quantidades de GA, levando a uma diminuição da expressão de *MdoCBF2* e *MdoCBF4*. Com o desligamento de *MdoDAM1* e a inibição da via dependente de CBF, a gema atinge a ecodormência, tornando-se apta a brotar após um período apropriado de exposição a condições favoráveis de crescimento. A exposição à luz e a temperaturas ótimas leva à retomada da atividade meristemática (mediada por CK) e à ativação do gene *MdoBRR10*, que mantem *MdoDAM1* desligado, liberando os fatores bloqueadores do crescimento por ele ativados (Figura 4).

Por fim, o presente trabalho visa contribuir com a adição de potenciais genes regulatórios à complexa rede molecular envolvida no ciclo da dormência, bem como sugerir o uso potencial dos genes *MdoBRR1*, *MdoBRR8* e *MdoBRR10* na geração de plantas geneticamente modificadas. Acredita-se que a superexpressão desses genes possa levar à obtenção de macieiras com menor requerimento de frio, uma vez que auxiliam no desligamento de *MdoDAM1*, um importante gene envolvido na manutenção da dormência. Embora estudos mais aprofundados sejam ainda necessários para comprovar tal hipótese, de maneira preventiva, um pedido de análise de patenteteabilidade (**Anexo I**) foi encaminhado ao setor responsável da EMBRAPA. O desenvolvimento dessa nova tecnologia visa propulsionar o setor produtivo e toda a cadeia vinculada a ele, de maneira rápida, eficaz e inovadora.

7 PERSPECTIVAS

Como principais perspectivas do presente trabalho estão a conclusão da caracterização funcional dos genes *MdoBRR1, MdoBRR2* e *MdoBRR10* em sistema de expressão heterólogo usando a planta modelo *Arabidopsis thaliana* do tipo selvagem. Para tal, as plantas foram transformadas utilizando *Agrobacterium tumefaciens* por meio do método de "*floral dip*" (CLOUGH & BENT, 1998). As regiões codificantes dos três genes foram inseridas no vetor binário pK7WG2D (KARIMIINZÉ & DEPICKER, 2002) que contém, na sua região de T-DNA, o promotor CaMV 35S e o gene de resistência a derivados da neomicina *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*). Atualmente, as plantas passam por seleção em T1 e caracterização fenotípica inicial considerando aspectos como comprimento de hipocótilos e raiz, tamanho de folhas e tempo de floração. Nas próximas gerações, serão confirmados os dados obtidos em T1 e ainda realizados tratamentos com diferentes concentrações de CK exógena para avaliar o nível de sensibilidade das plantas a esse estímulo, bem como as respostas desencadeadas por ele. Os dados serão comparados com plantas controles transformadas com pK7WG2D e o gene repórter *gusA*.

Além do sistema de expressão heterólogo, o potencial uso biotecnológico dos genes *MdoBRR* será avaliado em macieiras da cultivar Gala Brookfield®. Para tanto, plântulas introduzidas *in vitro* foram transformadas com o gene *MdoBRR1* inserido no vetor pK7WG2D por meio de *A. tumefaciens* baseado no protocolo descrito em CHEVREAU *et al.* (2019). Após cocultivo com *A. tumefaciens*, os explantes foram acondicionados na ausência de luz em meio seletivo contendo canamicina e estão sendo acompanhados mensalmente. Nessa etapa, ao longo de 6 meses, verificar-se-á a presença de estruturas caulinares com potencial formação de brotos que, então, serão transferidas para condições ótimas de cultivo (16 h luz, 23°C). Os brotos resistentes à canamicina serão micropropagados e a presença do gene será confirmada via PCR. As plantas positivas serão então enraizadas e aclimatadas em casas de vegetação. A avaliação fenotípica comparará plantas transformadas e não-transformadas em relação ao tempo necessário para que eventos fisiológicos como a senescência foliar, formação de gemas e brotação sejam estabelecidos.

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9 ANEXO I

Utilização dos genes *MdoBRR*s como potencial ativo biotecnológico na geração de plantas de macieia com menor requerimento de frio

Pedido de Patententeabilidade submetido pela Embrapa Uva e Vinho

OBS: Os anexos relatados no presente pedido de patenteabilidade podem ser encontrados no Capítulo II da presente tese.

FORMULÁRIO DE ANÁLISE DE PATENTEABILIDADE

Finalidade: orientar os gestores e os CLPI (Comitês Locais de Propriedade Intelectual) das Unidades da Embrapa no levantamento e encaminhamento de informações à Secretaria de Negócios - SNE para avaliação quanto à proteção intelectual de novo produto e/ou processo (inclusive método e sistema) tecnológico.

A. DOCUMENTAÇÃO JURÍDICO-TÉCNICA

Deve-se anexar ao presente formulário cópia de contrato/convênio, termos de sigilo, autorizações de acesso a recursos genéticos, autorizações relativas à legislação de biossegurança, bem como demais instrumentos jurídicos, além do próprio projeto de P&D, conforme o caso, relacionados à geração da tecnologia. Esta documentação é importante para que se avalie o contexto do trabalho que originou o produto e/ou processo, o que implica em efeitos sobre aspectos de propriedade intelectual e exploração econômica.

Relacionar os documentos anexados:

Tabela 1. Acesso no genoma de macieira dos 10 genes codificadores identificados como *Reguladores de Resposta do Tipo-B* (*MdoBRR*).
Tabela 2. Lista de iniciadores projetados utilizados nos ensaios.
Figura 1. Identificação de domínios conservados e análise filogenética dos MdoBRRs. **(A)** Ilustração gráfica representando a posição dos dois domínios conservados encontrados nas proteínas MdoBRR. O primeiro resíduo de metionina de cada proteína foi considerado como posição número 1. (B) A árvore filogenética foi inferida pelo método Bayesiano usando 68 sequências de proteínas anotadas como BRR de seis espécies diferentes (*A. thaliana, O. sativa* sub. Japonica, *P. trichocarpa*, *P. persica*, *P. bretschneideri* e *M. domestica*). Os MdoBRRs foram coloridos em azul.
Figura 2. Expressão gênica de *MdoBRR*s em 12 diferentes tecidos e órgãos de plantas de macieira. A expressão dos genes *MdoBRR*s foi avaliada por RT-qPCR nos estádios A, C, E2, I, J e M de acordo com a escala fenológica de Fleckinger (EPPO, 1984). A expressão relativa foi plotada em relação a *MdoBRR5* no estádio A. Barras de erros estão representadas. A análise estatística foi realizada com emprego do *software* GraphPad Prism v.6, utilizando ANOVA de uma via, seguido do teste de Tukey ($p < 0,05$). ND: Não detectado
Figura 3. Perfil transcricional dos genes *MdoBRR* durante a transição espontânea de endo- para a ecodormência em gemas da cultivar Castel Gala. A expressão relativa de sete *MdoBRRs* e *MdoDAM1* foi avaliada por RT-qPCR em gemas dormentes de 'Castel Gala' amostradas em quatro pontos de resfriamento diferentes (118, 325, 707 e 778 horas abaixo de 7,2 °C). A quantificação da *t*-zeatina foi realizada usando-se UPLC-ESI-MS / MS. ANOVA de uma via seguida pelo teste de Dunett com o *software* GraphPad Prism 6 (* $0,01 < p < 0,05$, ** $0,001 < p < 0,01$ ***) foi empregada na análise estatística**.** A expressão relativa foi plotada em relação a *MdoBRR1* no ponto 118. Barras de erros estão representadas. DM: massa seca.
Figura 4. Padrão de expressão dos genes *MdoBRR* ao longo da progressão da dormência de gemas de 'Royal Gala'. O ensaio de RT-qPCR foi realizado com gemas dormentes de 'Royal Gala' amostrados com 315 horas de frio (<7,2 °C) e condicionados em câmaras escuras controladas sob temperatura constante de 3 °C. Após 1.403 horas de frio, as brindilas contendo as gemas dormentes foram transferidas para condições permissivas de crescimento (25 ± 1,5 °C, 16 h de fotoperíodo e 70% de umidade relativa) por 24 a 360 h. A quantificação

da *t*-zeatina e a análise estatística foi realizada conforme descrito na Figura 3. A expressão relativa foi plotada em relação a *MdoBRR1* no ponto 315.

Figura 5. Tratamentos com citocinina exógena em gemas de maçã ecodormentes. A expressão relativa dos genes *MdoBRRs* e *MdoDAM1* foi avaliada em gemas ecodormentes tratadas por 16 h com 1, 2 ou 4 mM de BAP ou TDZ pelo método de spray (TOP) ou influxo (BASE) A análise estatística segue os mesmos padrões descritos na Figura 3.

Figura 6. Regulação de *MdoDAM1* por MdoBRRs e citocinina. A modulação do gene repórter *gusA* dirigido pelo promotor de *MdoDAM1* por meio da expressão transiente de *MdoBRR1*, *MdoBRR8* e *MdoBRR10* foi avaliada em protoplastos de Arabidopsis Col-0 (**A**) e no mutante triplo *arr* (**C**). O efeito da aplicação exógena de TZD variando de 0,0001 a 1,0 µM) também foi testado para o Col-0 (**B**) e o mutante triplo *arr* (**D**). DMSO foi usado como controle. O efeito dos efetores MdoBRR1, MdoBRR8 e MdoBRR10 também foi testado na versão do promotor mutado de *MdoDAM1* para os dois sítios de ligação à BRR (**E**). A atividade relativa de GUS / LUC foi calculada dividindo a atividade enzimática de GUS pela luminescência obtida através de rLUC. pGUSXX: controle negativo; pDAM1: pGUSXX-90 + região promotora de *MdoDAM1* fusionada a *gusA*. RR1, RR8 e RR10: Os efetores MdoBRRs clonados em pART7. pDAM1_M: construção similar ao promotor nativo de *MdoDAM1*, embora com mutações pontuais nos dois *cis*-elementos para BRR. ANOVA de uma via seguida pelo teste de Tukey com o *software* GraphPad Prism 6 (p <0,05) foi empregada para análise estatística.

Anexo 1. Sequência nucleotídica dos 10 *MdoBRRs* identificados

B. *TÍTULO DA PESQUISA/PROJETO*

Entendimento do papel dos Reguladores de Resposta do Tipo-B (BRR) da via de sinalização de citocinina durante o ciclo de dormência de gemas em macieira (*Malus* x *domestica* Borkh.).

C. *PALAVRAS-CHAVE*

Listar as principais referências bibliográficas relacionadas com a presente tecnologia.

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Bibliografia Patentária

Listar todas as patentes similares e associadas à presente tecnologia. Recomenda-se pesquisa preliminar em bases de patentes e pedidos de patentes: www.inpi.gov.br; www.uspto.gov; http://ep.espacenet.com. A busca nos sites de bases patentárias podem ser realizadas tanto através de palavras-chave quanto através da numeração do documento.

Não foram encontradas patentes similares em busca rápida nos bancos de dados acima mencionados.

Comparação com Produtos, Processos e Tecnologias Existentes

Verificar se há produtos/processos similares no mercado e descrevê-los, apresentando também as principais vantagens da presente tecnologia quando comparada aos produtos/processos já existentes no mercado.

> -

Desvantagens e/ou Limitações do Invento
Discorrer sobre as possíveis desvantagens e limitações da presente tecnologia

> O emprego de espécies vegetais que são naturalmente recalcitrantes a eventos de transgenia é um exemplo de limitação do presente invento, dado que ele parte da premissa que um gene codificador de *BRR* de macieira será incorporado no genoma da planta alvo, de modo a sintetizar maiores níveis transcritos que a planta selvagem. A necessidade de utilização de promotores tecidos/órgãos-específicos pode ser uma limitação da presente invenção, dado que nem todos os promotores desse tipo foram amplamente testados e validados numa ampla gama de espécies vegetais.

Usos Potenciais do Invento
Enumerar os principais usos futuros potenciais que esta tecnologia poderá apresentar

> Esta invenção tem como objetivo aumentar os níveis endógenos de transcritos de *BRR* por meio de técnicas de transgenia a fim de se obter potenciais plantas de macieiras com menor requerimento de frio em cultivares já comercialmente aceitas pelo mercado consumidor.

D. DESCRIÇÃO DETALHADA DA TECNOLOGIA
Trata-se de texto detalhado sobre a tecnologia de forma a atender o critério de suficiência descritiva. Deste modo, o texto deve permitir que qualquer especialista no assunto seja capaz de reproduzir a tecnologia. Evidenciar, ademais, quando for o caso, o melhor modo de concretização da tecnologia. Anexar ao formulário quaisquer arquivos de computador contendo figuras, fotografias, vídeos, texto e outros que tenham relação com a presente tecnologia e possam contribuir para a sua descrição.

> A presente invenção relata o potencial uso dos genes *MdoBRRs* na geração de plantas de macieira melhor adaptadas aos atuais e futuros cenários produtivos. Essas plantas terão potencialmente um menor requerimento de frio, resultando em uma brotação antecipada e mais uniforme.
>
> O método consiste na geração de plantas transgênicas da cultivar Gala que passarão a exibir níveis mais elevados de transcritos de *MdoBRR*s, auxiliando na repressão do gene *MdoDAM1* e possivelmente facilitando assim, a transição da endo- para a ecodormência.
>
> A presente invenção cobre todos os genes de macieira que codificam proteínas capazes de sintetizar os fatores de transcrição BRRs. Além disso, os genes *MdoBRR*s podem ser isolados a partir de qualquer tecido vegetal de macieira.
>
> A informação sobre as sequências nucleotídicas codificadoras desses genes pode ser obtida em diversos bancos de dados públicos de sequências disponíveis tais como GenBank, Genome Database for Rosaceae, The Apple Genome and Epigenome, entre outros. Exemplos de genes codificadores de *MdoBRRs* estão apresentados no Anexo 1 (*MdoBRR1* a *MdoBRR10*). Sequências nucleotídicas derivadas desses genes tais como por deleção, inserção, adição, inversão, substituição, entre outros, também estão englobadas na presente invenção, incluindo variações naturais das sequências

A presente invenção versa sobre sequências nucleotídicas oriundas de macieira capazes de sintetizar os fatores de transcrição BRRs. Exemplos de métodos para aumentar a atividade de BRRs incluem a incorporação de um gene de *MdoBRR* em um vetor, o qual permite a expressão desse gene em uma planta, ou partes de uma planta, após um evento de transformação ou edição genética.

EXEMPLO

A invenção está representada pelo exemplo a seguir. O exemplo a seguir é apenas uma forma representativa de utilização da tecnologia e não possui a intenção de limitar o seu uso para essa forma particular de representação.

Exemplo

1 Isolamento de genes codificadores de BRR em macieira

A busca por modelos gênicos preditos que codificam para BRRs foi realizada utilizando o genoma GDDH13 versão 1 descrito por Daccord et al. (2017) utilizando-se os parâmetros padrão de busca da ferramenta "Blastp" (Altschul et al., 1990). Como *input* do software os dois domínios essenciais para a funcionalidade dos BRRs denominados Domínio Receptor de Sinal (REC) e o domínio de ligação ao DNA (MYB) foram utilizados. Somente os genes anotados como reguladores de resposta e possuindo ambos os domínios foram selecionados para as análises posteriores. Ao todo, 10 modelos gênicos preditos codificadores de BRRs foram encontrados no genoma de macieira (Tabela 1). Análises de bioinformática foram realizadas visando caracterizar os modelos gênicos preditos identificados e todos apresentaram os domínios característicos dos fatores de transcrição (REC e MYB; Figura 1A). Além disso, os genes codificadores de BRRs identificados em macieira encontraram ortólogos no genoma de pereira e pessegueiro. As proteínas PpRR3, B-PpRR2, B-PpRR8, B-PpRR5 e B-PpRR9 estão relacionadas a MdoBRR1, MdoBRR2, MdoBRR5, MdoBRR8 e MdoBRR10, respectivamente. Entre os BRRs de pessegueiro, a proteína PpARR14-b está associada ao MdoBRR3 (Figura 1B).

2 Expressão de genes codificadores de BRRs em macieira em diferentes tecidos e órgãos

Para a análise dos níveis de expressão dos genes codificadores de BRRs em macieira, o RNA (ácido ribonucleico) total foi extraído de 12 diferentes órgãos da cultivar Gala Baigent compreendendo os estádios de floração até o amadurecimento do fruto durante os anos de 2009/2010 de acordo com a escala fenológica de Fleckinger (EPPO, 1984): gema fechada (estádio de desenvolvimento A); gema aberta (estádio C); botão floral (estádio E2); folha jovem (estádio E2); folha madura e frutificação com 10 mm de diâmetro (estádio I); folha, casca, polpa e semente de frutos verdes com 40 mm de diâmetro (estádio J); e casca, polpa e semente de frutos maduros com aproximadamente 70 mm de diâmetro (estádio M). As plantas da cultivar Gala Baigent foram provenientes de um pomar experimental da Estação Experimental de Fruticultura de Clima Temperado (EEFCT) da Embrapa Uva e Vinho em Vacaria/RS, com três anos de idade enxertadas em porta-enxerto Marubakaido com filtro M.9, e seguiram todas as etapas tradicionais de manejo. O RNA total foi isolado conforme descrito em Zeng & Yang (2002) e Falavigna et al. (2014), sendo o mesmo posteriormente tratado com DNase utilizando a TURBO DNA-free Kit (Ambion). O *kit* GeneAmp RNA

PCR Core Kit (Thermo Scientific) foi utilizado para a síntese de DNA complementar (cDNA).

O padrão de expressão dos genes codificadores para BRRs foi obtido por meio da técnica de RT-qPCR utilizando o aparelho StepOnePlus Real-Time PCR System (Thermo Scientific). A amplificação utilizada foi 10 minutos a 95°C para ativação da enzima, seguido de 40 ciclos de (1) desnaturação a 95°C por 15 segundos e (2) 60 segundos a 60°C para anelamento dos iniciadores e extensão das fitas. Ao final, foi realizada uma curva de dissociação entre as temperaturas de 60 a 95°C. A especificidade dos iniciadores foi avaliada pela presença de pico único na curva de dissociação. Na Figura 2 está ilustrado o resultado de expressão obtido nas diferentes amostras. Os resultados mostraram que, em geral, entre todos os tecidos, os maiores níveis de expressão foram observados com os genes *MdoBRR6-10*. O *MdoBRR6* foi expresso preferencialmente nas sementes (JS) e nas folhas, independentemente do estádio, e o *MdoBRR7* revelou maior quantidade de transcritos nos botões florais (E2IN). A expressão de *MdoBRR8* parece ser importante no florescimento (E2IN) e no desenvolvimento de frutos (J). O *MdoBRR9* e o MdoBRR10, juntamente com o *MdoBRR1*, apresentaram níveis de expressão notáveis em gemas fechadas (A), especialmente o *MdoBRR10*. O perfil transcricional de *MdoBRR5* mostrou níveis mais elevados de transcrição na casca e polpa de frutos maduros de maçã de 40 mm (M). *MdoBRR2*, *MdoBRR3* e *MdoBRR4* exibiram baixos níveis de transcrição nas condições avaliadas. Além disso, a expressão de *MdoBRR4* foi apenas detectada em sementes de frutos em desenvolvimento (JS), de forma extremamente reduzida.

3 Efeito do frio na expressão dos *MdoBRRs* e avaliação dos níveis de CK ao longo do ciclo da dormência de gemas de macieira

A influência do acúmulo de horas de frio na expressão gênica de *BRRs* em gemas de macieira foi avaliada à campo e em condições controladas. As amostragens de gemas à campo foram realizadas na cultivar Castel Gala com diferentes quantidades de horas de frio acumulados. Para cada ponto foram amostradas aleatoriamente 60 gemas e as mesmas foram divididas em 3 parcelas biológicas. Elas foram congeladas em nitrogênio líquido imediatamente após a coleta para posterior extração de RNA, síntese de cDNA e ensaios de RT-qPCR (conforme acima descrito no item 2). O acúmulo de horas de frio foi monitorado por estações automáticas de coleta de dados controladas pelo Instituto Nacional de Meteorologia (INMET) e localizadas na EEFCT, local onde ocorreu a amostragem. Foram contabilizadas como horas de frio somente aquelas que registraram temperaturas iguais ou inferiores a 7,2 °C.

O efeito do frio sob condições controladas foi avaliado a partir da amostragem de brindilas contendo gemas terminais da cultivar 'Royal Gala' em pomares experimentais da EEFCT. As brindilas (20 cm de comprimento, em média) foram higienizadas com hipoclorito de sódio seguida de sucessivas lavagens com água e acondicionadas em sacos plásticos pretos, conforme descrito em Falavigna et al. (2014). O tratamento de frio foi realizado colocando os sacos plásticos dentro de câmaras de crescimento no escuro, de forma vertical, posicionando a gema terminal para cima. A temperatura constante de 3 °C foi utilizada. A cada sete dias (168 HF) 60 gemas terminais foram coletadas, congeladas em nitrogênio líquido, dividas em 3 parcelas biológicas (20 gemas cada) e armazenadas a -80 °C até posterior extração de RNA, síntese de cDNA e ensaios de RT-qPCR (subitem 2). Em ambas as condições (campo/controlado), ocorreu a avalição do perfil de brotação das gemas utilizadas. Para tal, mais 40 brindilas de cada ponto amostral foram acondicionadas em condições ótimas de crescimento (23 °C, 70% umidade e 16 h de luz). O percentual de brotação foi avaliado a cada 7 dias durante 35 dias.

A extração do hormônio *t*-zeatina (CK endógena) foi realizada adicionando 4,0 mL de solução de extração (metanol: água: ácido fórmico, 75: 20: 5, v: v: v) em um tubo contendo 500 mg de material vegetal. As amostras foram incubadas a -20 °C por 3 h. Depois disso, foram condicionados em banho de ultrassom (frequência de 40kHz) por 25 minutos a 4 °C e os sobrenadantes foram recuperados por centrifugação a 1.750 g a 4 °C por 30 min. O passo de extração foi repetido duas vezes. O sobrenadante foi transferido para um novo tubo de microcentrífuga e concentrado em concentrador centrífugo à vácuo. Após a secagem, 1,0 mL de água foi adicionado, homogeneizado e transferido para as colunas Oasis MCX (Waters Corporation, EUA). A eluição da coluna foi realizada usando um gradiente de hidróxido de amônio (de 0,004 a 0,4 M). Na solução eluída foram adicionados 1,7% p / v de PVPP, homogeneizados e os sobrenadantes foram recuperados por centrifugação a 10.000 g por 45 min a 4 ºC. O sobrenadante foi transferido para um novo tubo de microcentrífuga 1,5 e concentrado em concentrador centrífugo à vácuo. Após a secagem, as amostras foram ressuspendidas 75 μL de metanol e filtradas através de um PTFE de 0,22 μm. A análise de quantificação foi realizada em UPLC-ESI-MS / MS. Os dados são representados em ng de *t*-zeatina / g de massa seca.

Os resultados revelaram maiores quantidades de transcrição de *MdoBRR1*, *MdoBRR8* e *MdoBRR9* de 700 a 778 horas de frio, o mesmo ponto em que a *t*-zeatina começou a se acumular (Figura 3), sugerindo a indução de *MdoBRR*s por meio do acúmulo de CK endógena na fase de ecodormência. De maneira oposta, os níveis mais altos de *MdoDAM1* foram observados de 118 a 325 horas de frio, durante a endodormência, seguidos de uma redução significativa em 700 e 778 h. A expressão dos outros quatro *MdoBRR*s (*MdoBRR5-7*; *MdoBRR10*) permaneceram constantes ao longo do ciclo de dormência.
A avaliação completa do ciclo de dormência de gemas de 'Royal Gala' revelou que o *MdoBRR1* e o *MdoBRR8* apresentaram um pico de expressão após a transição da endo- para a ecodormência (após 1.000 horas de frio). No mesmo momento, a expressão do *MdoDAM1* começou a diminuir (Figura 4), de maneira semelhante às observações feitas nas gemas de 'Castel Gala' (Figura 3). As horas iniciais em condições permissivas de crescimento (25 °C) aumentaram a transcrição de *MdoBRR10*, que permaneceu constante durante todo o tempo avaliado. A expressão de *MdoBRR9* também foi aumentada de 24 para 96 h durante a exposição a temperaturas amenas (25 °C). Em uma resposta posterior, após 168 h em condições permissivas de crescimento, as gemas ecodormentes começaram a acumular transcritos de *MdoBRR7*. Picos de acúmulo de CK foram observados antes da fase de ecodormência, diminuindo depois disso e retomando após exposição prolongada a temperaturas quentes, próximo à brotação (Figura 4).

4 Tratamentos com CK exógena

Visando avaliar se os *MdoBRR*s identificados são capazes de responder a tratamentos de CK exógena, diferentes CKs sintéticas e métodos de aplicação foram testados em gemas dormentes de macieira. Brindilas da cultivar Royal Gala de aproximadamente 15 cm foram coletadas nos pomares experimentais da EEFCT. O tratamento ocorreu em dois estágios distintos da dormência das gemas: endo- e ecodormência. Dois tipos de CK sintéticas foram utilizadas: BAP e thidiazuron (TDZ) em três concentrações distintas: 1, 2 ou 4 mM. As CKs foram solubilizadas no diluente apropriado e o volume final foi acertado com uma solução 0,05% de Tween 20, utilizada também como controle. Dois métodos de aplicação foram testados: O método "TOP" consistiu em mergulhar a gema terminal de cada brindila na solução apropriada de CK por 10 min. Após o tratamento, elas foram acondicionadas em espumas fenólicas e armazenadas em câmaras de crescimento (23 °C, 70% umidade e 16 h de luz). Após 16 h, as gemas apicais foram coletadas, congeladas em nitrogênio líquido e

armazenadas a -80°C. O método "BASE" consistiu em submergir a base da brindila (10 cm) em 10 mL da solução de CK a ser avaliada por 16 ou 48 h. Durante o período de tratamento elas foram armazenadas em câmaras de crescimento como descrito anteriormente, e nos tempos apropriados as gemas apicais foram coletadas da mesma maneira descrita acima para posterior extração de RNA, síntese de cDNA e ensaios de RT-qPCR (subitem 2).

Em uma primeira abordagem, usando gemas ecodormentes, o tratamento mais eficaz na ativação de *MdoBRR* foi alcançado pelo método TOP. Nesse caso, os genes *MdoBRR9* e o *MdoBRR10* demonstraram uma quantidade quase quatro vezes maior de transcritos em comparação com o controle quando BAP 4 mM foi aplicado. Nessas mesmas amostras, também foi observada a repressão de *MdoDAM1* (Figura 5A). Por outro lado, os tratamentos com TDZ mostraram uma clara tendência de indução de *MdoBRRs* com uma repressão significativa de *MdoDAM1*. Esses achados indicam que, nas gemas ecodormentes, a ativação do *MdoBRR9* e *MdoBRR10* é mediada pelo estímulo de CK. Além disso, a aplicação exógena de CK é capaz de regular negativamente a expressão de *MdoDAM1*. O tratamento pela BASE resultou em níveis mais baixos de ativação de *MdoBRRs* (Figura 5B) e o uso de gemas endodormente não resultou em grandes modulações na transcrição de *MdoBRRs* (dados não apresentados).

5 Construção dos vetores utilizados nos ensaios de transativação

As sequências codificantes dos genes *BRRs* de macieira foram amplificadas usando a enzima de alta fidelidade iProof™ High-Fidelity DNA Polymerase (Bio-Rad, EUA) a partir de amostras de cDNA da cultivar de macieira Royal Gala. Iniciadores específicos de cada sequência foram desenhados baseados na predição do genoma (DACCORD; CELTON; LINSMITH; et al., 2017) e utilizando-se o *software* Vector NTI (Invitrogen; Tabela 2).

Para a clonagem via metodologia de GATEWAY®, os iniciadores foram acrescidos de quatro nucleotídeos (CACC) na sua extremidade de forma a possibilitar a clonagem no vetor de entrada pENTER™/D-TOPO (Invitrogen, EUA). A confirmação da clonagem foi realizada por meio de digestão enzimática com enzimas de restrição e posterior sequenciamento. Confirmada a sequência, a CDS do genes *MdoBRR* foi transferida do vetor de entrada para os vetores de destino apropriados via recombinação utilizando-se o *kit* Gateway® LR Clonase® II (Invitrogen, EUA). A clonagem no vetor final foi confirmada por meio de digestão enzimática com endonucleases de restrição e posterior sequenciamento. Para o ensaio de transativação, o vetor de destino foi o pART7 (GLEAVE, 1992) contendo o promotor forte CaMV 35S de forma a garantir altos níveis de expressão do gene. Para os ensaios *in vivo*, o promotor de *MdoDAM1*, compreendendo a região de -614 a -111 pb, foi amplificado a partir de amostras de DNA da cultivar Royal Gala, utilizando enzimas de alta fidelidade. Após isso, a região de aproximadamente 500 pb foi clonada em vetor pGEM®-T Easy (Promega), conforme instruções do fabricante. A clonagem foi confirmada por meio de digestão enzimática com endonucleases de restrição e posterior sequenciamento. Em seguida, a sequência promotora foi removida do vetor pGEM®-T Easy por digestão enzimática dupla com endonucleases de restrição apropriadas para a construção. Após a obtenção do fragmento, o mesmo foi fusionado ao gene repórter *gusA* no vetor final pGUSXX-90 (PASQUALI *et al.*, 1994) empregando-se a enzima T4 DNA ligase conforme descrito pelo fabricante (New England BioLabs, EUA). A clonagem foi confirmada por meio de digestão enzimática com endonucleases de restrição e posterior sequenciamento. Uma segunda versão do promotor *MdoDAM1* foi utilizada contendo mutações pontuais nos sítios de ligação aos BRRs. Para a inserção das mutações nas regiões -518 e -179 pb, o promotor foi sintetizado pela empresa Thermo Fisher Scientific (EUA) e clonado em pGUSXX-90 conforme descrito na versão nativa.

6 Ensaios *in vivo* de interação DNA-proteína via transativação

Plantas de 4 a 5 semanas de *Arabidopsis thaliana* crescidas em condições ótimas (23 °C, 70% umidade e 16 h luz) foram utilizadas para o isolamento de protoplastos utilizando o método descrito por Wu et al. (2009). Simplificadamente, esse método baseia-se na remoção da epiderme das folhas e na utilização de enzimas para a degradação da parede celular de células do mesófilo foliar. O DNA é introduzido nas células por meio de transfecção, utilizando-se uma solução a 40% de PEG-Cálcio (Yoo & Sheen, 2007). Três vetores independentes foram transfectados em 1×10^5 células: o primeiro p2rL7 (DE SUTTER; VANDERHAEGHEN; TILLEMAN; et al., 2005) contendo o gene *Renilla Luciferase (rLuc),* utilizado como controle da transfecção; o segundo compreende a região promotora de 500 pb nativa ou mutada do gene *MdoDAM1* fusionado ao gene repórter *gusA* (*β-glicuronidase*) inserido no vetor pGUSXX-90 (PASQUALI *et al.*, 1994); e o terceiro contendo a proteína efetora (BRR) clonada no vetor pART7 (GLEAVE, 1992). Para cada experimento, 4 replicadas biológicas e 3 técnicas foram utilizadas. Os níveis de fluorescência (indicados pela atividade de *gusA*) e luminescência (indicados por *rLuc*) foram avaliados como descrito em Yoo et al. (2007), utilizando o equipamento SpectraMax® i3 Multi-Mode Detection Platform (Molecular Devices, EUA). Os dados gerados foram analisados pela relação entre as atividades enzimáticas de *gusA* e *rLuc*

Com base no padrão de expressão consistente relacionado a um possível papel regulador durante a dormência de gemas (Figuras 3 a 5), os genes *MdoBRR1*, *MdoBRR8* e *MdoBRR10* foram escolhidos para os ensaios de transativação. A disponibilidade do mutante triplo de Arabidopsis insensível à tratamento exógeno de CK (*arr1-3 arr10-5 arr12-1*) nos permitiu realizar o mesmo ensaio usando plantas de dois *backgrounds* genéticos distintos, o selvagem (Col-0) e mutante triplo *arr* (Argyros et al. 2008).

Os ensaios utilizando os protoplastos de Arabidopsis do tipo selvagem revelaram que todos os MdoBRRs testados foram capazes de regular negativamente a expressão do gene repórter (Figura 6A). Além disso, a aplicação exógena de TDZ em protoplastos transfectados apenas com o promotor de *MdoDAM1* fusionado à *gusA* levou a uma perda significativa na atividade do gene repórter, mesmo na menor concentração testada (Figura 6B).

Consistentemente, os ensaios usando o mutante triplo reforçam o papel de repressão de MdoBRRs sobre *MdoDAM1* quando a superexpressão transitória de *MdoBRR1*, *MdoBRR8* e *MdoBRR10* foi testada (Figura 6C). Quando TDZ foi aplicado, não foram encontradas diferenças significativas entre os tratamentos e o grupo controle (DMSO) (Figura 6D). Para confirmar que a regulação negativa do *gusA* direcionada pelo promotor de *MdoDAM1* é derivada da ação de MdoBRRs, mutações pontuais foram incorporadas nos dois locais de ligação de BRR encontrados ao longo da região promotora. Os resultados mostraram que a perda do sítio de ligação nativo de BRR interrompeu a capacidade de MdoBRR1, MdoBRR8 e MdoBRR10 em reprimir a expressão do gene repórter (Figura 6E). Finalmente, nossos achados indicam um mecanismo interessante de regulação negativa de *MdoDAM1* mediado por CK e pela ligação do MdoBRR1, MdoBRR8 e MdoBRR10.

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E. DIVULGAÇÃO DA TECNOLOGIA

A proteção patentária apresenta como premissa os seguintes requisitos diretos: (i) novidade; (ii) atividade inventiva; e (iii) aplicação industrial. Sob o ponto de vista indireto, não é deferível pedido de patente que não apresente suficiência descritiva.

Conforme consta no Art. 11° da Lei da Propriedade Industrial (LPI - Lei 9.279, de 14/05/1996), apresenta novidade a invenção que não está compreendida pelo estado da técnica. O estado da técnica é definido por "tudo aquilo tornado acessível ao público antes da data de depósito do pedido de patente, por descrição escrita ou oral, por uso ou qualquer outro meio, no Brasil ou no exterior...". Exceção é dada pelo Art. 12°, da mesma Lei, que prevê: "Não será considerada como estado da técnica a divulgação de invenção ou modelo de utilidade, quando ocorrida durante os 12 (doze) meses que precederem à data de depósito (...), se promovida: I - pelo inventor; (...)". Este período de 12 meses é conhecido como período de graça e é o prazo dado a um inventor para que, tendo divulgado sua pesquisa, assegure a novidade de um pedido de patente que trate do mesmo assunto. No entanto, salienta-se que a maioria dos países apresentam legislação patentária que não prevê este artifício, ou seja, uma vez tornada pública a tecnologia entra em domínio público (não é mais passível de patenteamento).

A invenção é dotada de atividade inventiva sempre que, para um técnico no assunto, não decorra de maneira evidente ou óbvia do estado da técnica.

Ademais, a invenção é suscetível de aplicação industrial quando puder ser utilizada ou produzida de maneira seriada.

Considerando o requisito novidade, solicita-se relacionar todas as divulgações (anexar cópias), internas ou externas, seja em formato oral, impresso, dentre outros, se houver, sobre a presente tecnologia:

CATTANI, A. M; SILVEIRA, C. P.; PASQUALI, G.; REVERS, L. F. Type-B response regulators of the cytokinin-signaling pathway link hormonal stimulus and molecular responses to overcome dormancy in apple. In: XXII International Congress of Genetics, Foz do Iguaçu, Brazil. Abstract, 2018
REVERS, L. F.; CATTANI, A. M.; SARTOR, T.; SILVEIRA, C. P.; PASQUALI, G. Apple FLC and TBRR genes regulate dormancy integrating hormonal stimulus and molecular responses. In: 13th Congress of the International Plant Molecular Biology, Montpellier, France. Oral Presentation, 2018.
CATTANI, A. M.; SILVEIRA, C. P.; PASQUALI, G.; REVERS, L. F. Reguladores de resposta do tipo B envolvidos na via de sinalização de citocininas auxiliam na integração entre estímulo hormonal e resposta molecular durante o ciclo de dormência de macieira. In: 16º Encontro de Iniciação Científica e 12º Encontro de Pós-graduandos da Embrapa Uva e Vinho, Bento Gonçalves, Brasil. Livro de Resumos 16º Encontro de Iniciação Científica e 12º Encontro de Pós-graduandos da Embrapa Uva e Vinho, p. 23, 2018.
CATTANI, A. M.; PASQUALI, G; REVERS, L. F. Regulação da dormência em macieira via reguladores RRTB. In: 15º Encontro de Iniciação Científica e 11º Encontro de Pós-graduandos da Embrapa Uva e Vinho, Bento Gonçalves, Brasil. Livro de Resumos 15º Encontro de Iniciação Científica e 11º Encontro de Pós-graduandos da Embrapa Uva e Vinho, p. 35, 2017
CATTANI, A. M.; FALAVIGNA, V. S.; ARENHART, R. A.; PASQUALI, G.; REVERS, L. F. Caracterização estrutural e funcional da subfamília gênica ARR-tipo B em macieira. In: 14º Encontro de Iniciação Científica e 10º Encontro de Pós-graduandos da Embrapa Uva e Vinho, Bento Gonçalves, Brasil. Livro de Resumos 14º Encontro de Iniciação Científica e 10º Encontro de Pós-graduandos da Embrapa Uva e Vinho, p. 19, 2016

F. ESTÁGIO DE DESENVOLVIMENTO DA TECNOLOGIA COM RELAÇÃO A COMERCIALIÇÃO

Relate as fases de geração e desenvolvimento da tecnologia, bem como o atual estádio de desenvolvimento com foco em sua aplicação econômica.

⊠	*Estágio embrionário* (Foi desenvolvida no laboratório apenas)
☐	*Parcialmente desenvolvida* (planta piloto – necessário desenvolvimento para sua comercialização)
☐	*Desenvolvida* (Existe um protótipo preparado para seu desenvolvimento e comercialização)
(Utilizar o espaço abaixo para, se necessário, descrever o que deverá ser feito para completar o desenvolvimento da tecnologia.)	
-	

G. ENCAMINHAMENTO

Este formulário deve ser assinado por pelo menos um dos inventores da tecnologia a ser avaliada. Enviar para o CLPI (Comitês Locais de Propriedade Intelectual).

H. DÚVIDAS E SUGESTÕES

Entrar em contato com o CLPI (Comitês Locais de Propriedade Intelectual).

10 ANEXO II

Winter is coming: genetic analyses of the bud break date locus reveal candidate genes from the cold perception pathway to dormancy release in apple (*Malus × domestica* Borkh.)

Artigo publicado no periódico "Frontiers in Plant Science"

MIOTTO, Y. E., TESSELE, C., CZERMAINSKI, A. B. C., PORTO, D. D., FALAVIGNA, V. da S., SARTOR, T., CATTANI, A. M.;...Revers, L. F. Spring Is Coming: Genetic Analyses of the Bud Break Date Locus Reveal Candidate Genes From the Cold Perception Pathway to Dormancy Release in Apple (*Malus × domestica* Borkh.). *Frontiers in Plant Science*, 10 (33), 2019.

Spring Is Coming: Genetic Analyses of the Bud Break Date Locus Reveal Candidate Genes From the Cold Perception Pathway to Dormancy Release in Apple (*Malus × domestica* Borkh.)

Yohanna Evelyn Miotto[1†‡], Carolina Tessele[1‡], Ana Beatriz Costa Czermainski[2],
Diogo Denardi Porto[2†], Vítor da Silveira Falavigna[2,3†], Tiago Sartor[2,3],
Amanda Malvessi Cattani[2,3], Carla Andrea Delatorre[1], Sérgio Amorim de Alencar[4],
Orzenil Bonfim da Silva-Junior[5], Roberto Coiti Togawa[5], Marcos Mota do Carmo Costa[5],
Georgios Joannis Pappas Jr.[6], Priscila Grynberg[5], Paulo Ricardo Dias de Oliveira[2],
Marcus Vinícius Kvitschal[7], Frederico Denardi[7], Vanessa Buffon[2] and
Luís Fernando Revers[2,3*]

[1] *Department of Crop Science, Agronomy School, Federal University of Rio Grande do Sul, Porto Alegre, Brazil,* [2] *Embrapa Uva e Vinho, Bento Gonçalves, Brazil,* [3] *Graduate Program in Cell and Molecular Biology, Centro de Biotecnologia, Federal University of Rio Grande do Sul, Porto Alegre, Brazil,* [4] *Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil,* [5] *Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil,* [6] *Department of Cell Biology, Universidade de Brasília, Brasília, Brazil,* [7] *Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina – Epagri – Estação Experimental de Caçador, Caçador, Brazil*

Chilling requirement (CR) for bud dormancy completion determines the time of bud break in apple (*Malus × domestica* Borkh.). The molecular control of bud dormancy is highly heritable, suggesting a strong genetic control of the trait. An available Infinium II SNP platform for genotyping containing 8,788 single nucleotide polymorphic markers was employed, and linkage maps were constructed in a F_1 cross from the low CR M13/91 and the moderate CR cv. Fred Hough. These maps were used to identify quantitative trait loci (QTL) for bud break date as a trait related to dormancy release. A major QTL for bud break was detected at the beginning of linkage group 9 (LG9). This QTL remained stable during seven seasons in two different growing sites. To increase mapping efficiency in detecting contributing genes underlying this QTL, 182 additional SNP markers located at the locus for bud break were used. Combining linkage mapping and structural characterization of the region, the high proportion of the phenotypic variance in the trait explained by the QTL is related to the coincident positioning of Arabidopsis orthologs for *ICE1*, *FLC*, and *PRE1* protein-coding genes. The proximity of these genes from the most explanatory markers of this QTL for bud break suggests potential genetic additive effects, reinforcing the hypothesis of inter-dependent mechanisms controlling dormancy induction and release in apple trees.

Keywords: apple, bud dormancy, chilling requirement, linkage mapping, *MdoFLC*, *MdoICE1*, *MdoPRE1*

INTRODUCTION

The domesticated apple (*Malus* × *domestica* Borkh.), as other temperate fruit trees, has developed the ability to enter a dormant state, which is a mechanism that enables plants to survive seasonal changes and protect sensitive meristems from unfavorable climatic conditions (Rohde and Bhalerao, 2007). Bud dormancy is usually divided into three consecutive phases: para-, endo-, and ecodormancy, where budburst inhibition is caused by signals derived from outside of the bud (but from the same plant), from the bud itself, or from environmental conditions, respectively (Lang, 1987). Endodormancy establishment is characterized by growth cessation, bud set, and leaf senescence, and the process is gradually overcome by prolonged exposure to cold. Each species and/or cultivar need a certain amount of low temperature exposure called chilling requirement (CR), in order to remove the physiological blocks that inhibit budburst and culminates into ecodormancy. In the ecodormant phase, the meristems achieve full developmental capacity and resume growth after a certain amount of warmth, which is also genotype-dependent (Dennis, 2003; Legave et al., 2008; Fu et al., 2014).

The physiological aspects of dormancy control have been partially elucidated and there are evidences that the mechanisms regulating dormancy release are highly heritable, suggesting a strong genetic control (reviewed in Falavigna et al., 2015). In fruit trees, the comprehension of molecular control of dormancy has been improved by the study of the *evergrowing* (*evg*) peach mutant, which fails to enter dormancy and maintains constant growth (Bielenberg et al., 2008). Mapping of the *evg* locus revealed six tandemly arranged MADS-box genes that were named *Dormancy-Associated MADS-box* (*DAM*). Further, functional characterization of some Rosaceous *DAM* orthologs proved their importance in the control of bud dormancy (Sasaki et al., 2011; Yamane and Tao, 2015; Wu et al., 2017). In apple, different strategies have been explored so far in order to understand the genetics of bud break date (BBD) and flowering time. Employing linkage map analysis on a bi-parental family, the first two quantitative trait loci (QTLs) for time of bud break were identified (Segura et al., 2007). In a similar approach, the end of the linkage group 9 (LG9) has been identified as a major QTL for time of bud break and flowering in other four progenies (van Dyk et al., 2010; Celton et al., 2011). Using a multi-parental population and pedigree-based analysis, Allard et al. (2016) detected a strong QTL for bud break and flowering time in the same chromosomal position, as well as another five QTLs of small effect coincident with the position of the *DAM* genes, the florigen *FLOWERING LOCUS T* (*FT*), or the flowering repressor *FLOWERING LOCUS C* (*FLC*). More recently, a genome-wide association (GWAS) study explored an apple core collection and confirmed the position of the major QTL for time of bud break on LG9, besides narrowing the confidence interval to ∼360 kb and emphasizing the importance of two major candidate genes encoding transcription factors containing NAC or MADS-box (putative *FLC*) domains (Urrestarazu et al., 2017).

In Arabidopsis, floral initiation can be induced by long-term chilling exposure, which is able to trigger the epigenetic suppression of *FLC* in a process called vernalization (Hepworth and Dean, 2015). Although the vernalization process somewhat resembles dormancy in temperate plants, the *FLC* role in perennial plants is still not fully understood. In apple, a putative *FLC* ortholog was identified and had a proposed role in dormancy release (ecodormancy) based on its differential expression during dormancy along with its co-localization in a QTL related to bud break (Porto et al., 2015). One of the positive regulators of Arabidopsis *FLC* expression is the CBF/DREB (C-repeat binding factor/dehydration-responsive element-binding protein) class of transcription factors, which are major integrators of plant cold response (Seo et al., 2009; Thomashow, 2010). Interestingly, ectopic expression of the peach *CBF1* gene in apple rendered increased freezing tolerance, early bud set and leaf senescence, and delayed bud break (Wisniewski et al., 2015). *CBF* gene expression is under direct regulation of ICE1 (Inducer of CBF Expression 1), a MYC-like basic-helix-loop-helix (bHLH) transcription factor that acts as a convergence point integrating cold and several other signaling pathways (Ding et al., 2015). Higher expression of a putative *ICE1* gene was found during pear endodormancy stage suggesting that it is importance in endormancy maintenance (Takemura et al., 2015). Additionally, an apple *ICE1* gene has been previously identified as differentially expressed during dormancy when comparing contrasting CR cultivars (Falavigna et al., 2014). These evidences strongly suggest that the cold sensing pathway might be acting during bud dormancy in apple. The same strategy employed to identify apple *FLC*, i.e., differential gene expression and co-localization in a bud break QTL, also yielded the identification of another gene that might play a role during apple dormancy, *PACLOBUTRAZOL RESISTANCE 1* (*PRE1*; Porto et al., 2015). As ICE1, PRE1 is a bHLH transcription factor, and its role is to integrate signals from brassinosteroids, gibberellin (GA), and light pathways. In addition, *PRE1* overexpression renders early flowering in Arabidopsis (Lee et al., 2006; Bai et al., 2012).

Despite of the significant advances, the genetic mechanisms of dormancy induction and release in apple trees are not fully understood. Therefore, it is still necessary to continue exploring the biological relevance of beginning of chromosome 9 in dormancy regulation. Within this context, the purpose of this study is to better characterize the LG9 QTL for BBD, a trait directly linked to CR and flowering, by enriching the LG9 with a set of newly developed SNPs for genotyping. Combining linkage mapping and a detailed structural characterization of the beginning of chromosome 9, we confirmed the position of the major QTL for BBD in LG9 and identified a new highly relevant candidate gene, the cold perception gene *MdoICE1*. The identification of *MdoICE1*, along with the already known *MdoFLC* and *MdoPRE1* genes previously identified by our group (Porto et al., 2015), provides us clues to propose that these genes are fully integrated in pathways leading from cold perception to responses associated to growth resumption after dormancy fulfillment. We postulate that these three genes may have additive effects on dormancy-associated phenotypes, reinforcing the hypothesis of inter-dependent mechanisms controlling dormancy induction and release in apple trees.

MATERIALS AND METHODS

Plant Materials

The plant material in our study consisted of one full sib (FS) family, including a total of 190 progeny individuals. This FS family (mapping population) was derived from a controlled pollination F1 cross between the low CR "M13/91" and the moderate CR "Fred Hough." The parental germplasm and the FS family were developed by Epagri – Caçador Experimental Station (Santa Catarina, Brazil), grafted on the widely used clonal apple rootstock M7 and planted in 2010 in two locations: 161 individuals were planted in a site located in Bento Gonçalves (BG, Rio Grande do Sul, Brazil; 29° 09′S, 51° 31′W; altitude 623 m) and 114 individuals in Vacaria (VC, Rio Grande do Sul, Brazil; 28° 33′S, 50° 57′W; altitude 970 m), Southern Brazil. The two site locations have distinct climate conditions that provide contrasting scenarios for the phenotypic segregation of CR and related traits. VC represents the condition of the Brazilian commercial orchards (type Cfb, Alvares et al., 2014) and BG as the condition of more mild/warm winters (type Cfa; Alvares et al., 2014; **Supplementary Tables S1, S2**).

Phenotyping

The main phenological stage evaluated was vegetative BBD (Bud Break Date). The phenotypic value of the trait corresponds to the date of the green point in the Fleckinger's apple phenological classification (EPPO, 1984). Phenotyping trait assessments were performed three times a week (day 1 being July 1st) over a period of seven growing seasons in BG (2011–2017) and five growing seasons in VC (2011, 2012, 2014, 2015, and 2017). Phenotypic and genotypic association was tested employing a chi-square independency test, separately for planting sites and year of growing cycle.

SNP Genotyping

IRSC (International Rosaceae SNP Consortium) SNP Markers

DNA from the parents and the FS family were extracted from samples of young leaf tissue, following Lefort and Douglas (1999) method modified to fit 2 mL microcentrifuge tubes. The DNA quality and concentration was checked using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Genomic DNA from samples of the individuals was amplified with no allelic partiality, followed by enzymatic fragmentation, precipitation, and DNA resuspension accordingly to Infinium II whole-genome genotyping assay protocol (Illumina, Inc., San Diego, CA, United States). Samples were applied to BeadChips containing sequence probes to interrogate 8,788 SNP positions distributed across the "Golden Delicious" apple genome assembly, *Malus* × *domestica* v1.0 (Velasco et al., 2010). These SNP makers were developed by the IRSC SNP Consortium as described previously (IRSC 9k Apple SNP array; Chagné et al., 2012). SNP genotyping was performed at the Genotyping and Genetic Diagnosis Unit (University of Florida, Gainesville, FL, United States). SNP genotypic data analysis and quality control were performed using GenomeStudio V2011.1 pipeline (Illumina, Inc.) using a SNP call rate filtering threshold of 0.90.

Kompetitive Allele Specific PCR (KASP) SNP Markers and Genotyping

To increase the SNP density for our QTL mapping study, we developed a new set of SNP markers. Whole-genome sequencing was performed using DNA samples from four individuals of "M13/91" (2) and "Fred Hough" (2). Sequencing was made using Illumina GA II platform, 1 × 100 bp, and generated 38 million reads (average coverage 6×) and 60 million reads (average coverage 10×), respectively, with reliable mapping on the genome assembly of "Golden Delicious," *Malus* × *domestica* v1.0 (Velasco et al., 2010). The data set of sequences generated was deposited under the access number PRJNA479978. With this sequence data, a large number of SNPs shared by the plant lines were detected (Alencar et al., 2011). These SNPs were further inspected for the development of genotyping assays targeting high-density mapping of apple LG9 based on the integration of the apple genetic map (Muranty et al., 2014) with contigs in the apple genome sequence. SNP positions deemed polymorphic between the two plant cultivars and located within predicted exons in the gene content annotation were considered primary targets for the design of genotyping assay using KASP technology. To provide enough sequence flank, we extracted a 70-bp string of nucleotides toward each direction from the target SNP using BedTools (Quinlan and Hall, 2010) and the reference genome. The set of sequences with size of 141 bp each were screened for features that could compromise the genotyping such as low complexity stretches of DNA and the presence of small units of repeats using MISA (Beier et al., 2017). The final set of 182 sequences containing putative new SNPs was used as source to the design of KASP markers for genotyping (**Supplementary Table S3**). Leaf discs with 10 mm diameter were freeze-dried and genotyped using the KASP technique at LGC genomics (Teddington, Middlesex, United Kingdom). Homozygous SNPs for both parents were discarded.

Genetic Linkage Map Construction and QTL Analysis

Genetic mapping of the parents was performed using JoinMap®4 (Van Ooijen, 2006). Segregation distortion of individual markers was revealed by chi-square test. Markers showing distorted segregation ($P < 0.05$) were removed from analysis. Linkage analysis was performed using the regression approach implemented for cross-pollinator (CP) with a minimum logarithm of the odds (LOD score) of >8 used to define LG. The Kosambi mapping function was applied for map distance calculation (Kosambi, 1944). LGs were numbered in accordance with Maliepaard et al. (1998) and graphs were prepared using MapChart 2.2 (Voorrips, 2002).

Quantitative trait loci analysis was performed with MapQTL®6 (Van Ooijen, 2009) using the phenotypic traits assessment from BG and VC. Regions with potential QTL effects were identified using interval mapping (IM) and restricted multiple QTL mapping (rMQM) functions. QTLs were declared significant if the maximum LOD, obtained after multiple rounds

of rMQM mapping, exceeded the LG and genome-wide (GW) LOD threshold (calculated with 1,000 permutations, mean error rate of 0.05). QTLs were characterized by the maximum LOD score and percentage of phenotypic variation explained. Broad sense heritability (H^2) for BBD was estimated based on the components of variance from the phenotypic analysis for both. Analyses were performed using the GLM procedure (Proc GLM) of the statistical software SAS/STAT® software (SAS Institute Inc., 2008).

In silico Candidate Gene Search

We listed the predicted gene structures in the contigs containing the markers of potential QTL effects in the chromosome 9 contributing to the trait value differences in the F1 cross. Gene Ontology terms for these genes were collected from the annotations in the genome assembly of "Golden Delicious," *Malus × domestica* v1.0, downloaded from PLAZA v2.5 platform (Van Bel et al., 2012). The gene list and the corresponding ontology terms were used to test if genes that belong to one ontology term do not differ in their ranks from genes that do not belong to that term, namely, the null hypothesis. The average LOD score for each marker in the MapQTL6 analysis using the phenotypic traits assessment from site locations BG and VC was used as the associated variable to rank the genes. Taken this continuous variable as the likelihood ratio of map a gene to the BBD QTL, we computed the Wilcoxon rank sum statistic, which is the sum of the ranks of the ontology term as a group for all corresponding genes in the whole chromosome. Approximate *p*-values for the rank test were obtained based on the method implemented in the program *func_wilcoxon* in the FUNC package (Prüfer et al., 2007). Significance of the tests was assessed in terms of a global *p*-value derived using a sensitive estimator in detecting a deviation from the null hypothesis. We used a cut-off of 0.05 for the global *p*-value to detect ontology terms that differs in their ranks. *P*-values declaring significance for one side of the test statistic were used to detect enrichment of gene-associated variables among the terms. Markers in the genes containing ontology terms that show enrichment were considered anchors to delimitate candidate regions of the BBD QTL in the chromosome 9 for *in silico* candidate gene search.

Physical Mapping of SNPs to the Assembly of a "Golden Delicious" Doubled-Haploid Tree (GDDH13) and Characterization of Candidate Regions of the BBD QTL

The positions of the SNP markers used in genotyping were physically mapped on the sequence of the pseudomolecule in the recently published genome assembly of Daccord et al. (2017). Sequences for the pseudomolecules were retrieved from the apple genome and epigenome project website ([1]GDDH13 v1.1). As the SNPs are originally described using a previous assembly (*Malus × domestica* v1.0), we mapped the v1.0 SNPs to the GDDH13 genome following UCSC same species lift over

procedure[2]. In summary, UCSC software tools (Kuhn et al., 2013) were used to create the alignment chain file between the two assemblies, and then we used CrossMap (Zhao et al., 2014) to convert the coordinates of the SNPs to the GDDH13 assembly. Using the coordinates of the markers highlighted in the QTL and Gene Ontology analyses, we defined candidate regions of the BBD QTL to perform careful inspection for prioritizing candidate genes (see section "Results" for the criteria utilized). We extracted and summarized functional annotation associated with individual genes and with group of genes with related ontology terms. Additionally, we performed scans of the sequences in the 5′ flanking sequence of the individual genes to define *cis*-acting elements putatively involved to chilling response in plants. PlantPAN 2.0 (Chow et al., 2016) database and tools were used for all the plant promoter analysis.

RESULTS

Phenotypic Trait Assessment

Apple BBD is mostly regulated by exposure to low temperatures (Heide and Prestrud, 2005). Therefore, we decide to use BBD measurements as the main indicator for CR in the mapping population. The observed BBD for the individuals in each year and at both sides showed remarkable differences and provided insights on genotype and environment interactions (**Figure 1**). Large phenotypic variation was observed between both sites, and BBD window was generally earlier and wider in BG (mean of observed extremes: 25 to 118 days) when compared to VC (mean of observed extremes: 32 to 87 days). Despite of this trait variability, the estimated broad sense heritability (H^2) was consistently high (H^2 = 0.54 in BG and 0.73 in VC), reinforcing the strong genetic effect for bud phenology traits linked to CR.

Linkage Mapping Construction

The F_1 progeny was genotyped using the IRSC 9k Infinium II array (Chagné et al., 2012). In total, 2,733 SNP markers (30.37%) passed the SNP calling and filtering from the GenomeStudio V2011.1 pipeline (Illumina, Inc.) and were used for linkage analysis. From the 182 KASP-SNPs previously selected for the LG9 high density mapping, 114 generated polymorphic amplification, and were further used, totaling 2,847 genotyped SNP markers. Following the double pseudo-testcross strategy, marker sets from either parent were separately processed for both maps. The genetic linkage map constructed for the parental "M13/91" covered 1,293.77 cM and consisted of 784 markers (**Supplementary Table S4**). Out of the 114 KASP-SNP markers tested, 34 markers were successfully integrated into the LG9 end (**Supplementary Figure S1**). LG10 was the longest LG on the map, spanning 101.68 cM, while LG17 was the shortest, spanning 32.42 cM. The highest number of markers mapped to a single LG was 75 on LG2, the smallest number of markers on a single LG was 10 on LG17 (**Supplementary Figure S1** and **Supplementary Table S4**). The map had an average marker density of one marker

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[1]https://iris.angers.inra.fr/gddh13/

[2]http://genomewiki.ucsc.edu/index.php/Same_species_lift_over_construction#Same_Species_Lift_Over
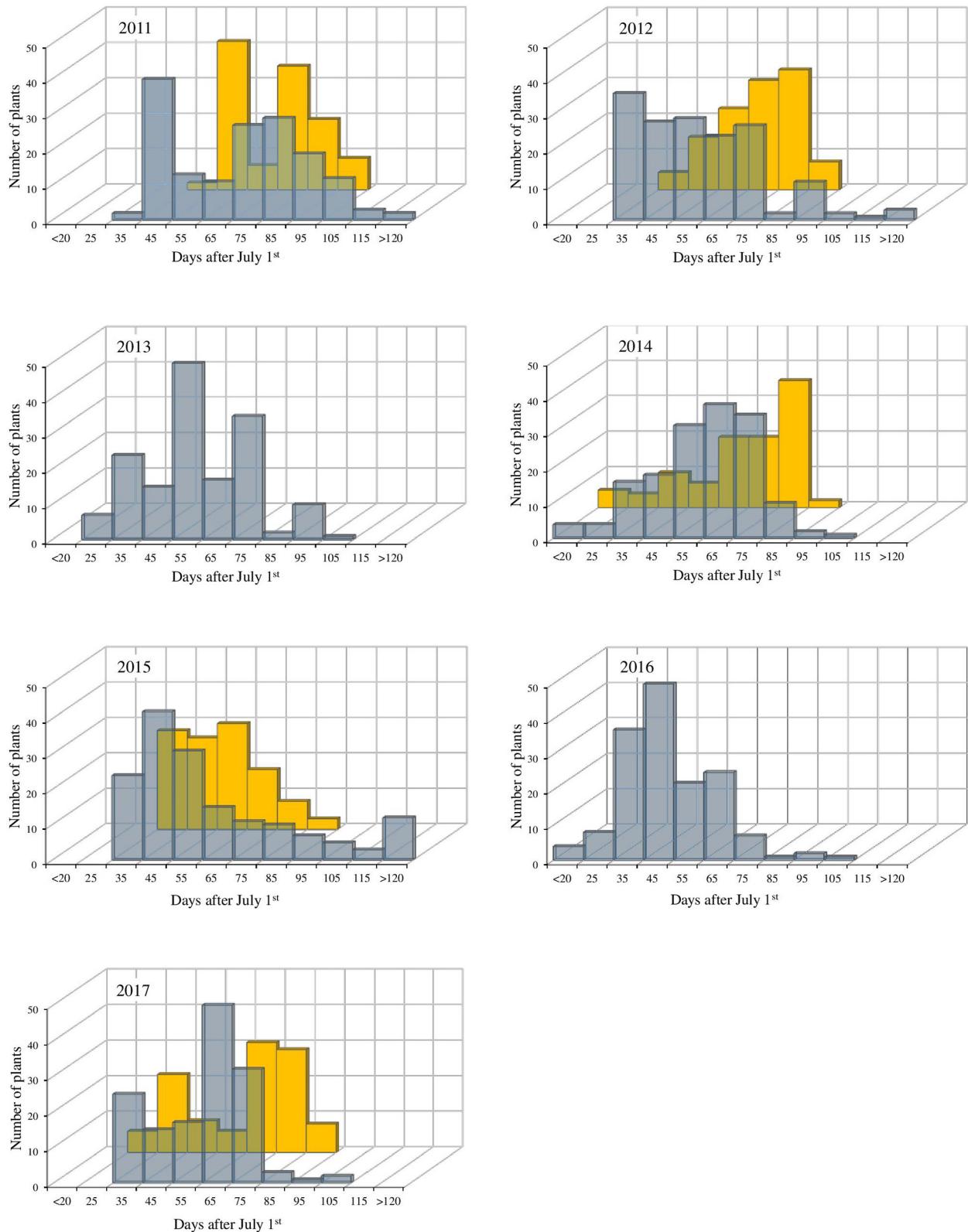
FIGURE 1 | Distribution of the bud break date (in days, starting from July 1st) for the population "M13/91" × "Fred Hough" in the evaluated years. Gray bars represent the experimental site in BG and yellow bars represent the experimental site in VC.

every 1.99 cM. The linkage map contained 18 regions in excess of 10 cM that contained no mapped molecular markers.

The genetic linkage map constructed for the parental "Fred Hough" covered 1,013.99 cM and consisted of 791 markers. Thirty-four KASP-SNP markers were successfully integrated in LG9 (**Supplementary Figure S1**). LG3 was the longest LG on the map, spanning 70.84 cM while LG11 was the shortest, spanning 27.52 cM. The highest number of markers mapped to a single LG was 91 on LG3 and the smallest number was 16 markers on LG11 (**Supplementary Figure S1** and **Supplementary Table S4**). The map had an average marker density of one marker every 1.55 cM and enclosed a total of nine regions in excess of 10 cM that contained no mapped molecular markers. Parental maps were aligned using common markers (**Supplementary Figure S1**) and exhibited 17 LGs corresponding to the number of chromosomes in the apple genome assembly (Velasco et al., 2010; Daccord et al., 2017). The order of the markers on the genetic maps was thoroughly checked, and their correspondence to the apple doubled haploid genome was performed (Daccord et al., 2017). Overall, the SNP marker order was coherent. However, the following main issues were identified that are common to other reports in the literature: (i) inconsistencies in LG assignment for 17% of the markers; (ii) regions of inversion (max ∼ 2.5 Mbp) involving 3% of the markers; and (iii) misplaced regions of markers within the same pseudo-chromosome for 2% of the markers.

QTL Detection

Quantitative trait loci analysis was performed separately on the parental maps for each set of phenotypical data obtained (BG and VC), and are shown in **Supplementary Table S5** and **Figure 2**. In all analyzed years, a single QTL region for BBD was detected for both parents at the beginning of LG9. Analysis performed on the BBD data for the two different population sites showed that this QTL exceeded the LG and GW LOD thresholds for all evaluated years. The BBD QTL located at the LG9 explained 24.5–45.6% of the phenotypic variance in BG population and 31–48% in VC population across different years (**Supplementary Table S5**). This result strongly suggests that a major effect locus contributing to trait value differences of vegetative BBD is located at the beginning of LG9. Overall, the LODs distribution across mapped intervals in the parental maps are reasonable conserved over years and sites, showing always a peak at the extremity of the LG9 on both parents. The distribution of significant LODs for the BBD QTL on "M13/91" LG9 was stable and restricted to the first 10–11 cM (**Supplementary Table S5**) when compared to the "Fred Hough" progenitor, which spanned a wider and more variable region. Examining the BBD QTL on "M13/91" progenitor, depending on the year and the LOD threshold, two remarkable peak regions are observed: the first at the extremity (marker TC_465054_Lg9) and the second starting around 9–11 cM (markers drm_qtl_snprnt113 and drm_qtl_snprnt137).

KASP-based SNP genotyping increased the number of markers anchored at the end of LG9. In M13/91, 10 exclusive markers were mapped in this region, spanning 9.3 cM more toward the end when compared to "Fred Hough." This shift is also clearly visible in the LOD and phenotypic variance

distribution observed in all evaluated years (**Supplementary Table S5**). When overlapping the LG9 BBD QTL on "M13/91" and "Fred Hough" linkage maps, it is observed that the "Fred Hough" BBD QTL starts at nearly the marker position where the "M13/91" BBD QTL approximately ends (see markers drm_qtl_snprnt113 and drm_qtl_snprnt137; **Supplementary Table S5**). These observations were used to define the region considered for detailed inspection in the search for candidate genes.

In order to demonstrate the markers contribution to the phenotypic variation for BBD, the top 15 markers associated with the QTL interval detected for both parents were tested. All the tested markers segregated for BBD in both sites (**Supplementary Figure S2** and **Supplementary Table S6**). Remarkably, the heterozygous markers for the "M13/91" parent, showed clear phenotypic distribution, with significant value of chi-square calculated in BG and VC. The separation of individuals into three distinct groups, early, intermediate, and late BBD, highlights the association between "low CR alleles" and the precocity of the growth resumption in this population.

In silico Candidate Gene Identification

We mapped the SNPs used in genotyping to the recently published high-quality doubled haploid genome assembly (Daccord et al., 2017). The distribution of the significant LODs in the QTL analysis for variation in the evaluated phenotype for both parents was fully investigated using the new coordinates. Markers that passed the LOD thresholds (LG and GW) for each parent were selected (**Supplementary Table S5** and **Figure 2**). Taking into account the narrower LOD distribution, the stability over the years, the low chilling heritability and the mapping of 10 additional exclusive markers, the most likely segment of DNA (the locus) representing the BBD QTL detected for "M13/91" was selected.

Using the relevant markers, we performed gene ontology analysis on the base of Wilcoxon rank sum tests taken the significant LODs as gene-associated variables (**Supplementary Table S7**). Noteworthy, enriched terms for genes target by markers or in close proximity to them, i.e., located in the same contig, included transmembrane transport (GO:0055085) and anion transport (GO:0006820) in the biological process ontology, ATPase activity coupled to transmembrane movement of substances (GO:0042626) in the molecular function ontology, and integral component of membrane (GO:0016021) and mitochondrial outer membrane (GO:0005741) in the cellular component ontology. Sequence coordinates for the eight gene models highlighted in the analysis of differences of rank sum test for ontology terms (**Supplementary Table S7**) were adjusted to include 200,000 bp in each direction (Brodie et al., 2016). All book-ended ("touching") entries were then merged into a single interval using, respectively, slopBed and mergeBed utilities from the BedTools v2.25 package (Quinlan and Hall, 2010). A set of three non-overlapping intervals encompassing a region that ranges from 204,479 to 3,926,269 bp in the chromosome 9 of the GDDH13.1.1 assembly was defined as loci of interest to mine the BBD QTL for candidate genes.
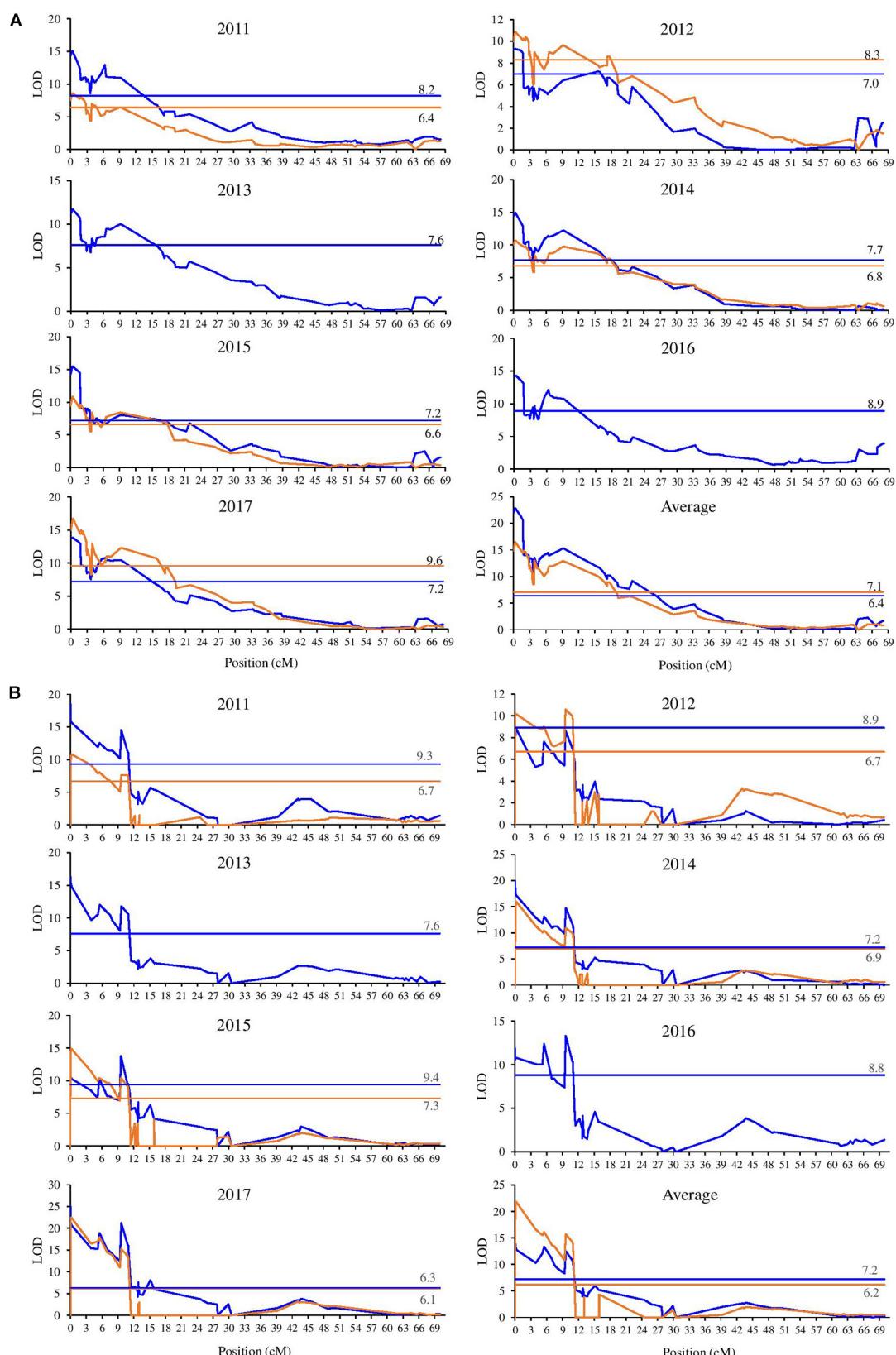
FIGURE 2 | Position of the QTL for bud break date detected in LG9 of the "M13/91" × "Fred Hough" maps. (Blue lines represent BG and orange lines represent VC. The threshold values are indicated.) **(A)** Fred Hough. **(B)** M13/91.

These intervals underlying the BBD QTL in our analysis comprised 17 markers (**Supplementary Table S5**; highlighted) and 219 predicted protein-coding gene structures, which were evaluated for prioritizing candidate genes (**Supplementary Table S8**). The list of gene models was carefully inspected giving priority to predicted genes that fitted in one or more of the following criteria: (i) related to cold response; (ii) functioning in dormancy and/or flowering time regulation; (iii) related to hormonal pathways, including absisic acid (ABA), GAs, and auxin-mediated signaling (Beauvieux et al., 2018); and (iv) having putative *cis*-acting elements in the promoter sequences thought to respond to chilling, such as those containing MYB and MYC motifs (Zhang et al., 2016).

In addition to relevant genes previously described in the literature at the top of the major QTL in LG9, like *MdoFLC* (MD09G1009100) and *MdoPRE1* (MD09G1049300) (Porto et al., 2015), we identified a predicted gene model (MD09G1003800, Chr09:335,088-338,411) that shares similarity to Arabidopsis *ICE1* (**Supplementary Figure S3**). The bHLH type transcription factor ICE1 binds to the promoter of the *CBF* genes to activate the *ICE1-CBF-COR* (COld Responsive genes) transcriptional cascade in plants, also known as *CBF* regulon (Chinnusamy et al., 2007; Thomashow, 2010; Ding et al., 2015; Park et al., 2015). The association of an apple *ICE1* (*MdoICE1*) to dormancy traits (CR, BBD, and flowering) agrees and complements the results observed when a peach *CBF* (*PpCBF1*) gene was constitutively expressed in an apple rootstock variety, resulting in altered expression of apple dormancy-related genes (*DAM* genes), early senescence in the autumn and delayed bud break in the spring (Wisniewski et al., 2015). Within this context, in order to investigate the *MdoICE1* participation in dormancy regulation, we determined its expression in different tissues and among a collection of individuals from the mapping population that showed stable early and late BBD across the years evaluated (**Supplementary Figure S4**). The data obtained demonstrated that *MdoICE1* is expressed in young/mature leaves; however, the transcriptional profiles did not reveal potential associations with the early neither the late bud break phenotypes. These results were somehow expected because activation of the *CBF* regulon relies on *ICE1* post-translational modifications triggered by cold stimulus (Ding et al., 2015). Taking into account that the transcriptional activity of the *ICE1* locus in itself cannot be associated with its biological function, we tested the CBF regulon transcriptional activity in response to cold stimulus using *in vitro* apple plants. The results gathered from this assay showed a slight but not significant induction of *MdoICE1*, followed by a significant sequential induction of *MdoCBFs* and *MdoDAM1*, a major candidate gene that has been associated with dormancy regulation in apple (Porto et al., 2016; **Supplementary Figure S5**). These data reinforce the findings reported by Wisniewski et al. (2015) and suggest an important role of the *MdoICE1*/*CBF* regulon in the control of bud dormancy in apple.

In the same region, we identified another gene (MD09G1004400, Chr09:368,274-371,719) characterized as mitochondrial phosphate transporter (MPT). The mRNA sequence for this gene showed high similarity with the *MPT* transcript EU072922, which was previously reported to be involved in accelerating bud dormancy release during chilling treatment in tree peony (*Paeonia suffruticosa*) (Huang et al., 2008; Zhang et al., 2016). The evidence for correlation of apple *ICE1* (*MdoICE1*) and MPT (*MdoMPT*) to traits associated to CR, BBD, and flowering reinforces a new perspective on the balance between different pathways in the control of dormancy release, integrating cold temperature perception, flowering, carbohydrate metabolism, and mitochondrial respiration. The relative positions of the main candidate genes within the BBD QTL on LG 9 are depicted in **Supplementary Figure S6**.

DISCUSSION

We present a genetic analysis of the BBD locus in a Full-Sib family derived from a controlled pollination F_1 cross between contrasting genotypes for CR. The observed phenology for BBD suggests a strong genetic control of trait variation. In our study, broad sense heritability values were found high for BBD, 0.54 at BG and 0.73 at VC, reinforcing the appreciate contribution of underlying genes to the observed phenotypic variance. Albeit the heritability estimate was shown to be specific to the population and environment analyzed (de Souza et al., 1998), there are previous studies that have produced similar estimates for the heritability of the BBD trait in apple and peach (Fan et al., 2010; van Dyk et al., 2010; Celton et al., 2011). The similarity of values calculated from different progenies in different environments suggests a common genetic mechanism contributing for the phenotypic variance of traits associated with CR. Furthermore, despite the lower heritability and the lower explanation observed in BG, it is worth to note that sites with mild winters made it possible to exploit the genotype in such environment, favoring the observation of early BBD genotypes.

The constructed genetic linkage maps showed 17 LGs in accordance with Maliepaard et al. (1998) and corresponding to the haploid number of chromosomes in apple (Velasco et al., 2010). Using *whole-genome* sequencing of parental germplasm "M13/91" and "Fred Hough" and mapping short sequence reads to the reference, 182 new SNP markers were developed to be used in KASP assay for SNP genotyping. Using these markers for genotyping, we successfully increased the marker density to improve QTL mapping efficiency in detecting putative novel contributors to the BBD trait variability. The 784 grouped markers for "M13/91" and the 791 for "Fred Hough" revealed good marker saturation in all LGs. Linkage maps constructed using SSR or SNP markers from different apple (van Dyk et al., 2010; Celton et al., 2011; Antanaviciute et al., 2012; Zhang et al., 2012; Clark et al., 2014) and pear (Yamamoto et al., 2014; Chen et al., 2015; Gabay et al., 2017) populations showed similar coverage.

The linkage maps of both parents enabled an efficient detection of a major QTL affecting BBD. This QTL explained 24.4–53.30% of the variability at BG and 31–63% at VC, besides co-localizing with the genomic region associated with BBD and flowering time on LG9 beginning specified by van Dyk et al. (2010), Celton et al. (2011), Allard et al. (2016), and

Urrestarazu et al. (2017). The identification of a common QTL in different segregating populations, climatic conditions, and years is an unusual finding. This result reinforces the importance of this region for the genetic control of BBD in apple trees and incentive investigations of the underlying genes contributing effects to the trait variance. Although this region explains more than 50% of the phenotypic variation observed for BBD, other important regions have also been reported on LG7, LG10, and LG12, as well as QTL with minor effects on LG8 and LG15 (Allard et al., 2016). Among the progress toward the search for candidate genes putatively associated with dormancy traits, the interval of this QTL was initially defined as the first 4.04 Mb (Celton et al., 2011) and subsequently narrowed to 1.8(Allard et al., 2016), 1.7 (Trainin et al., 2016), and 0.36 Mb (Urrestarazu et al., 2017). The segments indicated in these contributions overlap with the physical interval defined in our study (3.28 Mb), which was defined based on the stability of the QTL across years and sites.

The genetic control of dormancy in Rosaceae is a complex process and the identification of major genes controlling this trait are challenging. In the beginning of apple chromosome 9 (position 655,000), a sequence with similarity to *FLC* (MD09G1009100; *MdoFLC*) was already described as a strong candidate to have a functional role during flowering (Porto et al., 2015; Allard et al., 2016; Urrestarazu et al., 2017). *MdoFLC* was shown to be seasonally expressed during dormancy (Porto et al., 2015), and recently, this gene was shown to be differentially expressed when comparing ecodormant buds to bud break and fruit set stages (Kumar et al., 2017), suggesting a putative role during endo- to ecodormancy transition. In Arabidopsis, the *FLC* gene is the main repressor of flowering under unfavorable conditions (reviewed in Amasino and Michaels, 2010).

Besides *MdoFLC*, genes from other functional classes were identified inside the confidence intervals of the LG9 BBD QTL such as NAC-domain protein (MD09G1006400), putative WRKY transcription factor (MD09G1008800), and chromatin-remodeling complex (MD09G1011500; MD09G1011600) (Celton et al., 2011; Allard et al., 2016; Trainin et al., 2016; Urrestarazu et al., 2017). Although intense efforts have been made to trace candidate genes that can play roles in the genetic control of dormancy induction and release, a biological integration of these findings is still missing.

Within this context, we carefully inspected the physical interval of the LG9 BBD QTL for new candidate genes that could be primarily associated with cold response, dormancy or ABA-mediated signaling. We identified a gene model located close to the highest significant LOD scores (**Supplementary Tables S5, S8**) that shows similarity to an Arabidopsis gene encoding the bHLH transcription factor ICE1 (**Supplementary Figure S3**). Apples and pears diverge in bud dormancy regulation from other Rosaceae species because instead of being triggered by photoperiodic changes, the main regulator of this process is exposure to cold temperatures (Heide and Prestrud, 2005). Therefore, the identification of *MdoICE1* inside the apple BBD QTL gives us the opportunity to add new evidence to the elucidation of dormancy control: a candidate gene associated with cold perception and flowering regulation.

Apart from being located close to the highest significant LOD scores across the years evaluated in two different sites (**Supplementary Tables S5, S8**), it is well stablished in the literature that upon cold stimulus, ICE1 and its targets *CBF* transcription factors activate downstream cold-responsive gene targets, including COLD-REGULATED (COR) genes also known as the CBF regulon (Gilmour et al., 1998; Chinnusamy et al., 2003, 2007; Thomashow, 2010; Kim et al., 2015; Park et al., 2015; Zhan et al., 2015). In addition, ICE1 can also directly integrates cold signals into *FLC*-mediated flowering pathways, and ICE1 activity is post-translationally modulated by OST1, a key component in ABA signaling (Ding et al., 2015; Lee et al., 2015). Under floral promoting conditions, ICE1 binding to *FLC* and *CBF* promoters is inhibited and leads to flowering induction (Lee et al., 2015). In apple, MdCIbHLH1 (MD14G1148600), an ICE-like protein has the ability to promote chilling tolerance in transgenic plants; however, this gene has not been assigned to QTL positions for dormancy traits (Feng et al., 2012). This collection of evidences suggests *MdoICE1* as an appealing candidate gene to mediate cold and ABA responsiveness by activating the CBF regulon during the bud dormancy process in apple and that a balance between different pathways are acting to mediate adaptive responses to cold perception.

Interestingly, CBFs were shown to modulate apple dormancy by altering the expression of genes responsible for the molecular control of dormancy progression such as the *DAM* genes (Wisniewski et al., 2015; Artlip et al., 2016). Similarly, the pear CBF2 was shown to interact with the promoter of the *DAM* ortholog *PpMADS13-1* (Saito et al., 2015), a trend that was already suggested for *DAM* genes of other species (Horvath et al., 2010; Sasaki et al., 2011; Yamane et al., 2011; Porto et al., 2016). The context of these evidences together with the data gathered in this work can also contribute to the understanding of the genetics behind dormancy traits. As part of the CBF regulon, *MdoDAM* genes function may be directly affected by the genetic variance of their upstream regulators, therefore establishing a potential epistatic effect. This might be one of the explanations why QTL associated with *MdoDAM* chromosomal positions in apple were not found in bi-parental progenies (this study; van Dyk et al., 2010; Celton et al., 2011) and even using a large and powerful GWAS study (Urrestarazu et al., 2017). Most *MdoDAM* genes were found at LG8 in a region highly syntenic to the peach chromosome 1, where the *PpDAM* genes are located (Porto et al., 2016). Hence, if conserved genetic mechanisms involving *DAM* genes in peach were to be found in apple, they are expected to map to apple LG8. Allard et al. (2016) exploring a pedigree-based analysis in multi-parental populations were able to detect QTLs for bud phenology traits associated with *MdoDAM* chromosomal positions on LG8 and LG15 in two of the three years evaluated; however, they were considered of minor contribution.

In addition, we identified in the same QTL interval a gene model showing high similarity to *MPT* (*MdoMPT*), a gene involved in accelerating bud dormancy release during chilling treatment in tree peony (Huang et al., 2008; Zhang et al., 2016). Cold-responsive genes are able to encode a diverse array of

FIGURE 3 | The proposed hypothetical model for the apple bud dormancy process. The model depicts schematically the dormancy stages from dormancy establishment to bud break. Environmental stimuli are presented above the dormancy stages. Potential regulatory roles of candidate genes are shown in dark blue boxes. Green arrows indicate putative transcriptional activation while T end red bars represent repression activity. The orange star-shaped form represents source of energy. Post-translational modification of MdoICE1 (phosphorylation and stabilization) upon stimuli is indicated with P embedded in red.

proteins involved in respiration and metabolism of carbohydrates (Chinnusamy et al., 2003). Noteworthy, the carbohydrate metabolism appears essential in the transition from dormancy to active bud growth in response to cold. The bud capacity to burst was suggested to be tightly linked to its carbohydrates supply due to increasing carbohydrate uptake in the bud after dormancy release, with an increase in the expression and activity of membrane transporters (Beauvieux et al., 2018). Remarkably, the *MPT* expression regulation in dormancy release is thought to be essential to promote respiratory rate and energy metabolism, which is a process that requires delivering of inorganic phosphate to the mitochondrial ATP synthase complex (Beauvieux et al., 2018). The identification of *MdoMPT* in the LG9 QTL adds carbohydrate metabolism and mitochondrial respiration pathways into the likely players of dormancy in apple.

Taking advantage of the known functional pathways of ICE1, CBF, and FLC in Arabidopsis, we suggest a hypothetical model for the apple bud dormancy process based on the findings of the LG9 BBD QTL (**Figure 3**). Early cold waves and ABA stimuli activate the MdoCBF regulon through MdoICE1. *MdoDAM* genes would be activated leading to dormancy establishment. Medium- to long-term cold exposure have a role during endo- to ecodormancy transition by activating *MdoFLC* through the same pathway, but in a CR-dependent manner. The balance of both *MdoDAM* and *MdoFLC* levels would guarantee that dormancy release and bud break only occur during optimal growth promoting conditions after heat requirement fulfillment. However, how the same pathway would induce these genes in different time points is yet to be discovered.

Complementarily, the examination of the BBD QTL on the M13/91 progenitor revealed two remarkable peak regions dividing the QTL depending on the threshold adopted and year evaluated (see **Supplementary Table S5**): the first at the extremity (marker TC_465054_Lg9) and the second one starting around 9–11 cM (markers drm_qtl_snprnt113 and drm_qtl_snprnt137). The marker drm_qtl_snprnt137 lies within the MD09G1049300 gene model, which was annotated as an ortholog of *PRE1*. The LOD distribution drops to non-significant values immediately after this marker's position, except in the atypical winter of 2017 (see **Figure 1** and **Supplementary Tables S1, S2, S5**). This finding suggests a relevant role of *MdoPRE1* locus for the phenology of dormancy-associated traits (bud break and flowering). In agreement to the genetic analysis, differential expression of *MdoPRE1* during bud break was observed in apple (Porto et al., 2015; Kumar et al., 2017), reinforcing a potential role in growth resumption during bud dormancy release. This set of observations also accounts for a potential additive effect between the loci located at the borders of the LG9 BBD QTL, emphasizing the complex genetic control of this trait. The additive effect hypothesis is also consistent because time of bud break and flowering are indirect measures of CR, and genetic analysis approaches based on phenotyping of these traits failed to identify loci responsible for the maintenance of the dormancy process (growth repressors) in apple trees (Celton et al., 2011; Allard et al., 2016; Trainin et al., 2016; Urrestarazu et al., 2017).

In the present study, the use of phenological data collected during 7 years in two locations with different climatic conditions allowed the dissection of different loci contributions to BBD. The results obtained allowed us to revisit the

genetic characterization of the corresponding BBD QTL chromosomal segment, reinforcing the strong genetic effects over dormancy-associated traits. The proximity of candidate genes associated with chilling perception, ABA signaling, and flowering (*MdoICE1*, *MdoFLC*, and *MdoPRE1*) from the most explanatory markers suggests potential complementary roles of these genes functions in dormancy establishment and release. Several models for bud dormancy control have been proposed (Horvath, 2009; Campoy et al., 2011; Rinne et al., 2011; van der Schoot and Rinne, 2011). However, they are based on species in which photoperiod plays a major role in dormancy induction. These models do not address the peculiarities of this process in pipfruits, such as apples and pears. Therefore, a tentative summarizing model is proposed in **Figure 3**. The hypothetical model postulates that: *MdoICE* is important for cold perception to start the signaling involved in dormancy establishment and adaptive responses; the *MdoDAM* genes are important to induce bud set and to repress growth resumption until CR fulfillment; *MdoFLC* is important to inhibit growth after the transition from endo- to ecodormancy; *MdoPRE1* is important for bud break, possibly by mediating GA promotion of vegetative growth; and *MdoMPT* represents the energy source necessary for growth as being associated with carbohydrate metabolism. In the breeding context, the interpretation of the results presented in this work may help the development of strategies that could support the generation of new cultivars better adapted to each regional cultivation scenario, either by biotechnological or by conventional practices.

AUTHOR CONTRIBUTIONS

YEM was involved in the phenotypic analysis and KASP genotyping, as well as drafting the manuscript. CT was involved in phenotypic analysis and SNP genotyping using RosBREED markers. ABCC assisted in the management of the orchards, phenotypic and statistical analysis. DDP worked in the KASP markers development. DDP, VSF, OBdS-J, RCT, PG, MMCC and TS realized the *in silico* analysis of the candidate genes. SAdA, OBdS-J, RCT, MMCC, GJP and PG performed the *de novo* SNP discovery. VB and AMC performed the gene expression analysis. MVK and FD developed the F1 population. PRDO, CAD and LFR conceived and supervised the work, edited and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

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11 CURRICULUM VITAE

CATTANI, A. M.

DADOS PESSOAIS

Nome: Amanda Malvessi Cattani

Nascimento: 08/03/1992, Garibaldi, RS, Brasil

E-mail: amanda.cattani@gmail.com

FORMAÇÃO

Doutorado em Biologia Celular e Molecular.

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil.

Título: Compreendendo a dormência de gemas de macieira (*Malus × domestica* Borkh) por meio da integração de estímulos externos, sinalização hormonal e mecanismos moleculares

Orientadores: Dr. Luís Fernando Revers e Dr. Giancarlo Pasquali

Bolsista: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Período: 2016 – 2020

Mestrado em Biologia Celular e Molecular.

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil.

Título: Elementos repetitivos na regulação da transcrição de *Mycoplasma hyopneumoniae*

Orientadora: Dra. Irene Silveira Schrank

Bolsista: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Período: 2014 – 2016

Graduação em Biotecnologia

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil.

Título: Clonagem e expressão de genes relacionados com a biossíntese de NAD e FAD de *Mycoplasma hyopneumoniae*

Orientadora: Dra. Irene Silveira Schrank

Período: 2010 – 2014

Curso Técnico em Enologia e Viticultura
Instituto Federal do Rio Grande do Sul, IFRS, Bento Gonçalves, RS, Brasil
Período: 2007 – 2010

ESTÁGIOS

Estágio Obrigatório – Graduação em Biotecnologia (UFRGS)
Laboratório da empresa BEIFIUR Ltda, Garibaldi, RS, Brasil.
Projeto: Avaliar o comportamento dos micro-organismos *Azospirillum brasilense, Bradyrhizobium japonicum, Bacillus subtilis*, *Bacillus thuringiensis* e *Bacillus sphaericus*, importantes no melhoramento e desenvolvimento agrícola, quando combinados com herbicidas ou fungicidas comumente utilizados nas lavouras. Além de avaliar métodos para a produção e manutenção desses micro-organismos.
Período: 08/2013 – 12/2013

Estágio de Iniciação Cientifica
Laboratório de Micro-organismos Diazotróficos – Centro de Biotecnologia
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil.
Projeto: Caracterização de proteínas envolvidas em rotas metabólicas de *Mycoplasma hyopneumoniae.*
Orientação: Dra. Irene Silveira Schrank
Período: 2012 – 2013
Bolsista: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Estágio de Iniciação Cientifica Voluntário
Laboratório de Micro-organismos Diazotróficos – Centro de Biotecnologia
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil.
Projeto: Caracterização de proteínas envolvidas em rotas metabólicas de *Mycoplasma hyopneumoniae.*
Orientação: Dra. Irene Silveira Schrank

Período: 2011 – 2012

Estágio Obrigatório – Técnico em Enologia (IFRS)

Laboratório de análises da empresa Chandon Brasil Ltda, Garibaldi, RS, Brasil.

Projeto: Atividades gerais no controle laboratorial dos processos fermentativos da produção de espumantes. Período: 2009

ARTIGOS COMPLETOS PUBLICADOS

SIQUEIRA, F. M.; WEBER, S. S.; CATTANI, A. M.; SCHRANK, I. S. Genome organization in *Mycoplasma hyopneumoniae*: identification of promoter-like sequences, *Molecular Biology Reports*, 41 (8): 5395-402, 2014.

CATTANI, A. M.; SIQUEIRA, F. M.; GUEDES, R. L. M.; SCHRANK, I. S. Repetitive elements in *Mycoplasma hyopneumoniae* transcriptional regulation. *PLoS One*, 11(12): 4–7, 2016.

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CAPÍTULOS DE LIVRO PUBLICADOS

CATTANI, A. M., SARTOR, T., DA SILVEIRA FALAVIGNA, V., PORTO, D. D., SILVEIRA, C. P., DIAS DE OLIVEIRA, P. R., REVERS, L. F. The Control of Bud Break and Flowering Time in Plants: Contribution of Epigenetic Mechanisms and Consequences in Agriculture and Breeding. *Advances in Botanical Research*, 88, 277–325, 2018.

PRÊMIOS E TÍTULOS

2° Lugar - Melhores Trabalhos - Categoria Pós Graduação no 16° Encontro de Iniciação Científica e 12° Encontro de Pós-Graduandos da Embrapa Uva e Vinho, Embrapa Uva e Vinho (2018).

1° Lugar - Melhores Trabalhos - Categoria Pós Graduação no 15° Encontro de Iniciação Científica e 11° Encontro de Pós-Graduandos da Embrapa Uva e Vinho, Embrapa Uva e Vinho (2017).

PARTICIPAÇÃO EM BANCAS DE TRABALHOS DE CONCLUSÃO

TRABALHOS DE CONCLUSÃO DE CURSO DE GRADUAÇÃO

CATTANI, A. M.; TRAMONTINA, A. N.; MAFFESSONI, D.; TRAMONTINA, F. F. Participação em banca de Amanda de Souza."Detecção de fungos causadores de podridão de tronco de videira por PCR Nested e PCR Multiplex. 2018. Trabalho de Conclusão de Curso (Graduação em Engenharia de Bioprocessos e Biotecnologia) - Universidade Estadual do Rio Grande do Sul.

ORIENTAÇÕES E SUPERVISÕES CONCLUÍDAS

Luiza Rathke. Estágio Obrigatório. Estudante de Agronomia da Universidade Federal do Rio Grande do Sul, 2019.

Cássia Beatriz de Souza. Estágio de Iniciação Científica. Estudante de Graduação em Engenharia de Bioprocessos e Biotecnologia da Universidade Estadual do Rio Grande do Sul, 2019-2020.

RESUMOS PUBLICADOS EM EVENTOS E CONGRESSO

CATTANI, A. M; SILVEIRA, C. P.; PASQUALI, G.; REVERS, L. F. Type-B response regulators of the cytokinin-signaling pathway link hormonal stimulus and molecu

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