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CARACTERIZAÇÃO MOLECULAR E FUNCIONAL DO GENE *ATPLAC811.1* NA MORTE CELULAR PROGRAMADA (*PROGRAMMED CELL DEATH*) EM PLANTAS

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Porto Alegre, outubro de 2022.

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como parte dos requisitos necessários para a obtenção do grau de Doutora em Genética e Biologia Molecular.

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“A vida sem ciência é uma espécie de morte’’

(Sócrates)

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LISTA DE ABREVIATURAS E SIGLAS

ABA: Ácido abscísico (do inglês, *Abscisic Acid*)

Basta: Glufosinato de amônio (do inglês, *Glufosinate Ammonium*)

CAT: Catalase

cDNA: DNA complementar

DNA: Ácido Desoxirribonucleico (do inglês, *Deoxyribonucleic Acid*)

dNTP: Desoxirribonucleosídeo Trifosfatado

dPCD: Morte Celular Programada do Desenvolvimento (do inglês, *Developmental* PCD)

ePCD: Morte Celular Programada do Ambiente (do inglês, *Environmental* PCD)

ET: Etileno

HR: Resposta de Hipersensibilidade

GFP: Proteína Fluorescente Verde (do inglês, *Green Fluorescent Protein*)

JA: Ácido Jasmônico (do inglês, *Jasmonic Acid*)

LSD1: *Lesion Simulating Disease 1*

MS: Meio de cultivo Murashige and Skoog

PAR: Radiação Fotossinteticamente Ativa (do inglês, *Photosynthetically Active Radiation*)

PCD: Morte Celular Programada (do inglês, *Programmed Cell Death*)

PCR: Reação em Cadeia da Polimerase (do inglês, *Polimerase Chain Reaction*)

PLAC8: *Placent specific 8*

PPI: Protein-Protein Interacting

RNA: Ácido Ribonucleico (do inglês, *Ribonucleic Acid*)

ROS: Espécies Reativas de Oxigênio (do inglês, *Reactive Oxygen Species*)

RT-qPCR: Transcrição Reversa seguida da Reação Quantitativa da DNA Polimerase em Cadeia (do inglês, *Reverse Transcription-Quantitative Polymerase Chain Reaction*)

SA: Ácido salicílico (do inglês, *Salicylic Acid)*

TAIR: The Arabidopsis Information Resource

WT: Tipo selvagem (do inglês, *Wild Type*)

YFP: Proteína Fluorescente Amarela (do inglês, *Yellow Fluorescent Protein*)

Y2H: Duplo híbrido em levedura (do inglês, *Yeast Two-Hybrid*)

RESUMO

O processo celular denominado de morte celular programada (do inglês, *Programmed Cell Death*) corresponde ao suicídio celular de maneira geneticamente controlada e regulada em organismos eucarióticos e procarióticos, de modo a eliminar células danificadas, senescentes ou desnecessárias. Em plantas, a PCD ocorre durante etapas do desenvolvimento, nas respostas de defesa e frente a condições ambientais adversas. Ao contrário dos animais, o conhecimento sobre a PCD em plantas é limitado, com trabalhos realizados principalmente utilizando a planta *Arabidopsis thaliana* como modelo. Nessa planta foi sugerida uma rede que controla a morte celular mediada por resposta hipersensível (do inglês, *Hypersensitive Response*), o "*AtLSD1 - deathosome*". O gene codificador de proteína contendo o domínio PLAC8 (*Placenta-specific 8*), At1g52200 (*AtPLAC8-11*), foi descrito como componente do "*AtLSD1-deathosome*". O presente trabalho tem como objetivo o estudo funcional do gene *AtPLAC8-11*, especialmente em relação a PCD. Para isso, foram realizados estudos de localização subcelular dos produtos do gene *AtPLAC8-11*: AtPLAC8-11.1 (transcrito canônico) e AtPLAC8-11.2 (transcrito alternativo) em protoplastos e verificou-se um perfil diverso de localização das proteínas em ambas as variantes de acordo com a fusão C ou N-terminal com GFP (do inglês, *Green Fluorescent Protein*). Porém, foi possível obter co-localização com o marcador de retículo endoplasmático para ambas as formas. A expressão tecido-específica de AtPLAC811.1 e AtPLAC811.2 sob controle do promotor nativo em *Arabidopsis* foi observada em raiz e folha, com diferente localização subcelular nesses tecidos, como nas bases dos tricomas apenas para AtPLAC811.1. A análise da expressão de AtPLAC811.1 e AtPLAC811.2 sob controle do promotor nativo em diferentes partes da flor mostrou que AtPLAC811.1 é altamente expresso em sépalas e estames, em comparação com AtPLAC811.2. O tratamento com o indutor de resistência ou elicitor Pep1 aumentou a expressão tanto de AtPLAC811.1 quanto AtPLAC811.2 sob controle do promotor nativo nas raízes. O ensaio de duplo híbrido em levedura (YH2) (do inglês, *Yeast Two-Hybrid*) revelou que apenas AtPLAC811.1 interage com a proteína AtLSD1 e através do ensaio de complementação de fluorescência bimolecular (BiFC), observou-se reconstituição da YFP (do inglês, *Yellow Fluorescent Protein*) em formato indefinido em locais da célula. Os experimentos de transativação mostraram que a interação de AtPLAC811.1 com

AtLSD1 é capaz de interferir na atividade transcricional de AtLSD1. As plantas *knockout plac8-11* alteram a expressão de genes relacionados a PCD do desenvolvimento (dPCD) e do ambiente (ePCD). Um método promíscuo de biotina ligase TurboID foi utilizado para investigar os interatores de AtPLAC811.1 e AtPLAC811.2, resultando na identificação de diferentes proteínas interatoras, incluindo proteínas envolvidas em resposta a estresses para ambas as variantes. Nesse sentido, os resultados obtidos indicam o envolvimento de *AtPLAC8-11* no processo de PCD em *Arabidopsis*.

ABSTRACT

The cellular process denominated Programmed Cell Death (PCD) corresponds to cell suicide in a genetically controlled and regulated manner in eukaryotic and prokaryotic organisms, in order to eliminate damaged, senescent or unnecessary cells. In plants, PCD occurs during developmental stages, in defense responses and in adverse environmental conditions. Unlike animals, the knowledge about PCD in plants is limited, with works mainly performed using *Arabidopsis thaliana* as a model. In this plant, a network that controls cell death mediated by a Hypersensitive Response (HR) has been suggested, the "*AtLSD1 - deathosome*". The protein-coding gene containing the PLAC8 (Placenta-specific 8) domain, At1g52200 (*AtPLAC8-11*), has been described as a component of the "*AtLSD1-deathosome*". The present work aims at the functional study of the *AtPLAC8-11* gene, especially in relation to PCD. For this, studies of subcellular localization of *AtPLAC8-11* gene products: AtPLAC8-11.1 (canonical transcript) and AtPLAC8-11.2 (alternative transcript) in protoplasts were carried out and a diverse profile of protein localization was verified in both variants of according to C- or N-terminal fusion with Green Fluorescent Protein (GFP). However, co-localization with the endoplasmic reticulum marker was possible for both forms. Tissue-specific expression of AtPLAC811.1 and AtPLAC811.2 under native promoter control in *Arabidopsis* was observed in root and leaf, with different subcellular localization in these tissues, as in the trichome bases only for AtPLAC811.1. Analysis of the expression of AtPLAC811.1 and AtPLAC811.2 under native promoter control in different parts of the flower showed that AtPLAC811.1 is highly expressed in sepals and stamens, compared to AtPLAC811.2. Treatment with the resistance inducer or Pep1 elicitor increased the expression of both AtPLAC811.1 and AtPLAC811.2 under the control of the native promoter in the roots. The Yeast Two-Hybrid (YH2) assay revealed that only AtPLAC811.1 interacts with the AtLSD1 protein and through the Bimolecular Fluorescence Complementation (BiFC) assay, Yellow Fluorescent Protein (YFP) reconstitution was observed in undefined shape at locations in the cell. The transactivation experiments showed that the interaction of AtPLAC811.1 with AtLSD1 is capable of interfering with the transcriptional activity of AtLSD1. The p*lac8-11knockout* plants alter the expression of genes

related to developmental (dPCD) and environmental (ePCD) PCD. A promiscuous TurboID biotin ligase method was used to investigate the AtPLAC811.1 and AtPLAC811.2 interactors, resulting in the identification of different interacting proteins, including proteins involved in stress response for both variants. In this sense, the results obtained indicate the involvement of *AtPLAC8-11* in the PCD process in *Arabidopsis*.

1. INTRODUÇÃO

1.1 Estresses Ambientais: estresses bióticos e abióticos

As plantas são organismos sésseis que estão continuamente expostas ao seu ambiente, e, portanto, sofrem com as condições ambientais adversas, denominadas como estresses ambientais abióticos e bióticos (CRAMER et al., 2011; MISHRA et al., 2018). Os estresses abióticos são os principais responsáveis pela perda das principais plantas cultivadas em todo o mundo e incluem seca ou alagamento, baixa ou alta temperatura, toxicidade por metais pesados, elevada salinidade do solo e condições ácidas, intensidade luminosa, anaerobiose e carência de nutrientes (HE; HE; DING, 2018a; WANG; VINOCUR; ALTMAN, 2003; ZHANG et al., 2022a). Por outro lado, os estresses bióticos nas culturas ocorrem devido ao ataque de organismos vivos, incluindo a infecção por patógenos como fungos, bactérias, vírus, oomicetos e nematoides e ataque de herbívoros, causando prejuízos financeiros às culturas quando os limites para a ocorrência do estresse são ultrapassados (ATKINSON; URWIN, 2012; SUZUKI et al., 2014).

As plantas desenvolveram ao longo da evolução diferentes mecanismos de proteção que as permitem lidar com uma ampla gama de estresses bióticos e abióticos. Esses mecanismos incluem mudanças morfológicas, fisiológicas e bioquímicas durante os diferentes estágios de crescimento, tanto vegetativo como reprodutivo, possibilitando a sobrevivência frente a condições adversas (FUJITA et al., 2006a; HRMOVA; HUSSAIN, 2021; SIGNORELLI et al., 2019). Em condições de seca, por exemplo, as plantas promovem o fechamento estomático, enrolamento das folhas, indução de enzimas responsivas ao estresse, indução da síntese de osmólitos, diminuição do potencial hídrico, diminuição da fotossíntese e a inibição do transporte de água (MEENA et al., 2017). Ao lidarem com o calor excessivo, as plantas induzem a aclimatação, a síntese de proteínas de choque térmico e ativam os mecanismos de reparo de proteínas (UL HAQ et al., 2019). Quando expostas a metais pesados, ocorre a geração de espécies reativas de oxigênio (*Reactive Oxygen Species* - ROS) e deposição do excesso de metal em vacúolos, através da bioacumulação (SHARMA; DIETZ; MIMURA, 2016).

As respostas bioquímicas das plantas frente aos diversos estresses ambientais ocorrem a partir da transdução dos estímulos recebidos dos sensores presentes na superfície celular ou citoplasma, para a maquinaria transcricional localizada no núcleo, com o auxílio das vias de transdução de sinal (GULL; AHMAD LONE; UL ISLAM WANI, 2019). A Figura 1 ilustra as interações (*crosstalk*) das vias de sinalização entre os principais estresses abióticos e bióticos, trazendo como principais moléculas sinalizadoras os fitohormônios vegetais como o ácido salicílico (SA), ácido jasmônico (JA), etileno (ET) e ácido abscísico (ABA), além da geração de ROS. Após o estresse, as moléculas sinalizadoras como os fitohormônios, poliaminas (PAs), proteína quinase ativada por mitógeno (MAPK) e íons de cálcio (Ca^{2+}), promovem a mobilização dos efetores a jusante, principalmente proteínas quinases e fatores de transcrição, acarretando na alteração da expressão gênica e das atividades protéicas e enzimáticas, iniciando os sistemas de defesa (HE; HE; DING, 2018b; LAMERS; VAN DER MEER; TESTERINK, 2020).

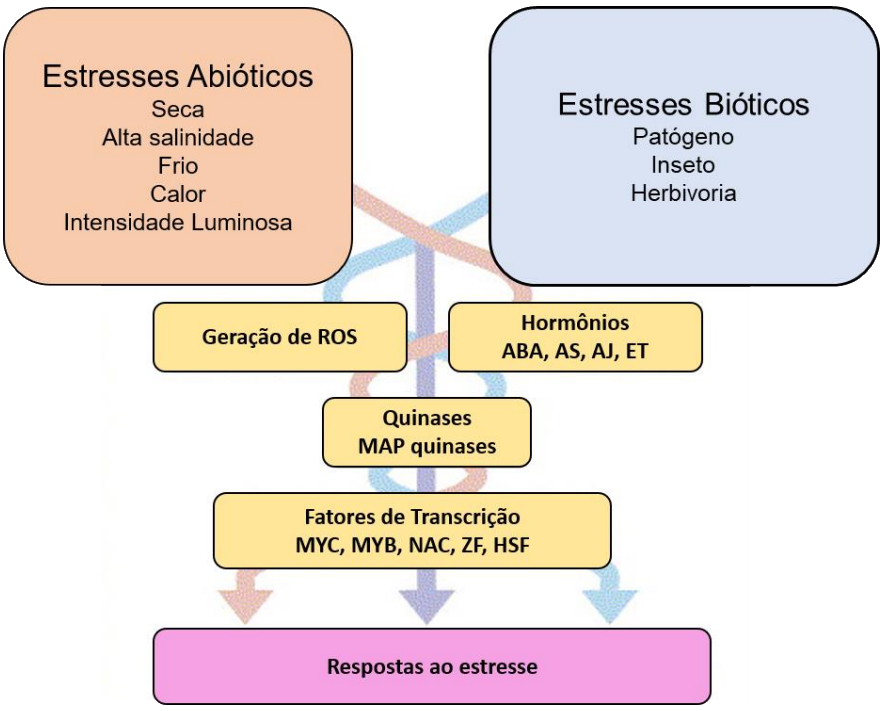

Figura 1: Vias de sinalização compartilhada entre os estresses ambientais. *Crosstalk* entre

a via de sinalização hormonal regulada por hormônios vegetais como o ácido salicílico (AS), ácido jasmônico (AJ), etileno (ET) e ácido abscísico (ABA) e a via sinalização de ROS nas respostas aos estresses ambientais. Adaptado de (FUJITA et al., 2006b).

Além dos mecanismos citados anteriormente, as respostas das plantas aos estresses bióticos envolvem mecanismos moleculares relacionados a resistência, tolerância, suscetibilidade e sensibilidade (CHACÓN-CERDAS et al., 2020). A morte celular programada (*Programmed Cell Death* - PCD) é um processo geneticamente controlado que leva à destruição celular organizada e pode atuar como parte das respostas de defesa contra os estresses ambientais abióticos e bióticos (BURKE et al., 2020; DANEVA et al., 2016a; WITUSZYNSKA; KARPINSKI, 2013a). Um exemplo clássico da PCD frente a estresses ambientais é a chamada resposta de hipersensibilidade (*Hypersensitive response* - HR), caracterizada por ser um tipo de morte celular utilizada como mecanismo de defesa frente ao ataque de patógenos (GREENBERG, 1997; LAM; KATO; LAWTON, 2001a).

Os estresses ambientais são considerados fatores limitantes no crescimento vegetal e na produtividade agrícola (COHEN; LEACH, 2019). Projeções globais envolvendo estudos de cenários quantitativos de segurança alimentar indicam que a demanda global total de alimentos deverá aumentar de 35% a 56% entre 2010 e 2050, podendo haver mudanças nesses intervalos com a inserção do fator mudanças climáticas, com um aumento de +30% a +62% para a demanda total de alimentos (VAN DIJK et al., 2021). Nesse sentido, o estudo dos mecanismos envolvidos nas respostas das plantas frente a esses estresses, bem como a descoberta e o desenvolvimento de variedades de culturas tolerantes a diversos estresses ambientais são de extrema importância para garantir a segurança alimentar global (DHANKHER; FOYER, 2018).

1.2 Morte Celular Programada (PCD) em Plantas: PCD do desenvolvimento e PCD ambiental

O processo de PCD se caracteriza como um mecanismo controlado para eliminar células específicas, ocorrendo em todos os organismos vivos (LOCATO; DE GARA, 2018). Em

plantas, distintos tipos de PCD executam papéis fundamentais de acordo com o estímulo recebido: a resposta a estímulos externos, como os estresses ambientais, se define como PCD ambiental (*Environmental* - ePCD), e a resposta a estímulos internos, como PCD relacionada ao desenvolvimento vegetativo e reprodutivo (*Developmental* PCD – dPCD) (LAM, 2004; OLVERA-CARRILLO et al., 2015). Como exemplos dos principais sistemas modelos bem estabelecidos e caracterizados para os diferentes tipos de PCD em plantas, pode-se citar as respostas de hipersensibilidade, observadas durante as interações planta-patógeno e o desenvolvimento de elementos traqueais no xilema de plantas vasculares, cruciais para o transporte de solutos e água (FUKUDA, 2000; LAM; KATO; LAWTON, 2001b).

Várias formas de PCD são executadas durante o desenvolvimento da planta por diversos tipos de células em função de seus programas de diferenciação (VAN DURME; NOWACK, 2016). Após o processo germinativo, na qual a PCD atua no desenvolvimento de sementes e embriões com morte celular no endosperma, no embrião e suspensor, a PCD prossegue com suas importantes funções durante o crescimento e o desenvolvimento vegetativo da planta (ROGERS, 2005). A PCD passa a atuar em processos envolvendo a formação de sistemas de transporte, como a xilogênese, no controle do tamanho do órgão, diferenciação de elementos traqueais, formação de aerênquima, regulação da morfogênese da folha, abscisão e deiscência de órgãos (DANEVA et al., 2016b).

O xilema caracteriza-se por ser uma via simples, de baixa resistência e de grande eficiência no transporte de água em plantas vasculares (BOLLHONER; PRESTELE; TUOMINEN, 2012). As células meristemáticas procambiais estabelecem o tecido vascular do xilema condutor de água no decorrer do crescimento primário, enquanto que o câmbio vascular, irá se desenvolver durante o crescimento secundário com base no tecido procâmbio remanescente (DEVILLARD; WALTER, 2014; FOSKET, 1994). As células procambiais e cambiais são capazes de se diferenciar em três tipos de células do xilema: fibras alongadas com paredes celulares espessas fornecendo suporte mecânico ao tecido, células do parênquima que atuam no armazenamento de alimentos, e os elementos traqueais (*Tracheary Elements* - TEs) encarregados do fluxo de massa de água (SCHUETZ; SMITH; ELLIS, 2013). Os TEs, traqueídes e os elementos de vaso, são formados a partir de elementos traqueais mortos, sendo

seu processo de diferenciação um exemplo representativo de PCD em plantas superiores (ESCAMEZ; TUOMINEN, 2014). O processo de diferenciação dos TEs inclui: diferenciação precoce na zona cambial, rápida expansão celular, formação de parede secundária, alterações na permeabilidade do tonoplasto e ruptura vacuolar, degradação do DNA, indução de genes relacionados à autólise, e hidrólise parcial das paredes celulares primárias não lignificadas. Assim sendo, os TEs maduros perdem seus núcleos e conteúdo celular, formando células mortas ocas através das quais são transportados água e nutrientes (FUKUDA, 1997; TURNER; GALLOIS; BROWN, 2007).

Outro exemplo do papel da PCD no desenvolvimento vegetal refere-se ao processo de abscisão. A abscisão é um estágio natural do desenvolvimento da planta que ocorre especificamente no tecido da zona de abscisão (*Abscission Zone* - AZ) (ROBERTS; ELLIOTT; GONZALEZ-CARRANZA, 2002). Vários órgãos como folhas, flores, frutos são separados da planta mãe através da abscisão de uma maneira altamente regulada, de modo a permitir que as plantas percam órgãos envelhecidos, maduros ou doentes (GOREN, 2007; NAKANO et al., 2013). Marcas características da PCD como a perda de viabilidade celular, alteração da morfologia nuclear, DNA fragmentado, elevados níveis de ROS, aumento da atividade enzimática e indução de genes associados a PCD foram identificadas em folhas e flores de tomate, especialmente nos estágios terminais do processo de abscisão, quando ocorre a separação celular (BAR-DROR et al., 2011).

Além dos processos de desenvolvimento da planta, a PCD atua também na resposta aos estímulos abióticos (WITUSZYNSKA; KARPINSKI, 2013b). Um dos exemplos de fatores abióticos que desencadeiam a PCD é a hipóxia, condição em que não há suprimento de oxigênio, comum em situações de alagamento. Em resposta a hipóxia, a PCD em plantas promove a formação de aerênquima e a degradação de células do parênquima para facilitar as trocas gasosas (DREW; HE; MORGAN, 2000; NI et al., 2019). O déficit hídrico em *Arabidopsis* induz a PCD na ponta primária da raiz e possui como características morfológicas o aparecimento de várias vesículas dentro do espaço vacuolar, resultando no aumento no tamanho do vacúolo, condensação da cromatina, degradação de organelas, colapso do tonoplasto e da membrana plasmática. A modificação da arquitetura do sistema radicular mediada pela PCD da ponta da

raiz primária promove a formação de raízes laterais e adventícias como mecanismo adaptativo ao estresse hídrico severo (DUAN et al., 2010). As plantas, por muitas vezes, são expostas a intensidades de luz que ultrapassam as necessidades da fotossíntese (ORT, 2001). Essa absorção excessiva de energia luminosa nos cloroplastos provoca a produção de ROS que desencadeia diversas vias de sinalização, levando ao processo de PCD nas folhas (SHUMBE et al., 2016). As plantas sacrificam suas folhas velhas/maduras quando elas estão fotodanificadas de maneira a recuperar nutrientes importantes para a planta ao invés de investir em reparo. Além disso, as condições foto-oxidativas excessivas causam danos nas membranas celulares levando à perda de água. Nesse sentido, a PCD pode atuar de maneira a evitar perdas dramáticas de água de folhas fotodanificadas para conservar o conteúdo hídrico. Por fim, os tecidos danificados ficam mais expostos ao ataque de patógenos e, portanto, a PCD nas folhas pode auxiliar na sobrevivência das plantas (D'ALESSANDRO; BEAUGELIN; HAVAUX, 2020).

Outro papel da PCD em plantas frente a condições de estresse ambiental é a resposta de hipersensibilidade, caracterizada como um tipo específico e único de morte celular que atua como um importante mecanismo de defesa local e sistêmica contra o ataque de patógenos, visando limitar a progressão do mesmo (BALINT-KURTI, 2019; COLL; EPPLE; DANGL, 2011). Uma ampla gama de patógenos pode desencadear a HR após poucas horas da infecção, sendo a resposta hipersensível normalmente condicionada pela existência de um gene de avirulência (*avr*) no patógeno, cujo produto será reconhecido por uma planta que possui o gene de resistência (*R*) compatível (KOURELIS; VAN DER HOORN, 2018). Algumas moléculas do patógenos chamadas de elicitoras também são capazes de desencadear a HR. Posteriormente ao reconhecimento do patógeno, as plantas passam por vários tipos de sinais bioquímicos e celulares, tal como pelas respostas de defesa, que incluem fluxo iônico, ativação de cascatas de quinase, geração de ROS, variações nos níveis dos hormônios vegetais, síntese de metabólitos secundários antimicrobianos, síntese de proteínas e reprogramação transcricional (HEATH, 2000; JONES; DANGL, 2006; MOREL; DANGL, 1997; ZURBRIGGEN; CARRILLO; HAJIREZAEI, 2010).

Em plantas, foi proposto pela primeira vez o termo AtLSD1 morteossomo (*Lesion Simulating Disease1 deathosome*), apresentando uma rede regulatória da PCD mediada por HR

em *Arabidopsis thaliana* (COLL; EPPLE; DANGL, 2011). Através dos resultados obtidos pelo emprego da técnica duplo-híbrido em levedura (*Yeast two-hybrid* - Y2H) foi apresentado um diagrama representando as interações entre os principais reguladores de morte celular conhecidos, como AtLSD1 (At4g20380) e a Metacaspase-1 (At1g02170) com seus parceiros de interação. O número de publicações sobre genes relacionados à PCD vem aumentando nos últimos dez anos e importantes famílias gênicas vêm sendo descritas como reguladoras do processo de morte celular (VALANDRO et al., 2020).

1.3 Família PLAC8

Os genes da família PLAC8 (*Placenta-specific genes* - PLAC) codificam proteínas com regiões ricas em cisteína: CXXXXCPC ou CLXXXXCPC, que se mantiveram conservadas ao longo da evolução (SONG et al., 2011). As proteínas contendo o domínio PLAC8, originalmente identificadas na camada de espongiotrofoblasto da placenta de mamíferos, constituem uma grande família e seus membros são encontrados em uma ampla gama de organismos eucarióticos, como fungos, algas, plantas superiores e mamíferos (CABREIRA-CAGLIARI et al., 2018; GALAVIZ-HERNANDEZ et al., 2003; LIBAULT; STACEY, 2010). As proteínas PLAC8 desempenham importantes papéis no controle do ciclo celular em animais; regulação da evolução tumoral e da maquinaria autofágica em humanos; resistência ao cádmio (Cd), detoxificação de Zinco (Zn) e no influxo de íons cálcio (Ca^{2+}) em plantas; e resistência ao cádmio (Cd) em fungos (ABBÀ et al., 2011; KINSEY et al., 2014; ROGULSKI et al., 2005; SONG et al., 2004).

Devido à desorganização na nomenclatura para denominar os genes pertencentes a família PLAC8, como Onzin and Cornifelin para a proteína PLAC8 humana e PCR (*Plant Cadmium Resistance* - PCR) para proteínas PLAC8 de plantas, uma nova classificação e nomenclatura foi proposta (CABREIRA-CAGLIARI et al., 2018; MICHIBATA et al., 2004; SONG et al., 2004). Os genes PLAC8 configuram uma única família gênica e são divididos em três tipos: os genes do tipo I são encontrados em mamíferos, fungos, plantas e algas, enquanto os tipos II e III são exclusivos de plantas. Referente à caracterização funcional dos genes

PLAC8, os do tipo I são os mais bem descritos atualmente, enquanto que os genes do tipo III ainda não foram caracterizados funcionalmente (CABREIRA-CAGLIARI et al., 2018). A figura 2 ilustra as principais funções da família PLAC8 em plantas e animais, bem como a separação da família nos tipos I, II e III de acordo com o organismo em questão.

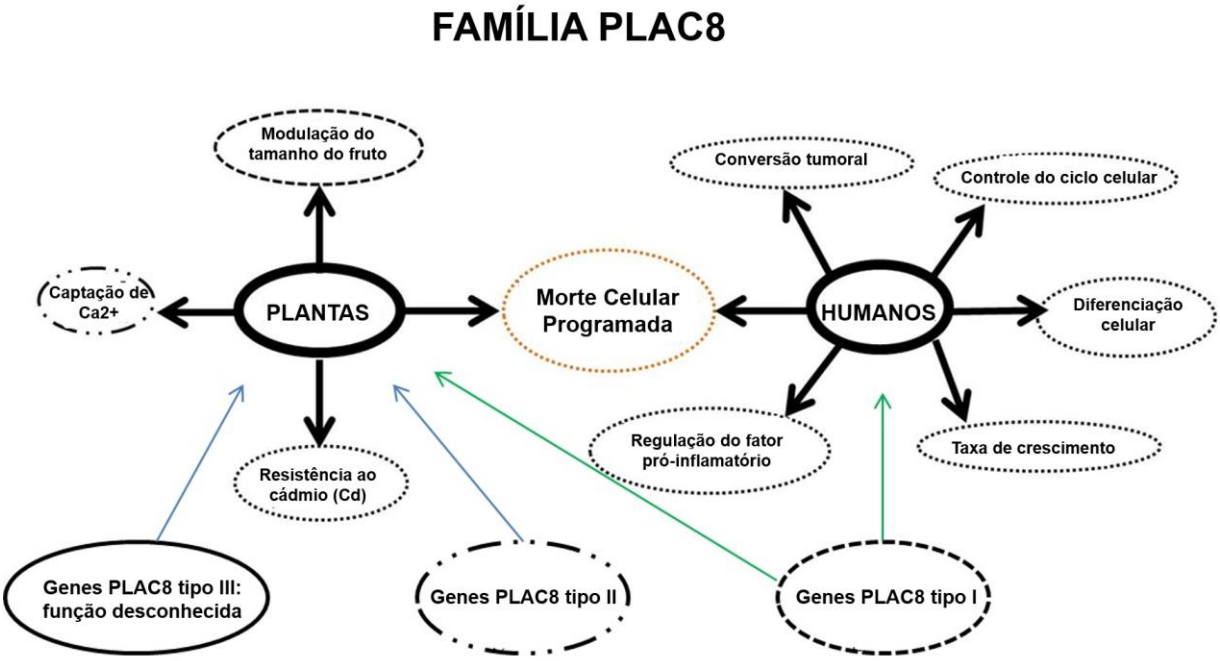

Figura 2: Principais funções da família gênica PLAC8 em plantas e humanos. Os círculos pontilhados retratam as funções dos genes PLAC8 do Tipo I, enquanto os círculos pontilhados duas vezes mostram a função encontrada nos genes do Tipo II. Os genes do tipo III possuem função desconhecida até o momento. A seta azul refere-se aos genes PLAC8 exclusivos de plantas e a seta verde assinala os genes PLAC8 observados em ambos os organismos. Figura adaptada de (CABREIRA-CAGLIARI et al., 2018).

As proteínas PLAC8 possivelmente evoluíram a partir de uma proteína ancestral comum e, ainda que desempenhem variadas funções nos diferentes organismos e tipos de células em que são expressas, uma função molecular comum nos sistemas biológicos provavelmente possa ser atribuída ao domínio PLAC8. Como exemplo disso, a levedura *Saccharomyces cerevisiae*

foi utilizada como organismo modelo para o estudo da função do domínio PLAC8 em dois organismos taxonomicamente distantes: mamíferos e fungos. Para isso, as proteínas PLAC8 de mamíferos e fungos, representada respectivamente por Onzin murino e FCR1 (*Fungal Cadmium Resistance* - FCR) fúngico foram expressas na levedura *S. cerevisiae*, que não possui genes codificantes das proteínas PLAC8. Após exposição a cádmio, ambas proteínas PLAC8 conferiram maior crescimento e viabilidade celular em comparação com as leveduras que não expressam esses genes. Assim como a PLAC8 fúngica, Onzin também foi capaz de reduzir o dano ao DNA e aumentar a tolerância ao cádmio por uma via dependente de DUN1 (*DNA-damage uninducible* – DUN1). Além disso, a expressão de ambas proteínas PLAC8 em *S. cerevisiae* foi capaz de ativar vias mitocondriais caracterizadas por regular a proliferação celular e o reparo de DNA em leveduras, sugerindo uma função ancestral comum das proteínas contendo domínio PLAC8 (DAGHINO et al., 2019).

Em *A. thaliana* são encontrados dezessete membros da família PLAC8. Entre esses, dois membros, com função desconhecida, estão presentes no morteossomo indicando o potencial desta família gênica no processo de PCD vegetal (COLL; EPPLE; DANGL, 2011). De acordo com as interações indicadas no morteossomo, o gene AtPlac811 (At1g52200) interage diretamente com AtLSD1 enquanto AtPlac817 (At4g23470) interage com Metacaspase-1 e AtLSD1 (CABREIRA-CAGLIARI et al., 2018; COLL; EPPLE; DANGL, 2011). Em *Arabidopsis thaliana*, os eventos de polinização que iniciam com a adesão do pólen à superfície do estigma culminando com a germinação e alongamento do tubo polínico, ocorrem nas células da papila (MATSUDA et al., 2015). Através de análises de transcritoma, AtPlac811 mostrou estar envolvido na PCD das células da papila, tendo expressão aumentada à medida em que as células da papilla envelhecem em direção a senescência (YE et al., 2020). Efeito oposto foi observado em plantas superexpressando CLE42 (*CLAVATA3/Embryo Surrounding Region-Related* - CLE), um regulador negativo da senescência em *Arabidopsis*, em que AtPLAC811.1 é regulado negativamente (ZHANG et al., 2022b).

1.4 Explorando a função gênica por estudos de Interações Proteína-Proteína (PPIs)

A proteômica pode ser definida como a análise de todo o conjunto proteico e suas

isoformas presentes em uma célula, tecido, biofluido ou organismo sob um conjunto específico e definido de condições (YU; STEWART; VEENSTRA, 2010). No que se refere aos mecanismos intrínsecos das respostas vegetais aos estresses ambientais, a proteômica oferece umas das melhores opções para se obter informações detalhadas de tal processo (KOMATSU, 2019). No trabalho de Khoza et al., 2019, experimentos utilizando técnicas de proteômica como cromatografia líquida acoplada com espectrometria de massa em tandem (*Liquid chromatography coupled with tandem mass spectrometry* - LC-MS/MS), permitiram a identificação de proteínas relacionadas às defesas das plantas, bem como os complexos de interações que são formados. Outro estudo utilizou uma abordagem proteômica baseada em espectrometria de massa quantitativa emergente chamada aquisição independente de dados (*Data-independent acquisition* - DIA). Essa técnica foi empregada para rastrear quantitativamente a mudança no proteoma do tomate (*Solanum lycopersicum*) durante as respostas da patogênese, incluindo o estágio inicial até o final da progressão da patogênese (FAN et al., 2019). Na cana-de-açúcar (*Saccharum officinarum*), um estudo investigou as mudanças no proteoma que ocorrem em duas variedades de cana-de-açúcar com suscetibilidade contrastante após a infecção por *S. scitamineum* visando identificar genes críticos para a resistência a doença denominada carvão (SINGH et al., 2019).

As proteínas não costumam agir sozinhas e se unem formando complexas conexões físico-químicas para desempenhar suas funções biológicas (DE LAS RIVAS; FONTANILLO, 2010a). Nesse sentindo, o mapeamento das interações proteína-proteína (*Protein–protein interactions* - PPIs) contribuem trazendo importantes informações sobre a regulação dos processos relacionados ao desenvolvimento das plantas e sobre as interações das plantas com seu meio ambiente. A rede de interações de proteínas em um organismo vivo é denominada interatoma e vem sendo considerada como poderosa ferramenta para descobrir funções desconhecidas de proteínas ao nível molecular, bem como para adquirir informações sobre complexas redes celulares (STRUK et al., 2019a; XING et al., 2016).

1.4.1 Bancos de dados de interação proteína-proteína

Após anos de pesquisa e estudos evolvendo diversas áreas da biologia como bioquímica,

biologia estrutural/celular/molecular, etc., foi possível a criação de bancos de dados completos e confiáveis para se obter informações sobre a função das diferentes proteínas e seus complexos, sendo uma importante ferramenta para o estudo das funções de inúmeros genes (SHOEMAKER; PANCHENKO, 2007). A tabela 1 a seguir descreve alguns dos principais bancos de dados de interação proteína-proteína.

Tabela 1: Bancos de dados de interação proteína-proteína.

Nome	URL	Espécie	Descrição	Referência
STRING	http://string.embl.de/	Todas	Disponibiliza informações de interação proteína-proteína, incluindo associações diretas (físicas) e indiretas (funcionais).	(SZKLARCZYK et al., 2019)
BioGRID	http://www.thebiogrid.org/	Todas	Banco de dados de interações protéicas, genéticas e químicas das principais espécies de organismos modelo.	(STARK, 2006)
IntAct	http://www.ebi.ac.uk/intact/	Todas	Disponibiliza ferramentas de análise de dados	(ORCHARD et al., 2014)

			de interação molecular.	
DIP	http://dip.doe-mbi.ucla.edu/dip/	Todas	Cataloga pares de proteínas que interagirem entre si.	(XENARIOS, 2000)

Fonte: Adaptado de (DE LAS RIVAS; FONTANILLO, 2010b).

1.4.2 Metodologias para estudo de interações proteína-proteína

Os experimentos para o estudo das interações entre proteínas são realizados em grande ou pequena escala, utilizando-se de duas principais tecnologias que geram distintos tipos de dados de PPI. O primeiro método é denominado como binário e é utilizado para medir interações físicas diretas entre os pares de proteínas, ao passo que, os métodos que medem interações físicas entre grupos de proteínas são denominados de métodos co-complexos (DE LAS RIVAS; FONTANILLO, 2010b). Dentre os métodos binários, o mais utilizando é o duplo-híbrido em levedura (*Yeast two-hybrid* - Y2H), e referente a metologia co-complexa, um dos métodos mais utilizados é a purificação por afinidade em combinação com espectrometria de massa (*Affinity purification mass spectrometry* - AP-MS) (BERGGÅRD; LINSE; JAMES, 2007; BRÜCKNER et al., 2009; YU et al., 2008). O sistema duplo-híbrido em levedura foi uma das primeiras metodologias desenvolvidas para o estudo de possíveis interações entre proteínas, sendo extensivamente utilizado na pesquisa em plantas (STRUK et al., 2019b). O método possibilita a detecção de proteínas que interagem utilizando o fator de transcrição Gal4 na levedura *Saccharomyces cerevisae* (FIELDS; SONG, 1989). Para tal, proteínas de interesse são fusionadas separadamente formando diferente híbridos. O híbrido 1 é formado pela fusão da proteína conhecida, chamada de isca, com o domínio de ligação ao DNA (*DNA-binding domain* - BD), à medida que no híbrido 2 ocorre a fusão da proteína de interesse, denominada de presa, com o domínio de ativação (*Activation domain* - AD) do fator de transcrição GAL4. Se houver interação entre as proteínas que

estão sendo testadas, ocorre a reconstrução de GAL4, seguido pela transcrição de um gene repórter, possibilitando a visualização da interação protéica, via crescimento da levedura em meios específicos ou através de reação de cor, dependendo do gene repórter utilizado (FIELDS; SONG, 1989; SEREBRIISKII et al., 2021). O sistema Y2H pode ser modificado e utilizado para os estudos de alto rendimento de interações de proteínas em uma grande escala genômica (BRÜCKNER et al., 2009). A purificação por afinidade combinada com espectrometria de massa depende da expressão de uma proteína isca marcada por afinidade a uma matriz de afinidade sólida (TIAN et al., 2017). A isca e determinada amostra biológica são misturadas para que ocorra a interação das proteínas da presa e consequente ligação à isca. Não ocorrendo a interação, não ocorre a ligação das proteínas à isca, sendo as mesmas lavadas da amostra. Quando a proteína isca (marcada) interage com seus parceiros de ligação (presas) se forma o complexo protéico que pode ser purificado a partir do lisado celular usando a matriz que reconhece especificamente o marcador de afinidade (DUNHAM; MULLIN; GINGRAS, 2012; GINGRAS et al., 2007; OEFFINGER, 2012). O complexo formado é então digerido enzimaticamente e posteriormente, a espectrometria de massas é empregada de maneira a identificar e quantificar as proteínas que foram purificadas por afinidade (CHOWDHURY et al., 2020). Dentre as principais vantagens dessa metodologia, tem-se que a mesma pode ser aplicada a estudos em grande escala além de fornecer informações quantitativas (MEYER; SELBACH, 2015; NESVIZHSKII, 2012).

Além dos métodos acima descritos, a tabela 2 abaixo lista e compara outras importantes técnicas de PPI que são utilizadas no estudo das funções protéicas.

Table 2: Principais técnicas para detecção e validação de PPI.

Método	**Aplicação**	**Ensaio**	**Propriedades de Leitura**	**Modo de interação**
Y2H	Detecção/Validação	*In vivo*	Ativação transcricional do gene	Binário

			repórter	
Microarranjo de proteínas	Detecção	*In vitro*	Marcação ou sem marcação	Binário
AP-MS (Purificação por afinidade acoplada a espectrometria de massa)	Detecção	*In vitro*	Espectrometria de massa	Co-complexo
Bio-ID (Identificação por biotinilação)	Detecção	*In vitro*	Espectrometria de massa	Co-complexo
BiFC (Complementação de fluorescência bimolecular)	Validação	*In vivo*	Fluorescência	Binário
Split-Luciferase	Validação	*In vivo*	Luminescência	Binário
Co-IP (co-imunoprecipitação)	Validação	*In vitro*	Western blot	Co-complexo

Fonte: Adaptado de (STRUK et al., 2019c).

2 OBJETIVOS

2.1 Objetivo geral

Ao contrário dos animais, o conhecimento sobre morte celular programada (*Programmed Cell Death* - PCD) em plantas é limitado e as redes moleculares que controlam a PCD vegetal são pouco compreendidas. Nesse sentido, a presente tese de doutorado tem por objetivo atualizar a rede de controle da PCD denominada como morteossomo, e através de estudos funcionais, caracterizar funcionalmente o gene *AtPLAC8-11.1* utilizando a planta modelo *Arabidopsis thaliana*.

2.2 Objetivos específicos

Os objetivos específicos são:

a) Através de pesquisas na literatura analisar e descrever as principais famílias gênicas envolvidas no processo de morte celular programada em plantas;

b) Caracterizar funcionalmente o gene *AtPLAC8-11.1* e o produto de seu splicing alternativo *AtPLAC8-11.2;*

c) Verificar a localização subcelular de AtPLAC8-11.1 e AtPLAC8-11.2 em *Arabidopsis thaliana*;

d) Realizar análises fenotípicas das plantas mutantes *plac8-11* cultivadas sob condições normais de crescimento e frente a diferentes estresses abióticos;

e) Identificar parceiros de interação para AtPLAC811.1 e AtPLAC811.2 através de experimento de interação proteína-proteína utilizando o sistema TurboID-marcação de proximidade.

3 RESULTADOS

Os resultados desta tese serão apresentados em três capítulos, abrangendo os objetivos específicos descritos anteriormente:

Capítulo I - Controle da morte celular programada (PCD) em plantas: Novas percepções do deathosome de *Arabidopsis thaliana*. Artigo científico publicado na revista Plant Science.

Capítulo II - Caracterização molecular e funcional do gene At1g52200 (*AtPLAC8-11.1*) envolvido na morte celular programada. Capítulo redigido em formato de artigo científico.

Capítulo III – Emprego do método TurboID-marcação de proximidade para a busca dos interatores de AtPLAC8-11.1 e AtPLAC8-11.2. Capítulo referente aos resultados do Doutorado Sanduíche.

Capítulo I

Controle da morte celular programada (PCD) em plantas: Novas percepções do deathosome de *Arabidopsis thaliana*

Este capítulo é referente ao artigo “Programmed cell death (PCD) control in plants: “New insights from the *Arabidopsis thaliana* deathosome”, publicado em 2020 na revista Plant Science. Nesse trabalho apresentamos uma atualização do chamado “AtLSD1 - deathosome”, proposto anteriormente por Coll, Epple e Dangl (2011) como uma rede composta por vários genes em *Arabidopsis* relacionados ao controle da morte celular mediada pela Resposta de Hipersensibilidade (HR) em plantas.

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Review article

Programmed cell death (PCD) control in plants: New insights from the *Arabidopsis thaliana* deathosome

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ABSTRACT

Programmed cell death (PCD) is a genetically controlled process that leads to cell suicide in both eukaryotic and prokaryotic organisms. In plants PCD occurs during development, defence response and when exposed to adverse conditions. PCD acts controlling the number of cells by eliminating damaged, old, or unnecessary cells to maintain cellular homeostasis. Unlike in animals, the knowledge about PCD in plants is limited. The molecular network that controls plant PCD is poorly understood. Here we present a review of the current mechanisms involved with the genetic control of PCD in plants. We also present an updated version of the AtLSD1 deathosome, which was previously proposed as a network controlling HR-mediated cell death in *Arabidopsis thaliana*. Finally, we discuss the unclear points and open questions related to the AtLSD1 deathosome.

1. Introduction

Plant development progresses in different phases: vegetative growth, reproduction phase, seed production, and senescence. All these events are governed by biochemical and genetic circuits resulting from endogenous alterations and in response to environmental stresses [1–3]. Eukaryotic and prokaryotic organisms have a fundamental biological cellular process known as programmed cell death (PCD) that controls cell suicide in a conserved and genetically regulated manner, causing the death of single cells, specific tissues, or whole organs [4–6]. PCD acts controlling the number of cells by eliminating damaged, old, or unnecessary cells to maintain cellular homeostasis. PCD in plants triggers the expression or activation of proteases, lipases, nucleases, and transporters, which act in relevant events such as signaling pathways, cell differentiation, nutrient mobilization, protein maturation, synthesis, and degradation of hormones [5,7–9].

PCD is an integral and fundamental component of the plant life cycle, occurring during its development (dPCD) and in response to the environment (ePCD). dPCD is present during different stages of cell differentiation, as well as occurring during cell aging. In this sense, dPCD encompasses reproductive and vegetative processes, such as differentiation of tracheal components, xylem formation, embryogenesis, pollen maturation, seed maturation, and leaf senescence [9–12]. As part of its defense mechanism, plant cells use ePCD to program plant cell death when exposed to different abiotic and biotic stress [4,13–15]. Due to the sessile condition of plants, PCD is extremely important, allowing plants to overcome adverse environmental conditions, and for defense responses to microbial pathogens and herbivorous insects [5,6,15–17].

Hypersensitive response (HR) is a form of PCD that occurs specifically under stress conditions linked to plant resistance [18,19]. HR is a type of cell death used as a defense mechanism triggered by several

Abbreviations: PCD, programmed cell death; dPCD, developmental programmed cell death; ePCD, environmental programmed cell death; HR, hypersensitive response; ROS, reactive oxygen species; TFs, transcriptional factors; Y2H, yeast-two-hybrid assay; LSD1, lesion simulated disease1; EDS1, enhanced disease susceptibility1; PAD4, Phytoalexin deficient4; H_2O_2, hydrogen peroxide; MC1, metacaspase1; CAT, catalase

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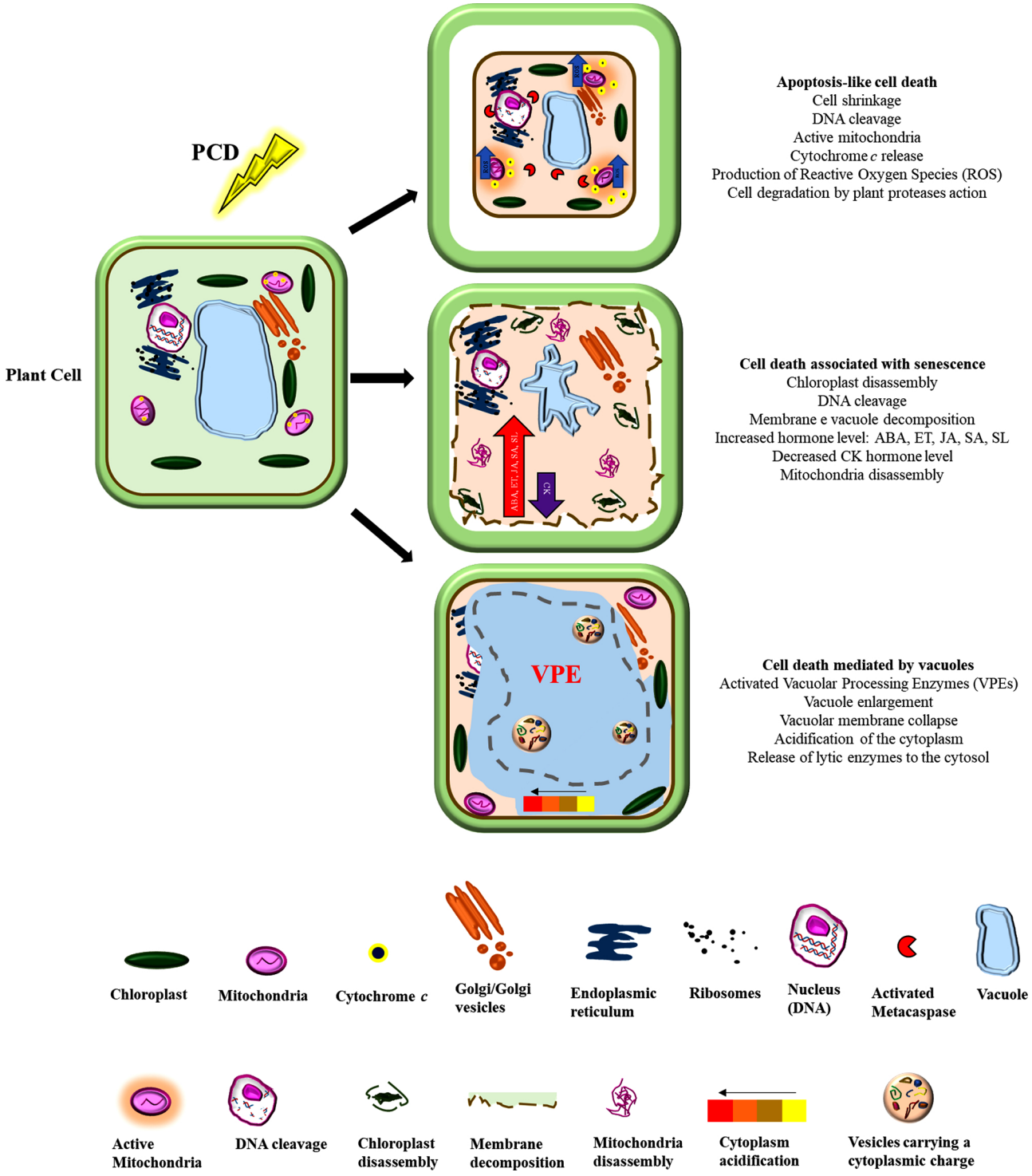

Fig. 1. Three types of Programmed Cell Death in plants. Apoptosis-like cell death involves features like cell shrinkage, maintaining the vacuolar and plasma membrane intact, DNA cleavage into smaller fragments, active mitochondria with cytochrome c release, and production of ROS ending with the cascade of event-mediated cell degradation by metacaspases [38–45]. In cell death associated with senescence, the first organelles to be disassembled are chloroplasts. In addition, it is also characterized by DNA cleavage, membrane, and vacuole decomposition, increased levels of some hormones (ABA, ET, JA, SA, SL), decreased cytokinin hormone level, and mitochondria disassembly [48,54,56–58]. When cell death is mediated by vacuoles occurs the activation of vacuolar Processing Enzymes (VPEs), enlargement and vacuolar membrane collapse, releasing of lytic enzymes to the cytosol having acidification of the cytoplasm [59–61,63].

pathogens, such as bacteria, fungi, insects, nematodes, and viruses [20]. In HR occurs the limitation of pathogen growth and confinement by rapid local death of plant tissues, which extend to the surroundings of the pathogen infection site [20–22]. After induction by abiotic elicitors and chemical inducers (salicylic acid and acetylsalicylic acid), the hypersensitive cell produces antimicrobial molecules such as phytoalexins and synthesizes pathogenesis-related proteins (PR) (chitinase, glucanase, defensin) [23–26]. HR is considered a specialized form of PCD which share symptomology similar to different types of PCD [19], including plant apoptosis-like cell death and vacuolar cell death features (Fig. 1). Notable hallmarks of apoptosis in HR response include membrane blebbing, chromatin condensation, genomic DNA fragmentation,

cell-wall alterations, ion fluxes modifications, and generation of reactive oxygen species (ROS), reactive oxygen intermediates (ROI), and nitric oxide (NO) [27–30]. Besides, HR presents features of vacuolar cell death with growth of lytic vacuoles and tonoplast rupture [31].

In contrast to the regulation of animal PCD, the molecular network that controls the distinct forms of plant PCD are poorly understood [13,32]. Coll et al. (2011) proposed a diagram depicting interactions between known cell death regulators in *A. thaliana* and their yeast-two-hybrid interacting partners. The called AtLSD1 deathosome highlights the known central regulator of HR-related PCD, LESION SIMULATING DISEASE1 (LSD1 - At4g20380), and its interaction partners. Here we firstly review the general aspects involved with PCD in plants. Then we further present an updated version of AtLSD1 deathosome [33], with the addition of interaction partners recently described in the literature []. We also describe the gene families involved with the regulation of the HR-related PCD in plants, using AtLSD1 as central component of this network. Finally, we discuss the unclear points and open questions about AtLSD1 deathosome in HR-mediated PCD.

2. General aspects of programmed cell death (PCD) in plants

Plant PCD classification is based on the similarities with cellular and biochemical markers of death in animal cells [5,11,34]. Therefore, plant PCD can be identified as (1) apoptosis-like cell death, (2) cell death associated with senescence, and (3) vacuolar cell death [35] (Fig. 1).

Plant apoptosis-like cell death (Fig. 1) counts with the presence of a rigid cell wall, and differ from animal apoptosis by the lack of apoptotic bodies, phagocytes, and macrophages [36,37]. The main characteristics resulting from the apoptotic process in plants are the condensation of the nucleus, DNA cleavage, cell shrinkage, cytochrome *c* release from mitochondria, production of reactive oxygen species (ROS) by mitochondria and chloroplast, and finally cell degradation linked to plant proteases activation [38–43]. Due to the onset of cell death, activation of caspase-like proteases (CLPs) occurs, resulting in the proteolytic activation of other plant cell death proteases (PCDPs) or transcriptional factors (TFs) involved in their expression. The processes proceed with the inactivation of endogenous proteinase inhibitors or in the immediate processing of proteins involved in orderly cell cleavage and degradation, which leads to cell death [44,45].

Senescence acts on the remobilization and recycling of accumulated nutrients in senescent organs to assist the growth of new vegetative and reproductive or storage organs, like seeds [46,47]. Some authors describe senescence (Fig. 1) as a slow form of PCD that represents the end of the development of plant organs, tissues, and cells [48–50]. However, senescence is also discussed as a fundamental cell differentiation process that occurs before PCD. This position is based on the fact that leaf yellowing can be reversible if the process was not too far ahead and the leaves can then greenish again. Also, the senescent process can result in cell death by PCD, but this is not mandatory [51,52]. Important hallmarks of PCD, such as DNA laddering and retracted protoplast were observed following the senescence process showing that the senescence and cell death are not synonymous but the senescence can trigger PCD [53].

Initially, the onset of the senescence process is caused by the perception and transduction of signals that trigger alterations in gene expression, affecting the levels of regulatory plant hormones, leading to increased concentration of senescence promoting hormones, such as abscisic acid (ABA), ethylene, jasmonic acid (JA), salicylic acid (SA) and strigolactones (SLs), and decreased cytokines that suppress the senescence process [48,54]. During early senescence, there is an increase in antioxidants compounds such as tocopherol, ascorbic acid (AsA), and glutathione [55]. There is also an increase in the level of other regulators such as sugars, calcium, and ROS that may act in the regulation of senescence-associated gene (SAGs) expression, allowing the progression of senescence [54,56,57]. Significant metabolic and structural changes occur during senescence, including chloroplast decomposition, chlorophyll degradation, reduction of photosynthetic activity and expression of genes associated with photosynthesis, and loss of cellular integrity. All these changes are associated with the remobilization of recovered nutrients. At the end of senescence, there is a decrease in antioxidant compounds, loss of cell viability, degradation of organelles, and cell death [54,57,58].

During vacuolar cell death (VCD) (Fig. 1), plants use their vacuoles and vacuolar lytic contents to gradually digest all or most all of their cell materials that are terminally differentiated [59,60]. Vacuolar processing enzymes (VPEs) are responsible for the maturation and activation of vacuolar proteins and the conversion of inactive hydrolytic enzymes to active forms, leading to vacuolar disruption and thus initiating the proteolytic cascade that culminates with vacuole-mediated cell death [61–63]. VCD occurs in all plant cells and tissues being involved not only in the immune response but also in plant development and response to stress inducers [64–66]. The main cytological features of VCD are vacuole enlargement by fusion of vesicles carrying a cytoplasmic content. Rupture of vacuolar membranes, releasing of lytic enzymes to the cytosol leading to intracellular acidification that attacks organelles, rapidly degrading residual cellular content [59,60,63].

3. HR-mediated cell death regulation: an update on the AtLSD1 deathosome

Information on the genetic, molecular, and physiological mechanisms involved in PCD in plants can be carried out through the identification of *A. thaliana* mutants that exhibit uncontrolled cell death [67]. One of the best-studied mutants, in terms of PCD, is *lsd1* [68]. Characterized by having uncontrolled cell death in contact with HR-eliciting bacteria, LSD1 has been useful in identifying genes with essential roles in signal transduction pathways for pathogen recognition, HR, and systemic acquired resistance (SAR) [69].

AtLSD1 deathosome was proposed in 2011 to describe genes related to PCD control in plants based on protein-interacting experiments, through yeast-two-hybrid assay (Y2H), revealing complementary putative LSD1 interaction partners in Arabidopsis [33]. AtLSD1-deathosome highlights the known central regulators of HR-related PCD in plants: METACASPASE-1 (At1g02170) and LESION SIMULATING DISEASE1 (LSD1 - At4g20380), which act antagonistically regulating PCD in plants [70–72].

In the original AtLSD1-deathsome, sixteen proteins belonging to eight main gene families (Metacaspase, LSD1, PLAC8, bZIP, MYB, NF-Y, AUX/IAA and PRP) were presented, some of them are well documented in plant PCD. The updated version of AtLSD1-deathsome, proposed in this work, brings ten new partners and adds important new gene families in HR-related PCD, such as EDS1/PAD4 and Catalase. The number of publications regarding proteins related to PCD has been increasing in the last ten years. So, we compiled in Table 1 some of the most relevant publications from gene families found in the updated AtLSD1-deathsome.

3.1. Lesion simulated disease (LSD1)

Efforts to understand genes regulating the PCD mechanism have resulted in the discovery of a mutant class that spontaneously showed lesion, even without pathogen infection. This mutant class presented histochemical and molecular markers of plant disease, hence the family name Lesion Simulating Disease (LSD) [68]. LSD genes encode a well-characterized class of zinc finger proteins composed by one, two, or three conserved zinc finger LSD domain CxxCRxxLMYxxGASxVxCxxC [73,74]. The consensus sequence of LSD domains is widely maintained], especially in the C2C2 arrangement, and is fundamental for protein-protein interactions [70,75–79]. In *A. thaliana*, three LSD genes are described: LSD1, LOL1 (LSD-like 1), and LOL2 (LSD-like 2) [71].

The involvement of the LSD family in several biological processes,

Table 1
Published articles of gene families related with PCD in plants.

Gene family	Article	Reference	Year
LSD1	It is the first report on the Arabidopsis mutant class *lsd* that spontaneously form necrotic lesions on leaves.	[68]	1994
	The Arabidopsis LSD1 is a zinc finger protein that monitors a superoxide-dependent signal and negatively regulates plant cell death pathway via either repression of a prodeath pathway or activation of an antideath pathway, in response to signals from cells suffering pathogen-induced hypersensitive cell death.	[73]	1997
	LSD1 regulates salicylic acid-dependent induction of copper-zinc superoxide dismutase responsible for detoxification of accumulating superoxide before it can trigger a cell death cascade in *Arabidopsis thaliana*.	[298]	1999
	EDS1 and PAD4 are essential regulators of reactive oxygen intermediates and SA-dependent signaling in a plant defense potentiation circuit controlled by LSD1 in Arabidopsis.	[84]	2001
	Broccoli florets express similar genes during detached (harvest-induced) senescence to those expressed in Arabidopsis leaves during senescence, in particular, the broccoli LSD1 and BI homologues are upregulated at 12 and 24 h, prior to DNA degradation and before the onset of visible senescence.	[299]	2004
	The Arabidopsis LSD1 prevents photooxidative damage controlling PAD4-, EDS1-, and SA-dependent stomatal closure and subsequent photorespiratory production of ROS.	[81]	2004
	OsLSD1, a rice zinc finger protein, plays a negative role in regulating plant PCD, whereas it plays a positive role in callus differentiation.	[300]	2005
	The Arabidopsis transcription factor bZIP10 shuttles between the nucleus and the cytoplasm and can be retained outside the nucleus by LSD1. *AtbZIP10* and *LSD1* act antagonistically in both pathogen-induced hypersensitive response and basal defense responses.	[78]	2006
	The formation of lysigenous aerenchyma in Arabidopsis depends on the plant defense regulators *LSD1*, *EDS1*, and *PAD4* that operate upstream of ethylene and reactive oxygen species production.	[301]	2007
	Molecular phylogeny, evolution, and functional divergence of the LSD1-like gene family: inference from the rice genome	[302]	2007
	The rice *OsLOL2*, a *LSD1*-related gene in rice, encodes a zinc finger protein involved in rice growth and disease resistance.	[303]	2007
	Overexpression of rice *OsLOL2* gene, a *LSD1*-related gene in rice, confers disease resistance to *Pseudomonas syringae* pv. *Tabaci in tobacco* through the induction of PR proteins and HR-like reaction.	[304]	2008
	LSD1, *EDS1* and *PAD4* constitute a ROS/ethylene homeostatic switch, controlling light acclimation and pathogen defense holistic responses. *LSD1* suppresses ROS production and photorespiration via direct or indirect regulation of SOD and CAT gene expression and activity. *PAD4*-and EDS1-dependent cellular ethylene production, together with *EIN2*, modulate ethylene-induced programmed cell death.	[82]	2008
	Arabidopsis LSD1 plays an important role in ROS responses to repress cell death under low temperature. Cell death phenotype requires *EDS1* and *PAD4*.	[83]	2010
	The LSD1-type zinc finger motifs of PsLSD1 are a novel nuclear localization signal and directly bind to importin α, suggesting that the nuclear import of LSD1 may rely on the interaction between its zinc finger motifs and importin α.	[76]	2011
	The bamboo *BohLOL1*, a homolog of Arabidopsis *LSD1* and *LOL1*, is upregulated during growth, which uniquely occurs in growing bamboo, and in response to biotic stress.	[305]	2011
	The Arabidopsis AtGILP functions as a plasma membrane anchor, bringing other regulators of PCD, such as AtLSD1, to the plasma membrane, and negatively regulate hypersensitive cell death.	[77]	2011
	The expression of the *Glycine max LSD* genes can be regulated by biotic (*Phakopsora pachyrhizi* infection) and abiotic (dehydration) stresses.	[75]	2013
	The wheat zinc finger protein TaLSD1 negatively regulates the plant hypersensitive cell death and is involved in disease resistance against the stripe rust pathogen.	[306]	2013
	LSD1 physically and genetically interacts with catalases in the light-dependent runaway cell death and hypersensitive responses cell death processes in Arabidopsis. The accumulation of SA is required for PCD regulated by LSD1 and catalases.	[79]	2013
	Apart from playing an important role in abiotic and biotic stress responses, LSD1, EDS1, and PAD4 also participate in the regulation of photosynthesis, transpiration, cellular signaling, and seed yield.	[80]	2013
	Gm-LSD1 − 2 is involved in the defense response of *Glycine max* to *Heterodera glycines* parasitism through the establishment of an environment whereby the protected, living plant cell could secrete materials in the vicinity of the parasitizing nematode to disarm it.	[307].	2015
	LSD1 and HY5 perform opposite roles to regulate excess red light (RL)-triggered PCD associated with ROS and SA production. LSD1 suppresses the *EDS1* expression by upregulation of *SR1*, whereas HY5 promotes the *EDS1* expression under RL.	[268]	2015
	LSD1 and EDS1 are antagonistic regulators of UV-C-induced PCD in *Arabidopsis thaliana*.	[147]	2015
	A rice LSD1-like-type ZFP gene OsLOL5 enhances saline-alkaline tolerance in transgenic *Arabidopsis thaliana*, yeast and rice	[308]	2016
	Arabidopsis and poplar PAD4, LSD1 and EDS1 constitute a molecular hub, which is able to regulate plant survival during drought stress, vegetative biomass production and generative development, cell division and cell death, as well as affect the cell wall structure and physical properties of wood in poplar.	[309]	2016
	LSD1 constitutes a condition-dependent molecular regulator of diverse cellular processes that balance between cell division and cell death pathways by acting as a redox-sensing scaffold protein and transcription regulator.	[67]	2017
	Molecular regulators belonging to the LSD1 family play an important role in the precise balancing of diverse PCD players during syncytium development required for successful nematode parasitism.	[85]	2018
	The proposed mathematical models and biological experiments presented in this study prove that SA and H_2O_2 are conditionally regulated by LSD1/EDS/PAD4 to govern WUE, biomass accumulation and seed yield in Arabidopsis.	[310]	2019
	Biotechnological potential of LSD1, EDS1, and PAD4 in the improvement of crops and industrial plants.	[311]	2019
	The uncoupled expression of nuclear genes associated with photosynthesis (PhANGs) and plastid genes (PhAPGs) contributes to cell death in the *lsd1* mutant.	[312]	2019
MC	The LeMCA1, a type-II metacaspase, is upregulated upon infection of tomato leaves with *Botrytis cinerea*, a fungal pathogen that induces cell death in several plant species, but is not regulated during chemical-induced PCD in suspension-cultured tomato cells.	[313]	2003
	The first experimental evidence for metacaspase function in the activation and/or execution of PCD in plants, and its requirement in plant embryogenesis.	[314].	2004
	Two Arabidopsis metacaspases AtMCP1b and AtMCP2b are arginine/lysine-specific cysteine proteases and activate apoptosis-like cell death in yeast.	[72]	2005
	The Arabidopsis *MCA8* is part of an evolutionary conserved PCD pathway activated by oxidative stress, being strongly up-regulated by UVC, H_2O_2, or methyl viologen. A recombinant metacaspase-8 cleave after arginine and complement the H_2O_2 no-death phenotype of a yeast metacaspase knockout.	[94]	2008
	The AtMC1 and AtMC2, type-I metacaspases, antagonistically control PCD in Arabidopsis. AtMC1 is a positive regulator of cell death and requires conserved caspase-like putative catalytic residues for its function, whereas AtMC2 negatively regulates cell death and is independent of the putative catalytic residues.	[70]	2010
		[102]	2011

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Table 1 (*continued*)

Gene family	Article	Reference	Year
	AtMCP2d, the most abundantly expressed member of the type II metacaspase subfamily in Arabidopsis, is a positive mediator of cell death triggered by some forms of fungal toxin, bacterial pathogen- or herbicide-induced stress.		
	Vitis vinifera has six metacaspases genes. The Thompson seedless grapes, which present abnormal PCD in ovule cells and subsequent ovule abortion, upregulates *MC1*, *MC3*, and *MC4* at 35 days after flowering, during ovule development.	[315]	2013
	The pepper metacaspase 9 (Camc9) is a positive regulator of pathogen-induced cell death via the regulation of reactive oxygen species production and defense -related gene expression in plants.	[316]	2013
	AtMC9 is specifically expressed in developing xylem vessel elements, and is required for efficient progression of autolysis during vessel cell death.	[103]	2013
	The plant metacaspase AtMC1 in pathogen-triggered programmed cell death and aging: Functional linkage with autophagy	[101]	2014
	The rice *MC2*, *MC5*, *MC7* and *MC8* are preferentially expressed in mature tissues, when compared to young tissues, indicating a role in senescence. *OsMC1*, *OsMC5*, *OsMC6* and *OsMC8* are regulated by multiple biotic and abiotic stresses such as *Magnaporthe oryzae* infection, pest damaged, cold stress and drought stress, which usually lead to PCD. *OsMC4* and *OsMC6*, especially *OsMC6*, might have been selected during cultivated rice domestication.	[317]	2014
	Phylogenetic analysis of the metacapase gene family in *Viridiplantae* showed that the metacaspases are divided into three principal groups: type I with zinc finger domain, type I without zinc finger domain, and type II metacaspases. The algae and moss are presented as outgroup, suggesting that these three classes of metacaspases originated in the early stages of Viridiplantae, being the absence of the zinc finger domain the ancient condition.	[95]	2015
	Members of the *OsMC* family display differential expression patterns in response to abiotic and biotic stresses and stress-related hormones. Protein-protein interaction analyses show OsMC1 (localized in the nucleus) interacting with OsLSD1 and OsLSD3 while OsMC3 (evenly distributed in the cells) only interacting with OsLSD1, and that the zinc finger domain in OsMC1 is responsible for the interaction activity.	[318]	2015
	Tomato possesses eight metacaspase genes (*SlMC1−8*), *SlMC1−6* belong to type I metacaspases, and *SlMC7−8* type II metacaspases. *SlMCs* have distinct expression patterns in various tomato tissues, and most of them are regulated by drought, cold, salt, methyl viologen, and ethephon treatments.	[319]	2016
	Rubber tree has nine metacaspase genes (*HbMC1−9*), *HbMC1−7* belong to type I metacaspases, and *HbC9−8* type II metacaspases. *HbMCs* have distinct expression patterns in various tissues and developmental stages, and most of them are regulated by drought, cold, and salt stress, implying their possible functions in tissue development and plant stress responses.	[320]	2016
	Metacaspases versus caspases in development and cell fate regulation.	[321]	2017
	Cucumber has five metacaspase genes (*CsMC1−5*), *CsMC1−3* belong to type I metacaspases, and *CsMC4−5* type II metacaspases. *CsMCs* have distinct expression patterns in various tissues and developmental stages, and the transcript levels of *CsMC* genes are regulated by multiple abiotic stresses such as NaCl, PEG, and cold, implying their possible functions in tissue development and plant stress responses.	[322]	2018
	PttMC13 and PttMC14, AtMC9 homologs in hybrid aspen (*Populus tremula* × *tremuloide*), are involved in downstream proteolytic processes and cell death of xylem elements.	[323]	2018
	Around 90 metacaspase genes were identified in the genomes of four cotton species (*Gossypium raimondii*, *Gossypium barbadense*, *Gossypium hirsutum*, and *Gossypium arboreum*), and classified as type-I or type-II genes. During developmentally regulated PCD, type-II *MC* genes may play an important role related to fiber elongation, while type-I genes may affect the thickening of the secondary wall; during environmentally induced PCD, the expression levels of four genes were affected in the root, stem, and leaf tissues within 6 h of an abiotic stress treatment.	[324]	2019
	Seventy metacaspase genes were identified in Rosaceae genomes, including 8, 7, 8, 12, 12, and 23 MC genes in the genomes of *F. vesca*, *P. mume*, *P. persica*, *P. communis*, *P. bretschneideri* and *M. domestica*, respectively. The vast majority of MC gene of *P. communis*, *P. bretschneideri* and *M. domestica* was expanded by large-scale gene duplication, and expression profiling revealed that *PbMC01* and *PbMC03* are detected in all four pear pollen tube and seven fruit development stages.	[325]	2019
	Potato has eight metacaspase genes (*SotubMC1−8*), *SotubMCs* are differentially regulated in various developmental tissues and by multiple stresses and plant hormones, suggesting their distinct and essential role in plants. Some of the tissues, such as leaf undergoing senescence, displayed higher relative expression of some of the metacaspases, implying their involvement in leaf senescence.	[326]	2019
	The type I metacaspase gene in peanut (*Arachis hypoganea* L.), *AhMC1*, is a positive factor in aluminum (Al)-induced PCD.	[327]	2020
	The crystal structures of Metacaspase 4 from Arabidopsis, AtMC4, were determined and are characterized by a Ca^{2+}-dependent metacaspase self-cleavage and activation that mediates plant PCD and immune response.	[328]	2020
	The relationship between molecular responses of barley (*Hordeum vulgare* L.) to drought tolerance and PCD was verified in this study. Tolerant barley modulates PCD to alleviate drought stress damages probably via *MC1* (*Metacaspase 1*) and *TSN1 (Tudor-SN 1)* hub genes.	[329]	2020
PLAC8	The tomato fruit weight gene *fw2.2* governs a quantitative trait locus that accounts for 30% of fruit size variation, with increased fruit size chiefly due to increased carpel ovary cell number.	[117]	2000
	The QTL *fw2.2* directly affects the size of developing tomato fruit, with secondary effects on fruit number and photosynthate distribution.	[330]	2001
	Arabidopsis PCR1 is located at the plasma membrane and reduces the Cd levels in Cd-treated Arabidopsis cells and AtPcr1-transformed yeast.	[112]	2004
	The tomato fw2.2 interacts with the highly conserved regulatory unit of CKII kinase and thus may affect the regulation of cell division via CKII- mediated pathways in common with yeast and animals.	[331]	2006
	MCA1 is a plasma membrane protein that correlates Ca^{2+} influx with mechanosensing in *Arabidopsis thaliana*.	[121]	2007
	Members of the *fw2.2* gene family in *Zea mays* have been named *CNR* (Cell Number Regulator) and two of them, *CNR1* and *2*, exert their effect on plants and organ size by modulating cell number.	[118]	2010
	Plant *FW2.2-like* gene family and the larger related family of proteins with the PLAC8 domain are critical components of the cellular regulatory schema to control cell division and organogenesis.	[115]	2010
	The soybean plasma membrane protein FWL1 is specifically expressed in root hair cells in response to rhizobia and in nodules, and is critical in nodule development.	[116]	2010
	Arabidopsis PCR2 is a zinc (Zn) exporter implicated in two processes, namely, the detoxification of Zn in the presence of high concentrations of Zn and the transfer of Zn from the root to the shoot.	[120]	2010
	The avocado *FW2.2-like* transcript level is considerably higher in small fruit tissues than in the same normal fruit tissues at all examined stages of fruit growth, this gene may have a role as negative regulator of fruit cell division.	[332]	2010
	The plasma membrane protein MCA1 and its paralog MCA2 from *Arabidopsis thaliana* are involved in mechanical stress-induced Ca^{2+} influx, with overlapping roles in plant growth, but distinct when related to Ca uptake in roots.	[122]	2010
		[333]	2011

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Table 1 (*continued*)

Gene family	Article	Reference	Year
	Brassica juncea PCR1 is a Ca^{2+} efflux transporter that is required for the efficient radial transfer of Ca^{2+} in the root and is implicated in the translocation of Ca to the shoot.		
	Plasma membrane protein OsMCA1 is involved in regulation of hypo-osmotic shock-induced Ca^{2+} influx and modulates generation of reactive oxygen species in cultured rice cell	[334]	2012
	The sweet cherry CNR family members PavCNR12 and PavCNR20 are potential candidates to control fruit size in both sweet and sour cherry.	[335]	2013
	Rice has eight *FW2.2-like* (*FWL*) genes (*OsFWL1*−*8)*, and it was reported that *OsFWL3* and *OsFWL5* regulate grain size and plant height, respectively.	[336]	2013
	Physalis floridana CNR1 encodes a cell membrane-anchored protein that functions as a negative regulator of cell division through molecular interactions of CNR1with AG2 and of AG2 with CyclinD2;1, thus directing cell division and contributing to the natural variation of berry size within the *Physalis* species.	[337]	2015
	OsPCR1 (*Oryza sativa*) is localized at the plasma membrane, enhancing grain weight and size as well as influencing the Zn concentration in the brown rice and husks.	[124]	2015
	An SNP in rice *MCA1* generates a loss-of-function mutation resulting in plant architecture defect by upregulation of genes related to GA deactivation, which decreased bioactive GA levels.	[338]	2015
	The PbFWLs are fw2.2-like genes in pear fruit, localized in the plasma membrane being negatively related to the cell division, once they are more expressed in small-sized fruit cultivar.	[119]	2016
	GmFWL1 affect nodule organogenesis by interacting with membrane microdomains in soybean plants infected with the nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum*.	[339]	2017
	It provides the first report comprising the existence of different groups of PLAC8 proteins (types I, II, and III) based on domain composition. It suggests the first unified nomenclature based on the initially characterized *PLAC8* gene.	[108]	2018
	Heterogeneous expression of the rice genes *OsFWL3*−*7* affects Cd resistance in yeast, and OsFWL4 mediates the translocation of Cd from roots to shoots.	[340]	2018
	Activation of *OsFWL5* expression in rice triggers H_2O_2 accumulation and cell death, and increases resistance to *Xanthomonas oryzae pv. Oryzae*, which is associated with increased expression of genes involved in jasmonic acid-related signaling.	[123]	2019
	The rice *OsFWL4* gene belongs to the FW 2.2-Like family and acts as a negative regulator of the tiller number and plant yield.	[341]	2020
GILP	The plasma membrane protein AtGILP interacts with AtLSD1, and negatively regulates hypersensitive cell death in Arabidopsis.	[77]	2011
	The GILP family is composed of one (minimum) or three (maximum) genes in each *Viridiplantae* species. The expression modulation of GILP genes associated with their plasma membrane location suggests that they could act in the signaling of biotic/abiotic stress response in plants.	[126]	2018
NF-Y	The Arabidopsis transcription factor AtNF-YB1 confer drought tolerance and its orthologous in maize ZmNF-YB2 lead to improved corn yields on water-limited acres.	[171]	2007
	The wheat genome has 10 *TaNF-YA*, 11 *TaNF-YB*, and *14 TaNF-YC* genes, and expression analysis revealed that some of the wheat NF-Y genes are expressed ubiquitously, while others are in an organ-specific manner, and that nine NF-Y members are potentially involved in plant drought adaptation.	[342]	2007
	The Arabidopsis *NFYA5* is regulated by drought stress at both transcriptional and posttranscriptional level, and is important for drought resistance in an ABA-dependent manner.	[172]	2008
	In Arabidopsis, the response to ER stress involves the proteolytic activation of bZIP28, the upregulation of the *NF-YC2*, and the translocation of the NF-YB3 from the cytosol to the nucleus to form a transcriptional complex that upregulates the expression of ER stress-induced genes.	[152]	2010
	The Arabidopsis miR169 is strongly down-regulated, whereas its targets, NFYA family members, are strongly induced by nitrogen starvation.	[176]	2011
	Functional and transcriptome analysis reveals an acclimatization strategy for abiotic stress tolerance mediated by Arabidopsis NF-YA family members.	[343]	2012
	Homologous NF-YC2 subunit from Arabidopsis and tobacco are activated by photooxidative stress and induces flowering.	[153]	2012
	The NF-YB family has 20 members in *Populus trichocarpa* and the expression of four homologs (*PeNF-YB7*, *-8*, *-11*, *−13*) in *P. euphratica* is upregulated in the leaves in response to drought stress.	[344]	2013
	NFYA1 is involved in the regulation of postgermination growth arrest under salt stress and ABA treatment in Arabidopsis.	[345]	2013
	GmNFYA3, a target gene of miR169, functions in positive modulation of drought stress tolerance and has potential applications in molecular breeding to enhance drought tolerance in crops.	[173]	2013
	The transcription factor PdNF-YB7 isolated from fast-growing poplar clone NE-19 [*Populus nigra* × *(Populus deltoides* × *Populus nigra*)] positively confers drought tolerance and improves water-use efficiency in Arabidopsis.	[346]	2013
	AtHAP5A as a transcription factor interact with CCAAT motif in vivo, and AtXTH21, one direct target of AtHAP5A, is involved in freezing stress resistance in Arabidopsis.	[175]	2014
	The transcription factor DPB3−1, key for drought and heat stress tolerance in Arabidopsis, form a transcriptional complex with NF-YA and NF-YB subunits, and the identified trimer enhances heat stress-inducible gene expression during heat stress responses in cooperation with DREB2A in Arabidopsis.	[347]	2014
	In *Brassica napus*, *NF-Y* members are regulated by abiotic stresses in ABA-dependent or ABA-independent manner, and *BnNF-Y* promoter analysis shows that multiple members contain abiotic stress-responsive elements.	[348]	2014
	The soybean genome has 21 *GmNF-YA*, 32 *GmNF-YB*, and 15 *GmNF-YC* genes. Expression analysis revealed that certain groups of soybean *NF-Y* genes are likely involved in specific developmental and stress responses.	[349]	2015
	Rice *NF-YA7* is induced by drought stress and its overexpression in transgenic rice plants improve their drought tolerance in an ABA-independent manner.	[174]	2015
	TaNF-YA10–1 presents dual transcriptional control via its role in activating target drought-responsive genes or repressing target salt-responsive genes, probably due to the interaction with different NF-YB/NF-YC heterodimers in wheat.	[350]	2015
	The over expression of an *Amaranthus hypochondriacus NF-YC* gene modifies growth and confers water deficit stress resistance in Arabidopsis.	[351]	2015
	Transgenic Arabidopsis plants overexpressing P*icea wilsonii NF-YB3* gene show increased tolerance to salt, osmotic, and drought stress, probably through modulating gene regulation in the CBF-dependent pathway.	[352]	2015
	Foxtail millet has 39 *NF-Y* genes in which 11 are drought upregulated; additionally, *SiNF-YA1* and *SiNF-YB8* are highly activated in leaves and/or roots by drought and salt stresses. Both genes act as key components of stress tolerance by being involved in both ABA-dependent and ROS signal pathways.	[353]	2015
	The wheat *NF-YB3-1* is crucial for plant drought tolerance mostly through its function in modulating an ABA-associated signaling pathway, which impacts on the physiological processes associated with stomata movement, reactive oxygen species (ROS) metabolism, osmolyte accumulation, and root system architecture establishment.	[354]	2017

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Table 1 (*continued*)

Gene family	Article	Reference	Year
	Plant NF-Y transcription factors: key players in plant-microbe interactions, root development and adaptation to stress.	[355]	2017
	Transgenic tobacco plants overexpressing the garlic *NF-YC8* gene show increased tolerance to salt and drought stresses, the last is probably achieved through the improved antioxidative stress ability conferred by AsNF-YC8.	[356]	2017
	The soybean genome has 21 *GmNF-YA*, 23 *GmNF-YB*, and 24 *GmNF-YC* genes, and expression analysis revealed that certain groups of soybean *NF-Y* genes are likely involved in stress responses conditions and nodulation.	[357]	2018
	The *Prunus persica* genome has 6 *NF-YA*, 12 *NF-YB*, and 6 *NF-YC* genes, and expression analysis revealed that some groups of peach *NF-Y* genes are likely involved in specific developmental and drought responses.	[358]	2019
	The *Sorghum bicolor* genome has 8 *NF-YA*, 11 *NF-YB*, and 14 *NF-YC* genes, and *in-silico* expression profiling based on the available rice transcriptome data for different abiotic stress conditions revealed several potential *SbNF-Ys* showing higher expression level under drought, salt, cold and heat stresses.	[359]	2019
	NF-YB2 and NF-YB3, which have the greatest sequence similarity to each other among NF-YB family proteins in Arabidopsis are functionally diversified and specifically activate dehydration-inducible and heat-inducible genes, respectively.	[360]	2019
	The barley genome has 8 *HvNF-YA*, 11 *HvNF-YB* and 4 *HvNF-YC* genes, and co-expression network analysis indicated the potential functions of *HvNF-Ys* in photosynthesis, starch biosynthesis and osmotic stress tolerance.	[361]	2019
	NF-YA genes control tolerance to abiotic stress: the overexpression of *GmNFYA5*, an *NF-YA* gene in soybean (*Glycine max* L.) results in enhanced drought tolerance to transgenic Arabidopsis and soybean plants.	[362]	2020
	PdNF-YB21 is a root-specific NF-Y family transcription factor in *Populus* acting as a positive regulator of root growth and increased drought resistance.	[363]	2020
	The characterization of NF-Y transcription factor families in industrial rapeseed (*Brassica napus* L.) was able to identify 9 *NF-YAs*, 6 *NF-YBs*, and 5 *NF-YCs* related to abiotic stresses. The expression of *BnNF-YA3* in Arabidopsis plants showed affect processes such as germination and root elongation under abiotic stress.	[364]	2020
EDS1 PAD4	EDS1 gene is a necessary component of the resistance response specified by several RPP genes.	[133]	1996
	PAD4 participates in a positive regulatory loop that increases SA levels, thereby activating SA-dependent defense responses.	[136]	1999
	EDS1 functions upstream of salicylic acid-dependent PR1 mRNA accumulation and is not required for jasmonic acid-induced PDF1.2 mRNA expression.	[135]	1999
	RPP13-Nd is the first Arabidopsis *R* gene product reported to act via a novel signaling pathway that is independent of salicylic acid-mediated responses and is completely independent of *NDR1* and *EDS1*.	[365]	2001
	EDS1 and PAD4, two signaling genes that mediate some R responses, are required for runaway cell death in the lsd1 mutant.	[84]	2001
	EDS1 present two functions: the first is required early in plant defense, independently of PAD4, while the second recruits PAD4 in the amplification of defences, possibly by direct EDS1–PAD4 association	[132]	2001
	Actin cytoskeletal function and EDS1 activity, in combination, are major contributors to NHR (Non-Host Resistance) in *Arabidopsis* against wheat powdery mildew	[366]	2003
	RAC1, unlike other previously described *A. thaliana* TIR-NB-LRR resistance genes, does not require *PAD4* for induction of defense, but is dependent of *EDS1*.	[367]	2004
	Coregulation between individual EDS1 complexes, suggest that dynamic interactions of EDS1 and its signaling partners in multiple cell compartments are important for plant defense signal relay.	[140]	2005
	EDS1 in tomato is required for both basal and *R* gene-mediated resistance.	[368]	2005
	EDS1, PAD4 and SAG101 provide a major barrier to infection by both host-adapted and non-host pathogens.	[134]	2005
	FMO1 positively regulates the EDS1 pathway, while NUDT7 exerts negative control of EDS1 signaling.	[146]	2006
	EDS1 and PAD4 (negatively influenced by MPK4 activit) are central to the antagonism between the SA and ET/JA defense pathways, acting as positive regulators of SA accumulation and negative regulators of ET/JA defense signaling.	[369]	2006
	PAD4 is a key gene in green peach aphid (GPA) *Myzus persicae* (Sülzer) without its signaling partner EDS1.	[370]	2007
	EDS1 and SA play a redundant role in plant defense mediated by R proteins and in signaling induced by low 18:1 fatty acid levels.	[371]	2009
	EDS1 acts as an important regulator of transcriptional reprogramming in the immune response by allowing the induction and repression of particular defense-related genes in the cytoplasm and nucleus.	[144]	2010
	PAD4 is required for phloem-localized antibiosis, in addition to antixenotic defenses against green peach aphid (GPA) *Myzus persicae* (Sülzer).	[372]	2010
	EDS1 responds to the status of $O_2^{\bullet -}$ or $O_2^{\bullet -}$-generated molecules to coordinate cell death and defense outputs.	[373]	2010
	A differential susceptibility to powdery mildew is correlated with differences in *EDS1* expression instead differences in *EDS1* function, considering a resistant and a susceptible variety.	[374]	2010
	EDS1, PAD4, SAG101 function independently as well as in a ternary complex to mediate plant defense signaling against turnip crinkle virus.	[137]	2011
	EDS1 behaves as an effector target and activated TIR-NB-LRR signal transducer for defenses, which require nucleo-cytoplasmic coordination.	[375]	2011
	Whereas EDS1 dissociated from PAD4 is able to confer wild-type local resistance and cell death, an EDS1–PAD4 complex is necessary for the reinforcement of basal resistance through salicylic acid (SA).	[139]	2011
	Natural variation in the TIR-NB-LRR gene VICTR (for VARIATION IN COMPOUND TRIGGERED ROOT growth response) induces root growth arrest via association with EDS1 and PAD4 in a nuclear protein complex.	[376]	2012
	In silico docking between rice EDS1 and PAD4 suggest a dimeric protein complex, which is perhaps important for triggering the salicylic acid signalling pathway in plants.	[377]	2012
	PAD4 protein is necessary for phloem-based resistance against the green peach aphid (GPA; *Myzus persicae* Sülzer) acting at the site of penetration of the vasculature by the insect stylet.	[378]	2012
	Senescence-associated ubiquitin ligase1 (*saul1*) senescence depends on the PAD4-dependent salicylic acid pathway but does not require NPR1 signaling.	[379]	2012
	LSD1/EDS1/PAD4 hub is important in the integration and regulation of acclimatory and defense responses that underpin plant fitness during growth and development.	[80]	2013
	Distinct functional EDS1 heterodimers with SAG101 or PAD4 might explain the central importance and versatility of this regulatory node in plant immunity.	[138]	2013
	Overexpression of *AtPAD4* in roots of soybean confers resistance to cyst and root knot nematodes.	[380]	2013
	Silencing of NbEDS1 from *Gossypium barbadense* resulted in increased susceptibility to *Verticillium dahliae* infection in *Nicotiana benthamiana*.	[381]	2014
	PAPS1 suppresses the salicylic acid-independent immune response downstream of EDS1/PAD4.	[382]	2014
	EDS1 isoforms and one PAD4 protein are required for bacterial resistance derived from the soybean resistance to Pseudomonas syringae.	[383]	2014
	Constitutive defense phenotypes of *cir1* require both EDS1 and PAD4, indicating that CIR1 lies upstream of the EDS1-PAD4 regulatory node of the immune response.	[384]	2014

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Table 1 (*continued*)

Gene family	Article	Reference	Year
	A dominant allele of *WAK2* constitutively activates the stress response and is dependent upon EDS1 and PAD4.	[385]	2014
	Vitis EDS1 and EDL2 proteins interact with *Vitis* PAD4 and AtPAD4, suggesting that *Vitis* EDS1/EDL2 forms a complex with PAD4 to confer resistance in *Vitis* species native to the North American continent.	[386]	2014
	PAD4 acts as a signalling hub to provide resistance to phloem-feeding aphids.	[387]	2014
	Arabidopsis BIK1 confers susceptibility to green peach aphid infestation through suppression of PAD4 expression.	[388]	2014
	OsPAD4 present an important role in wound-induced systemic resistance.	[389]	2014
	The combined action of EDS1, PAD4 and SAG 101 induces SA accumulation to limit *Fusarium graminearum* infection.	[390]	2015
	PAD4 gene of *Populus tremula* × *tremuloides hybrid* might be involved in the regulation of cellular ROS homeostasis and in the cell division and cell death balance.	[391]	2015
	Molecular evidence of stable complex of EDS1-PAD4 supporting SA defense pathway in response to biotic stress in grape.	[392]	2015
	SAG101, *EDS1*, and *PAD4* are involved in the freezing response in *Arabidopsis* by modulating the homeostasis of salicylic acid (SA) and diacylglycerol (DAG).	[393]	2015
	EDS1 of Gossypium barbadense played a fundamental role in Verticillium wilt resistance responses by regulating the accumulation of SA and H_2O_2.	[394]	2016
	Misexpression of *AtTX12* (Toll/interleukin-1 receptor domain) induces growth defects and expression of defense-related genes partially in EDS1-independent manner.	[395]	2016
	DM2h locus (a known hotspot for deleterious epistatic interactions) and nuclear EDS1 cooperate to drive cells into an immune response at the expense of growth.	[396]	2016
	The root growth response mediated by the small molecule named DFPM is equally activated by EDS1 pools enriched in the nucleus or the cytoplasm.	[397]	2016
	PAD4, LSD1 and EDS1 constitute a molecular hub, integrating plant responses to water stress, vegetative biomass production and generative development.	[309]	2016
	EDS1 with PAD4 promoting SA biosynthesis, which maintains important SA-related resistance programs, increasing robustness of the innate immune system.	[142]	2017
	GBF1 is an important component of the plant defense, which oppositely regulating *CAT2* and *PAD4*, promoting disease resistance in *Arabidopsis thaliana*.	[270]	2017
	VfEDL1 (*Vitis flexuosa* Enhanced Disease Susceptibility1-like1), VfEDL2 and VfEDL respond differently against *Elsinoe ampelina* infection.	[398]	2017
	AtPAD4 overexpression in *Brachypodium distachyon* plants led to SA accumulation and induced PR gene expression, which enhanced resistance to *Puccinia brachypodii*.	[399]	2017
	Bacillus velezensis YC7010-induced systemic resistance to the Aphids mainly dependent on higher expression of *PAD4*.	[400]	2017
	The treatment of Arabidopsis plants with Thaxtomin A (TXA), a phytotoxin from plant pathogenic *Streptomyces scabies*, led to enhanced disease resistance to bacterial and oomycete infection, which was dependent on *EDS1* and *PAD4*, as well as on *FMO1* and *ICS1*.	[141]	2018
	PLDα1 and PLDδ oppositely modulate basal, post-penetration resistance against powdery mildew through a non-canonical mechanism that is independent of EDS1/PAD4, SA, and JA.	[401]	2018
	TaEDS1 could acts as a positive regulator and confers resistance against powdery mildew in common wheat.	[402]	2018
	Arabidopsis TNL/EDS1 signaling restricts bacterial pathogen growth inside leaf tissues by interfering with the hub MYC2.	[403]	2018
	Antagonism of transcription factor MYC2 by EDS1/PAD4 complexes bolsters salicylic acid defense in Arabidopsis effector-triggered immunity.		
	SA and H_2O_2 are conditionally regulated by LSD1/EDS/PAD4 to govern WUE, biomass accumulation and seed yield.	[310]	2018
	LSD1, EDS1 and PAD4 features are potentially important for agricultural and industrial use.	[311]	2019
	A Coevolved EDS1-SAG101-NRG1 module mediates cell death signaling by TIR-domain immune receptors.	[404]	2019
	PAD4/SAG101-unbound AtEDS1 is stable as a monomer and does not form homodimers, suggesting that the AtEDS1 monomer represents an inactive or pre-activated ground state.	[405]	2019
	EDS1-SAG101 complex is essential for TNL-mediated resistance responses in *Nicotiana benthamiana*.	[145]	2019
	EDR1 directly associates with EDS1 and PAD4 and inhibits their interaction in yeast and plant cells, and an EDS1 mutation (S135 F) likely alters an EDS1-independent function of PAD4..	[148]	2020
	A *Phytophthora capsici* effector (PcAvh103) interacts with the lipase domain of EDS1 and can promote the disassociation of EDS1–PAD4 complex to suppress plant immunity.	[406]	2020
CAT	Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide.	[69]	1996
	Regulation of catalases in Arabidopsis	[194]	1997
	Cata1-deficient tobacco plants showed disorders in elevated light developing white necrotic lesions on the leaves.	[407]	1997
	Senescence induced new isoforms of catalase in the peroxisomes and combined loss of SOD and CAT activities led to enhanced oxidative stress and cell death.	[408]	2001
	The level of DNA damage in tobacco catalase-deficient (CAT1AS) and wild-type (SR1) plants was analyzed by indirect and direct acting mutagens.	[409]	2003
	Catalase plays a central role in the suppression of mitochondrial $\Delta\Psi_m$ breakdown and PCD induced by β-glucan elicitor.	[188]	2005
	Oxoglutarate-dependent dioxygenase presents a role in H_2O_2 induced cell death.	[189]	2005
	CATs and other scavenger enzymes may be coordinately regulated during development, but differentially expressed in response to different stresses for controlling ROS homeostasis.	[195]	2008
	Transgenic CAT-deficient plants in tobacco (*Nicotiana tabacum* L.) showed a higher sensitivity to membrane damage and cell death when leaf disks were exposed to cadmium-induced cell death.	[410]	2010
	Tobacco (*Nicotiana tabaccum* L.) leaf discs from transgenic plants with a basal reduced CAT activity was analyzed after paraquat-induced cell death.	[411]	2012
	nca1 mutants present reduced activities of all three catalase isoforms in Arabidopsis and loss of *NCA1* (NO Catalase Activity1) function led to high suppression of avrRPM1- dependent cell death.	[177]	2013
	LSD1-catalase interaction plays an important role in regulating PCD in Arabidopsis.	[79]	2013
	Heat stress leads to PCD, which is mediated by the long duration of the oxidant state and accompanied by epigenetic changes across the genome, including alterations in the expression of PCD-related genes and caspase-like-encoding genes.	[187]	2015
	Catalase in Scots pine (*Pinus sylvestris* L.) is involved in zygotic embryogenesis and cell death processes.	[412]	2015
	The plant pathogenic oomycete, *Phytophthora sojae* secretes effectors (PsCRN63 or PsCRN115) to control plant PCD and H_2O_2 homeostasis through direct interaction with catalases for plant resistance.	[413]	2015
	In tobacco BY-2 cell suspension cultures, the levels of peroxisomal catalase protein and CAT activity modulate the onset of cell death via ROS levels and autophagy.	[414]	2018
		[415]	2018

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Table 1 (*continued*)

Gene family	Article	Reference	Year
	Rosmarinic acid inhibits two of the main ROS scavenging enzymes, causing strong ROS accumulation that induces several alterations on mitochondrial ultrastructure and activity through the dissipation of $\Delta\Psi_m$, TCA-cycle alteration, cell starvation and consequently cell death of Arabidopsis seedlings.		
MYB	*AtMYB30* acts in the initiation of cell death rather than in the limitation of its extent.	[240]	1999
	A pathogen- and JA-induced rice gene (*JAmyb*) encodes a Myb transcription factor is closely to host cell death and is involved in the JA-mediated, SA-independent signaling pathways.	[416]	2001
	AtMYB30 is a positive regulator of the hypersensitive cell death.	[241]	2002
	The expression of *HbMyb1* in *Hevea brasiliensis* is likely associated with TPD (tapping panel dryness) and was propose that HbMyb1 acts as a negative regulator for PCD-induced genes.	[417]	2003
	AtMYB30 is involved in an amplification loop or signalling cascade that modulates SA synthesis, which in turn modulates cell death.	[242]	2006
	The silencing of *AtCDC5*, a Myb-related protein in *Arabidopsis thaliana* induces accelerated cell death.	[418]	2007
	MYB30 modulates hypersensitive response via VLCFAs (very-long-chain fatty acids) by themselves, or VLCFA derivatives, as cell death messengers in plants.	[243]	2008
	The timing of tapetal programmed cell death is critical for pollen development, and the MYB80/UNDEAD system may regulate that timing.	[244]	2011
	HbMyb1, a Myb transcription factor from *Hevea brasiliensis* was overexpressed in tobacco and in these transgenic plants, the cell death induced by abiotic and biotic agents was suppressed with a close correlation between *HbMyb1* protein levels and the extent of suppression.	[419]	2011
	Evaluation of R2R3-MYB genes in canola presents an overview of the action of this family in PCD.	[245]	2016
	Transcriptomic profiling identifies differentially expressed genes associated with programmed cell death of nucellar cells in *Ginkgo biloba* L.	[246]	2019
	BnaMYB111 l from rapeseed encodes an R2R3-type MYB transcription factor and its expression promotes ROS accumulation and cell death in tobacco and rapeseed protoplasts.	[420]	2020
	OsMYB80 regulates male fertility in rice by directly targeting multiple biological processes, such as cell death gene expression associated with the tapetum PCD.	[421]	2020
bZIP	TGA2 or TGA5 are members of the TGA subclass of bZIP transcription factors that interact with NIM1/NPR1 and the overexpression of *TGA5* provide SAR-independent resistance in *Arabidopsis thaliana* to *Peronospora parasitica*.	[422]	2002
	GBF1 binds to two overlapping DNA fragments of the *CAT2* promoter, suggesting a role of GBF1 in the regulation of *CAT2* expression and H_2O_2 levels.	[269]	2010
	During endoplasmic reticulum stress (ER), *NAC089* is up-regulated promoting ER-stress-induced PCD and this is directly controlled by bZIP28 and bZIP60.	[423]	2014
	LSD1 and HY5 antagonistically modulated EDS1-dependent ROS and SA signaling, acting in PCD induced in response to red light.	[268]	2015
	GBF1 is an important component of the plant defense, which oppositely regulating *CAT2* and *PAD4*, promoting disease resistance in Arabidopsis.	[270]	2017
SERPIN	The barley (*Hordeum vulgare*) protein Zx gene was isolated and sequenced, showing 30% similarity to the animal members of the serpin superfamily.	[424]	1993
	From grains of hexaploid bread wheat (*Triticum aestivum* L.) six serpins were identified and five major molecular forms of them were cloned and characterized, being suicide substrate inhibitors of chymotrypsin and cathepsin G.	[425]	2000
	Expression studies for suicide-substrate serpins in vegetative and grain tissues of barley (*Hordeum vulgare* L.).	[426]	2003
	AtMC9 is blocked by AtSerpin1 and them were localized in the extracellular space, suggesting an in vivo interaction.	[219]	2006
	Serpins in plants and green algae.	[427]	2008
	The Arabidopsis Serpin genes *AtSRP2* and *AtSRP3* have high levels of transcripts in reproductive tissues and are necessary for normal responses of plants following exposure to alkylating genotoxins such as methyl methanesulfonate (MMS).	[215]	2009
	Serpin protease inhibitors in plant biology.	[197]	2012
	Serpins encoded in the rice genome go through protein sequence analysis indicating a diversity of reactive-center sequences and a low identity with Arabidopsis serpins. Phylogeny denotes two main clades and transcriptional analysis showed the rice serpins expression during development.	[428]	2012
	AtSerpin1 controls the pro-death function of compartmentalized protease RD21 by determining a set-point for its activity and limiting the damage induced during cell death.	[214]	2013
	OsSRP-LRS negatively regulates stress-induced cell death in rice.	[216]	2015
	AtSRP4 and AtSRP5 are negative regulators of stress-induced cell death and *AvrRpt2*-triggered immunity.	[213]	2017
	Serpin acting as a suicide inhibitor in plants.	[198]	2018
	Plant serpin protease inhibitors: specificity and duality of function.	[429]	2019
	Wheat serpins are involved with grain development.	[202]	2019

such as plant growth, seed yield, photosynthesis, water use efficiency, and cellular signaling homeostasis, has been described [80]. However, the most prominent role of LSD members is in the PCD mechanism. The synchronized function of the three LSD genes in *A. thaliana* coordinatesthe PCD occurrence: LSD1 acting as a negative and LOL1 as a positive regulator that is itself negatively regulated by LOL2 [33]. LSD1 and LOL1 also have opposite effects on copper-zinc superoxide dismutase and reactive oxygen intermediates accumulation [71].

AtLSD1 is the well-characterized gene of the LSD family. AtLSD1 enhances ROS-scavenging capacity in the oxidative stress response after conditions that promote excess excitation energy [81]. Together with EDS1 (Enhanced disease susceptibility1) and PAD4 (Phytoalexin deficient4), LSD1 acts as a ROS/ethylene homeostatic switch in the crosstalk between light acclimation and immunity [82]. LSD1, EDS1, and PAD4 also mediate PCD response in chilling occurrence [83]. This triad is likewise essential to disease resistance signaling components to the cell death pathway [84]. In biotic conditions, the transcriptomic dynamics of the LSD1-dependent network pointed to several genes, which are co-regulated with it [85]. In summary, AtLSD1 has been proposed to act as a cellular hub in PCD machinery [78]. In the deathosome, LSD1 appears interacting with several proteins (Fig. 2) [33].

3.2. Metacaspase

In animals, caspases are known for their crucial role in the regulation of apoptosis. In general, caspases are divided into initiators and executioners. The initiator caspases are related to the early stages of apoptosis and have a protein-protein interaction motif at the N-terminal prodomain. Caspase executioners do not have the protein-protein interaction motifs and are activated by initiator caspases to perform the

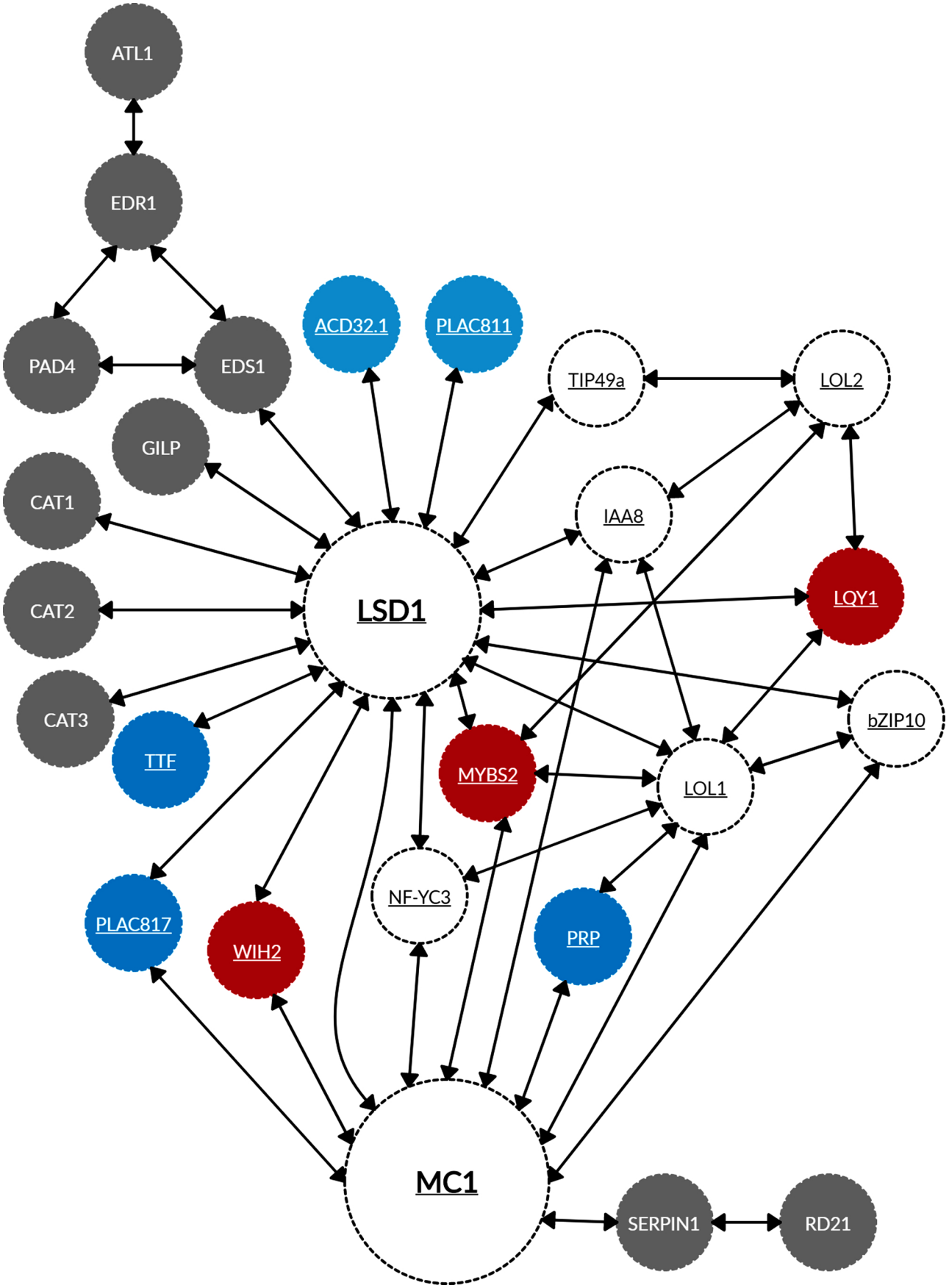

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Fig. 2. Update of AtLSD1 deathosome. Underlined genes were originally described by Coll et al. (2011). Deathosome: LSD1 (Lesion Simulated Disease1); LOL1 (LSD-like 1); LOL2 (LSD-like 2); MC1: (Metacaspase1); bZIP10 (Basic Leucine-region Zipper); PLAC811 and PLAC817 (Placenta-specific 8); MYBS2 (Myeloblastosis); IAA8 (Indole-3-Acetic Acid 8); LQY1 (Low Quantum Yield of photosystem II 1); TIP49a (49-kDa TBP-Interacting Protein); PRP (Proline-rich Protein); NF-YC3 (Nuclear Factor-Y C3); ACD32.1 (Alpha-Crystallin Domain 32.1); WIH2 (Windhose2); TTF (Transcription Termination Factor). Among them, genes in red were characterized after the first deathosome publication, and genes in blue have not been characterized so far. New members of the regulatory network are shown in gray: SERPIN1 (Serine proteases inhibitor1); MC9 (Metacaspase9); RD21 (Responsive to Desiccation-21); CAT1 (Catalase1); CAT2 (Catalase2); CAT3 (Catalase3); GILP (GSH-Induced LITAF Domain Protein); WSCP (Water-Soluble Chlorophyll-binding Protein); PDI5 (Protein Disulfide Isomerase-5); EDR1 (Enhanced Disease Resistance1); ATL1 (Arabidopsis Toxicos en Levadura1); EDS1 (Enhanced Disease Susceptibility1); and PAD4 (Phytoalexin Deficient4). Adapted from Coll et al. (2011) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cleavage of cellular substrates necessary to make an orderly cell death [6,86].

Caspase-3 activity was identified in mammalian apoptosis pathways acting as an executor [87,88]. In *A. thaliana,* caspase-3-like protease participated in the Cd^{2+}-induced PCD, and its activation was detected during PCD after exposure to ultraviolet C (UV-C) [89,90]. The identification and characterization of metacaspases, arginine/lysine-specific cysteine proteases with structural homology with animal caspases, defined the existence of caspase-type proteases in the control of plant PCD activation [6,43,91,92].

Characterized by being a family of cysteine proteases, metacaspases are found only in caspase-deprived eukaryotic organisms such as protists, fungi, and plants [6,93–96]. Metacaspases are characterized by a conserved caspase-like catalytic domain, which contains a small 10 kDa (p10) and a large 20 kDa (p20) catalytic subunits [97,98]. Besides, according to the presence or absence of an N-terminal prodomain, two types of metacaspases can be recognized [96]. In the N-terminus region, type I metacaspases, present in protists, fungi, and plants, have a prodomain similar to the found in metazoan caspases, rich in proline-rich repeats (PRR) and, in plants, there is also a zinc-finger motif in this region. Type II metacaspases, plant-related, resembling executioner caspases does not have an extended prodomain and a zinc-finger motif; however, they have a linker between the p20 and p10 subunits, where the catalytic dyad of histidine/cysteine is located [6,95,96,99].

Nine metacaspase genes are found in *A. thaliana*, three belonging to type I (*AtMC1* to *AtMC3*), and six to type II (*AtMC4* to *AtMC9*). The metacaspases type I, AtMC1, AtMC2 and AtMC3 have an important plant-specific LSD1-like zinc-finger N-terminal motif (CxxCRxxLMYxxGASxVxCxxC) [70]. *AtMC* genes are involved in biological defense-related to cell death processes and also in developmentally regulated PCD [96,100].

MC1 (At1g02170) is a positive regulator of pathogen-induced PCD, acting as a positive mediator of *lsd1* runaway cell death. This MC1 function requires the caspase-like catalytic activity and is negatively controlled by its N-terminal prodomain. The N-terminal prodomain of metacaspase is necessary for interacting with LSD1 protein, and this interaction occurs via their zinc-finger domains. The interaction between MC1 and LSD1 leads to a block in AtMC1-dependent PCD, by the prodomain removal [33,70,101]. MC2 does not interact with MC1 or LSD1 proteins, but it can negatively regulate MC1 posttranscriptionally via an unknown mechanism [33,70]. MC4 positively regulates cell death via oxidative stress signaling and is essential in response to fungal toxin fumonisin B-1 [80]. MC8 is indispensable for PCD regulation induced by UVC, hydrogen peroxide (H_2O_2), or methyl viologen [94]. MC9 has a function in xylem cell death related to degradation of vessel cell contents [103]. The induction of cell death by a secreted plant GRIM REAPER (GRI) peptide requires activation of MC9, performing proteolytic cleavage of GRI, which binds to the receptor kinase POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 5 (PRK5) and promotes ROS-induced cell death [104]. MC9 is also involved in cell death during vascular tracheary elements (TEs) differentiation since reduced *MC9* expression in TEs of *MC9*-RNAi lines results in higher levels of autophagy [105]. MC1 is a positive regulator of HR-cell death, and *A. thaliana* genes with unknown functions were presented as interaction partners of MC1 (Fig. 2), and this becomes a gateway for several studies to understand the role of these interactors [33].

3.3. Placenta-specific 8 (PLAC8)

PLAC (Placenta-specific) genes were first identified from microarray analyses of mouse placental and embryonic RNAs [106]. PLAC8 family proteins are characterized by containing conserved cysteine-rich regions: CXXXXCPC or CLXXXXCPC, found in a wide range of eukaryotic organisms: mammals, plants, fungi, and algae [107,108]. Different roles of PLAC8 proteins are described, including cell cycle control in animals, an essential regulator of tumor evolution and autophagic machinery in humans, resistance to heavy metals in plants, and cadmium resistance in fungus [109–112]. The first PLAC8 genes were characterized and studied in humans [106]. The fact that PLAC8 proteins in humans are related to cell differentiation and proliferation, apoptosis, control of the progression of several diseases, made them candidates for diagnostic and therapeutic targeting [109,113,114]. All these works reinforce their regulatory role in humans, mainly in mammal's apoptosis networks. Therefore, the study of this gene family with conserved cysteine-rich regions in other organisms becomes an interesting issue [108].

In plants, PLAC8 motif proteins are related to developmental processes such as fruit size regulation, coordinating plant cell division, and nodule development [108,115,116]. An important quantitative trait loci (QTLs), the tomato FW2.2 (Fruit Weight-2.2), works in the regulation of cell division and fruit size [117]. FWL proteins have a highly conserved cysteine-rich motif. The FW2.2 gene was cloned and showed to contain a PLAC8 domain, evidencing the conservation of the biochemical function between them [115]. Fw2.2 orthologs genes in *Zea mays* were denominated Cell Number Regulator (CNR) genes. When ectopically overexpressed, CNR1 reduces plant size, while CNR2 expression is negatively related to tissue growth activity [118]. In pears (*Pyrus spp*), a study performed the cloning, localization, and expression experiments of two fw2.2-like genes (PbFWL). PbFWLs encode Cys-rich proteins belonging to the PLAC8 family and were localized in the plasma membrane and during the cell division period. Their expression is increased in small-fruited pear species, which might be negatively involved in the cell division in pear fruit [119]. The GmFWL1 (*Glycine max* FW2.2-like 1) is fundamental for soybean nodule organogenesis, increased its expression when inoculating with the *Bradyrhizobium japonicum*, that is a nitrogen-fixing symbiotic bacterium. The silencing of GmFWL1, by RNA interference (RNAi), reduces nodule number, and affects heterochromatinization [116].

Other functions of PLAC8 proteins in plants are in the metal resistance, Ca^{2+} uptake, and in PCD [112,120–123]. Members of the PCR (Plant Cadmium Resistance) protein family share the PLAC8 domain and mediate resistance to heavy metals [112,120]. Arabidopsis PCR1 localizes in the plasma membrane and plants overexpressing AtPCR1 showed increased cadmium (Cd) resistance, as well reduction in Cd uptake [112]. AtPCR2 is a zinc (Zn) exporter implicated in two processes, the detoxification of Zn in the presence of high concentrations of Zn and the transfer of Zn from the root to the shoot [120]. OsPCR1 (*Oryza sativa*) is localized at the plasma membrane, enhancing grain weight and size as well as influencing the Zn concentration in the brown rice and husks [124]. Other PLAC8 proteins in Arabidopsis have roles in Ca^{2+} uptake, such as MCA1 (Mid1-complementing activity) and their paralog, MCA2 [121,122]. Ultimately, the overexpression of OsFWL5, a fruit-weight 2.2-like gene, enhanced H_2O_2 accumulation, and cell death [123].

PLAC8 nomenclature was recently unified, and a new classification was proposed. Using the cysteine-rich domain that characterizes PLAC8 proteins (Pfam PF04749), 445 sequences similar to the protein encoded by the first characterized PLAC8 gene were recovered [108]. These sequences allowed the classification of these genes in three types: type I (337 genes), II (48 genes), III (71 genes). Type I genes are found in mammals, fungi, plants, and algae, while types II and III are restricted to plants. Type I PLAC8 genes are the best characterized so far, whereas genes classified as type III have not been functionally characterized [107,108]. Seventeen members of the PLAC8 family are found in *A. thaliana*, and two members of the PLAC8 family are present in the deathosome, evidencing the possible role of this gene family in plant PCD. Plac811 (At1g52200) gene interacts directly with LSD1 while Plac817 (At4g23470) interacts with both MC1 and LSD1 (Fig. 2) [33,108]. Another evidence of *PLAC811* involvement in PCD is the upregulation in papilla cells as it ages from young to senescent. [125].

3.4. GSH-induced LITAF domain protein (GILP)

First characterized in humans, the LITAF domain [Lipopolysaccharide (LPS)-Induced Tumor Necrosis Factor Alpha (TNF-a) Factor] is present in viruses, fungi, plants, and metazoans [77]. In plants, the LITAF motif-containing proteins are called GILP (GSH-Induced LITAF Domain Protein), and the motif comprises 66 amino acids, formed by an N-terminal CxxC knuckle, a hydrophobic region, and a C-terminal HxCPxC knuckle. Also, the initial (PAPF) and final (DFEK) amino acid positions are well conserved. The majority of GILP proteins have a transmembrane domain located inside the LITAF motif [126].

In humans, *LITAF*, *SIMPLE* (Small Integral Membrane Protein of the Lysosome/Late Endosome), and *PIG7* (p53-Inducible Gene 7) refer to the same gene, which expression is dramatically induced during p53-mediated apoptosis, suggesting that it may be involved in the regulation of PCD [127–129]. LITAF is a transcription factor, critical for the activation of numerous immune cells via classical pathways, but it can also regulate protein degradation when located in the membranes of late endosomes and lysosomes [130].

A single report has investigated the function of GILP in plants [77]. The *AtGILP (AT5G13190)* is upregulated in response to avirulent pathogen and fumonisin B1 (FB1), which are known to trigger PCD. Also, the overexpression of *AtGILP* in Arabidopsis can suppress avirulent pathogen-triggered PCD. The Arabidopsis protein containing the LITAF motif is localized at the plasma membrane, whereas both the N and C-terminal domains are involved in the interaction with LSD1, a negative regulator of PCD (see above) [77]. The authors suggest that AtGILP is a negative regulator of PCD by acting as a membrane anchor, bringing other proteins to the membrane via protein-protein interaction (Fig. 2) [77].

3.5. Enhanced disease Susceptibility1 and phytoalexin Deficient4 (EDS1 and PAD4)

Resistance(R) genes mediate recognition of specific molecular components of invasive pathogens [131]. EDS1(Enhanced diseasee Susceptibility1) and PAD4 (Phytoalexin Deficient4) are important regulators of *R* gene-mediated plant defense [132]. The Arabidopsis *EDS1* (Enhanced Disease Susceptibility1) gene encodes a lipase-like protein and participates in the pathways of resistance responses by various RPP (Resistance to *Peronospora Parasitica*) genes, being essential for the recognition of the molecular components [84,133–135]. The PAD4 peptide sequence has similarities regions to eukaryotic triacylglycerol lipases and esterases, participates positively in a regulatory loop to increases SA levels, and is necessary for the expression of several defense responses after pathogen infection [136]. Both EDS1 and PAD4 positively regulates SA accumulation and are necessary for resistance conditioned by an equal spectrum of *R* genes [132].

The signaling and regulation of plant innate immunity against virulent biotrophic and hemibiotrophic pathogens require proteins such as EDS1, PAD4, and another EDS1 interaction partner known as SAG101 (Senescence Associated Gene101) [137–139]. These three proteins present a conserved EP domain (EDS1 and PAD4-defined) at the C-terminal [132,140]. Arabidopsis has two functionally isoforms of EDS1, and either of them can physically interact with PAD4 and SAG101 to form different complexes in the nucleus and cytoplasm. Also, both isoforms can form ternary complexes with PAD4 and SAG101 [132,137,140]. EDS1 facilities the interaction between SAG101 and PAD4 once they do not interact directly [137]. While the EDS1-SAG101 heterodimer exists mainly in the nucleus, EDS1–PAD4 associations occur in the nucleus and cytoplasm [140]. The complex EDS1, PAD4, and SAG101 is predominantly nuclear-localized, and this suggests that SAG101 and PAD4 can control the subcellular localization of EDS1 [137]. The complexes formed by EDS1 with SAG101 or PAD4 activate defense gene expression and accumulation of stress signaling molecules, such as SA [134,141–144]. The complex EDS1-PAD4 is essential for efficient immune response, whereas the *pad4* mutant exhibits function compromised. This does not occur in the absence of the EDS1-SAG101 complex because of the presence of the EDS1-PAD4 heterodimer [134,138,145]. There are distinct immune responses in the EDS1/PAD4 association. One is dependent on the accumulation of SA as a defense molecule, and the other pathway occurs independently of SA with other signal intermediates such as *ICS1* (Isochorismate Synthase1) [132,141,146].

EDS1, but not PAD4, interacts with LSD1 in the nucleus and cytoplasm [67]. LSD1, EDS1, and PAD4 act together in the regulation of various molecular and physiological processes that influence Arabidopsis fitness, such as water use efficiency, photosynthetic efficiency, seed yield, and control of the H_2O_2 content and cellular levels of SA [80]. The formation of this core is vital for pathogen resistance, and studies have demonstrated that EDS1 (At3g48090) and PAD4 (At3g52430) are necessary for runaway cell death in the *lsd1* mutant [67,84,141,142]. LSD1 and EDS1 act antagonistically, controlling the UV-C-induced cell death. While the *lsd1* mutant showed increased cell death, *eds1* presents reduced cell death in response to UV-C stress [147]. Another recent study identified Arabidopsis EDR1 (Enhanced Disease Resistance1) as an interactor of both PAD4 and EDS1 [148]. EDR1 plays roles in stress response, ethylene signaling, and cell death, acting as a negative regulator [149]. It has been proposed that EDR1 negatively regulates defense responses by interfering with the heteromeric association of EDS1 and PAD4 to inhibit cell death. The yeast three-hybrid showed inhibition of the EDS1-PAD4 interaction when EDR1 is coexpressed with them [148]. As new members of the deathosome, EDS1 (At3g48090) interacts with LSD1 and PAD4 (At3g52430), while EDR1 (AT1G08720) interacts with EDS1 and PAD4 (Fig. 2).

3.6. Nuclear factor-Y (NF-Y)

The Nuclear Factor-Y (NF-Y) TFs, initially discovered by its ability to bind to the conserved Y box element present at the promoter of the mouse MHC Class II Ea, belong to the CCAAT-binding factor (CBF) family, also known as the Heme Activator Protein (HAP) family in yeast. The NF-Y is a heterocomplex formed by NF-YA, NF-YB, and NF-YC subunits, all of which are necessary for binding to the CCAAT box-containing promoters in eukaryotes [150,151]. In yeast, there is a fourth subunit necessary for complex formation and transcriptional activation . NF-YB and NF-YC have a histone-fold domain (HFD), which mediate their association in the cytoplasm [151]. Formation of NF-YB/YC heterodimer is necessary for the translocation of NF-YB into the nucleus since the NF-YC family member contains a nuclear localization signal (NLS). NF-YA, which provides a sequence-specific contact to the CCAAT box, assembles with NF-YB/YC to form the NF-Y heterotrimer in the nucleus [152,153]. In yeast and mammals, each subunit of NF-Y is usually encoded by a single gene with multiple splicing isoforms [150,154]. In contrast, plants have gene families encoding each NF-Y

subunit type, providing the potential for multiple alternative forms of NF-Y complexes in plants [155].

In mammals, the NF-Y complex plays essential roles in cell proliferation, apoptosis, stress response, DNA damage, and tumor development [156]. Several pieces of evidence suggest that NF-Y affects apoptosis and proliferation by influencing the cell cycle, and this function of NF-Y is often related to the p53 tumor suppressor whose activation can lead to growth arrest or programmed cell death [157–159]. Activation of p53 can result not only from physical and chemical stresses but also from oncogene imbalance [156,160]. The activated p53 interacting with NF-Y leads to the recruitment of histone-deacetylases (HDACs) to repress many critical genes required for cell-cycle progressions such as cyclin A, cyclin B1, cyclin B2, cdc25A, cdc25C, and cdk1 through binding to CCAAT boxes in their promoters, which results in cell apoptosis [156,158,161,162].

At1g54830, also known as NF-YC3, interacts with the three regulators of Arabidopsis HR-PCD: LSD1, LOL1 and MC1, and was proposed to be part of the deathosome [33] (Fig. 2). However, this is the only study linking NF-Y plant proteins in PCD to date. NF-YC3 is also involved in many aspects of plant growth and development as flowering and seed germination [163,164]. Given the number and diversity of plant NF-Y encoding genes, the NF-Y- subunits have acquired specific functions in diverse processes such as embryo development, flowering time control, ER-stress, drought stress, freezing stress, nitrogen nutrition, and nodule development [165,166,175,176,167–174[430]].

3.7. Catalase (CAT)

ROS, especially H_2O_2, is involved in all steps of the plant PCD, including induction, signaling, and execution [177]. Since PCD is closely related to ROS generation, the activity of antioxidant enzymes, including catalases (CATs), is necessary leading to the conversion of H_2O_2 to H_2O and O_2, removing the excess of H_2O_2 [177–180]. CAT is an important H_2O_2-scavenger enzyme found in any aerobic organisms. A single gene encodes catalase in animals, but in plants, a small, conserved multigenic family encodes catalases, which have varied physiological functions [181–186].

The control of the level of intracellular ROS contributes to plant PCD defense responses to adverse biotic and abiotic environmental stimuli [79,177,178,181,186,187]. When catalase and alternative oxidase (AOX) are inhibited in potato (*Solanum tuberosum L.*) suspension cultures treated with β-glucan elicitor, released from the cell wall of the phytopathogenic fungus *Phytophthora megasperma* by soybean glucanases, it was observed an enhanced H_2O_2 accumulation, affecting the mitochondrial membrane potential and PCD [188]. Also, Arabidopsis plants sprayed with the catalase inhibitor aminotriazole (AT) present a reduction in the catalase activity, with a consequent accumulation of H_2O_2 and cell death. [189].

In *Arabidopsis*, the *CAT* family is formed by three members, CAT1, CAT2, and CAT3, of 492 amino acid residues with 87–94% similarity [178,183]. Catalases can be divided into three classes based on their gene structure and expression. In *Arabidopsis*, CAT2 belongs to Class I, CAT3 to Class II, and CAT1 to Class III [185,190,191]. *CAT2* is highly expressed in photosynthetic tissues, while *CAT3* appears in leaves, strongly related to the vascular system having increased expression with age, and *CAT1* is low expressed in leaves, being associated with seeds and reproductive tissues [181,184,185,190,192]. Catalases can be controlled in a coordinated manner during development, but they are differentially expressed in response to adverse conditions [193]. In *Arabidopsis* seedlings light induce *CAT1* and *CAT2* mRNA abundance, but *CAT3* is negatively regulated. The expression of *CAT1* is not dependent on circadian control but the expression of *CAT2* and *CAT3* is regulated by circadian rhythm [194]. Different expression profiles and functional analysis of the three Arabidopsis catalases under various abiotic stresses revealed that CAT1, CAT2, and CAT3 were relevant for the removal of H_2O_2 generated, contributing to ROS homeostasis. The expression of *CAT1* was induced in all treatments performed (drought, cold, oxidative stresses, abscisic acid, and salicylic acid). CAT2 was induced in cold and dry stresses, and CAT3 enhanced by oxidative stress, exposure to ABA, and in the senescent stage of the plant [195]. Catalases expression is also regulated by senescence. During leaf senescence, *CAT2* expression was down-regulated, while the *CAT3* expression was enhanced by senescence and age [192].

The *LSD1* cell death has been related to oxidative stress, and in the loss-of-function *lsd1*, there was a high accumulation of superoxide, which was sufficient to create a lesion and initiate runaway cell death [69]. Genetic studies demonstrated that LSD1 interacts with three catalases: CAT1 (At1g20630), CAT2 (At4g35090), and CAT3 (At1g20620) to regulate light-dependent runaway cell death and hypersensitive-type cell death. This interaction was confirmed *in vitro* and *in vivo* with the three catalases and is dependent on zinc fingers from LSD1. Reduced catalase activity was found in the *lsd1* mutant, showing that its activity is in part dependent on *LSD1*. Under treatment with 3-amino-1,2,4-triazole (3AT), which is a catalase inhibitor and consequently results in an increase of peroxisomal H_2O_2 concentrations leding to cell death, *lsd1* proved to be more affected by the treatment than wild type seedlings. This showed the relation in the interaction between LSD1 and catalases, which control ROS produced in the peroxisomal compartment [79]. Therefore, LSD1-catalase interaction in Arabidopsis has a role in controlling PCD and is part of the deathosome (Fig. 2).

3.8. Serine proteases inhibitor (SERPIN)

Plant proteases have critical regulatory functions in the most diverse biological processes [196,197]. Serine protease inhibitors (Serpin) gene superfamily are ubiquitous in the vegetal kingdom, representing the most abundant family of plant protease inhibitors, distributed in countless species, encompassing all domains of life [196–199]. Serpins have high structural conservation, peptide sequences composed of approximately 340–440 amino acids, and are characterized by the presence of nine α helices, three β sheets, and one flexible, reactive central loop (RCL) [200,201]. The proteins in this family act as inhibitors by forming covalent complexes with target proteases, such as cysteine and serine proteases [196,197,202].

The human genome contains 37 SERPIN genes, and 30 of them are functional inhibitors, implicated in many physiological processes, such as blood coagulation, fibrinolysis, immune response, cell growth and maturation, extracellular matrix remodeling, hormone transporter, tumor suppression, inflammation, and regulation of cell death, and the impairment of their function is related to various diseases [203–206]. Intracellular serpins are present in the nucleocytoplasmic compartment and are known as regulators of cell survival because they inhibit diverse key serine proteases and executioner proteases that initiate cell death [207]. Granzyme is a granule cytotoxin formed by cytotoxic T lymphocytes (CTLs) to exterminate virus-infected and malignant cells via apoptosis through the extravasation of the content of its cytolytic granules [208,209]. Humans have five distinct granzymes (GrA, GrB, GrH, GrK, and GrM) [210]. Tumor cells, through the intracellular expression of serpins, prevent cell death via cytotoxic lymphocytes. When SERPINB9 intracellular expression occurs by malignant cells, they become resistant to GrB-induced apoptosis [211]. Cell death induced by GrM, as well as by natural killers (NK), was inhibited by overexpression of SERPINB4 in a manner dependent on its central reactive loop [212].

In plants, serpins have been involved in growth, development, defense against pathogens, and controlling of stress-induced cell death [213–216]. Eight genes belonging to the Serpin family are present in the Arabidopsis genome, playing different roles as regulators of cell death [213]. Both AtSRP4 and AtSRP5 negatively regulates UV-induced cell death and repress the HR-induced cell death, caused by bacterial effector AvrRpt2, but the influence of AtSRP4 was more remarkable than AtSRP5 [213]. In rice, OsSRP-LRS is the closest homolog of

Arabidopsis Serpin1, and RNAi lines showed enhanced cell death upon a necrotrophic fungal pathogen, UV, or salt treatment. In this sense, OsSRP-LRS is a negative regulator of stress-induced cell death, and this goes according to the role of the abolishment of cell death for this class of proteases inhibitors [216]. Furthermore, AtSerpin1 has the potential to act in pest control [217].

A recent study showed that Serpin1 of *A. thaliana* acts as a suicide inhibitor on immune/cell death regulators, blocking the self-processing of MC1 and restricting cell death mediated by MC1 [198]. Serpin1 also regulates the set-point of the RD21 (RESPONSIVE-TO-DESICCATION-21) protease activity. Serpin 1 interacts in vivo with RD21, a pro-death protease, controlling HR-cell death and thereby reducing the damage [214,218]. Due HR-PCD function, Serpin1 (At1g47710), and RD21(At1g47128) were included as new members of the AtLSD1 deathosome (Fig. 2) [198,214,218].

AtSerpin1 is also a potent inhibitor of AtMC9 activity in vitro through cleaving its central reactive loop and binding to AtMC9. It was also demonstrated that AtMC9 and AtSerpin1 are co-located in the extracellular space, allowing the physical interaction between the two proteins. Although there is evidence of the interaction between these two proteins, no functional description resulting from this interaction was reported so far [219].

3.9. Myeloblastosis (MYB)

The members of the MYB superfamily of transcriptional activators are widely distributed in eukaryotes having a highly conserved amino-terminal MYB domain [220–222]. MYB proteins are characterized by a conserved DNA binding domain, which consists of one to three (R1, R2, R3) imperfect repeats, each of them comprising a sequence of approximately 50–53 amino acid residues and encoding three α-helices. The second and third helices create a helix–turn–helix (HTH) structure that, when linked to DNA, it intersperses in the main groove [223,224].

The vertebrates express three related Myb proteins: MYB (c-Myb), MYBL1 (A-Myb), and MYBL2 (B-Myb), which have been linked to the control of important cell process, such as growth, proliferation, and differentiation [225–227]. The B-MYB expression is increased in cells with rapid division and during development, having a critical role as a regulator of cell cycle progression, cell differentiation, and regulating the expression of targets in senescence and apoptosis [228–234]. B-MYB enhances cell survival since it activates anti-apoptotic genes like BCL2 and ApoJ/clusterin [232]. c-Myb also induces the expression of BCL-2, which protects T lymphocytes from apoptosis [235]. A-MYB acts as a male-specific master regulator of male meiosis [236].

In plants, the MYB family comprises one of the largest TFs, evidencing their essential role in the regulation of plant-specific processes [224]. According to the number of adjacent repeats, MYB proteins are divided into subfamilies called R1-MYB, R2R3-MYB, 3R-MYB, and 4R-MYB [220]. *A. thaliana* has more than a hundred R2R3-MYB genes that play essential roles controlling plant-specific processes, such as secondary metabolism, biosynthetic pathways, trichome differentiation, cellular morphogenesis, meristem formation, and in response to biotic and abiotic stresses [221,222,237–239].

MYB TFs also have involvement in plant PCD, such as AtMYB30, a type of R2R3-MYB TF, which participates in the establishment of cell death [240]. AtMYB30 is a positive regulator of HR-PCD, [241–243]. Another member of the MYB family related to PCD is the AtMYB80 TF, that participates in the regulation of tapetal PCD, necessary for pollen development, via MYB80/UNDEAD system [244]. In canola (*Brassica napus L.*), the expression of the BnaMYB78 gene, a member of the R2R3-MYB TF and, homolog to AtMYB78 (At5g49620), modulates ROS accumulation and hypersensitive response-like cell death, by the control of transcription of ROS-and defense-related genes [245]. Moreover, *MYB* genes are upregulated at the stage of nucellar PCD initiation during pollen chamber formation in *Ginkgo biloba* L. ovules [246]. In the deathosome revision presented in this work, At5g08520 (MYBS2), already described as an activator in sugar and ABA signaling pathways, appears interacting with MC1, LSD1, LOL1, and LOL2 (Fig. 2) [33,247].

3.10. Basic leucine-region zipper (bZIP)

The basic leucine zipper (bZIP) family of DNA-binding proteins is evolutionarily conserved in diverse eukaryote organisms, such as animals, plants, and fungi [248]. bZIP TFs can homodimerize and heterodimerize to form several dimers, regulating transcription. The proteins of the bZIP TFs family have a leucine zipper dimerization interface, the leucine zipper, and a conserved DNA-binding domain (BD) rich in basic amino acid residues, such as lysine and arginine [249,250]. The leucine zipper motif consists in the presence of hydrophobic amino acid residues, mostly leucine, which are spaced seven amino acids, with repetition of at least three times (LxxxxxxLxxxxxxL) [250,251].

In humans there are seven bZIP protein families, identified as Jun (v-jun avian sarcoma virus 17 oncogene homolog), Fos (FBJ murine osteosarcoma viral oncogene homolog), ATF (activating transcription factor), CREB (cAMP-responsive element binding protein), C/EBP (CCAAT/enhancer binding protein), Maf (avian musculoaponeurotic fibrosarcoma virus homolog), and PAR (proline and acidic rich), being related to PCD [248,249,252]. PAR subfamily of bZIP TFs have a role in the transcriptional regulation of aBH3-only proapoptotic gene [253]. A transmembrane bZIP Transcription Factor, BBF2H7 (BBF2 human homolog on chromosome 7)/CREB3L2 (cAMP-responsive element binding protein 3-like 2) is an endoplasmic reticulum (ER) stress transducer which protects neurons from ER stress-induced cell death. Neuroblastoma cell lines overexpressing BBF2H7 presented attenuated ER stress-induced cell death, while knockdown of BBF2H7 can enhance the ER stress-induced cell death [254]. In the nematode *Caenorhabditis elegans*, CES-2 (cell death specification-2) encodes a bZIP transcription factor, which regulates PCD [255]. CES-2 is an upstream regulator of the core apoptotic cell death pathway, directly regulating the expression of key autophagy-related genes [256]. In the filamentous fungus, *Podospora anserine,* IDI-4 (Induced During Incompatibility) was characterized as a gene encoding a bZIP transcription factor that regulated autophagy and cell death [257].

In plants, the members of the bZIP TFs are involved in the regulation of many biological processes, such as plant growth and development, encompassing morphogenesis and seed formation, hormone signaling, light signaling, senescence, vascular development, as well in response to abiotic and biotic stress [258–266]. The Arabidopsis genome contains 78 members of the bZIP family classified into 13 groups []; some of them are involved in PCD [267]. Under excess of red light (RL), HY5 (elongated hypocotyl 5), a bZIP TF acts as a positive regulator of RL-triggered PCD. HY5 interacts with phytochrome B (phyB) and directly binds the promoter of EDS1 to enhance its expression, which leads to ROS release, SA accumulation, and light-induced PCD [268]. The member of bZIP TFs, G-BOX BINDING FACTOR1 (GBF1), has a negative effect on *CAT2* expression and a regulatory function in leaf senescence in Arabidopsis, likely controlling the intracellular H_2O_2 level [269]. GBF1 also negatively regulates the *CAT2* expression induced by *Pseudomonas syringae*, and, therefore, is a positive regulator of the pathogen-induced HR. Binding to the intron of *PAD4*, GBF1 positively regulates its expression. Elevated expression of *PAD4* and reduced expression of *CAT2* enhanced disease resistance in Arabidopsis [270].

AtbZIP10 (At4g02640) is a transcription factor that shuttles between the nucleus and the cytoplasm, acting as a positive mediator in basal defense, and hypersensitive response via expression of target genes. AtbZIP10 is a positive regulator of cell death. LSD1 acts as an anchor protein retaining AtbZIP10 in the cytosol. Without the nuclear import of bZIP10, its activity in HR responses and basal defense is restricted. In the deathosome, AtbZIP10 appears interacting with LOL1, MC1, and LSD1(Fig. 2) [33,78].

3.11. Additional AtLSD1 deathosome gene family partners

Some of the interaction partners described in AtLSD1 deathosome belong to families with no direct relationship with PCD. However, they have been identified as interaction partners of essential and well-known regulators of HR-related PCD in plants. Therefore, they are considered as part of the process, even though the mechanism of their actions is not known.

The plant hormone auxin is an essential regulator of several developmental processes. Changes in auxin levels lead quick responses involving many classes of early auxin-responsive genes, including the Auxin/Indole-3-Acetic Acid (Aux/IAA) family, the auxin response factor (ARF) family and others [271]. The *Aux/IAA* gene family are primary auxin-responsive genes characterized by encoding short-lived proteins that have nuclear localization [272–275]. Mutations in Aux/IAA proteins affect a variety of developmental processes, showing the essential roles these proteins have in plant development, such as root initiation, elongation and number, hypocotyl and shoot growth, organ development, and fruit ripening [271,276–279]. IAA8 (AT2G22670) interacts in the deathosome with LOL1, LOL2, LSD1, and MC1 (Fig. 2), and is involved in auxin-dominated developmental processes, related to the formation of lateral roots [33,272].

The plant proteins PRPs (proline-rich proteins) are cell wall proteins characterized by the presence of repetitive proline-rich sequences [280,281]. PRP proteins are expressed in a spatially and temporally regulated manner in diverse plant species playing significant roles during plant growth, development, and in response to adverse environmental stimuli [281–284]. When plants suffer pathogens attack, PRPs act by interconnecting with other cell wall components to form a protective layer at the site of the infection [280]. At5g45350 (PRP), a proline-rich protein with unknown function, was identified as a member of the deathosome interacting with Metacaspase1 and LOL1 (Fig. 2) [33,285]. At2g41420, known as WIH2 (WINDHOSE2), is a proline-rich protein interacting in the deathosome with LSD1 and Metacaspase1 (Fig. 2) [33]. WIH2 and WIH1encode small peptides present in plants and fungi, acting together in the transition from somatic to reproductive cell fate. These genes have relevant importance in the formation of Megaspore Mother Cell (MMC) [286].

In addition to the families above, there are other interaction partners described in the deathosome that belong to distinct families not well characterized until now. Initially discovered in rat liver, 49-kDa TBP-Interacting Protein (TIP49) is similar to the bacterial DNA helicase RuvB and serve as an autoantigen in human [287–289]. In Arabidopsis, the T-DNA insertion lines that present low mRNA levels of *AtTip49a* (AT5G22330), which interacts with LSD1 and LOL2 in deathosome (Fig. 2), increases the function of RPP5 and RPP2, acting as a negative regulator of some R-dependent responses. Also, it was showed that ATip49a is necessary to normal Arabidopsis development, since morphological defects, especially in the viability of the sporophyte and the female gametophyte, were observed in tip49a lines [33,290]. At1g75690, LQY1 (Low Quantum Yield of photosystem II 1) is a zinc finger protein with disulfide isomerase activity that under high light operates to protect the PSII activity controlling the restoration and reassembly of photodamaged PSII proteins [291]. LQY1 has as interaction partners LOL1, LOL2, and LSD1 (Fig. 2) [33]. Other two proteins that interact only with LSD1 in the deathosome, At1g06460, ACD32.1 (Alpha-Crystallin Domain 32.1), and At5g63135, TTF (Transcription Termination Factor), have no defined function so far (Fig. 2) [33].

EDR1 has already been mentioned previously as a new member of the deathosome interacting with EDS1 and PAD4 (Fig. 2) [148]. Another interaction partner of ERD1 is ATL1 (Arabidopsis Toxicos en Levadura1), a RING-finger E3 ligase. In Arabidopsis, overexpression of ATL1 (At1g04360) restricts growth while in *Nicotiana benthamiana* promotes cell death and tissue collapse. ATL1 is a positive regulator of cell death by ubiquitination events upon negative regulators (unknown proteins) of PCD. The ATL1-EDR1 interaction occurs in the TGN/EE (trans-Golgi network/early endosome), and perhaps by direct phosphorylation, EDR1 inactivates ATL1. Therefore, EDR1 negatively regulates ATL1 to repress ATL1-mediated cell death in *A.thaliana* and *N. benthamiana*. ATL1 is also necessary for *edr1*-mediated resistance, controlling plant cell death, defenses, and growth [292]. In the new version of the AtLSD1 deathosome, ATL1 is interacting with EDR1 (Fig. 2).

RD21 works as a negative regulator of FB1-induced cell death in Arabidopsis [293]. RD21 also interacts with other proteins acting in other types of PCD, such as AtWSCP (Water-Soluble Chlorophyll-binding Protein), which is a Kunitz-type protease inhibitor, that regulates cell death during flower development in *A. thaliana*. Early flower cell death was observed in *wscp* knockout mutant. In this sense, WSCP (At1g72290) is related to cell death control in the female reproductive organ by physically interacting with RD21. This interaction blocked the activity of RD21 as cysteine protease and pro-death protein [294]. Another interactor of RD21 identified by the Y2H screen was PDI5 (Protein Disulfide Isomerase-5), encoded by At1g07960 [295]. The expression of PDI5 occurs in endothelial cells fated to die in developing seed tissues. The subcellular location of PD5I is the endoplasmic reticulum (ER), protein storage vacuoles (PSVs), and lytic vacuoles (LVs) of endothelial cells. PDI5 function was related to the correct development of seeds and in regulating the timing of PCD. The control of PCD in endothelial cells occurs via the role of PDI5 as a chaperone, associating with RD21 and, inhibiting their protease activity, until the onset of PCD. With this, PDI5 could protect organelles from degradation [295].

In addition to the genes mentioned above, in a recent study, 38 LSD1 interaction partners were identified through Tandem Affinity Purification (TAP) [67]. In this experiment, the authors induced PCD responses by exogenously applying H_2O_2, which is a hallmark of cell death by acting as a signaling molecule [296,297]. It was observed that LSD1-interactome is highly condition-dependent, once LSD1 was able to interact with 25 proteins in non-oxidative (0 mM H_2O_2) condition, 16 proteins (AT5G26830, AT5G56680, AT3G62120, AT4G09000, AT1G65930, AT2G47510, AT3G29360, AT3G09820, AT3G23810, AT4G13930, AT5G16990, AT3G48730, AT4G20850, AT3G54470, AT5G56350, AT1G10390) when cells were treated with 10 mM H_2O_2 and only the threonyl-tRNA synthetase (AT5G26830), UDP-glucose 6-dehydrogenase (AT3G29360), and the pyruvate kinase (AT5G56350) were found in the combination of both conditions. The oxidative treatment used in the TAP experiment resulted in the purification of LSD1 interaction partners related to abiotic and biotic stresses, transport, and energy, indicating its role in the intensive regulation of signaling pathways when under stress conditions and in the restriction of plant growth and development. Moreover, in control conditions, the identified proteins are related to several distinct biological functions, which may indicate that LDS1 is involved in other pathways besides PCD, such as cell division, cell wall formation, and embryo development as well in ubiquitination and methylation, being important to plant growth [67].

4. Advances in AtLSD1 deathosome network

Here we present the updated version of AtLSD1 deathosome, based on interaction data of proteins related to the genetic control of PCD in Arabidopsis. Both LSD1 and MC1 possibly present a key role in AtLSD1 deathosome, physically interacting with highly diverse protein families involved with HR-PCD in Arabidopsis (Fig. 2). However, the number of genes belonging to AtLSD1 deathosome is probably bigger than currently described in the literature. Besides the advances in the understanding of the genetic control of PCD in plants, several unsolved questions still need to be experimentally investigated.

It is not clear if dPCD and ePCD are under the same genetic control. If these two categories of PCD in plants share the same mechanisms of action is unclear. The identification of the central genes that are

coregulated would be assisted by investigating the transcriptome data related to the particular type of PCD [13]. The transcriptome profiles of the various stages of development and adverse environmental conditions that lead to PCD were compared [10]. Different sets of regulated genes were identified during the environmental and development-induced conditions of the plant. It was observed that the dPCD marker genes, e.g., CEP1, PASPA3, BFN1, MC9, and SCPL48 are not up-regulated during ePCD [10]. In the uptade deathosome, EDR1 and RD21 are examples of genes that, according to their protein interaction partner, have functions related to ePCD and also dPCD.

Therefore, additional experiments to to define if dPCD and ePCD are under the same regulatory mechanisms or not are required.

Several genes described in AtLSD1 deathosome belong to large gene families containing a high number of members. The genetic and physiological redundancy effect of genes belonging to the same gene family was not thoroughly studied. Contrary, members of the same gene family might be involved in specific PCD responses, not even evaluated.

PCD, as an essential developmental process in plants, is a tightly regulated process. The majority of fine-tune regulation is not currently known. We still have a long way to understand the PCD process in plants. That is a considerable challenge for the plant science community to understand the regulatory mechanisms involved with the PCD process. The understanding of PCD provides useful raw material for crop breeding programs, once PCD is necessary for normal plant growth and development. As a result, the development of plants with high adaptability potential against biotic and abiotic stresses through genetic engineering may be favored.

Declaration of Competing Interest

None.

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Capítulo II

Caracterização molecular e funcional do gene At1g52200 (*AtPLAC8-11.1*) envolvido na morte celular programada.

Neste capítulo é apresentado o manuscrito de um artigo que está em processo de preparação e refere-se ao estudo funcional do gene At1g52200 (*AtPLAC8-11.1*). O título desse artigo é "MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF *Arabidopsis thaliana AtPLAC811*, a PLAC8 Protein encoding gene", e será submetido a uma revista científica na área de genética vegetal.

Research: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF *Arabidopsis thaliana AtPLAC811,* a PLAC8 Protein encoding gene.

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Abstract

Several studies were performed to understand the mechanisms related to Programmed Cell Death (PCD) in plants, mainly using *Arabidopsis thaliana* as a model. The "AtLSD1 - deathosome" presents a network controlling Hypersensitive Response-mediated cell death in *Arabidopsis thaliana*, and a gene encoding protein with PLAC8 motif (Placenta-specific 8), *AtPLAC811*, has been described as "AtLSD1-deathosome" component. Here we found that the subcellular localization of AtPLAC8-11.1 and its splicing variant AtPLAC8-11.2 in protoplasts showed a profile varied in both variants according to C- and N-terminal GFP fusion, having co-localization with the Endoplasmic reticulum marker for both forms. The expression and localization of AtPLAC811.1 and AtPLAC811.2 under native promoter in Arabidopsis showed the same tissue-specific expression regarding root and leaf, but different subcellular localization was observed, as at the bases of trichomes to AtPLAC811.1. Furthermore, AtPLAC811.1 was highly expressed in sepal and stamen in comparison to AtPLAC811.2 and both forms under native promoter had increased expression in roots after treatment with the Pep1 elicitor compared to their respective controls. Yeast two-hybrid assay (Y2H) showed that only AtPLAC811.1 interacted with AtLSD1 protein and by bimolecular fluorescence complementation (BiFC) assay, the reconstructed YFP was visualized in spots in undefined format spread across the cell. Transactivation experiments showed that AtPLAC811.1 interaction with AtLSD1 interfered with the AtLSD1 activity as a transcriptional regulator. In addition, the absence of AtPLAC8 in the *plac8* knockout line alters the expression of genes related to dPCD and ePCD.

Keywords: PLAC8 (Placent-specific), *Arabidopsis thaliana*, Deathosome Programmed Cell Death

Abbreviations. PCD: programmed cell death dPCD: development programmed cell death ePCD: environmental programmed cell death ROS: reactive oxygen species Y2H: yeast-two-hybrid assay LSD1: lesion simulated disease1

1. Introduction

Plant cells use a set of regulatory pathways to program cell death during plant development, and/or in response to pathogens and stress signals, in order to control cell number, eliminate damaged cells and maintain cellular homeostasis [1–3]. Cell death can be driven by an active, conserved, and genetically programmed signaling pathway known as programmed cell death (PCD) [4,5]. Researchers have proposed a network called "LESION SIMULATING DISEASE1 (LSD1 - At4g20380) deathosome", composed of several genes related to controlling hypersensitive response-mediated cell death in *Arabidopsis thaliana* [6]. Results from yeast two-hybrid assay have previously described the interactions between the main known cell death regulators, AtLSD1 and AtMetacaspase1, with their interaction partners [6]. Recently, an updated version of AtLSD1 deathosome was proposed with a description of the gene families involved with the regulation of the HR-related PCD in plants [7]. Two genes encoding proteins belonging to PLAC8 family (Placenta-specific 8), At1g52200 (*AtPLAC811*) and At4g23470 (*AtPLAC817*), interact with AtLSD1 and have been described as "AtLSD1 deathosome" components [6].

Proteins containing PLAC8 motif form a large family whose members can be found in fungi, algae, higher plants and animals [8]. Through the cysteine-rich domain that characterizes PLAC8 proteins (Pfam PF04749), 445 sequences were recovered and a new classification of the PLAC8 family was proposed into three types: type I, II and III. Type I genes are found in mammals, fungi, plants and algae, and are the best characterized to date. PLAC8 type II and III genes are unique to plants, and type III genes have not yet been functionally characterized [9]. Genetic alignment between PLAC8 types I, II and III proteins, encoded by the 17 members of the PLAC8 family found in *Arabidopsis thaliana*, At1g52200 appears as number 11, belonging, therefore, to the PLAC8 type I genes, receiving the name of *AtPLAC811* [9].

Several studies have found some members of PLAC8 family, such as *AtPLAC811*, in experiments involving stress and PCD. The peptide CLE42 is a negative regulator of leaf senescence, and in the transcriptome analysis of 21-d-old leaves of Col-0 and plants overexpressing CLE42, four genes belonging to the PLAC8 family were differentially expressed in plants overexpressing CLE42 compared to wild-type [10]. AtPLAC811 was up-regulated in

aging *Arabidopsis* papilla cells transcriptome analysis, indicating possible involvement in the regulation of papilla cell death [11].

Arabidopsis thaliana phototropins, *PHOT1* and *PHOT2,* have a novel role in UV-C induced photooxidative stress responses, and cell death [12]. In response to UV-C treatment, they observed the induction of genes, such as AtPLAC811, which were also up-regulated after blue light treatment in *phot1* and *phot2* mutants [12]. A study was concerned in known how gene expression is altered in space [13]. Microarray results showed that 249 genes were differentially expressed in samples in spaceflight with 1g centrifugal force (F 1g) in comparison to samples in ground 1g control (G 1g), including AtPLAC811, suggesting that plants might respond to space radiation via PCD[13]. *AtPLAC811* was also significantly regulated with increased expression by ozone (O_3) treatment, which is used to study apoplastic ROS signaling and verify its role in cell death and defense signaling [14].

In the present study, we investigate the possible role of the plant PLAC8 protein encoding gene, *AtPLAC811* in the PCD process. We confirm that AtPLAC811.1 directly interacts with AtLSD1, and partially co-localizes with AtLSD1 in *Arabidopsis* mesophyll protoplast. Overexpression of *AtPLAC811* was only possible using an inducible overexpression system. Therefore, we present evidence that the AtPLAC811 protein is involved in the regulation of PCD process in plants.

2. Results

2.1 Identification of the plant PLAC8 domain

The alignment between PLAC8 proteins of types I, II, and III, encoded by the 17 members of the PLAC8 family found in *Arabidopsis thaliana*, allowed the classification of the At1g52200 gene as number 11, called then *AtPLAC8-11.1* [9]. There was a deposit of a splicing variant predicted sequence for the At1g52200 gene, designated At1g52200.2 or *AtPLAC811.2* [15]. Both variants of *AtPLAC8-11* were amplified from *Arabidopsis thaliana* cDNA using specific primers (Supplementary Figure 1). To our knowledge, this is the first experimental confirmation of the existence and amplification of the alternative splicing form *AtPLAC8-11.2*. The genomic sequence of the AtPLAC811 gene has four exons and three introns (Figure 1a), which splicing results in the canonical transcript *AtPLAC8-11.1*. This canonical transcript encodes a peptide sequence of 190 amino acids (Figure 1b). The alternative splicing resulted in a transcript (*AtPLAC8-11.2*) in which the last intron has been retained during mRNA processing and introduces a stop codon (TGA) to the open reading frame. As a result, the alternative splicing coding region has a peptide sequence of 187 amino acids (Figure 1c). The domain that characterizes PLAC8 proteins is found in both the AtPLAC811.1 and AtPLAC811.2 proteins, between amino acids 53 and 151 (Figures 1b and c). A transmembrane region was predicted between amino acids 94 and 113 in both forms (Figures 1b and c). The transmembrane domain prediction combined with transmembrane topology and signal peptide predictor reveals in both forms of the AtPLAC811 proteins a non-cytoplasmic region between amino acids 1 and 93 and a cytoplasmic region between amino acids 114 and 190 (Supplementary Figure 2).

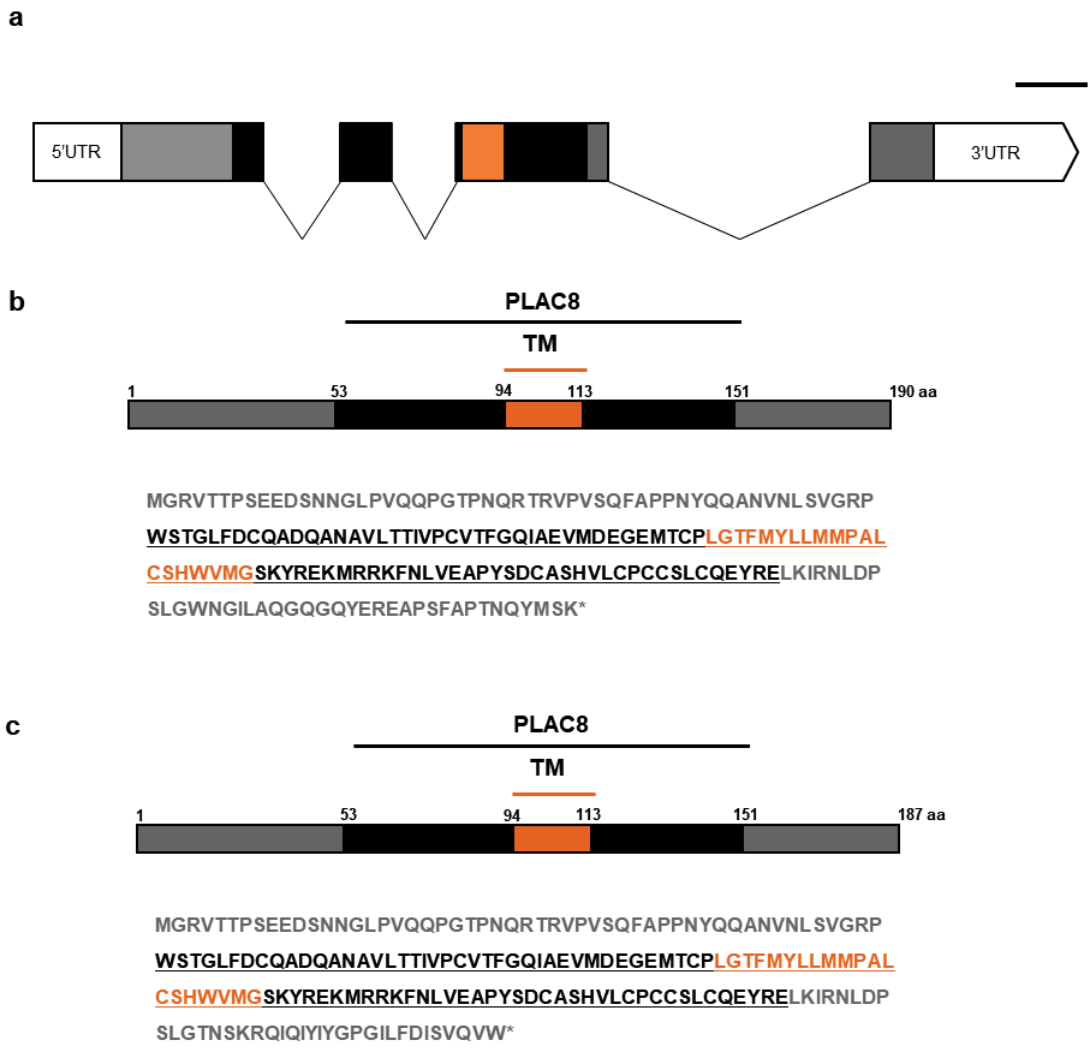


Figure 1. AtPLAC811 gene structure and peptide sequence. (a) Schematic diagram of AtPLAC811 genomic sequence. Exons are represented by gray boxes and introns by gray lines. **(b)** AtPLAC811.1 amino acid sequence. PLAC8 domain, predicted, is represented in black and it comprises the amino acid residues 53 to 151. The putative transmembrane domain is represented in orange and it comprises the amino acid residues 94 to 113. **(c)** Splicing variant AtPLAC811.2 amino acid sequence. PLAC8 domain was predicted by the SMART program (http://smart. embl-heidelberg.de/) and the transmembrane domain was predicted by the TMHMM – 2.0 program (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0).

2.2 AtPLAC811 subcellular localization in *Arabidopsis* protoplasts

The subcellular localization of AtPLAC8-11.1 and AtPLAC8-11.2 in *A.thaliana* protoplasts was investigated. *In silico* prediction using ePLANT (http://bar.utoronto.ca/eplant/) indicates AtPLAC811 subcellular localization in the plasma membrane [16]. To test this hypothesis, firstly, *Arabidopsis* protoplasts were cotransformed using a plasma membrane organellar marker (pBIN20) with an RFP fusion, and plasmids containing N- and C-terminal GFP fusion (PMDC43 and PMD83 respectively), as described in Figure 2a. We observed no colocalization between AtPLAC8111.1 and AtPLAC811.2 with the plasma membrane marker (Supplementary Figure 3a). The observed subcellular localization profile varied in both variants, according to tagging GFP at the N terminus or C terminus. With N-GFP fusion, the fluoresce was spread throughout the protoplast with non-uniform structures dispersed in the cell (Figure 2 i and ii). A single large structure or multiple undefined structures in the cell was observed with C-GFP fusion (Figure 2 iii and iv). Hereupon, to continue the investigation of the AtPLAC811 subcellular localization, we use other pBIN20 organelle-specific markers. It was possible to see co-localization with the Endoplasmic Reticulum (ER) marker for both AtPLAC811.1 and AtPLAC811.2 using GFP at the N terminus (Figure 2c). Golgi was also unable to co-localize with the proteins in the study (Supplementary Figure 3b).

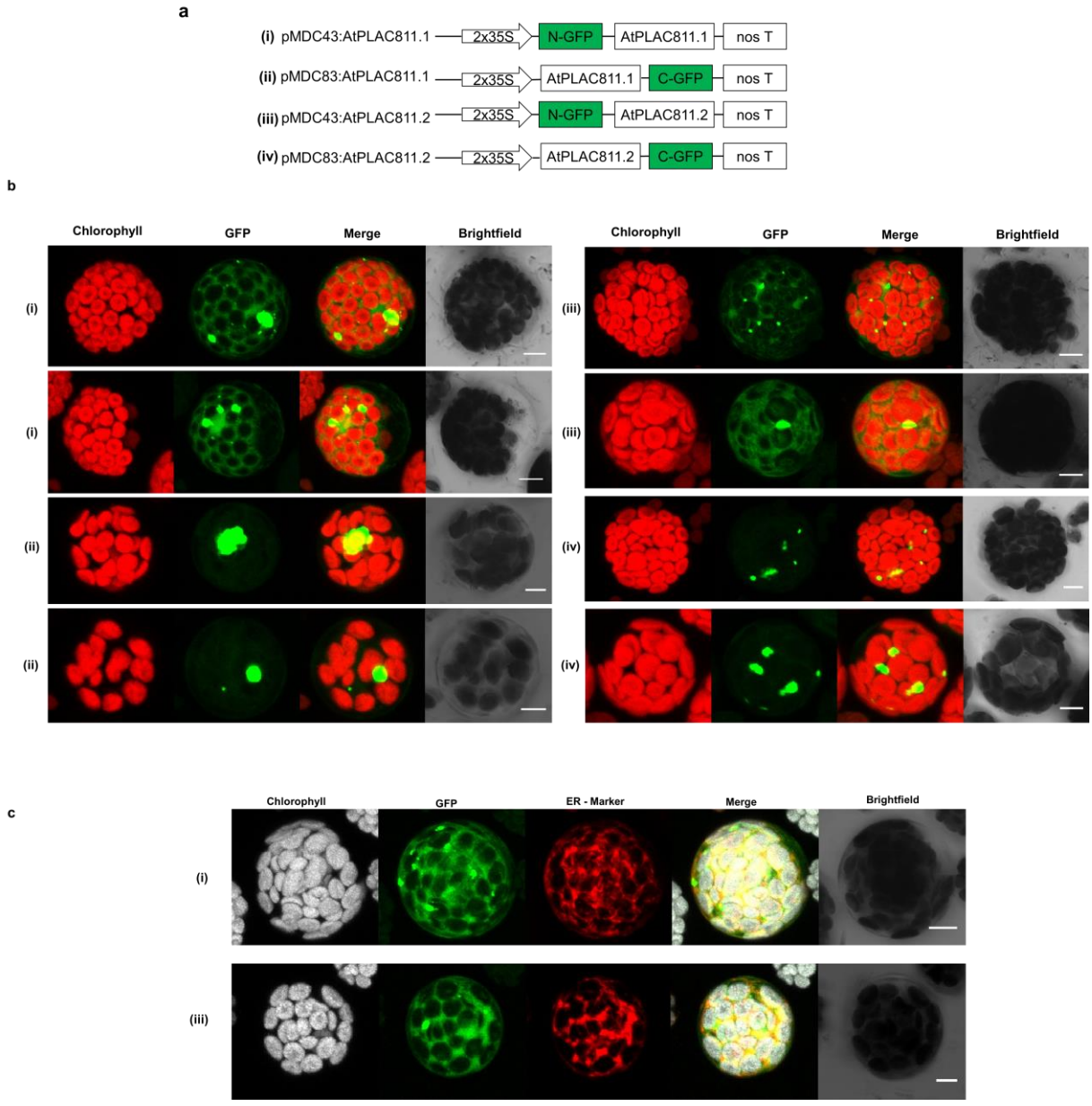


Figure 2. AtPLAC811 subcellular localization. (a) Schematic diagram of pMDC43 and pMD83 vectors. AtPLAC811.1 and AtPLAC811.2 were fused to pMDC43 and pMDC83 vectors with an expression of amino-terminal GFP fusion and carboxi GFP fusion respectively. **(b) Protein subcellular localization of AtPLAC811.1 and AtPLAC811.2.** Confocal microscope images of transient expression assay performed on *A. thaliana* mesophyll protoplasts isolated from wild-type plants. Protoplasts were co-transformed with vectors

described in (a). The chlorophyll autofluorescence is represented in the red columns, the GFP fluorescence in the green column, and the combination of chlorophyll autofluorescence and GFP fluorescence is shown in the merge column. **(c) AtPLAC811.1 and AtPLAC11.2 co-localizes with endoplasmic reticulum.** Constructs showed in (a-ii) and (a-iv), and an endoplasmic reticulum organelle-specific marker (pBIN20 ER) were transfected into *Arabidopsis* protoplasts. The GFP signal was detected with a confocal microscope. The merge indicates the co-localization between AtPLAC811.1 and AtPLAC811.2 with the endoplasmic reticulum marker. Scale bars 5µM.

2.3 Localization of AtPLAC811.1 and AtPLAC811.2 under native promoter in *Arabidopsis* cells

To perform the visualization in live tissues of AtPLAC811 proteins, we generated transgenic plants expressing the genomic sequence of AtPLAC811.1-YFP and AtPLAC811.2-YFP fusion driven by its native promoter transformed into *plac811* knockout background (Figure 3a). The construction also has an engineered promiscuous biotin ligase TurboID (TbID) method for the investigation of protein-protein interactions. The protein levels in homozygous T3 transgenic plants were analyzed by western blotting, and 35S:YFP-TbID empty vector was used as a positive control (Figure 3b). The results demonstrate that AtPLAC811.1 has a higher abundance level in comparison to AtPLAC811.2 in leaves of 20-day-old transgenic plants (Figure 3b). Furthermore, the AtPLAC811.1 transgenic plant driven by the 35S promoter shows the lowest level of expression, possibly evidencing gene silencing (Figure 3b). According to Figure 3 (i, ii, and iii), confocal microscopic analyses of stable *Arabidopsis* transformant *promoPLAC811:PLAC811.1-YFP-TbID* showed tissue-specific gene expression in root and leaf, located surrounding the membrane in the root (3c i) with granular points spread throughout the cell. Fluorescence was also detected surrounding membranes in the leaf epidermal and stomata guard cells (3c ii) and also at the bases of trichomes (3c iii and Supplementary Figure 4). The construction *promoPLAC811:PLAC811.2-YFP-TbID* has expression in the root (3c iv) and interestingly inside of pair of guard cells (3c v), and no expression was detected in trichomes (3c vi).

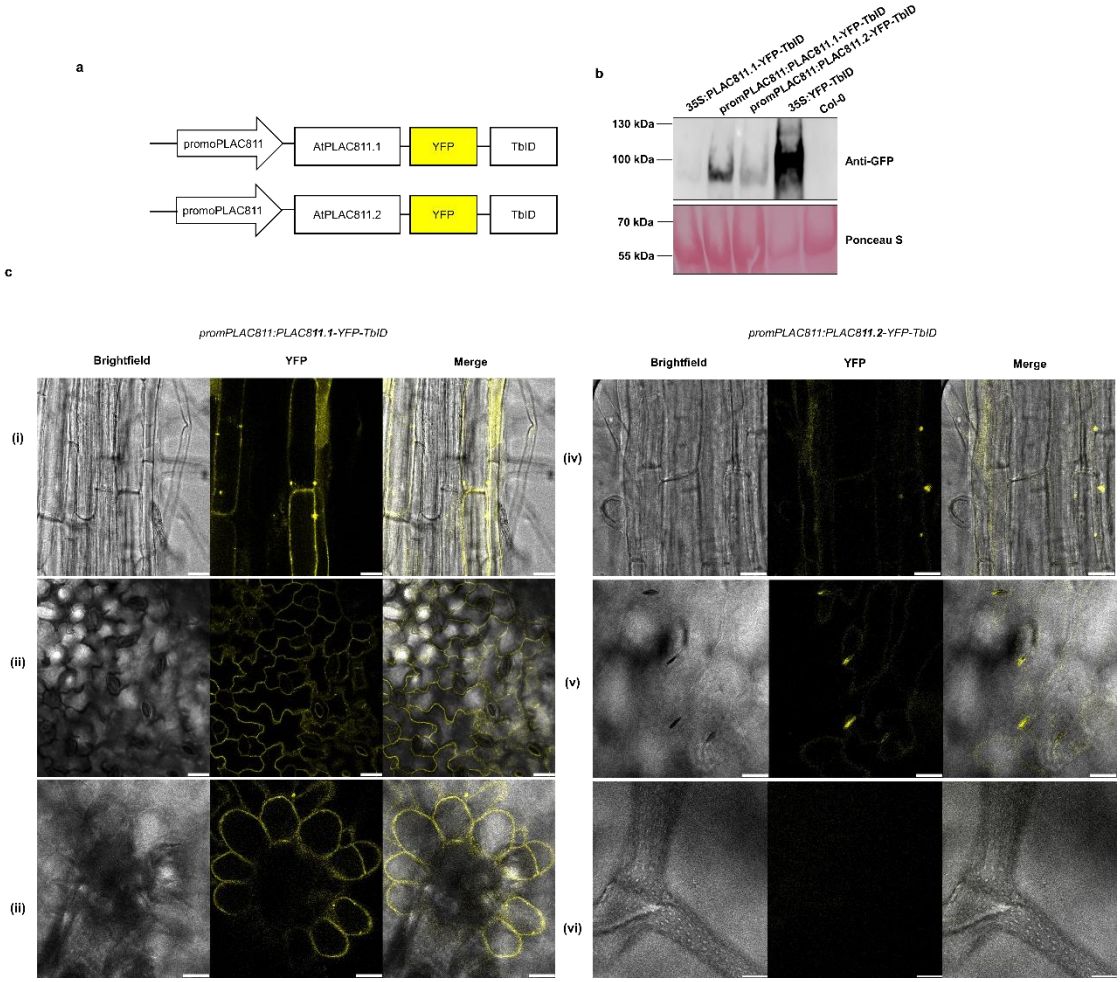


Figure 3. Subcellular localization of AtPLAC811.1 and AtPLAC811.2 under native promoter. (a) Schematic diagram of the TurboID vectors. Full-length genomic AtPLAC811.1 and AtPLAC811.2 driven by 2kb of its native promoter were fused to YFP-tagged TurboID. **(b) Western blot analysis for protein expression in the leaves relative to construction in letter (a).** Western blot analysis of protein extracts from leaves of 20-day-old transgenic plants using an anti-GFP antibody. The Ponceau staining of the Rubisco band (55 kDa) was used as a loading control. **(c) Subcellular localization of AtPLAC811.1-YFP and AtPLAC811.2-YFP in different tissues of *Arabidopsis* thaliana seedlings.** Seedlings of 10-day-old grown in MS medium were observed by confocal microscope. Localization of AtPLAC811.1-YFP in the root (i), leaf epidermal and stomata guard cells (ii) and trichome (iii).

Localization of AtPLAC811.2-YFP in leaf stoma guard cells (v) and trichome (vi). Scale bars 20µM.

We also examined the root subcellular localization of the fusion protein in the *promoPLAC811:PLAC811.1-YFP-TbID* and *promoPLAC811:PLAC811.2-YFP-TbID* *Arabidopsis* plants exposed to the plant elicitor peptide 1 (Pep1). Pep1 is an endogenous peptide elicitor that promotes the expression of pathogen defense genes[17,18]. A higher fluorescence was observed in AtPLAC811.1 and AtPLAC811.2 roots treated with 100 mM Pep1 compared to their respective controls (Figure 4), indicating the responsiveness to treatment and its possible involvement in defense responses. The literature also indicates that Pep1 promotes morphological alterations in roots, as cells welling in the root transition zone (TZ) [19]. Transgenic plants harboring *promoPLAC811:PLAC811.1-YFP-TbID* showed more induced swollen cells in the TZ in comparison with its splicing variant (Supplementary Figure 5).

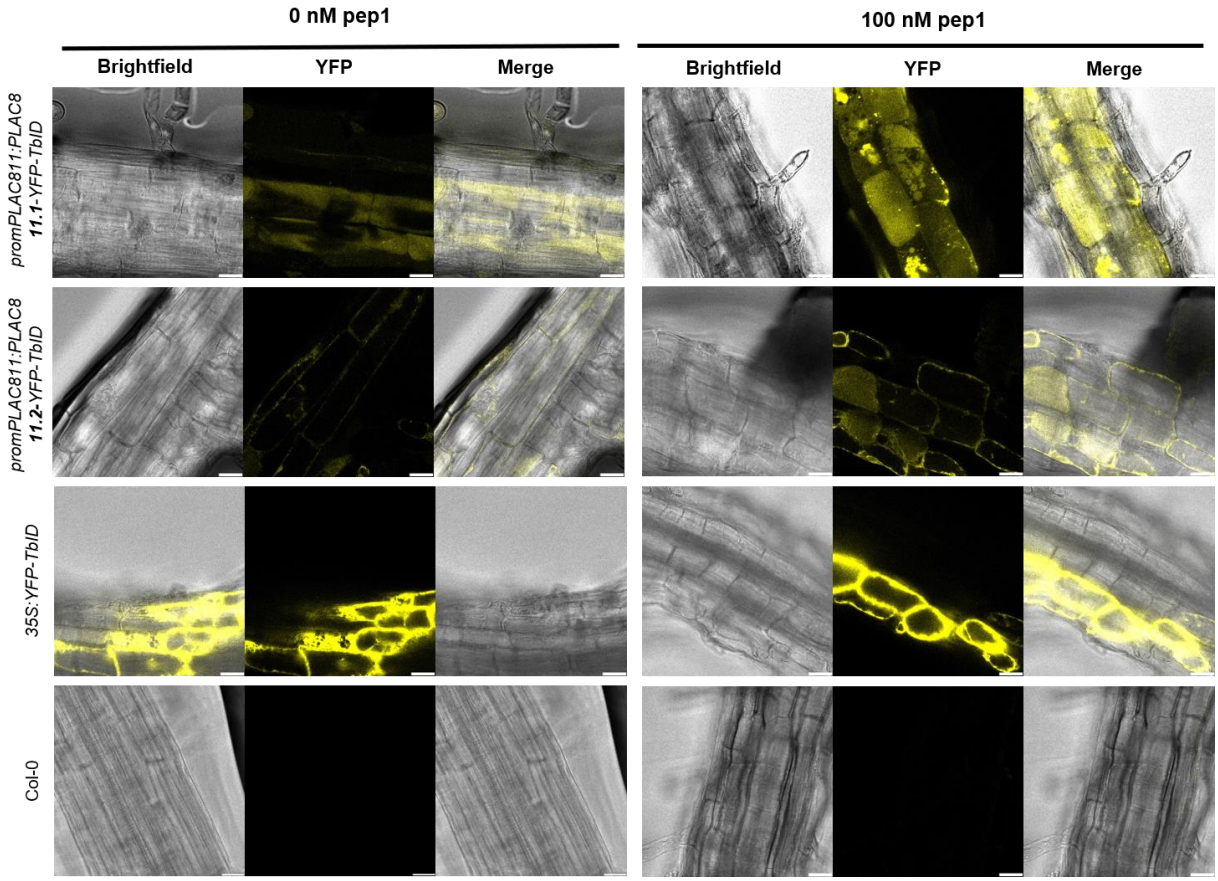


Figure 4. Root subcellular localization of AtPLAC811.1 and AtPLAC811.2 in response to Pep1. Seedlings of 10-day-old grown on half-strength MS agar medium without or with 100 nM of pep1 were observed by confocal microscope. *35S:YFP-TbID* was used as a positive control and Col-0 as a negative control. The experiments were repeated two times with similar results. Scale bars 20µM.

2.4 Expression of *AtPLAC811.1* and *AtPLAC811.2* under native promoter in *Arabidopsis* flowers

The literature indicates that *AtPLAC811* was differentially expressed in the sepal as compared to the petal [20]. In order to gain further insight into the AtPLAC811.1 expression pattern in *Arabidopsis* flowers, the flower of 5-week-old transgenic plants harboring *promoPLAC811:PLAC811.1-YFP-TbID* was separated into their different floral parts and was visualized at confocal microscopy (Figure 5). We observed strong fluorescence at sepal (i), anther + filament of stamen (ii), and stigma (iii). A weaker fluorescence was observed on the leaf (iv) and no fluorescence was observed on the petal (v). Interestingly, in the flower of the plant expressing the splicing variant, *promoPLAC811:PLAC811.2-YFP-TbID* was observed fluorescence only at the stigma (Figure 5 viii) and leaf (Figure 5 ix).

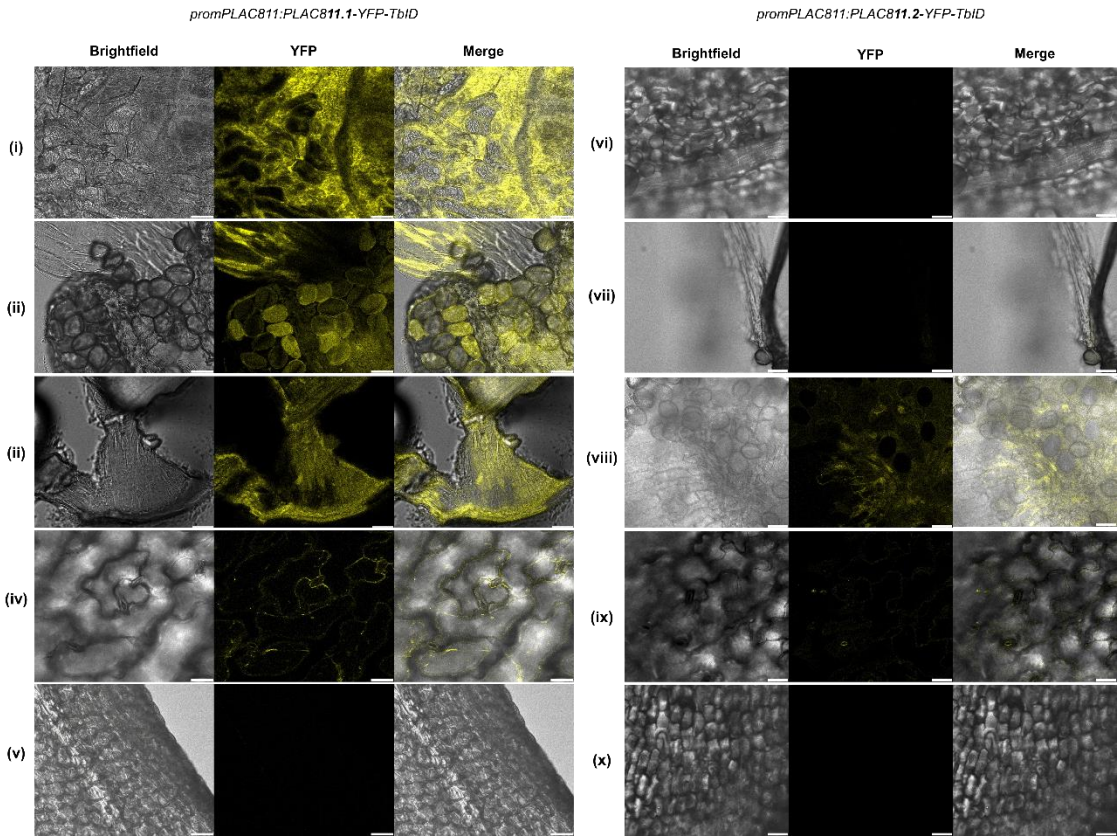


Figure 5. *AtPLAC811.1* and *AtPLAC811.2* expression patterns in flowers. Confocal observation in different types of floral organs (i-x). Flower of 5-week-old grown in soil was observed by confocal microscope. **(i-v)** Expression of AtPLAC811.1 driven by native promoter in sepal (i), anther + filament (stamen) (ii), stigma (iii) and leaf (iv). No fluorescence on the petal (v). **(vi-x)** Expression of AtPLAC811.2 driven by native promoter in stigma (viii) and leaf (iv). No fluorescence was detected in sepal (vi), anther + filament (stamen) (vii), and petal (x). Scale bars 20µM.

2.5 AtPLAC811 partially co-localizes with AtLSD1 and interacts with AtLSD1

The literature indicates that the LSD1 subcellular location is nucleo-cytoplasmic [21]. To understand how AtPLAC811.1 and AtLSD1 interact, we studied the subcellular locations in *Arabidopsis* protoplasts coexpressed with AtPLAC811.1:GFP and AtLSD1: YFP (Figure 6A).

Arabidopsis protoplasts were co-transformed and confocal images reveal partial colocalization of AtPLAC811.1 and AtLSD1 in the cytoplasm and non-uniform structure(s) in the cell. Our results are in agreement with the literature, regarding the LSD1 subcellular location, which is in the nucleus and cytoplasm (Figure 6B). However, the AtPLAC811.1 and AtLSD1 co-localization in the nucleus was not observed as shown in (Figure 6B see the white arrows). It is important to note that the non-co-localization between AtPLAC811.1 and AtLSD1 was observed in control conditions, without any treatment, as evaluated in this experiment.

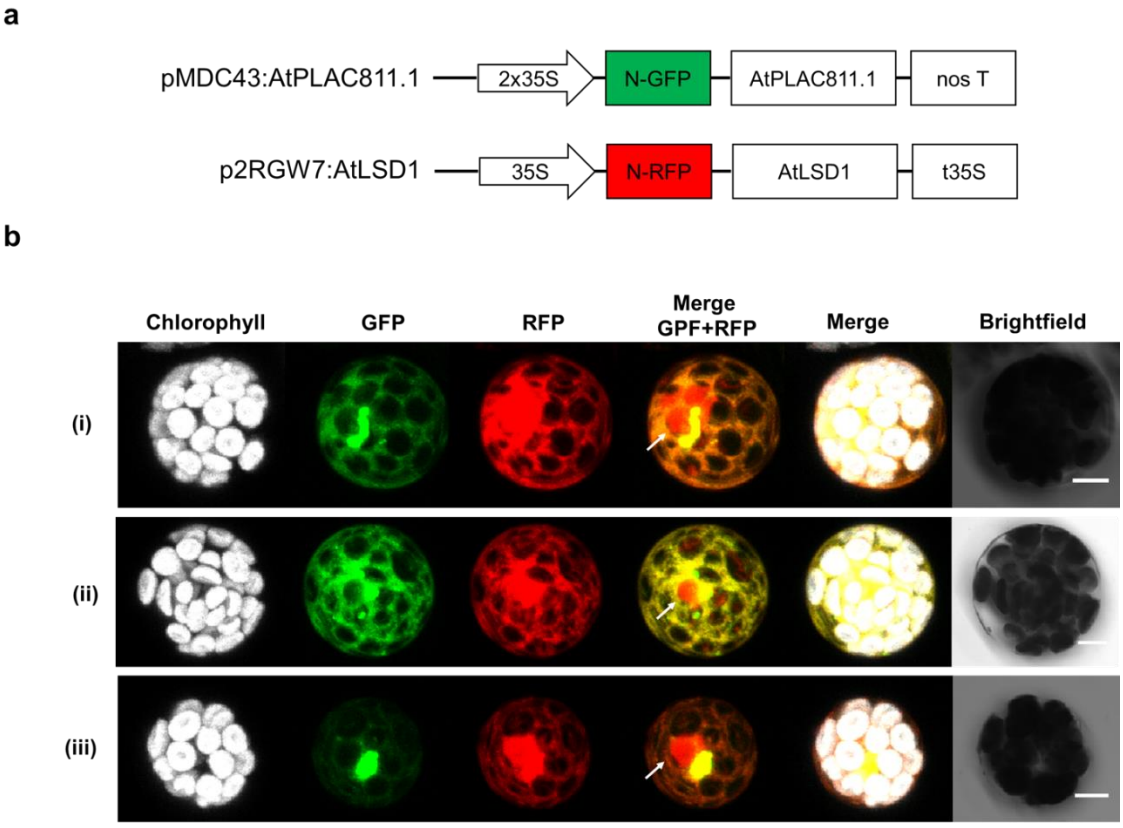
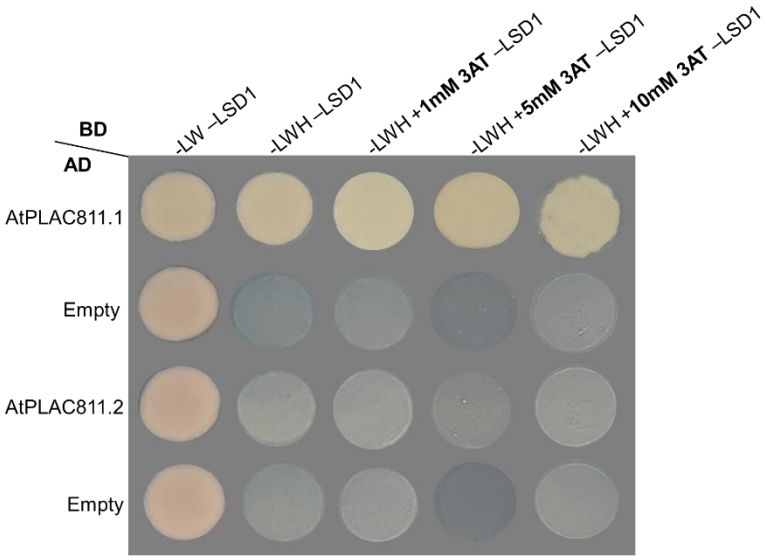


Figure 6. AtPLAC811.1 partially co-localizes with AtLSD1. (a) Schematic diagram of pMDC43:AtPLAC811.1 and p2RGW7 vectors. AtPLAC811.1 was fused to pMDC43 vector with expression of amino-terminal GFP fusion and AtLSD1 was fused to p2RGW7 vector with expression of amino-terminal RFP fusion. **(b) Protein co-localization of AtPLAC811.1 and AtLSD1.** Replicates of confocal microscope images of transient expression assay performed on *A. thaliana* mesophyll protoplasts isolated from wild-type (WT) plants. Protoplasts were co-

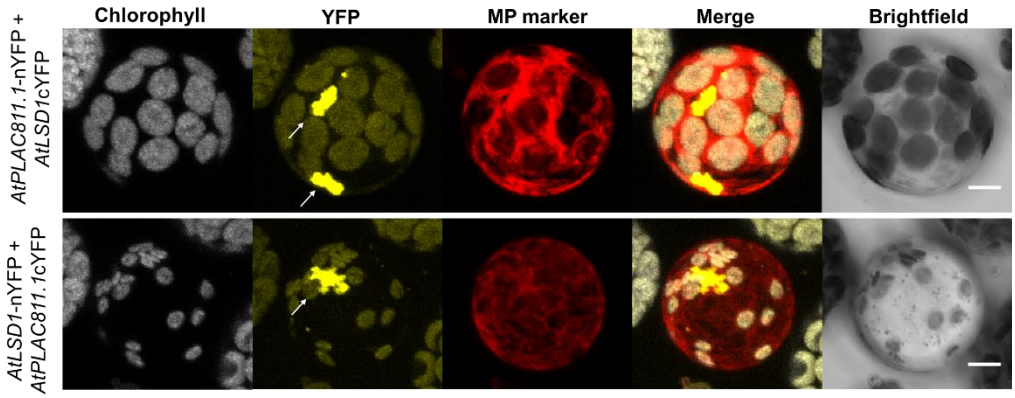
transformed with vectors described in (a). The fluorescence signal of AtPLAC811.1 is represented by GFP signals and AtLSD1 by RFP signals. The merge indicates partial co-localization between these two proteins in the cytoplasm. White arrows possibly indicate the nucleus, where there is no AtPLAC811.1 co-localization. Scale bar 5µM.

It is known that alternative splicing can generate isoforms with diverse interaction profiles regarding the interactome context, acting as a distinct protein [22]. To confirm the interaction between AtPlac811.1 and AtLSD1 revealed in the AtLSD1 deathosome proposed by [23] and verify a possible interaction between AtPlac811.2 and AtLSD1, we performed a yeast two-hybrid assay (Y2H). AtPLAC811.1 interact strongly with AtLSD1 protein (Figure 7a). Interestingly, in the alternative form of splicing, Atplac811.2, the retention of 80bp of the last intron and the exclusion of the fourth exon is sufficient to impair the interaction. These results suggest that AtPLAC8 interacts with AtLSD1 and the C-terminal region is essential to the interaction. We next performed bimolecular fluorescence complementation (BiFC) assay by co-expressing AtPLAC811.1-YFP^{N} and AtLSD1-YFP^{C} and the opposite in *Arabidopsis thaliana* leaves. As shown in Fig. 7b, reconstructed YFP was visualized in spots in undefined format spread across the cell. BiFC confirms the interaction between PLAC811.1 and LSD1 and the interaction occurs when YFP is reconstructed in the N-terminal portion. Also, the possible interaction between AtLSD1 and the other protein belonging to the PLAC8 family, AtPLAC817 (At4g23470), described as a component of the AtLSD1-deathosome was evaluated. AtPLAC817 and AtLSD1 interact in yeast cells, but the interaction weakens with the increasing addition of the competitive inhibitor of histidine synthesis (Supplementary Figure 6).

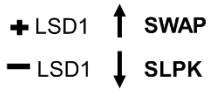
a


BD
AD
-LW –LSD1
-LWH –LSD1
-LWH +1mM 3AT –LSD1
-LWH +5mM 3AT –LSD1
-LWH +10mM 3AT –LSD1
AtPLAC811.1
Empty
AtPLAC811.2
Empty

b


Chlorophyll
YFP
MP marker
Merge
Brightfield
AtPLAC811.1-nYFP + AtLSD1cYFP
AtLSD1-nYFP + AtPLAC811.1cYFP

c


+ LSD1
SWAP
– LSD1
SLPK

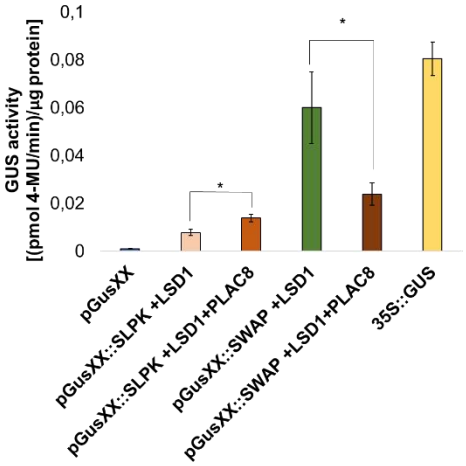

GUS activity
[(pmol 4-MU/min)/µg protein]
0,1
0,08
0,06
0,04
0,02
0
*
*
pGusXX
pGusXX::SLPK +LSD1
pGusXX::SLPK +LSD1+PLAC8
pGusXX::SWAP +LSD1
pGusXX::SWAP +LSD1+PLAC8
35S::GUS

Figure 7. AtPLAC811.1 interacts with AtLSD1. (a) Yeast two-hybrid assay. The CDS of AtPlac811.1 or AtPlac811.2 were cloned in the pDEST22 (AD = Activation Domain) destination vector and were used as preys. The destination vector pDEST32 (BD = Binding Domain) containing the AtLSD1 CDS was used as bait. Co-transformations of the empty vector with pDEST vectors containing the CDS of AtPlac811.1 or AtPlac811.2 were used as negative controls. Yeast cells grown on SD plates deficient in Tryptophan and Leucine (-LW) indicate successful co-transformations. Yeast cells were grown on SD plates deficient in Tryptophan, Leucine, and Histidine (-LWH) and supplemented with either 1, 5 or 10 mM of 3-Amino-1,2,4-triazole (3AT). **(b) Bimolecular fluorescence complementation (BiFC) assays of the interaction between AtPLAC811.1 and AtLSD1 in *Arabidopsis* protoplast cells.** Confocal microscope images of BiFC assay show fluorescence signal in non-uniform structure(s) in the cell (white arrows). First panel: AtPLAC811.1 fused to the N-terminus of pSAT4 (YFP) and AtLSD1 fused to the N-terminus of pSAT4 (YFP) were co-transformed and transient expression assay was performed on *A. thaliana* mesophyll protoplasts. Second Panel: AtLSD1 fused to the N-terminus of pSAT4 (YFP) and AtPLAC8 fused to the N-terminus of pSAT4 (YFP) were co-transformed and a transient expression assay was performed on *A. thaliana* mesophyll protoplasts. pBIN20 membrane marker was co-expressed as a membrane marker. Scale bar 5µM. **(c) Transactivation assay in *Arabidopsis thaliana* protoplasts.** GUS enzymatic activity (mM MU/min) was determined by the 4-MUG analysis method. Asterisks (*) represent a significantly significant difference in Student's t-test compared to the control ($p<0.05$). pGusXX::SLPK: 2k bp fragment of the AtSPLK promoter fused to the GUS gene; pGusXX::SWAP: 2k bp fragment of the AtSWAP promoter fused to the GUS gene; LSD1 = CDS of the AtLSD1 gene fused to the CaMV35S promoter for overexpression of the LSD1 protein; PLAC8 = CDS of the AtPLAC811.1 gene fused to the CaMV35S promoter for overexpression of the AtPLAC811.1 protein.

AtLSD1 is very well studied in relation to both biotic and abiotic stress responses. There is evidence that indicates AtLSD1 acts as a transcriptional regulator [21]. To get insights into the AtLSD1 and AtPLAC811.1 interaction, AtLDS1 target genes with opposite regulation were chosen to carry out transactivation experiments in *A.thaliana* protoplasts, using AtLSD1 and

AtPLAC811.1 as effector and interactor, respectively. AtLSD1 regulates positively AtSWAP (Suppressor-of-White-APricot)/surp domain-containing protein) and negatively AtSLPK (S-locus lectin protein kinase family protein). In presence of AtPLAC811.1, the AtSWAP expression was weakened and the AtSLPK expression was enhanced as measured by the transactivation assay (Figure 7c). This result indicates that AtPLAC811.1 interaction with AtLSD1 interferes with the AtLSD1 activity as a transcriptional regulator.

2.6 Absence of *AtPLAC811* alters the expression of genes related to dPCD and ePCD

The literature indicates conserved regulated genes involved in diverse plant PCD processes, discriminating genes involved in Developmentally (dPCD) and Environmentally (ePCD) PCD in plants [24]. We have examined the expression level of key genes involved in dPCD and ePCD in *plac811* knockout plants. Concerning dPCD genes, we found the expression of *CEP1* (AT5G50260), *RNS3* (AT1G26820), and *PASP3* (AT4G04460) did not differ significantly between the WT and *plac811* knockout plants. However, *DMP4* (AT4G18425), *BFN1* (AT1G11190), *MC9* (AT5G04200), and notably *SCPL48* (AT3G45010) expression was higher in *plac811* knockout plants in comparison to in comparison to wild-type plants (Figure 8a). *DMP4* and *BFN1* which are involved in plant organ senescence [25], *AtMC9* acts regulating autolysis of the xylem vessel elements [26] and *SCLP48* is related to cellular developmental process [25]. Significant differences in the expression of ePCD genes were also observed (Figure 8b) between WT and *plac811*, with higher *LSD1* (AT4G20380) and *MC1* (At1g02170) expression in the mutant, indicating that AtPLAC811.1 may have a role in both dPCD and ePCD processes.

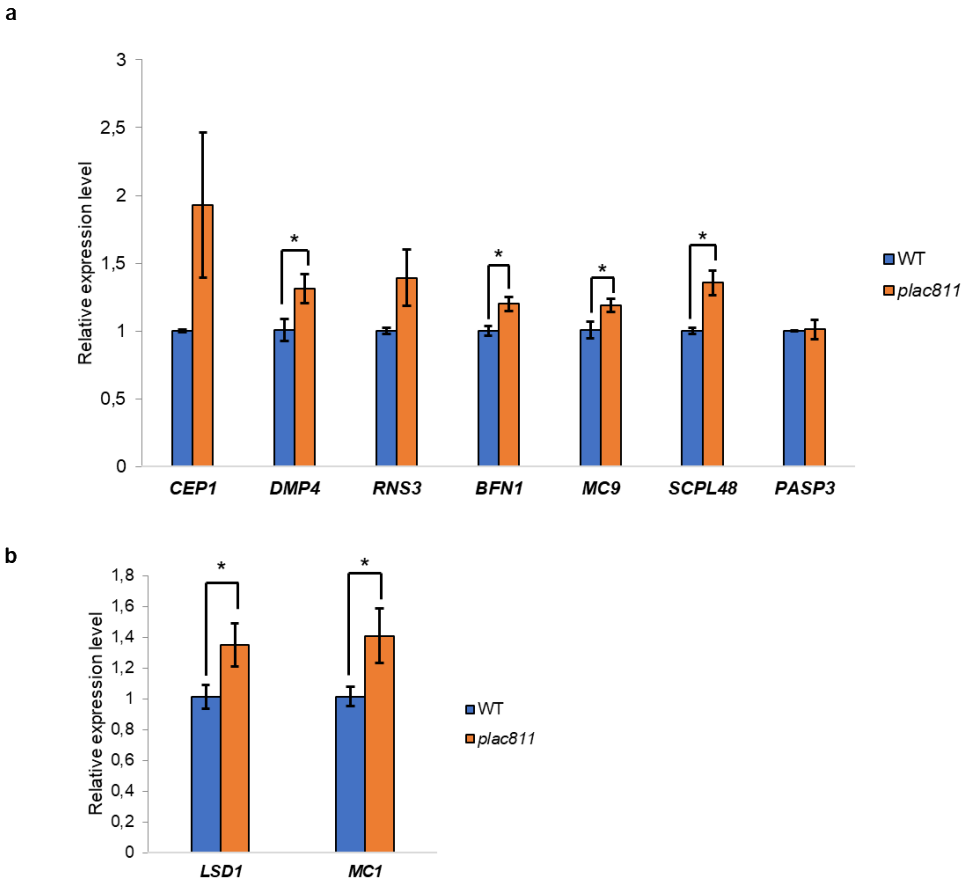


Figure 8. Expression of dPCD and ePCD genes in *Arabidopsis* WT and *plac811* knockout plants. (a) Expression levels of seven dPCD genes were analyzed by qRT-PCR. The leaf of three-week-old wild-type *Arabidopsis* and *plac811* knockout plants grown on soil were used. Results represent means (± error) from three lines of each genotype with two biological replicates for each line. Ubiquitin was used as an internal control. **(b) Expression level of two selected dPCD genes were analyzed by qRT-PCR**. Seedlings of ten-days-old wild-type *Arabidopsis* and *plac811* knockout plants grown on MS medium were used. Results represent means (± error) from three lines of each genotype with three biological replicates for each line. Ubiquitin and PP2A were used as internal controls. Asterisks indicate significant differences from the WT Col-0 (t-test: *, $P < 0.05$).

Catalases (*CAT*) play an important role in performing the decomposition of hydrogen peroxide (H_2O_2) to produce H_2O and O_2 in plants [27,28]. We analyzed the expression of *LSD1* and *MC1* in response to 3-amino-1,2,4-triazole (3-AT) which promotes inhibition of the catalase activity thus leading to an increase in peroxisomal H_2O_2 concentrations and cell death process [29,30]. In response to 3-AT, no phenotypic difference was observed between *plac811* and wild-type plants (Figure 8a). In *Arabidopsis*, it's known that AtLSD1 interacted physically and genetically with catalases in cell death processes and that the catalase activity is decreased in the *lsd1* mutant [30]. The expression of LSD1 was lower after germination with 3-AT in both wild-type and *plac811* plants in comparison to their controls (Figure 8b). The *MC1* expression level was reduced only in *plac811* seedlings germinated in 3-AT (Figure 8c). Taken together the results indicate that the absence of *AtPLAC811* alters the expression of important PCD genes in control conditions and in response to stress.

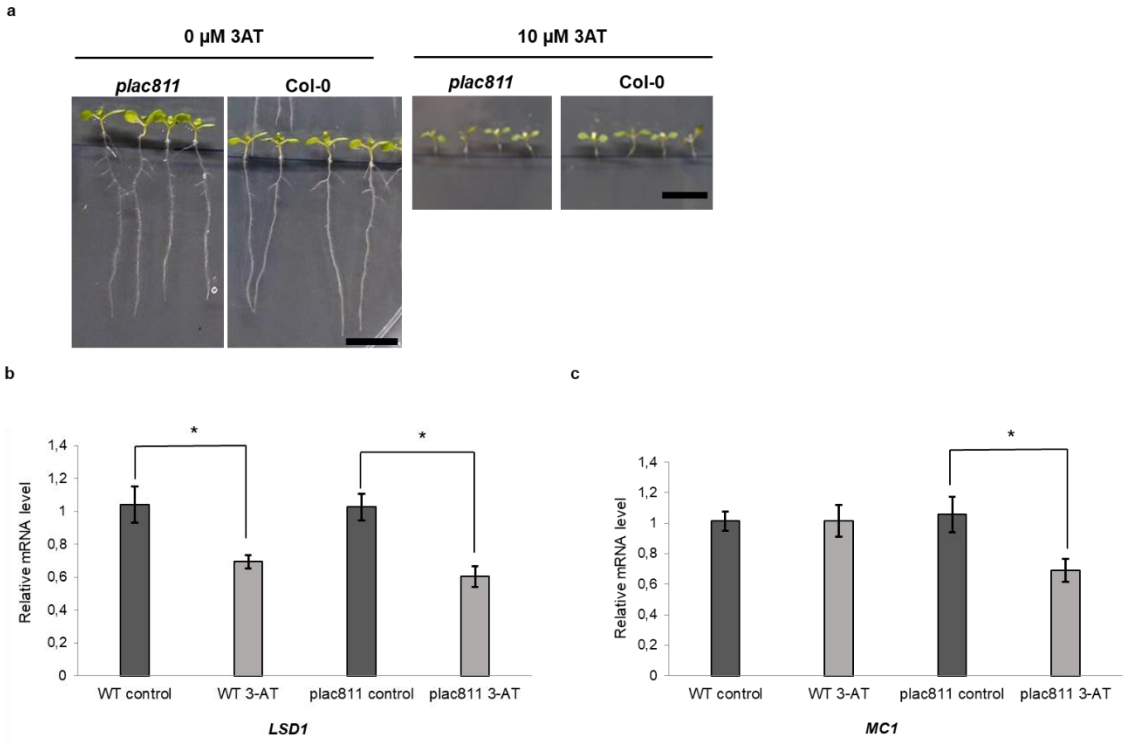


Figure 8. Expression of *LSD1* and *MC1* genes in Arabidopsis WT and in *plac811* knockout plants in response to 3-AT. (a) Seedlings of ten-days-old *Arabidopsis* and *plac811* knockout plants germinated and grown on ½ MS medium or ½ MS medium with 10µM 3-AT. **(b) The**

expression level of *LSD1* corresponding to the seedlings in letter (a) were analyzed by qRT-PCR. (c) The expression level of *MC1* corresponding to the seedlings in letter (a) were analyzed by qRT-PCR. Results represent means (± error) from three lines of each genotype with three biological replicates for each line. Ubiquitin and PP2A were used as internal control. Asterisks indicate significant differences from the WT Col-0 (t-test: *, $P < 0.05$). Scale bar 1cm.

Exposure of *plac811* plants to abiotic stresses such as heat, senescence and salt, was performed separately in a manner to try to understand the function of AtPLAC811 (Supplementary Figure 7). For that, mutants with T-DNA insertions in the promoter (CS852038) and in the first exon (CS378853) of *AtPLAC8-11* were acquired from the Arabidopsis Stock Center (ABRC) seed bank. Expression analysis confirmed that CS852038 is a knockdown mutant line, while CS378853 is a knockout (Supplementary Figure 7a). Despite significant results among mutant seedlings compared to wild ones, the results were not replicated, which is the case in heat treatment, where the heat stress showed to greatly affect the development of *plac811* mutant seedlings (Supplementary Figure 7b). In the senescence experiment, the reduction of the chlorophyll content in the *plac811* knockout mutant was bigger in comparison to wild-type seedlings (Supplementary Figure 7c). Interestingly, the saline abiotic stress experiment has a significant effect on the relative inhibition of root size in the *plac811* knockout mutant in 140mM NaCl but not in 100mM NaCl (Supplementary Figure 7d). Other abiotic stresses and also some biotic (Fumonisin B1 and Pep1) elicitors stresses were also performed but no consistent and significant difference was observed between wild-type and *plac811* plants (Supplementary Figure 8). These results suggest that in some cases, rather than the significant phenotypic differences, we have the genetic compensation by paralogous, once that *Arabidopsis* has seventeen members of the PLAC8 family with a conserved domain.

2.7 Shoot and root enhanced growth of *Arabidopsis* DEX-inducible *AtPLAC811.1* overexpresion lines.

Arabidopsis plants (Col-0) overexpressing *AtPLAC8-11.1* driven by CaMV 35S promoter were generated using pEarleyGate vectors (Supplementary Figure S7a). Despite many attempts, it

was not possible to obtain a large number of homozygous T3 transformants, since transformant seedlings have reduced root size, and development problems, resulting in difficulty in obtaining seeds. It was common to obtain silenced plants indicating a process of co-suppression of the endogenous gene occurred (Supplementary Figure S7b). Two homozygous T3 lines overexpressing *AtPLAC811.1* were obtained (Supplementary Figure S7c). The first one presented an unhealthy phenotype, with leaf yellowing, resulting in developmental problems. Differently, the second line overexpression *AtPLAC811.1* showed big green leaves, possibly due to an insertion into a region that promotes the observed phenotype, reflecting the phenotype and not the overexpression itself (Supplementary Figure S7d). Difficulties in obtaining plants overexpressing *AtPLAC8-11.1* indicate the possibility that high transcriptional levels of *AtPLAC8-11.1* may be detrimental to the plant.

As an alternative strategy we performed overexpression of *AtPLAC8-11.1* using a dexamethasone inducible gene expression [31]. We obtained three homozygous T3 lines, but only two lines (G and H) were shown to be overexpressing *AtPLAC811.1* upon dexamethasone application (Figure 9a). The *DEX:AtPLAC811.1* (#G) is the line with a higher *AtPLAC811.1* expression level and after grown with (+) DEX, seedlings of this transgenic line showed a significant increase in shoot and root fresh weight compared to wild-type seedlings (Figure 9b, c and d). This result suggests that the overexpression of *AtPLAC8-11.1* is involved in vegetative growth.

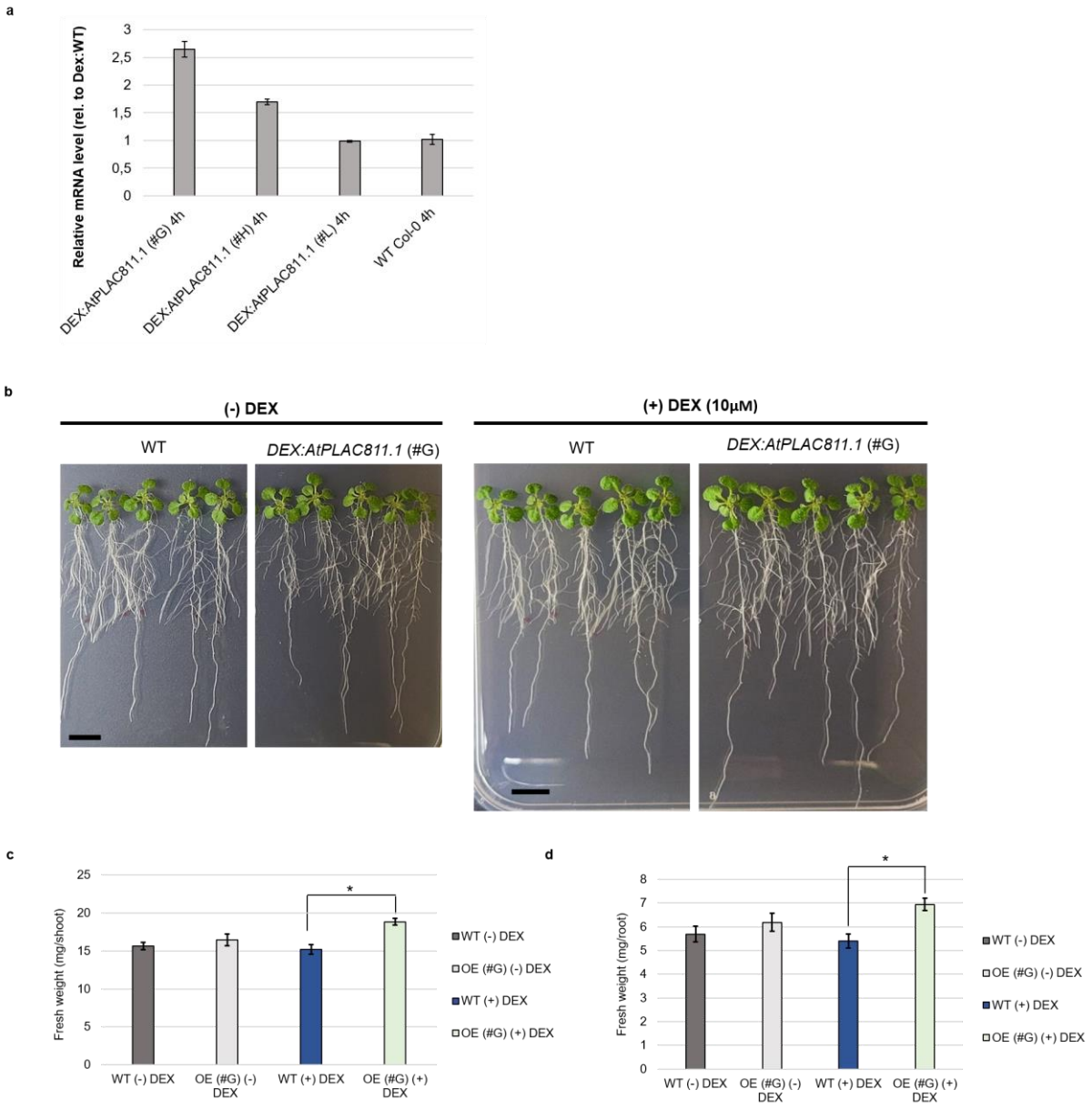


Figure 9. INDUCIBLE *PLAC811.1-IOE* promotes shoot and root development. (a) Relative expression levels of AtPLAC811.1 protein in WT, OE(#G), OE(#H) and OE(L) seedlings. WT and transgenic seedlings were grown on MS medium for 7 days transferred to new MS medium plates containing 10 µM DEX and incubated for 4 hours. Total RNAs from each sample were isolated and subjected to RT-PCR analysis for AtPLAC811.1. Results represent means (± error) from three lines of each genotype with three biological replicates for each line. Ubiquitin was employed as an endogenous internal control. **(b) Phenotype of**

***DEX:AtPLAC811.1* (#G) transgenic plants.** WT and *DEX:AtPLAC811.1* (#G) transgenic seedlings were grown on MS medium for 10 days and then were transferred to new MS medium plates containing DMSO (–) DEX or 10 μM DEX (+) DEX for another 5 days. Plants were photographed (15 d old). Scale bars 1cm. **(c-d) Measurements of shoot and root weights after treatment in letter b.**

To better understand the role of AtPLAC811.1 in leaf size, *DEX:AtPLAC811.1* (#G), *DEX:AtPLAC811.1* (#H) and WT seedlings were directly germinated in ½ MS medium plates containing DMSO (–) DEX or 10 μM DEX (+) DEX and photographs taken under a stereoscopic microscope (Figure 10). According to Figure 10 (i) and (ii), the *DEX:AtPLAC811.1* transgenic lines when grown in ½ MS medium containing DEX showed an early leaf primordia development in comparison to seedlings grown in ½ MS medium without DEX. The wild-type seedling showed no difference between the DEX treatment and the control. Our results indicate that *AtPLAC811.1* has an important role in leaf growth and development.

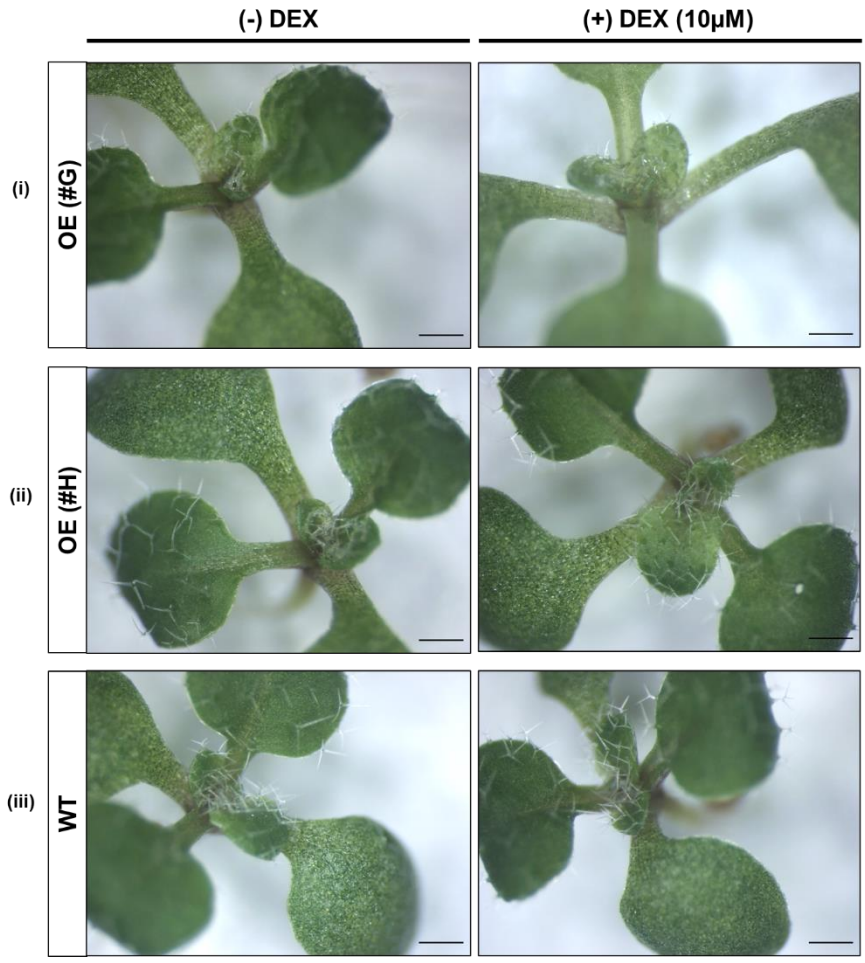


Figure 10. INDUCIBLE *PLAC811.1-IOE* leaf phenotypes. 14-day-old plants of **(i)** *DEX:AtPLAC811.1* (#G), **(ii)** *DEX:AtPLAC811.1* (#H), **(c)** WT grown on ½ MS medium plates containing DMSO (–) DEX or 10 μM DEX (+) DEX. Scale bars 0.5 mm.

3. Discussion

The *Arabidopsis* annotated genome sequence affords detailed information regarding the gene content of a genomic segment of interest, providing an opportunity to determine the functions of several protein-coding genes [32,33]. Until now, there are no directly experimental studies in the literature indicating the functionality of the *AtPLAC8-11* gene, although it appears in transcriptome results in several articles [10–14,20]. We have identified the *Arabidopsis AtPLAC811*

gene, which encodes a predicted protein containing a PLAC8 domain and its variant encoded by an alternatively spliced transcript (Figs. 1 and Supplementary 1).

There is a prediction that the AtPLAC811 protein is an integral component of the plasma membrane [25], but was not possible to observe fluorescence in the membrane in the protoplasts expressing the fusion AtPLAC811-GFP (Supplementary Figure 3). N- or C-terminal fusions can interfere in some posttranslational modification sites essential for membrane protein targeting. In this sense, some plasma membrane proteins lose out to localize to the plasma membrane using N- or C-terminal fusion [34]. In our experimental conditions, the AtPLAC811.1 and AtPLAC811.2 subcellular localization varied according to C- and N-terminal GFP fusion proteins. The AtPLAC811.1 and AtPLAC811.2 constructions with N-terminal GFP fusion showed a pattern of fluorescence in the cytoplasm with non-uniform structures dispersed throughout the cell (Figure 2b i and iii and Figure 6b). While the constructions with C-terminal GFP fusion showed a different pattern of fluorescence within a single large structure (Figure 2b iii) or in several spots in undefined format spread across the cell (Figure 2b iv). Besides, AtPLAC811.1:GPF and AtPLAC811.2:GFP fusion proteins were found to be associated with endoplasmic reticulum marker (Figure 2c). N- terminal fusions with fluorescent protein have interference in the plastid and mitochondrial localization signals and, can also abolish endoplasmic reticulum signal peptides[35]. C-terminal fusions might cause proteins to mislocalize, especially regarding peroxisomal proteins. Finally, it seems that proteins fusion with GFP at the C-terminal is normally better in preserving the localization of the native protein [35]. In this sense, the non-uniform structures seen in the GFP C-terminal fusion localization seem to represent the possible subcellular localization of AtPLAC811.1 and its splicing variant.

Our results also demonstrated that AtPLAC811.1 interact with AtLSD1 (Figure 7a), which is a very well-known negative regulator of plant PCD [36–38]. Furthermore, AtPLAC811.1 affected the transcription factor role of AtLSD1 in the regulation of target genes (Figure 7c), suggesting that AtPLAC811.1 may be involved in the regulation of PCD. It is known that the PLAC8 human protein, also known as Ozin, is involved in differentiation, proliferation, and apoptosis processes, further evidencing the important role of this gene family in PCD [39,40]. The interaction between AtPLAC817, another member of PLAC8 family in *Arabidopsis*, and AtLSD1 was also confirmed (Figure S6), but with a weaker interaction. Although both forms, AtPLAC811.1 and

AtPLAC811.2 have the PLAC8 domain, which characterizes the proteins belonging to this gene family, only the canonical form was able to interact with AtLSD1, indicating possible distinct functions and the importance of the C-terminal region for the interaction to occur, since part of this region is not in the splicing variant. Alternative splicing acts to diversify the transcriptome and proteome of an organism contributing in a varied manner to the regulation of cellular responses to environmental conditions, as well as to developmental stimuli [41,42].

There are also differences in the floral expression pattern between the canonical form and the splicing variant, which can be also observed by confocal imaging of plants expressing a translational fusion of *AtPLAC811.1* and *AtPLAC811.2* and YFP under native promoter (Figure 5). Our results indicate that *AtPLAC811.1* is higher expressed in floral organs, mainly in sepal. This is an interesting result since the specific defense gene expression patterns in *Arabidopsis* flower can influence the resistance to pathogens, indicating that in the flower, resistance to the pathogen is likely dependent on the up-regulation of stress-responsive genes, such as *AtPLAC811.1* [20]. Besides, the role of the sepal against biotrophic pathogens is acting as a chemical defense barrier during the development of reproductive structures [20]. Differences were also seen regarding the tissue-specific gene expression of *AtPLAC811.1* and *AtPLAC811.2* driven by the native promoter in root and leaf (Figs. 3 and S4), where only *AtPLAC811.1* appears surrounding the bases of trichomes, which acts as a protective barrier against pathogen attacks [43]. AtPep1 promotes a severe inhibition of root growth inducing callose and lignin depositions as protective barriers against pathogens [44] . *AtPLAC811.1* and *AtPLAC811.2* showed to be more responsive to Pep1 treatment (Figure 4) with important histological changes in the root (Supplementary Figure 5), indicating the possible role of AtPLAC811 protein in innate immunity responses.

The knockout of *AtPLAC811* in *plac8* plants has effects on the expression of some dPCD genes, such as *DMP4, BFN1, AtMC9* and *SCLP48*. The promoter of *DMP4* showed an expression pattern in the degenerating endosperm, anther tapetum layer before tapetum cell death, lateral root cap cells differentiation and tracheary elements, and in senescing petals [24]. The *BFN1* promoter analyses revealed *GUS* expression in senescent leaves, developing anthers and seeds, and also in floral organs after fertilization [45]. *Metacaspase 9* (*MC9*) acts in xylem cell death [26]. The *SCPL48* promoter exhibit a wide spatial and temporal expression pattern as in petals at

anthesis and in the entire lateral root cap, tracheary elements and in their neighboring cells, developing anthers, tapetum and into the outer anther layers and during seed development [24]. Furthermore, ePCD genes such as LSD1 and MC1, essential regulators of plant PCD, also increased their expression in absence of *AtPLAC811*. The opposite effect was observed in stress conditions, when plants were germinated with the catalase inhibitor 3-AT, leading to increasing of peroxisomal H_2O_2 concentrations and cell death in *Arabidopsis* [46]. In this case, the expression level of LSD1 and MC1 were reduced in *plac8* plants (Figure 8b and c).

Since it is not possible to obtain transgenic plants overexpressing *AtPLAC811.1* driven by a constitutive promoter (Figure S9), the overexpression using an inducible promoter seems to be an alternative to performing overexpression of *AtPLAC8-11.1*, and thus to study its function (Figure 9). More studies should be performed using transgenic lines overexpressing *AtPLAC811.1*, but initial studies reveal that *AtPLAC811.1* may be involved in leaf and root size regulation (Figs. 9c and 10). This is an interesting result because the PLAC8 domain is involved in the regulation of fruit size and cell number in tomato plants [47,48] . Besides that, *AtPLAC811.1* appears in the microarray results as genes affected in GROWTH-REGULATING FACTOR 9 (GRF9)-IOE lines after estradiol (EST) induction. GRF9 negatively regulate *Arabidopsis* leaf growth by controlling *OBP3-RESPONSIVE GENE 3* (ORG3) and thus restricting cell proliferation in leaf primordia[49].

In conclusion, we have shown that AtPLAC811.1 but not its splicing variant interacts with AtLSD1, and this interaction may affect the AtLSD1 function as a t transcription factor, which may contribute to the hypothesis of the involvement of AtPLAC811 in the cell death process. In addition, *plac811* plants showed to interfere in the expression of some dPCD and ePCD markers.

4. Material and methods

Plant materials and growth conditions

Arabidopsis thaliana plants used as the wild-type in this work are all in the Col-0 ecotype. The T-DNA insertional mutants *plac811* knockout (CS378853) and *plac811* knockdown (WiscDsLox344D04 /CS852038), for the AtPLAC811 gene, with insertion of T-DNA in the

first exon and in the promoter of the gene, respectively, were selected and acquired from the Arabidopsis Biological Resource Center (ABRC) seed bank. The plants were grown in a greenhouse in a climate-controlled manner, under long day conditions with 16 h light/8 h dark at 22°C for general growth and seed harvesting. For seedlings grown on the ½ MS medium in plates, *Arabidopsis thaliana* seeds were surface sterilized with 70% EtOH, with addition of 0.1% Trinton-100x and then inoculated into plates containing ½ MS medium-strength (Murashige and Skoog Basal Salt Mixture - Sigma-Aldrich) with 1% (m/v) of sucrose, 0.5% (m/v) of phytagel and pH 5.7– 5.8. The seeds were stratified in the dark at 4 °C for 72 h before exposing them to white light under long-day conditions with 16 h light/8 h dark, 22 °C and, 130 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). For the stress response assays, genotypes were grown on ½ MS medium supplied with or without the indicated concentration of 100 nM of Pep1 (1 mM stock in distilled deionized H_2O), 10 uM 3-AT, 2.5 mM H_20_2, 0.3 uM FBI or 100 and 140 mM NaCl.

Plasmid construction

The oligonucleotides used to amplify DNA-specific sequences are in Supplementary Table 1. All the fragments cloned into the vectors were confirmed by DNA sequencing. To perform subcellular studies, the CDS of *AtPLAC811.1* and *AtPLAC811.2* were amplified with gene-specific primers using cDNA (complementary DNA) obtained from leaves of *A. thaliana* as a template. The amplified products were separately cloned into entry vector pENTR/D-TOPO (Invitrogen®), and then into a Gateway-compatible pMDC43 and pMD83 destination vector through an LR Clonase II reaction (Gateway™ LR Clonase™ II Enzyme Mix - Invitrogen) [50]. To expression studies involving the *AtPLAC811* promoter region, about 2 kb upstream region from the translational start site of *AtPLAC811* plus the genomic sequence of *AtPLAC811.1* or *AtPLAC811.2* were amplified with gene-specific primers using *A. thaliana* gDNA as a template. The amplified products were cloned separately into pENTR/D-TOPO (Invitrogen®), and after were subcloned into a Gateway-compatible promo-YFP-TurboID by LR reaction (Invitrogen). The construction *promoPLAC811:PLAC811.1-YFP-TbID* and *promoPLAC811:PLAC811.2-YFP-TbID* were transformed into *plac811* plants using the floral dip method with *Agrobacterium tumefaciens* strain EHA105 [51]. The YFP fluorescence of seedlings, root and

flower segments were visualized using an SP8 confocal microscope (Leica Microsystems, Heerbrugg, Germany). For the generation of *Arabidopsis* plants inducible overexpressing *AtPLAC811.1*, the coding sequence of *AtPLAC811.1* was cloned into the pINDEX1 vector between *Xho*I and *Spe*I restriction sites [52]. *pINDEX1:AtPLAC811.1* construct was transformed into *A. thaliana* plants using the floral dip method with *Agrobacterium tumefaciens* strain EHA105. The T1 seeds of the plants used in the process of genetic transformation by the floral dip method were selected on ½ MS-agar plates supplemented with 50 µg/µl of hygromycin antibiotic, and the resistant seedlings were transferred into individual pots containing a soil:vermiculite mixture (1:2 by volume). The inducible overexpressing transgenic lines were confirmed by PCR using hygromycin (HTP) and specific primers. The selection of hygromycin-resistant plants was carried out until the T3 homozygous stage, being confirmed by expression analysis.

Yeast Two-Hybrid Analysis

For yeast two-hybrid experiments, pENTR/D-TOPO:*AtPLAC811.1*, pENTR/D-TOPO:*AtPLAC811.2, and* pENTR/D-TOPO:*AtPLAC817* were fused through an LR Clonase II reaction (Invitrogen) into the yeast two-hybrid destination vector pDEST22, which encodes the GAL4 transcription activation domain, and pENTR/D-TOPO:*AtLSD1* to pDEST32, which encodes the DNA binding domain of Gal4. The constructions *pDEST22:AtPLAC811.1*, *pDEST22:AtPLAC811.2*, *pDEST22:AtPLAC817* and *pDEST32:AtLSD1* were cotransformed into the *Saccharomyces cerevisiae* strain AH109 using the lithium acetate method[53]. The yeast transformants were plated on a SD medium (synthetic minimal medium) lacking leucine (−Leu) and tryptophan (−Trp) and lacking leucine (−Leu) tryptophan (−Trp), and Histidine (-His). To test the high-affinity binding between bait and prey, increasing concentrations of 3-amino-1,2,4-triazole (3-AT) were used (1, 5, and 10mM).

BiFC

BiFC constructs were generated by cloning CSD region of *AtPLAC811.1* (AT1G52200) and *AtLDS1* (AT4G20380) into the Gateway BiFC Vectors: pSAT4-DEST-n(1-174)EYFP-C1 and pSAT5-DEST-c(175-end)EYFP-C1(B) [54]. *AtPLAC811.1*-nYFP was co-transformed with

AtLSD1-cYFP and *AtLSD1*-nYF was co-transformed with *AtPLAC811.1*-cYFP into *Arabidopsis* mesophyll protoplasts, which were isolated and transfected following a previous method [55]. The protoplasts were examined for BiFC signals using an Olympus FLUOVIEW FV1000 confocal microscope (Olympus, Tokyo, Japan).

Transactivation assay

The SWAP (LOC_AT5G06520), and SLPK (LOC_ AT4G11900) promoter regions (2.0 kb upstream of the ATG) were amplified by PCR from *Arabidopsis* gDNA. The promoter regions were then cloned into the target vector pGUSxx [56] between Sac*II* and Nco*I*, fusing the promoter regions to the GUS reporter gene (β-D-glucuronidase). Plasmids (pSAT) containing the *AtLSD1* and *AtPLAC811.1* CDS under the control of the CaMV35S promoter were used for overexpression of AtLSD1 effector protein and AtPLAC811.1 interactor protein. *Arabidopsis* mesophyll protoplast cells were co-transfected with 10 µg of each plasmid (plasmid with promoter region, and effector plasmid) plus 10 µg of 35S::AtPLAC811.1 and incubated for 16h. Protoplasts were harvested via centrifugation and lysed in 100 µl of Extraction buffer (0.1 volume of 10X Na-phosphate/EDTA buffer, 0.1% sodium lauryl sarcosine, 10 mM 2-mercaptoethanol, 0.1 % Triton X-100). GUS activity was measured using MUG (4-methylumbelliferyl-β-D-glucuronide) and MU (4-methylumbelliferone). Negative (empty pGusXX), positive (pGusSH) controls were used in the assay.

Western blot

Leaves of *Arabidopsis* plants expressing *promoPLAC811:PLAC811.1-YFP-TbID*, *promoPLAC811:PLAC811.2-YFP-TbID* and *35S:PLAC811.1-YFP-TbID* were harvested in liquid nitrogen and homogenized with SDS sample buffer (2X) (500 mM Tris–HCl, pH 6.8, 10% SDS, 20% Glycerol, 2% 2-β- mercaptoethanol, and 0.01% bromophenol blue). After boiling for 10 min with SDS sample buffer (2X), the protein extracts were loaded onto an SDS-PAGE gel, and then proteins were transferred to nitrocellulose membrane (Bio-rad) using the Trans-Blot® SD Semi-Dry Blotting (Bio-Rad). Membranes were blocked for 1h in 5% fat-free milk in PBST and then incubated with the primary antibody Anti-GFP Mouse mAb 1:1000 (TransGen Biotech) followed by incubation with the secondary antibody Goat anti-Mouse IgG

1:3000 (Bio-rad).

Extraction of RNA and RT-qPCR analysis

To analyze the expression level of dPCD and ePCD genes in *Arabidopsis* WT and in *plac811* knockout plants, the leaf of three-week-old *Arabidopsis* grown on soil were frozen in liquid nitrogen. To verify the expression level of AtLSD1 and AtMC1, seedlings of ten-days-old *Arabidopsis* and *plac811* knockout plants germinated directly on ½ MS medium or ½ MS medium with 10µM 3-AT were frozen in liquid nitrogen. In both cases, the total RNA was isolated using the Direct-zol RNA Kit (Zymo Research). RNA was converted into first-strand cDNA using M-MLV Reverse Transcriptase (Promega), with oligo(dT) as a primer in previous treatment with DNase (Promega). The resultant cDNAs were used as a template for quantitative PCR amplification in using as a fluorescent reporter the intercalation dye SYBR-GREEN (Molecular Probes), and Platinum Taq DNA Polymerase (Invitrogen). For dPCD and ePCD genes, ubiquitin-10 (AT4G05320) was used as a reference gene and the experiments were performed in biological duplicates and in experimental triplicates. For 3-AT experiment, the primers PP2A (AT1G13320) and ubiquitin-10 (AT4G05320) were used for normalization and the experiments were performed in biological triplicate and in experimental triplicates. To verify the expression level of *AtPLAC811.1* in transgenic plants harboring a dexamethasone inducible gene expression system, the leaves of 7-days-old Col-0, *DEX:AtPLAC811.1* (#G), *DEX:AtPLAC811.1* (#H), and *DEX:AtPLAC811.1* (#L) plants incubated for 4 hours with 10 µM DEX were collected and frozen. The RNA was isolated using the RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. The commercial kit RQ1 RNase-Free DNase (Promega) was used to remove possible contamination with gDNA and cDNA synthesis was performed using the enzyme M-MLV Reverse Transcriptase (Invitrogen). The qRT-PCR was performed using the SensiMix™ SYBR® & Fluorescein Kit (BioLine). The endogenous gene Ubiquitin-10 (AT4G05320) was used as an internal control. The experiments were performed in biological triplicate and in experimental triplicates. Expression data were normalized with the expression level of the endogenous gene Ubiquitin-10 (AT4G05320). All experiments were performed using the $2^{-\Delta\Delta Ct}$ method [57] and the primers used are in Supplementary Table 1.

Supplementary Table 1

Supplementary Table S1.

Primers used for Gateway cloning

Primer name	**Sequence 5'-3'**
PLAC811.1/11.2-FWD	CACCATGGGTCGTGTCACTACTCCAT
PLAC811.1_stop-REV	CTTAGACATATATTGATTTGTAGGAGCAAA
PLAC811.1_no_stop-REV	CTTAGACATATATTGATTTGTAGGAGCAAA
PLAC811.2_stop-REV	TCACCACACTTGAACACTTATATCAA
PLAC811.2_no_stop-REV	CCACACTTGAACACTTATATCAA
PLAC817-FWD	CACCATGCCGAAACAGGATATGGA
PLAC817_stop-REV	CTTTGGGTAAGCGGGAGGA
LSD1-FWD	CACCATGCAGGACCAGCTGGTG
LSD1-REV	CTTTTTGTCAGTTGTCACTCCAAC
promoPLAC811-FWD	CACCTGGTAGAAGGTAATCTGTCGC

Primers used in Transactivation assay

Primer name	**Sequence 5'-3'**
SWAP-FWD	ATACCGCGGTCCTGCTGCTGTTCATGAGG
SWAP-REV	ATACCATGGTCAGATTCAGAGAGAAGGGACA
SLPK-FWD	ATACCGCGGTGAGGGGTAGCAAATTGTAGA
SLPK-REV	ATACCATGGCTTGGCTCACGAGTCAACTAG

Primers used in OE inducible

Primer name	**Sequence 5'-3'**
PLAC811.1-FWD	ATACTCGAGCGGATGGGTCGTGTCACTACTCCAT
PLAC811.1-REV	AAAACTAGTCGGTTACTTAGACATATATTGATTTGTAGGAGCA

Primers used for RT-qPCR

Primer name	**Sequence 5'-3'**
PP2A-FWD	TAACGTGGCCAAAATGATGC
PP2A-REV	ACCAAGCGGTTGTGGAGAAC
UBIQUITIN10-FWD	GGCCTTGTATAATCCCTGATGAATAAG
UBIQUITIN10-REV	ACTATGTTTCCGTTCCTGTTATCTCTTT
PLAC811.1-FWD	ACTGGGTGATGGGATCAAAG
PLAC811.1-REV	AGGAGCAAAACTTGGTGCTT
CEP1-FWD	TACTCCGAGGGAGTGTTTACCG
CEP1-REV	TCCATACCCTACTACCGCAACTC
DMP4-FWD	CGTGTTTGGTGCGGTGGTTT
DMP4-REV	CGCCTACCGGCAAAGCTGTA
RNS3-FWD	CTACAAAACCGGTGGATGGC
RNS3-REV	CACGTACCGTGTTTCTCCCA
BFN1-FWD	ATCGTTGGACCAGCCATCTC
BFN1-REV	TGCTGAAGCTGAGACGTGAA
SCPL48-FWD	ACGAACAAAGCCGATCCAGT
SCPL48-REV	CCGGTTGGTCCACGTAGATT

MC9-FWD	CTCAAGCCGGATCTGGAGAC
MC9-REV	GGAAGTTGATTGACCAATTCTCGG
PASPA3-FWD	AGCTCAGGAGGATCCGAAGAA
PASPA3-REV	GCTCAGCAGCATAAGCGAGT
LSD1-FWD	CATTGTCGGACGACCCTCAT
LSD1-REV	ATTTGGCCGGTTAGTTGGGA
MC1-FWD	TCCACATGGTGTCAAGCTCC
MC1-REV	CTCCCACACATACTGCCCA
PLAC811.1-FWD	ACTGGGTGATGGGATCAAAG
PLAC811.1-REV	AGGAGCAAAACTTGGTGCTT

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7. Supplementary figures

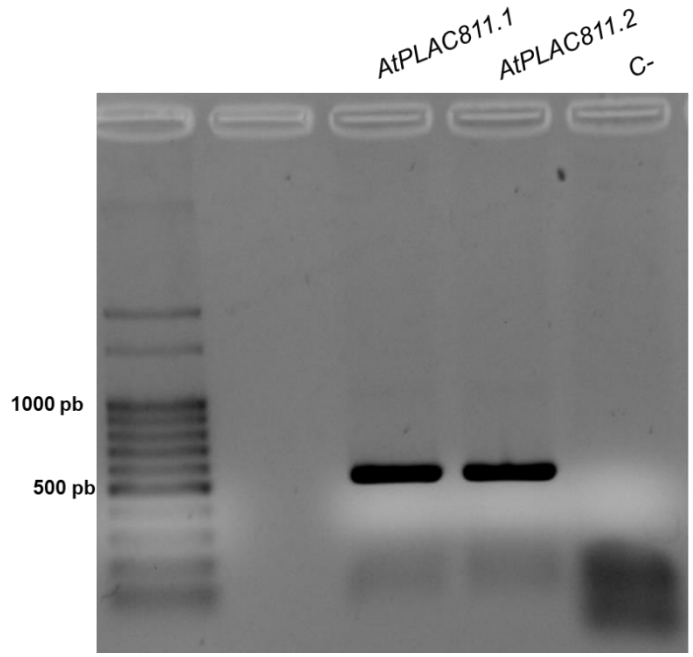
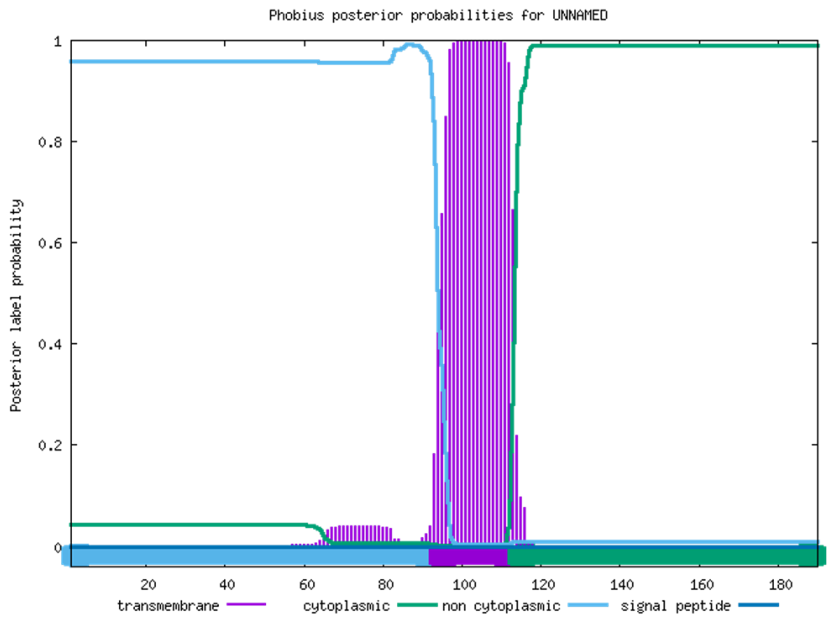


Figure S1. Amplification of *AtPLAC8-11.1 and AtPLAC811.2* from *Arabidopsis thaliana* cDNA. Visualization in 1.2% agarose gel stained with red gel.

a



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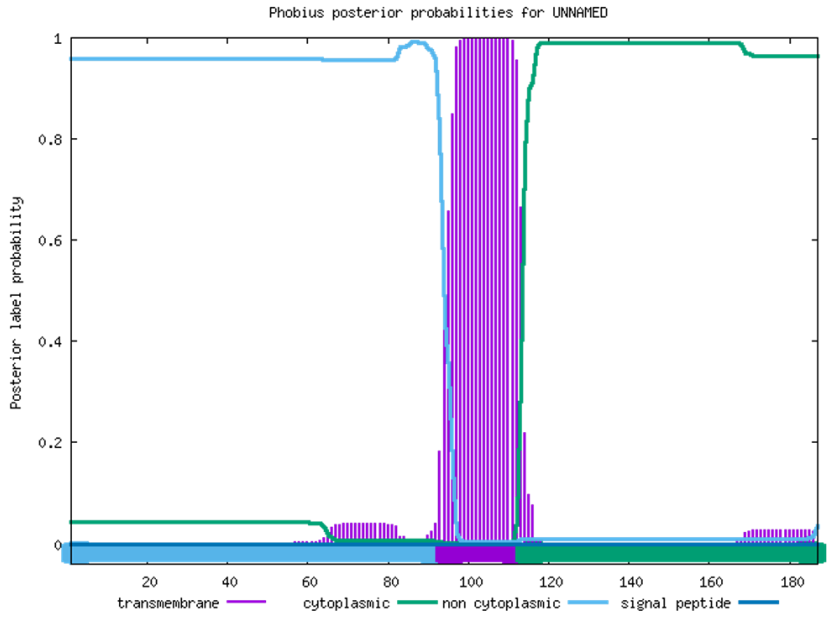


Figure S2. AtPLAC811 transmembrane topology and signal peptide predictor. (a) AtPLAC811.1 prediction of transmembrane segments and signal peptide: identification of the non-cytoplasmic region between 1 and 93 amino acids; the transmembrane region between

amino acids 94 and 113 and a cytoplasmic region between amino acids 114 and 190. **(b)** AtPLAC811.2 prediction of transmembrane segments and signal peptide: identification of the non-cytoplasmic region between 1 and 93 amino acids; the transmembrane region between amino acids 94 and 113 and a cytoplasmic region between amino acids 114 and 187. The predictions were using the Phobius program (https://phobius.sbc.su.se/index.html).

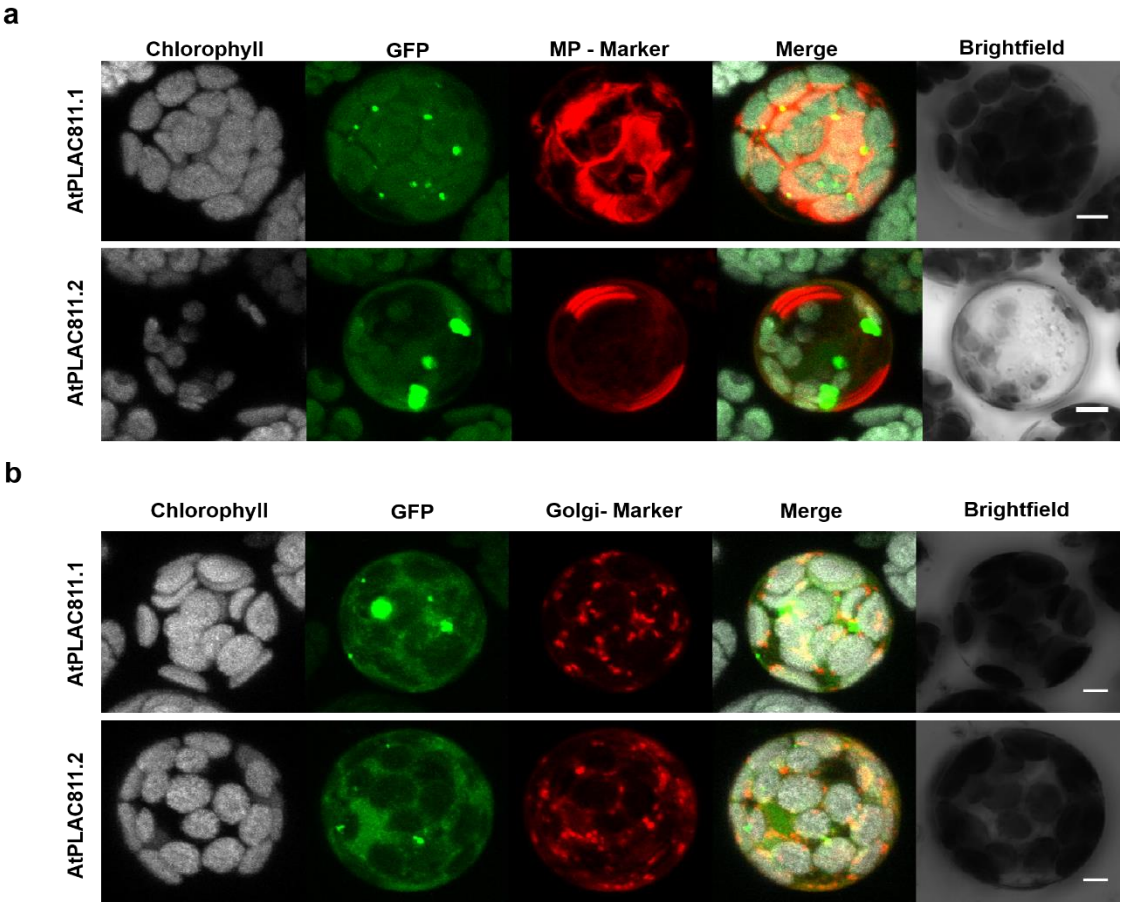


Figure S3. AtPLAC811.1 and AtPLAC811.2 subcellular localization in Arabidopsis leaf protoplasts. Confocal microscope images of transient expression assay performed on *A. thaliana* mesophyll protoplasts isolated from wild-type (WT) plants. **(a)** The fluorescence signal of AtPLAC811.1 and AtPLAC811.2 are represented by N-terminal GFP signals and pBIN20 membrane marker to RFP signals. **(b)** The fluorescence signal of AtPLAC811.1 and AtPLAC811.2 are represented by N-terminal GFP signals and pBIN20 golgi marker by RFP signals. Scale bar 5µM.

*promPLAC811:PLAC8**11.1**-YFP-TbID*

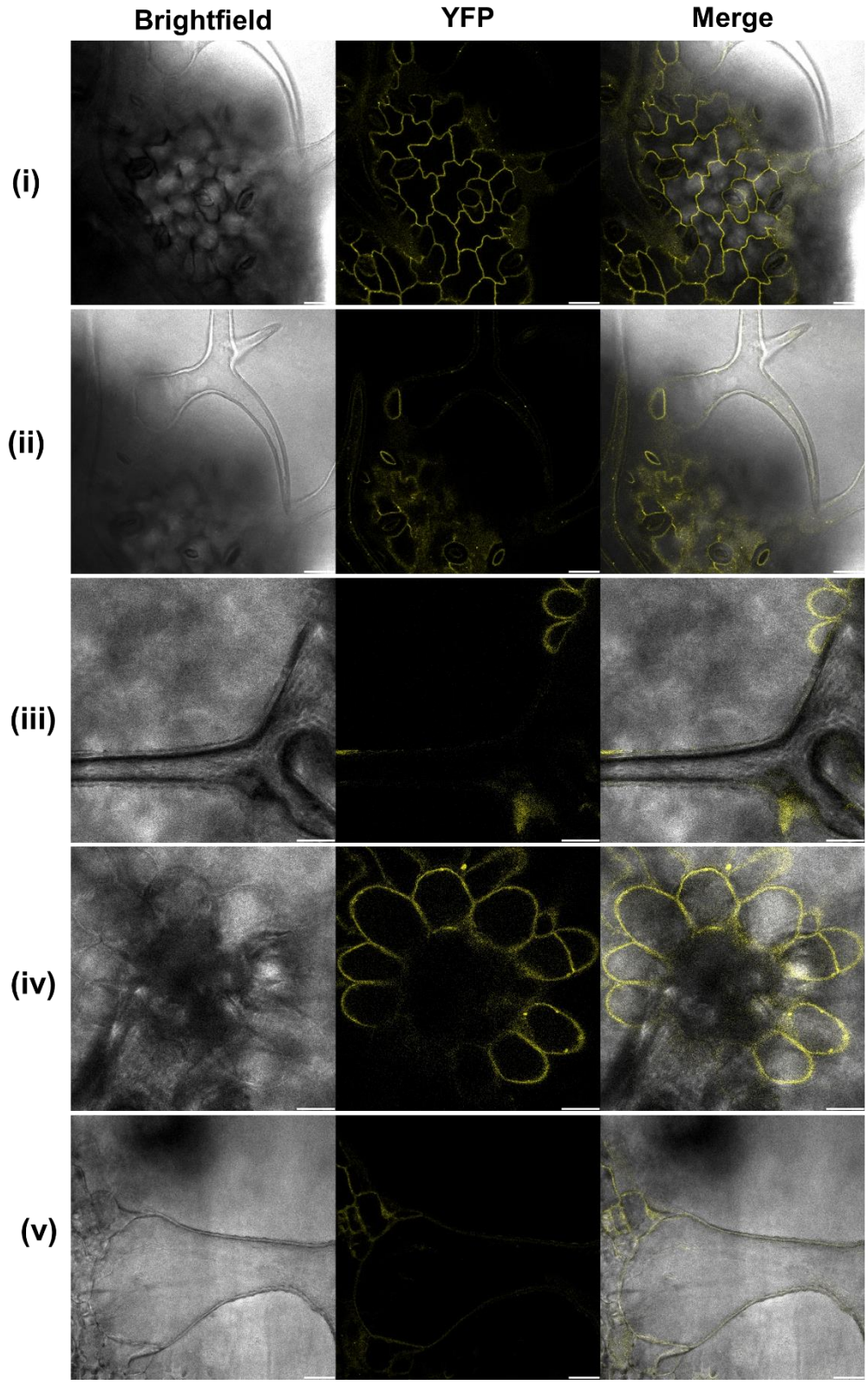


Figure S4. AtPLAC811.1 subcellular localization in *Arabidopsis* leaf. Seedlings of 10-day-old grown in MS medium were observed by confocal microscope. Localization of *promoPLAC811:AtPLAC811.1-YFP-TbID* appears in the membranes of *Arabidopsis* leaves (i) and especially in the trichomes bases (ii, iii, iv and v). Scale bars 20µM.

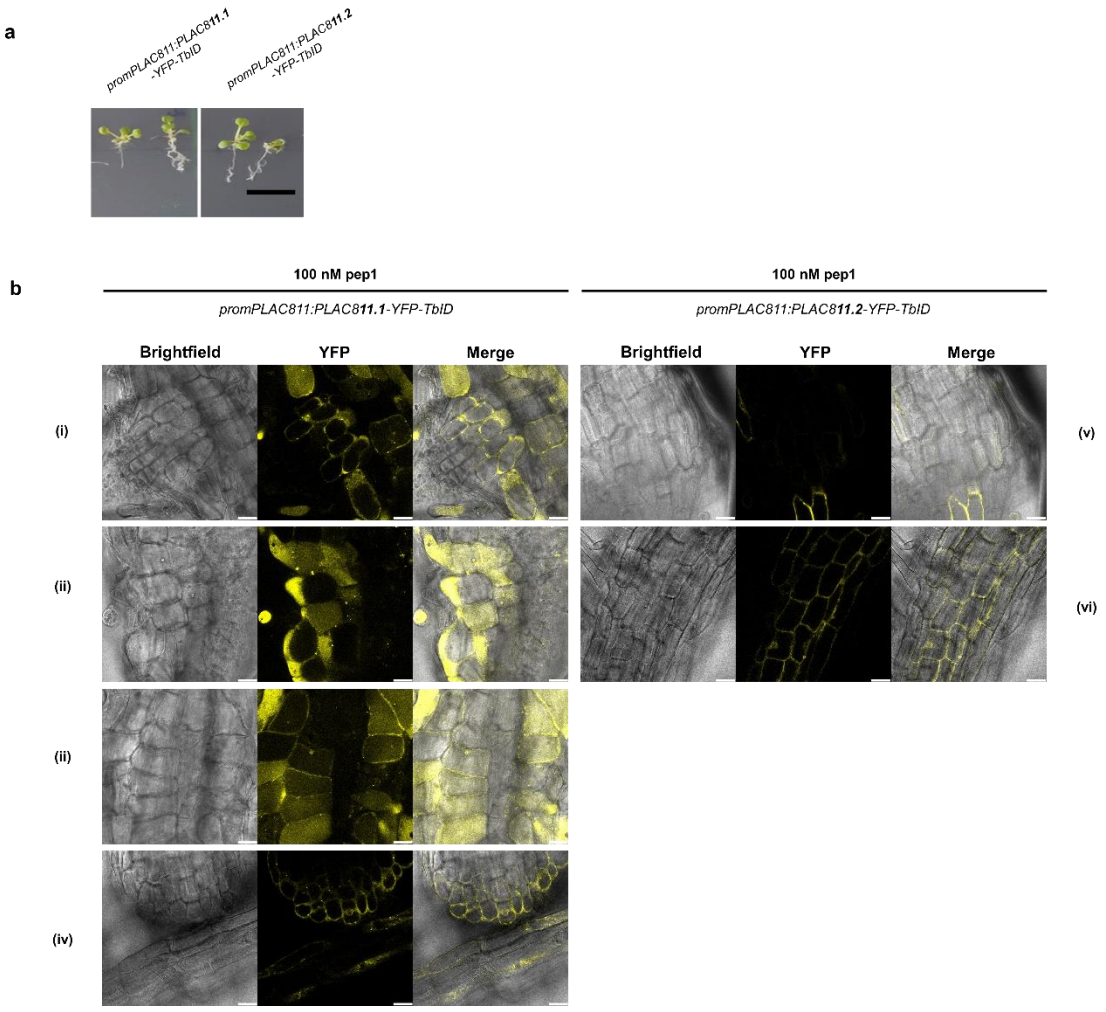


Figure S5. Histological changes in AtPLAC811.1 and AtPLAC811.2 roots in response to Pep1. (a) The 10-day-old transgenic *plac811* seedlings harboring *promoPLAC811:PLAC811.1-YFP-TbID* and *promoPLAC811:PLAC811.2-YFP-TbID* grown in half-strength MS agar medium with 100nM Pep1. **(b)** Seedlings in letter (a) photographed under a confocal laser-

scanning microscope (i-iv): *promoPLAC811:PLAC811.1-YFP-TbID* roots and (v,vi): *promoPLAC811:PLAC811.2-YFP-TbID* roots. Scale bars 20µM.

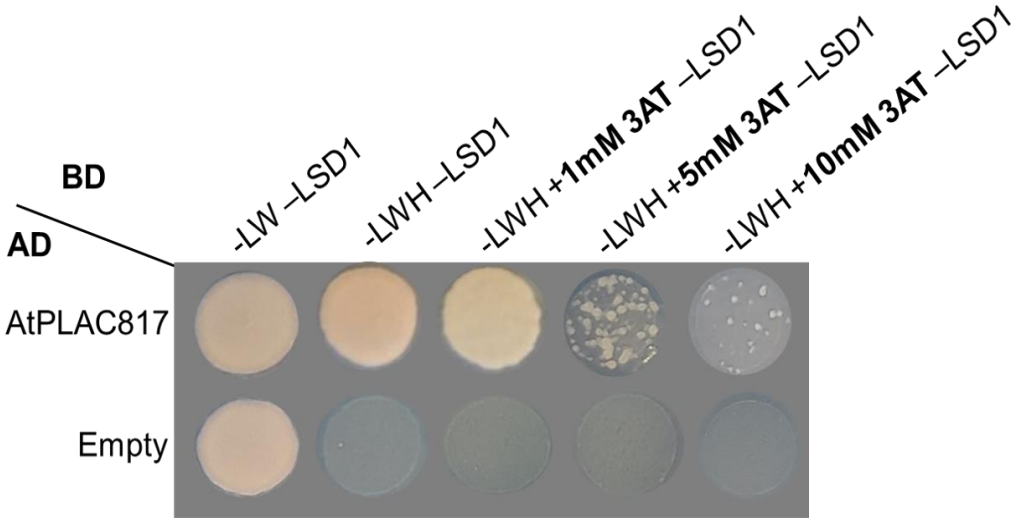


Figure S6. Yeast two-hybrid assay: AtPLAC817 interacts with AtLSD1. The CDS of AtPlac817 was cloned in the pDEST22 (AD = Activation Domain) destination vector and was used as prey. The destination vector pDEST32 (BD = Binding Domain) containing the AtLSD1 CDS was used as bait. Co-transformation of the empty vector with pDEST vector containing the CDS of AtPlac817 was used as a negative control. Yeast cells grown on SD plates deficient in Tryptophan and Leucine (-LW) indicate successful co-transformations. Yeast cells were grown on SD plates deficient in Tryptophan, Leucine, and Histidine (-LWH) and supplemented with either 1, 5 or 10 mM of 3-Amino-1,2,4-triazole (3AT).

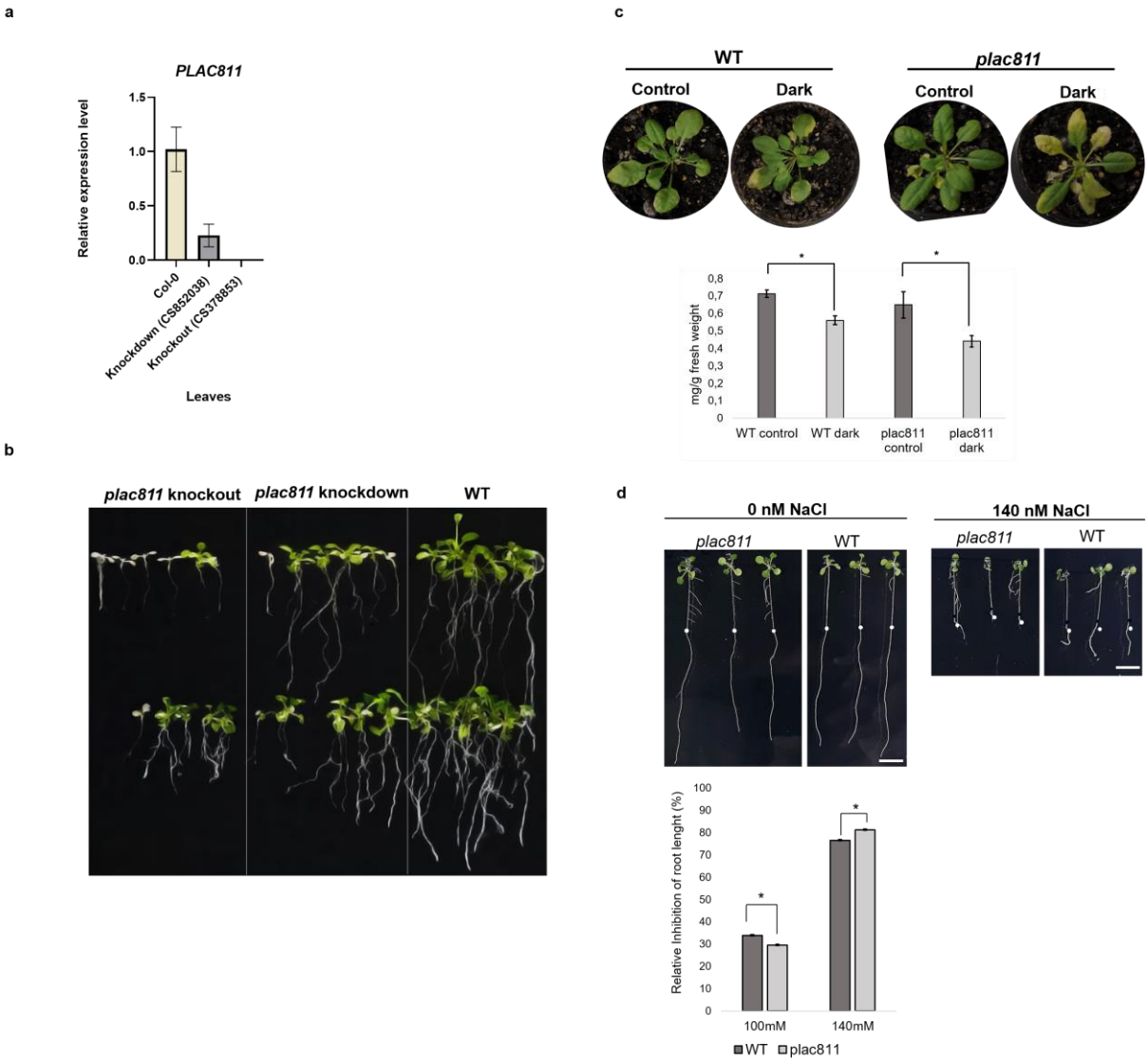


Figure S7. Phenotypic analysis of *plac811* mutant against abiotic stress. (a) Analysis of *AtPLAC8-11.1* gene expression in shoot of CS852038 and CS378853 mutant lines with insertion of T-DNA in the promoter region and exon 1, respectively. The columns present the mean of the biological triplicates and the bars the standard error. (n = 3). The standardizer used was Ubiquitin-10 (AT4G05320) and the analyzes were performed using the 2-ΔΔCt method (Livak and Schmittgen, 2001). **(b) Heat Stress experiment**. 1-week-old *plac811* knockout, *plac811* knockdown and wild-type seedlings were subject to heat stress at 45 °C for 1 hour. Photographs were taken 10 days after the heat treatment. **(c) Senescence experiment.**

3-week-old *plac811* knockout and wild-type seedlings were exposed to dark conditions for 10 days and chlorophyll content was measured. **(d) Salt stress experiment.** Relative inhibition of root length of WT and *plac8-11* knockout mutant seedlings grown on ½ MS agar plates with 140 mM NaCl after growing vertically at 22˚C for 10 days. n= 20. Scale bars 1cm.

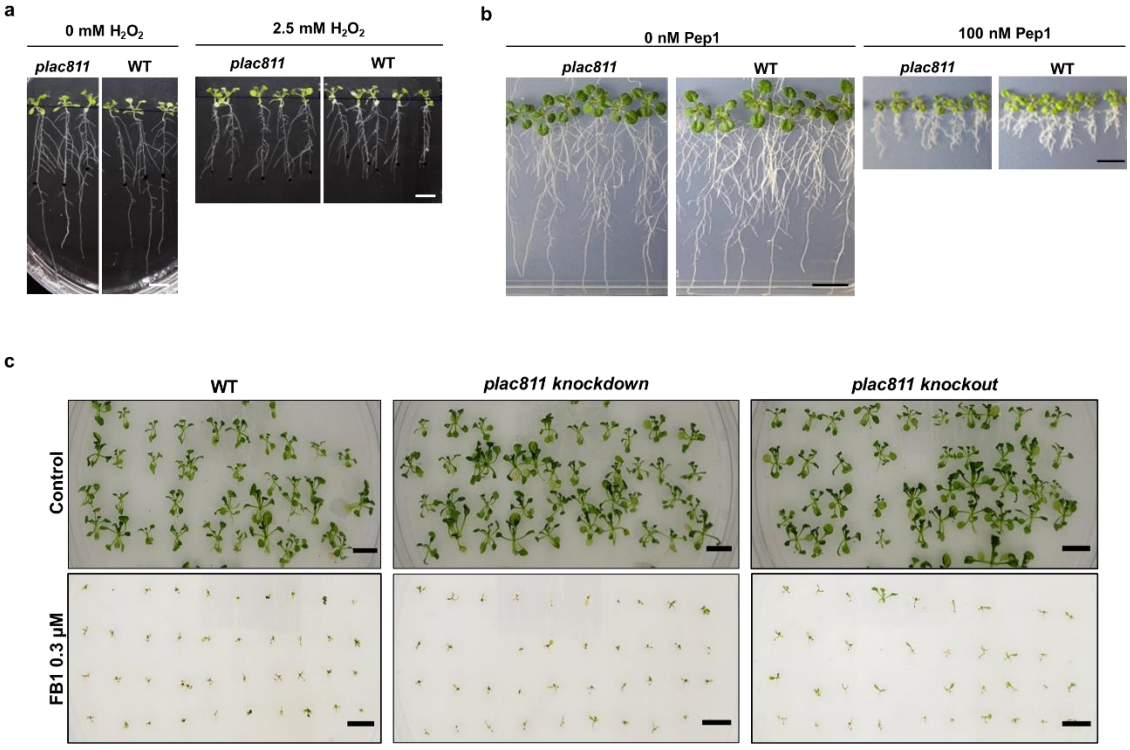
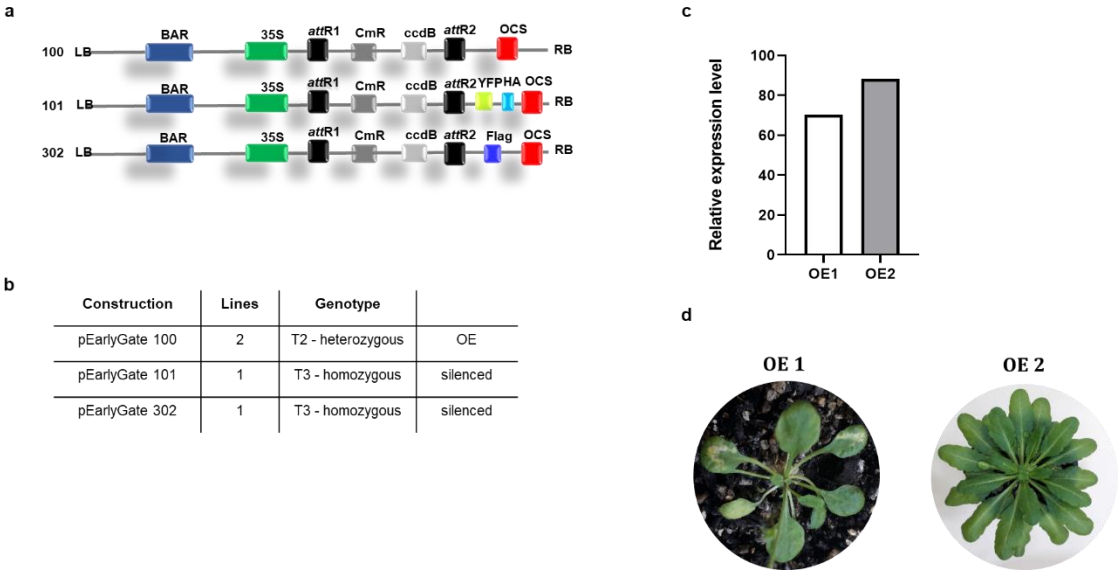


Figure S8. Phenotypic analysis of *plac811* mutant against abiotic stress and biotic stress. (a) Hydrogen Peroxide experiment. 1-week-old *plac811* knockout and wild-type plants were grown on ½ MS medium were transferred to ½ MS medium without or with 2.5 mM H_2O_2. Photographs were taken 7 days after H_2O_2 treatment. **(b) Pep1 elicitor experiment.** Seedlings of *plac811* knockout and wild-type directly germinated on half-strength ½ MS agar medium with or without 100 nM of pep1. Photographs were taken 14 days after the Pep1 treatment. **(c) Fumonisin B1 (FB1) treatment.** Seedlings of *plac811* knockout and wild-type directly germinated on half-strength ½ MS agar medium with or without 0.3 µM of FB1. Photographs were taken 20 days after FB1 treatment. Scale bars 1cm.



b

Construction	Lines	Genotype	
pEarlyGate 100	2	T2 - heterozygous	OE
pEarlyGate 101	1	T3 - homozygous	silenced
pEarlyGate 302	1	T3 - homozygous	silenced

Figure S9. Transgenic plants overexpressing (OE) the *AtPLAC8-11.1* present an unhealthy phenotype. (a) pEarleyGate vectors. Different pEarleyGate vectors were used to express *AtPLAC811.1* fused in frame with FLAG (302) and yellow fluorescent proteins, like YFP (101) at either the amino-terminal or carboxy-terminal end of the target proteins. **(b) pEarleyGate constructions.** Summary of T2 and T3 *Arabidopsis* plants overexpressing *AtPLAC8-11.1* generated by the Floral Dipping method. **(c) Lines overexpression *AtPLAC811.1*.** Expression of *AtPLAC8-11.1* in the shoot of *Arabidopsis thaliana* plants grown under control conditions and evaluated by RT-qPCR. *AtPP2A* (AT1G13320) was used as reference gene. **(d) Phenotypes of transgenic *Arabidopsis* overexpressing *AtPLAC811.1* fused in frame with YFP (pEarleyGate 100).** Phenotypes of two transgenic plants T2 in the soil after plate selection with the herbicide Basta [10 mg/L].

Capítulo III

O terceiro capítulo apresenta parte dos resultados obtidos durante doutorado sanduíche realizado em Carnegie Institution of Science, Departament of Plant Science na Califórnia sob orientação do Dr. Zhiyong Wang, durante o período de dezembro de 2021 – maio de 2022. Durante esse período buscou-se identificar os parceiros de interação de AtPLAC811.1 e de sua variante de splicing, AtPLAC811.2 através de experimentos de interação proteína-proteína utilizando o sistema TurboID marcação de proximidade.

INTRODUÇÃO

O estudo das interações proteína-proteína (*protein-protein interaction* - PPI) são essenciais para entender as relações funcionais entre proteínas (FUKAO, 2012). Essas interações atuam amplamente em diversos processos biológicos, controlando sinais que afetam a homeostase geral do organismo, agindo no controle metabólico, na regulação das etapas do desenvolvimento, atuando em vias de transdução de sinal e em resposta a estresses (BRACHA-DRORI et al., 2004; CÁNOVAS et al., 2004; FUKAO, 2012; PAN et al., 2021). Nesse sentido, descrever as redes moleculares complexas que atuam em processos biológicos específicos, através das redes de sinalização em PPIs, permite prever os processos biológicos nos quais uma proteína de função desconhecida está envolvida (ZHANG; GAO; YUAN, 2010). Assim, a descoberta de novos parceiros de interação para proteínas chaves da morte celular programada pode auxiliar no entendimento dos mecanismos regulatórios envolvidos nesse processo rigidamente regulado, que hoje é considerado como um desafio para a comunidade científica de plantas (VALANDRO et al., 2020).

A abordagem de "marcação de proximidade catalisada por enzimas" (*proximity labeling* - PL) foi desenvolvida para identificar proteínas que interagem com uma proteína alvo, superando algumas desvantagens de métodos tradicionais como a purificação por afinidade acoplada com espectrometria de massa (*Affinity purification coupled with mass spectrometry* - AP-MS), de difícil identificação de proteínas que interagem fracamente entre si (ROUX et al., 2012a; ZHANG et al., 2019). Nas abordagens de marcação de proximidade, a proteína alvo de interesse é fusionada traducionalmente com uma enzima catalisadora da biotinilação de proteínas endógenas de maneira dependente da proximidade, permitindo que proteínas próximas e que estejam interagindo sejam marcadas na presença de biotina (BRANON et al., 2018; ZHANG et al., 2019). As principais enzimas utilizadas para o estudo de PL foram uma ascorbato peroxidase de soja modificada (denominada APEX2) e um mutante promíscuo da biotina ligase de *Escherichia coli* BirA (denominada BioID) (CHOI-RHEE; SCHULMAN; CRONAN, 2008; RHEE et al., 2013). A biotina ligase BirA* possui uma mutação que desestabiliza a ligase, permitindo que moléculas ativas de biotina se dissociem da ligase e se liguem a lisinas expostas nas proteínas vizinhas. Assim, para capturar complexos proteicos, é possível expressar a

proteína de interesse (isca) fundida com BirA* em plantas de *A. thaliana* e realizar o tratamento com biotina exógena (ROUX et al., 2012b).

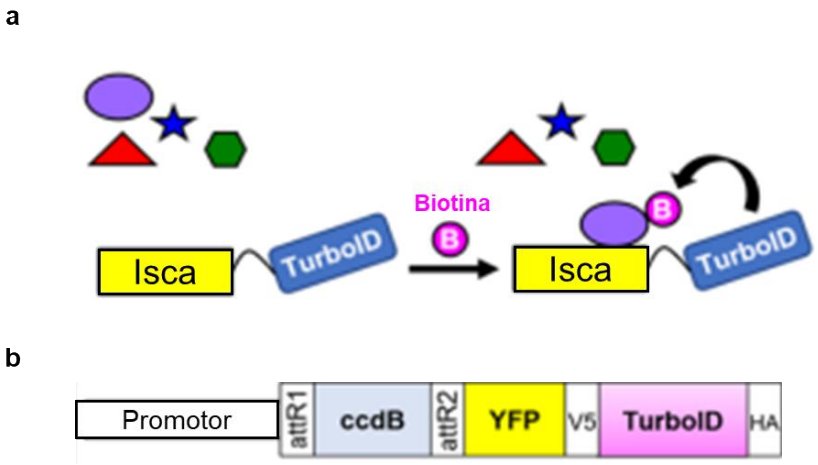


Figura 1. Marcação de proximidade por TurboID. (a) Diagrama representativo da marcação de biotina dependente da proximidade de proteínas que interagem. **(b)** Diagrama do vetor binário compatível com o sistema *Gateway* para expressar a proteína de fusão TurboID (TbID) marcada com YFP (*ccdB* será substituído pela sequência de codificação de interesse, como AtPLAC811.1 e AtPLAC811.2). Adaptado de (KIM et al., 2019).

Nos últimas anos, avanços científicos permitiram melhorar significativamente a cinética de marcação de BioID, originando um novo mutante promíscuo BirA, denominado de TurboID (Figura 1) (BRANON et al., 2018). A metolodogia TurboID tem como vantagens a marcação de proximidade de maneira rápida e não tóxica, atingindo um nível alto de biotinilação em apenas 10 minutos (BRANON et al., 2018). De maneira geral, após algumas horas com alimentação de biotina, as proteínas biotiniladas (interatores) podem ser podem ser isoladas por captura com esferas ou matrizes de afinidade por estreptavidina e identificadas por espectrometria de massa (ROUX et al., 2012).

OBJETIVOS

Identificar novos parceiros de interação para AtPLAC811.1 e AtPLAC811.2 através de experimento de interação proteína-proteína utilizando o sistema TurboID-marcação de proximidade.

MATERIAIS E MÉTODOS

Clonagem da região promotora de *AtPLAC811* e sequência genômica de *AtPLAC8-11.1* e *AtPLAC8-11.2* no vetor de entrada pENTR/D-TOPO

Cerca de 2 kb da região a montante do sítio de início da tradução de *AtPLAC811* mais a sequência genômica de *AtPLAC811.1*, ou *AtPLAC811.2* foram amplificados com um conjunto de primers específicos (Tabela 1) a partir de gDNA de *A. thaliana.* Para a realização de amplificação foi utilizada a enzima *Q5 Hot Start High-Fidelity* DNA Polimerase (New England Biolabs), de acordo com as especificações do fabricante. As amplificações foram verificadas em por eletroforese em gel de agarose 1% corado com GelRed® diluído 1:500 (Biotium), e após a corrida dos fragmentos as bandas contendo os *amplicons* desejados foram cortadas do gel e purificadas com o kit de purificação de DNA em banda de gel (Ludwig Biotec). Os fragmentos purificados foram clonados separadamente no vetor pENTR/D-TOPO® (Invitrogen®) de 2580 pb, de maneira a gerar clones de entrada para o sistema Gateway (Invitrogen). Para tal, utilizou-se o kit pENTR Directional TOPO® Cloning (Introvigen), de acordo com as instruções do fabricante. Mediante choque térmico, o produto da clonagem foi inserido em células bacterianas termocompetentes de *E. coli* OmniMAX. Após a confirmação por PCR, os clones positivos tiveram seus plasmídeos extraídos com kit de *miniprep* (PureYield™ Plasmid Miniprep System - Promega) e foram confirmados por sequenciamento.

Tabela 1. *Primers* utilizados nas reações de clonagem.

	Nome	Sequência	Produto
a)	***a) promoPLAC811:PLAC811.1***	F: CACCTGGTAGAAGGTAATCTGTCGC R: CTTAGACATATATTGATTTGTAGGAGCAAA	3.247 pb
b)	***promoPLAC811:PLAC811.2***	F: CACCTGGTAGAAGGTAATCTGTCGC R: CCACACTTGAACACTTATATCAA	2.872 pb

F: *Forward*; R: *Reverse*. * Em vermelho está destacado a sequência de 4 pares de bases (CACC) necessária para clonagem direcional da região de interesse no vetor de entrada pENTR/D-TOPO (Invitrogen®).

Recombinação com o vetor de destino pEG-TW-Turbo e transformação de *Agrobacterium tumefaciens* EHA105

Os plasmídeos *pENTR:promoPLAC811:PLAC811.1* e *pENTR:promoPLAC811:PLAC811.2* foram linearizados com a enzima de restrição ApaLI (New England Biolabs®), purificados com kit de purificação de DNA em banda de gel (Ludwig Biotec) e recombinados no vetor de destino pEG-TW-Turbo, cedido pelo professor Zhiyong Wang, utilizando o kit Gateway® LR Clonase™ Enzyme Mix (Invitrogen) de acordo com as instruções do fabricante, para obter as seguintes construções: *promoPLAC811:PLAC811.1-YFP-TbID* e *promoPLAC811:PLAC811.2-YFP-TbID*. Células eletrocompetentes de *A. tumefaciens* EHA105 foram submetidas a transformações com os vetores acima descritos através do protocolo de eletroporação, utilizando o eletroporador (BTX) com a aplicação de um pulso elétrico de 2500 volts. A suspensão bacteriana foi recuperada adicionando-se 500 µL de meio LB (Luria-Bertani - Invitrogen) líquido nas células transformadas e a reação foi incubada a 28 ºC por 2 horas. Após a recuperação, a suspensão contendo as bactérias eletrotransformadas foi cultivada em meio LB sólido contendo os antibióticos rifampicina (100 mg/L) e canamicina (50 mg/L). Estas placas foram incubadas em estufa por 48 horas a 28 ºC para o aparecimento de colônias.

Análise por PCR das colônias de *Agrobacterium tumefaciens* EHA105 transformadas com o vetor de destino pEG-TW-Turbo

Os conjuntos de primers *PLAC811.1* e *PLAC811.2* (Tabela 2a e b) foram utilizados em reações de PCR de modo a confirmar a correta inserção das versões gênicas de *AtPLAC811* nas construções com o vetor pEG-TW-Turbo e visam a amplificação da região genômica de *AtPLAC811.1* e *AtPLAC811.2*. Para tal, utilizou-se o mesmo *primer* forward para ambas as versões, começando no códon de iniciação da síntese protéica e com *primers* reversos específicos para ambas as versões, abrangendo o final das respectivas regiões codificantes. Visto que as construções conferem resistência em plantas ao herbicida Basta, o conjunto de primers do gene de resistência ao BASTA (Tabela 2c) também foi utilizado como controle de transformação. As reações de PCR de confirmação foram realizadas com os produtos das

extrações do DNA plasmidial de colônias de *Agrobacterium tumefaciens* EHA105 transformadas com o vetor de destino pEG-TW-Turbo, que foram utilizadas para realizar o floral dip. As reações de PCR foram realizadas em aparelho Veriti™ 96-Well Thermal Cycler (Applied Biosystems®) e com a enzima Platinum® Taq DNA Polymerase (Invitrogen®).

Tabela 2. *Primers* utilizados nas reações de confirmações.

	Nome	Sequência	Produto
a)	***PLAC811.1***	F: ATGGGTCGTGTCACTACTCCAT R: CTTAGACATATATTGATTTGTAGGAGCAAA	1.134pb
b)	***PLAC811.2***	F: ATGGGTCGTGTCACTACTCCAT R: CCACACTTGAACACTTATATCAA	749pb
c)	**BASTA**	F: GCACCATCGTCAACCACTAC R: GTACCGGCAGGCTGAAGTC	552pb

F*: Forward;* R*: Reverse.*

Floral Dipping e Seleção de plantas transgênicas de *A. thaliana*

Plantas *knockout plac8-11* foram transformadas geneticamente com as construções *promoPLAC811:PLAC811.1-YFP-TbID* and *promoPLAC811:PLAC811.2-YFP-TbID* através do método de floral dipping (ZHANG et al., 2006). A assepsia das sementes T1 oriundas da transformação genética via *floral dipping* foi realizada adicionando-se 1 mL de etanol 70% + Triton 100-x 0,1% por 5 min, seguindo pela adição de hipoclorito 4% por mais 8 minutos, sob constante agitação. As sementes foram lavadas 5 vezes com água destilada estéril, sendo ressuspendidas em solução estéril de agarose 0,1%. As sementes transformadas foram inoculadas em placas de Petri contendo meio MS meia-força (Murashige and Skoog Basal Salt Mixture - Sigma-Aldrich) com 1% (m/v) de sacarose e 0,5% de fitagel (m/v). O pH da solução nutritiva foi ajustado entre 5,7 - 5,8 pela adição de KOH. Para evitar contaminação pelo crescimento de *Agrobacterium tumefaciens*, as placas foram suplementadas com o antifúngico nistatina (50 µg/mL) e o antibiótico carbenicilina (250 µg/mL). O herbicida glufosinato de amônio, comercialmente denominado por basta (10 mg/L) foi empregado para realizar a seleção das sementes transgênicas. Após estratificação no escuro a 4 °C por 72h, as placas foram

transferidas para sala de cultivo sob condições de dia longo com 16h de luz/8h escuro, 24°C, 130 µmol m^{-2} s^{-1} de radiação fotossinteticamente ativa (PAR) e 34% de umidade relativa (UR). As plântulas transgênicas resistentes em placa foram transferidas para uma mistura de 1:2 de solo e vermiculita expandida, e crescidas na sala de cultivo até a coleta das sementes. A partir das plantas T2, a seleção das plantas transgênicas ocorreu com a aplicação do herbicida basta (diluído 1:500) nas plantas germinadas diretamente em solo. A seleção de plantas resistentes ao herbicida basta foi realizada até o estágio homozigoto T3, sendo confirmada por *western blot*.

Western Blot para análise de proteínas

Após a seleção de plantas transgênicas resistentes ao herbicida basta, folhas de plantas de *Arabidopsis* expressando as construções *promoPLAC811:PLAC811.1-YFP-TbID* e *promoPLAC811:PLAC811.2-YFP-TbID* foram coletadas e armazenadas em nitrogênio. As plantas contendo a construção *35S:PLAC811.1-YFP-TbID*, cedidas pelo professor Zhiyong Wang foram usadas como controle positivo. Para a realização de *western blots*, aproximadamente 50 mg de cada material vegetal foi macerado e homogeneizado com 100 µL do tampão de amostra SDS (*Sodium Dodecyl Sulfate* – SDS) (2X) (500 mM Tris-HCl pH 6,8, 10% SDS, 20% Glicerol, 2% 2-β-mercaptoetanol e 0,01% azul de bromofenol). Após aquecimento em bloco térmico a 90°C por 10 min com tampão de amostra SDS (2X), os extratos proteicos foram centrifugados por 10 minutos a 12.500 rcf e então os sobrenadantes foram cuidadosamente transferidos para os poços do gel SDS-PAGE 7.5% (Mini-PROTEAN® TGX™ Precast Protein Gels – BIO-RAD). Para a corrida do gel, utilizou-se cuba vertical de eletroforese preenchida com o tampão de solubilização SDS (1X) (EDTA 500 mM pH 8.0, 10% SDS, e Tris-Cl 1M pH 7.5). Inicialmente a corrida do gel deu-se a 80 V por 30 minutos e após, a 120 V por aproximadamente 1 hora. Após a corrida, as proteínas foram transferidas para membrana de nitrocelulose (Bio-rad) com auxílio do tampão de transferência (192 mM glicina, 25mM tris base e 20% metanol) e a transferência para membrana deu-se a 12 V por 12 minutos utilizando o aparelho Trans-Blot® SD Semi-Dry Blotting (Bio-Rad). Após a transferência, a membrana foi bloqueada por 1h em uma solução 1x PBST (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4 e 0.1% Tween® 20 detergente) contendo 5% de leite desnatado. Após o bloqueio, a membrana foi incubada 16 horas a 4°C sob agitação com o anticorpo

primário Anti-GFP Mouse mAb 1:1000 (TransGen Biotech). A membrana então foi lavada três vezes com tampão PBST acima descrito e, após, incubada com a solução PBST com o anticorpo secundário Goat anti-Mouse IgG 1:3000 (Bio-rad) por 1 h à temperatura ambiente sob agitação. Após o período de incubação com o anticorpo secundário, a membrana foi lavada três vezes com tampão 1x PBST. Para a detecção das proteínas foi utilizado o kit SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher).

Emprego do método TurboID-marcação de proximidade para busca de interatores de AtPLAC8-11.1 e AtPLAC8-11.2

Nesse experimento foi utilizada a marcação por afinidade *in vivo* por biotinilação baseada em proximidade a partir de adaptações do procedimento experimental descrito por KIM et al., 2019; MAIR et al., 2019. Após o *western blot* para verificação das linhagens canditadas para o realizar o experimento TurboID, as linhagens T3 homozigotas com maior expressão proteica das construções *promoPLAC811:PLAC811.1-YFP-TbID* e *promoPLAC811:PLAC811.2-YFP-TbID* foram utilizadas no experimento, sendo a linhagem *35S:PLAC811.1-YFP-TbID* empregada como controle. Inicialmente realizou-se a preparação dos meios MS (Tabela 3) para germinação e crescimento das sementes.

Tabela 3. Meio MS para germinação e crescimento das linhagens selecionadas para o experimento TurboID.

1 L	Meio MS N14	Meio MS N15 * reagentes marcados com N15
MS sem nitrogênio (PhytoTech Labs®)	0.78 g	0.78 g
Nitrato de amônio, NH_4NO_3 (Merk)	0.5 g	-
Nitrato de potássio, KNO_3 (Thermo Fisher)	0.5 g	-
* Nitrato de amônio, NH_4NO_3 (Cambridge isotope laboratories)	-	0.5 g
* Nitrato de potássio, KNO_3 (Cambridge isotope laboratories)	-	0.5 g
Phytoblend (Caisson labs)	9 g	9 g

* Os asteriscos em vermelho significam reagentes marcados com N15.

Após a preparação dos meios MS N14 e MS N15, os mesmos foram vertidos em placas quadradas com dimensões de 115x115 mm. As sementes das construções acima citadas foram esterilizadas com uma solução de etanol 70% + Triton 100x 0,1% por 10 minutos com agitação. Após as sementes foram lavadas com etanol 100% cinco vezes e colocadas sobre papel filtro estéril para secagem na capela de fluxo laminar. Todos os experimentos foram realizados em triplicatas conforme Figure 2.

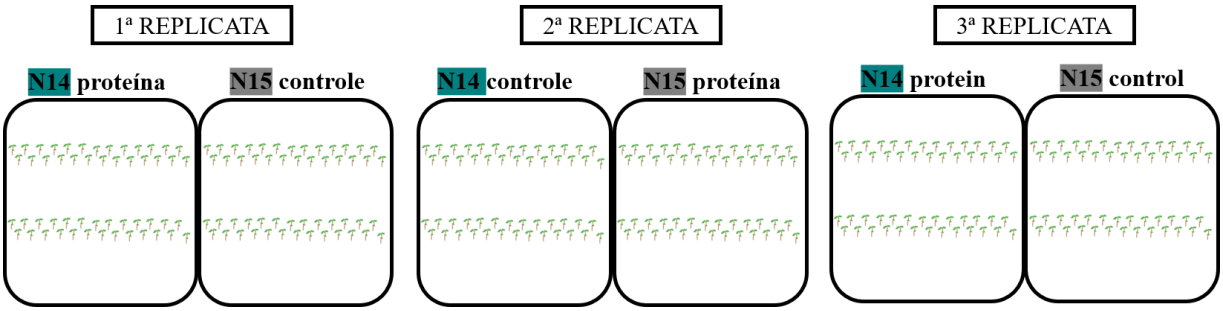


Figura 2. Desenho experimental de preparação das replicatas das amostras utilizando os meios MS N14 e N15. Aproximadamente 50 sementes das respectivas construções foram semeadas em cada placa.

De maneira a obter os interatores de AtPLAC811.1 e AtPLAC811.2, foram realizados dois experimentos independentes, sendo que no primeiro experimento a construção *promoPLAC811:PLAC811.1-YFP-TbID* foi utilizada como proteína isca e a construção *35S:PLAC811.1-YFP-TbID* como controle. O segundo experimento buscou identificar os interatores de AtPLAC811.2 e para isso, a construção *promoPLAC811:PLAC811.2-YFP-TbID* foi utilizada como proteína isca e a construção *promoPLAC811:PLAC811.1-YFP-TbID* como controle. Após a preparação das placas com sementes nos meios MS contendo isótopos de nitrogênio diferentes, denominado de meio MS N14 e MS N15, as sementes foram estratificadas no escuro a 4 °C por 72 h antes de serem colocadas em posição vertical em uma câmara de crescimento sob a condição de luz constante a 21-22 °C. Após quinze dias de crescimento, plântulas das construções acima citadas (*promoPLAC811:PLAC811.1-YFP-TbID*, *promoPLAC811:PLAC811.2-YFP-TbID* e *35S:PLAC811.1-YFP-TbID*) foram infiltradas com uma solução de 50 µM de biotina com a aplicação de vácuo através de uma seringa, até que os espaços de ar fossem preenchidos com líquido (aproximadamente 5 minutos). Após a

infiltração, as plântulas foram incubadas à 22 ℃ com a solução de biotina por 3 horas. Posteriormente ao tratamento com a biotina, o material vegetal foi seco, congelado em nitrogênio líquido e armazenado em ultrafreezer -80°C para posterior utilização.

Os procedimentos experimentais a partir de agora serão descritos conforme realização progressiva ao longo dos dias. **Dia 1 - extração de proteína:** O material vegetal foi macerado com Tissue Lyser® (30/s, 2m). Em um falcon de 50 mL previamente resfriado com auxílio de nitrogênio líquido, foi adicionado igualmente 1g de material vegetal referente as plântulas crescidas em meio MS N14 e 1 g das plântulas crescidas em meio MS N15. Após, foi adicionado 2 mL do tampão de extração (50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 0.5 % Na-deoxycholate, 1 mM EGTA, 1 mM DTT, 1x coquetel inibidor de protease (Roche) e 1 mM PMSF) e o material vegetal agitado em vortex em câmara fria, sendo posteriormente incubado a 4°C por 10 minutos. O material foi então sonicado no gelo (Branson, 20% de amplitude, 10sON/10s OFF por 2 min). A suspensão foi centrifugada a 1.500 rpm por 5 minutos a 4°C e o sobrenadante foi transferido para um novo tubo, sendo novamente centrifugado a 12.000 rpm por 10 minutos a 4°C para remover os detritos celulares. Para remover a biotina livre, o sobrenadante foi passado através da coluna de dessalinização PD-10 usando o protocolo de gravidade de acordo com as instruções do fabricante. As proteínas dessalinizadas foram eluidas da coluna com tampão de equilíbrio (50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % SDS, 1 % Triton-X-100, 0.5 % Na-deoxycholate, 1 mM EGTA e 1 mM DTT) e uma alíquota de 30 uL foi reservada para medir a concentração do extrato protéico por Bradford com o kit Pierce™ BCA Protein Assay (Thermo Scientific). O eluato coletado foi incubado com esferas de estreptavidina Dynabeads™ M-280 (Invitrogen), que foram pré-lavadas com tampão de extração duas vezes. O extrato proteico foi incubado com as esferas de estreptavidina em rotor a 4 °C *overnight*. Aproximadamente 150 µL de esferas de estreptavidina foram usadas para 2 g de tecido inicial.

Dia 2 - purificação de afinidade: No dia seguinte, as esferas foram centrifugadas a 1500 rpm por 2 minutos a 4°C. O sobrenadante foi removido e então foram adicionados 14 mL de tampão de extração gelado e as esferas foram novamente centrifugadas a 1500 rpm por 2 minutos a 4°C. O sobrenadante foi novamente removido e as esferas lavadas com 1 mL de cada

uma das seguintes soluções: 2x com tampão de extração gelado sem inibidor de protease (as esferas foram transferidas para um novo tubo na primeira vez), 2x com tampão de equilíbrio gelado, 1x com KCl 1M gelado (as esferas foram transferidas para um novo tubo), 1x com Na_2CO_3 100mM gelado, 1x com Ureia 2M em Tris 10mM pH8 a temperatura ambiente. Cada lavagem foi realizada em câmara fria incubando a respectiva solução com as esferas em rotor por 8 minutos. **Digestão tríptica nas esferas:** As esferas de estreptavidina foram lavadas por 3 minutos duas vezes cada com 1 mL de Tris 50 mM pH 7,5 (as esferas foram transferidas para um novo tubo na primeira lavagem). Após a última lavagem a solução foi removida e substituída por 80 µl de tampão de tripsina [50 mM Tris pH 7,5, 1M de ureia, 1 mM de DTT e 0,4 µg de tripsina (Sigma-Aldrich)]. As esferas foram então incubadas por 3h a 25°C com agitação, e após o sobrenadante foi transferido para um novo tubo. As esferas foram lavadas duas vezes rapidamente com 60 µl de ureia 1M em 50 mM de Tris pH 7,5 e todos os sobrenadantes foram combinados (volume final de 200 µl (60 uL + 60 uL + 80 uL), sendo chamados como peptídeos eluídos **(e)**. As esferas foram armazenadas a 4°C para posterior utilização. Os eluídos combinados foram primeiro reduzidos, adicionando DTT a uma concentração final de 4 mM e incubados a 25°C por 30 minutos com agitação, e depois alquilados pela adição de iodoacetamida a uma concentração final de 10 mM e incubação a 25°C por 45 minutos no escuro. Finalmente, outros 0,5 µg de tripsina foram adicionados e a digestão foi completada por incubação *overnight* a 25°C com agitação.

As esferas que estavam a 4°C, denominadas como peptídeos biotinilados ligados a estreptavidina **(b)** foram lavadas 3x com água destilada estéril. O tampão de eluição (80% acetonitrila/0,2% ácido trifluoroacético/0,1% ácido fórmico) foi pré-aquecido a 65°C e utilizando para eluir os peptídeos biotinilados das esferas, adicionando-se 200 µL do tampão de eluição às esferas e incubando a 75 °C por 5 minutos. O processo de eluição foi repetido 3x e os eluatos (600 µL) foram combinados e secos em SpeedVac (~ 2h). Por fim, adicionou-se 200 µL de uma solução contendo 5% acetonitrila e 0,1% ácido trifluoroacético ao tubo seco e o os peptídeos enriquecidos foram dessalinizados com colunas C18 (Pierce® C18 Tips, Thermo Scientific) da seguinte maneira: Os *tips* de dessalinização C18 foram primeiro ativados aspirando duas vezes e descartando 200 µl de tampão B2 (0,1% de ácido fórmico, 50% de acetonitrila) e equilibradas por quatro vezes aspirando e descartando 200 µl de tampão A2 (0,1%

de ácido fórmico). Os peptídeos foram ligados aos *tips* aspirando e dispensando a amostra oito vezes. Em seguida, o *tip* foi lavado 10 vezes aspirando e descartando 200 µl de tampão A2 e os peptídeos foram eluídos aspirando e dispensando 200 µl de tampão B2 em um novo tubo por oito vezes e então secos em SpeedVac (~1hr). Após, adicionou-se 100 uL tampão de tripsina (1M ureia em Tris 50 mM pH 7,5) aos peptídeos secos, sendo posteriormente reduzidos adicionando DTT a uma concentração final de 4 mM e incubando a 25°C por 30 minutos com agitação e depois alquilados adicionando iodoacetamida a uma concentração final de 10 mM e incubando a 25°C por 45 minutos no escuro. Por fim, outros 0,5 µg de tripsina foram adicionados e a digestão foi completada por incubação *overnight* a 25°C com agitação.

Dia 3: Adicionou-se mais 0,5 µg de tripsina pela manhã aos produtos do dia anterior, que foram posteriormente acidificados adicionando-se a digestão 10% de ácido fórmico para obter uma concentração final de 0,1%, sendo então dessalinizados com colunas C18 (Pierce® C18 Tips, Thermo Scientific) conforme descrição do dia anterior. Os peptídeos (e) e (b) dessalinizados foram secos em SpeedVac (~1h) e armazenados a -80°C até o processamento na espectrometria de massas.

Análise LC-MS/MS para detecção de peptídeo biotinilado

Para análise LC-MS/MS, os peptídeos foram ressuspensos em ácido fórmico a 0,1%. As amostras foram analisadas em um espectrômetro de massa quadrupolo-Orbitrap híbrido Q-Exactive HF (Thermo Fisher), equipado com um sistema de cromatografia líquida Easy LC 1200 UPLC (Thermo Fisher). Os peptídeos foram primeiro capturados usando a coluna de captura Acclaim PepMap 100 (75 uM x 2cm, nanoViper 2Pk, C18, 3 µm, 100A), depois separados usando a coluna analítica Aura Ultimate nanoflow UHPLC coluna (25cm x 74 um, C18, 1,7 µm) (IonOptiks). Os peptídeos foram eluídos por um gradiente de 3% a 28% de solvente B (80% de acetonitrila, 0,1% de ácido fórmico) ao longo de 100 min e de 28% a 44% de solvente B ao longo de 20 min, seguido de lavagem curta a 90% de solvente B.

Processamento e análise de dados para detecção dos interatores de AtPLAC811.1 e AtPLAC811.2

A identificação e quantificação de proteínas foi realizada em quatro etapas principais através do software *ProteinProspector* v 6.2.1 seguindo as orientações conforme descrito no artigo de Shrestha et al., 2022. **Primeira etapa:** Na primeira etapa, ocorreu a identificação das proteínas marcadas com nitrogênio ^{14}N e nitrogênio ^{15}N através de pesquisas em banco de dados, PA.TAIR10_pep_20101214.fasta.random.concat para Arabidopsis, utilizando os respectivos parâmetros de ^{14}N e ^{15}N separadamente, conforme Tabela 4.

Tabela 4. Parâmetros de pesquisa para ambas as marcações leves de 14N e com marcações pesadas de 15N.

Configuração do nome do parâmetro
Banco de dado: *TAIR10_pep_20101214.fasta.random.concat*
Taxonomia: todas
Massas: monoisotópicas
Digestão: tripsina
Máx. Clivagens perdidas: 1
Máx. Mods: 2
Parâmetros de pesquisa específicos para N^{14} (pesquisa ^{14}N [leve])
Modificação constante: Carbamidometil (C)
Modificação variável: Acetil (Proteína N-termo); Acetil+Oxidação (Proteína N-term M); Gln → piro-Glu (N-term Q); Perda de met (proteína N-termo M); Met-loss+Acetil (Proteína N-term M); Oxidação (M).
Parâmetros de pesquisa específicos para N^{15} (pesquisa ^{15}N [pesado])
Modificação constante: Marcador: 15N(1) (A); Marcador: 15N(1) (D); Marcador: 15N(1) (E); Marcador: 15N(1) (F); Marcador: 15N(1) (G); Marcador: 15N(1) (I); Marcador: 15N(1) (L); Marcador: 15N(1) (M); Marcador: 15N(1) (P); Marcador: 15N(1) (S); Marcador: 15N(1) (T); Marcador: 15N(1) (V); Marcador: 15N(1) (Y); Marcador: 15N(1)+Carbamidometil(C); Marcador: 15N(2) (K); Marcador: 15N(2) (N); Marcador: 15N(2) (Q); Marcador: 15N(2) (W); Marcador: 15N(3) (H); Marcador: 15N(4) (R).
Modificação variável: Acetil (Proteína N-termo); Perda de met (proteína N-termo M); Met-loss+Acetil (Proteína N-term M); Marcador: 15N(1)+Acetil+Oxidação (Proteína N-term M); Marcador: 15N(1)+Gln → piro-Glu (N-term Q); Marcador: 15N(1)+Oxidação (M).

Adaptado de (SHRESTHA et al., 2022).

Segunda etapa: Na segunda etapa foi determinada a eficiência da marcação ou enriquecimento de peptídeos marcados com ^{15}N. A eficiência da marcação é realizada com cerca de 8 a 10 peptídeos de diferentes proteínas abundantes, comparando o perfil isotópico experimental com o pico teórico para peptídeos com eficiência de marcação diferente. Para o cálculo, utiliza-se o software ImageJ, onde a intensidade do pico monoisotópico (M) é setada como distância conhecida de 1, e então a intensidade do pico M-1 é medida. A razão percentual de M-1/M é calculada e utilizada para comparar com as razões teóricas, visando determinar a eficiência da marcação. **Terceira etapa:** Na terceira etapa, após o cálculo da eficiência da marcação do experimento, realiza-se o "*Search Compare*" no software ProteinProspector com os parâmetros encontrados e especificados pelo usuário para extrair informações de quantificação. **Quarta etapa:** Recuperação do relatório para a quantificação e realização da análise informática.

RESULTADOS E DISCUSSÃO

Amplificação e confirmação das sequências promotoras + genômicas de *AtPLAC8-11* e do splicing alternativo *AtPLAC8-11.2*.

Através de PCR, buscou-se realizar a amplificação da região promotora de *AtPLAC811* (~2kb) em conjunto com a região genômica de *AtPLAC811.1* (3.247 pb) (Figura 3a), bem como a amplificação da região promotora de *AtPLAC811* (~2kb) em conjunto com a região genômica do splicing variante *AtPLAC811.2* (2.872 pb) (Figura 3b). A sequência genômica do gene *AtPLAC811.1* possui quatro éxons e três íntrons (Figura 3a), conforme representado pela marcação em azul e em branco após o início do códon de iniciação ATG, que está representado em vermelho. A sequência genômica de *AtPLAC811.2* é composta por três éxons e três íntrons (Figura 3b).

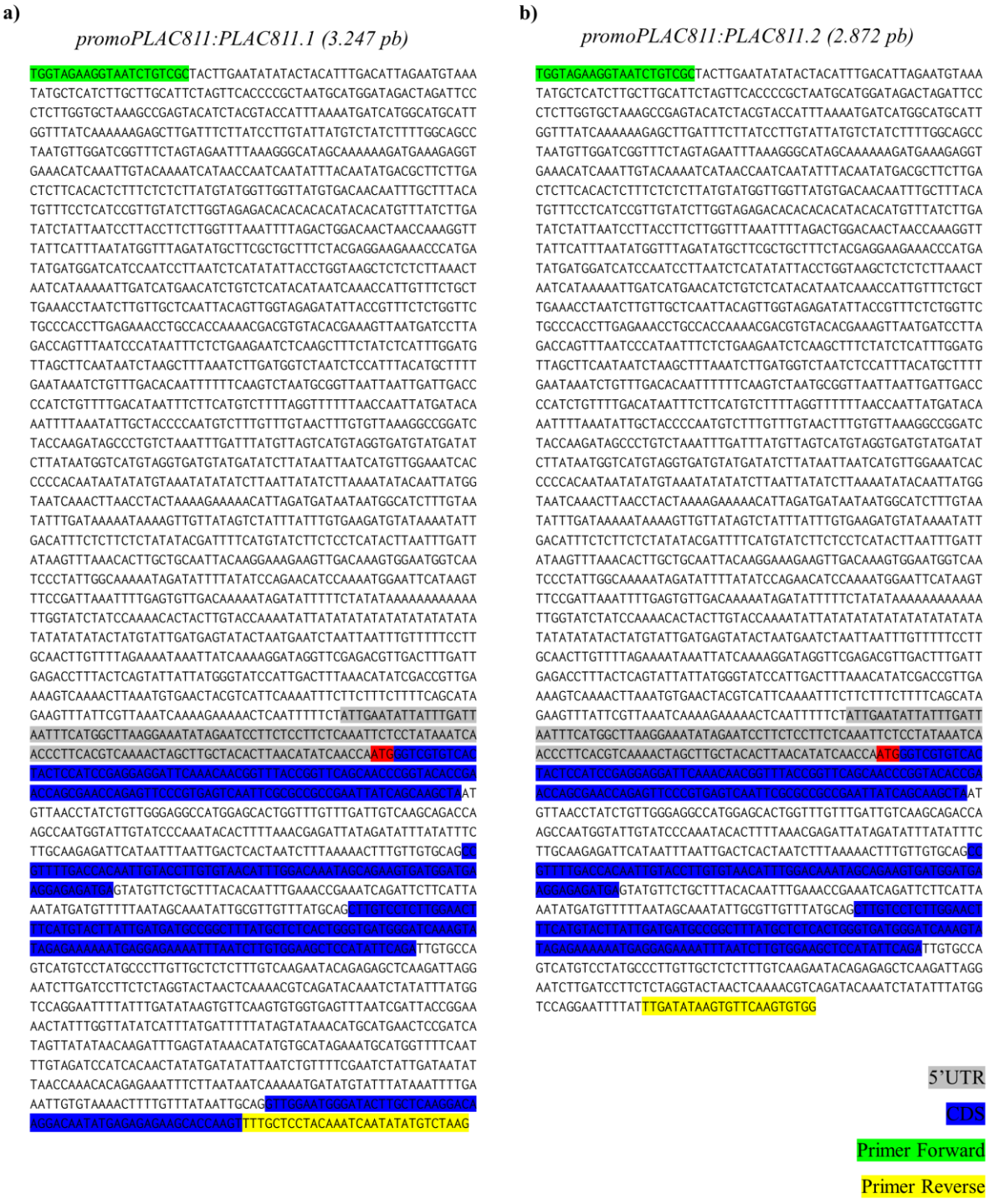


Figura 3: Sequência promotora e sequência genômica do gene *AtPLAC811.1* e do transcrito alternativo *AtPLAC811.2*. Para a amplificação das respectivas sequências genômicas de cada versão e da região promotora, os primers utilizados estão marcados nas

sequências nas cores verde para o primer direto e amarelo para o primer reverso. A marcação em azul corresponde a região de codificante de *AtPLAC811.1* e de *AtPLAC811.2.*

Nas reações de amplificação da região promotora e sequência genômica de *AtPLAC811.1* e *AtPLAC811.2* foram utilizados os *primers* listados na Tabela 1 que eliminam o códon de finalização (*stop codon*) para que houvesse a continuação da síntese protéica e fusão à proteína fluorescente YFP na recombinação com o vetor de destino pEG-TW-Turbo. Foi possível observar uma banda menor na amplificação da sequência referente a variante de splicing (~ 2.872 pb) em comparação com a forma canônica (3.247 pb) conforme esperado (Figura 4a). Nas reações de confirmação, o DNA plasmidial de colônias de *A. tumefaciens* EHA105 transformadas com o vetor de destino pEG-TW-Turbo foram utilizados bem como os *primers* listados na Tabela 2. Novamente, na figura 4a, foi observado uma banda menor na amplificação da sequência referente a variante de splicing (~ 749 pb) em comparação com a forma canônica (1.134 pb), visto que os *primers* utilizados amplificam do códon de iniciação da síntese protéica (ATG) até o final das respectivas regiões codificantes, incluindo as regiões intrônicas presentes na construção com o vetor pEG-TW-Turbo. Por fim, ambas as colônias contêm o gene de resistência ao herbicida BASTA presente no vetor pEG-TW-Turbo (Figura 4c), indicando transformação positiva.

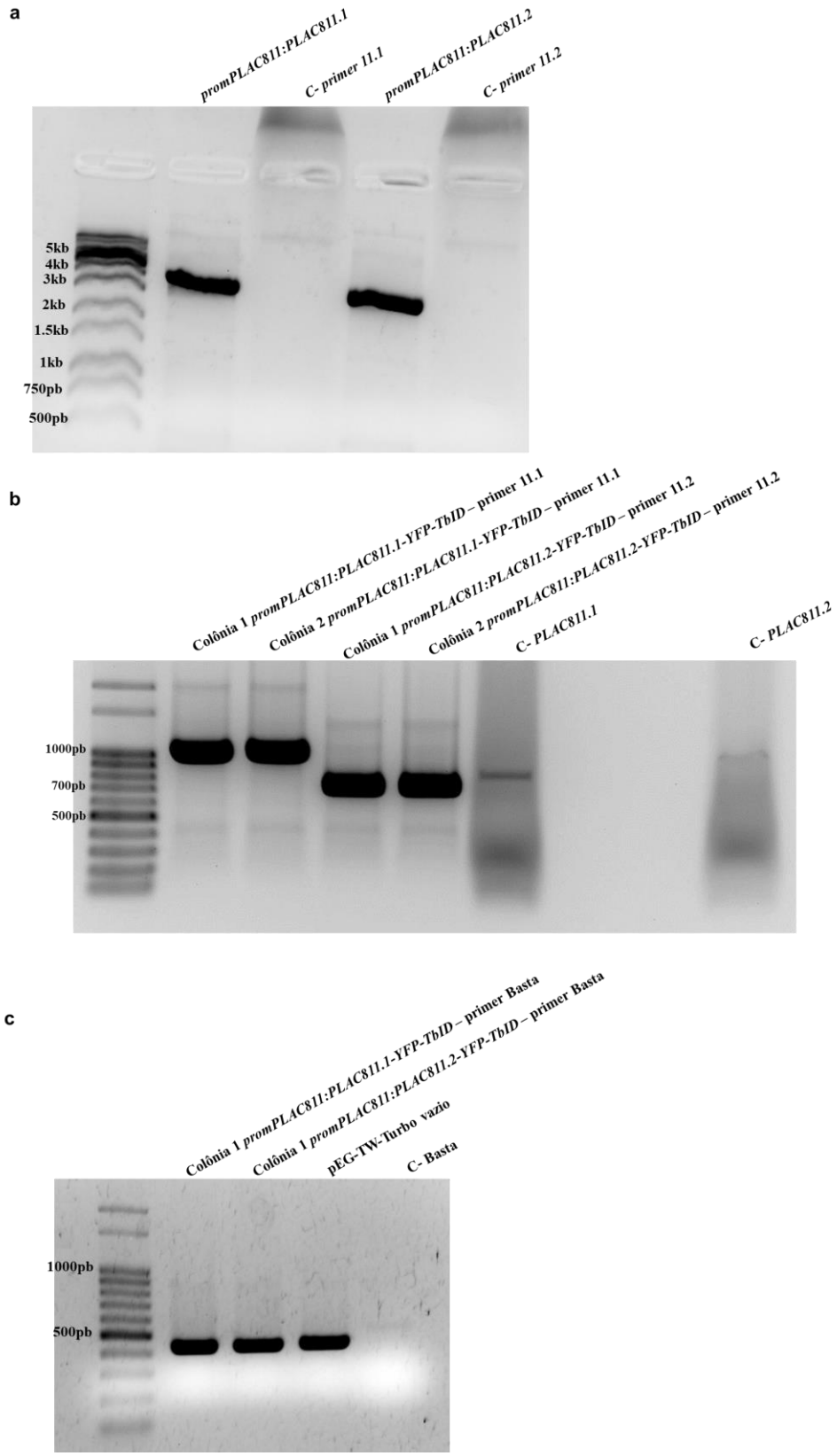

a
promPLAC811:PLAC811.1
C- primer 11.1
promPLAC811:PLAC811.2
C- primer 11.2
5kb
4kb
3kb
2kb
1.5kb
1kb
750pb
500pb
b
Colônia 1 promPLAC811:PLAC811.1-YFP-TbID – primer 11.1
Colônia 2 promPLAC811:PLAC811.1-YFP-TbID – primer 11.1
Colônia 1 promPLAC811:PLAC811.2-YFP-TbID – primer 11.2
Colônia 2 promPLAC811:PLAC811.2-YFP-TbID – primer 11.2
C- PLAC811.1
C- PLAC811.2
1000pb
700pb
500pb
c
Colônia 1 promPLAC811:PLAC811.1-YFP-TbID – primer Basta
Colônia 1 promPLAC811:PLAC811.2-YFP-TbID – primer Basta
pEG-TW-Turbo vazio
C- Basta
1000pb
500pb

Figura 4: (a) Amplificação da sequência promotora + gene *AtPLAC8-11* (3.247 pb) e sua variante de splicing *AtPLAC8-11.2* (2.872 pb) para clonagem molecular. As respectivas regiões de interesse foram amplificadas utilizando gDNA de *Arabidopsis thaliana* e utilizadas para as reações de clonagem. **(b) Amplificação da sequência genômica de *AtPLAC8-11* (1.134pb) e seu variante de splicing *AtPLAC8-11.2* (749pb) em colônias de *A. tumefaciens* transformadas com o vetor de destino pEG-TW-Turbo. (c) Amplificação da Sequência do gene de resistência ao herbicida Basta (552 pb) em colônias de *A. tumefaciens* transformadas com o vetor de destino pEG-TW-Turbo.** Os produtos resultantes das reações de PCR foram visualizados em gel de agarose 1,2% corado com gel red. Marcador de peso molecular de 100 pb e de 1kb (Ludwig®).

***Western Blot* para análise de proteínas**

Após o processo de clonagem das construções *promoPLAC811:PLAC811.1-YFP-TbID* e *promoPLAC811:PLAC811.2-YFP-TbID*, ocorreu a transformação genética de plantas de *plac811* e seleção para obtenção de plantas T3 homozigotas. Posteriormente, realizou-se *western blot* para identificar as linhagens com o maior nível de expressão proteica a fim de serem utilizadas no experimento TurboID-marcação de proximidade. Para a construção abrangendo a forma canônica de AtPLAC811, *promoPLAC811:PLAC811.1-YFP-TbID,* foi possível obter apenas uma linhagem transgênica T3 homozigota, porém, a análise de *western blot* de duas plantas T3 dessa linhagem, mostrou que a mesma possui um bom nível protéico para realização do experimento (Figura 5a). Para a construção *promoPLAC811:PLAC811.2-YFP-TbID*, que abrange a variante de splicing, foi possível obter cinco linhagens transgênicas T3 homozigotas, sendo que a linhagem número 2 foi a que apresentou o maior nível protéico em comparação com as demais e assim sendo, foi escolhida para a realização do experimento TurboID-marcação de proximidade (Figura 5b).

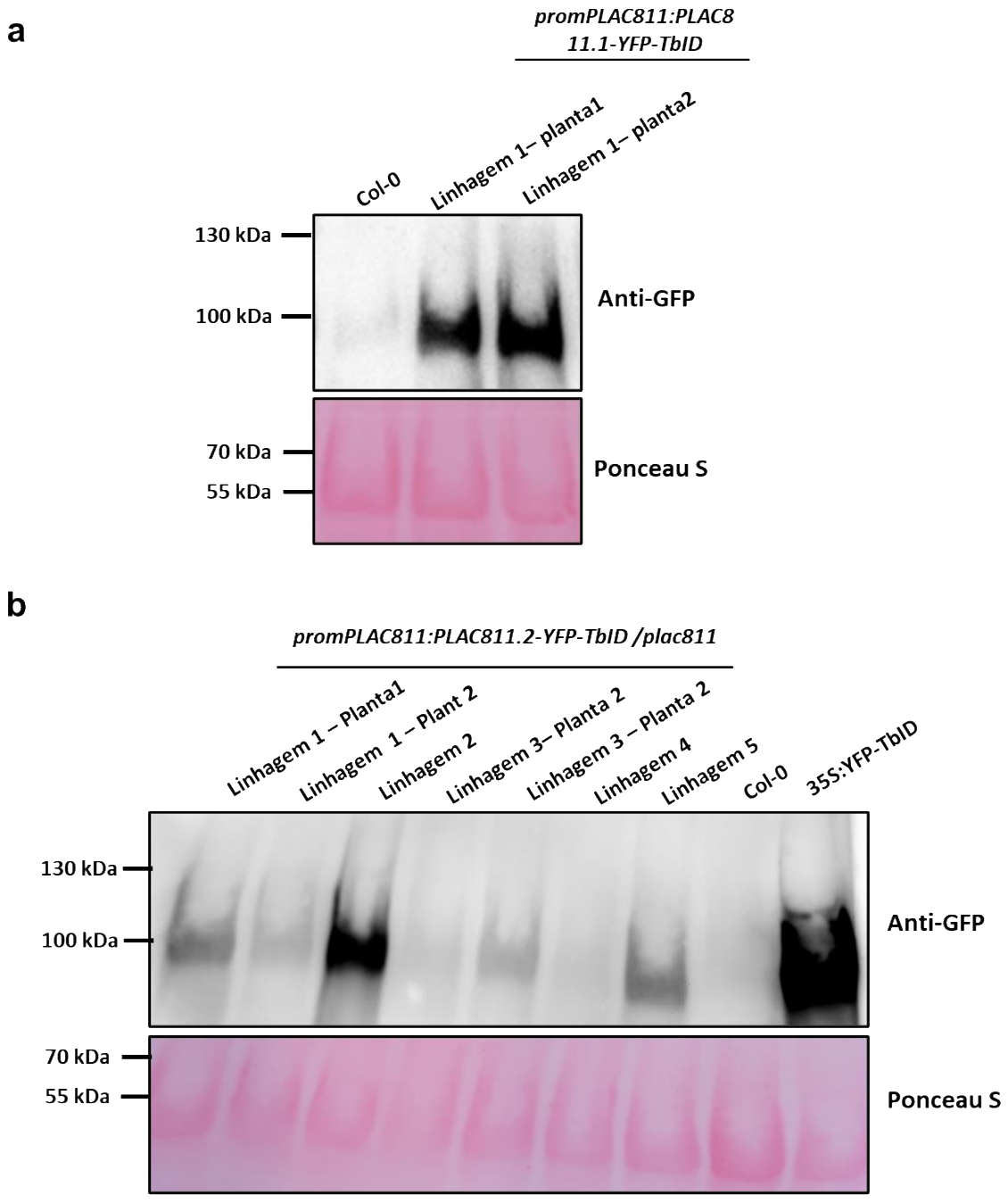


Figura 5. Análise de *western blot* para expressão de proteínas. (a) *Western blot* de extratos protéicos de folhas de uma linhagem transgênicas da construção *promoPLAC811:PLAC811.1-YFP-TbID* com 20 dias de idade usando anticorpo anti-GFP. **(b)** *Western blot* de extratos protéicos de folhas de cinco linhagens transgênicas da construção *promoPLAC811:PLAC811.2-*

YFP-TbID com 20 dias de idade usando anticorpo anti-GFP. A coloração de Ponceau corresponde às proteínas Rubisco (55 KDa), sendo utilizadas como controle.

Dosagem de proteínas pelo método de Bradford

No primeiro dia de experimento TurboID-marcação de proximidade (extração das proteínas), uma alíquota de 30 uL das proteínas das replicatas dessalinizantes que foram eluídas da coluna com tampão de equilíbrio foi reservada para determinar a concentração do extrato protéico por Bradford com o kit Pierce™ BCA Protein Assay (Thermo Scientific). O experimento envolvendo *promoPLAC811:PLAC811.1-YFP-TbID* e *35S:PLAC811.1-YFP-TbID* apresentou maiores valores protéicos (mg/mL) nas duas primeiras replicatas (Figura 6a) em comparação com o experimento envolvendo *promoPLAC811:PLAC811.2-YFP-TbID* e *promoPLAC811:PLAC811.1-YFP-TbID* (Figura 6b).

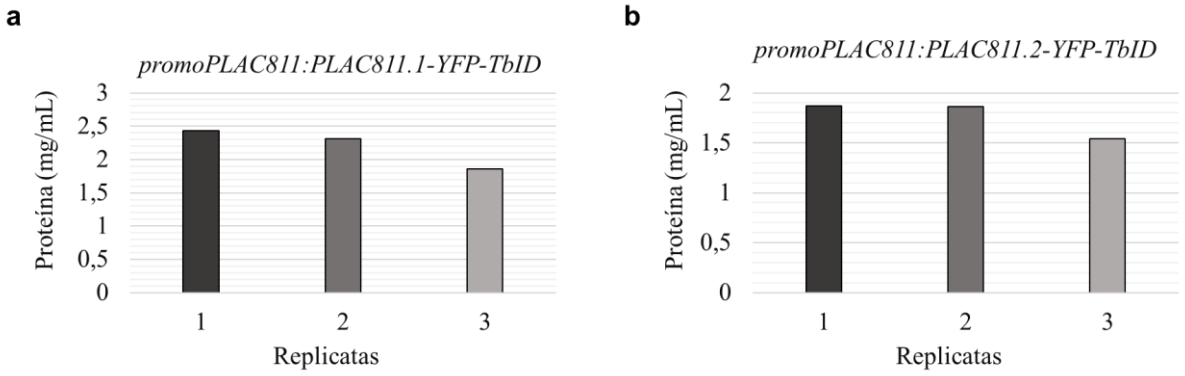


Figura 6. Dosagem de proteínas totais. O teor de proteína foi determinado com o ensaio de Bradford usando albumina do soro bovino (BSA) como padrão. **(a)** Determinação quantitativa de proteína total das três replicatas do experimento *promoPLAC811:PLAC811.1-YFP-TbID* e *35S:YFP-TbID*. **(b)** Determinação quantitativa de proteína total das três replicatas do experimento *promoPLAC811:PLAC811.2-YFP-TbID* e *promoPLAC811:PLAC811.1-YFP-TbID*.

TurboID-marcação de proximidade para busca de interatores de AtPLAC8-11.1 e AtPLAC8-11.2

Sabe-se que a eficiência de marcação entre diferentes experimentos pode variar entre 93-99% após 14 dias de marcação em placas ou em cultura líquida para plantas de Arabidopsis. Nesse sentido, se uma amostra possuir 95% de enriquecimento de marcação, quer dizer que o peptídeo marcado com ^{15}N tem 95% de ^{15}N e 5% de nitrogênio ^{14}N padrão (SHRESTHA et al., 2022). A tabela 5 abaixo descreve as diferentes eficiências de marcação encontrados nos seis experimentos realizados, bem como o número total de proteínas identificadas em cada replicata, sem filtragem e comparação com seu respectivo controle.

Tabela 5. Resumo dos dados de identificação e quantificação de seis experimentos biológicos TurboID-marcação de proximidade.

Experimentos		Eficiência da Marcação	Proteínas identificadas (^{14}N e ^{15}N combinados)
1	1ª replicata (^{14}N PLAC811.1/^{15}N C+)	97.9%	2705
	2ª replicata (^{14}N C+/^{15}N PLAC811.1)	98%	2822
	3 ª replicata (^{14}N PLAC811.1/^{15}N C+)	98.1%	2574
2	1ª replicata (^{14}N PLAC811.2/^{15}N PLAC811.1)	97.8%	3441
	2ª replicata (^{14}N PLAC811.1/^{15}N PLAC811.2)	98%	3092
	3ª replicata (^{14}N PLAC811.2/^{15}N PLAC811.1)	97.8%	3869

É possível observar que nos dois experimentos foram obtidos valores de eficiência de marcação semelhantes, variando na faixa de 97.8% - 98.1%. O trabalho de SHRESTHA et al., 2022 obteve uma eficiência de marcação de 98,5% e sugere que para se obter dados de alta qualidade, recomenda-se obter 97% ou mais de eficiência de marcação para que mais proteínas sejam identificadas e quantificadas de forma reprodutível entre as diferentes amostras. A eficiência de marcação de alto nível depende de alguns fatores como: o sal contendo ^{15}N precisa

ter mais de 99% de pureza; o tempo de marcação, no qual recomenda-se o cultivo de Arabidopsis por 14 dias para obter alta eficiência de rotulagem e por fim, a disponibilidade do sal ^{15}N, no qual as sementes não devem ser semeadas em excesso em placas de meio sólido ou no meio líquido (SHRESTHA et al., 2022). Na Tabela 2 é possível também verificar o número de proteínas que foram identificadas (^{14}N e ^{15}N combinados**)** após a incorporação do valor da eficiência de marcação no programa *ProteinProspector*. O número de proteínas identificadas foi similar entre as replicatas do mesmo experimento. Porém, o segundo experimento, que buscou identificar os parceiros de interação de *AtPLAC811.2* apresentou um maior número de proteínas. O trabalho de referência de SHRESTHA et al., 2022 identificou em seus experimentos no mínimo 5361 proteínas e no máximo 6751 proteínas, mostrando que um maior número de proteínas pode ser identificado, variando a quantidade nas replicatas.

A seguir serão descritos os interatores de AtPLAC811.1 e AtPLAC811.2 após as filtragens em relação aos seus respectivos controles e os pontos de corte que foram utilizados.

Interatores de AtPLAC8-11.1

Para identificar as proteínas que interagem com AtPLAC811.1, analisou-se em cada replicata separadamente, a razão da média da intensidade de sinal entre as amostras das proteínas provenientes de plantas crescidas em meio com ^{14}N (*light*) e ^{15}N (*heavy*): Med L/H. No caso da primeira e da terceira replicata do experimento em questão, as plantas superexpressando *AtPLAC811.1* foram crescidas no meio contendo ^{14}N. Nesse sentido, o ponto de corte em relação a lista de todas as proteínas obtidas nessas replicadas foi de 2, visto que na divisão de L/H $\frac{2}{1}$, obtemos a lista de proteínas que estavam no mínimo duas vezes (100%) mais enriquecidas no meio ^{14}N. Já para a segunda replicata, as plantas superexpressando *AtPLAC811.1* foram crescidas no meio contendo ^{15}N. Nesse sentido, o ponto de corte em relação a lista de todas as proteínas obtidas nessas replicadas foi de 0,5, visto que na divisão de L/H $\frac{1}{0,5}$,obtemos a lista de proteínas que estavam no mínimo duas vezes (100%) mais enriquecidas no meio ^{15}N.

Após a filtragem das proteínas em cada replicata, obteve-se as proteínas proximais de AtPLAC811.1 sendo enriquecidas mais de 2 vezes em *promoPLAC811:PLAC811.1-YFP-TbID*

em relação ao controle *35S:YFP-YFP-TbID.* Conforme a figura 7, na replicata 1 obteve-se 126 proteínas, na replicata 2 obteve-se 155 proteínas e na replicata 3 obteve-se 144 proteínas. Análises utilizando o diagrama de Venn foram realizadas de modo a estudar esses conjuntos de proteínas. Apenas duas proteínas foram encontradas em comum nas três replicatas. Nas replicatas 1 e 2 foram encontradas 4 proteínas em comum, bem como nas replicatas 2 e 3. A replicatas 1 e 3 compartilham 26 proteínas em comum (Figura 7).

Replicatas	N° de proteínas
1	126
2	155
3	144
1 apenas	94
2 apenas	145
3 apenas	112
1 & 2 & 3	2
1 & 2 apenas	4
2 & 3 apenas	4
1 & 3 apenas	26

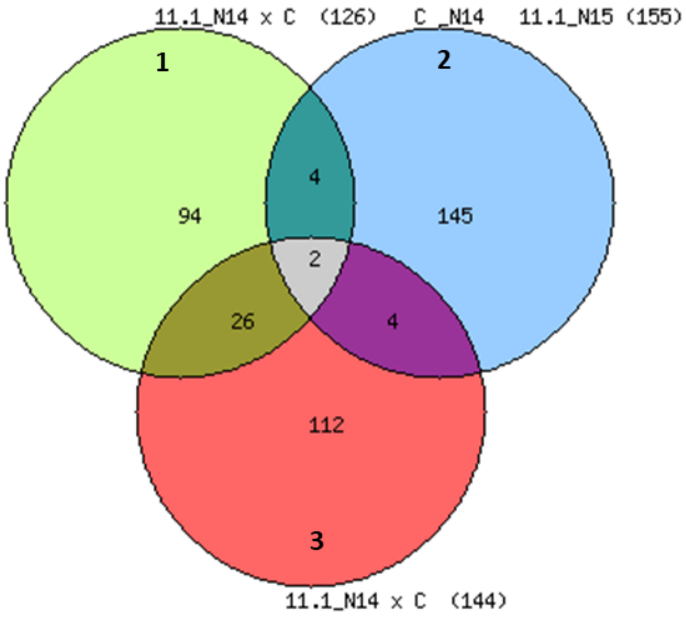


Figura 7: Diagrama de Venn ilustrando a sobreposição de proteínas proximais de AtPLAC811.1. As proteínas biotiniladas identificadas nas replicatas 1, 2 e 3 são 126 proteínas, 155 proteínas e 144, respectivamente.

Na Tabela 6 abaixo são descritas as proteínas identificadas nas três replicatas (1, 2 e 3) e também as proteínas de dois experimentos replicados onde os isótopos foram trocados, ou seja, ora a construção *promoPLAC811:PLAC811.1-YFP-TbID* no meio ^{14}N e a construção *35S:YFP-YFP-TbID* no meio ^{15}N (replicatas 1 e 2) e o contrário (replicatas 2 e 3).

Tabela 6. Identificação baseada em TurboID-marcação de proximidade de proteínas que interagem com AtPLAC811.1 em *Arabidopsis.*

Replicatas	Descrição	1ª replicata	2ª replicata	3ª replicata
1 & 2 & 3		Med L/H	Med L/H	Med L/H
AT3G46430.1	Mitochondrial F1F0-ATP synthase (AtMtATP6): confers tolerance to several abiotic stresses in *Saccharomyces cerevisiae* and *Arabidopsis thaliana.*	2,48	0,0737	4.07
AT1G80070.1	Pre-mRNA-processing-splicing factor: Encodes a factor that influences pre-mRNA splicing and is required for embryonic development. Mutations result in an abnormal suspensor and embryo lethality.	2	0,0512	4,78
1 & 2				
AT5G27350.1	SFP1: Encodes a sugar-porter family protein that is induced during leaf senescence.	100	0,469	-
AT1G74070.1	Cyclophilin member of the peptidyl-prolyl cis-trans isomerase protein family: involved in cellular processes such as protein folding, maturation and trafficking.	9,52	0,0773	-
AT2G37660.1	NAD(P)-BINDING ROSSMANN-FOLD superfamily protein.	8,25	0,01	-
AT3G57150.1	HOMOLOGUE OF NAP57: Encodes a putative pseudouridine synthase.	8,2	0,585	-
2 & 3				
AT5G47480.1	RGPR-RELATED: Part of endomembrane trafficking system.	-	0,108	100
AT3G13110.1	SERINE ACETYLTRANSFERASE 2;2: Encodes a mitochondrial serine O-acetyltransferase involved in sulfur assimilation and cysteine biosynthesis. Expressed in the vascular system.	-	0,197	2,17
AT1G23130.1	POLYKETIDE CYCLASE/DEHYDRASE AND LIPID TRANSPORT SUPERFAMILY PROTEIN	-	0,588	2,36

AT3G62910.1	RF1: Encodes a plastid-localized ribosome release factor 1 that is essential in chloroplast development. Mutant seedlings are pale green albino.	-	0,596	2,72

Em relação às duas proteínas comuns a todas as replicatas do experimento, o produto do gene AT3G46430.1, também conhecido como *AtMtATP6*, é descrito como uma subunidade de 6 kDa do complexo mitocondrial F_1F_0 (ATP sintetase), caracterizado por ser um complexo enzimático chave do metabolismo energético, fornecendo ATP para a célula (MOGHADAM et al., 2012). Em Arabidopsis, a expressão gênica de *AtMtATP6* em células cultivadas em suspensão foi induzida mediante diversos estresses abióticos, como sal, seca e frio. Além disso, a superexpressão do gene *AtMtATP6* em leveduras e plantas de Arabidopsis aumentou a resistência a sal, seca, estresse oxidativo e frio, sugerindo que a indução da F_1F_0-ATPase desempenha um papel importante na tolerância a estresses ambientais (ZHANG; LIU; TAKANO, 2008). O produto do gene AT1G80070.1, também conhecido como fator de processamento de pré-mRNA 8 (Prp8) auxilia no equilíbrio da seleção precisa do local de junção e a excisão rápida e eficiente de íntrons e união de éxons (CABEZAS-FUSTER et al., 2022). O gene AT1G80070.1 é também conhecido como SUS2, sendo essencial para o desenvolvimento embrionário em *Arabidopsis thaliana* (SASAKI et al., 2015; SCHWARTZ; YEUNG; MEINKE, 1994). Sendo assim, as duas proteínas identificadas no experimento TurboID-marcação de proximidade levam a sugerir o envolvimento de *AtPLAC811.1* frente a condições de estresse e seu papel regulatório em mecanismos que envolvem o processamento de RNA.

Dentre as proteínas identificadas nas replicatas 1 e 2, o gene AT5G27350.1, denominado *SFP1*, tem sua expressão aumentada durante a senescência foliar, sendo esse aumento acompanhado por um acúmulo de monossacarídeos. Nesse sentido, SFP1 possivelmente atua no transporte de açúcar durante a senescência foliar (QUIRINO; REITER; AMASINO, 2001). Esse é um resultado interessante e sugere possível envolvimento de AtPLAC811.1 no processo de senescência, visto que em outro estudo, quatro genes pertencentes à família PLAC8, incluindo AtPLAC811.1, foram diferencialmente expressos em plantas superexpressando um regulador negativo da senescência foliar em relação a plantas selvagens (ZHANG et al., 2022c).

Como membro mais representativo encontrado nas replicatas 2 e 3, o produto do gene AT5G47480.1, também denominado como MAG5 ou MAIGO 5 atua na exportação de proteínas dos locais de saída do retículo endoplasmático para cisternas de Golgi, sendo considerado um componente-chave da exportação a partir do retículo endoplasmático em plantas (TAKAGI et al., 2013). Esse dado, pode estar em acordo com estudos anteriores do nosso grupo (dados não publicados) onde verificou-se que AtPLAC811 possui uma localização subcelular difícil de ser corretamente identificada, porém, ambas as formas, AtPLAC811.1 e AtPLAC811.2 colocalizaram com um marcador de retículo endoplasmático, reforçando dessa maneira a possível localização subcelular e o envolvimento de AtPLAC811.1 com proteínas reticulares.

Interatores de AtPLAC8-11.2

Para identificar as proteínas que interagem com AtPLAC811.2, realizou-se os mesmos procedimentos descritos para a identificação dos interatores de AtPLAC8-11.1. Após a filtragem das proteínas em cada replicata, obteve-se as proteínas proximais de AtPLAC811.2 sendo enriquecidas mais de 2 vezes em *promoPLAC811:PLAC811.2-YFP-TbID* em relação a *promoPLAC811:PLAC811.1-YFP-TbID*. Conforme a figura 8, na replicata 1 obteve-se 2216 proteínas, na replicata 2 obteve-se 1455 proteínas e na replicata 3 obteve-se 3002 proteínas. Análises utilizando o diagrama de Venn revelaram que 1065 proteínas foram encontradas em comum nas três replicatas. Nas replicatas 1 e 2 foram encontradas 74 proteínas em comum; nas replicatas 2 e 3 148 proteínas e as replicatas 1 e 3 compartilharam 721 proteínas em comum (Figura 8).

Replicatas	N° de proteínas
1	2216
2	1455
3	3002
1 apenas	356
2 apenas	168
3 apenas	1068
1 & 2 & 3	1065
1 & 2 apenas	74
2 & 3 apenas	148
1 & 3 apenas	721

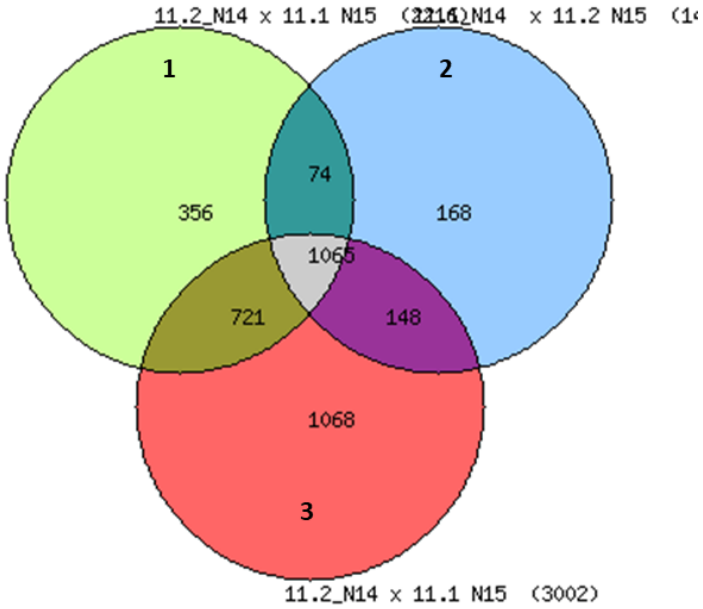


Figura 8: Diagrama de Venn ilustrando a sobreposição de proteínas proximais de AtPLAC811.2. As proteínas biotiniladas identificadas nas replicatas 1, 2 e 3 são 2216 proteínas, 1455 proteínas e 3002, respectivamente.

Nas três replicatas do experimento 2, houve a identificação de uma grande quantidade de proteínas enriquecidas (1065) na variante de splicing em comparação com o controle utilizado no experimento, que foi a construção com a forma canônica. Nesse sentido, buscou-se realizar uma segunda filtragem com essas proteínas. Para isso, a presença das proteínas encontradas nas três replicatas foi verificada nas tabelas geradas do experimento 1, visando excluir as proteínas que aparecerem enriquecidas no controle *35S:YFP-YFP-TbID* (vetor vazio), visto que apesar de serem experimentos diferentes, possuíamos os dados de tais proteínas. Assim sendo, após a segunda filtragem para o experimento 2, nas três replicatas obteve-se uma lista de 97 proteínas sendo enriquecidas mais de 2 vezes em *promoPLAC811:PLAC811.2-YFP-TbID* em relação ao controle do experimento *promoPLAC811:PLAC811.1-YFP-TbID* e também sendo ausentes na lista de proteínas referente ao controle padrão *35S:YFP-YFP-TbID* (Tabela 7). É importante salientar que devido a grande quantidade de proteína presente nas três triplicatas, os dados das proteínas encontradas em duas replicatas (1 e 2 ou 2 e 3) ainda serão analisados com maior detalhe, e, por isso, não constam na tabela a seguir.

Tabela 7. Identificação baseada em TurboID-marcação de proximidade de proteínas que interagem com AtPLAC811.2 em *Arabidopsis.*

Replicatas	Descrição	1ª Replicata	2ª Replicata	3ª Replicata
1 & 2 & 3		Med L/H	Med L/H	Med L/H
AT5G53130.1	CNGC1: *CYCLIC NUCLEOTIDE GATED CHANNEL 1*.	100	0,0263	66,9
AT5G55850.3	Proteína NOI: envolvida na resposta a nematóide.	100	0,0396	41
AT4G37880.1	Proteína contendo domínios LisH/CRA/RING-U-box.	100	0,0368	26,1
AT1G72690.1	Proteína pesada de neurofilamento.	100	0,0271	21,6
AT5G16030.1	Proteína ativadora de GTPase.	100	0,01	19
AT3G11330.1	PIRL9: classe distinta e específica de plantas de LRRs (*Leucine rich repeat proteins*) intracelulares que provavelmente medeiam interações de proteínas, possivelmente no contexto de transdução de sinal. Necessário para a diferenciação de micrósporos em pólen.	100	0,598	13,8
AT4G31800.1	AtWRKY18: Fator de transcrição induzido por patógeno. WRKY18 aumentou a resistência a *P. syringae*.	100	0,01	13,7
AT1G13690.1	ATPase E1: estimula a atividade ATPase de DnaK/DnaJ.	100	0,116	11,1
AT3G09350.1	FES1A: Codifica um dos ortólogos de Arabidopsis da proteína 1 de ligação a Hsp70 humana (HspBP-1) e Fes1p de levedura, sugerindo o envolvimento de Fes1A na termotolerância adquirida.	100	0,077	10,9
AT1G72630.1	ELF4-L2: envolvido no arrastamento do relógio circadiano, fotoperiodismo, regulação positiva do ritmo circadiano.	100	0,297	10,9
AT3G51920.1	AtCML9: codifica um membro divergente da calmodulina. Este gene é expresso em folhas, flores e síliquas.	100	0,0683	9,67
AT4G11740.1	SAY1: Isolado como um supressor de um mutante dominante no gene Ara4 que causa severa inibição do crescimento quando expresso em cepas mutantes de levedura *ypt1*.	100	0,01	9,51
AT3G47890.1	Hidrolase do terminal carboxi da ubiquitina.	100	0,01	6,83
AT2G42490.1	CUAO-ZETA: Amina oxidase de cobre localizada em peroxissomo envolvida na formação de raízes laterais.	100	0,228	3,91
AT3G10260.1	RTNLB8: Proteína da família *Reticulon.*	100	0,574	4,25
AT1G73230.1	Complexo associado ao polipeptídeo nascente (NAC).	100	0,0532	4,81
AT1G67950.1	BPL2: Parceiro de ligação ACD11, regula negativamente a resposta de defesa mediada por ROS.	100	0,0347	3,91
AT3G49800.1	Proteína contendo o domínio BSD.	100	0,0945	3,9

AT5G64830.1	Morte celular programada 2 - proteína contendo domínio C-terminal.	100	0,01	3,72
AT2G40080.1	Floração precoce 4 (ELF4): promove a precisão do relógio e é necessário para ritmos sustentados na ausência de ciclos diários de luz/escuridão.	100	0,0926	2,74
AT2G45200.1	GOS12: Codifica um membro da família de genes GOS1 (Golgi SNARE).	99,7	0,01	7,01
AT1G22870.1	SCYL2A: Um dos dois parálogos em Arabidopsis. A perda de SCYL2B e SCYL2A resulta em graves defeitos de crescimento.	74,4	0,0357	16,9
AT3G58170.1	AtBET11: Codifica uma proteína SNARE semelhante a Bet1/Sft1 que suprime totalmente o defeito de crescimento sensível à temperatura em células de levedura *sft1-1*.	64,9	0,0261	59,7
AT2G17480.1	AtMLO8: Membro de uma grande família de proteínas de sete domínios transmembranares específicos para plantas, homólogas da proteína do locus o (MLO) de resistência ao míldio da cevada.	58,8	0,461	32,9
AT1G53510.1	AtMPK18: Membro da família MAP Kinase. O crescimento da raiz mutante é sensível à orizalina e sugere um papel na sinalização durante a organização dos microtúbulos.	58,2	0,0319	36
AT1G61690.1	FYVE4: proteína de ligação ao fosfoinositídeo.	46,8	0,16	8,87
AT3G22190.1	IQD5: Proteína de domínio IQ67 (IQD) que são uma das maiores famílias de proteínas de ligação CaM.	39,1	0,0494	40,7
AT1G76140.1	POQR-LIKE: Putativa Prolil oligopeptidase.	37,3	0,111	8,63
AT4G33650.1	APEM1: Codifica uma proteína com alta similaridade de sequência com a superfamília da dinamina. Localiza-se no cloroplasto, mitocôndria e peroxissomo. Envolvido na fissão de peroxissomos e mitocôndrias em combinação com DRP3B.	37,2	0,147	10,6
AT1G06210.1	TOL2: Codifica um membro da família Arabidopsis TOL de proteínas de ligação à ubiquitina que atua de forma redundante no reconhecimento e posterior classificação endocítica de uma proteína transportadora de auxina do tipo PIN-FORMED (PIN) na membrana plasmática, modulando a distribuição dinâmica de auxina e respostas de crescimento associadas.	35,7	0,01	5,48
AT4G38930.2	Proteína da família UFD1 de degradação por fusão de ubiquitina.	32,7	0,153	30,5
AT3G05420.1	ACBP4: proteína de ligação de acil-CoA com alta afinidade para oleoil-CoA. Envolvido no transporte de ácidos graxos. Desempenha um papel na determinação do teor de óleo da semente.	32,4	0,353	3,98
AT1G61100.1	Proteína de resistência a doenças (classe TIR).	32,3	0,01	13,4
AT4G02590.1	BHLH059: regulador transcricional BHLH. Mostra efeitos específicos do ecótipo na	32,1	0,0768	17,9

	acumulação e imunidade de ácido salicílico dependentes da temperatura. Governa a competência das células do periciclo para iniciar a formação do primórdio radicular lateral.			
AT1G27850.1	BPP5: Codifica uma proteína associada a microtúbulos envolvida na organização de microtúbulos corticais durante o desenvolvimento da folha.	30,4	0,0343	21,5
AT4G02200.1	AtDIL9-5: proteína da família responsiva à seca.	29,6	0,01	5,19
AT5G62865.1	Proteína hipotética.	28,7	0,0126	9,9
AT1G67890.1	RAF11: Codifica uma proteína com semelhança com as RAF MAP quinases. Com base no fenótipo de perda de função, o RAF11 parece estar envolvido na mediação de respostas ABA, como dormência e estresse ambiental.	28,1	0,0913	20,1
AT1G17360.1	Proteína fosfatase 1 semelhante à subunidade reguladora.	27,8	0,0211	11,2
AT2G43560.1	Proteína da família de peptidil-prolil cis-trans isomerase do tipo FKBP	26,9	0,362	5,91
AT1G55300.1	TAF7: fator 7 associado a TBP.	25	0,025	11,3
AT5G11580.1	Proteína da família reguladora da condensação cromossômica (RCC1).	22,1	0,01	9,73
AT3G18040.1	Codifica uma proteína com semelhança com MAP quinase (MAPK9). Expressa-se preferencialmente em células-guarda e parece estar envolvida na sinalização ABA mediada por espécies reativas de oxigênio.	21	0,0319	8,44
AT2G26590.1	RPN13: partícula reguladora não ATPase 13.	20,7	0,0898	9,02
AT5G49230.1	AtDIL9-7: codifica uma proteína localizada no núcleo com semelhança com proteínas induzidas pela seca.	20,2	0,01	19,1
AT5G51390.1	Proteína hipotética.	19,6	0,0345	9,55
AT2G40060.1	CLC2: Codifica uma clatrina que está localizada na zona de divisão cortical e na placa celular.	18,6	0,228	10,6
AT4G38810.2	Sensor de cálcio: Sensor de cálcio interativo SnRK2. Codifica duas isoformas diferentes que podem inibir SnRK2. A forma mais longa (AT4G38810.2) é dependente de cálcio, a outra não.	17,2	0,037	9,45
AT5G13560.1	Manutenção estrutural da proteína cromossômica.	15,6	0,01	10,3
AT1G69220.1	SIK1: codifica serina/treonina quinase 1 (SIK1). Regula a proliferação e expansão celular.	15,3	0,0858	9,42
AT5G53280.1	PDV1: proteína integral da membrana do envelope externo (como seu homólogo PDV2), componente da maquinaria de divisão plastidial.	14,3	0,01	8,78
AT4G19180.1	AtAPY7: Codifica uma apirase putativa envolvida na formação do padrão de exina do pólen e na deiscência de anteras.	14,2	0,0407	7,65
AT3G10220.1	EMBRIÃO DEFEITUOSO 2804: Codifica um cofator de ligação à tubulina. Plantas mutantes homozigotas são letais para o embrião.	13	0,01	4,3
AT5G41810.1	Avr9/Cf-9 proteína eliciada rapidamente.	13	0,182	2,91

AT1G80840.1	AtWRKY40: Fator de transcrição induzido por patógeno.	12,1	0,206	9,95
AT3G46960.1	O gene codifica uma RNA helicase de box DExD/H, envolvida na regulação da resposta ao estresse por privação de K+.	9,89	0,0823	13,2
AT4G10470.1	Proteína hipotética.	9,88	0,01	4,89
AT5G46020.1	MUSE7: codifica um substrato putativo de quinase evolutivamente conservado de função desconhecida que afeta o acúmulo de proteínas NLR.	9,1	0,01	14,1
AT3G11950.1	Proteína da superfamília TRAF-LIKE.	8,42	0,202	11,6
AT5G52200.1	ATI-2: codifica um inibidor da proteína fosfatase 1 (PP1).	8,34	0,282	5,25
AT5G56950.1	NAP1;3: Codifica um membro de uma pequena família de genes de proteínas com semelhança com proteínas de montagem de nucleossomos. Pode funcionar no reparo por excisão de nucleotídeos.	8,21	0,294	6,54
AT1G76030.1	ATVAB1: um dos três genes que codificam a subunidade vacuolar ATP sintase B1. A proteína se liga e co-localiza com F-actina, agrupando F-actina para formar uma estrutura de ordem superior e estabiliza os filamentos de actina *in vitro*.	8,12	0,171	9,06
AT4G29810.1	ATMKK2: codifica uma MAP quinase 2 que regula MPK6 e MPK4 em resposta a estresses de frio e sal.	8,1	0,0721	7,89
AT3G02540.1	RAD23C: codifica um membro da família RADIATION SENSITIVE23 (RAD23). As proteínas RAD23 desempenham um papel essencial no ciclo celular, morfologia e fertilidade das plantas através da entrega de substratos UPS (sistema de proteassoma ubiquitina/26S) ao proteassoma 26S.	7,53	0,0336	13,7
AT3G48860.2	SCD2: Citocinese estomática defeituosa 2-proteína de bobina enrolada.	7,43	0,226	11,7
AT2G39900.1	WLIM2A: codifica um membro das proteínas LIM de Arabidopsis: uma família de empacotadores de actina com padrões de expressão distintos. Regula o órgão do citoesqueleto de actina.	6,99	0,01	5,83
AT1G06640.1	Codifica uma proteína cuja sequência é semelhante a uma dioxigenase dependente de 2-oxoglutarato.	6,89	0,1	13,2
AT2G26210.1	Proteína da família de repetição da anquirina.	6,88	0,163	40,3
AT5G26980.1	ATSYP41: membro da família de genes SYP4. O grupo SYP4 regula as vias de transporte secretora e vacuolar na rede pós-Golgi e mantém a morfologia do aparelho de Golgi e TGN. Consistente com um papel secretor, as proteínas SYP4 são necessárias para respostas de resistência extracelular a um patógeno fúngico.	6,6	0,323	11,7

AT4G18950.1	BHP: é uma proteína quinase do tipo Raf envolvida na mediação da abertura estomática dependente da luz azul.	6,41	0,0512	12,2
AT1G02090.1	COP9 SIGNALOSOME SUBUNIT 7: codifica uma fosfoproteína que é uma subunidade do COP9 signalossomo. Mutantes exibem fenótipo fotomorfogênico constitutivo.	6,35	0,162	14,8
AT5G63950.1	CHR24: remodelação da cromatina 24.	6,11	0,107	10,8
AT1G06640.2	Codifica uma proteína cuja sequência é semelhante a uma dioxigenase dependente de 2-oxoglutarato.	5,92	0,306	7,96
AT5G17330.1	AtGAD1: Codifica uma das duas isoformas de glutamato descarboxilase.	5,84	0,271	9,61
AT2G41620.1	Proteína da família do componente que interage com a nucleoporina (Nup93/Nic96-like).	5,63	0,196	21,9
AT4G24370.1	Regulador positivo de SKD1.	5,4	0,142	6,69
AT2G40070.1	Codifica uma proteína associada a microtúbulos envolvida na organização de microtúbulos corticais durante o desenvolvimento da folha.	5,27	0,209	8,26
AT2G02000.1	GAD3: Glutamato descarboxilase 3. O GABA desempenha um papel fundamental na aclimatação das plantas ao estresse de luz + calor, potencialmente promovendo a autofagia.	5,25	0,271	9,84
AT1G62380.1	ACO2: codifica uma proteína semelhante à 1-aminociclopropano-1-carboxílico oxidase (ACC oxidase). A expressão dos transcritos de AtACO2 é afetada pelo etileno.	5,09	0,424	5,39
AT5G14250.1	COP13: codifica a subunidade 3 do sinalossoma COP9.	4,88	0,293	4,33
AT1G63700.1	EMB71: membro da subfamília MEKK, um componente da via reguladora do desenvolvimento estomático. Mutações neste locus resultam em letalidade do embrião.	4,77	0,0654	17,2
AT4G17330.1	AtG2484-1: gene de função desconhecida expresso em plântulas, botões florais e caules.	4,75	0,393	9,39
AT1G20670.1	BRD1: proteína contendo bromodomínio de ligação ao DNA, interage com os componentes centrais do complexo SWI/SNF.	4,32	0,01	9,08
AT5G43130.1	TAF4B: fator 4 associado a TBP.	4,13	0,424	4,35
AT3G60660.1	Proteína semelhante ao fuso/cinetócoro.	3,95	0,385	2,52
AT1G70410.1	ATBCA4: codifica uma anidrase beta carbônica betaCA4 putativa. Juntamente com betaCA1 (At3g01500) regula os movimentos estomáticos controlados por CO_2 nas células-guarda, bem como atenua a imunidade.	3,87	0,473	4,56
AT1G26550.1	Proteína da família de peptidil-prolil cis-trans isomerase do tipo FKBP.	3,86	0,45	3,66
AT1G61620.1	CSU1: Codifica uma ubiquitina ligase E3 que desempenha um papel importante na manutenção da homeostase do COP1 ao direcionar o COP1 para ubiquitinação e degradação em mudas cultivadas no escuro.	3,82	0,333	6,64

AT1G75310.1	AUL1: proteína tipo auxina 1.	3,69	0,168	8,36
AT5G08120.1	MPB2C: Proteína de ligação à proteína de movimento viral e associada a microtúbulos. Envolvido no alinhamento dos microtúbulos corticais, na padronização dos estômatos e na restrição de infecções tobamovirais.	3,57	0,01	4,97
AT3G52920.2	Ativador transcricional (DUF662).	3,44	0,17	24
AT2G36410.3	Ativador transcricional (DUF662).	3,44	0,17	24
AT5G47210.3	Hialuronano /família de ligação ao mRNA.	3,04	0,315	3,73
AT2G36480.1	Complexo de clivagem de pré-mRNA 2 proteína semelhante a PCf11.	2,96	0,405	4,21
AT3G06300.1	AT-P4H-2: codifica uma prolil-4 hidroxilase que pode hidroxilar poli(L-prolina) e outros peptídeos ricos em prolina.	2,51	0,47	2,86
AT2G29290.2	DEG14: proteína da superfamília Rossmann-fold de ligação a NAD(P).	2,32	0,163	3,32
AT1G73600.1	ATPMT3: codifica uma fosfoetanolamina N-metiltransferase dependente de S-adenosil-L-metionina cuja expressão responde tanto ao fosfato (Pi) quanto ao fosfito (Phi) nas raízes.	2,07	0,438	4,25

Dentre as proteínas identificadas no experimento 2, a que é codificada pelo gene AtCNGC1 é umas das mais representativas, sendo altamente enriquecida nas amostras referentes à variante de splicing. O gene AtCNGC1 denota canais de cátions não seletivos controlados por nucleotídeos cíclicos (CNGC) e faz parte de uma família de proteínas de transporte de íons vegetais (LENG et al., 2002). Os CNGC possuem também a capacidade de se ligar a à calmodulina, que é uma proteína ligante ao cálcio, importante para diversas funções celulares (KÖHLER; NEUHAUS, 2000). Resultados, mediante análise do comprimento da raiz primária, sugerem que AtCNGC1 desempenha um papel importante na toxicidade a metais pesados tóxicos, como o chumbo (Pb). A análise do conteúdo iônico verificou que as mutações em AtCNGC1 resultaram em acúmulo reduzido de íons de chumbo (Pb^{2+}). Nesse sentido, AtCNGC1 parece estar envolvido na absorção e transporte de íons Pb^{2+} em plantas (MOON et al., 2019). Nos experimentos foi também identificado uma variante de splicing, AT4G38810.2, que atua como sensor de cálcio e inibe a atividade da quinase SnRK2 de forma dependente de cálcio. A regulação negativa da atividade de SnRK2, indica envolvimento de AT4G38810.2 na resposta ao ácido abscísico durante a germinação das sementes (BUCHOLC et al., 2011).

O possível envolvimento de AtPLAC811.2 na defesa da planta frente a estresses bióticos pode ser sustentada pela identificação de proteínas envolvidas em tais processos, caso da proteína NOI (AT5G55850.3), envolvida na reposta a nematoide. As proteínas do domínio NOI possuem funções conservadas na regulação das respostas de defesa das plantas sendo alvo das proteínas efetoras de *Pseudomonas syringae*, como AvrRpt2 (KIM et al., 2005). Além disso, uma proteína contendo o domínio NOI em *Nicotiana benthamiana* (ortóloga a At5g55850; NOI4) foi necessária para induzir uma resposta de hipersensibilidade (LU, 2003). Outra proteína identificada com papel semelhante foi AtWRKY18 (AT4G31800.1), que é um fator de transcrição induzido por patógeno, atuando no aumento da resistência a *Pseudomonas syringae* (XU et al., 2006). A proteína codificada pelo gene AT1G61100.1, descrita como proteína de resistência a doenças da classe TIR (*Toll/interleukin-1 Receptor*), também foi identificada.

Interessantemente, foram identificadas proteínas que estão relacionadas com a organização e o alinhamento dos microtúbulos corticais, principalmente durante o desenvolvimento da folha. Os genes AT1G53510.1, AT1G27850.1, AT2G40070.1 e AT5G08120.1 codificam tais proteínas. Os microtúbulos corticais das plantas encontram-se ancorados lateralmente à membrana plasmática e regulam o direcionamento do complexo de celulose sintase para a membrana plasmática, realizando importante papel na determinação da forma e função da célula (ODA, 2015). Além disso, os genes (AT1G76030.1 e AT2G39900.1) que codificam proteínas envolvidas na regulação do órgão do citoesqueleto de actina e estabilização dos filamentos de actina foram identificados no experimento.

Proteínas relacionadas com o Complexo de Golgi também foram identificadas. AT2G45200.1 codifica um membro da família de genes Golgi SNARE, que têm um papel central no tráfego de vesículas, no reconhecimento e fusão entre a vesícula e as membranas alvo (KIM; BASSHAM, 2013). AT5G26980.1 faz parte do grupo SYP4, que regula as vias de transporte secretora e vacuolar na rede pós-Golgi e mantém a morfologia do aparelho de Golgi e da rede trans-Golgi (TGN) (UEMURA et al., 2012). Em Arabidopsis, o gene que codifica AT1G22870.1 corresponde a um dos ortólogos do gene animal SCYL2 e foi encontrado no experimento de interação. Os genes SCYL2 estão envolvidos no tráfego de vesículas mediado por clatrina, sendo essenciais para o crescimento das plantas. As proteínas SCYL2 localizam-se

no Golgi, rede trans-Golgi e compartimento pré-vacuolar e co-localizam com Cadeia Pesada de Clatrina1 (CHC1) (JUNG et al., 2017). O envolvimento de *AtPLAC811.2* no tráfego de membrana ganha suporte adicional com a identificação do gene AT2G40060.1, que codifica uma clatrina, CLC2, com papel crítico no tráfego de membrana da rede trans-Golgi/endossomo precoce (TGN/EE) e membrana plasmática durante o desenvolvimento da planta (WANG et al., 2013).

Constatou-se a presença de proteínas com funções distintas e alto nível de enriquecimento no experimento 2, como por exemplo as proteínas coindicadas pelos genes AT3G10220.1 e AT1G63700.1, que estão relacionadas com letalidade e defeitos do embrião em seus mutantes. AT5G64830.1 está relacionada à morte celular programada e ambos AT1G67890.1 e AT3G18040.1 parecem estar envolvidos na mediação de respostas e sinalização de ácido abscísico. AT5G17330.1 (*GAD1*) e AT2G02000.1 (*GAD3*) estão relacionados com a enzima glutamato descarboxilase, que desempenha importante papel no crescimento, desenvolvimento, resposta e adaptação das plantas a estresses ambientais (QIU et al., 2020). A inoculação com *Pseudomonas syringae* expressando o gene efetor *avrRpt2* induziu a expressão de AT5G17330 (*GAD1*) e aumentou os níveis de ácido gama-aminobutírico (GABA). Além disso, o padrão de expressão dos promotores GAD foi analisado em tecidos de plântulas utilizando o repórter β-glucuronidase (GUS) indicaram que *GAD1* foi expresso principalmente em folhas e raízes na fase vegetativa e *GAD3* nas anteras e embriões (DENG et al., 2020).

CONCLUSÕES E PERSPECTIVAS

O experimento 2 revelou um número significativamente maior de proteínas (98 proteínas) em comum nas três replicatas em comparação ao experimento 1 (2 proteínas). De acordo com a figura 5, que retrata a análise de *western blot* para a expressão de proteína das linhagens candidatas ao experimento TurboID-marcação de proximidade, ambas construções *AtPLAC811.1* e *AtPLAC811.2* expressam nível protéico similar. A figura 6, que mostra a dosagem de proteínas totais, realizada no primeiro dia de experimento (Dia 1 - extração de

proteína) mostrou que as replicatas do experimento 1 obtiveram um maior teor de proteína. Nesse sentido, uma possível degradação protéica ou perda de parte das amostras a partir do segundo dia de experimento, em etapas envolvendo a purificação de afinidade e a digestão tríptica nas esferas pode ter ocorrido no experimento 1, visto o número expressivamente menor de proteínas. Assim sendo, uma possível alternativa para identificar um maior número de interatores da forma canônica, seria o de realizar filtragens nos dados obtidos do experimento 2, identificando as proteínas que não foram enriquecidas na variante de splicing e comparando com a lista de proteínas do controle (vetor vazio) do experimento 1. Outra possível explicação seria a de que AtPLAC811.1 interaja com um número menor de proteínas. Através dos experimentos de marcação de proximidade, foi possível observar a importância de um controle para os experimentos, a fim de eliminar falsos positivos. No caso dos experimentos desse trabalho, foi utilizada a construção *35S:YFP-YFP-TbID* (vetor vazio). Porém, no caso do vetor escolhido para os experimentos (pEG-TW-Turbo), que permite a inclusão da região promotora fusionada ao gene de interesse, o ideal seria utilizar como vetor vazio a construção *promoPLAC811:YFP-YFP-TbID*.

De qualquer maneira, as perspectivas futuras para ambos os experimentos seriam a validação da interação com os principais alvos candidatos, utilizando a técnica de duplo híbrido em levedura, e após a confirmação da interação, realizar a investigação do papel dessa interação e sua relevância biológica. Nesse momento, se o interator das proteínas PLAC811 for um fator de transcrição, como AtWRKY18, que é um fator de transcrição induzido por patógeno encontrado no experimento 2, seria interessante realizar o ensaio de transativação. A abordagem de realizar um cruzamento entre mutantes, incluindo *plac811* e o interator também é válida, desde que o interator possua sua caracterização fenotípica estabelecida. De maneira geral, o experimento TurboID-marcação de proximidade com marcação metabólica usando isótopos estáveis como o ^{15}N mostrou possuir grande potencial para identificação de parceiros de interação em plantas.

4. CONSIDERAÇÕES FINAIS

Ao longo da evolução as plantas desenvolveram diferentes mecanismos de sobrevivência frente aos inúmeros estresses abióticos e bióticos impostos pelo meio ambiente (MARERI; PARROTTA; CAI, 2022). A via geneticamente controlada de suicídio celular, conhecida como morte celular programada (PCD), é um dos mecanismos que pode auxiliar as plantas frente às condições ambientais adversas (LOCATO; DE GARA, 2018). Além de atuar no desenvolvimento das plantas, a PCD pode ser ativada como uma resposta de defesa essencial ao enfrentamento dos estresses ambientais. Porém, o conhecimento sobre PCD em plantas é limitado (EBEED; EL-HELELY, 2021).

O artigo de revisão apresentado no capítulo I intitulado "Controle da morte celular programada (PCD) em plantas: Novas percepções do deathosome de *Arabidopsis thaliana"*, contribui significativamente para um melhor entendimento do processo de PCD vegetal, utilizando a planta modelo *Arabidopsis thaliana*. Nesse trabalho de revisão, os aspectos gerais da PCD em plantas foram examinados na literatura, discutindo a classificação da PCD em plantas em três tipos diferentes: i) morte celular semelhante à apoptose, ii) morte celular associada à senescência e iii) morte celular mediada por vacúolos. A morte celular semelhante à apoptose recebe esse nome devido a algumas diferenças importantes encontradas nas células vegetais em comparação com os animais no que se refere a presença de uma parede celular rígida e a falta de corpos apoptóticos, fagócitos e macrófagos por parte das plantas (VAN DOORN, 2011). A morte celular associada à senescência é conhecida por desempenhar importante papel na remobilização e reciclagem de nutrientes de órgãos senescentes, auxiliando o crescimento de novos órgãos vegetativos, reprodutivos e de armazenamento (BUCHANAN-WOLLASTON et al., 2002). No caso da morte celular vacuolar, foi verificado que as plantas fazem uso de seus vacúolos e do conteúdo lítico vacuolar para digerir gradualmente todos ou grande parte dos seus materiais celulares que estão destinados a sofrer o processo de PCD (HARA-NISHIMURA; HATSUGAI, 2011).

Além da classificação da PCD vegetal em três tipos, propusemos uma atualização do "AtLSD1 – deathosome", descrito primeiramente por Coll et al., 2011 como uma rede composta

por vários genes em *Arabidopsis* relacionados ao controle da morte celular mediada pela resposta de hipersensibilidade em plantas. No AtLSD1-deathosome proposto por Coll e colaboradores (2011), baseado em experimentos de duplo híbrido em levedura, dezesseis proteínas pertencentes a oito famílias gênicas, como Metacaspase, LSD1, PLAC8, bZIP, MYB, NF-Y, AUX/IAA e PRP foram apresentadas como parceiros de interação complementares de AtLSD1, sendo algumas dessas famílias bem documentadas em PCD vegetal. A atualização do AtLSD1-deathosome, realizada neste trabalho, apresenta dez novos parceiros de interação e adiciona novas famílias gênicas importantes na PCD, principalmente famílias relacionadas com a resposta de hipersensibilidade, como é o caso das famílias gênicas EDS1/PAD4 e Catalase (LI et al., 2013; RUSTÉRUCCI et al., 2001). Devido ao aumento das publicações sobre proteínas com envolvimento no processo de PCD, além da inclusão das proteínas identificadas como interatoras do AtLSD1-deathosome, reunimos em uma tabela algumas das publicações mais relevantes das famílias gênicas encontradas no AtLSD1-deathosome atualizado.

No capítulo II dessa tese, foram apresentados os resultados relacionados com a caracterização molecular e funcional do gene At1g52200 (*AtPLAC8-11.1*) no processo de morte celular programada. Nesse estudo de caracterização funcional buscou-se incluir a variante de splicing *AtPLAC811.2,* visto que o *splicing* alternativo pode atuar na diversificação do transcritoma e o proteoma de um organismo, resultando em uma proteína com função diferente da forma canônica (SHANG; CAO; MA, 2017). Inicialmente, estudos de localização subcelular foram realizados com ambas as formas e observou-se padrão de localização similar em ambas as versões, havendo co-localização com o marcador de retículo endoplasmático. Este achado é interessante visto que além de ser uma organela altamente dinâmica, servindo como local de produção de proteínas destinadas a vacúolos, membrana plasmática ou apoplasto, o retículo endoplasmático vem sendo relacionado com a imunidade de plantas e morte celular (EICHMANN; SCHÄFER, 2012). A localização de AtPLAC811.1 e AtPLAC811.2 sob controle do promotor nativo também foi avaliada e permitiu verificar algumas diferenças entre as variantes. Ambas as construções *promoPLAC811:PLAC811.1-YFP-TbID* e *promoPLAC811:PLAC811.2-YFP-TbID* levaram a expressão tecido-específica na raiz e folha. Interessantemente, a fluorescência de *AtPLAC811.1-YFP* foi observada nas membranas circundantes nas células-guarda da epiderme, dos estômatos das folhas e sobretudo nas bases

dos tricomas, enquanto que a fluorescência de *AtPLAC811.2-YFP* foi verificada dentro do par de células-guarda e sem expressão nos tricomas. Sabe-se que os tricomas protegem as plantas contra o ataque de herbívoros, insetos e patógenos, através da secreção de substâncias tóxicas (WANG et al., 2021). Esse resultado pode então ser um indicativo do possível envolvimento de *AtPLAC811.1* nas respostas de defesa das plantas. A expressão de *AtPLAC811.1* sob controle do promotor nativo em flores de *Arabidopsis* está de acordo com a literatura no que diz respeito a expressão diferencial de AtPLAC811.1 na sépala em comparação com a pétala (EDERLI et al., 2015). A sépala é descrita como sendo o órgão floral verde mais externo, envolvendo e protegendo os órgãos reprodutivos que estão em desenvolvimento antes do desabrochar da flor (ROEDER et al., 2012). Além desse resultado, nossos achados indicaram que a construção abrangendo a forma variante de splicing apresentou fluorescência apenas no estigma e na folha, enquanto na forma canônica, além de forte fluorescência na sépala, foi possível observar fluorescência na folha, estame e estigma, indicando o forte envolvimento de AtPLAC811.1 no órgão floral.

Em relação ao envolvimento de *AtPLAC811* no processo de morte celular programada, obtivemos inicialmente a confirmação da interação de AtPLAC811.1 com o principal regulador negativo da PCD em plantas, AtLSD1. Através de experimentos de duplo híbrido em levedura, demonstramos que apenas AtPLAC811.1 foi capaz de interagir com a proteína AtLSD1, evidenciando a importância da região C-terminal de AtPLAC811 para que a interação ocorra, visto que tal região é modificada na proteína codificada pela variante de splicing. O ensaio de BiFC confirmou a interação de AtPLAC811.1 e AtLSD1 mostrando a reconstituição da YFP em manchas espalhadas pela célula em localização indefinida. Foi verificado também, que AtPLAC811.1 co-localiza parcialmente com AtLSD1 em protoplastos de *Arabidospis*. No entanto, nesse experimento, a co-localização AtPLAC811.1 e AtLSD1 no núcleo não foi observada. AtLSD1 é bem caracterizado em relação às respostas frente aos estresses bióticos e abióticos. Porém, um estudo recente mostrou o papel AtLSD1 como um regulador transcricional (CZARNOCKA et al., 2017). Experimentos de transativação descritos no capítulo II mostraram que a interação de AtPLAC811.1 com AtLSD1 é capaz de interferir na atividade de AtLSD1 como regulador transcricional, evidenciando o papel de AtPLAC811.1 na rede regulatória de AtLSD1. O papel de *AtPLAC811* na morte celular programada e nas respostas de defesa das

plantas foi evidenciado através do experimento utilizando o elicitor Pep1 que promove a expressão de genes de defesa de patógenos (HUFFAKER et al., 2013). Tanto AtPLAC811.1 quanto AtPLAC811.2 tiveram suas expressões aumentadas nas raízes após o tratamento com o elicitor de Pep 1 em comparação com seus respectivos controles. Por fim, a ausência de *AtPLAC811* no mutante nocaute *plac811* altera a expressão de genes relacionados a dPCD e ePCD e a dificuldade em obter plantas superexpressando AtPLAC811.1 utilizando um promotor constitutivo indica o envolvimento de AtPLAC811.1 em processos importantes na planta, e abre espaço para o estudo das linhagens transgênicas de superexpressão de AtPLAC811.1 induzidas por dexametasona.

No capítulo III, intitulado "Emprego do método TurboID-marcação de proximidade para a busca dos interatores de AtPLAC8-11.1 e AtPLAC8-11.2", experimentos de proteômica foram realizados e os possíveis parceiros de interação de ambas as variantes foram identificados. Nossas análises revelaram que para a forma canônica AtPLAC811.1 foram identificados um número significativamente menor de parceiros de interação, porém com relevantes funções envolvendo a resistência frente a diversos estresses abióticos, como é o caso de *AtMtATP6* (ZHANG; LIU; TAKANO, 2008), e também *AtSFP1* que tem sua expressão aumentada durante a senescência foliar (QUIRINO; REITER; AMASINO, 2001). *AtPLAC811* e outros membros da família PLAC8 foram diferencialmente expressos em plantas superexpressando um regulador negativo da senescência foliar em comparação com plantas selvagens, reafirmando o papel de *AtPLAC811.1* na senescência (ZHANG et al., 2022c). Foi identificada também a proteína AtMAIGO5, considerada componente-chave da exportação de proteínas a partir do retículo endoplasmático (TAKAGI et al., 2013). Esse é um resultado interessante visto que tanto AtPLAC811.1 quanto AtPLAC811.2 obtiveram co-localização com o marcador de retículo endoplasmático.

Em relação aos interatores de AtPLAC811.2, foram identificados um número elevado de proteínas que interagem com a proteína codificada pela variante de splicing, estando as mesmas relacionadas a funções celulares distintas. Dentre as proteínas identificadas, observa-se a presença de proteínas relacionadas ao transporte de íons, envolvimento na organização e no alinhamento dos microtúbulos corticais, proteínas relacionadas com a organela de Golgi,

associadas com a letalidade e defeitos do embrião, sinalização de ácido abscísico, mediação da abertura estomática, formação do padrão de exina do pólen e na deiscência de anteras. Em relação a morte celular programada e as respostas de defesa da planta, foram identificadas algumas proteínas interatoras, como a proteína AT5G64830.1 que está relacionada à morte celular programada e com os fatores de transcrição AtWRKY18 (AT4G31800.1) e AtWRKY40 (AT1G80840.1) que são induzidos por patógenos (ABEYSINGHE; LAM; NG, 2019; BINIAZ et al., 2022). Esses resultados indicam que apesar de AtPLAC811.2 não interagir com AtLSD1, conforme demonstrado nos experimentos do capítulo II dessa tese, a forma derivada da variante de splicing possui envolvimento nas respostas aos estresses ambientais possivelmente interagindo com outras proteínas importantes.

Como perspectivas deste trabalho, pretendemos avaliar fenotipicamente e molecularmente as respostas das plantas transgênicas superexpressando *AtPLAC811.1* e *AtPLAC811.2* sob controle do promotor nativo e das plantas superexpressando *AtPLAC811.1* de maneira induzível frente a diversas condições de estresses abióticos e bióticos. Além disso, pretendemos confirmar através de experimentos de duplo híbrido em levedura, a interação entre as principais proteínas enriquecidas apresentadas no capítulo III com AtPLAC811.1 e AtPLAC811.2. Esses resultados darão maior segurança sobre a participação de AtPLAC811 em diferentes processos celulares, permitindo a abertura de novos campos de estudos para a caracterização de *AtPLAC811.1* e *AtPLAC811.2*, baseado na descrição funcional dos interatores, quando existente.

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