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ANÁLISE FUNCIONAL E POTENCIAL BIOTECNOLÓGICO DE DESIDRINAS E GALACTINOL SINTASES DE MACIEIRA

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LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

ABA - ácido abscísico

CAMTA - "Calmodulin Binding Transcription Activator"

CBF/DREB1 - "C-repeat binding factor/dehydration-responsive element binding 1"

COR - "cold responsive"

DAM - "Dormancy-associated MADS-box"

DHN - desidrinas

DREBs - "Dehydration-Responsive Element-Binding proteins"

EBB - "Early bud-break"

FAO - "Food and Agriculture Organization of the United Nations"

ISH - hibridização *in situ*

GA - ácido giberélico

GH17 - 1,3-β-glucanases 17

GolS - galactinol sintase

GT8 - "glycosyltransferase 8"

ICE1 - "Inducer of CBF Expression 1"

LEA - "late embryogenesis abundant"

MAA - milhões de anos atrás

RFO - "raffinose family oligosaccharides"

WGD - "whole genome duplication"

RESUMO

A macieira (*Malus* x *domestica* Borkh.) é uma frutífera de clima temperado de grande importância econômica, e sua produtividade está diretamente relacionada à dormência. Além dos genes responsáveis pelo controle molecular, uma série de proteínas e metabólitos também é recrutada para proteger a integridade da gema dormente, destacando-se as desidrinas (DHN) e as enzimas galactinol sintases (GolS). As DHNs são proteínas que atuam na resposta adaptativa vegetal a estresses abióticos, enquanto que GolS são enzimas responsáveis pela síntese de galactinol, essencial à síntese de oligossacarídeos da família da rafinose (RFOs), os quais se acumulam em resposta a estresses abióticos. O objetivo do presente trabalho foi explorar a adaptação das gemas a condições de estresse a que são submetidas na dormência, visando identificar genes com potencial uso biotecnológico. Para tal, foram identificados e caracterizados os genes codificadores de DHNs e GolS no genoma da macieira por meio da utilização de ferramentas *in silico* para estudar a evolução, experimentos a campo e sob condições controladas, análises de expressão, localização subcelular, e geração de plantas transgênicas. As análises evolutivas sugerem que eventos de duplicação do genoma inteiro (WGD) foram responsáveis por moldar a evolução e diversificação dos genes *GolS* em macieira, enquanto que no caso das *DHN* eventos de duplicação em tandem e WGD nortearam a sua evolução. Nossos resultados sugerem que DHNs, galactinol e rafinose integram uma série de mecanismos que agem em conjunto durante a dormência a fim de proteger a integridade da gema, além dos carboidratos constituírem uma fonte de energia para a brotação. Ao longo da evolução, o aparecimento de novas estruturas e programas de desenvolvimento, tais como a gema e a dormência, necessitaram de adaptação de vias moleculares já estabelecidas, o que ajuda a explicar por que as dormências de gemas e de sementes compartilham rotas moleculares comuns. Finalmente, o gene *MdDHN11* foi funcionalmente caracterizado e nossos resultados fornecem evidências de que MdDHN11 desempenha importantes papéis durante o desenvolvimento da semente de maçã, protegendo o embrião e o endosperma de alterações no status da água. Além disso, apenas a planta superexpressando *MdDHN11* sobreviveu ao ensaio de simulação de seca, confirmando o potencial uso biotecnológico de DHNs de macieira no aumento da tolerância ao déficit hídrico.

ABSTRACT

Apple tree (*Malus* x *domestica* Borkh.) is a temperate fruit crop of great economic importance worldwide and its productivity is related to bud dormancy. Besides genes responsible for the molecular control of the process, a number of proteins and metabolites are also recruited to protect bud integrity, such as dehydrins (DHN) and galactinol synthases (GolS). DHNs are proteins that act on plant adaptive responses to abiotic stresses, while GolS are enzymes that catalyze for the synthesis of galactinol, an essential carbohydrate in the synthesis of raffinose family oligosaccharides (RFOs), which also accumulate in response to abiotic stresses. The objective of this work was to explore bud adaptation to stress conditions that occur during dormancy to identify genes with potential biotechnological applications. *DHN* and *GolS* genes were identified and characterized in the apple genome employing *in silico* tools, experiments under field and controlled conditions, expression analysis, subcellular localization assays, and the generation of transgenic plants. Evolutionary analyses suggest that whole genome duplication (WGD) events were responsible for shaping the evolution and diversification of *GolS* genes in apple, whereas WGD and tandem duplication events could be held accountable for *DHN* evolution. Our results suggest that DHNs, galactinol and raffinose integrate a series of mechanisms that act together during dormancy in order to protect bud integrity, besides the carbohydrates being an energy source for budbreak. During evolution, the appearance of new structures and developmental programs, such as buds and dormancy, required the adaptation of already established molecular pathways, partially explaining why bud and seed dormancy share common pathways. Finally, the *MdDHN11* gene has been functionally characterized and our results provide evidences that MdDHN11 plays important roles during apple seed development by protecting the embryo and the endosperm from water deficit. Moreover, only the plant overexpressing *MdDHN11* survived the water withholding assay, confirming the potential biotechnological use of apple DHNs in increasing tolerance to drought.

1 INTRODUÇÃO

Plantas frutíferas de clima temperado, tais como a macieira (*Malus* x *domestica* Borkh.), possuem grande importância econômica, e sua produtividade está diretamente relacionada ao processo de dormência, uma vez que um ciclo de dormência bem ajustado é essencial para o alcance integral do seu potencial genético (FALAVIGNA *et al.*, 2015b). Este processo ocorre em resposta a sinais ambientais cíclicos, principalmente temperatura e fotoperíodo, que coordenam o seu estabelecimento, manutenção e superação (SHIM *et al.*, 2014). A dormência é caracterizada pela presença da gema dormente, uma estrutura receptora dos sinais ambientais e que carrega um programa de desenvolvimento de proteção ao meristema (ROHDE & BHALERAO, 2007). Apesar do processo de dormência ser bem caracterizado do ponto de vista fisiológico, os seus principais reguladores genéticos e moleculares ainda são desconhecidos ou foram apenas parcialmente explicados (ROHDE & BHALERAO, 2007; CAMPOY *et al*., 2011; VAN DER SCHOOT & RINNE, 2011). Estudos recentes demonstraram que um dos maiores integradores da resposta ao frio, o fator de transcrição CBF/DREB1 ("C-repeat binding factor/dehydration-responsive element binding 1"), também é responsável por modular a dormência (WISNIEWSKI *et al.*, 2015; ARTLIP *et al.*, 2016). Além dos genes responsáveis pelo controle molecular da progressão da dormência, uma série de proteínas e metabólitos também é recrutada para proteger a integridade da gema durante o inverno. Assim, diversos estudos de expressão gênica durante a dormência identificaram a indução de genes de reposta ao frio e a desidratação que integram a cascata de resposta dos CBFs, destacando-se a presença de desidrinas (DHN) e de galactinol sintases (GolS; DERORY *et al.*, 2006; YAMANE *et al.*, 2008; LEIDA *et al.*, 2010; SANTAMARÍA *et al.*, 2011; LIU *et al.*, 2012; UENO *et al.*, 2013; FALAVIGNA *et al.*, 2014; PORTO *et al.*, 2015).

As DHNs são polipeptídeos pertencentes ao grupo II das proteínas LEA ("late embryogenesis abundant") e possuem papel importante na resposta adaptativa vegetal a estresses abióticos, principalmente os que envolvem desidratação (HANIN *et al.*, 2011). Embora diversas funções já tenham sido descritas, tais como atividade antioxidante, ligação a membranas, metais pesados e lipídeos, e proteção ao calor e frio, o exato mecanismo de ação dessas proteínas ainda é desconhecido (revisado em HANIN *et al*.,

2011; GRAETHER & BODDINGTON, 2014; BANERJEE & ROYCHOUDHURY, 2016). Por sua vez, a GolS (EC 2.4.1.123) é uma enzima chave na via de biossíntese dos oligossacarídeos da família da rafinose (RFO - "raffinose family oligosaccharides"), catalisando a formação de galactinol por meio de UDP-galactose e mio-inositol (ELSAYED *et al*., 2014). O galactinol é um carboidrato essencial na síntese de RFOs, tais como a rafinose e a estaquiose, os quais atuam como osmoprotetores em resposta ao frio, congelamento e déficit hídrico (SENGUPTA *et al.*, 2015). Além disso, galactinol e RFOs atuam como solutos compatíveis desempenhando um papel importante no armazenamento de carbono, ajuste osmótico, transdução de sinal, estabilização de membranas e proteínas, entre outros (ELSAYED *et al*., 2014; SENGUPTA *et al.*, 2015). A expressão ectópica de *DHN* (CHENG *et al.*, 2002; XING *et al.*, 2011; RUIBAL *et al.*, 2012; SHEKHAWAT *et al*., 2011; XIE *et al.*, 2012; YANG *et al.*, 2014) ou de *GolS* (TAJI *et al.*, 2002; SUN *et al.*, 2013) em diversas espécies vegetais leva ao aumento da tolerância à seca.

Para a agricultura, a deficiência hídrica é o principal estresse abiótico causador de perda de produtividade (MAHAJAN & TUTEJA, 2005). Em geral, as plantas desenvolveram sistemas elaborados e sensíveis que permitem a rápida sinalização, resposta e adaptação ao estresse. Entretanto, o seu grau de adaptabilidade dependerá da sua fisiologia, bioquímica e capacidade molecular de adaptação (UMEZAWA *et al.*, 2006). Levando-se em conta os modelos recentes de aquecimento global, os quais prevêem um aumento médio das temperaturas e alterações no regime de distribuição de chuvas, uma maior perda na produção de alimentos poderá ocorrer a médio e longo prazo devido a influência das mudanças climáticas (KIRTMAN *et al.*, 2013). Neste contexto, juntamente com esforços para a conservação da água e da terra, novas tecnologias agrícolas serão necessárias, tais como a geração de plantas geneticamente modificadas melhor adaptadas a condições adversas de cultivo (MAHAJAN & TUTEJA, 2005; UMEZAWA *et al.*, 2006; TAKEDA & MATSUOKA, 2008).

A compreensão dos mecanismos que possibilitam que as plantas superem condições ambientais adversas é de grande importância para a manutenção de culturas vegetais de interesse agronômico. Deste modo, a exploração da adaptabilidade da gema às condições de estresse que ocorrem durante a dormência, como a limitação hídrica, torna-se um interessante modelo de estudo visando identificar genes com potencial uso biotecnológico. Neste contexto, estudos prévios de análise de expressão gênica diferencial durante a

progressão da dormência de gemas em macieira permitiram identificar genes responsáveis pelo controle molecular deste processo, bem como genes com potencial protetor à integridade da gema durante o inverno, como *DHN* e *GolS* (FALAVIGNA *et al.*, 2014).

1.1 O PROCESSO DE DORMÊNCIA

A identificação e a compreensão dos processos que regem o desenvolvimento de espécies perenes, em especial a mudança da fase juvenil à reprodutiva, a progressão da dormência e a floração, são essenciais para a obtenção de culturas comerciais melhor adaptadas a cada cenário regional de cultivo (HORVATH *et al.*, 2003). As plantas perenes podem responder a condições ambientais sazonais com o estabelecimento de um período de dormência, o qual é definido como a incapacidade de iniciar o crescimento meristemático sob condições favoráveis (ROHDE & BHALERAO, 2007). Em resposta a diferentes fatores, três tipos de dormência de gemas foram previamente descritos (Figura 1): (i) paradormência – inibição do crescimento provocada por outro órgão da planta; (ii) endodormência – dormência regulada por sinais internos das gemas; e (iii) ecodormência – dormência devido a condições ambientais temporariamente desfavoráveis (LANG *et al.*, 1987). A entrada do processo de endodormência, tipo de dormência com maior apelo comercial, ocorre em resposta à exposição ao frio e fotoperíodo curto, sendo caracterizada pela paralisação do crescimento vegetativo, queda das folhas e formação da gema dormente. Esta última é uma estrutura integradora dos sinais ambientais, carregando um programa de desenvolvimento de proteção ao meristema (ROHDE & BHALERAO, 2007). Na presente Tese, o termo dormência será utilizado para se referir ao processo de endodormência.

Diferentes fatores ambientais, fisiológicos e moleculares já tiveram o seu papel parcialmente caracterizado no processo de dormência. Contudo, a hipótese de que apenas um sinal atue diretamente nas gemas, sendo responsável pela ativação e manutenção do processo, é improvável (JACKSON, 2003; SHIM *et al.*, 2014). A interação entre esses sinais regulatórios ainda é pouco conhecida, apesar dos recentes avanços conquistados na área (FALAVIGNA *et al.*, 2015b).

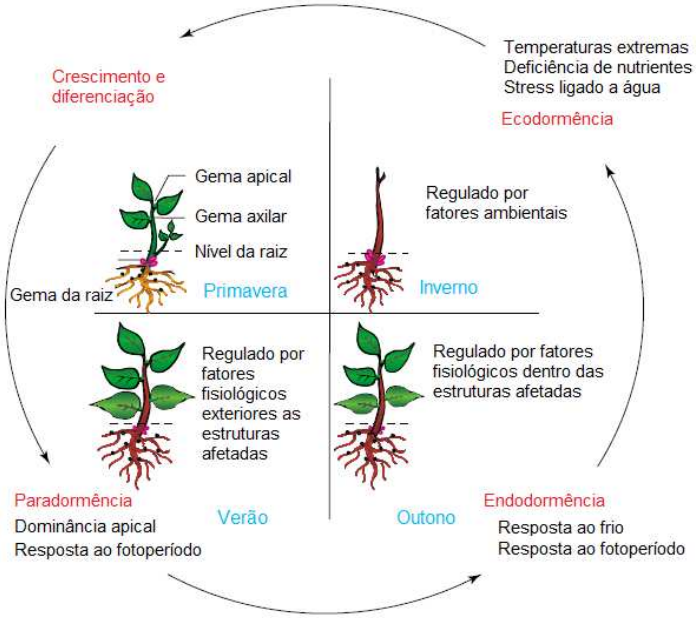

Figura 1. Diagrama relacionando as quatro estações do ano com o correspondente tipo de dormência de gemas. Adaptado de HORVATH *et al.* (2003).

1.1.1 Mecanismos fisiológicos da dormência

Os principais componentes fisiológicos associados com a perpeção das mudanças fenotípicas que regulam o processo de dormência são os receptores de sinais ambientais, os hormônios vegetais e os açúcares (CHAO *et al.*, 2016). Os principais sinais ambientais que induzem as plantas perenes a entrar em dormência são dias consecutivos com fotoperíodo curto e a exposição a baixas temperaturas. O estabelecimento do processo de dormência consiste na paralisação do crescimento vegetativo, na formação da gema apical e no início da senescência das folhas ainda no outono (HORVATH *et al.*, 2003; JACKSON, 2003; SHIM *et al.*, 2014). Uma vez que o crescimento vegetativo cessa, as células meristemáticas ficam inabilitadas a responder aos sinais de promoção de crescimento (ROHDE & BHALERAO, 2007). A progressão e a manutenção da dormência está diretamente relacionada à exposição da planta ao frio prolongado. Assim, a superação deste fenômeno, com a consequente entrada na ecodormência, envolve a exposição da planta a temperaturas abaixo de 7,2ºC. Entretanto, cada espécie vegetal possui um requerimento de exposição ao frio específico de sua região de origem, sugerindo um forte controle genético desta

característica (revisado em FALAVIGNA *et al.*, 2015b). Uma vez ecodormente, a planta estará apta a reiniciar o seu ciclo vegetativo e reprodutivo quando houver condições ambientais favoráveis (JACKSON, 2003).

Embora diversos estudos tenham identificado a atuação de hormônios vegetais no processo de regulação da dormência, o papel dos fitormônios nesse processo ainda não foi totalmente elucidado (HORVATH *et al.*, 2003; CHAO *et al.*, 2007). O ácido giberélico (GA) é um hormônio vegetal responsável por controlar o alongamento e a diferenciação celular. Uma diminuição na concentração de GA foi observada em plantas expostas a fotoperíodo curto, levando a paralisação do crescimento vegetativo e da divisão celular nos meristemas subapicais (RINNE *et al.*, 2011). Tal observação é sustentada pela regulação negativa imposta ao gene *GA-20 oxidase* em folhas, o qual codifica uma enzima chave na biossíntese de GA (ALLONA *et al.*, 2008; VAN DER SCHOOT & RINNE, 2011). A aplicação exógena de GA é capaz de quebrar a dormência de gemas em substituição ao frio, sugerindo que o frio é capaz de estimular a biossíntese de GA, além de ativar genes de sua síntese no ápice da gema. Uma semelhança entre a exposição ao frio e a aplicação de GA consiste no fato de que ambos são capazes de remover depósitos de calose presentes no meristema apical, os quais inibem a comunicação do meristema com o restante da planta. O mecanismo de ação da aplicação de GA consiste no recrutamento de enzimas GH17 (1,3-β-glucanases), as quais são capazes de degradar a calose e restituir a comunicação do meristema com a planta (PAUL *et al*., 2014). O ácido abscísico (ABA), por sua vez, é um fitohormônio envolvido na resposta a estresses abióticos, na maturação de sementes e na paralisação do crescimento. Contudo, o seu papel durante o processo de dormência de gemas ainda é controverso (ROHDE & BHALERAO, 2007), apesar de ABA já ter sido descrito como indutor da dormência por facilitar a formação da gema (RUTTINK *et al.*, 2007). Embora o nível de ABA alcance um pico após três a quatro semanas de exposição a fotoperíodo curto, resultando na ativação de genes da sua rota de síntese e de transdução de sinal, nenhuma correlação foi comprovada entre a paralisação do crescimento vegetativo, a dormência de gemas e os níveis apicais de ABA em plantas perenes. O seu envolvimento na dormência parece estar mais relacionado ao controle da aclimatação ao frio e a tolerância à desidratação (ARORA *et al*., 2003; ALLONA *et al.*, 2008; VAN DER SCHOOT & RINNE, 2011). Recentemente, um estudo demonstrou uma diminuição dos níveis de ABA, jasmonato e etileno, com o concomitante aumento dos

níveis de GA, citocininas e auxinas após 72 horas da superação da paradormência (CHAO *et al.*, 2016). Estes resultados sugerem que diferentes fitormônios atuam em conjunto para direcionar mudanças celulares envolvidas na diferenciação da gema, bem como na retomada do crescimento, sendo que o papel definitivo de cada um deles ainda não foi estabelecido, principalmente quando considerada a dormência de gemas.

Os açúcares, além de servirem como fontes de energia, são moléculas sinalizadoras que regulam a expressão de diversos genes durante o desenvolvimento vegetal (RIOU-KHAMLICHI *et al.*, 2000). A transição de gemas vegetativas da paradormência para a endodormência possui rotas de sinalização que envolvem açúcares como moléculas sinalizadoras (CHAO *et al.*, 2007). Em pereiras, demonstrou-se que o aumento da entrada e catabolismo de sorbitol em gemas ocorre antes da brotação e de uma maneira sincronizada com a progressão da dormência, possuindo um papel sinalizador do status do processo (ITO *et al*., 2012; ITO *et al.*, 2013). Especula-se que alguns açúcares, tais como a sacarose, o amido, o galactinol e a rafinose, possuem um papel importante na regulação da dormência de gemas por meio da sua interação com fitormônios, como ABA e auxinas (CHAO *et al.*, 2007; RUTTINK *et al.*, 2007). Recentemente, foi demonstrado que o fluxo de açúcares é responsável por regular o processo de dominância apical, uma forma de paradormência no ramo, e não de auxina (MASON *et al.*, 2014). Esta descoberta evidencia o quão pouco compreendido são os mecanismos de regulação da dormência de gemas, o que gera uma oportunidade para que diversos outros mecanismos ainda sejam descobertos.

1.1.2 Mecanismos moleculares da dormência

A compreensão dos mecanismos moleculares que controlam o estabelecimento, a manutenção e a superação do processo de dormência em gemas é de grande importância para a geração de plantas mais adaptadas aos diferentes cenários regionais de cultivo. A transição do ciclo de dormência deve ser finamente sincronizada com as variações climáticas sazonais, uma vez que os sinais ambientais, tais como temperatura e fotoperíodo, auxiliam a planta a regular este processo de sincronização (SHIM *et al.*, 2014). Entretanto, uma vez que a descoberta de genes reguladores da dormência teve início

apenas recentemente, ainda não foi estabelecido como estes se integram com os sinais ambientais e com as rotas fisiológicas conhecidas. Uma completa e atualizada revisão dos mecanismos genéticos e moleculares que regulam este processo em plantas perenes, com foco especial na macieira, está descrita no Capítulo I da presente Tese. Esta seção faz parte de um capítulo publicado no livro "Advances in Plant Dormancy" de 2015.

1.1.2.1 Percepção do frio

As variações de temperatura que acontecem ao longo das mudanças das estações do ano possuem papel regulatório em diversos estádios de desenvolvimento vegetal, uma vez que a planta necessita perceber tais oscilações de modo a responder satisfatoriamente (PENFIELD, 2008). O processo de dormência pode ser entendido como a sincronização da fenologia da planta à exposição a baixas temperaturas e fotoperíodo curto, condições ambientais que ocorrem nos invernos das regiões de climas frios e temperados, havendo a necessidade de aclimatação e de resposta a temperaturas extremas de frio (CAMPOY *et al*., 2011). Macieiras e pereiras possuem a indução da dormência exclusivamente controlada pela exposição a baixas temperaturas (HEIDE & PRESTRUD, 2005), independentemente de alterações no fotoperíodo, o qual é o principal indutor da dormência em outras plantas perenes como álamo e pessegueiro (FALAVIGNA *et al*., 2015b). Além disso, estudos recentes demonstraram a interação entre a percepção ao frio e o processo de dormência, o que torna estes fenômenos interdependentes, sendo necessário que sejam estudados e compreendidos conjuntamente (WISNIEWSKI *et al.*, 2011; WISNIEWSKI *et al*., 2015; ARTLIP *et al.*, 2016).

A exposição ao frio possui diferentes efeitos deletérios sobre as plantas, podendo promover danos celulares irreversíveis caso a planta não passe por um processo de aclimatação ao frio, através do qual após prévia exposição a baixas temperaturas as plantas ganham tolerância a temperaturas extremas de frio (PENFIELD, 2008; KURBIDAEVA & NOVOKRESHCHENOVA, 2011; EREMINA *et al*., 2016). Desta forma, a percepção ao frio por parte das plantas é de suma importância, uma vez que antecede a paralisação do crescimento vegetal por meio de uma grande reprogramação transcricional (FOWLER &

THOMASHOW, 2002; PENFIELD, 2008; TANINO *et al.*, 2010). Diversos mecanismos moleculares de percepção ao frio já foram descritos em plantas, apesar de muitas rotas de sinalização ainda necessitarem de maiores estudos (EREMINA *et al*., 2016).

Uma das rotas de resposta ao frio mais estudadas em Arabidopsis envolve os fatores de transcrição CBFs, também conhecidas como DREBs ("Dehydration-Responsive Element-Binding proteins"), os quais possuem função central na indução de genes de aclimatação ao frio (Figura 2; EREMINA *et al.*, 2016). A exposição a baixas temperaturas leva ao aumento de íons cálcio no citoplasma, gerando uma cascata de sinalização onde proteínas quinases são ativadas e, por meio de fosforilação, ativam fatores de transcrição de resposta ao frio (CHINNUSAMY *et al*., 2007; KURBIDAEVA & NOVOKRESHCHENOVA, 2011). Entre estes, destacam-se os fatores de transcrição ICE1 ("Inducer of CBF Expression 1") e CAMTA ("Calmodulin Binding Transcription Activator"), os quais possuem sua forma ativa apenas após modificações pós-traducionais induzidas pelo frio. Após ICE1 ser ativado por fosforilação e sumoilação, a sua proteína entra no núcleo e forma dímeros capazes de interagir com o DNA e induzir a expressão dos genes *CBF*s (EREMINA *et al*., 2016). A sua regulação negativa também é realizada por modificações pós-traducionais, uma vez que sua forma ativa é marcada para degradação no proteossoma por meio da ubiquitinase HOS1 ("High expression of osmotically responsive genes"; Figura 2; CHINNUSAMY *et al*., 2007). A família de fatores de transcrição CAMTA interage com calmodulinas e íons cálcio, formando um complexo capaz de interagir com a região promotora dos genes *CBF*s (DOHERTY *et al.*, 2009). Outras rotas de aclimatação ao frio independentes dos genes *CBFs* já foram descritas, apesar de a grande maioria desses mecanismos ainda ser pouco conhecida (revisado em CHINNUSAMY *et al.*, 2007; KURBIDAEVA & NOVOKRESHCHENOVA, 2011; EREMINA *et al.*, 2016).

Os fatores de transcrição CBFs regulam a expressão dos genes *COR* ("cold responsive"), os quais estão envolvidos em diversas funções protetoras celulares, tais como detoxificação de espécies reativas de oxigênio, metabolismo e sinalização hormonal, biossíntese de osmoprotetores, transporte de membranas, metabolismo de açúcares solúveis, entre outros (Figura 2; CHINNUSAMY *et al*., 2007; TARKOWSKI & VAN DEN ENDE, 2015). Entre os principais genes *COR* já identificados, destacam-se desidrinas, galactinol sintases, GA oxidases, α- e β-amilases, entre outros (MARUYAMA

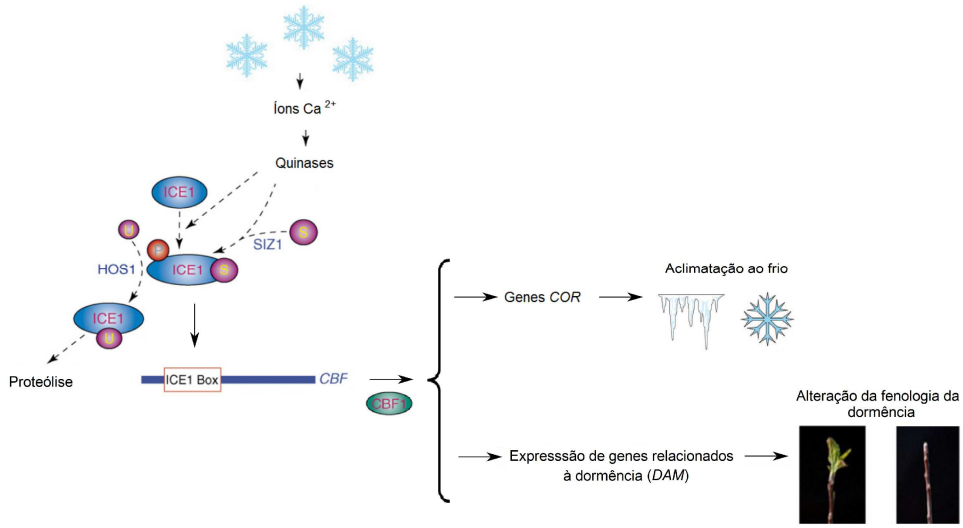

Figura 2. Cascata de sinalização dos CBFs. A exposição a baixas temperaturas leva ao aumento de íons cálcio no citoplasma e ativa as proteínas quinases. Por sua vez, ICE1 é ativado por meio de fosforilação e sumoilação por meio de quinases e SIZ1 ("SAP and MiZ1"), respectivamente. HOS1 é uma ubiquitinase responsável por marcar ICE1 para degradação no proteossoma. Os CBFs ativam tanto os genes *COR* quanto genes relacionados ao processo de dormência, tais como os genes *DAM*, resultando em aclimatação ao frio e modulando a dormência, respectivamente. Setas tracejadas indicam modificações pós-traducionais; setas sólidas indicam ativação. Adaptado de CHINNUSAMY *et al.* (2007) e WISNIEWSKI *et al.* (2015).

et al., 2009; PARK et al., 2015; TARKOWSKI & VAN DEN ENDE, 2015). Diversos estudos analisaram os efeitos da expressão ectópica de *CBF*s no aumento da tolerância a estresses abióticos. Apesar da obtenção de plantas transgênicas com aumentada tolerância ao estresse salino, hídrico e por baixas temperaturas, as plantas obtidas apresentavam fenótipo anão, sugerindo que a expressão ectópica de *CBF* altera diversas rotas metabólicas na planta (KURBIDAEVA & NOVOKRESHCHENOVA, 2011). Recentemente foi demonstrado que a superexpressão de *CBF* de pessegueiro em macieira alterou a dinâmica do processo de dormência, tornando-a responsiva a entrar em dormência após exposição à fotoperíodo curto (WISNIEWSKI *et al.*, 2011), o que naturalmente não ocorre em macieira (HEIDE & PRESTRUD, 2005). Essas plantas transgênicas foram avaliadas no campo durante três ciclos vegetativos e demonstraram maior tolerância ao frio, atraso no crescimento, além de ter sido confirmada a alteração da fenologia da dormência (ARTLIP *et al.*, 2014). Estes resultados evidenciam que os CBFs, além de recrutarem uma série de proteínas e metabólitos para proteção da gema durante o

inverno, também são capazes de modular o processo de dormência por meio da regulação de genes chave desse processo, tais como os genes *DAM* ("*Dormancy-associated MADS-box*") e *EBB* ("*Early bud-break*"; Figura 2; WISNIEWSKI *et al*., 2015; ARTLIP *et al*., 2016). Concordando com estes resultados, diversos estudos de expressão gênica durante a dormência de gemas identificaram a indução de genes *COR* que integram a cascata de resposta dos CBFs, destacando-se a presença de desidrinas e de galactinol sintases (DERORY *et al.*, 2006; YAMANE *et al.*, 2008; LEIDA *et al.*, 2010; SANTAMARÍA *et al.*, 2011; LIU *et al.*, 2012; UENO *et al.*, 2013; FALAVIGNA *et al.*, 2014; PORTO *et al.*, 2015).

1.1.2.1.1 Desidrinas

As desidrinas (DHNs) são proteínas caracterizadas pela presença de um domínio altamente conservado de 15 aminoácidos ricos em lisina, o segmento K, o qual pode estar presente em uma ou mais cópias próximo à região carboxi-terminal (KOSOVÁ *et al*., 2007). Complementariamente pode ou não haver a presença de outras duas sequências conservadas, uma rica em tirosina, o segmento Y, e outra rica em serina, o segmento S (HANIN *et al.*, 2011). Em geral, as DHNs são classificadas conforme a ordem e o número de repetições desses segmentos (KOSOVÁ *et al*., 2007).

As DHNs foram inicialmente identificadas durante o processo de dessecação de sementes, uma vez que se acumulam nos últimos estágios do desenvolvimento de sementes concomitantemente com o início da dormência (BANERJEE & ROYCHOUDHURY, 2016). Posteriormente, foram identificadas em plantas submetidas a estresses abióticos que envolvem desidratação, tais como baixas temperaturas, déficit hídrico ou alta salinidade (GRAETHER & BODDINGTON, 2014; BANERJEE & ROYCHOUDHURY, 2016). A indução de *DHN* em resposta a exposição a estresses abióticos se deve ao fato desses genes integrarem o conjunto de genes *COR*, sendo alvos diretos dos fatores de transcrição CBF (Figura 2; KURBIDAEVA & NOVOKRESHCHENOVA, 2011).

Embora diversas funções já tenham sido descritas para as DHNs, tais como ação antioxidante, ligação a membranas, metais pesados e lipídeos, e proteção ao calor e frio, o

seu preciso mecanismo de ação ainda é desconhecido (HANIN *et al.*, 2011; GRAETHER & BODDINGTON, 2014). Devido as DHNs serem proteínas intrinsecamente desordenadas, acredita-se que elas possam alterar o seu padrão conformacional em resposta a mudanças de disponibilidade de água, protegendo outras moléculas por meio de interações com partes parcialmente desidratadas (HOEKSTRA *et al*., 2001; TOMPA *et al.*, 2006; GRAETHER & BODDINGTON, 2014). Além disso, sugere-se que seu padrão conformacional desordenado é requerido para a sua função crioprotetiva (HUGHES *et al.*, 2013). Diversos estudos demonstraram que a expressão ectópica de *DHN* em plantas leva ao aumento da tolerância à seca, ao frio e à salinidade (revisado em HANIN *et al.* 2011; BANERJEE & ROYCHOUDHURY, 2016), o que as torna uma interessante ferramenta para obtenção de plantas melhor adaptadas a condições adversas de cultivo.

Estudos de expressão gênica durante o processo de dormência de gemas demonstraram a indução de *DHN* (DERORY *et al.*, 2006; YAMANE *et al.*, 2008; LEIDA *et al.*, 2010; SANTAMARÍA *et al.*, 2011; LIU *et al.*, 2012; UENO *et al.*, 2013; FALAVIGNA *et al.*, 2014; PORTO *et al.*, 2015). Estudos em diferentes espécies vegetais lenhosas demonstraram que o acúmulo de *DHN* se inicia durante o outono e alcança seu maior nível no inverno, coincidindo com a máxima tolerância da planta a temperaturas extremas de frio e, em geral, desaparecem quando o crescimento vegetativo é restabelecido (KEILIN *et al.*, 2007; FERNANDEZ *et al.*, 2012). Um estudo recente identificou e caracterizou onze DHNs de macieira sob o contexto da dormência de gemas (FALAVIGNA *et al.*, 2015a). Nesse trabalho foi demonstrado que as *MdDHN* possuem funções altamente divergentes, com níveis de sobreposição, e que os seus padrões de expressão são finamente ajustados por sinais ambientais durante a dormência de gemas. Além disso, sugere-se que o acúmulo de *MdDHN2-6* durante o inverno é um dos mecanismos parcialmente responsáveis pela tolerância da gema ao frio, enquanto a *MdDHN11* desempenha importantes papéis na tolerância a dessecação de sementes (FALAVIGNA *et al.*, 2015a).

1.1.2.1.1 Galactinol sintases

As enzimas galactinol sintases (GolS; EC 2.4.1.123) são uma subfamília das "glycosyltransferases 8" (GT8), uma grande família de enzimas envolvidas na biossíntese de açúcares de função estrutural, de armazenamento, de energia e de sinalização celular (SENGUPTA *et al.*, 2012). A GolS catalisa a formação de galactinol por meio de UDP-galactose e mio-inositol, sendo uma enzima chave na via de biossíntese dos RFOs, tais como rafinose, estaquiose, verbascose, entre outros, uma vez que o galactinol é um carboidrato essencial para a síntese de cada elemento da série da rafinose (Figura 3; ELSAYED *et al.*, 2014). O galactinol e os RFOs são solutos compatíveis que desempenham papéis importantes no armazenamento de carbono, ajuste osmótico, transdução de sinal, estabilização de membranas e proteínas, entre outros (ELSAYED *et al*., 2014; SENGUPTA *et al.*, 2015). Esses solutos podem ser acumulados em grandes quantidades na célula sem afetar processos metabólicos primários (UNDA *et al.*, 2012). Galactinol e RFOs são acumulados em resposta a estresses abióticos, tais como frio, congelamento, salinidade e déficit hídrico (SENGUPTA *et al.*, 2015). Em ervilha, o acúmulo destes carboidratos é importante para a proteção do embrião contra a dessecação durante os últimos estágios de desenvolvimento de sementes e também para a correta germinação (BLÖCHL *et al*., 2007). Entretanto, pouco se sabe sobre os mecanismos de ação dos RFOs. Estudos sugerem que esses metabólitos funcionem como sinalizadores moleculares, integrando vias de resposta a estresses e atuando como eliminadores de espécies reativas de oxigênio (NISHIZAWA *et al*., 2008; VALLURU & VAN DEN ENDE, 2011).

A GolS é uma enzima citosólica e com pH ótimo de atuação entre 5,6 e 7,5, possuindo várias isoformas codificadas por genes com variações significativas nos seus padrões de expressão (SCHNEIDER & KELLER, 2009). Os genes GolS são alvos diretos dos fatores de transcrição CBF, WRKY e *heat shock*, sendo induzidos em diversos tecidos e, em geral, sob condições de estresses abióticos (ELSAYED *et al*., 2014; SENGUPTA *et al.*, 2015). Além disso, a expressão de *GolS* nos estádios finais de maturação da semente também já foi relatada (DOWNIE *et al.*, 2003; ZHAO *et al.*, 2004; BLÖCHL *et al*., 2007;). Recentemente, genes *GolS* apresentaram uma maior quantidade de transcritos em gemas

endodormentes em relação a gemas paradormentes, resultando na mobilização sazonal de RFOs e levando a aclimatação ao frio no inverno (SANTAMARÍA *et al.*, 2011; UNDA *et al.*, 2012; IBÁÑEZ *et al.*, 2013; FALAVIGNA *et al.*, 2014). Finalmente, a expressão ectópica de *GolS* gerou um aumento da tolerância à seca e ao congelamento (TAJI *et al.*, 2002; SONG *et al.*, 2013; SUN *et al.*, 2013), o que torna estes genes bons candidatos para caracterização visando à geração de plantas transgênicas melhor adaptadas a condições adversas de cultivo.

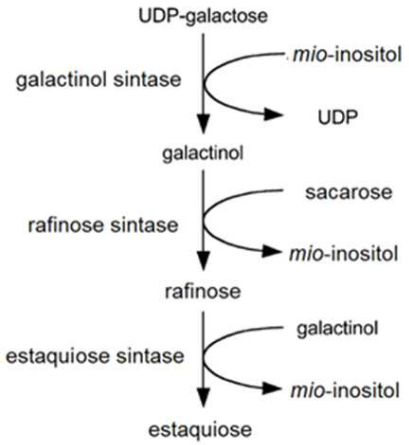

Figura 3. Rota de biossíntese de galactinol, rafinose e estaquiose. Adaptado de NISHIZAWA *et al.* (2008).

1.2 DÉFICIT HÍDRICO

A característica séssil das plantas as torna constantemente suscetíveis a exposição a estresses abióticos, tais como seca, frio, salinidade, metais pesados, entre outros, os quais frequentemente impõem restrições ao seu crescimento e produtividade (LAMB, 2012). Estima-se que cerca de 50% da produção mundial de alimentos é perdida anualmente em razão da exposição aos mais diversos tipos de estresses abióticos, sendo que, para a agricultura, a deficiência hídrica é o principal causador de perdas de produtividade (MAHAJAN & TUTEJA, 2005). Por outro lado, ao longo da evolução as plantas desenvolveram mecanismos específicos que lhes permitem detectar mudanças ambientais e

responder a condições complexas de estresse, minimizando os danos ao mesmo tempo em que conservam recursos para o crescimento e reprodução (ATKINSON & URWIN, 2012). Entre esses mecanismos destacam-se o fechamento de estômatos, a repressão de crescimento e fotossíntese, a alteração na taxa de respiração, o acúmulo de osmólitos e proteínas, entre outros (SHINOZAKI & YAMAGUCHI-SHINOZAKI, 2007). Entretanto, o grau de adaptabilidade dependerá da sua fisiologia, e capacidade bioquímica e molecular de adaptação (Umezawa *et al.*, 2006). Levando-se em conta os modelos recentes de previsão de aquecimento global, os quais prevêem um aumento médio das temperaturas e alterações no regime de distribuição de chuvas, uma maior perda na produção de alimentos poderá ocorrer a médio e longo prazo devido a influência das mudanças climáticas (KIRTMAN *et al.*, 2013). Neste contexto, juntamente com esforços para a conservação da água e da terra, novas tecnologias agrícolas serão necessárias, tais como a geração de plantas geneticamente modificadas melhor adaptadas a condições adversas de cultivo (MAHAJAN & TUTEJA, 2005; UMEZAWA *et al.*, 2006; TAKEDA & MATSUOKA, 2008).

Ao longo das últimas décadas, ocorreu uma grande busca em diversos programas de melhoramento vegetal em todo o mundo por plantas com maior tolerância à seca, principalmente "commodities". Muitos desses programas utilizaram diversas abordagens genéticas por meio da exploração de cultivares naturalmente mais tolerantes (revisado em CATTIVELLI *et al.*, 2008). Nos últimos anos, com a evolução das técnicas moleculares de exploração de genomas, transcriptomas e metabolomas, diversas rotas moleculares envolvidas na resposta, sinalização e mitigação dos efeitos da exposição ao déficit hídrico foram descritas (revisado em ZHU, 2002; VALLURU & VAN DEN ENDE, 2011; KRASENSKY & JONAK, 2012; LAWLOR, 2013; OSAKABE *et al.*, 2014; DUTTA & ROY, 2016), principalmente explorando espécies já adaptadas a condições extremas de privação de água (e.g. ITURRIAGA *et al.*, 1992). Além disso, os avanços gerados por esses estudos fomentaram a produção de plantas geneticamente modificadas visando avaliar os efeitos da expressão heteróloga destes genes em plantas-modelo. Neste contexto, diversos artigos de revisão já descreveram os avanços oriundos da inserção por meio de transgenia de diferentes classes de genes, muitos dos quais eficientemente conferiram maior tolerância à seca às plantas transformadas (revisado em CATTIVELLI *et al.*, 2008; KRASENSKY & JONAK, 2012; DUTTA & ROY, 2016). Entretanto, muitos destes genes

são responsáveis por integrar grandes rotas de sinalização de mais de uma característica, além do estresse hídrico. É o caso dos genes *CBF*, cuja superexpressão aumenta a tolerância à exposição a diferentes estresses, mas altera a arquitetura das plantas tornando-as anãs (CATTIVELLI *et al.*, 2008; KURBIDAEVA & NOVOKRESHCHENOVA, 2011). Assim, novas estratégias para identificar genes com potencial utilização na geração de culturas geneticamente modificadas são essenciais. A exploração da adaptabilidade da gema às condições de estresse que ocorrem durante a dormência em macieira é uma abordagem inovadora buscando a identificação de genes com potencial uso biotecnológico, principalmente no contexto da limitação hídrica.

1.3 A CULTURA DA MACIEIRA

A macieira (*Malus* x *domestica* Borkh.) é uma planta perene de clima temperado pertencente à tribo Pyreae, da família das Rosáceas, dentro da ordem das Rosales (CANTINO *et al.*, 2007). As montanhas Tian Shan na Ásia central são consideradas o seu centro de origem e dispersão, o que influenciou diversos aspectos fisiológicos do seu desenvolvimento, inclusive a dormência (CORNILLE *et al.*, 2014). Nesse aspecto, a macieira possui baixas temperaturas como o principal regulador do processo de dormência (HEIDE & PRESTRUD, 2005), diferentemente do que ocorre com outras plantas perenes onde alterações no fotoperíodo atuam como o principal regulador (FALAVIGNA *et al.*, 2015b). Acredita-se que este tipo de regulação seja um padrão entre plantas originárias de regiões onde as variações de temperatura, mais do que a qualidade da luz, marcam as estações do ano com maior precisão, possuindo mecanismos evolutivamente direcionados à sensibilidade e à percepção de temperaturas para a sincronização da sua fenologia com o ambiente (CAMPOY *et al*., 2011). Outra característica peculiar é o seu pseudofruto, o pomo, o qual é formado por um receptáculo carnudo com tecidos acessórios contendo cinco carpelos com sementes (JACKSON, 2003).

A macieira é uma das principais frutíferas cultivadas mundialmente, sendo a quarta mais importante atrás apenas de citros, videira e bananeira (TROGGIO *et al.*, 2012). No ano de 2013, de acordo com a FAO ("Food and Agriculture Organization of the United

Nations"), foram produzidas 81 milhões de toneladas de maçãs no mundo, sendo que o Brasil ocupou o 11º lugar na produção com mais de 1,2 milhões de toneladas. Grandes variações fenotípicas são encontradas entre as suas cultivares, incluindo produtividade, características do fruto, resistência a pragas, arquitetura e tamanho da planta, entre outros (TROGGIO *et al.*, 2012). No Brasil, as principais cultivares comerciais são Gala, Fuji e clones de seus mutantes somáticos, os quais agradam ao consumidor brasileiro devido às suas propriedades organolépticas (FIORAVANÇO *et al.*, 2010).

A comunidade científica conta desde 2010 com a disponibilização pública do genoma de macieira da cultivar Golden Delicious, a qual teve 81,3% do seu genoma sequenciado (VELASCO *et al.*, 2010). O sequenciamento revelou grandes porções cromossômicas duplicadas, provavelmente oriundas de um evento recente de duplicação do genoma inteiro (WGD - "whole genome duplication"). A macieira e outras espécies da tribo Pyreae, como a pereira, apresentam 17 cromossomos haplóides, enquanto outros membros da família das Rosáceas, tais como o pessegueiro e morango, apresentam de sete a nove cromossomos haplóides (TROGGIO *et al.*, 2012). Corroborando este achado, análises de mapeamento genômico indicaram forte colinearidade entre os cromossomos das espécies da tribo Pyreae, sugerindo que o evento recente de WGD foi responsável pelo seu maior número de cromossomos, partindo de 9 cromossomos ancestrais para os atuais 17 (VELASCO *et al.*, 2010; WU *et al.*, 2013). O evento recente de WGD ocorreu há cerca de 50 milhões de anos atrás (MAA) e é responsável por algumas características únicas das espécies desta tribo, tais como o fruto em formato de pomo (VELASCO *et al.*, 2010). Acredita-se que um evento de extinção em massa ocorreu neste período, sendo que as plantas contendo este evento de WGD se mostraram melhor adaptadas aquele cenário, perpetuando os seus descendentes (TROGGIO *et al.*, 2012). Neste contexto, a genômica comparativa auxilia na compreensão da estrutura do genoma e de sua função, bem como das forças evolutivas que levaram às configurações cromossômicas atuais e os mecanismos responsáveis pela diversidade de cada espécie. Um estudo comparando os genomas das Rosáceas macieira, pessegueiro e morango identificou que cada espécie apresenta diferentes modos evolutivos após terem divergido de um ancestral comum (JUNG *et al.*, 2012). Entretanto, todas as três espécies compartilham um evento de triplicação comum a todas as espécies Eudicotiledôneas, o qual ocorreu cerca de 125 MAA e contribuiu para a

diversificação molecular e filogenética dessas espécies (JIAO *et al.*, 2012; JUNG *et al.*, 2012).

A disponibilidade de acesso público ao genoma da macieira, bem como ao de diversas outras espécies vegetais (e.g. bancos de dados do NCBI, PHYTOZOME, Rosaceae.org, GigaDB, PLAZA, entre outros), é uma importante ferramenta para a caracterização de conjuntos de genes de interesse. Além disso, a partir do sequenciamento dos genomas é possível descrever a história evolutiva de uma determinada espécie e de suas espécies parentais, possibilitando uma maior compreensão sobre os diferentes eventos de duplicação genômica e de genes que ajudaram a moldar as características fenológicas atualmente encontradas nas espécies.

Neste contexto, a presente Tese de Doutorado partiu da premissa de que é possível transferir a adaptabilidade a estresses abióticos presente em gemas e sementes dormentes de macieira para estruturas naturalmente sensíveis a tais fenômenos, tais como flores, folhas e raízes. Para tal, foram identificados e caracterizados os genes de *DHN*s e de *GolS*s de macieira, buscando explorar aqueles com maior potencial de uso biotecnológico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Identificar e caracterizar os genes que codificam desidrinas e galactinol sintases no genoma de macieira, de modo a identificar genes candidatos a terem o seu potencial uso biotecnológico avaliado buscando gerar plantas transgênicas mais tolerantes ao estresse hídrico, bem como identificar regiões promotoras capazes de dirigir a expressão transgênica tecido-específica.

2.2 OBJETIVOS ESPECÍFICOS

a. Identificar e caracterizar as desidrinas de macieira durante a dormência de gemas;
b. Identificar e caracterizar as galactinol sintases durante a dormência de gemas;
c. Obter plantas transgênicas superexpressando genes com potencial de conferir maior tolerância ao estresse hídrico;
d. Caracterizar regiões promotoras capazes de dirigir a expressão tecido-específica em plantas-modelo.

Os resultados dos estudos relativos ao objetivo específico **a** foram publicados na forma de artigo e estão apresentados no **Capítulo II**; o conjunto de resultados do objetivo **b** está reunido no **Capítulo IV** sob a forma de artigo a ser submetido para publicação; e os dados referentes aos itens **c** e **d** compõem o **Capítulo III** também sob forma de artigo a ser submetido para publicação.

3 CAPÍTULO I

Chapter 5: Recent advances in genetics and molecular control of bud dormancy in pipfruits

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Chapter 5
Recent Advances in Genetics and Molecular Control of Bud Dormancy in Pipfruits

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Introduction

Temperate fruit crops have great economic importance worldwide and their production is closely related to bud dormancy, given that a well-adjusted dormancy cycle is crucial for the achievement of their full genetic potential. This process is regulated by environmental inputs, mainly chilling temperatures and photoperiodic changes, which are required for dormancy establishment and release (Horvath et al. 2003; Rohde and Bhalerao 2007). Bud dormancy is usually divided into paradormancy, endodormancy and ecodormancy, which refers to a failure of meristem growth under favorable conditions caused by signals derived from outside of the bud (but from the same plant), from the bud itself or from the environment, respectively (Lang et al. 1987). Dormancy entrance is characterized by growth cessation, bud set and leaf senescence. Once dormant, plants often need to be exposed to extended periods of cold (temperatures below 7.2 °C) to overcome it and the fulfillment of this chilling requirement (CR) culminates in ecodormancy (Horvath et al. 2003). The mechanisms regulating dormancy release are highly heritable and finely tuned, with each genotype being strongly influenced by its region of origin, suggesting a

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strong genetic control of this trait (Dennis 1987; Howe et al. 2000; Labuschagné et al. 2002; Jackson 2003; Campoy et al. 2011).

The direct relationships between bud dormancy and cold exposure gain importance when considering the recently proposed models for global warming. These models predict a rise in global mean temperatures and milder winters, which could result in difficulties for the production of temperate fruit crops (Arora et al. 2003; Campoy et al. 2011; Kirtman et al. 2013). Thereby, the importance of understanding the regulation of dormancy progression is gaining momentum with the main objective of maintaining sustainable crop yields in a changing environment. In this context, a wide range of approaches, from the genetic to the genomic perspective, are being used in several perennial crops as study models. In fact, although the main controlling mechanisms are still unknown or only partially explained, research advances in plant dormancy, especially in peach and poplar, are unveiling key regulators of this process.

Despite worldwide efforts applied to studying the dormancy process, only recently have two of the most economically important temperate fruit crops, apples and pears (FAO 2012), been explored for this agronomic trait. These pipfruits gain their name because of the small hard seeds (pips) in the center of the fruit (Palmer 2012), which differ from seeds of other Rosaceae species, such as peaches and strawberries. In addition, pipfruits also diverge in bud dormancy regulation because instead of being triggered by photoperiodic changes the main regulator of this process is exposure to low temperatures (Heide and Prestrud 2005). The Central and Western Asian origin of the pipfruits could explain their partial insensitivity to photoperiod, given that temperature in these regions varies more strongly than day length in comparison with other latitudes. Therefore, temperature would be a more reliable marker of the cold season than light quality to synchronize their phenology to the environment (Campoy et al. 2011).

Several advanced molecular models for bud dormancy control have been proposed (Horvath 2009; Campoy et al. 2011; van der Schoot and Rinne 2011; Rinne et al. 2011). However, they are based on species in which photoperiodic changes play a major role in dormancy induction and the peculiarities of this process in pipfruits are not addressed by these models. This review intends to help fill this gap, discussing the recent findings in genetics and genomics of bud dormancy control in pipfruits. The better understanding of this process may permit the development of new strategies that could help the generation of cultivars better adapted to each regional cultivation scenario.

Linkage Mapping of Dormancy-Related Traits

A major approach in the discovery of genes controlling phenological characteristics, such as bud dormancy, is to determine the association between the presence or absence of the trait of interest (phenotypes) and the profiles of molecular markers (genotypes) across individuals of a segregating population, a strategy known as linkage mapping (Mackay et al. 2009). Linkage mapping from experimental populations is very widespread in herbaceous crops, such as wheat and rice, but this is

not the case for tree crops, such as apple and pear (Troggio et al. 2012). The main reasons are the high costs of maintaining a population of trees suitable for linkage mapping and their long juvenile period, especially when working with adult traits such as fruit quality or dormancy of reproductive buds (Flachowsky et al. 2009; Grattapaglia and Kirst 2008; Neale and Kremer 2011; Myles 2013).

One of the first attempts to assess the heritable components of tree bud phenology was done using populations of *Populus* sp. hybrids (Bradshaw and Stettler 1995). At the time, the consensus among geneticists was that characters with broad phenotype distributions, such as time of bud flush, were controlled by a large number of genes, each one with small effects. The authors found that most of the variation for bud phenology observed in their experimental population (84.7%) was explained by five quantitative trait loci (QTL) distributed in five linkage groups. However, it remained an open question whether each identified QTL represented one gene with a major effect or a cluster of genes with minor effects. This question was addressed by the refinement of the QTL analysis and the mapping of candidate genes for the control of bud phenology (Frewen et al. 2000). The authors found two genes potentially related to dormancy regulation to be coincident with the confidence intervals of two major QTLs, namely *PHYTOCHROME B* (*PHYB*) and *ABSCISIC ACID INSENSITIVE* (*ABI*) homologs. Both were shown to be involved in timing of bud set and bud development (Olsen et al. 1997; Rohde et al. 2002). These first studies demonstrated that most of the genetic control of bud phenology could be mapped to a few genomic intervals.

Dormancy-associated traits, due to their quantitative nature, are often a subject of quantitative genetics disciplines. Bud dormancy-related phenotypes exhibiting a classical Mendelian segregation, which are more straightforward to map than QTLs (Mackay 2001), are very rare. An invaluable research opportunity was explored from the mapping of the *evergrowing* (*evg*) locus of peach (Bielenberg et al. 2008). The *evg* mutants are non-dormant, i.e., they do not stop growing even when exposed to short photoperiods or low temperatures, and the *evg* trait segregates as a single recessive gene. Sequencing of the *evg* locus revealed a cluster of six MIKC-type MADS-box genes, thereafter called *Dormancy-Associated MADS-box* (*DAM*) genes.

When the genetic control of bud dormancy in peach was characterized by quantitative genetics approach, two major QTLs (explaining more than 30% of the phenotypic variation) were found, and one of them overlapped with the *evg* locus on linkage group one (LG1, Fan et al. 2010). Further high-resolution mapping of this QTL and next-generation resequencing of the genomes from extreme phenotype individuals indicated *DAM* genes as the most probable genetic elements underlying the effects of the LG1 QTL (Zhebentyayeva et al. 2014).

Both peach and poplar are self-compatible and fast-growing trees; hence, true F2 populations can be established in relatively short timeframes (Fan et al. 2010; Faria et al. 2011). Linkage mapping in F2 generation is virtually impossible for self-incompatible species; therefore, alternative cross strategies are needed to obtain segregant populations. The high level of heterozygosity commonly found in self-incompatible species can be used as leverage for the generation of linkage maps by

the two-way pseudo-testcross approach (Grattapaglia and Sederoff 1994). The main idea behind this strategy is to follow the 1:1 segregation of genotypes from markers that are heterozygous in only one parent. It follows that two linkage maps are constructed, one for each parent, and the maps can be integrated through markers that are present in both parental lines. The two-way pseudo-testcross is a convenient, simple-to-implement and robust strategy for linkage mapping of tree species in the F1 generation and does not depend on prior genetic information from the parental lines.

Apple and pear are self-incompatible species with a long juvenile period, and these limitations have hampered genetic understanding and improvement of both crops (Jackson 2003). The first controlled crosses of apple trees for breeding purposes date from 1806, and apple breeders usually select genotypes carrying desired traits from the F1 progenies (Kellerhals 2009). Many of the target traits to be introgressed to apple cultivars are related to disease resistance, tree architecture, flowering and fruit quality (Korban and Tartarini 2009). Pear breeding also typically involves generation of genetic variation by crossing, aiming to improve fruit quality, disease resistance, storage ability, among other traits (Yamamoto and Chevreau 2009).

Breeding and academic research of slow growing trees, such as apple and pear, can benefit greatly from the knowledge obtained using molecular markers linked to heritable traits. For apple, a considerable range of molecular and genetic data is publicly available, as well as a high-quality whole genome draft (Velasco et al. 2010). Among the many tools and databases available, a noteworthy resource is the apple 8K single nucleotide polymorphism (SNP) array developed by the International RosBREED Consortium (Chagné et al. 2012). The SNPs that compose the chip were chosen after analyzing the resequencing data from 27 cultivar accessions, representing most of the genetic variation available for apple germplasm. Afterwards, due to the lack of SNP markers described for pear and the high collinearity between apple and pear genomes, approximately 1000 newly discovered SNPs from pear were added to the chip, collectively totaling nearly 9000 markers (Montanari et al. 2013). One limitation of this platform, however, is the unexpected segregation patterns for a large number (more than half) of markers (Troggio et al. 2013). The reason for this anomaly is the high level of paralogy exhibited by the apple genome, probably caused by a recent whole genome duplication event (Velasco et al. 2010). In practical terms, a great number of probes anneal in paralogous sites, resulting in distorted genotype proportions in the experimental population. This can be minimized by the use of stringent quality filtering of observed genotype distributions, in order to select only reliable markers.

In apple, several linkage mapping studies have already been done specifically for the characterization of dormancy traits. The experimental population for dormancy-related QTL analysis in apple is set up from the offspring of a crossing between individual cultivars differing in CR. There are many apple cultivars with various ranges of CR, and this trait, as for *Populus* sp., is largely genetically controlled, most likely as a single dominant gene for the low CR trait (Hauagge and Cummins 1991). In an early QTL identification attempt following the two-way pseudo-

testcross strategy, Conner et al. (1998) found eight regions distributed in seven linkage groups as highly associated with timing of bud break. However, the linkage map constructed did not include markers that could be transferred to the reference apple genetic map and, hence, the numbering of linkage groups is not the standard for apple genetic studies.

In a more recent study, van Dyk et al. (2010) performed map construction and QTL analysis for dormancy traits from populations in South Africa derived from crosses between individuals from 'Anna' (very low CR) and 'Golden Delicious' (high CR) and from 'Anna' and 'Sharpe's Early' (high CR). The maps, constructed from F1 genotypes employing 320 simple sequence repeat (SSR) markers, were composed of 17 linkage groups (LGs), corresponding to the number of apple chromosomes. The single QTL found was positioned on LG9 and explained around 40 % of the variation in the timing of both vegetative and floral bud break (van Dyk et al. 2010). In a similar approach, Celton et al. (2011) constructed maps from crossings between 'Starkrimson' and 'Granny Smith' and between X3263 and 'Belrène,' the last consisting of a population of more than 300 individuals. The QTL analysis of timing of bud break revealed several associations for this trait across the genome, the major one being on LG9, in close agreement with the confidence interval found by van Dyk et al. (2010). The region of interest was defined as the first 4 million base pairs from chromosome 9 in the apple genome, a region identical to the one found in an independent linkage mapping of dormancy-related traits performed by our own group (Tessele et al. manuscript in preparation). Candidate gene analysis of this region revealed enrichment for functional classes such as stimulus, biological regulation, signaling, programmed cell death and cell cycle control (Celton et al. 2011). These segregant populations were established in very divergent climatic conditions, yet shared the same genomic region as containing most of the genetic control of the timing of bud break. These findings suggest that variation in dormancy-related traits in apple has a strong genetic component. In addition, the overlap of genomic intervals for QTLs identified from different progenies suggests a common underlying genetic mechanism as responsible for the variation of the trait. The next step, therefore, is to further characterize the major QTLs for apple bud dormancy-related traits, as already carried on for peach (Zhebentyayeva et al. 2014). A consensus approach among molecular geneticists is to genotype the same population used for QTL identification using a high number of markers located in the region of interest, which is often called fine mapping or high-resolution mapping (Mackay 2001).

The availability of the next-generation sequencing technologies and high-quality genomes now allows the discovery of new molecular markers with low relative cost. DNA resequencing was carried out for parental individuals from the population segregating for dormancy traits established by our group, and as a result, more than 80,000 SNPs were discovered (Alencar et al. 2011). After validation, these new markers will be fundamental for the fine characterization of the apple dormancy-related traits QTLs.

Despite its significant economic importance, pear does not benefit from the same range of genetics and genomics resources as apple. Molecular markers have been

used in pear for the determination of genetic diversity, association with genes of agronomical interest, and construction of linkage maps (Yamamoto and Chevreau 2009). The first pear genetic map was constructed from a cross between Japanese (*P. pyrifolia*) cultivars using random amplified polymorphic DNA (RAPD) markers (Iketani et al. 2001). Yamamoto et al. (2002) assembled a pear map including simple sequence repeats (SSR) markers shared between apple and pear. The transferability of these markers allowed the comparison of maps from apple and pear, and indicated a high level of synteny between the two genomes. The close evolutionary relationship between the two species was clearly demonstrated with the recent publication of the genomes of the Japanese (Wu et al. 2013) and the European (Chagné et al. 2014) pears. In fact, the high transferability of molecular markers between pear and apple allowed a combination of SNPs from both species to be arrayed in the same platform for the genotyping of the two crops interchangeably (Montanari et al. 2013).

Various QTL identification attempts have been made in pear and yielded DNA markers closely associated with disease resistance, fruit storage and leaf traits (Yamamoto and Chevreau 2009; Sun et al. 2009). However, to the best of our knowledge, no QTL mapping for dormancy-related traits were performed in pear to date. Indeed, due to their genetic similarities, much of what is being discovered in apple may be applied to dormancy in pear. This statement is in agreement with the findings reported by Celton et al. (2009), which confirmed the ready transferability of SSR markers from *Malus* to *Pyrus*.

Molecular Control of Bud Dormancy Progression

Bud dormancy is a complex process that includes a range of states, degrees of development and the outgrowth that is tightly synchronized with seasonal changes. The elucidation of molecular networks responsible for the control of bud dormancy progression has been almost exclusively done on systems induced by photoperiodic changes (Böhlenius et al. 2006; Li et al. 2009; Jiménez et al. 2010; Doğramaci et al. 2010). Some components of photoperiod perception are known to play roles in dormancy regulation, such as PHYA (PHYTOCHROME A), CONSTANS (CO) and FT (FLOWERING LOCUS T). In annual plants such as *Arabidopsis thaliana*, flowering occurs in response to long-day photoperiods, with CO and FT controlling photoperiod perception and flowering time, respectively (Amasino and Michaels 2010). In *Populus* trees, Böhlenius et al. (2006) reported that *PtFT1* also controls the short-day-photoperiod-induced growth cessation and bud set. In an independent study, Hsu et al. (2011) identified two *FT* paralogs (*FT1* and *FT2*) in poplar and indicated that their expressions are temporally and spatially separated. These authors demonstrated that *FT1* expression during winter coincides with the transition of vegetative to reproductive phases, whereas *FT2* promotes vegetative growth and inhibition of bud set in response to warm temperatures and long days. In agreement to these findings, Kotoda et al. (2010) reported that apple also has two *FT* genes,

and Srinivasan et al. (2012) showed that the overexpression of a poplar *FT* in plum (*Prunus* x *domestica*) impaired dormancy entrance.

The expression of genes regulated by photoperiod is interconnected in a cascade of events, where PHYA, along with other circadian clock components, regulates CO, which in turns induces *FT* transcription leading to flowering. Furthermore, the signaling cascade regulated by photoperiod perception is closely connected to the cold temperature perception pathway, involving several related transcription factors (Amasino and Michaels 2010). However, the role of temperature perception in bud set and in induction of bud dormancy is still poorly understood. Some genes that play key roles in photoperiod perception involved in crosstalk with the temperature pathway could act as temperature sensors, such as the phytochromes (Franklin 2009). In *A. thaliana*, temperature regulates flowering through the vernalization pathway, which is mediated by the FLOWERING LOCUS C (FLC). FLC is a MADS-domain transcriptional regulator that represses two floral integrators, *FT* and *SOC1*, inhibiting flowering at low temperatures (Helliwell et al. 2006). Interestingly, there is a feedback loop involving SOC1 and FLC regulation, which may prevent premature flowering under cold conditions (Seo et al. 2009). Hereupon, SOC1 negatively regulates the cold response pathway through the direct repression of C-repeat binding factor/dehydration-responsive element-binding protein (CBF/DREB1) transcription factors, which are responsible for most of the cold-induced gene expression in plants (Seo et al. 2009; Thomashow 2010). On the other hand, the expression of *CBF/DREB1* increases *FLC* expression that in turn represses *FT* and *SOC1*, thereby delaying flowering (Seo et al. 2009). The crosstalk between temperature and photoperiod pathways in dormancy regulation was markedly demonstrated by Wisniewski et al. (2011), which reported that the ectopic expression of a peach *CBF* in apple triggered dormancy induction by short-day photoperiod. The same transgenic plants were further evaluated over three growing seasons demonstrating increased cold tolerance, delayed growth and altered dormancy phenology under field conditions (Artlip et al. 2014).

Horvath (2009) proposed a schematic model of how cold temperatures putatively mediate dormancy induction, suggesting that CBF transcription factors promote expression of *DAM* genes, possibly by chromatin remodeling (Horvath 2009). *DAM* genes are classified as belonging to the SVP/StMADS11 clade of MADS-box transcription factors, and due to protein sequence similarities, genes closely related to *DAM* are sometimes referred to as *SVP*-like genes. In *A. thaliana*, *SVP* is a *MADS-box* gene that regulates floral transition and contributes to the specification of floral meristems (Gregis et al. 2013). The *DAM* genes were first described in peach and presented distinct seasonal expression patterns (Bielenberg et al. 2008). From the six genes described, only *PpDAM5* and *PpDAM6* were regulated by cold exposure (Li et al. 2009). Moreover, the transcript accumulation pattern identified for these genes, e.g., induction during autumn and declining through the winter, suggests a growth repressing role (Li et al. 2009; Yamane et al. 2011). Additionally, it was recently shown that the silencing of *PpDAM6* is preceded by changes in the methylation status of H3K27 residues of histones bound to its chromatin (Leida et al. 2012), as well as occurs in the silencing of *FLC* and other genes that regulate vernalization

in *A. thaliana* (Angel et al. 2011). Although putative *DAM* orthologues were identified in pear (Ubi et al. 2010; Saito et al. 2013), apple (Falavigna et al. 2014), raspberry (Mazzitelli et al. 2007), kiwifruit (Wu et al. 2012), leafy spurge (Horvath et al. 2010) and apricot (Sasaki et al. 2011), a complete functional characterization of *DAM* genes remains to be reported.

Among efforts made to elucidate the involvement of *DAM* genes in bud dormancy and flowering, Horvath et al. (2010) reported that the overexpression of a leafy spurge *DAM* gene in *Arabidopsis* delayed flowering, as was also observed in *SVP* overexpressing lines (Gregis et al. 2013). Furthermore, Horvath et al. (2010) demonstrated that *DAM* genes are preferentially expressed in response to cold temperatures, causing a negative-regulation of *FT* or *FT*-like genes, leading to growth cessation and dormancy entrance. Interestingly, Sasaki et al. (2011) reported that overexpressing *PmDAM6* in poplar resulted in variable *FT* transcript levels, induction of growth cessation and precocious bud formation. On the other hand, Bai et al. (2013) found no correlation between the expression patterns of *DAM* and *FT* genes in pear, suggesting that both genes are not regulated in the Rosaceae family in the same manner as in leafy spurge. Finally, in the perennial kiwifruit, *SVP*-like genes were identified and functionally characterized in *Arabidopsis*. Out of four genes (*SVP1*, *SVP2*, *SVP3* and *SVP4*), only *SVP3* was able to rescue the flowering phenotype in *Arabidopsis svp* mutant lines (Wu et al. 2012). Distinct roles were therefore suggested for kiwifruit *SVP*-like genes in bud dormancy and flowering. Paradoxically, a report from the same authors showed that the ectopic expression of *SVP3* in kiwifruit and tobacco did not have any effect on growth and dormancy (Wu et al. 2014).

Several models have been devised for the regulation of dormancy induction and release. For example, Horvath (2009) proposed a model for bud dormancy induction where *DAM*, *FT* and a *FT*-like gene named CENTRORADIALIS (*CENL*) play key roles. The *DAM* genes would be induced after a short exposure to cold, probably through the action of CBF and chromatin remodeling mechanisms, as well as by the short-day photoperiod output from the circadian clock mediated by PHYA. Once induced, the DAM transcription factors would repress *FT/CENL*, causing growth cessation and dormancy induction. After long-term cold exposure, likely via chromatin modification, the down-regulation of *DAM* genes occurs leading to dormancy release. Similarly, Jiménez et al. (2010) proposed a simple conceptual model to explain the putative roles of DAM5/DAM6 in the endodormancy-to-ecodormancy transition. According to this model, the expression of *DAM5* and *DAM6* is triggered by short photoperiods. On the other hand, chilling exposure disrupts the circadian perception of photoperiodic stimuli, resulting in repression of *DAM5* and *DAM6*, and allowing the expression of the genes required for growth under permissive environmental conditions. Finally, Campoy et al. (2011) proposed a similar model integrating all this information combined with data generated studying dormancy in other species, such as chestnut and hybrid aspen.

An elegant mechanism to explain dormancy cycling was proposed by Rinne et al. (2001) based on low-temperature mediated enhancement of 1-3-ß-D-glucanases production. Removal of 1-3-ß-glucan from the plasmodesmata restores the symplasmic

communication network, leading to chilling-induced release from dormancy by the assumption of a proliferation-competent state. The same authors identified three groups of genes, members of *GLUCAN HYDROLASE 17* family (*GH17*), that are upregulated by chilling temperatures and GA biosynthesis in *Populus*. The group 1 *GH17* genes are transiently upregulated by short-term photoperiodic exposure in order to maintain the symplasmic paths to facilitate bud formation. On the other hand, group 2 and 3 *GH17* genes are upregulated by GA_3 and long-term chilling exposure, allowing callose removal and, thereby, enabling reopening of signaling conduits for *FT* transport to the apex. After sufficient chilling, growth-related genes are upregulated by elevated temperatures, mediated by GA_4, leading to bud burst (van der Schoot and Rinne 2011; Rinne et al. 2011). The models proposed for peach and poplar helped to better understand the dormancy processes in perennial trees; however they rely on advances made on species for which photoperiodic changes are the main inductor of bud dormancy (Horvath 2009; Campoy et al. 2011; van der Schoot and Rinne 2011; Rinne et al. 2011). Thus, the major findings related to dormancy progression in pipfruits are often neglected and therefore need to be better addressed.

Bud Dormancy in Pipfruits

Pipfruits differ from other plant models used to study bud dormancy, such as peach and poplar, at the physiological level because the most important environmental trigger for dormancy induction is low-temperature exposure (Heide and Prestrud 2005), instead of photoperiodic changes. Thus, it can be expected that different molecular pathways are being influenced during dormancy entrance in pipfruits. In this sense, several studies have been conducted to identify similarities as well as peculiarities of this process in apples and pears.

Pioneering work has been performed in apple exploring the contrasting phenotypes between ‘Gala’ and its spontaneous mutation ‘Castel Gala’. This last cultivar requires only 50% of the CR for dormancy release in comparison with the original cultivar, resulting in earlier bud break. Using suppression subtractive hybridization as a gene discovery tool and RT-qPCR for validation, Falavigna et al. (2014) identified 17 candidate genes, with transcripts coding for DAM, dehydrins, GAST1, LTI65, NAC, HTA8, HTA12 and RAP2.12-like proteins presenting major differences in gene expression between cultivars through the winter. One of the most noteworthy results was the transcriptional profile obtained for a *DAM*-like gene, whose expression was very similar to peach *PpDAM5* and *PpDAM6* genes (Li et al. 2009; Yamane et al. 2011). In an independent approach, Porto et al. (2015) carried out a transcriptomic assay aiming to analyze changes in apple gene (~57,000) expression in response to chilling accumulation in the field and under controlled conditions using a microarray chip. Cold exposure mainly repressed the expression of transcripts related to photosynthesis, whereas long-term cold exposure repressed flavonoid biosynthesis genes. These results indicate that photosynthesis and

auxin transport are major regulatory nodes of apple dormancy and unveil strong candidates for the control of bud dormancy. Genes related to the circadian clock, hormonal signaling, and regulation of growth and flower development were annotated, including the *MdFT1* gene. Interestingly, apple trees overexpressing *MdFT1* displayed early flowering despite a lack of any chilling exposure (Tränkner et al. 2010). Several studies overexpressing *FT* homologous genes in apple reported precocious flowering (Kotoda et al. 2010; Flachowsky et al. 2012; Wenzel et al. 2013), but the authors have not addressed its effects of dormancy process. These findings suggest the existence of common pathways (e.g., DAM family, *FT* homologs and hormone signaling) in the regulation of dormancy progression in apple in comparison with other better characterized species, such as peach and poplar. However, the identification of new pathways whose relationships to dormancy still need to be unveiled remains a possibility.

The availability of the pear genome sequence will likely become a very important tool to improve the genomics of many agronomic traits, including bud dormancy (Chagné et al. 2014; Wu et al. 2013). In fact, despite this advance, several efforts were performed trying to discover the molecular mechanisms underlying bud dormancy progression in pear. Two remarkable and independent pear transcriptomes were generated using RNA-seq to explore endo- and eco-dormant flower buds (Liu et al. 2012; Bai et al. 2013). Interestingly, both studies identified pathways already related to dormancy in other species, but also reported, for the first time, other dormancy-related pathways, such as endocytosis, glycerophospholipid metabolism, and biosynthesis of phenylpropanoids, stilbenoids, diarylheptanoids, gingerols and ether lipids. These data, along with those reported in apple (Falavigna et al. 2014; Porto et al. 2015), suggest that we are far from fully understanding bud dormancy in pipfruits and new research approaches must be explored.

Additionally, besides the whole RNA-seq data generated by Liu et al. (2012) and Bai et al. (2013), both authors presented transcript accumulation patterns for *DAM* genes and their results coincided with the first findings reported for this gene family in pear (Ubi et al. 2010; Saito et al. 2013). Two putative *DAM* genes were identified (namely *PpMADS13–1* and *PpMADS13–2*) and their expression pattern was analyzed by RT-qPCR during dormancy. They showed that both genes are gradually down-regulated concomitantly with endodormancy release (Ubi et al. 2010). After that, a third *DAM* gene was also isolated (*PpMADS13–3*), and its transcript levels showed a decrease near and after endodormancy release (Saito et al. 2013).

Two additional reports also investigated dormancy regulation in pear. Nishitami et al. (2012) identified two putatively novel dormancy-related transcription factors, NAC2 and PRR5, using a microarray chip to study the transition from endodormancy to ecodormancy in pear buds. Both genes displayed a sharp increase in the transcript accumulation levels during the end of endodormancy until ecodormancy. Likewise, Takemura et al. (2013) identified several genes that may play a role in regulating endodormancy release, highlighting the transcriptional profile obtained for clone 245 (*Auxin influx carrier component*), which was induced near and after bud break.

Another approach to investigate bud dormancy in pear was the characterization of carbohydrate metabolism. Marafon et al. (2011) demonstrated that the exposure of branches to cold temperatures affects starch and soluble sugar contents in wood and bud tissues of Japanese pears. Sufficient chilling supply during winter increased the activities of cell wall acid invertase and sucrose-6-phosphate synthase, yielding increased levels of reducing sugars and starch contents in bud tissues that are then used for budburst and blooming in spring (Marafon et al. 2011). Additionally, another study showed that endodormancy release occurred concomitantly with the accumulation of sorbitol in xylem sap, and the increase of sorbitol influx and catabolism in flower buds occurred only after bud break (Ito et al. 2012). Finally, trying to elucidate which physiological events were involved in the seasonal changes of carbohydrate dynamics during winter, the results found by Ito et al. (2013) suggest that carbohydrates in the shoot tissues may be converted to sorbitol and loaded into xylem sap. Therefore, sorbitol accumulation patterns could be synchronized with the progression of dormancy, whereas the total carbohydrate transported into shoots from other storage organs may be related to freezing tolerance acquisition rather than dormancy progression (Ito et al. 2013).

A groundbreaking discovery by Mason et al. (2014) uncovered fundamental roles of sugar signaling in bud dormancy. According to their report, lateral dormant buds under the effect of apical dominance, which is a form of paradormancy, resume growth upon receiving an extra amount of sugar supply. Sugar surplus in the phloem is a direct consequence of shoot decapitation, and this signal is much faster than auxin depletion across the stem. Lateral bud outgrowth induced by sugars is independent from auxin signaling, long regarded as the main regulator of apical dominance. This new and exciting evidence indicates that carbohydrate metabolism will probably have an increasing importance in studies involving bud dormancy progression in perennial species.

Concluding Remarks

Bud dormancy, especially dormancy release, remains one of the less understood processes in plant biology. This delay in relation to other well-characterized plant phenomena can be due to methodological issues inherent to the study of dormancy itself, as it is one of the most hermetic subjects at the experimental point of view. However, current approaches available in the fields of plant physiology and molecular biology may provide significant advances in the genetics and genomics of this trait. New technologies, such as high throughput data generation and functional analysis in heterologous systems, hold promise for unraveling the inner circuits of dormancy regulation. At the moment, quantitative genetics and comparative genomics seem to be the most fruitful paths toward the identification of components of dormancy regulation. Functional characterization of these components in their original species background is the next challenge, which can reveal how independently described nodes assemble into a full regulatory mechanism.

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4 CAPÍTULO II

Functional diversification of the dehydrin gene family in apple and its contribution to cold acclimation during dormancy

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Functional diversification of the dehydrin gene family in apple and its contribution to cold acclimation during dormancy

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Dehydrins (DHN) are proteins involved in plant adaptive responses to abiotic stresses, mainly dehydration. Several studies in perennial crops have linked bud dormancy progression, a process characterized by the inability to initiate growth from meristems under favorable conditions, with *DHN* gene expression. However, an in-depth characterization of DHNs during bud dormancy progression is still missing. An extensive in silico characterization of the apple *DHN* gene family was performed. Additionally, we used five different experiments that generated samples with different dormancy status, including genotypes with contrasting dormancy traits, to analyze how *DHN* genes are being regulated during bud dormancy progression in apple by real-time quantitative polymerase chain reaction (RT-qPCR). Duplication events took place in the diversification of apple *DHN* family. Additionally, *MdDHN* genes presented tissue- and bud dormant-specific expression patterns. Our results indicate that *MdDHN* genes are highly divergent in function, with overlapping levels, and that their expressions are fine-tuned by the environment during the dormancy process in apple.

Introduction

Dormancy in plants has been defined as the growth inability of meristems under favorable conditions (Rohde and Bhalerao 2007). Some perennial trees from temperate climates, such as apple (*Malus* × *domestica* Borkh.), display all three stages of bud dormancy progression: paradormancy, endodormancy and ecodormancy (Faust et al. 1997). In paradormancy, bud growth is inhibited due to signals produced in distal parts of the plant. Endodormancy, whose signal constraint to growth is within each bud, is triggered by low temperatures (LT)

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Abbreviations – ARC5, accumulation and replication of chloroplast 5; CBF, C-repeat binding factor/DREB1; CH, chilling hours; CR, chilling requirement; DHN, dehydrin; LEA, late embryogenesis abundant; LT, low temperature; MBP, maximum budbreak percentage; MDH, malate dehydrogenase; RACE, rapid amplification of cDNA ends; RT-qPCR, real-time quantitative polymerase chain reaction; TMp1, type 1 membrane protein-like; WD40, transcription factor WD40-like repeat domain; WGD, whole-genome duplication.

and short days during autumn and winter (Horvath 2009). In order to overcome endodormancy, plants require a prolonged LT exposure to fulfill genotype-specific chilling requirements (CR) in order to promote the transition to the ecodormant state (Rohde and Bhalerao 2007). In ecodormancy, growth is temporarily inhibited by unfavorable environmental conditions, which is restored when plants are able to resume growth (Horvath 2009).

Dehydrins (DHNs) are a class of polypeptides that belong to group II of late embryogenesis abundant (LEA) proteins and are characterized by the presence of three characteristic motifs (K-, S- and Y-segments). These motifs are involved in responses to changes in water availability, mainly caused by abiotic stresses. Although many possible functions were reported for DHNs, their precise mechanisms of action remain unknown (reviewed in Rorat 2006, Hanin et al. 2011, Graether and Boddington 2014).

It has been suggested that the reduction of free water content in apple dormant buds coincides with DHN protein accumulation during winter (Faust et al. 1997). Indeed, several reports identified seasonal accumulation patterns of *DHN*s during winter in perennial plants (Wisniewski et al. 1996, Welling et al. 2004, Yamane et al. 2006, Yakovlev et al. 2008, Garcia-Bañuelos et al. 2009). In an attempt to compare low and high CR for apple cultivars, our group has recently reported a seasonal transcript accumulation pattern for *MdDHN2*, *MdDHN4* and *MdDHN6* in dormant buds (Falavigna et al. 2014). These cultivars displayed elevated steady-state mRNA levels during winter in comparison to samples harvested in spring and summer. However, the apple cultivar with low CR showed an early decline of transcript levels in the end of winter compared to high CR cultivars, prior to growth resumption. Additionally, nine apple *DHN*s (*MdDHN1–9*) were characterized according to their transcription levels in different organs and under drought, LT and abscisic acid treatments, but their relationship with the bud dormancy process was not assessed (Liang et al. 2012).

In the present work, we investigate the expression of *MdDHN*s within the context of bud dormancy progression and in selected growth developmental stages. The expression of all *MdDHN* genes was analyzed by real-time polymerase chain reaction (RT-qPCR) using samples representing dormancy, and from flowering to fruit ripening stages, including seeds. These samples provided an important resource to analyze how gene expression is being regulated during dormancy in apple. We identified five *MdDHN*s regulated during the dormancy process, likely through the CBF (C-repeat binding factor/DREB1) pathway, which may contribute to the bud tolerance to cold during dormancy.

Materials and methods

Sampling strategy and controlled LT treatments

Plant material was obtained from three apple orchards located in the cities of Vacaria, RS (−28.513777, −50.881465 and 972 m altitude), Caçador, SC (−26.836971, −50.975246 and 935 m altitude) and Papanduva, SC (−26.434870, −50.106103 and 788 m altitude), in Southern Brazil. All apple trees were in adult stage and underwent standard orchard management practices.

The experimental orchard in Vacaria consisted of three blocks with 10 'Gala Baigent' apple trees (3-year-old) each. The Gala Baigent trees were grafted on Marubakaido rootstocks with M.9 as interstock. Six apple developmental stages were partitioned in different tissues and organs resulting in 13 samples. The sample stages were defined according to the Fleckinger scale (EPPO 1984): closed terminal buds (A stage); buds at initial bursting (C stage); flower buds and young leaves (E2 stage); whole set-fruits approximately 10 mm in diameter and leaves (I stage); mature leaves and unripe fruits approximately 40 mm in diameter which were divided into pulp, seed and peel (J stage). Additionally, we sampled mature fruits approximately 70 mm in diameter partitioning them into pulp, seed and peel (named M). Samples were immediately frozen in liquid nitrogen in the field and stored at −80°C. Sampling dates and images of the developmental stages selected are presented in Table S1, Supporting Information.

The orchard in Caçador consisted of 7-year-old 'Fuji Standard' plants grafted on M.7 rootstocks. Three biological replicates consisted of pooled samples from four trees each. Forty closed terminal buds from each plant were harvested at eight time points from January 2009 to February 2010. Samples were immediately frozen in liquid nitrogen in the field and stored at −80°C until use. Sampling dates and chilling hours (CH, number of hours below 7.2°C) accumulated by these samples are presented in Table S2.

Samples from Papanduva were taken from three blocks per cultivar (Castel Gala and Royal Gala), each block containing 20 plants. The selection of these genotypes was based on the contrasting CR of 'Royal Gala' (600 CH) in relation to its natural bud sport 'Castel Gala' (300 CH). The new cultivar has a precocious cycle usually starting growth a month earlier, being otherwise identical to the original cultivar (Denardi and Seccon 2005, Anzanello et al. 2014a). 'Castel Gala' plants were grafted on M.9

rootstocks in 2006. 'Royal Gala' plants were grafted on Marubakaido rootstocks with M.9 as interstock in 2003. Sampled twigs (20 cm long) were disinfected with ethanol 70% for 45–60 s and sodium hypochlorite 2.5% for 20 m, rinsed, air-dried and wrapped in black plastic bags. Treatments were performed by placing the bags inside growth chambers in the dark with the terminal bud upwards. Maximum budbreak percentages (MBP) of 40 twigs per treatment were determined as described by Anzanello et al. (2014b). Briefly, twigs were cut at the basis and fixed in floral foam inside a growth chamber under forcing conditions (25 ± 1.5°C, 12 h photoperiod and 70% relative humidity). MBP was calculated by the total number of terminal buds showing green tips divided by the total number of viable terminal buds after 56 days in the growth chamber. These procedures were repeated on all twigs subjected to controlled temperature conditions (Fig. 6 and Figs S4 and S5). On April 5, 2011, a total of 420 twigs were sampled from each cultivar without CH exposure before that date. Twigs were exposed to a daily cycle of 12 h at 3°C and 12 h at 15°C until they reached 0, 24, 48, 96, 336 and 600 CH for 'Royal Gala' or 0, 24, 48, 96, 240 and 408 CH for 'Castel Gala'. At each point, MBP was determined and 30 additional closed terminal buds were frozen in liquid nitrogen and stored at −80°C. Additionally, two experiments under controlled LT conditions, performed with samples of the same experimental area, are described in Figs S4 and S5.

In silico analysis of *MdDHN* genes and deduced amino acid sequences

To identify predicted gene models coding for DHNs in the apple genome version 1.0 (http://rosaceae.org/; Velasco et al. 2010), we performed BLASTP searches using the conserved K-segment as query (Altschul et al. 1990). All hits obtained had their sequences annotated by comparison with the NCBI non-redundant protein database using the BLAST2GO software with an *E*-value cutoff of $1e^{-6}$ (Conesa et al. 2005).

Deduced amino acid sequences of 12 MdDHNs were used for searching conserved domains using MEME Suite v.4.9.0 (Bailey and Elkan 1994). Default parameters were used, except motif distribution among sequences was set to any number of repetitions, maximum number of motifs was set to 5 and maximum motif width was defined between 6 and 16 amino acids.

Full-length protein sequences of DHNs from *Arabidopsis thaliana*, *Hordeum vulgare*, *Glycine max*, *Malus* × *domestica*, *Oryza sativa*, *Populus trichocarpa*, *Prunus mume* and *Vitis vinifera* were aligned using ClustalW (Higgins et al. 1994). References and accession numbers of all sequences used are presented in Table S3. The phylogenetic tree was inferred using MRBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001) employing the mixed amino acid substitution model in default settings. Four million generations were run, sampled every 100 generations and the first 25% trees were discarded as burn-in. The remaining ones were summarized in a consensus tree, which was visualized and edited using FIGTREE v.1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Collinear block analysis was performed by comparing the whole-genome protein sequences from apple against themselves and against those from *P. mume* using BLASTP (*E*-value < $1e^{-10}$, top five matches). The results and gene positions were used as inputs to determine the collinear blocks using MCSCANX (Wang et al. 2012).

Nucleic acid extraction and cDNA synthesis

Approximately 200 mg of frozen plant material was used for nucleic acid isolation. DNA was purified from mature leaves of 'Gala Baigent' trees according to Lodhi et al. (1994) and Lefort and Douglas (1999) modified protocols adapted to 2 ml tubes. Total RNA of each sampled material was isolated as described in Falavigna et al. (2014) and DNase-treated using TURBO DNA-free Kit (Ambion, Austin, TX). Complementary DNA was synthesized using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. DNA-free RNA samples from mature seeds were also employed in 5′ and 3′ rapid amplification of cDNA ends (RACE) for *MdDHN10* and *MdDHN11* cDNA synthesis using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. RACE products were sequenced at ACTGene Ltd. (Porto Alegre, Brazil) using an automatic ABI-PRISM 3100 Genetic Analyzer and associated chemistry (Applied Biosystems).

Real-time quantitative polymerase chain reaction

Gene-specific primers were designed using the PRIMER3 v0.4.0 software (Table S4; Rozen and Skaletsky 2000). RT-qPCR was performed as described in Falavigna et al. (2014). Biological samples (n = 3) were analyzed in four technical replicates. PCR efficiency was calculated using LINREGPCR v.2012.0 (Ruijter et al. 2009). Mean relative gene expression was calculated by the Pfaffl method (2001). Reference genes used for organ and tissue samples were *MDH* (malate dehydrogenase), *TMp1* (type 1 membrane protein-like) and *WD40* (transcription factor WD40-like repeat domain), whereas *ARC5* (accumulation and replication of chloroplast 5), *MDH* and *WD40*

were employed as reference genes for closed terminal buds as described by Perini et al. (2014).

Results

Identification and classification of *MdDHN* gene family

In order to identify genes coding for MdDHNs, a BLASTP search was performed in the apple genome (http://rosaceae.org/) using the consensus sequence of the K-segment, a motif ubiquitously present in the DHN family of proteins in plants (Rorat 2006). We identified 16 predicted gene models and their deduced peptide sequences were annotated using the BLAST2GO software. The gene sequence represented by the accession number MDP0000156140 was excluded based on low similarity to LEA proteins. Multiple alignments of the remaining 15 sequences revealed that MDP0000126135 and MDP0000770493 were identical and four gene models seemed to be artifacts of the genome assembly. MDP0000595270 and MDP0000595271 were predicted in the genome contig MDC011430.191, whereas MDP0000868044 and MDP0000868045 were derived from contig MDC016760.214. The gene model MDP0000595271 exhibited a predicted start codon within the intron of MDP0000868045 and the remaining sequences were identical. Moreover, MDP0000595270 and MDP0000868044 were identical in their 3′ portion. These two results suggested that one of these two contigs was misplaced in the genome assembly. To test this hypothesis, we performed a PCR spanning a common region of both contigs. The analysis showed that only the amplicon from the contig MDC016760.214 was amplified. Sequencing of the amplicon confirmed the identity of the fragment, supporting the idea that only contig MDC016760.214 is properly positioned within apple chromosome 2. We, therefore, excluded MDP0000126135, MDP0000156140, MDP0000595270 and MDP0000595271 from further analyses.

Our genome-wide survey identified nine genes (*MdDHN1–9*) previously identified by Liang et al. (2012), and three additional family members, named *MdDHN10–12* (Fig. 1). *MdDHN10* and *MdDHN11* were amplified by RACE and sequenced (GenBank KF578380 and KF578381). The obtained sequence for

Gene Name	Genome Accession Code	GenBank Accession Code	Chromosomal Localization	Type	Motif Distribution
MdDHN1	MDP0000868045/ MDP0000595271	JQ649456	chr2:12477545..12478867/ chr2:8259119..8259842	Y_2SK_3	
MdDHN2	MDP0000698024	JQ649457	chr2:12484455..12485624	Y_2SK_3	
MdDHN3	MDP0000689622	JQ649458	chr2:12500269..12500520	K_4	
MdDHN4	MDP0000360414	JQ649459	chr2:12500767..12501879	Y_2SK_3	
MdDHN5	MDP0000862169	JQ649460	chr2:12503902..12504672	YK_4	
MdDHN6	MDP0000265874	JQ649461	chr2:12701912..12702484	YK_3	
MdDHN7	MDP0000196703	JQ649462	chr2:12736190..12736886	K_3	
MdDHN8	MDP0000529003	JQ649463	chr12:20761988..20764699	SK_3	
MdDHN9	MDP0000770493/ MDP0000126135	JQ649464	chr15:6960886..6962054/ chr15:6977891..6979059	SK_3	
MdDHN10	MDP0000868044/ MDP0000595270	KF578380	chr2:12470746..12472298/ chr2:8255278..8257253	Y_3SK_3	
MdDHN11	MDP0000629961	KF578381	chr10:10172519..10178352	Y_3SK_2	
MdDHN12	MDP0000178973	-	chr9:21206400..21207388	K_6	

Fig. 1. Identification and classification of apple *DHN* genes. Names were attributed following Liang et al. (2012). Genome and GenBank accession codes are provided by the '*Malus domestica* Genome' (http://rosaceae.org/) and NCBI (http://www.ncbi.nlm.nih.gov/) databases, respectively. Chromosomal localization and DHN classification are shown. Schematic view of the conserved motifs between deduced protein sequences of MdDHNs performed by MEME (Bailey and Elkan 1994) is shown. Each colored box represents a conserved motif: cyan blue represents K-segments; red represents S-segments; pink represents Y-segments; dark blue and yellow represent putative novel motifs. The height of the motif box is proportional to –log (*P* value), with the maximum height composed by a *P* value of $1e^{-10}$. Gray lines represent non-conserved sequences. See Fig. S2 for individual motif details. GenBank accession codes for *MdDHN1–9* were provided by Liang et al. (2012).

MdDHN10 was identical to the model predicted in the apple genome. However, alignments for *MdDHN11* revealed that, out of four predicted exons in the genome, only the second and the third are truly transcribed (Fig. S1). No transcript for *MdDHN12* was detected in our RACE attempts (see section 'MdDHN transcript levels in apple tissues and organs').

Predicted amino acid sequences for all 12 MdDHNs were screened for conserved domains using the MEME software. The analysis identified five significantly conserved motifs (Fig. 1), including those classified as K-segment (Fig. S2A), S-segment (Fig. S2C) and Y-segment (Fig. S2D). Two putative DHN conserved motifs (Fig. S2B, E) were additionally found and considered as novel because no matches with known domains in Pfam (Punta et al. 2012), PROSITE (Sigrist et al. 2013) and SMART (Letunic et al. 2012) databases were found. As a result, we assigned the 12 MdDHNs into four of five DHN subclasses (Hanin et al. 2011). The subgroups Y_nSK_n and K_n were the most represented with five and three members, respectively. Subgroups SK_n and YK_n displayed two members each, whereas no MdDHN was assigned to the K_nS subclass (Fig. 1).

Evolutionary relationships of *MdDHN*s

Deduced peptide sequences from all *MdDHN*s and those from seven other plant species (Table S3) whose DHN family members have been previously described were used in a phylogenetic analysis employing MRBAYES (Fig. 2). The resulting tree topology indicated that the majority of the proteins could be grouped into six major clusters of orthologous and paralogous proteins (A–F), with apple members distributed into four of them (groups A, C, D and F). Groups A and B were mainly composed of Y_nSK_n subclass, group E with K_n and K_nS subclasses and group F with SK_n subclass. Interestingly, groups C and D comprised all *MdDHN*s from chromosome 2 together with their counterparts in *P. mume* and *G. max*. Additionally, we identified putative orthologs between apple and *P. mume*, such as MdDHN11/PmLEA20 and MdDHN12/PmLEA19. The remaining MdDHNs grouped as paralogs. Only At3g50970, HvDHN5, PtDHN10 and VvDHN1 did not group with other members in the tree.

In order to infer the evolutionary history of the *DHN* genes in the Rosaceae family, a synteny study was performed between the genomes of apple and *P. mume* using the MCSCANX software. This algorithm is able

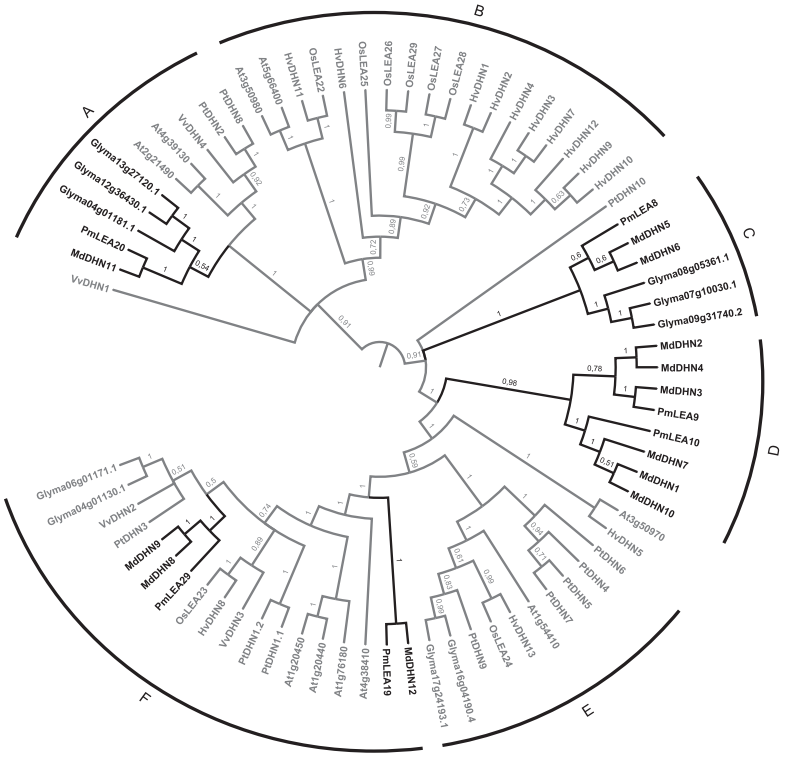

Fig. 2. Phylogenetic relationships for full-length amino acid sequences of DHNs from *Arabidopsis thaliana*, *Hordeum vulgare*, *Glycine max*, *Malus* × *domestica*, *Oryza sativa*, *Populus trichocarpa*, *Prunus mume* and *Vitis vinifera*. The six groups (A–F) of orthologous and paralogous proteins identified are shown. The tree was inferred using MRBAYES v.3.1.2 (Huelsenbeck and Ronquist 2001). Nodal support is given by a posteriori probability values shown next to the corresponding nodes (when >0.50). All accession codes used in the phylogenetic analysis are depicted in Table S3.

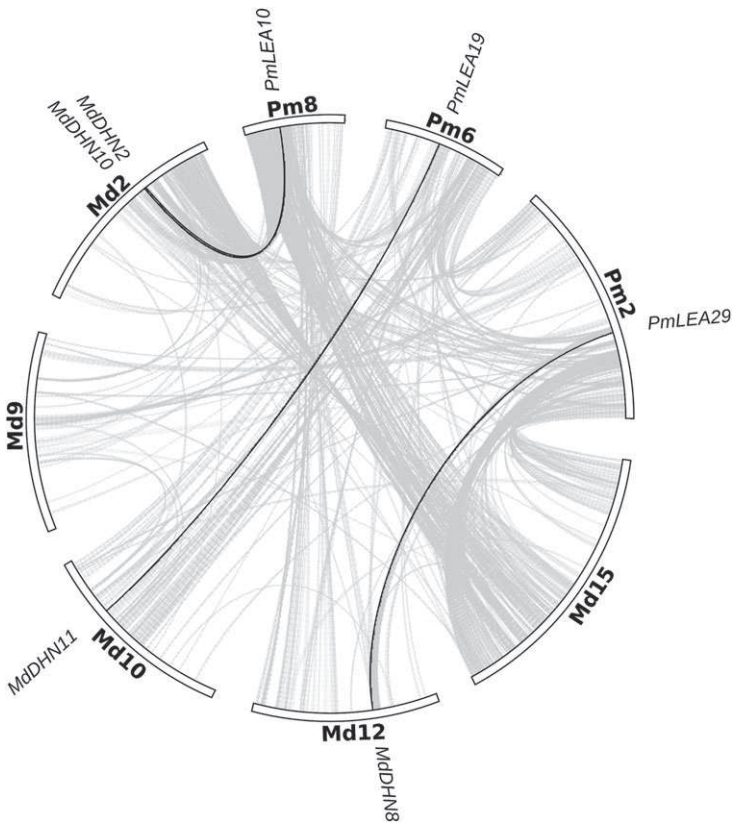

Fig. 3. Synteny analysis of *DHN* genes from apple and *Prunus mume*. Chromosomes containing apple (Md2, 9, 10, 12 and 15) and *Prunus mume* (Pm2, 6 and 8) *DHN* genes are depicted. Lines connecting two chromosomal regions indicate collinearity relationships with (black) or without (gray) the involvement of *DHN* genes.

to scan genomes recognizing putative homologous chromosomal regions using genes as anchors (Wang et al. 2012). This analysis identified many collinear regions between the genomes (gray lines in Fig. 3), with four collinear blocks containing *DHN*s (black lines in Fig. 3). Of these, two were between chromosome 8 from *P. mume* and chromosome 2 from apple (*PmLEA10*/*MdDHN2* and *PmLEA10*/*MdDHN10*), one was between chromosome 6 from *P. mume* and chromosome 10 from apple (*PmLEA19*/*MdDHN11*) and one was found between chromosome 2 from *P. mume* and chromosome 12 from apple (*PmLEA29*/*MdDHN8*). In summary, these results indicate the presence of conserved genomic regions containing *DHN*s of apple and *P. mume*, suggesting that genome duplication events likely played important roles in the expansion of this gene family in Rosaceae species.

MdDHN transcript levels in different tissues and organs

The transcript accumulation of *MdDHN*s was investigated by RT-qPCR in 13 different organs and tissues. Tissue/organ sampling dates and developmental stages are presented in Table S1. Four different patterns of expression were identified (Fig. 4). *MdDHN1* and *MdDHN11* were expressed mainly in seeds of unripe and ripe fruits, with very low relative levels of transcripts in other organs or tissues analyzed. While *MdDHN1* exhibited more than 120-fold higher transcript accumulation in mature seeds compared to closed terminal buds, strikingly, *MdDHN11* expression was about 6000-fold higher in mature seeds relative to closed terminal buds. *MdDHN2*, *MdDHN3* and *MdDHN4* were expressed mainly in closed terminal buds, with some expression in pulp and peel of mature fruits. Additionally, *MdDHN2* showed low level of expression in mature seeds in comparison to closed terminal buds. *MdDHN5* and *MdDHN6* presented similar transcript accumulation in closed terminal buds and mature seeds. *MdDHN7*, *MdDHN8*, *MdDHN9* and *MdDHN10* were detected in nearly all tissues and organs analyzed. While *MdDHN7* presented higher transcript amounts in young leaves and seeds, *MdDHN8* and *MdDHN9* showed higher transcript accumulation in pulp and peel

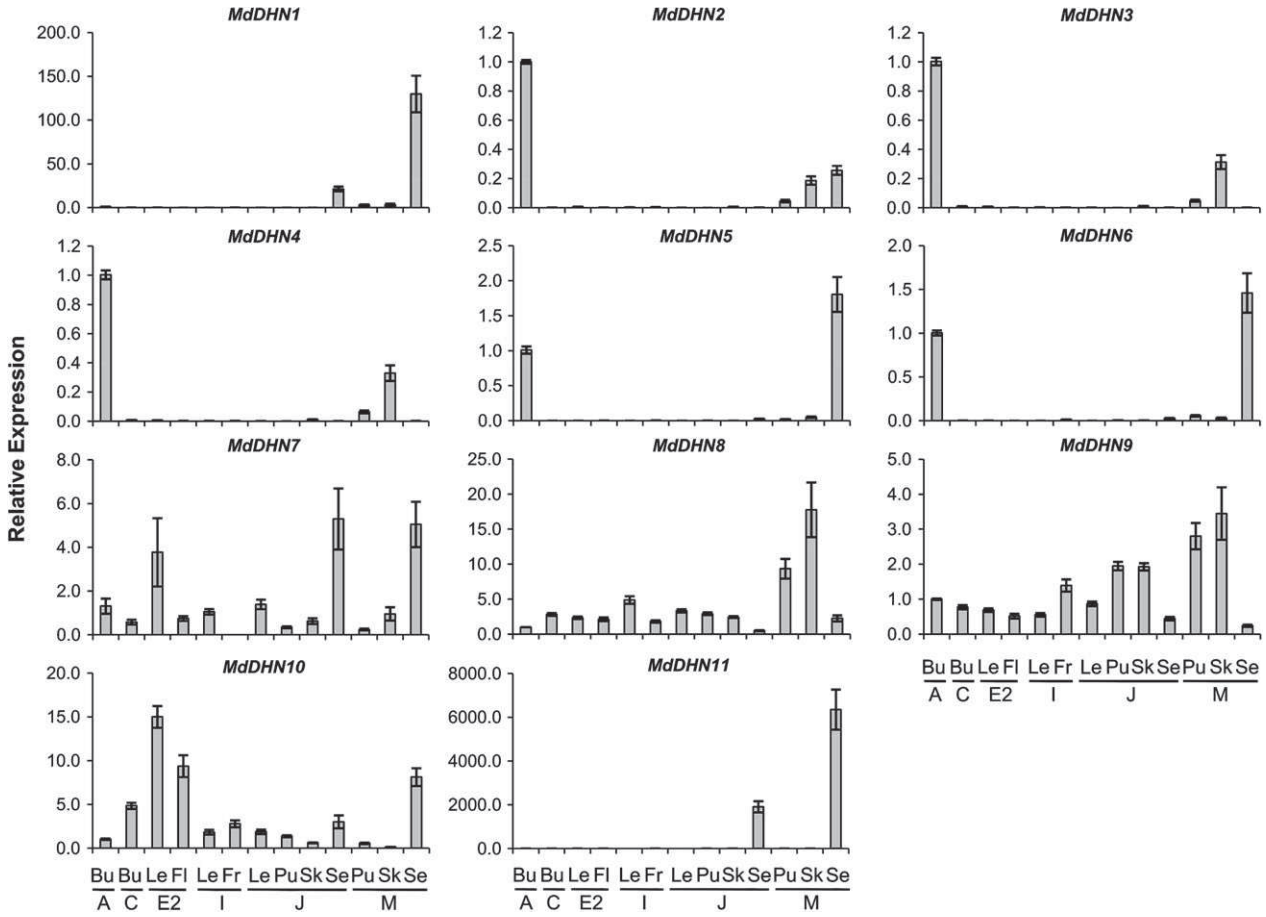

Fig. 4. Mean relative expression levels in 13 different apple tissues and organs. Stages A, C, E2, I and J were sampled according to the Fleckinger phenological scale (EPPO 1984). 'M' stands for mature fruits. Sampling dates and images of the developmental stages considered are presented in Table S1. Bu, bud; Le, leaf; Fl, flower; Fr, fruit; Pu, fruit pulp; Sk, fruit peel and Se, seed. Relative transcript levels in closed terminal buds (Bu/A) were set to 1. Standard error bars are shown.

of mature fruits and *MdDHN10* was detected mainly in young flowers and young leaves. Finally, *MdDHN12* transcripts were not detected in any of the tissues or organs analyzed under our conditions. We nevertheless confirmed the presence of the *MdDHN12* sequence in the apple genome by PCR amplification from genomic DNA (Fig. S3) and by sequencing of the amplicon (data not shown).

Transcript accumulation of *MdDHN* genes through the annual growth cycle

We analyzed the transcript accumulation for *MdDHN1–11* by RT-qPCR in closed terminal buds sampled from the high CR cultivar Fuji Standard during a complete growing cycle from January 2009 to February 2010 (Fig. 5). We carefully monitored chilling exposure (Table S2), growth cessation and the onset of growth resumption (50% of buds in green tip stage) as markers for dormancy establishment and completion. Because budbreak occurred on September 15, 2009, which corresponds to the end of winter in Southern Brazil (September 21 equinox), we therefore considered samples from May 27 (growth cessation) to September 9, 2009 (near growth resumption) as representing the complete bud dormancy progression.

The *MdDHN* gene family displayed a clear seasonal pattern of transcript accumulation (Fig. 5). The expression timing of *MdDHN1*, *MdDHN9* and *MdDHN10* coincides most closely with the growth resumption, with a gradual transcript decline during spring and summer. *MdDHN2*, *MdDHN3*, *MdDHN4*, *MdDHN5*, *MdDHN6*, *MdDHN8* and *MdDHN11* showed a noticeable peak of transcript accumulation during winter and their expressions drastically decreased near budbreak, maintaining low levels through spring and summer. *MdDHN2* and *MdDHN4* showed the highest levels of transcriptional induction, and *MdDHN3*, *MdDHN4*,

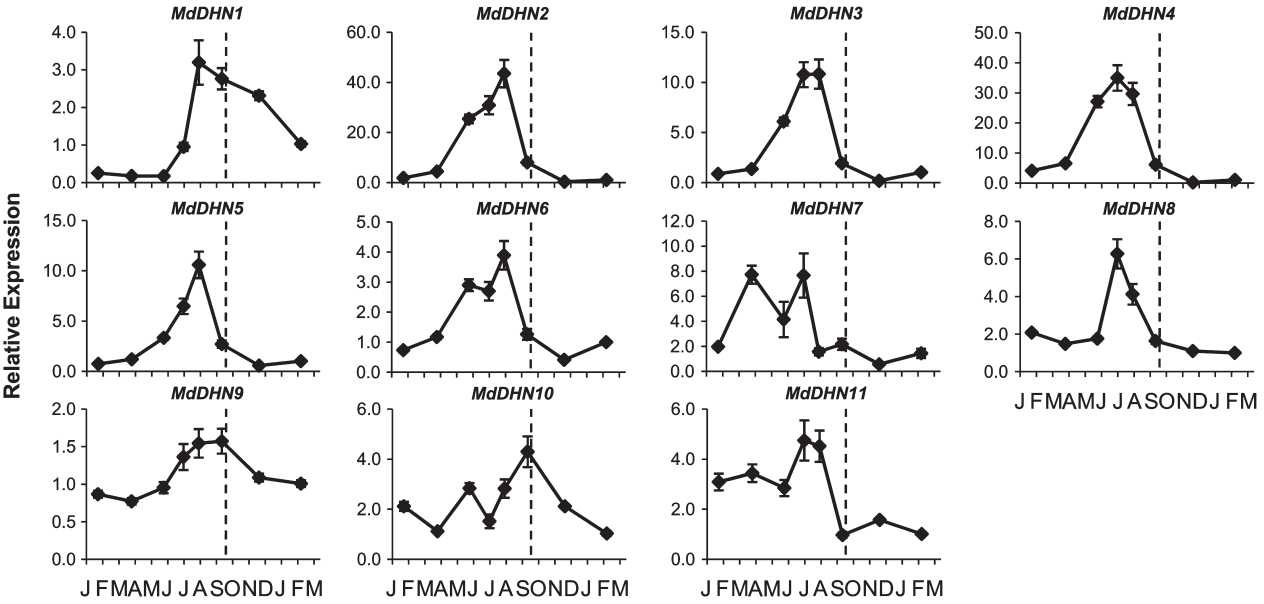

Fig. 5. Kinetics of the relative transcript levels for *MdDHN1–11* during 2009/2010. RT-qPCRs were performed with RNA samples isolated from closed terminal buds harvested from 'Fuji Standard' trees grown in Caçador (SC). Dashed lines correspond to budbreak date (50% of buds in green tip stage), which occurred on September 15, 2009 (end of winter). Months are represented by their initial letters on the x-axis. Relative transcript levels in February 2010 were set to 1. Standard error bars are shown. Graphics for *MdDHN2*, *MdDHN4* and *MdDHN6* genes were reproduced from Falavigna et al. (2014), with permission of the publisher.

MdDHN8 and *MdDHN11* presented their expression peaks occurring concomitantly with winter solstice (June 21). Finally, *MdDHN7* was the only member that presented an oscillatory pattern of expression in the beginning of autumn and winter combined with the same fast reduction to low levels of expression prior to growth resumption.

Gene expression of *MdDHN*s in samples with contrasting dormancy status

We performed three experiments to produce samples with contrasting MBP. Two experiments used contrasting CR cultivars with similar genetic backgrounds, 'Castel Gala' and 'Royal Gala'. The first experiment was performed in 2009, and the MBP analysis revealed that 'Castel Gala' (86.4%) showed a much higher percentage of budburst than 'Royal Gala' (7.7%), confirming the expected behavior of 'Castel Gala' (Fig. S4A). After 504 h of exposure to LT6 (6°C), both cultivars displayed similar MBP, which persisted until the end of the treatments. In the second experiment, both cultivars had the endodormancy process induced and released by treating sampled twigs with a daily cycle of 12 h at 3°C and 12 h at 15°C (Fig. 6A, B). After 96 CH, 'Royal Gala' decreased the MBP from 60.4 to 14.1%. The MBP almost doubled after 336 CH and, at the end of the treatment, 46.6% of budbreak was obtained. 'Castel Gala' twigs showed a decrease in MBP from 67.2 to 33.5% after 48 CH. After 240 CH, the growth competence almost returned to field samples levels (61%). At the end of the treatment, 83.5% of budbreak was obtained.

Despite the evident difference in MBP (Fig. 6A, B and Fig. S4A), similar transcriptional responses were identified between cultivars. *MdDHN2–6* showed a continuous increase in gene expression during the treatments, with *MdDHN2*, *MdDHN3* and *MdDHN5* presenting the highest transcript amounts (Fig. 6C, D). Despite the relative baseline expression, *MdDHN7–10* presented a peak of transcripts during 24 and 48 CH and then restored similar levels to the ones observed in field samples in the rest of the experiment. Finally, *MdDHN1* and *MdDHN11* displayed a slight increase in transcript levels in the first part of the treatment, which persisted until the end of the analysis. In 2009 samples (Fig. S4B), 168 h of LT6 exposure increased gene expression of all *MdDHN*s in both cultivars, except for *MdDHN7*. After 840 h of LT6, three distinct responses were observed: *MdDHN1* and *MdDHN11* were further induced; *MdDHN2*, *MdDHN5*, *MdDHN6* *MdDHN8* and *MdDHN9* maintained the same level of transcript accumulation observed at 168 h and *MdDHN3*, *MdDHN4* and *MdDHN10* showed a decline in transcript levels. *MdDHN7* showed similar levels of

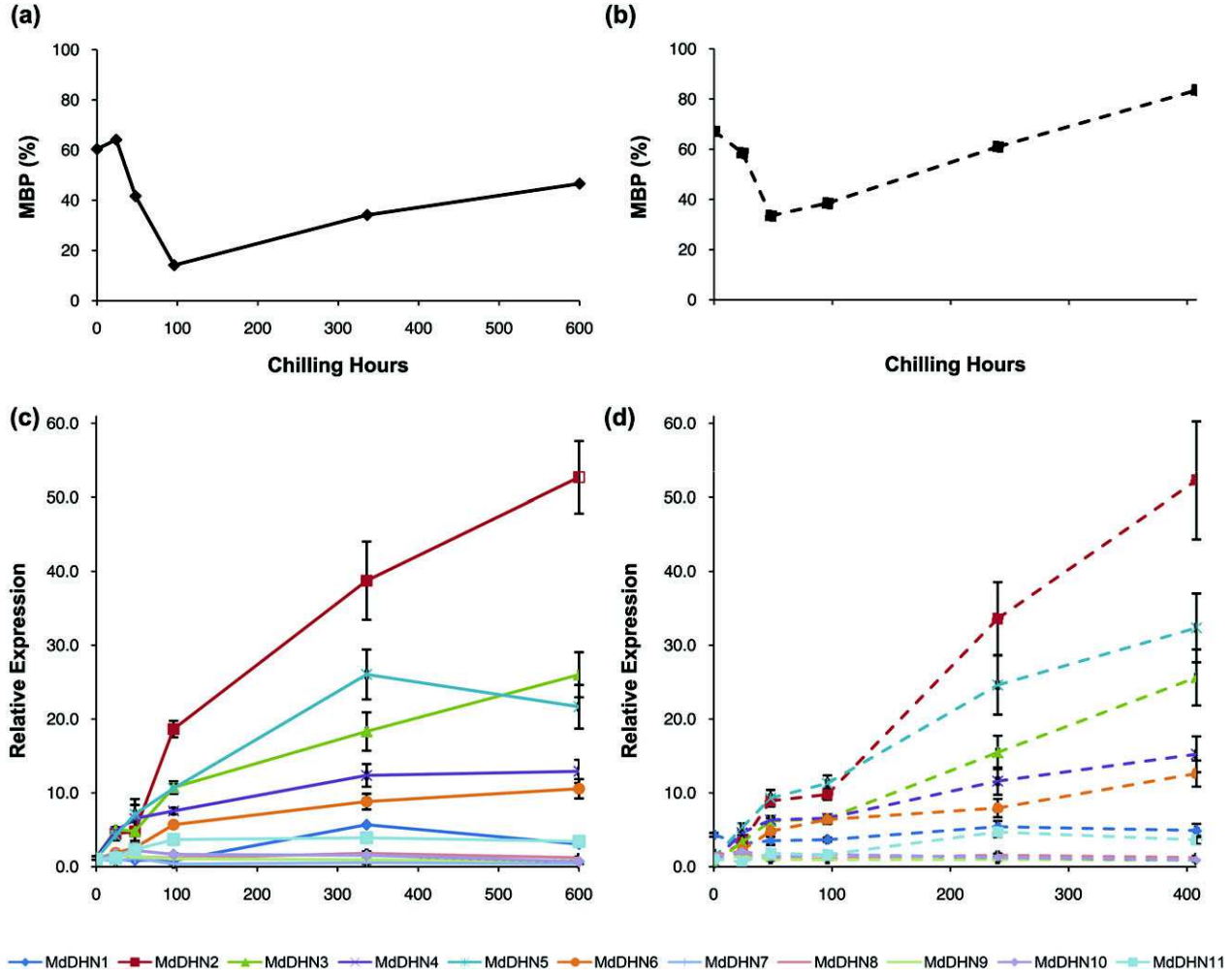

Fig. 6. Endodormancy analysis and relative expression levels of *MdDHN1–11* genes. MBP analysis of 'Royal Gala' (A) or 'Castel Gala' (B) twigs after treatments (3°C/15°C for 12/12 h) in a growth chamber. Mean relative expression levels were performed by RT-qPCR of RNAs isolated from closed terminal buds of 'Royal Gala' (C) or 'Castel Gala' (D) twigs. Solid lines, 'Royal Gala'; dashed lines, 'Castel Gala'. Relative transcript levels in the field sample (0 CH) were set to 1. Standard error bars are shown in the gene expression graphs.

transcript accumulation during cold treatments for 'Royal Gala' and a slight decrease in 'Castel Gala' samples.

The last experiment explored the effects of cold (3°C, LT3) and growth permissive (25°C) temperatures on *MdDHN* transcript accumulation profiles during endodormancy of 'Royal Gala'. After 168 and 438 h under LT3, MBP initially at 6.6%, increased to 16.6 and 61.6%, respectively (Fig. S5A). However, exposure to 25°C diminished MBP to 9.3%. Re-exposure to LT3 recovered MBP to some extent (14.4%). The twigs were maintained for 168 h under cold because this treatment was able to induce *MdDHN*s expression (Fig. S4B). Two distinct patterns of transcript accumulation were identified (Fig. S5B). *MdDHN1*, *MdDHN7*, *MdDHN8*, *MdDHN10* and *MdDHN11* were clearly induced by growth permissive temperature. Re-exposure to LT3 further induced their expressions, except for *MdDHN7*, which showed a slight repression. Conversely, *MdDHN2*, *MdDHN3*, *MdDHN4*, *MdDHN5*, *MdDHN6* and *MdDHN9* showed a drastic decrease in transcript levels after acclimation at 25°C, with re-exposure to LT3 being able to restore transcript levels. In control samples, no substantial alteration of transcript levels was observed, except for *MdDHN7* and *MdDHN9* that were down-regulated.

Discussion

DHNs are proteins known to play important roles in plant adaptive responses to abiotic stresses, mainly dehydration (Rorat 2006). Several studies linking *DHN* expression to bud dormancy (Welling et al.

2004, Yamane et al. 2006, Yakovlev et al. 2008, Garcia-Bañuelos et al. 2009, Porto et al. 2015), including a previous work from our group (Falavigna et al. 2014), have been reported. Recently, nine gene models coding for DHNs in apple were identified but not using bud dormancy as subjected (Liang et al. 2012). Therefore, the identification of *MdDHN* genes being expressed during bud dormancy in our previous work (Falavigna et al. 2014) and the recent description of the gene family in apple (Liang et al. 2012) prompted us to better characterize how *MdDHN* genes are regulated during bud dormancy progression.

The identification of gene families in a genome search can lead to divergent results according to the employed methodology, and it was observed when comparing our data to the results reported by Liang et al. (2012). In their analyses, an HMM (hidden Markov models)-based (Finn et al. 2011) search was employed to identify putative *DHN*s in the apple genome retrieving 12 candidates for the family. In our screening, the conserved DHN K-segment was used as query in BLASTP searches against the same apple genome. Our analysis rendered 16 matches, comprising the 12 putative *MdDHN*s previously described and four novel candidates (Fig. 1). Following the identification of 12 *MdDHN* members, Liang et al. (2012) screened apple EST (expressed sequence tag) databases to verify whether these genes were expressed. Sequences without any matches were discarded, yielding nine genes (*MdDHN1–9*) that were used in their further analysis. In our manual approach to remove artifacts from the candidate list, we were able to precisely select predicted gene models, revealing three novel apple *DHN*s (*MdDHN10–12*; Fig. 1). Furthermore, our analysis showed that *MdDHN10* and *MdDHN11* are expressed (Figs 4–6).

We performed a motif-based analysis to search conserved motifs previously described for DHN proteins (Rorat 2006), using the MEME software (Bailey and Elkan 1994). Our classification for MdDHN1–9 matched with the one described by Liang et al. (2012), with the exception of MdDHN1 and MdDHN4. Because a statistical significance was found for two Y-segments in these sequences, both putative proteins were therefore classified as Y_2SK_3 instead of YSK_3 (Fig. 1). Additionally, all MdDHNs were assigned into four of five subclasses reported for DHNs. No MdDHN belonging to the K_nS subclass was found. DHNs from this specific subclass were proposed to exhibit hydroxyl and peroxyl radical-scavenging activity under cellular dehydration stresses (Hara et al. 2003, Hara et al. 2004). This might suggest that other genes or even MdDHNs assigned to other subclasses may be responsible for this kind of reactive oxygen species detoxification in apple. In addition to the known conserved DHN domains, two novel DHN motifs were identified using sequences from Arabidopsis, barley and poplar (Liu et al. 2012b). In addition, two other highly conserved motifs were found in DHNs from maritime pine (Perdiguero et al. 2012). Finally, our analysis identified two distinct new motifs exploring apple DHNs (Fig. 1; Fig. S2B, E). These data suggest that uncharacterized domains of DHN proteins may exist and further investigation needs to be conducted to unveil their functional roles.

The number of *DHN* genes in apple (12) suggests that duplication events may have happened. Analyzing a 35 kb region of the apple chromosome 2, six *MdDHN*s are arranged in tandem, suggesting that this kind of gene duplication event also could be held accountable for the amount of *DHN* genes in apple. Additionally, as suggested by Velasco et al. (2010), the genome of the domesticated apple has undergone a recent whole-genome duplication (WGD) event that certainly contributed to the higher number of *DHN* genes in apple in comparison to its Rosaceae counterpart *P. mume*, which did not undergo a recent WGD (Zhang et al. 2012). In grapevine, four *DHN* genes were identified and no evidence for duplication events in this family was found (Yang et al. 2012). However, gene family expansion by means of duplication events was a recurrent feature for the *DHN* family in other species. In *P. mume* and rice, the *DHN* family is composed of six and eight members, respectively, with both species displaying genes arranged as tandem repeats (Wang et al. 2007, Du et al. 2013). In other species, besides tandem rearrangements, segmental duplication may have equally contributed to the expansion of *DHN* family, which is composed of 10 members in *Arabidopsis* (Hundertmark and Hincha 2008), 13 in barley (Battaglia et al. 2008), 11 in poplar (Liu et al. 2012b) and 10 in soybean (Yamasaki et al. 2013).

The construction of phylogenetic trees showed MdDHN members present into four of the six clusters identified. All *MdDHN*s that mapped to chromosome 2 clustered in groups C and D (Fig. 2). Three *PmDHN*s, which are disposed as tandem repeats in the genome (Du et al. 2013), are also present in these groups. The synteny analysis revealed that *PmLEA10* is one of a series of genes that show collinear relationships with two regions of apple chromosome 2, one region containing *MdDHN2* and other containing *MdDHN10* (Fig. 3). Additionally, *PmLEA29* and *MdDHN8* concomitantly clustered in the same branch of the phylogenetic tree and mapped to the same collinear region. Taken together, these results suggest that, at least for the *DHN* gene family, duplication events happened after the Rosaceae family diversification from *Prunus* and *Malus* ancestors,

with another round of gene duplication events occurring only in the *Malus* genus (Figs 2 and 3).

In order to quantify the transcript accumulation of *MdDHN1–11* in different apple tissues and organs, RT-qPCR was performed (Fig. 4). However, our results differed from those reported by Liang et al. (2012), who performed semi-quantitative RT-PCR in five organs: flower, fruit, leaf, root and seed. Only *MdDHN7*, *MdDHN8* and *MdDHN9* shared common expression patterns comparing both studies. These different results may derive from at least three possible reasons. First, we identified five genes mainly expressed in closed terminal buds, which were not analyzed by Liang et al. (2012). Moreover, because *DHN* expression is highly regulated by temperature and water availability, the differences between results may be due to environmental factors. Finally, RT-qPCR is a much more sensitive technique than semi-quantitative RT-PCR and permits an accurate quantification (Gachon et al. 2004).

Interestingly, 7 of 12 genes (approximately 64%) of this family were mostly expressed in dormant organs, i.e. seeds and closed terminal buds (Fig. 4). This finding is in agreement with transcript accumulation patterns described previously for the *LEA* gene family, which is known to be up-regulated during seed maturation and desiccation, phenomena related to seed dormancy in Arabidopsis (Rorat 2006, Holdsworth et al. 2008, Angelovici et al. 2010). Furthermore, a recent report identified common transcriptional pathways during dormancy release of buds and seeds in peach, including a *LEA*-like family member (Leida et al. 2012). Therefore, we suggest that some adaptive pathways analogous to seed dormancy may also be present in the bud dormancy process. Finally, the marked expression of *MdDHN11*, reaching 6000-fold higher levels in mature seeds relative to closed terminal buds, suggest that this gene has an important role in seed tolerance to desiccation (Fig. 4).

Comparative genomics aims to track characteristic features of orthologs in multiple genomes (Thornton and DeSalle 2000). Therefore, the identification of orthologous and paralogous sequences along with functional information may be used as a tool to predict gene function (Zhang 2003). In fact, some interesting relationships may be drawn by the comparison of the phylogenetic results with the RT-qPCR data (Figs 2 and 4). All genes that clustered in groups A and C were mostly expressed in seeds (Hundertmark and Hincha 2008, Liu et al. 2012b, Yang et al. 2012, Yamasaki et al. 2013, this work). *PmDHN*s were mainly expressed in flowers, although gene expression in seed was not analyzed (Du et al. 2013). We, therefore, propose that *DHNs* from groups A and C are mostly seed-expressed genes. MdDHN1, MdDHN7, MdDHN10 and PmLEA10 formed a branch in group D. Interestingly, *PmLEA10* was expressed in all five *P. mume* organs analyzed (Du et al. 2013) and a very similar expression pattern was also found for *MdDHN7* and *MdDHN10* (Fig. 4). Finally, *MdDHN8* and *MdDHN9*, which exhibited a transcript accumulation pattern throughout many tissues and organs of apple, formed a branch with PmLEA29 in group F. Accordingly, *PmLEA29* also presented a wide pattern of transcript accumulation in *Prunus* (Du et al. 2013). In conclusion, our phylogenetic analysis strongly agrees with our transcript accumulation data, reinforcing the importance of the combination of these analyses in the prediction of *DHN* gene function.

The gene expression of *MdDHN*s was analyzed through an annual cycle of 'Fuji Standard' closed terminal buds. Transcripts of *MdDHN1*, *MdDHN9* and *MdDHN10* accumulated after winter, in a similar trend to the results reported for pear and sessile oak (Liu et al. 2012a, Ueno et al. 2013). Moreover, Tompa et al. (2006) characterized water and ion binding of AtDHNs and showed that these proteins can bind large amounts of water and solute ions. In this sense, the DHN competence to retain water could be one possible explanation to our findings, given that water supply is a necessary attribute during normal plant growth. On the other hand, *MdDHN2*, *MdDHN3*, *MdDHN4*, *MdDHN5*, *MdDHN6*, *MdDHN8* and *MdDHN11* showed a seasonal transcriptional profile during winter allied with a remarkable repression near budbreak (Fig. 5). Similar seasonal patterns of *DHN* transcript accumulation was already found in field-harvested samples of birch and Norway spruce (Welling et al. 2004, Yakovlev et al. 2008), bark tissues of eight woody species and during bud dormancy in *P. mume* (Wisniewski et al. 1996, Yamane et al. 2006). DHN accumulation, even under the metabolically less active endodormant state, might be partially explained by the reduction of water availability observed in buds during winter (de Fay et al. 2000, Améglio et al. 2002, Rinne et al. 2011).

In an attempt to characterize the seasonal *MdDHN* expression previously identified in closed terminal buds (Fig. 5), three experiments using controlled temperatures were carried out to produce contrasting MBP samples. We gathered data that confirmed the contrasting CR between Castel Gala and Royal Gala cultivars (Fig. 6A, B and Fig. S4A; Denardi and Seccon 2005, Anzanello et al. 2014a); and the reversion of the chilling effect over endodormancy after exposure to growth permissive temperatures (Fig. S5A; Richardson et al. 1974, Erez et al. 1979, Young 1992).

In the first experiment, all *MdDHN* genes presented increased steady-state mRNA levels after exposure to LT6, suggesting that cold affects gene regulation of the

whole *MdDHN* family, with the exception of *MdDHN7* (Fig. S4B). However, studies analyzing gene expression of the *DHN* family in apple and grapevine seedlings under cold treatment found that only a few members were induced by this condition (Liang et al. 2012, Yang et al. 2012), suggesting that closed terminal buds impose a different regulation mechanism over *MdDHN* expression under cold (Fig. S4). Interestingly, when analyzing the influence of growth permissive temperatures during endodormancy, two patterns of gene expression were observed (Fig. S5). Prolonged exposure to 25°C decreased the transcript accumulation levels of *MdDHN2–6*. Furthermore, these genes restored their expression levels after re-exposure to cold, although no significant alterations in the MBP were measured. These profiles closely agree with the models proposed for almond, birch and peach *DHN*s (Welling and Palva 2008, Barros et al. 2012, Artlip et al. 2013). In these models, some members of the CBF family of transcription factors are involved in cold acclimation after endodormancy induction, and seasonally regulate the expression of *DHN*s. In addition, Arora et al. (1997) induced cold acclimation in blueberry floral buds followed by a dormancy neutral treatment that caused deacclimation. These authors observed an increased level of DHN proteins during acclimation followed by a reduction after deacclimation. Their results suggest that DHN changes are more closely related to cold hardiness rather than with dormancy. In agreement to Arora et al. (1997), our findings suggest that *MdDHN2–6* are environmentally regulated, probably mediated by CBFs, and our treatment likely triggered a deacclimation process in these bud samples.

Finally, our last experiment attempted to simulate the winter season in Southern Brazil, as we used a daily cycle of 12 h at 3°C and 12 h at 15°C to induce and release dormancy (Fig. 6). Once again, *MdDHN2–6* were differentially regulated in comparison to the other *MdDHN*s. Interestingly, buds kept accumulating *MdDHN2–6* transcripts while dormant, given that we analyzed gene expression before forcing budburst. Considering that these five genes present at least one C-repeat/DRE *cis* element in their promoter regions (data not shown), one hypothesis could be that the transcriptional regulation of these five genes during bud dormancy likely occurs through the CBF pathway. This result agrees with the findings reported by Artlip et al. (2013), who stated that peach *CBF*s are LT-inducible and directly regulate the expression of *PpDHN1*, the promoter of which contains two C-repeat/DRE *cis* elements. Furthermore, ectopic expression of a peach *CBF* in apple triggered dormancy induction by short days (Wisniewski et al. 2011). The same group further analyzed the expression of genes known to be associated with freezing tolerance and dormancy in these plants proposing a model where CBFs regulate the expression of apple dormancy-related genes while concomitantly induce cold-regulated genes, such as *DHN*s (Wisniewski et al. 2015). Within this context, MdDHN accumulation could be one of the mechanisms partially responsible for bud cold tolerance during winter. For instance, the ability of DHNs to shift their conformational status during changes in water availability, as also the ability to bind in partly dehydrated surfaces of other proteins (Tompa et al. 2006, Graether and Boddington 2014), could act protecting bud integrity. In summary, our results indicate that distinct *MdDHN*s play different functions in the cell, with overlapping levels, and that their expressions are fine-tuned by the environment during the dormancy process in apple.

Author contributions

V. S. F., Y. E. M. and D. D. P. performed all the sampling, RNA extraction and cDNA synthesis. V. S. F. also performed the RT-qPCR assay, the bioinformatic analysis, analyzed the data and drafted the manuscript. Y. E. M. and D. D. P. also carried out the RACE assays. R. A., H. P. S. and F. B. F. designed and executed the controlled temperature experiments. L. F. R., M. M. P. and G. P. contributed for the experimental design, discussion of the results and revision of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sampling dates and developmental stages of the different tissues and organs harvested.

Table S2. Sampling dates, corresponding season and chilling hours accumulated by closed terminal buds harvested in 2009 and 2010.

Table S3. Accession codes of *DHN* genes used in the phylogenetic analysis.

Table S4. Primers employed in RT-qPCR studies.

Fig. S1. Sequence alignment between genomic and RACE sequences of *MdDHN11*.

Fig. S2. Significantly enriched motifs of MdDHN deduced proteins identified with the MEME software.

Fig. S3. PCR analysis of *MdDHN12* in 'Gala Baigent' apple trees.

Fig. S4. Endodormancy analysis and relative expression levels of *MdDHN1–11* genes in apple twigs exposed to cold.

Fig. S5. Endodormancy analysis and *MdDHN1–11* relative expression in 'Royal Gala' twigs exposed to cold and growth permissive temperatures.

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5 CAPÍTULO III

Characterization of apple dehydrin 11 reveals its contribution to water deficit tolerance during seed development

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Title

Characterization of apple dehydrin 11 reveals its contribution to water deficit tolerance during seed development

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Abbreviations

ABI, ABSCISIC ACID INSENSITIVE; DAA, days after anthesis; DAPI, 4',6-diamidino-2-phenylindole; DHN, dehydrin; EYFP, enhanced yellow fluorescent protein; EGFP, enhanced green fluorescent protein; ISH, *in situ* hybridization; LEA, late embryogenesis abundant; MS, Murashige and Skoog.

Abstract

Water deficit is among the most important abiotic stress in terms of crop productivity losses, and new agricultural technologies, such as the generation of genetically modified crops, are needed to maintain growth and yield in a changing environment. Dehydrins (DHNs) are protective proteins known to accumulate during the last stages of seed development, conferring tolerance to desiccation. Apple dehydrin 11 (*MdDHN11*) is expressed during seed development, with putative roles in seed tolerance to desiccation. In the present work, a collection of experiments were performed to functionally characterize *MdDHN11*, including *in situ* hybridization, subcellular localization, *in silico* promoter analysis, ectopic expression of *MdDHN11* and its response to water deficit. The *MdDHN11* expression conferred drought-tolerance to Arabidopsis. Moreove, the results gathered in this work provide evidences that MdDHN11 protects the embryo and the endosperm during seed development by accumulatting in the nucellus layer of apple seeds. Its mechanism of action likely involves the interaction with other proteins and membranes in the cytoplasm and nucleus. These findings suggest that apple *DHN11* could represent a new gene source for the generation of crops with improved adaptation to adverse environmental conditions such as water deficit.

Keywords: Apple, dehydrin, drought-tolerance, seed, water stress

Highlights

- The functional characterization of *MdDHN11* is presented;
- MdDHN11 is expressed in the nucellus layer of apple seeds;
- MdDHN11 protein is localized in nucleus and cytoplasm;
- Ectopic expression of *MdDHN11* generated drought-tolerant plants;
- Apple *DHN*s are a new resource for the generation of transgenic crops.

1. Introduction

The sessile feature of plants makes them constantly subjected to abiotic stress exposure such as drought, cold, salinity, heavy metals, among others, which often impose restrictions to their normal growth and productivity. For agriculture, water deficit is among the most important abiotic stress in terms of loss of productivity [1]. In general, plants have developed elaborate and sensitive systems that allow fast signaling responses resulting in adaptation; however, the degree of adaptability will depend on their physiology, biochemistry and molecular mechanisms [2]. When considering the recently proposed models for global warming, which predict a rise in global mean temperatures and changes in rainfall distribution system, greater losses in food production in medium and long-term may occur due to the influence of climate changes [3]. In this context, along with efforts to conserve water and land, new agricultural technologies will be needed such as the generation of genetically modified crops better adapted to grow on adverse environmental conditions [1,2,4].

Dehydrins (DHNs) are a class of polypeptides that belong to group II of late embryogenesis abundant (LEA) proteins and play important roles in plant adaptive responses to abiotic stresses, mainly dehydration [5]. DHNs are characterized by the presence of a highly conserved domain, the K-segment, followed or not by the presence of other two conserved domains, the Y- and S-segment. Although many putative functions were reported for DHNs, such as membrane and lipid binding, heat and cold protection, binding to metals and radical-scavenging activity, their precise mechanisms of action remain unknown (reviewed in [5,6]). Moreover, several reports showed that ectopic expression of *DHN*s in plants can increase drought tolerance [7–12]. Besides their involvement with abiotic stresses, several DHNs are known to accumulate during the last stages of seed development, probably conferring tolerance to desiccation [5]. This protective role is due to polar residues present in their structure that act as water replenishers and interact with other proteins during dehydration [13].

In apple (*Malus* x *domestica* Borkh.), one of the most economically important perennials worldwide, nine *DHN*s were characterized according to their expression at transcript levels in response to drought and cold [14], and all eleven apple *DHN*s were analyzed during bud dormancy progression, flowering and fruit ripening stages [15]. A high functional diversification was identified among apple *DHN* genes. Interestingly,

MdDHN11 showed a seed-specific transcript accumulation pattern compared to other tissues and organs [15]. This result, combined with other comparative genomic analyzes, suggest that MdDHN11 has an important role in seed tolerance to desiccation. This process involves critical water losses in order to prepare seed for germination, and is an active stage in terms of gene expression and metabolism [16]. Thus, due to the putative biological role played by MdDHN11 during this process, the *MdDHN11* gene becomes a good candidate to be tested aiming to obtain plants tolerant to water stress. Additionally, the high transcript levels identified in seeds (6,000-fold) encourages the study of its promoter region for driving tissue-specific expression genes. Recently, the identification of tissue-specific promoters has gained agronomic importance, given that the use of constitutive promoters is not always a useful feature [17].

In the present work, the *MdDHN11* gene was functionally characterized by analyzing its spatio-temporal expression in apple seeds, its subcellular localization, the ability of its promoter to drive transgenic expression, and the performance of *MdDHN11* overexpressing plants under water deficit. The data gathered in this work confirms the protective role of MdDHN11 during seed development and provides a new gene source for the generation of crops better adapted to grow in adverse environmental conditions.

2. Material and methods

2.1 Apple plant material

Apple fruits were harvested in an experimental orchard located at Embrapa Uva e Vinho (-29.165762, -51.535147 and 614m above sea level) in Southern Brazil. The apple trees underwent standard orchard management practices. Plant material consisted of two-year-old 'Imperial Gala' trees grafted on M.7 rootstock. Flowers at the anthesis stage were identified at the field. Apple fruits were harvested at 22, 51 and 83 days after anthesis (DAA), which corresponded to fruits with 20, 40 and 60 mm diameter, respectively (Fig. 1). At each sampling point, at least six individual whole fruits were analyzed. Seeds were immediately removed and fixed in 4% formaldehyde overnight under vacuum (600 mmHg) at the first 30 min [18]. Samples were dehydrated through a graded series of ethyl alcohol from 30 to 100% and stored at -20°C until use.

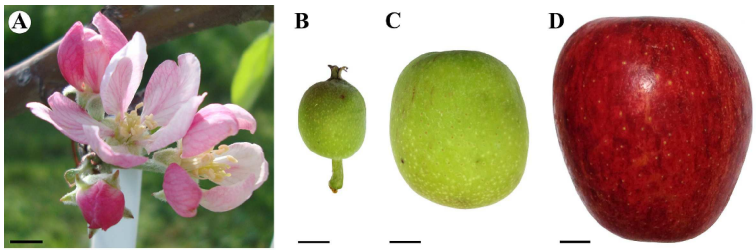

Fig. 1. Apple plant material. (A) Flowers identified at the field at the anthesis stage (0 DAA). (B) 20 mm fruits (22 DAA). (C) 40 mm fruits (51 DAA). (D) 60 mm fruits (83 DAA). Scale bar: 1 cm.

2.2. Seed morphological assay

The fixed and dehydrated samples were embedded in 2-hydroxyethyl methacrylate resin according to [19]. The 5 µm sections were obtained using a Leica RM 2255 microtome. The metachromatic reagent Toluidine blue O [20] was used to stain seed structures by submerging the slides with sections in the reagent for one minute. The stained tissues were photomicrographed in bright field using a Leica DM1000 LED microscope coupled with a Leica MC170 HD camera system.

2.3 In situ *hybridization*

Samples were embedded in paraffin and prepared for longitudinal and transversal sections (8-10 µm) using a Leica RM2125 RTS microtome. Tissue samples were mounted on silanized microscope slides. A gene-specific fragment of 225 pb from *MdDHN11* was amplified (Supplementary Table 1) and cloned into pGEM®-T Easy Vector Systems (Promega), according to manufacturer's instructions. The resulting vector was confirmed by sequencing. Sense and antisense labeled probes were generated by digoxigenin-labeling using T7 or SP6 RNA polymerase of the DIG RNA Labeling Kit (Roche), respectively. After the detection of the hybridization signals by immunostaining, slides were washed, dehydrated, and mounted using Entellan® (Merck). Photomicrographs were obtained as described above.

2.4 Gene expression analysis

DNA was purified from 100 mg of young leaves using modified protocols scaled down to 2 mL tubes [21,22], whereas total RNA was isolated [23,24] and DNase-treated using TURBO DNA-free Kit (Ambion). The SuperScript™ III Reverse Transcriptase

(Invitrogen) was used for cDNA synthesis, according to manufacturer's instructions. Real-time PCR was performed as described [15]. *Actin 2* was employed as reference gene.

2.5 Subcellular localization of MdDHN11

The subcellular localization of MdDHN11 was analyzed through a transient expression assay in Arabidopsis leaf protoplasts. The complete coding sequence of *MdDHN11* was amplified using Platinum® *Pfx* DNA polymerase (Invitrogen) from cDNA obtained from mature seeds of 'Imperial Gala' using gene-specific primers (Supplementary Table 1). The amplicon was cloned into pENTR™ Directional TOPO® (Invitrogen) and then transferred by recombination to the p2YGW7 vector [25] using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), resulting in the fusion of *EYFP* (enhanced yellow fluorescent protein) to *MdDHN11* (35S::*EYFP-MdDHN11*). Protoplast isolation and transformation was performed essentially as described [26]. For positive control, 35S::*EGFP* (enhanced green fluorescent protein) was used, whereas untransformed cells were used as negative control. Transformed protoplasts and negative control were incubated in the light at 24ºC for approximately 20 h prior to imaging. Cell nucleus was stained using 1 µg/mL of DAPI (4',6-diamidino-2-phenylindole). Fluorescence microscopy was performed with an Olympus FluoView 1000 confocal laser-scanning microscope (UFRGS Electron Microscopy Center, Porto Alegre, Brazil) equipped with a set of filters capable of distinguish chlorophyll autofluorescence, DAPI, GFP and YFP fluorescence. The assay was repeated twice.

2.6 Arabidopsis transformation

The promoter region of *MdDHN11* (2,050 pb length upstream of the start codon ATG) was amplified using PfuUltra II Fusion HS DNA Polymerase (Agilent) and gene-specific primers (Supplementary Table 1) from DNA purified from 'Imperial Gala' leaves. The promoter was cloned into pENTR™ Directional TOPO® (Invitrogen), and then recombined to the pHGWFS7 vector [25] using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), fusing the promoter to *GFP-GUS*. This cloning was confirmed by sequencing and then used to transform Arabidopsis ecotype Columbia plants using the *Agrobacterium tumefaciens* (EHA105) floral dip method [27]. Transformed Arabidopsis were selected on Murashige and Skoog (MS) medium supplemented with 1% sucrose on phytagel plates

182 containing 25 μg/mL of hygromycin. Two-week-old plate-grown plants were transferred to
183 soil pots. Transgenic plants were validated by amplifying a fragment of *GFP* from mature
184 leaves (Supplementary Table 1). Tissues and organs from different developmental stages
185 were incubated overnight in 1 mM X-Gluc, 100 mM phosphate buffer (pH 7.0), 2 mM
186 KH2Fe, and 0.5% Triton X-100 at 37ºC. The staining buffer was removed, the samples
187 were washed and incubated in 70% ethanol. Photographs were obtained in bright field
188 using a Leica M165FC stereomicroscope coupled with a Leica DFC 500 camera system.
189 To generate transgenic plants overexpressing *MdDHN11*, the complete coding
190 sequence of *MdDHN11*, previously cloned into pENTR™ Directional TOPO® (Invitrogen),
191 was transferred by recombination to the pH7WG2D.1 vector [25] using Gateway® LR
192 Clonase™ II Enzyme Mix (Invitrogen). The resulting vector (35S::*MdDHN11*) was
193 confirmed by sequencing and used for Arabidopsis transformation as previously described.
194 Transformed Arabidopsis seeds were selected and grown as described above. As the vector
195 also encodes *EGFP* under the control of the *RolD* promoter, a GFP selection was
196 performed using a Leica M165FC stereomicroscope equipped with a filter capable of
197 distinguishing GFP fluorescence. Molecular analyses were performed by amplifying a
198 fragment of *GFP* by PCR using DNA extracted from two-month-old leaves. Finally, to
199 assess the overexpression of the transgene, RT-qPCR was performed using gene-specific
200 primers for *MdDHN11*. PCR and RT-qPCR products were analyzed in 1% ethidium
201 bromide gel electrophoresis.
202
203 *2.7 Water withhold assay*
204 Two-month-old non-transformed and overexpressing *MdDHN11* plants were
205 transferred to vats without water for 36 days. After treatment, plants were rehydrated for
206 six days prior evaluation. Pictures before and after the experiment, were taken for visual
207 analysis.
208
209 *2.8* Cis-*elements and gene ontology analyses*
210 The promoter region of *MdDHN11* (2,050 pb length upstream of the start codon
211 ATG) was obtained from the apple genome v1.0 (http://www.rosaceae.org, [28]), and
212 searched for *cis*-elements using the AtPAN database (Arabidopsis thaliana Promoter
213 Analysis Net, http://atpan.itps.ncku.edu.tw/, [29]). The result was trimmed as follows:

given strand only, exclusion of repeated *cis*-elements at the same position, and only *cis*-elements equal or larger than six nucleotides were considered. The *cis*-element analysis of *MdDHN11* promoter was compiled into a figure using the Gene Structure Display Server 2.0 [30]. A genome-wide search of the ABI3 (ABSCISIC ACID INSENSITIVE 3) *cis*-element in the promoter regions of all predicted genes from apple was performed following the method described by [31]. Briefly, 36,991 apple promoter sequences were screened for the presence of ABI3 using FIMO (Find Individual Motif Occurrence, [32]), with a match *p* value of less than $1e^{-3}$ and given strand only. Genes containing two or more copies of this motif were scanned for enrichment of gene ontology (GO) terms using agriGO (Plant GO slim subset, [33]) and REVIGO [34].

3. Results

3.1 Morphoanatomical analysis of apple seeds

Morphoanatomical analyses of 'Imperial Gala' apple seeds were performed (Fig. 2). During the early development of the seed (seeds from 20 mm fruits), the nucellar tissue increased and the seed coat (testa) differentiated, while the young embryo (globular embryo) is immersed in the developing endosperm (Fig. 2A and B). In seeds of 40 mm fruits the further development of the endosperm and the embryo (torpedo embryo) was observed, limiting the nucellus to a small number of cell layers in contact with the integument already differentiated in seed coat, with mesotestal characteristics (Fig. 2C and D). In seeds of 60 mm fruits, a well-developed embryo can be observed and the endosperm is reduced to a few cell layers in contact with the testa (Fig. 2E and F).

3.2 Spatial and temporal accumulation of MdDHN11 transcripts and subcellular localization of the encoded protein

Transcript accumulation of *MdDHN11* was already analyzed during several developmental stages, and its main expression was identified during seed development [15]. Gene expression data in mature fruit seeds was reanalyzed aiming to identify which *MdDHN* gene presented the highest steady-state mRNA levels in this sample. *MdDHN11*, along with *MdDHN1*, showed the highest transcript accumulation in seeds among all apple *DHN*s (Supplementary Figure S1). This result prompt us to further characterize the

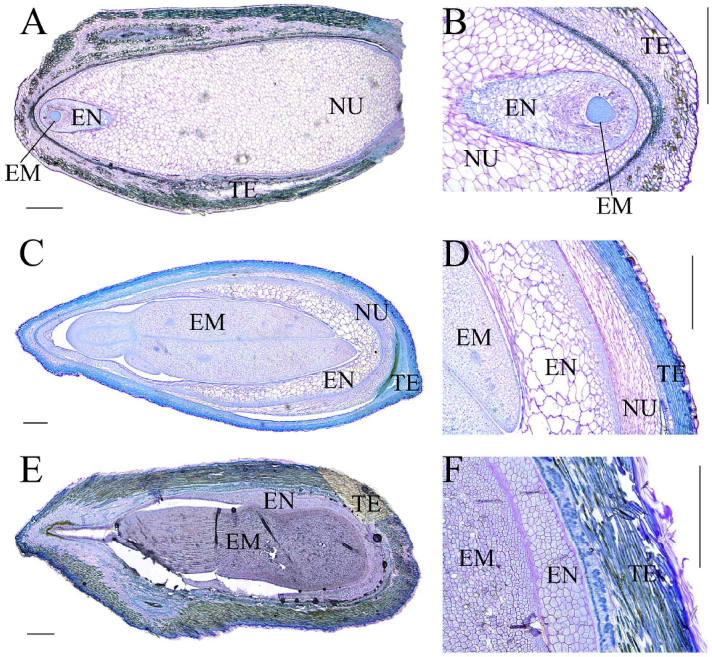

Fig. 2. Apple seed structure of fruits ranging from 20 mm to 60 mm diameter. Light micrographs of apple seed cross sections were stained with Toluidine blue O. (A-B) Seed of 20 mm fruit. (C-D) Seed of 40 mm fruit. (E-F) Seed of 60 mm fruit. EN, endosperm; EM, embryo; NU, nucellus; TE, testa. Scale bar: 500 µm.

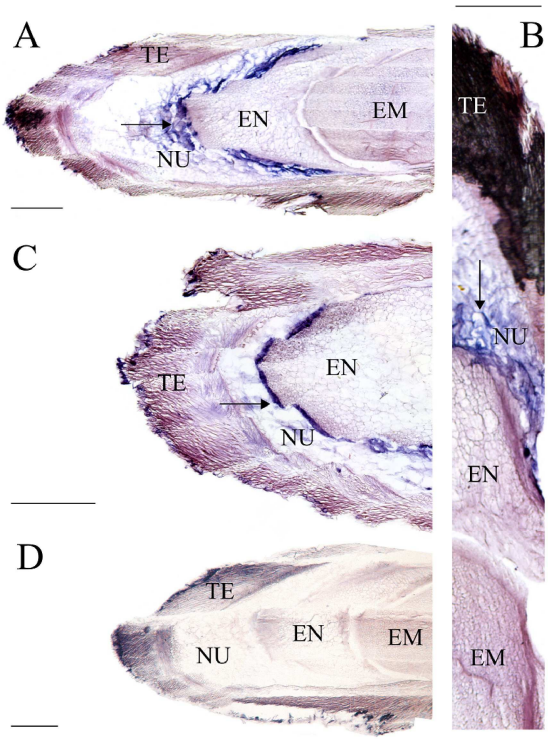

Fig. 3. Spatial and temporal accumulation of *MdDHN11* transcripts in apple seeds. Hybridization signal (black arrow) is visible in the nucellus (A, B and C) compared to the control slide (D). EN, endosperm; EM, embryo; NU, nucellus; TE, testa. Scale bar: 500 µm.

MdDHN11 spatial and temporal expression pattern using *in situ* hybridization (ISH) assays in apple seeds. The antisense *MdDHN11* probe signal was clearly visible in the nucellus layer from seeds of 40 mm fruits (Fig. 3A, B and C). ISH slides hybridized with the sense probe were used as control and showed no hybridization signal (Fig. 3D).

Complementarily, two web-based programs were used to predict the subcellular localization of MdDHN11. While the Plant-mPloc v.2.0 program predict it to be localized in the cytoplasm [35], the CELLO v.2.5 program predict it to be localized in the nucleus [36]. To confirm or refute these predictions, the full-length CDS of *MdDHN11* was fused to *EYFP* in order to identify the subcellular localization of MdDHN11. Transient expression analyses in Arabidopsis leaf protoplasts revealed that MdDHN11 was present in both predicted tissues, nucleus and cytoplasm (Fig. 4).

3.3 MdDHN11 *overexpressing plants and evaluation of their drought-stress tolerance*

Four independent Arabidopsis lines expressing *MdDHN11* were recovered and analyzed. From these plants, only three carried the construction based on the PCR analyses and GFP fluorescence (#1, #2 and #4). However, analyses by RT-qPCR revealed that only line #1 expressed the transgene (Fig. 5A). In order to investigate whether the overexpression of *MdDHN11* in Arabidopsis plants is able to increase tolerance to water deficit, transformed and non-transformed plants were grown under normal conditions for two months and then the water supply was removed. After 36 days of treatment, plants were rehydrated and only the transgenic line #1 showed drought stress tolerance (Fig. 5B). This transgenic line produced flowers and completed its normal growth cycle (data not shown). None of the control plants nor the transformed plants without ectopic *MdDHN11* expression survived to the assay (Fig. 5B).

3.4 Characterization of MdDHN11 *promoter region*

With the aim of investigating which *cis*-elements in the promoter region of *MdDHN11* may be responsible for its seed-specific expression [15], 2,050 bp upstream of the start codon ATG were analyzed using the AtPAN database. After trimming (see Material and Methods section), a total of 132 *cis*-elements were identified, whereas 21 were related to seed expression, namely ABI3 (three copies), ABI5 (eight copies), BPBF

(one copy), EBOXBNNAPA (six copies), GBF (one copy), and SEF3 (two copies). Dehydration-related *cis*-elements were also characterized, given that critical water losses occur to prepare seeds for germination [16]. A total of 18 *cis*-elements related to dehydration were identified, namely DREB1A (one copy), MYC (three copies) and MYC2 (14 copies). Interestingly, 14 *cis*-elements related to seed expression overlapped with dehydration-related *cis*-elements (Fig. 6A). Interestingly, the *MdDHN11* promoter displayed two ABI3 motifs near the start codon (red bars in Fig. 6A), and several reports showed the need of two copies of this *cis*-element in order to direct the proper seed-specific regulation [reviewed in 37]. Apple genes displaying two or more ABI3 motifs in their promoters were identified. The rate of occurrence of ABI3 in the apple genes changed from 24.6 (only one ABI3) to 5.3% (two or more). GO terms enrichment was performed to identify biological processes related to genes that possess two or more ABI3 *cis*-elements. Several enriched terms were annotated, with the most significant ones being "metabolism", "multi-organism process", "pollination", "reproduction" and "reproductive process" (Fig. 6B).

In order to analyze if the promoter region of *MdDHN11* is capable to drive seed-specific transgenic expression in Arabidopsis, the promoter region of *MdDHN11* was cloned and used to drive the expression of *GFP-GUS*. Eight independent lines were recovered and, after PCR validation, only four plants carried the Prom*MdDHN11*::*GFP-GUS* construction (Fig. 7A). Although several developmental stages were analyzed, no GUS staining was observed in any of the transgenic or non-transformed plants (Fig. 7B).

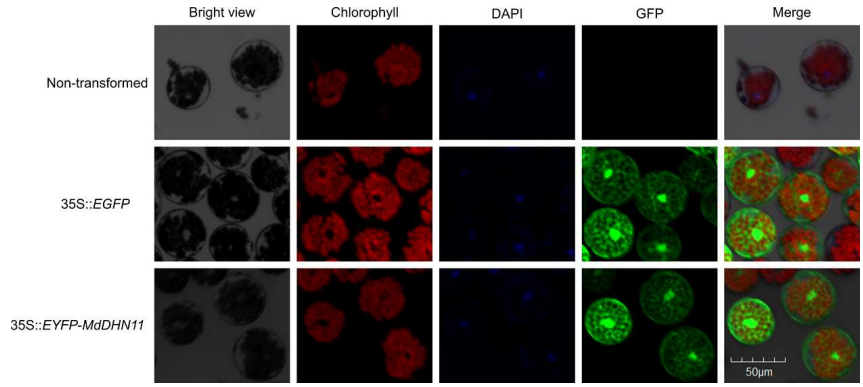

Fig. 4. Subcellular localization of MdDHN11 in Arabidopsis leaf protoplasts. Negative and positive controls were untransformed cells or cells transformed with 35S::*EGFP*, respectively. All images were captured with a confocal laser scanning system. Green, EYFP or EGFP; red, chlorophyll; blue, DAPI. All pictures are at the same scale.

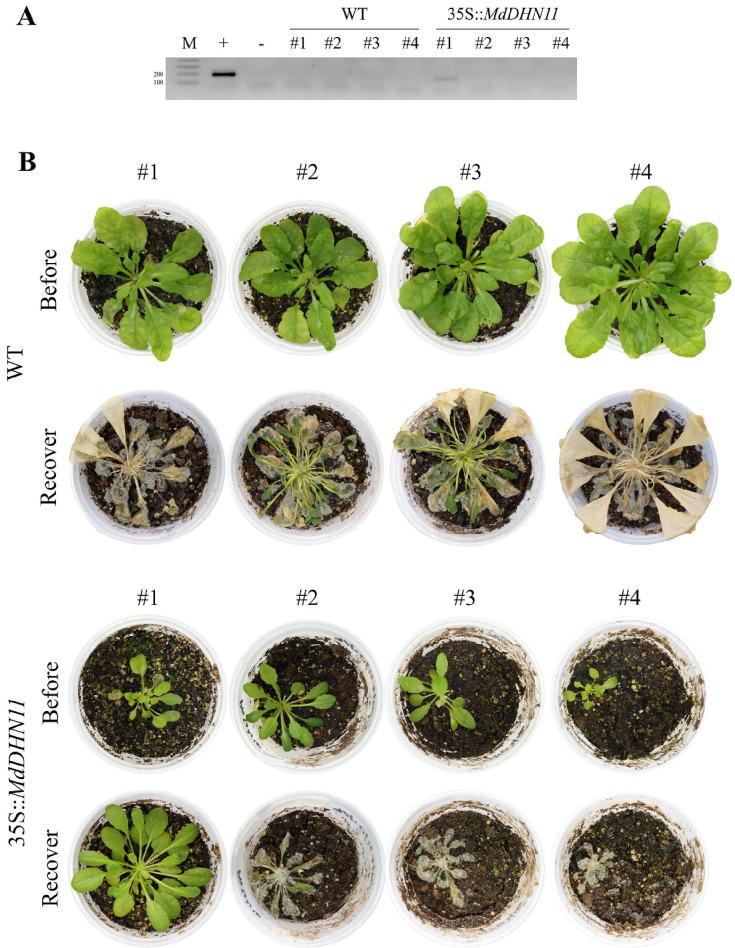

Fig. 5. Evaluation of transgenic plants overexpressing *MdDHN11*. (A) RT-qPCR of the transgene in Arabidopsis leaves of non-transformed and transformed plants. Positive control consisted of apple seed cDNA, while negative control was water. (B) Phenotype of plants exposed to drought stress. Two-month-old plants were stressed by withholding water for 36 days. Thereafter, plants were rehydrated for six days.

4. Discussion

Dehydrins have been extensively studied in various species and are important proteins in plant adaptive responses to abiotic stresses. Several studies in different plants, including apple [14], have shown their transcript accumulation in response to drought, salt, and cold, among others (reviewed in [5,6]). These studies have guided which *DHN* gene should be used in the generation of transgenic plants with enhanced tolerance to water stress exposure [8–12]. However, overexpression of *DHN*s in plants is not a guarantee of increased tolerance to abiotic stresses [5]. In this context, the identification of new genes

with potential in the generation of genetically modified crops is essential. It has been suggested that *MdDHN11* acts in seed tolerance to desiccation due to its seed-specific expression during apple fruit development [15]. This finding prompts us to functionally characterize *MdDHN11* aiming to better understand its protective role during seed development, as also to evaluate the potential biotechnological application of this gene in the generation of plants better adapted to drought conditions.

In flowering plants, seed development is a coordinated process of three genetically different components: maternal tissues, embryo and endosperm. The last two are originated by the double fertilization of the egg and the central cell in the female gametophyte and are responsible for the initiation of seed development [38,39]. Although the maternal tissues of the ovule do not participate in the fertilization process, they undergo several reprogramming events in response to it. One of these maternal tissues is a central mass of parenchyma called nucellus, which has a nutritive and protective role. Plants have shifted between endosperm and nucellus as the main nutrient storage system during angiosperm seed evolution [40]. All these main seed components, i.e. embryo, endosperm, nucellus and testa, were identified in apple seeds (Fig. 2). In apple, about four-to-six weeks after fertilization, the endosperm becomes cellular and soon fills much of the developing ovule as it grows at the expense of the nucellus. Afterwards, the embryo gradually consumes the endosperm and occupies most of the seed [41]. Embryo development from globular to torpedo, at the expense of the endosperm, is clearly visible through the stages analyzed (Fig. 2). The photomicrographs of the morphoanatomical analyses are up to now, the best images reporting the apple seed development in the literature, especially considering seeds of 40 mm fruits. These images can help the better understanding of analyses such as the ISH assay presented here.

The strong hybridization signal in the nucellus layer, in the border with the endosperm of fruits with 40 mm diameter (Fig. 3) is a very interesting result due to the maternal origin of the nucellus layer and the protective function described for DHNs. Given that this layer is located between the testa and the endosperm, the accumulation of MdDHN11 represent a protective source for the embryo and the endosperm during seed development. Within this context, the DHN ability to shift their conformational status and bind partly dehydrated surfaces of other proteins [6,42] could act protecting stored proteins and nutrients to support seed survival during maturation and desiccation. Such kind of

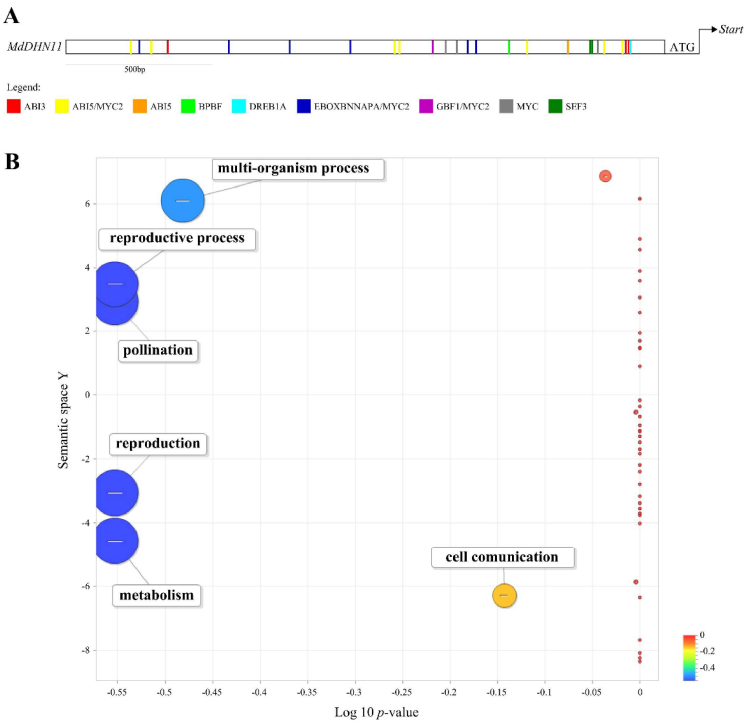

Fig. 6. Arrangement and analysis of the *cis*-elements in the *MdDHN11* promoter. (A) Representative image of the promoter region of *MdDHN11* with the location of the identified dehydration and seed-specific *cis*-elements. (B) GO enrichment of apple genes containing two or more ABI3 *cis*-elements in their promoters. The scatterplot shows the cluster representatives (i.e. terms remaining after redundancy reduction) in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms semantic similarities. Bubble color and size indicates the agriGO-provided *p* value (see the color legend in the bottom right corner).

Fig. 7. Histochemical GUS analysis of *MdDHN11* promoter in different organs. (A) PCR validation of the transgenic plants. (B) GUS staining of transgenic plant #8 carrying the Prom*MdDHN11*::*GFP-GUS* construction in inflorescence, flower, leaf, seed and young and mature silique.

activity would need the location of MdDHN11 in the cytoplasm, as demonstrated by the subcellular localization assay in Arabidopsis leaf protoplasts (Fig. 4). Several previous reports characterized DHNs locations inside the cell, finding then in nucleus, cytoplasm, mitochondria, chloroplast and plasma membrane [6]. However, a recent subcellular localization study of all Arabidopsis DHN proteins showed that these proteins are only localized in the cytoplasm or in the cytoplasm and nucleus [43]. Interestingly, phylogenetic analyses of DHNs from seven species, including apple and Arabidopsis, demonstrated that MdDHN11 is present in a seed-specific clade together with AtDHN14 and AtDHN45 [15], and these two AtDHNs are both localized in nucleus and cytoplasm [43]. Taken together, these results fit well with our subcellular localization data and add additional evidence of the protective role of MdDHN11 during apple seed development.

To gain insights about the capacity of MdDHN11 of conferring protection during restricted water availability, a functional characterization of apple *DHN*s by a transgenic expression assay was performed. To achieve this purpose, *MdDHN11* was expressed in Arabidopsis and the survival rate of these plants against a severe water deficit was analyzed. The sole plant able to survive this condition was exactly the one expressing *MdDHN11* (Fig. 5A). Interestingly, less than two hours after rehydration the transgenic plant was already fully recovered, demonstrating that MdDHN11 was able to maintain root integrity and functionality. Other reports also related better root growth and development of plants overexpressing *DHN*s in comparison to wild-type growing under stress-inducing agents such as PEG or mannitol [9,10]. The protective role of DHNs may occur due to their disordered protein structure allied with their inability to denature [6], which during dehydration events allow them to bind other proteins and interact with membranes [42,44], protecting tissues and cells under stress. Studies with *DHN* genes from banana, maize, medicago, rice, wheat, and the moss *Physcomitrella patens* also pointed their ability to enhance tolerance to drought and osmotic stresses in several different plants, such as Arabidopsis, banana, tobacco, and rice [5,7–12]. Interestingly, the overexpression of endogenous Arabidopsis *DHN*s were used to obtain plants more tolerant to freeze stress [45], but not dehydration.

In an attempt to better understand possible mechanisms that might regulate *MdDHN11* expression during seed development, an *in silico* characterization of its promoter region was performed. As expected, several known *cis*-elements related to seed

specificity and dehydration were identified (Fig. 6A). However, the presence of these motifs is not evidence that they are functional or are controlling *MdDHN11* expression. One of these *cis*-elements, ABI3, was already described as needing two or more copies to properly regulate seed specificity [37]. Only 5% of all searched apple genes possessed this structure, including *MdDHN11*. A GO terms enrichment of these genes identified biological processes mainly related to reproductive processes (Fig. 6B), which is an indicative of the conserved role of this *cis*-element in apple. Additionally, the occurrence of the six seed-specific elements found in this work in apple gene promoters was quite high, ranging from 24 to 40% (~9,100 to ~15,000 genes). In agreement to that, a whole transcriptome analysis of 54 grapevine samples of different vegetative and reproductive organs at various developmental stages showed that almost 19,000 genes are expressed during seed development [46]. In order to evaluate the ability of *MdDHN11* promoter driving transgenic seed-specific expression in Arabidopsis, we fused its promoter to *GFP-GUS*. Unfortunately, we did not identified GUS staining in any of the several different tissues analyzed from transgenic plants (Fig. 7). One possible explanation is the different seed structure between apple and Arabidopsis. In Arabidopsis, the nucellus is among the first tissues to degenerate during seed development by programmed cell death [40]. However, in apple the nucellus layer is still present at least 51 DAA (Fig. 2). Still, further experiments in plants with seed structures similar to apple are necessary to confirm or refute such hypothesis.

Taken together, the results gathered in this work provide evidence that MdDHN11 plays important roles during apple seed development by protecting the embryo and the endosperm from water deficit. The mechanism of action probably involves the interaction of MdDHN11 with proteins, membranes and other components in the cytoplasm and nucleus. Additionally, apple DHNs represent a new resource of genes for the generation of crops better adapted to grow in a changing environment under adverse environmental conditions.

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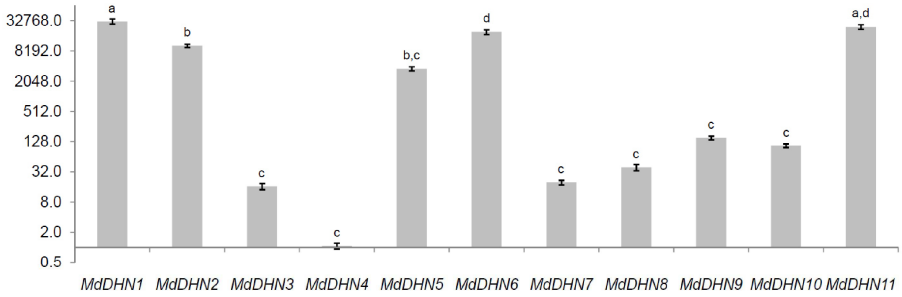
SUPPORTING INFORMATION

Supplementary Table 1. Primers employed in this work.

Primer name	Foward	Amplicon length (bp)
*MdDHN11*_CDS	Fw: CACCCTCGAGATGGCTCAAATTAGAGACGAGTACG	603
	Rv: AGATCTTCACTGGTGGCTATGATGACC	
Prom_*MdDHN11*	Fw: CACCAGAGGAGGGGGAACAAATATCC	2050
	Rv: TTGGATAATTTCCAAAAATGATCC	
*MdDHN11*_ISH/RT-PCR	Fw: CACCCTCGAGATGGCTCAAATTAGAGACGAGTACG	225
	Rv: CCAGAGCCGGGAACATG	
EGFP	Fw: CACATGAAGCAGCACGACTT	265
	Rv: AGTTCACCTTGATGCCGTTC	
AtACT2	Fw: GACCTTGCTGGACGTGACCTTAC	134
	Rv: GTAGTCAACAGCAACAAAGGAGAGC	



Supplementary Figure S1. Transcript accumulation levels of *MdDHN1-11* in seeds from apple mature fruits. Relative expression was plotted using *MdDHN4* as calibrator. Standard error bars are shown. Means with the same letter are not significantly different for $p<0.01$ by repeated measure ANOVA followed by Tukey's test.

6 CAPÍTULO IV

Evolutionary analysis of *galactinol synthase* genes in the Rosaceae family: new roles of galactinol and raffinose family oligosaccharides during bud dormancy

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Title

Evolutionary analysis of *galactinol synthase* genes in the Rosaceae family: new roles of galactinol and raffinose family oligosaccharides during bud dormancy

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Summary

1. Galactinol synthase (GolS) catalyses the first committed step in the biosynthetic pathway of raffinose family oligosaccharides (RFOs), which play key roles in carbon storage, signal transduction, and osmoprotection. Despite their importance, no relevant literature data accessing their full characterization during bud dormancy or in the Rosaceae family are available.
2. The evolutionary history of *GolS* genes was assessed in the Rosaceae family using several bioinformatic tools. *GolS* genes were identified and transcriptionally characterized during bud dormancy in apple, along with galactinol and raffinose metabolites. Subcellular localization of MdGolS2 was also determined.
3. Our evolutionary analyses revealed that *GolS* genes are constantly evolving within each order, family and species analyzed. Besides, the highest transcript amounts of apple *GolS1-2* occurred during bud dormancy, concomitantly with the highest levels of galactinol and raffinose. Therefore, transcript and carbohydrate quantifications showed a strong positive correlation.
4. Whole genome duplications have most probably driven the evolution and diversification of *GolS* genes in Fabidae and Rosaceae speciation. Moreover, the data gathered in this work allow us to suggest that RFOs are acting in the protection of bud integrity during adverse environmental conditions. The accumulation of RFOs in apple buds may serve both as protection as source of energy.

Key words: apple, dormancy, *Malus* x *domestica*, raffinose family oligosaccharides, whole-genome duplication

Introduction

Gene and genome duplication events yielded the raw genetic material for biological evolution, with major impacts on most eukaryotes, including plants (Zhang, 2003). Whole-genome duplication (WGD) has been a common feature during angiosperm speciation and is considered to be a driving force in evolution due to the innovations associated with these events (Jiao *et al*., 2011). In flowering plants, several rounds of WGD were already traced, including an ancient event occurred 200 million years ago (MYA) and shared even between basal angiosperms such as *Amborella trichopoda (Amborella* Genome Project, 2013). In the particular case of eudicots, a triplication event that occurred about 125 MYA has shaped their genomes, and contributed to molecular and phylogenetic diversification (Jiao *et al*., 2012). Apple (*Malus* x *domestica* Borkh.), the main fruit crop of temperate regions worldwide, belong to the Rosaceae family and was subjected to these duplication events during the course of its evolution. Moreover, apples also underwent a recent WGD event around 50 MYA that is restricted to the Pyreae clade, which encompasses pear and various Rosaceae genera (Velasco *et al*., 2010; Wu *et al*., 2013). These events triggered the emerging of specific subclades of genes, resulting in unique developmental and metabolic traits.

Temperate fruit crops such as apple are subjected to alternated growth and arrest periods along the year. The latter, called dormancy, is an adaptive trait for plant survival under adverse environmental conditions, and a well-adjusted dormancy cycle is crucial for the full achievement of their genetic potential (Falavigna *et al*., 2015b). This process is characterized by the formation of stress-tolerant specialized bud structures that carry a developmental program for the protection of meristems (Rohde & Bhalerao, 2007). Recent findings indicated that C-repeat binding transcription factors (CBF), major integrators of the cold response and freezing tolerance, may also modulate dormancy in apple (Wisniewski *et al*., 2015; Artlip *et al*., 2016). Besides genes responsible for the molecular control of dormancy progression, a series of proteins and metabolites were also shown to be recruited for the protection of bud integrity during winter. Dehydrins, sugars, detoxification enzymes, chaperones, protein kinases, and other compounds stand out in the process (Maruyama *et al*., 2009; Eremina *et al*., 2016). Supporting these findings, several gene expression studies carried out during bud dormancy have demonstrated the induction of many cold- and dehydration-related genes that integrate the CBF regulon, with

galactinol synthases (GolS) being recurrent in the reports (Derory *et al*., 2006; Santamaría *et al*., 2011; Liu *et al*., 2012; Falavigna *et al*., 2014).

GolS (EC 2.4.1.123) is a key enzyme catalyzing the first committed step in raffinose family oligosaccharides (RFOs) biosynthesis, responsible for galactinol formation using UDP-galactose and *myo*-inositol as substrates (Elsayed *et al*., 2014). GolS proteins integrate a subfamily of the GT8 family of glycosyltransferases that are involved in the synthesis of diverse sugar conjugates important in structure, storage, energy and signaling (Sengupta *et al.*, 2012). Galactinol is a building block used in the synthesis of raffinose, stachyose, and other RFOs. These sugars have been described to accumulate in response to cold, freezing, and water deficit (Sengupta *et al*., 2015). Moreover, galactinol and RFOs are compatible solutes that play roles in carbon storage, osmotic adjustments, signal transduction, membrane and protein stabilization, among other functions (Krasensky & Jonak, 2012; Elsayed *et al*., 2014; Sengupta *et al*., 2015). The accumulation of galactinol and RFOs during the last stages of seed development is responsible for protecting the embryo during seed desiccation, being necessary for the proper seed germination in pea (Blöchl *et al*., 2007). Finally, *GolS* induction during bud dormancy progression likely results in the seasonal mobilization of RFOs that lead to cold hardiness (Santamaría *et al*., 2011; Unda *et al*., 2012; Ibáñez *et al*., 2013; Falavigna *et al*., 2014).

In the present work, apple *GolS* genes were identified and characterized during bud dormancy and in several other developmental stages. We assessed the evolutionary history of GolS in a branch of the Fabidae clade, and more specifically in the Rosaceae family. The data gathered in this work allow us to suggest that protective roles are played by galactinol and RFOs during seed development and bud dormancy, acting on their protection and providing energy for germination and budbreak, respectively. Our evolutionary analyses showed that duplication events have driven the evolution and diversification of *GolS* genes during Fabidae speciation.

Materials and Methods

Plant material

Apple samples were harvested in two experimental orchards located in Vacaria/RS (-28.513777, -50.881465 and 972 m altitude) and Caçador/SC (-26.836971, -50.975246 and 935 m altitude), Brazil. In Vacaria, 3-year-old 'Gala Baigent' trees grafted on

Marubakaido rootstocks with M.9 as interstock were disposed in three sampling blocks of ten plants each. Six developmental stages were partitioned in different tissues and organs resulting in 13 samples according to the Fleckinger scale (EPPO, 1984): dormant buds (A stage; 07/21/2009); buds at initial bursting (C stage; 09/04/2009); flower buds and young leaves (E2 stage; 09/12/2009); 10 mm-diameter whole set-fruits and associated leaves (I stage; 10/28/2009); 40 mm-diameter unripe fruits that were divided into pulp, seed and skin, as well as leaves (J stage; 12/08/2009). Mature fruits with 70 mm in diameter were also sampled and partitioned into pulp, seed and skin (called M; 02/02/2010). Samples were immediately frozen in liquid nitrogen in the field and stored at -80ºC until use. See Falavigna *et al*., 2015a for images of the developmental stages. In Caçador, 7-year-old 'Fuji Standard' trees grafted on M.7 rootstocks were disposed in three sampling blocks of four plants each. Forty closed terminal buds from each plant were sampled at eight time points from January 2009 to February 2010 always at 11:00 am. Samples were immediately frozen in liquid nitrogen in the field and stored at -80ºC until use. Sampling dates and corresponding chilling hours (number of hours below 7.2ºC) accumulated by these samples are depicted in Table S1.

Gene expression analysis

DNA was purified from 200 mg of mature leaves of 'Gala Baigent' trees using modified protocols adapted to 2 mL tubes (Lodhi *et al*., 1994; Lefort & Douglas, 1999). Total RNA was isolated as described in Zeng & Yang (2002) and Falavigna *et al*. (2014), and DNase-treated using the TURBO DNA-free Kit (Ambion, Austin, USA). The GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, USA) was used for cDNA synthesis according to manufacturer's instructions. DNA-free RNA samples from mature seeds and dormant buds were also employed in 5' and 3' Rapid Amplification of cDNA Ends (RACE) synthesis using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, USA) according to the manufacturer's protocol. RACE products were sequenced at ACTGene Ltd. (Porto Alegre/RS, Brazil) using an automatic ABI-PRISM 3100 Genetic Analyzer and associated chemistry (Applied Biosystems). Real-time PCR was performed as described (Falavigna *et al*., 2015a). PCR efficiency and mean relative gene expression were calculated using LinRegPCR v.2012.0 (Ruijter *et al*., 2009) and Pfaffl (2001) equation, respectively. *ARC5* (Accumulation and Replication of

Chloroplast 5), *MDH* (malate dehydrogenase) and *WD40* (transcription factor *WD40*-like repeat domain) or *ARC5*, *MDH* and *TMp1* (*type 1 membrane protein-like*) were used as reference genes for closed terminal buds or for organ/tissue samples, respectively (Perini *et al*., 2014).

Carbohydrate analysis

Carbohydrates were extracted using modified protocols (Amaral *et al.*, 2007; Eyles *et al.*, 2013). Briefly, galactinol, raffinose and sucrose levels were determined from 100 mg of pulverized closed terminal buds from 'Fuji Standard'. Soluble carbohydrates were extracted with 1 mL of 80% ethanol in an 80ºC water bath for 30 min. After centrifugation (10,000 rpm for 5 min), supernatant was stored and samples were re-extracted 5-times. Three independent extractions were conducted with each biological replicate. In order to measure extraction efficiency, an additional independent extraction was conducted adding 100 mg/L of each analyte as internal standards. After extraction, a purification step was performed to remove lesser polar compounds. Volumes of 500 µL of chloroform and 400 µL of water were added to 1 mL of total supernatant followed by vigorous shaking (10 s) and resting (5 min). After centrifugation (13,000 rpm for 5 min), supernatant was collected and filtered (0.22 µm). An ultra-performance liquid chromatography (Waters Acquity UPLC system, Milford, USA) coupled to a triple quadrupole mass spectrometer (MS Waters Xevo TQ) was used for separation and quantitation of carbohydrates. A set of Acquity UPLC BEH amide pre-column (2.1 x 5.0 mm, 1.7 micron particles) and column (2.1 x 100 mm, 1.7 micron particles) was applied for the chromatographic separation. The mobile phase consisted of (A) 0.05% ammonia in water and (B) 0.05% ammonia in acetonitrile. A linear gradient condition from 20% A:80% B to 35% A:65% B at 8 min, which then returned to the initial condition for additional 7 min was established. The column was held at 35ºC and the sample compartment at 7ºC. An injection volume of 2 µL was employed and the flow rate was fixed at 0.3 mL/min. Under these conditions, retention times were: sucrose, 4.3 min; raffinose, 6.8 min; and galactinol, 7.4 min. MS was operated in negative ion electrospray mode with 2.5 kV of capillary voltage, 150ºC of ion source temperature, 800 L/h of desolvation gas (nitrogen), 20 L/h of cone gas flow and 600ºC of desolvation temperature. Data acquisition was conducted with MassLynx version 4.1 (Waters) and the multiple reaction monitoring (MRM) modes. The MRM transitions, cone

and collision voltages were, respectively: sucrose, 341->178.4 m/z, 35V and 15V; raffinose 504->221 m/z, 35V and 25V; galactinol 341->179, 33V and 25V. The quantification was done by calibration curves from eleven concentrations ranging from 0 to 50 mg/L of each sugar. Repeated measure analysis of variance (ANOVA) followed by Tukey's post hoc test with 99% confidence interval were used to evaluate the statistical significance of differences in carbohydrate levels between sampling points using GraphPad Prism 5.0a (La Jolla, USA). A correlation analysis was conducted using Pearson's two-tailed test at a 0.01 significance level using GraphPad Prism.

Subcellular localization of MdGolS2

In order to analyze the subcellular localization of MdGolS2, a transient expression assay was performed in *Arabidopsis thaliana* Col-0 leaf protoplasts. The complete coding sequence of *MdGolS2* was amplified with high fidelity enzymes (Invitrogen, Waltham, USA) using gene-specific primers (Table S2) and cloned into pENTR™ Directional TOPO® (Invitrogen). The *MdGolS2* gene was fused to *EYFP*-coding gene (enhanced yellow fluorescent protein) at its 5' portion through the p2YGW7 vector (Karimi *et al*., 2007) using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen) according to the manufacturer's instructions. The resulting vector (35S::*EYFP-MdGolS2*) was used in protoplast transformation. Protoplast isolation and transformation was performed as described (Wu *et al*., 2009). Negative controls were composed by untransformed cells, whereas positive controls were cells transformed with 35S::*EGFP* (enhanced green fluorescent protein). Transformed protoplasts and negative controls were incubated under light for 16-48 h at 24ºC prior to imaging. Cell nuclei were stained using 1 µg/mL of 4',6-diamidine2'-phenylindole dehydrochloride (DAPI). Fluorescence microscopy was performed using an Olympus FluoView 1000 confocal laser-scanning microscope (UFRGS Electron Microscopy Center, Porto Alegre/RS, Brazil) equipped with a set of filters capable of distinguishing chlorophyll autofluorescence, DAPI, EGFP and EYFP fluorescences. Protoplast isolation and transformation procedures were repeated twice in order to confirm obtained results.

In silico analysis

In order to identify predicted gene models coding for GolS in the apple genome version 1.0 (http://rosaceae.org/; Velasco *et al*., 2010), Blastp searches were performed using a sequence previously identified by our group as query (MDP0000209143; ABD15 in Falavigna *et al*., 2014; Altschul *et al*., 1990). Resulting sequences were annotated by comparison with the NCBI non-redundant protein database using Blast2GO version 3.0.9 (*E*-value cutoff of $1e^{-5}$; Conesa *et al*., 2005) and then screened for the presence of the GT8 domain (PF01501) in the SMART database (Letunic *et al*., 2012). Primers were designed using Primer3 v0.4.0 (Table S2; Rozen & Skaletsky, 2000). The exon/intron structures of *MdGolS* genes were compiled into a figure using Fancy Gene v1.4 (Rambaldi & Ciccarelli, 2009).

The available annotated genomes from Rosales, Fabales, Fagales and Curcubitales from the Fabidae clade were screened for the presence of GolS proteins in GigaDB (http://gigadb.org/), Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html), PLAZA (http://bioinformatics.psb.ugent.be/plaza/), NCBI (http://www.ncbi.nlm.nih.gov/) and Rosaceae (http://rosaceae.org/) databases using the same MdGolS query sequence previously identified by the group as query in Blastp searches (MDP0000209143; accessed in May 2016). All identified sequences were trimmed as previously described. Resulting GolS amino acid sequences from adzuki bean, apple, *Arachis duranensis*, *Arachis ipaensis*, bean, black raspberry, chick pea, Chinese white pear, cucumber, European pear, Japanese apricot, jujube, lotus, medicago, mulberry, mung bean, muskmelon, peach, pigeon pea, soybean, strawberry, watermelon, wild soybean and woodland strawberry (Table S3) were aligned using ClustalW (Thompson *et al*., 1994) and phylogenetic relationships were inferred using MrBayes version 3.2.6 (Ronquist *et al*., 2012). A glycogenin-like starch initiation protein-B (PGSIP-B) sequence from soybean was used as outgroup (Yin *et al*., 2010). The mixed amino acid substitution model was used in the default settings and 12 million generations were run, sampled every 100 generations, with the first 25% trees discarded as burn-in. The remaining ones were summarized in a consensus tree that was visualized and edited in FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Collinear block analyses were performed by intra- and interspecific genome-wide protein sequence comparison for Rosaceae species using Blastp (*E*-value < $1e^{-10}$). Results

and gene positions were used as inputs to determine the collinear blocks using MCScanX (Wang *et al*., 2012). Ka (nonsynonymous substitutions rate) and Ks (synonymous substitution rates) from paralogs were calculated using downstream analysis tools implemented in the MCScanX software. Additionally, Ks-dating was calculated from collinear blocks containing paralogous *GolS* genes. To this purpose, six gene pairs flanking each *GolS* gene were used, and if fewer than 12 homologous genes were found then all available gene pairs were used instead. Finally, the type of gene duplication was determined using MCScanX tools.

Results

GolS *genes in apple*

The apple genome was screened for GolS sequences by means of Blastp searches using MDP0000209143 as query (Falavigna *et al*., 2014). Resulting sequences were compared to SMART and NCBI non-redundant protein databases (Conesa *et al*., 2005; Letunic *et al*., 2012), and sequences lacking the GT8 domain (PF01501) or not annotated as GolS-like, respectively, were excluded. Additionally, multiple alignments of remaining sequences allowed us to exclude four hits due to their high amino acid sequence similarity (over 94%). This led to the identification of ten predicted gene models coding for GolS in apple (Table 1). Only five of them were successfully amplified when using a pool of cDNAs from 13 different apple tissues and organs (Fig. S1). These five genes were named *MdGolS1-5* and were amplified by RACE and sequenced (Table 1). Only *MdGolS2* and *3* showed differences in comparison to the genomic sequence, a misprediction in the second exon that resulted in 30 additional nucleotides and three nucleotide mismatches leading to two amino acids changes, respectively (Fig. S2).

The structures of *MdGolS* genes were obtained from the apple genome and curated with our sequencing data, when available (Fig. 1). *MdGolS1*, *2*, *3* and *5* showed four exons, *MdGolS4* and *10* presented three exons, and *MdGolS6* exhibited two exons. The remaining sequences displayed more than four exons in their structure, with *MdGolS8* and *9* showing the highest number of exons. The GT8 domain in apple of GolS-deduced proteins was composed by around 240 amino acids. All sequences showed one GT8 domain, except MdGolS7 that presented two. However, both domains were truncated in this sequence. MdGolS6 and 10 also presented a truncated version of the GT8 domain.

Table 1. Identification of *GolS* genes in apple.

Gene name	Genome accession	GenBank accession	Chromosomal location	Protein size (aa)
MdGolS1	**MDP0000208137**/ MDP0000231923	KDF578380	chr4:18788681..18790278/ chr4:18790825..18792432	333
MdGolS2	**MDP0000209143**	KDF578381	chr13:6216980..6218442	337
MdGolS3	**MDP0000412025**/ MDP0000251505	KDF578382	chr13:12198890..12200856/ chr13:12198044..12200010	328
MdGolS4	**MDP0000466683**/ MDP0000446914	KDF578383	chr17:24394945..24396399/ chr17:24409800..24411258	348
MdGolS5	**MDP0000587199**	KDF578384	chr16:4473935..4475280	302
MdGolS6	**MDP0000210400**	-	chr4:22368100..22369707	140
MdGolS7	**MDP0000213439**	-	chr16:7987604..7989954	312
MdGolS8	**MDP0000245723**	-	chr11:6156086..6161075	632
MdGolS9	**MDP0000267261**	-	chr11:6159157..6164973	615
MdGolS10	**MDP0000426442**	-	chr9:31566427..31567685	261

Genome and GenBank accession codes are provided by the '*Malus domestica* Genome' (http://rosaceae.org/) and NCBI (http://www.ncbi.nlm.nih.gov/) databases, respectively. Bold sequences were used in further analyses.

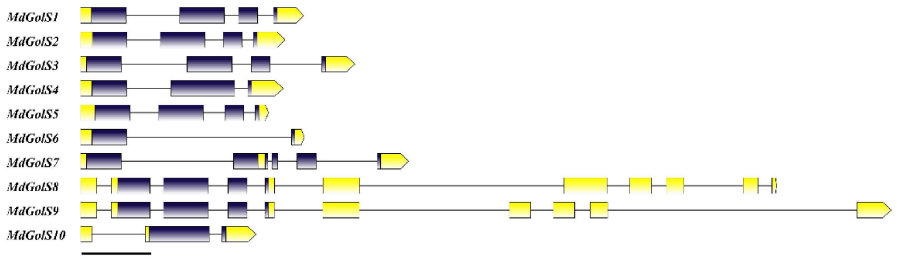

Fig. 1. Intron and exon structure of *MdGolS* genes. The GT8-coding domain is colored in blue and remaining exon sequences in yellow. Lines represent introns. Scale bar: 500 bp.

GolS evolution between Fabidae and Rosaceae

GolS were already characterized in wider phylogenetic scopes from green algae to plants (Yin *et al*., 2010; Sengupta *et al*., 2012). In this work, a deeper characterization was performed focusing on four orders that form a branch in the Fabidae clade, Curcubitales, Fabales, Fagales and Rosales based on Cantino *et al*. (2007) phylogenetic nomenclature of *Tracheophyta*. After an initial screening for the presence of *GolS* genes in the available genomes of these four orders, obtained sequences were trimmed, resulting in 130 putative genes coding for GolS (Table S3). The highest number of GolS members was identified among Rosales, with ten and twelve members in apple and European pear, respectively. The other species in Rosales displayed at least five GolS members. The species belonging to Curcubitales showed only three or four members, while species in Fabales ranged from

three to six members. No available annotated genomes from Fagales were found in the searched databases.

A phylogenetic tree was constructed in order to identify evolutionary relationships of GolS proteins among three orders of the Fabidea clade (Fig. 2). Based on previous phylogenetic analysis (Yin *et al*., 2010), a PGSIP-B from soybean was used to root the tree, given that this protein is evolutionarily close to GolS, although belonging to another subfamily of the GT8 family domain. The tree topology resulting from the alignment of 131 full-length amino acid sequences showed a clear segregation between the orders analyzed. Five clusters were composed only by Rosales sequences (Fig. 2, orange), four clusters showed the presence of Fabales sequences (grey), and four clusters were composed by Curcubitales sequences (yellow). The phylogenetic analysis allowed us to suggest that the evolution of *GolS* genes was highly influenced by duplication events that occurred within each order of the Fabidae clade. Moreover, even within the Rosales order, it was possible to identify segregation between members of the Rosaceae family (apple, black raspberry, Japanese apricot, peach, pears and strawberries) that grouped together, from members of the Moraceae (mulberry) and Rhamnaceae (jujube) families. Phylogenetic analyses allowed the identification of apple orthologs with European or Chinese pear sequences, MdGolS1 with PbGolS4 and PcGolS9, MdGolS2 with PcGolS4, and MdGolS7 with PcGolS12 (Fig. 2). MdGolS3 formed a cluster with European and Chinese pear orthologs. MdGolS5 was present in a cluster with European pear paralogs. MdGolS6 was present in a cluster with GolS sequences from apple, pear, peach and Japanese apricot. Finally, remaining apple sequences grouped as paralogous pairs: MdGolS4 with MdGolS10 and MdGolS8 with MdGolS9.

In view of the obtained results from the phylogenetic approach, which suggested an independent evolution of *GolS* genes within each order of the Fabidae clade, collinearity and Ks-dating analyses were performed aiming to trace GolS evolutionary history inside the Rosaceae family. Our strategy was based on the comparison of the apple genome with those from black raspberry, Chinese pear, European pear, Japanese apricot, peach, strawberry and woodland strawberry. These comparisons rendered several collinear regions (Fig. 3, gray lines), including *GolS* genes (black lines), which allowed us to estimate the evolutionary age of the duplication event of these genes in the Rosaceae family. It is worth to mention that some genomes, i.e. black raspberry, European pear and

strawberry, are not assembled in chromosomes (chr), which resulted in the presence of few collinear blocks for these species.

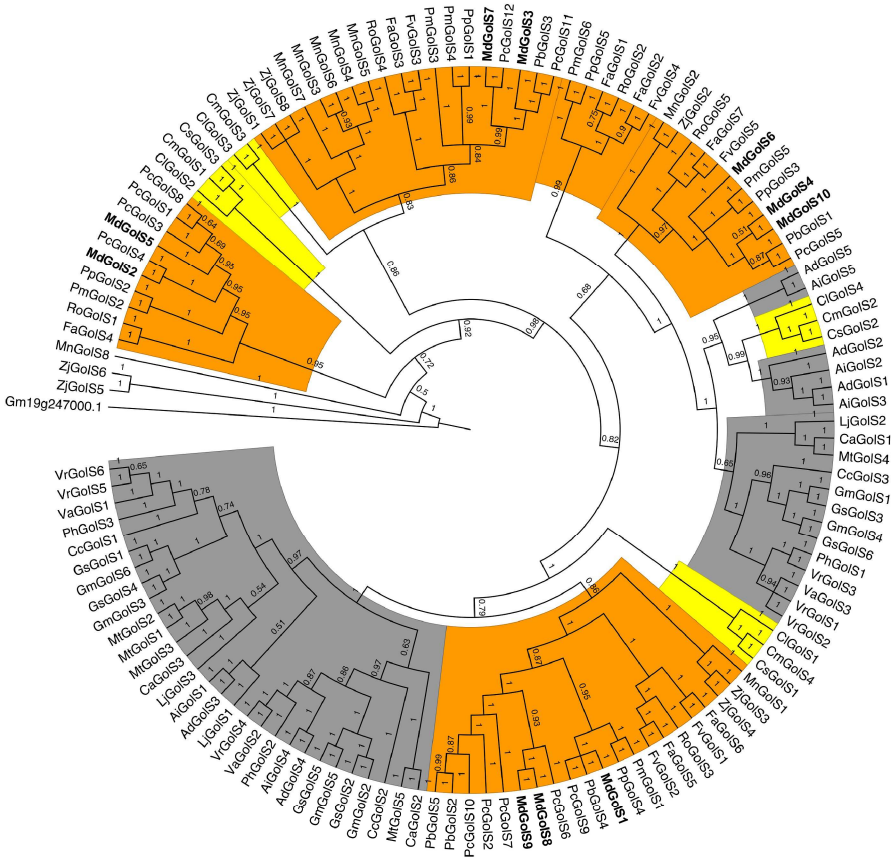

Fig. 2. Phylogenetic relationships of GolS protein sequences from 24 species belonging to Curcubitales (yellow), Fabales (gray) and Rosales (orange). The tree was inferred using MrBayes version 3.2.6 (Ronquist *et al*., 2012). Branch support is given by a posteriori probability values shown next to the corresponding branches (when > 0.5). A PGSIP-B sequence from soybean was used as outgroup. Accession codes used in the phylogenetic analysis are depicted in Table S3. Ad, *Arachis duranensis*; Ai, *Arachis ipaensis*; Ca, *Cicer arietinum*; Cc, *Cajanus cajan*; Cl, *Citrullus lanatus*; Cm, *Cucumis melo*; Cs, *Cucumis sativus*; Fa, *Fragaria* x *ananassa*; Fv, *Fragaria vesca*; Gm, *Glycine max*; Gs, *Glycine soja*; Lj, *Lotus japonicus*; Md, *Malus* x *domestica*; Mn, *Morus notabilis*; Mt, *Medicago truncatula*; Pb, *Pyrus bretschneideri*; Pc, *Pyrus communis*; Pm, *Prunus mume*; Pp, *Prunus persica*; Pv, *Phaseolus vulgaris*; Ro, *Rubus occidentalis*; Va, *Vigna angularis*; Vr, *Vigna radiata*; Zj, *Ziziphus jujuba*.

Within the apple genome, five pairs of collinear blocks were identified containing *GolS* genes, one between Mdchr4 and 16 (*MdGolS5*/*MdGolS6*), one between Mdchr9 and 16 (*MdGolS7*/*MdGolS10*), one between Mdchr9 and 17 (*MdGolS4*/*MdGolS10*), and two between Mdchr13 and 16 (*MdGolS2*/*MdGolS5* and *MdGolS3*/*MdGolS7*). Some of these

blocks are syntenic, given that they shared the same Pyreae ancestors such as Mdchr9 and 17 and Mdchr13 and 16 (Velasco *et al*., 2010). Only one block was identified between strawberry and apple (*MdGolS1*/*FaGolS5*, Fig. 3a). However, although woodland strawberry has fewer *GolS* members in comparison to strawberry (five and seven, respectively), the collinearity comparison with apple rendered five collinear blocks in addition to one block between Fvchr1 and 4 (Fig. 3b). *RoGolS3* shared four collinear blocks with three different apple chromosomes (Mdchr4, 13 and 16, Fig. 3c), whereas *RoGolS1* presented collinear regions with *MdGolS2* and *MdGolS5*. European pear, Japanese apricot and peach showed the highest number of collinear blocks with apple, eight, nine and ten, respectively (Fig. 3d-f). Several collinear genomic regions containing *GolS* genes were identified within each genome. Finally, although no collinear blocks were found between Chinese pear chromosomes for *GolS* genes, five blocks were identified linking pear and apple: one between Pbchr4 and Mdchr4; one between Pbchr11 and Mdchr11; one between Pbch13 and Mdchr13; one between Pbchr17 and Mdchr9; and one between Pbrchr17 and Mdchr17 (Fig. 3g). However, only five *GolS* genes were identified in the Chinese pear genome after trimming. This finding should be analyzed with caution, given that apple and European pear, species evolutionarily close to Chinese pear, presented 10 and 12 members, respectively.

Ks-dating was calculated aiming to estimate the time of gene duplications for all collinear blocks containing paralogous *GolS* genes in the Rosaceae family (Table 2). Interestingly, only apple and European pear paralogous sequences showed Ks values lower than 0.5, which corresponded to the recent WGD. *GolS* paralogs from Japanese apricot, peach and woodland strawberry showed Ks values ranging from 1.1 to 1.8. Two apple paralogs, *MdGolS5*/*MdGolS6* and *MdGolS7*/*MdGolS10*, also presented similar high Ks values. All these paralogs had their duplicated gene origin annotated as WGD events, suggesting that segmental duplications were the main contribution force to the expansion of *GolS* genes in the Rosaceae genomes. In order to identify if these Ks values higher than 1.1 corresponded to the triplication event or to the ancient WGD common to all angiosperms, we searched the genomes of grapevine and *Amborella* for *GolS* genes to gain insights (Jiao *et al*., 2011). Only two *GolS* genes were identified in *Amborella*, but no collinear blocks were identified between them (data not shown). Notwithstanding, 12 genes were identified in grapevine, with six collinear blocks containing *GolS* genes. The

Ks-dating of these collinear blocks ranged from 1 to 1.7 (Table S4). Interestingly, the six grapevine *GolS* genes outside the collinear blocks exhibited their duplicated gene origin annotated as tandem, instead of WGD.

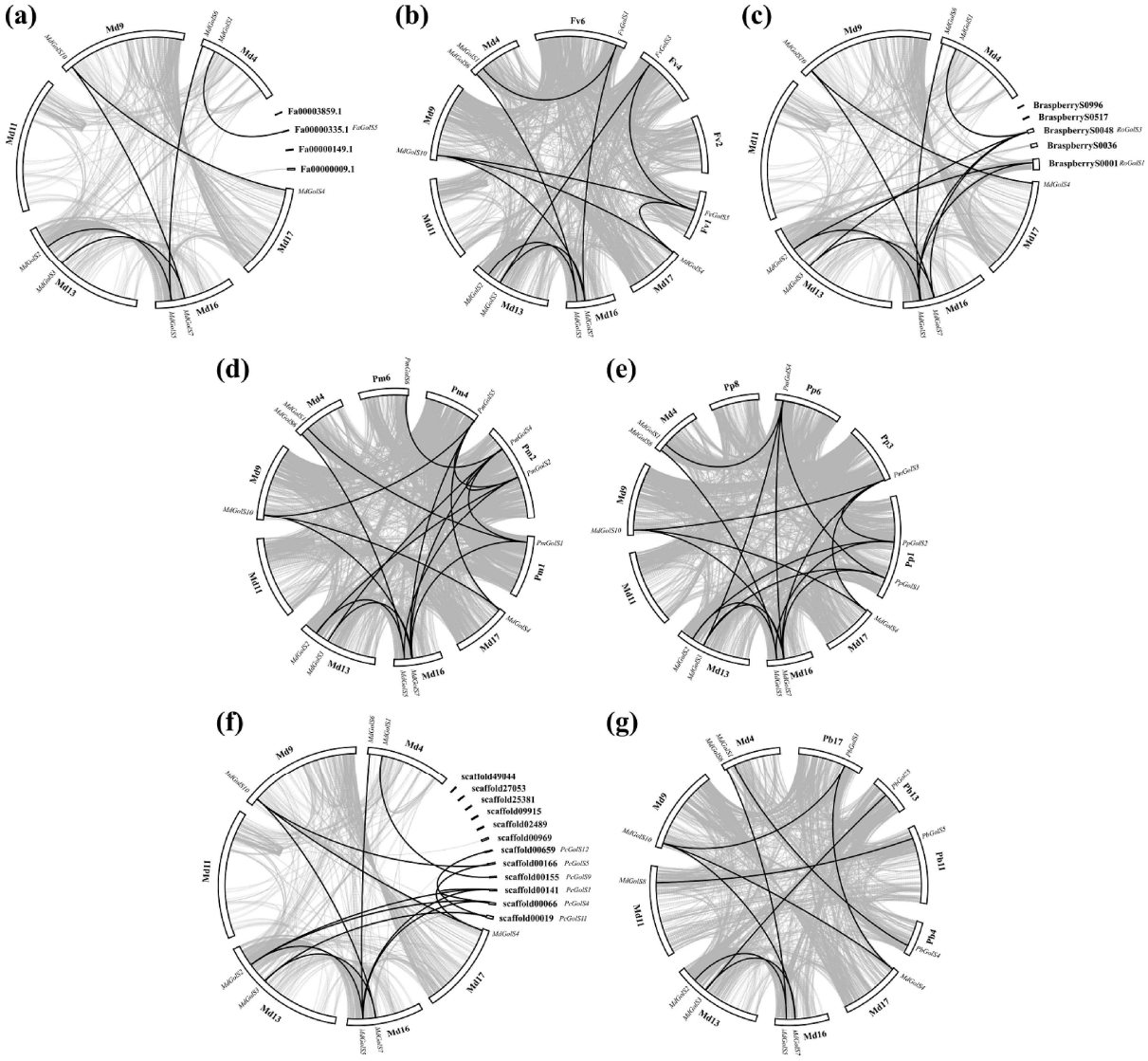

Fig. 3. Collinearity analyses among apple and other Rosaceae species. Chromosomes or scaffolds, when appropriate, containing *GolS* genes are shown. Collinear blocks with *GolS* genes are shown as black lines, whereas the remaining collinear blocks are shown in gray lines. Analysis performed included chromosomes/scaffolds from apple and **(a)** strawberry; **(b)** woodland strawberry; **(c)** black raspberry; **(d)** Japanese apricot; **(e)** peach; **(f)** European pear; and **(g)** Chinese pear.

Finally, the Ka/Ks ratio was calculated for all *GolS* paralogs in an attempt to identify the selection force that could be held accountable in these genes after the duplication events. The Ka/Ks ratio is used to identify positive selection, which is

399 indicated by values higher than 1. Ka/Ks ratios lower than 1 indicates negative selection,
400 whereas a ratio equal to 1 indicates neutral selection. The Ka/Ks ratios for the duplicated
401 *GolS* genes in the Rosaceae family were all lower than 0.55, suggesting that these genes
402 experienced purifying selection pressures with limited functional divergence after WGDs.
403 Taken together, these results suggest that WGD events played important roles in the
404 expansion of *GolS* genes in Rosaceae species, especially in apple.
405

406 **Table 2.** Ks-dating and Ka/Ks ratios of *GolS* gene duplication events.

Duplicated genes	Ks-dating of the collinear block	Ka/Ks ratio	Duplicated gene origin
MdGolS2/MdGolS5	0.450	0.173	WGD
MdGolS3/MdGolS7	0.299	0.415	WGD
MdGolS4/MdGolS10	0.192	0.538	WGD
MdGolS5/MdGolS6	1.234	0.458	WGD
MdGolS7/MdGolS10	1.337	0.280	WGD
FvGolS3/FvGolS5	1.116	0.359	WGD
PcGolS1/PcGolS4	0.183	0.141	WGD
PcGolS11/PcGolS12	0.180	0.321	WGD
PmGolS1/PmGolS4	1.156	0.101	WGD
PmGolS2/PmGolS4	1.462	0.140	WGD
PmGolS2/PmGolS5	1.572	-	WGD
PmGolS4/PmGolS6	1.559	0.154	WGD
PpGolS1/PpGolS4	1.148	0.118	WGD
PpGolS2/PpGolS3	1.797	0.054	WGD

407 Fv, *Fragaria vesca*; Ka, nonsynonymous substitutions rate; Ks, synonymous substitutions rate; Md, *Malus* x
408 *domestica*; Pc, *Pyrus communis*; Pm, *Prunus mume*; Pp, *Prunus persica*; WGD, whole-genome duplication
409

410 *Expressions patterns of* MdGolS *genes in apple tissues and organs*

411 Transcript analysis of ten *MdGolS* genes was conducted via real-time PCR in 13
412 apple tissues and organs during the progression of bud dormancy, flowering and fruit
413 ripening. *MdGolS1*, *3* and *4* were mainly expressed in mature seeds; *MdGolS2* was mainly
414 expressed in closed terminal buds; whereas *MdGolS5* was more highly expressed in buds
415 at initial bursting and leaves from I and J stages (Fig. 4). *MdGolS4* transcripts were not
416 found in closed terminal buds, although high transcript amounts were observed in buds at
417 initial bursting. Additionally, *MdGolS5* could not be detected in mature fruits, and
418 presented the lowest transcript levels over all samplings in comparison to other *MdGolS*

419 genes. Finally, no transcripts were detected for *MdGolS6-10* in any of the tissues or organs
420 analyzed (Fig. S1).
421
422 *Annual growth and dormancy cycle analysis*
423 Closed terminal buds from field-grown apple trees of 'Fuji Standard' were sampled
424 during a complete annual growth and dormancy cycle from summer 2009 (January 21st) to
425 summer 2010 (February 2nd). Chilling exposure, and growth cessation and resumption
426 (50% of buds in green tip stage) were tracked in order to determine dormancy
427 establishment, fulfillment and release (Table S1). The gene expression of *MdGolS1-5* was
428 quantified by real-time PCR throughout these sampling times (Fig. 5a). *MdGolS1* and *2*
429 clearly displayed a seasonal transcript accumulation during dormancy, with higher
430 amounts in the winter. Additionally, both genes were down-regulated near budbreak
431 (September 15th), with lower transcript levels during spring and summer. *MdGolS2*
432 reached the highest expression levels in comparison to other *MdGolS* genes in mid-winter
433 (July 30th). Contrastingly, *MdGolS3* and *5* presented low transcriptional variation during
434 the year. Finally, *MdGolS4* expression was completely abolished during dormancy
435 entrance (May 27th, autumn) and maintenance (June 30th, winter), restoring its
436 transcriptional levels during mid-winter (July 30th), spring and summer.
437 The seasonal expression profile observed for some *MdGolS* genes prompted us to
438 quantify galactinol, raffinose and sucrose levels via UPLC during the same annual
439 sampling cycle of 'Fuji Standard' apple buds (Fig. 5b). The sugar extraction efficiency was
440 above 95% for all three analytes. Galactinol and raffinose started to accumulate during
441 dormancy establishment (May), reaching their highest amounts during deep dormancy
442 (June and July), and restoring to low levels after dormancy overcoming (November 2009
443 to February 2010). Raffinose levels were approximately 180-fold higher in samples
444 harvested in July in comparison to summer samples, while galactinol levels were
445 approximately 14-fold higher in the same comparison. This seasonal accumulation was
446 statistically significant. Moreover, the transcript level of *MdGolS1-2* and the galactinol and
447 raffinose contend during the year showed a strong positive correlation (Table S5). Finally,
448 sucrose levels were higher during winter (June, July and September) in comparison to
449 autumn, spring and summer samples. The most contrasting points (September and March
450 2009) differed 3-times.
451

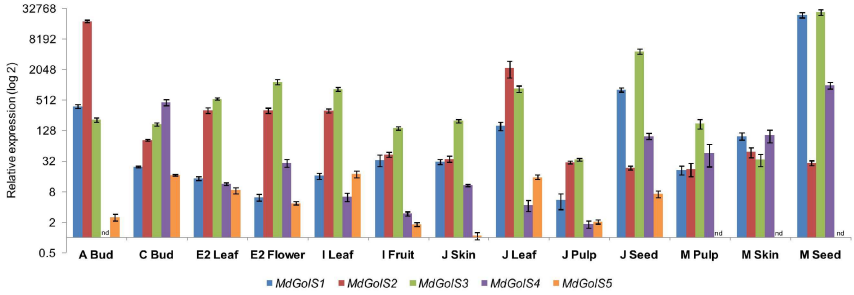

Fig. 4. Transcript accumulation kinetics of *MdGolS1-5* in 13 apple tissues and organs. Stages A, C, E2, I and J were sampled according to the Fleckinger phenological scale (EPPO, 1984), whereas 'M' stands for mature fruits. Nd, non-detected. Relative expression was plotted using *MdGolS5* gene expression levels in skin from J stage as calibrator. Standard error bars are shown.

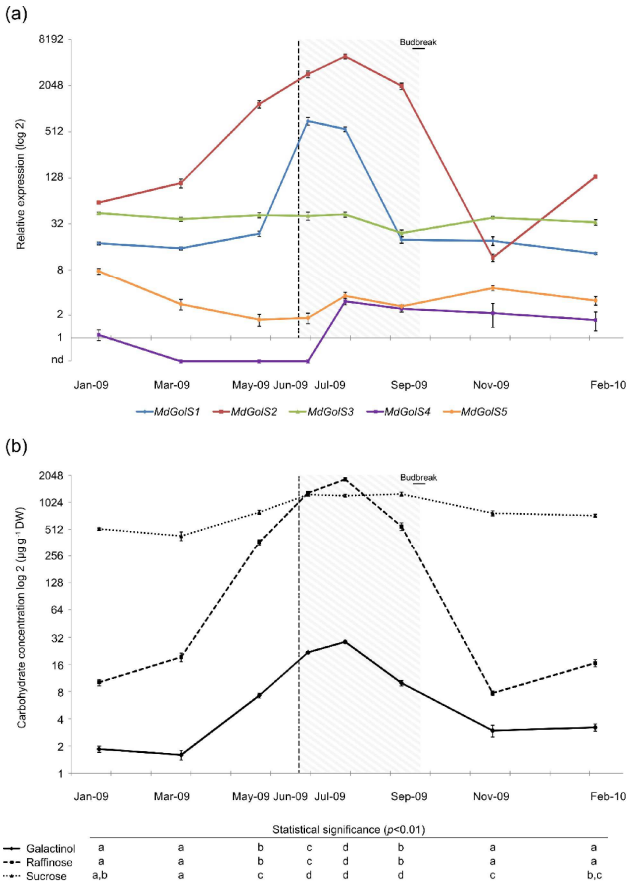

Fig. 5. Annual growth and dormancy cycle analysis of 'Fuji Standard' closed terminal buds. The vertical dashed line indicates the approximate winter solstice (June 21th); shaded area corresponds to the winter season; and the horizontal solid bar corresponds to the budbreak interval (50% of buds in green tip stage). Standard error bars are shown. **(a)** Seasonal *MdGolS* transcription analysis. Relative expression was plotted using *MdGolS4* transcript levels in January 2009 as calibrator. Nd, non-detected. *MdGolS2* graphic, with modifications, was reproduced from Falavigna *et al*. (2014) with permission of the publisher. **(b)** Galactinol, raffinose and sucrose levels in apple buds determined by UPLC. Means with the same letter are not significantly different for $p<0.01$ by repeated measure ANOVA followed by Tukey's test. DW, dry weight.

Subcellular localization of MdGolS2

The seasonal accumulation of *MdGolS2* transcripts, combined with the accumulation of galactinol and raffinose during winter, suggest that these metabolites may protect bud integrity during dormancy. In order to gain insights about *MdGolS2* function, a subcellular localization assay was performed. Transient expression analyses in Arabidopsis leaf protoplasts revealed that MdGolS2 was present in the nucleus and cytoplasm (Fig. 6). The best results were obtained after approximately 16 hours of incubation, whereas after 48 hours almost no EYFP signal was detectable in transformed cells. Besides, it is worth to mention that a low transformation efficiency with the 35S::*EYFP-MdGolS2* construction was achieved in relation to positive control. Positive controls showed similar subcelular localization, whereas negative control showed only DAPI and chlorophyll fluorescence.

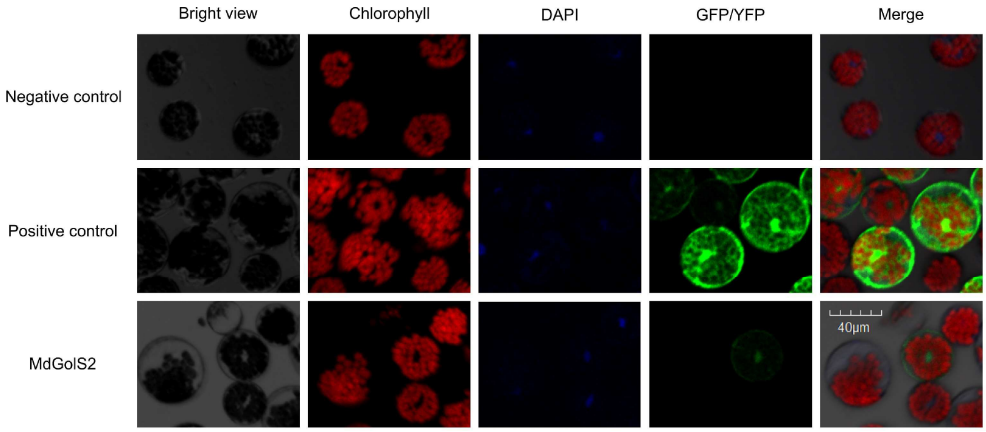

Fig. 6. Subcellular localization of MdGolS2 in Arabidopsis leaf protoplasts was obtained through transient expression of 35S::*EYFP-MdGolS2*. Negative and positive controls were composed by untransformed cells or cells transformed with 35S::*EGFP*, respectively. All images were captured with a confocal laser scanning system. Green, EYFP or EGFP; red, chlorophyll; blue, DAPI.

Discussion

Galactinol synthase (GolS) is a key enzyme in the biosynthetic pathway of RFOs, which are carbohydrates that play roles in carbon storage, signal transduction, osmeoprotection, among others (reviewed in Elsayed *et al*., 2014 and Sengupta *et al*., 2015). Several studies identified *GolS* genes being induced during bud dormancy and winter, and this likely result in a seasonal mobilization of carbohydrates leading to cold hardiness and protection (Derory *et al*., 2006; Santamaría *et al*., 2011; Liu *et al*., 2012; Unda *et al*., 2012; Ibáñez *et al*., 2013; Falavigna *et al*., 2014; Yue *et al*., 2015). Despite the

497 importance of *GolS* genes and the growing availability of sequenced plant genomes, few
498 reports in the literature characterized these genes (Taji *et al.*, 2002; Yin *et al.*, 2010;
499 Philippe *et al.*, 2010; Zhou *et al.*, 2012b; Filiz *et al.*, 2015). Given that no characterization
500 studies were found for *GolS* genes in the Rosaceae family, including apple, we decided to
501 gain advantage of the public access to several genomes and perform an evolutionary
502 analysis of these genes. In addition, considering that a previous work from our group
503 identified an apple *GolS* highly induced during bud dormancy (Falavigna *et al*., 2014), we
504 characterized *MdGolS* genes within the context of bud dormancy.

505 In apple, ten predicted gene models coding for GolS were identified (Table 1). This
506 amount of genes resembles that found in Arabidopsis and poplar genomes, with eight and
507 nine members, respectively (Yin *et al*., 2010). Interestingly, all three species were
508 subjected to recent WGD events (Velasco *et al*., 2010; Jiao *et al*., 2011). Moreover, the
509 only sequences that presented a complete GT8 domain and similar exon/intron structure
510 were *MdGolS1-5* (Fig. 1), exactly those expressed under the conditions analyzed (Fig. S1).
511 Interestingly, the prevailing structure of Arabidopsis and poplar *GolS* sequences is four
512 exons and three introns, the same structure observed for *MdGolS1*, *2*, *3* and *5*. In
513 agreement to the organ-selective *GolS* expression observed in apple, Arabidopsis *GolS5*, *6*,
514 *7*, *9* and *10* are almost undetectable in leaves (Nishizawa *et al*., 2008). Finally, all apple
515 GolS have a putative serine phosphorylation site around amino acid position 265, except
516 MdGolS6, and this is a characteristic feature of nearly all GolS proteins reported (Taji *et*
517 *al*., 2002).

518 With the aim of tracing the evolutionary history of GolS in a branch of the Fabidae
519 clade, and more specifically in the Rosaceae family, several experiments addressing the
520 evolution of GolS were conducted. Given that no orthologs were identified between
521 species of different orders (Fig. 2), we suggest that *GolS* genes are constantly evolving
522 within each order of the Fabidae clade. In a similar fashion, segregation between monocot
523 and dicot GolS sequences were already reported, even showing that the diversification in
524 dicots is greater (Sengupta *et al*., 2012; Elsayed *et al*., 2014). Our finding is corroborated
525 by the high range of GolS members in each order. In the specific case of Rosales, it was
526 possible to identify a separation between the Rosaceae family in comparison to the
527 Moraceae and Rhamnaceae families. In agreement, jujube and mulberry are evolutionary
528 close species, and presented a higher number of *GolS* genes (eight members each) in

comparison to Rosaceae species that did not undergo a recent WGD such as peach (five), Japanese apricot (six) and woodland strawberry (five). Interestingly, the jujube genome is enriched of gene families involved in sugar metabolism in relation to other Rosales species (Liu *et al*., 2014), which could explain the higher number of *GolS* genes in this species. When analyzing only the Rosaceae family, apple and pears GolS sequences grouped together in the phylogenetic tree, confirming that they are evolutionarily close (Wu *et al*., 2013; Chagné *et al*., 2014). Moreover, they experienced a recent WGD event that is not shared by other Rosaceae species such as black raspberry, Japanese apricot, peach, or strawberries (Jung *et al*., 2012; Zhang *et al*., 2012). This may partially explain the high number of *GolS* genes in apple and European pear, and confirms that *GolS* genes are constantly evolving within each species.

Collinearity analysis is a useful tool to determine the history of large chromosome blocks (Wang *et al*., 2012). However, one of the main problems of such analysis resides in genomes that are not fully assembled or anchored in genetic maps or individual chromosomes. This is a common trend that complicates the inference of syntenic relationships (Kellogg, 2016). Unfortunately, some Rosaceae genomes are yet not fully assembled, which results in incomplete analysis. Nevertheless, genomes from apple, Chinese pear, peach, Japanese apricot and woodland strawberry are well assembled and provided very interesting results. In our studies, several collinear regions containing *GolS* genes were identified between apple and Chinese pear, Japanese apricot, peach, or woodland strawberry (Table S6), representing good examples of the syntenic relationships that exists between these genomes (Jung *et al.*, 2012; Zhang *et al.*, 2012; Wu *et al.*, 2013). Interestingly, all duplicated gene origins in Rosaceae corresponded to WGDs (Table 2), supporting evidences that the expansion of *GolS* genes was strongly influenced by these kind of events. The recent WGD event that is shared between apples and pears (Velasco *et al*., 2010; Wu *et al*., 2013) could be traced by Ks values lower than 0.45 (Table 2). In *A. trichopoda*, the ancient WGD was traced to a Ks value around 1.84 (*Amborella* Genome Project, 2013), and it is estimated that Ks values lower than 1.5 were originated after the divergence of *Amborella* and the rest of the angiosperms (Jiao *et al.*, 2011). However, no collinear blocks were identified between both *Amborella GolS* genes (data not shown). Within this context, *GolS* paralogs from apple, Japanese apricot, peach, woodland strawberry and grapevine (Table 2 and S3) with Ks values higher than 1 were originated in

561 the triplication event. Specifically in apple, the expansion of *GolS* genes was possibly
562 driven by both triplication and recent WGD events (Table 2).

563 In general, paleopolyploidy events may provide opportunities for positive selection,
564 generating advantageous mutations that persist through evolution by retaining duplicated
565 copies of genes (Yang & Bielawski, 2000; Zhang, 2003). The Ka/Ks ratios of the
566 duplicated *GolS* genes in Rosaceae showed strong purifying selection after WGD events.
567 In several other plants, genes derived from older duplication events also tended to have
568 experienced stronger purifying selection (Zou *et al.*, 2009; Körbes *et al.*, 2016). This
569 finding suggests that *GolS* members are evolving together, with duplication events being
570 responsible for shaping the number of these genes within each species.

571 To gain insights over possible roles of *MdGolS* genes, their transcript accumulation
572 profiles were determined in samples from bud dormancy progression to flowering and fruit
573 ripening stages (Fig. 4). *MdGolS1*, *3* and *4* presented their highest transcript amounts in
574 mature seeds. This finding agrees with previous reports that identified *GolS* accumulation
575 during the last stages of seed development (reviewed in Elsayed *et al*., 2014 and Sengupta
576 *et al*., 2015). The proposed role of GolS in seeds would be increasing seed RFOs amounts,
577 offering seed desiccation tolerance and energy during germination (Blöchl *et al.*, 2007;
578 Elsayed *et al.*, 2014; Sengupta *et al.*, 2015). On the other hand, *MdGolS2* was mainly
579 expressed in dormant buds. Interestingly, almost all *MdGolS* genes were highly expressed
580 in dormant organs, i.e. buds and seeds. Common pathways between bud and seed
581 dormancy were already reported in peach, especially those involving adaptive responses
582 such as the accumulation of osmoprotectants (Leida *et al*., 2012). In apple, these
583 relationships were already found for dehydrin proteins, which are involved in plant
584 responses to dehydration (Falavigna *et al*., 2015a).

585 Transcript levels of *MdGolS* genes as well as carbohydrate contents were quantified
586 in apple buds during a typical year. *MdGolS1* and *2* displayed seasonal variation of
587 transcripts along the year, reaching their highest amounts during winter (Fig. 5a). In
588 agreement, galactinol and raffinose levels showed similar seasonal patterns (Fig. 5b) and a
589 strong positive correlation with *MdGolS1* and *2* gene expression (Table S5). Taken
590 together, our findings strongly suggest that the seasonal carbohydrate mobilization of
591 RFOs during winter is being performed by *MdGolS1* and *2*, and that this metabolic
592 pathway confers protection to bud integrity and yields energy for growth after budbreak.

Similar *GolS* transcript patterns during dormancy were already identified in chestnut, hybrid poplar, pear and sessile oak (Derory *et al*., 2006; Santamaría *et al*., 2011; Liu *et al*., 2012; Unda *et al*., 2012; Ibáñez *et al*., 2013), whereas similar raffinose content variation was identified in trembling aspen (Cox & Stushnoff, 2001). Additionally, the MdGolS2 protein localization was identified in the cytoplasm and nucleus (Fig. 6), and several evidences indicated that GolS proteins, like raffinose synthases, are also localized in the cytoplasm (Schneider & Keller, 2009). The localization and function of MdGolS2 in nuclei needs to be better characterized. We cannot rule out the possibility of protein diffusion or membrane binding rather than targeting MdGolS2 to the nucleus. The GolS1 from cotton, for example, was shown to be a membrane-associated protein (Zhou *et al.*, 2012a).

Our results suggest that galactinol and raffinose integrate a series of mechanisms that act together during dormancy in order to protect the bud from adverse conditions. Galactinol and RFOs function is probably based on their ability to scavenge hydroxyl radicals as well as their osmolyte property, maintaining cell turgor and stabilizing cell proteins (Nishizawa *et al*., 2008; Elsayed *et al*., 2014). Moreover, their reduced levels prior budbreak strongly suggest that these carbohydrates are being used as energy source. The accumulation of metabolites that can serve both as protection and source of energy is a highly advantageous evolutionary feature. However, further experiments are needed to confirm these assumptions. In addition, our data showed that WGD events have probably driven the evolution and diversification of *GolS* genes in angiosperms. Basal organisms such as *Amborella* showed only two *GolS* genes, whereas during eudicot evolution several rounds of duplication occurred with the generation and further maintenance of copies of these genes. It is tempting to speculate that the appearance of new structures and developmental programs, such as buds and dormancy, required the adaptation of already established molecular pathways. Finally, we believe that the different patterns of gene expression of *GolS* genes influenced the development of complex angiosperms.

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Author contributions

VSF, DDP and LFR performed field samplings. VSF and YEM performed nucleic acid extractions as well as real-time PCR assay. YEM and DDP carried out RACE assays. HPS, DDP and VSF performed the carbohydrate quantification analysis. VSF performed the evolutionary analyses, the subcellular localization assay, and compiled all the data into the manuscript. LFR, PRDO, MMP and GP contributed for the experimental design, discussion of the results and revision of the manuscript.

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Supporting Information

Table S1. Sampling dates, corresponding season and chilling hours accumulated by 'Fuji Standard' buds.

Date	Corresponding season	Accumulated hours below 7.2 °C
01/21/2009	Summer	0
03/26/2009	Autumn	0
05/27/2009	Autumn	76
06/30/2009	Winter	278
07/30/2009	Winter	450
09/09/2009	Winter	528
11/25/2009	Spring	0
02/01/2010	Summer	0

827 **Table S2.** List of primers.

Name	Primers	Amplicon size on cDNA samples (bp)	Amplicon size on gDNA samples (bp)
MDP0000208137	F: CGATCACCTCTTTGACTACCC*	87	87
	R: TACTGTGGGCTACTGCTCCA*		
MDP0000209143	F: CAATACAGCCCTGAGACCG*	99	99
	R: GCCTACGCAAAGTGAAGAGTG*		
	CGAGCCTAGCCGCTTGAC*	-	-
MDP0000412025	F: GCCACCCTGAGAATGTCG*	117	488
	R: TCTTGATGTCCTCTCTCTCCA*		
	CGCCTCCGTCGTTGTACTTC*	-	-
MDP0000466683	F: CCAAGGGGTTGAGAAAGGTA*	96	96
	R: CCTGAGATTCAAGAATGCGA*		
	CGAGGGCAATTTTTATGCTGT*	-	-
MDP0000587199	F: GGATTGGTTTGTACTTCTTTTGGA*	117	269
	R: AGCCTAGCCGCTTGACATAC*		
MDP0000210400	F: TCTCGGGTGGGTAAACGG	137	137
	CAAGGGATTGAGGAAGGTCA		
MDP0000213439	F: GAGAAGACATGGAGTGCGTCTC	156	185
	R: CGAGGAAGGAGGGGTAACTTC		
MDP0000245723/ MDP0000267261	F: CGTTTCCGTCCAAGAAGATC R: GCACAGTGAAAGAGATTGAGCC	114	212
MDP0000426442	F: GCTTCGCCACTGCTTACG	71	457
	R: ACCTGAATGTCCCGTCCA		
MdGolS2_CDS	F:CACCCTCGAGATGGCACCACCAGAAGTTCC	1014	-
	R:AGATCTTCAAGCAGCAGATGGAGCAG		

828 Primers with * were also employed in amplification and sequencing of RACE products.

829

830 **Table S3.** Sequences used in evolutionary analyses.

Name	Accession number	Plant	Database
MdGolS1	MDP0000208137	Apple	*Malus domestica* v1.0[a]
MdGolS2	MDP0000209143	Apple	*Malus domestica* v1.0[a]
MdGolS3	MDP0000412025	Apple	*Malus domestica* v1.0[a]
MdGolS4	MDP0000466683	Apple	*Malus domestica* v1.0[a]
MdGolS5	MDP0000587199	Apple	*Malus domestica* v1.0[a]
MdGolS6	MDP0000210400	Apple	*Malus domestica* v1.0[a]
MdGolS7	MDP0000213439	Apple	*Malus domestica* v1.0[a]
MdGolS8	MDP0000245723	Apple	*Malus domestica* v1.0[a]
MdGolS9	MDP0000267261	Apple	*Malus domestica* v1.0[a]
MdGolS10	MDP0000426442	Apple	*Malus domestica* v1.0[a]
FvGolS1	mrna00243.1-v1.0-hybrid	Woodland strawberry	*Fragaria vesca* v1.0 hybrid[b]
FvGolS2	mrna00244.1-v1.0-hybrid	Woodland strawberry	*Fragaria vesca* v1.0 hybrid[b]
FvGolS3	mrna05015.1-v1.0-hybrid	Woodland strawberry	*Fragaria vesca* v1.0 hybrid[b]
FvGolS4	mrna10568.1-v1.0-hybrid	Woodland strawberry	*Fragaria vesca* v1.0 hybrid[b]
FvGolS5	mrna24544.1-v1.0-hybrid	Woodland strawberry	*Fragaria vesca* v1.0 hybrid[b]
FaGolS1	FANhyb_icon00000194_a.1.g00001.1/partial	Strawberry	*F ananassa* FANhybrid r1.2[a]
FaGolS2	FANhyb_icon00033720_a.1.g00001.1/partial	Strawberry	*F ananassa* FANhybrid r1.2[a]
FaGolS3	FANhyb_rscf00000009.1.g00008.1	Strawberry	*F ananassa* FANhybrid r1.2[a]

FaGolS4	FANhyb_rscf00000149.1.g00001.1	Strawberry	*F ananassa* FANhybrid r1.2[a]
FaGolS5	FANhyb_rscf00000335.1.g00001.1	Strawberry	*F ananassa* FANhybrid r1.2[a]
FaGolS6	FANhyb_rscf00000335.1.g00002.1	Strawberry	*F ananassa* FANhybrid r1.2[a]
FaGolS7	FANhyb_rscf00003859.1.g00001.1	Strawberry	*F ananassa* FANhybrid r1.2[a]
MnGolS1	XP_010092949.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS2	XP_010094413.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS3	XP_010098778.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS4	XP_010098779.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS5	XP_010098781.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS6	XP_010098782.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS7	XP_010098783.1	Mulberry	*M. notabilis* ASM41409v2[c]
MnGolS8	XP_010100446.1	Mulberry	*M. notabilis* ASM41409v2[c]
PmGolS1	Pm003196	Japanese apricot	*Prunus mume* v1.0[c]
PmGolS2	Pm006697	Japanese apricot	*Prunus mume* v1.0[c]
PmGolS3	Pm008398	Japanese apricot	*Prunus mume* v1.0[c]
PmGolS4	Pm008430	Japanese apricot	*Prunus mume* v1.0[c]
PmGolS5	Pm012900	Japanese apricot	*Prunus mume* v1.0[c]
PmGolS6	Pm020004	Japanese apricot	*Prunus mume* v1.0[c]
PpGolS1	Prupe.1G111300.1.p	Peach	*Prunus persica* v2.1[a]
PpGolS2	Prupe.1G251600.1.p	Peach	*Prunus persica* v2.1[a]
PpGolS3	Prupe.3G005100.1.p	Peach	*Prunus persica* v2.1[a]
PpGolS4	Prupe.6G309400.1.p	Peach	*Prunus persica* v2.1[a]
PpGolS5	Prupe.8G258200.1.p	Peach	*Prunus persica* v2.1[a]
PbGolS1	Pbr008619.1	Chinese white pear	*P. bretschneideri* v1.0[d]
PbGolS2	Pbr011631.1	Chinese white pear	*P. bretschneideri* v1.0[d]
PbGolS3	Pbr014990.1	Chinese white pear	*P. bretschneideri* v1.0[d]
PbGolS4	Pbr028045.1	Chinese white pear	*P. bretschneideri* v1.0[d]
PbGolS5	Pbr033744.1	Chinese white pear	*P. bretschneideri* v1.0[d]
PcGolS1	PCP000396.1	European pear	*Pyrus communis* v1.0[a]
PcGolS2	PCP001555.1	European pear	*Pyrus communis* v1.0[a]
PcGolS3	PCP006472.1	European pear	*Pyrus communis* v1.0[a]
PcGolS4	PCP008315.1	European pear	*Pyrus communis* v1.0[a]
PcGolS5	PCP008443.1	European pear	*Pyrus communis* v1.0[a]
PcGolS6	PCP013831.1	European pear	*Pyrus communis* v1.0[a]
PcGolS7	PCP014143.1	European pear	*Pyrus communis* v1.0[a]
PcGolS8	PCP020723.1	European pear	*Pyrus communis* v1.0[a]
PcGolS9	PCP022796.1	European pear	*Pyrus communis* v1.0[a]
PcGolS10	PCP023911.1	European pear	*Pyrus communis* v1.0[a]
PcGolS11	PCP028844.1	European pear	*Pyrus communis* v1.0[a]
PcGolS12	PCP029528.1	European pear	*Pyrus communis* v1.0[a]
RoGolS1	Bras_G00307	Black raspberry	*R. occidentalis* v1.0.a1[a]
RoGolS2	Bras_G07040	Black raspberry	*R. occidentalis* v1.0.a1[a]
RoGolS3	Bras_G08334	Black raspberry	*R. occidentalis* v1.0.a1[a]
RoGolS4	Bras_G24221	Black raspberry	*R. occidentalis* v1.0.a1[a]
RoGolS5	Bras_G27322	Black raspberry	*R. occidentalis* v1.0.a1[a]
ZjGolS1	XP_015867141.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
ZjGolS2	XP_015870937.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
ZjGolS3	XP_015874745.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
ZjGolS4	XP_015874746.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
ZjGolS5	XP_015894691.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]

ZjGolS6	XP_015894735.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
ZjGolS7	XP_015900107.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
ZjGolS8	XP_015900108.1	Jujube	*Z. jujuba* ZizJuj_1.1[c]
AdGolS1	XP_015939405.1	*Arachis duranensis*	*A. duranensis* Aradu1.0[c]
AdGolS2	XP_015939406.1	*Arachis duranensis*	*A. duranensis* Aradu1.0[c]
AdGolS3	XP_015939595.1	*Arachis duranensis*	*A. duranensis* Aradu1.0[c]
AdGolS4	XP_015968696.1	*Arachis duranensis*	*A. duranensis* Aradu1.0[c]
AdGolS5	XP_015968864.1	*Arachis duranensis*	*A. duranensis* Aradu1.0[c]
AiGolS1	XP_016176717.1	*Arachis ipaensis*	*A. ipaensis* Araip1.0[c]
AiGolS2	XP_016176983.1	*Arachis ipaensis*	*A. ipaensis* Araip1.0[c]
AiGolS3	XP_016177387.1	*Arachis ipaensis*	*A. ipaensis* Araip1.0[c]
AiGolS4	XP_016205613.1	*Arachis ipaensis*	*A. ipaensis* Araip1.0[c]
AiGolS5	XP_016205726.1	*Arachis ipaensis*	*A. ipaensis* Araip1.0[c]
CcGolS1	C.cajan_06744	Pigeon pea	*Cajanus cajan*[d]
CcGolS2	C.cajan_10278	Pigeon pea	*Cajanus cajan*[d]
CcGolS3	C.cajan_10714	Pigeon pea	*Cajanus cajan*[d]
CaGolS1	XP_004494448.1	Chick pea	*C. arietinum* ASM33114v1[c]
CaGolS2	XP_004494525.1	Chick pea	*C. arietinum* ASM33114v1[c]
CaGolS3	XP_004495634.1	Chick pea	*C. arietinum* ASM33114v1[c]
GmGolS1	Glyma.03G222000.1	Soybean	*Glycine max* Wm82.a2.v1[b]
GmGolS2	Glyma.03G229800.1	Soybean	*Glycine max* Wm82.a2.v1[b]
GmGolS3	Glyma.10G145300.1	Soybean	*Glycine max* Wm82.a2.v1[b]
GmGolS4	Glyma.19G219100.1	Soybean	*Glycine max* Wm82.a2.v1[b]
GmGolS5	Glyma.19G227800.1	Soybean	*Glycine max* Wm82.a2.v1[b]
GmGolS6	Glyma.20G094500.1	Soybean	*Glycine max* Wm82.a2.v1[b]
GsGolS1	KHN02817.1	Wild soybean	*Glycine soja* W05v1.0[c]
GsGolS2	KHN03918.1	Wild soybean	*Glycine soja* W05v1.0[c]
GsGolS3	KHN14150.1	Wild soybean	*Glycine soja* W05v1.0[c]
GsGolS4	KHN16038.1	Wild soybean	*Glycine soja* W05v1.0[c]
GsGolS5	KHN43351.1	Wild soybean	*Glycine soja* W05v1.0[c]
GsGolS6	KHN43432.1	Wild soybean	*Glycine soja* W05v1.0[c]
LjGolS1	LJ1G068880	Lotus	*Lotus japonicus*[e]
LjGolS2	LJ1G070250	Lotus	*Lotus japonicus*[e]
LjGolS3	LJ5G026190	Lotus	*Lotus japonicus*[e]
MtGolS1	Medtr1g084660.1	Medicago	*M. truncatula* Mt4.0v1[b]
MtGolS2	Medtr1g084670.1	Medicago	*M. truncatula* Mt4.0v1[b]
MtGolS3	Medtr4g132185.1	Medicago	*M. truncatula* Mt4.0v1[b]
MtGolS4	Medtr7g109920.1	Medicago	*M. truncatula* Mt4.0v1[b]
MtGolS5	Medtr7g111850.1	Medicago	*M. truncatula* Mt4.0v1[b]
PvGolS1	Phvul.001G215300.1	Bean	*Phaseolus vulgaris* 1.0[b]
PvGolS2	Phvul.001G223700.1	Bean	*Phaseolus vulgaris* 1.0[b]
PvGolS3	Phvul.007G203400.1	Bean	*Phaseolus vulgaris* 1.0[b]
VaGolS1	KOM35442.1	Adzuki bean	*Vigna angularis* 1.1[c]
VaGolS2	KOM39289.1	Adzuki bean	*Vigna angularis* 1.1[c]
VaGolS3	KOM39376.1	Adzuki bean	*Vigna angularis* 1.1[c]
VrGolS1	XP_014495254.1	Mung bean	*Vigna radiata* ver6[c]
VrGolS2	XP_014495255.1	Mung bean	*Vigna radiata* ver6[c]
VrGolS3	XP_014495256.1	Mung bean	*Vigna radiata* ver6[c]
VrGolS4	XP_014495390.1	Mung bean	*Vigna radiata* ver6[c]
VrGolS5	XP_014498752.1	Mung bean	*Vigna radiata* ver6[c]

VrGolS6	XP_014513607.1	Mung bean	*Vigna radiata* ver6[c]
ClGolS1	CL01G13320	Watermelon	*Citrullus lanatus*[e]
ClGolS2	CL05G26300	Watermelon	*Citrullus lanatus*[e]
ClGolS3	CL06G04090	Watermelon	*Citrullus lanatus*[e]
ClGolS4	CL10G09890	Watermelon	*Citrullus lanatus*[e]
CmGolS1	XP_008447007.1	Muskmelon	*C. melo* ASM31304v1[c]
CmGolS2	XP_008447460.1	Muskmelon	*C. melo* ASM31304v1[c]
CmGolS3	XP_008458318.1	Muskmelon	*C. melo* ASM31304v1[c]
CmGolS4	XP_008463593.1	Muskmelon	*C. melo* ASM31304v1[c]
CsGolS1	Cucsa.180650.1	Cucumber	*C. sativus* ASM407v2[b]
CsGolS2	Cucsa.192640.1	Cucumber	*C. sativus* ASM407v2[b]
CsGolS3	Cucsa.286110.1	Cucumber	*C. sativus* ASM407v2[b]
Gm19G247000	Glyma.19G247000.1	Soybean	*Glycine max* Wm82.a2.v1[b]

[a] Rosaceae.org; [b] PHYTOZOME; [c] NCBI; [d] GigaDB; [e] PLAZA

Table S4. Ks-dating of grapevine *GolS* gene paralogs.

Duplicated genes	Ks-dating of the collinear block	Duplicated gene origin
VIT_01s0127g00470/VIT_07s0005g01970	1.68	WGD
VIT_14s0060g00730/VIT_07s0005g01970	1.11	WGD
VIT_05s0020g00330/VIT_07s0005g01970	0.99	WGD
VIT_05s0077g00430/VIT_07s0005g01970	1.33	WGD
VIT_14s0060g00730/VIT_05s0020g00330	1.49	WGD
VIT_14s0060g00730/VIT_05s0077g00430	1.14	WGD

Sequences were identified in the *Vitis vinifera* 12X Database V1 available at CRIBI (http://genomes.cribi.unipd.it/grape/).

Table S5. Correlation between the quantification of *MdGolS* genes expression via real-time PCR and carbohydrates content measured by UPLC in apple buds of 'Fuji Standard'.

	MdGolS1	*MdGolS2*	*MdGolS3*	*MdGolS4*	*MdGolS5*	Galactinol	Raffinose	Sucrose
MdGolS1	1.00							
MdGolS2	0.81	1.00						
MdGolS3	0.35	0.10	1.00					
MdGolS4	0.01	0.35	-0.31	1.00				
MdGolS5	-0.28	-0.34	0.36	0.28	1.00			
Galactinol	**0.91**	**0.98**	0.19	0.28	-0.34	1.00		
Raffinose	**0.90**	**0.99**	0.20	0.27	-0.33	**1.00**	1.00	
Sucrose	0.67	0.82	-0.29	0.40	-0.45	0.81	0.80	1.00

Results from the quantification of *MdGolS1-5* and sugar content during eight time points along the year (Fig. 5). Bold values showed positive correlation and are significant at the 0.01 level.

Table S6. List of syntenic chromosomes containing *GolS* genes.

Chromosome	Syntenic apple chromosome	Gene	Apple gene	Gene	Apple gene
Ppchr1	Mdchr13	*PpGolS1*	*MdGolS3*	*PpGolS2*	*MdGolS2*
	Mdchr16		*MdGolS7*		*MdGolS5*
Ppchr3	Mdchr9	*PpGolS3*	*MdGolS10*		
	Mdchr17		*MdGolS4*		
Ppchr6	Mdchr4	*PpGolS4*	*MdGolS1*		
Pmchr1	Mdchr4	*PmGoLS1*	*MdGolS1*		
	Mdchr16		*MdGolS7*		
Pmchr2	Mdchr13	*PmGolS2*	*MdGolS2*	*PmGolS4*	*MdGolS3*
	Mdchr16		*MdGolS5*		*MdGolS7*
Ppchr4	Mdchr9	*PpGolS5*	*MdGolS10*		
	Mdchr17		*MdGolS4*		
Fvchr1	Mdchr9	*FvGolS5*	*MdGolS10*		
	Mdchr17		*MdGolS4*		
Fvchr4	Mdchr13	*FvGolS3*	*MdGolS3*		
Fvchr6	Mdchr4	*FvGolS1*	*MdGolS1*		
Pbchr4	Mdchr4	*PbGolS4*	*MdGolS1*		
Pbchr11	Mdchr11	*PbGolS5*	*MdGolS8*		
Pbchr13	Mdchr13	*PbGolS3*	*MdGolS3*		
Pbchr17	Mdchr9	*PbGolS1*	*MdGolS10*		
	Mdchr17		*MdGolS4*		

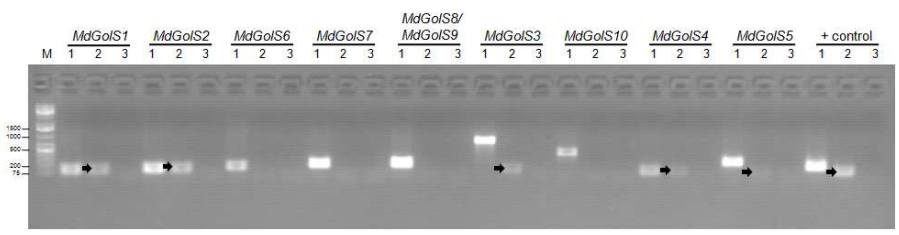

Fig. S1. PCR analysis of *MdGolS* genes in 'Gala Baigent' apple trees. Genomic DNA (lane 1), cDNA pool (lane 2) or water (lane 3), were submitted to RT-PCR. M, GeneRuler® 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, USA). Arrows indicate amplification on cDNA samples. *MDH* was used as positive control. Expected amplicon size for each gene is shown in Table S2.

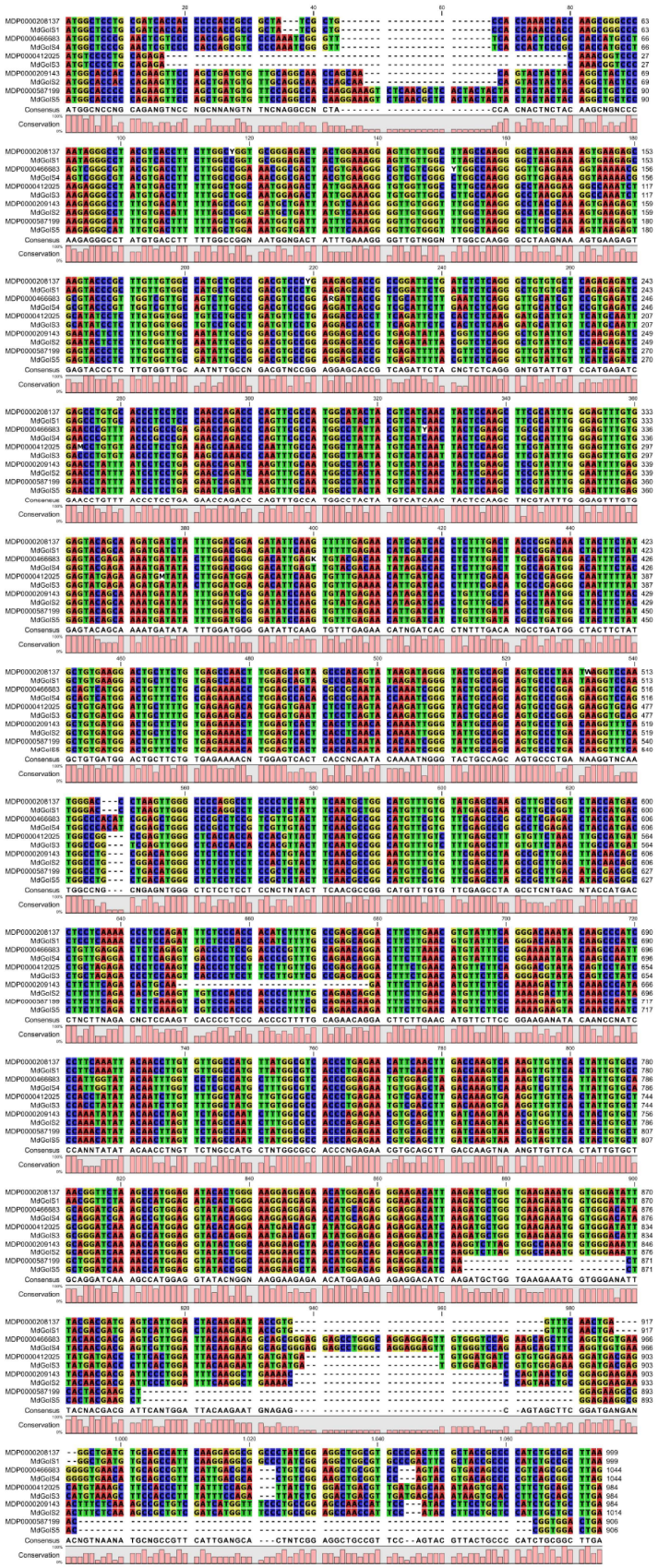

Fig. S2. Multiple alignments of *GolS* CDS predicted in the apple genome with those sequences experimentally amplified by RACE.

7 DISCUSSÃO

A compreensão dos mecanismos que possibilitam às plantas superarem condições ambientais adversas é de grande importância para a manutenção e a ampliação de culturas vegetais de interesse agronômico. A dormência de gemas é um processo complexo que envolve a reprogramação transcricional da planta como um todo, de modo a sincronizar a sua fenologia com as variações climáticas sazonais (SHIM *et al.*, 2014). A dormência é caracterizada pela presença de uma estrutura especializada, a gema, a qual integra os sinais ambientais e desempenha um importante papel na proteção do meristema (ROHDE & BHALERAO, 2007). A exploração da adaptabilidade da gema a condições de estresse que ocorrem durante o inverno é uma abordagem inovadora visando a identificação de genes com potencial uso biotecnológico. Para tal, um estudo de expressão gênica diferencial durante a progressão da dormência de gemas em macieira foi realizado previamente, permitindo identificar uma série de genes com potencial protetor à integridade da gema durante o inverno, destacando-se a presença de genes *DHN* e *GolS* (FALAVIGNA *et al.*, 2014). Pela presente Tese de Doutorado, objetivou-se caracterizar todos os genes de ambas as famílias gênicas no genoma da macieira, de modo a identificar aqueles com maior potencial de exploração biotecnológica, seja por meio do estudo da sua região promotora, seja por meio de sua expressão ectópica visando a obtenção de plantas mais tolerantes a estresses abióticos. Neste contexto, a presente Tese de Doutorado partiu da premissa de que é possível transferir a adaptabilidade a estresses abióticos presente em gemas e sementes dormentes de macieira para estruturas naturalmente sensíveis a tais fenômenos, tais como folhas, flores e raízes.

7.1 DESIDRINAS - DHN

As DHN são proteínas que atuam na resposta adaptativa vegetal a estresses abióticos, principalmente os que envolvem desidratação (HANIN *et al.*, 2011). Elas já foram identificadas durante o processo de dessecação de sementes, em resposta a estresses

abióticos como baixas temperaturas, déficit hídrico e alta salinidade, e durante o processo de dormência de gemas (UENO *et al.*, 2013; GRAETHER & BODDINGTON, 2014; FALAVIGNA *et al.*, 2015a; PORTO *et al.*, 2015; BANERJEE & ROYCHOUDHURY, 2016).

No genoma de macieira, inicialmente nove modelos gênicos preditos codificando DHN foram identificados, mas seu papel durante o processo de dormência não foi avaliado (LIANG *et al.*, 2012). Paralelamente, estudos do grupo demonstraram a existência de três genes *DHN* adicionais no genoma de macieira, além de perfis de expressão relacionando alguns genes dessa família ao processo de dormência. Neste contexto, a família de genes codificadores de DHNs em macieira foi caracterizada sob o enfoque do processo da dormência de gemas (Capítulo II; FALAVIGNA *et al.*, 2015a). A análise evolutiva dos genes *DHN* de macieira por meio de ferramentas de filogenia, sintenia e colinearidade permitiu identificar que tanto eventos de WGD quanto duplicações em *tandem* nortearam a evolução e a diversificação destes genes em macieira.

Os genes *MdDHN* tiveram seu perfil transcricional caracterizado em diversas amostras desde a dormência e o florescimento até o amadurecimento dos frutos. Um dos resultados mais interessantes foi obtido para o gene *MdDHN11*, o qual é cerca de 6.000 vezes mais expresso em sementes em relação a outros tecidos, sugerindo sua atuação na tolerância à dessecação de sementes. O processo de dessecação envolve perdas críticas de água de modo a preparar a semente para longos períodos de dormência e preservação até a germinação, com uma grande reprogramação transcricional e metabólica (ANGELOVICI *et al.*, 2010). Desta forma, devido ao provável papel biológico desempenhado por MdDHN11 durante este processo, o gene *MdDHN11* torna-se um bom candidato a ser caracterizado buscando a obtenção de plantas mais tolerantes ao estresse hídrico. Adicionalmente, 64% dos genes *MdDHN* foram majoritariamente expressos em órgãos dormentes, isto é, em gemas e sementes. Estudos em pêssego demonstraram rotas comuns entre a dormência de gemas e de sementes, principalmente para metabólitos envolvidos em respostas adaptativas, como é o caso das proteínas DHN (LEIDA *et al.*, 2012).

De modo a melhor caracterizar o papel dos genes *DHN* durante a dormência de gemas em macieira, quatro experimentos independentes foram realizados utilizando cultivares com diferentes profundidades de dormência: ciclo anual com amostragens de gemas a campo; superação da dormência sob condições controladas; indução e superação

do processo de dormência sob condições controladas; e simulação do efeito de temperaturas permissivas de crescimento durante a dormência. Os genes *MdDHN2-6* apresentaram perfis de expressão mais consistentes com um papel na tolerância da gema ao frio, provavelmente sob a direta influência dos fatores de transcrição CBFs. Durante a dormência, um estádio metabolicamente menos ativo, ocorre a redução da disponibilidade de água nas gemas (DE FAŸ *et al*., 2000; AMÉGLIO *et al.*, 2002), sendo que o acúmulo de moléculas capazes de se ligar a grandes quantidades de água e íons, como é o caso das proteínas DHN (TOMPA *et al.*, 2006), é uma interessante estratégia para minimizar os efeitos deletérios do déficit hídrico. Além disto, as proteínas DHN são capazes de alterar seu padrão conformacional em resposta a mudanças na disponibilidade hídrica, de modo a proteger outras moléculas por meio de interações com partes parcialmente desidratadas (HOEKSTRA *et al*., 2001; TOMPA *et al.*, 2006; GRAETHER & BODDINGTON, 2014).

No trabalho descrito no Capítulo II, identificou-se que os genes *MdDHN* possuem funções altamente divergentes, com níveis de sobreposição, e que os seus padrões de expressão são finamente ajustados por sinais ambientais durante a dormência de gemas. Complementariamente, sugere-se que o acúmulo de trascritos *MdDHN2-6* durante o inverno é um dos mecanismos parcialmente responsáveis pela tolerância da gema ao frio, enquanto que o gene *MdDHN11* desempenha importantes papéis na tolerância à dessecação de sementes (FALAVIGNA *et al*., 2015a). Estes resultados nortearam a escolha do gene *MdDHN11* para ensaios visando explorar o seu potencial uso biotecnológico.

Análises genômicas comparativas e de expressão sugerem que o gene *MdDHN11* é um dos responsáveis por conferir tolerância à dessecação durante o desenvolvimento de sementes de maçã. Este processo envolve perdas críticas de água visando preparar a semente para os longos períodos de dormência e preservação até a germinação (ANGELOVICI *et al.*, 2010). Assim, o gene *MdDHN11* tornou-se candidato a ser caracterizado buscando a obtenção de plantas mais tolerantes à exposição ao estresse hídrico e buscando desenvolver promotores semente-específicos. Recentemente, a identificação de promotores tecido-específicos ganhou importância agronômica, uma vez que a utilização de promotores constitutivos nem sempre é uma característica desejada (DUTT *et al.*, 2014). Neste contexto, a caracterização funcional de *MdDHN11* foi realizada por meio do emprego de técnicas de hibridização *in situ* (ISH), localização subcelular,

análise da habilidade do seu promotor guiar a expressão tecido-específica, e a resposta de plantas superexpressando este gene ao déficit hídrico (Capítulo III).

Devido à limitada literatura acerca da anatomia das sementes de maçãs (JACKSON, 2003), um ensaio morfoanatômico foi realizado durante o desenvolvimento destas. Todos os principais componentes da semente, isto é, embrião, endosperma, nucelo e testa, foram identificados. Foi possível identificar a expansão do endosperma às custas do nucelo, bem como o desenvolvimento do embrião, de globular para torpedo, às custas do endosperma. As fotomicrografias apresentadas no Capítulo III são as melhores imagens do desenvolvimento de sementes de maçãs presentes na literatura, e podem auxiliar na interpretação de resultados envolvendo sementes, tais como o ISH do presente trabalho.

A determinação da expressão espaço-temporal de *MdDHN11* por ISH em sementes de frutos de 40 mm de diâmetro mostrou o acúmulo de transcritos na camada do nucelo. O acúmulo de MdDHN11 na camada entre o endosperma e a testa sugere um papel protetor ao embrião e ao endosperma durante o desenvolvimento de sementes. Complementariamente, a localização desta proteína no núcleo e no citoplasma, como já havia sido demonstrado para DHNs de *A. thaliana* (CANDAT *et al.*, 2014), reforça a hipótese protetora. De modo a fornecer evidências diretas de que esta proteína é capaz de conferir maior tolerância ao déficit hídrico, o gene *MdDHN11* foi superexpressado em *A. thaliana* e as plantas transgênicas e controles foram submetidas a severos ensaios de simulação de seca. A única planta capaz de sobreviver aos ensaios de 36 dias de privação de água foi justamente a planta contendo o transgene. Menos de duas horas após a reidratação, a planta transgênica já estava totalmente recuperada, o que sugere que o gene MdDHN11 também ajudou a manter a integridade e a funcionalidade da raiz. Outros relatos também demonstraram um melhor desenvolvimento de raízes de plantas superexpressando genes *DHN* em relação a plantas não transformadas (SHEKHAWAT *et al*., 2011; YANG *et al.*, 2014).

Finalmente, a capacidade da região promotora de *MdDHN11* dirigir a expressão transgênica semente-específica foi avaliada. Entretanto, não foi possível identificar a atividade da proteína-repórter GUS em nenhum dos tecidos de plantas transgênicas analisados. Uma possível explicação se deve ao fato da diferença morfoanatômica existente entre as sementes de maçã e de *A. thaliana*. Em *A. thaliana*, o nucelo é um dos primeiros tecidos a ser degenerado durante o desenvolvimento da semente (XU *et al.*,

2016), enquanto que, em macieira, este tecido ainda está presente mais de 50 dias após a antese. Ainda assim, novos experimentos em plantas com estruturas semelhantes às da semente de maçã são necessários para refutar ou confirmar tal hipótese.

Em conjunto, os resultados obtidos forneceram evidências de que o gene *MdDHN11* desempenha importantes papéis durante o desenvolvimento da semente de maçã, protegendo o embrião e o endosperma de alterações no *status* da água. O seu mecanismo de ação provavelmente envolve a interação de MdDHN11 com outras proteínas, membranas e metabólitos presentes no citoplasma e no núcleo. Além disto, as DHNs de macieira são um novo recurso para a geração de plantas melhor adaptadas a condições adversas de cultivo.

7.2 GALACTINOL SINTASES - GolS

GolS é a enzima responsável pela síntese de galactinol, um carboidrato base para a síntese de RFOs (ELSAYED *et al*., 2014). Estes carboidratos foram descritos por se acumular em resposta ao frio, ao congelamento e ao déficit hídrico em plantas, sendo solutos compatíveis com importantes papéis no armazenamento de carbono, no ajuste osmótico, na transdução de sinal, na estabilização de membranas e proteínas, entre outros (ELSAYED *et al*., 2014; SENGUPTA *et al.*, 2015). Além disto, genes *GolS* são induzidos nos últimos estádios de desenvolvimento de sementes, atuando na mobilização de RFOs de modo a gerar tolerância à dessecação e fornecer energia para a germinação (BLÖCHL *et al*., 2007; PETERBAUER & RICHTER, 2007).

Um total de dez genes potencialmente codificadores de GolS foram encontrados no genoma de macieira. Estes genes putativos foram evolutivamente caracterizados dentro de um ramo do clado Fabidae e dentro da família das rosáceas. Nenhum ortólogo foi identificado entre os genes *GolS* das diferentes ordens analisadas (Curcubitales, Fabales e Rosales), indicando uma forte segregação evolutiva entre estas ordens do clado Fabidae. Resultados similares foram encontrados quando proteínas deduzidas GolS de monocotiledôneas foram comparadas com sequências peptídicas GolS de dicotiledôneas (SENGUPTA *et al.*, 2012; ELSAYED *et al*., 2014). Complementariamente, mesmo dentro

da ordem Rosales houve segregação entre os genes *GolS* oriundos de diferentes famílias. Macieira e pereira apresentaram um número superior de genes *GolS* em relação a outras espécies de rosáceas, o que pode ser explicado pelo recente evento de duplicação do genoma inteiro (WGD) compartilhado por ambas (VELASCO *et al.*, 2010; CHAGNÉ *et al.*, 2014). As análises de colinearidade e sintenia confirmaram estas afirmações, além de demonstrarem que o evento mais antigo de WGD (triplicação) também colaborou para moldar a evolução dos genes *GolS*s em rosáceas. No caso particular de macieira, ambos os eventos de WGD, o evento recente e o de triplicação, colaboraram para a evolução e diversificação dos genes *GolS*.

A análise do perfil de expressão dos genes *MdGolS* demonstrou que apenas os genes *MdGolS1-5* são normalmente expressos durante o ciclo vegetativo e reprodutivo de macieira. Destes, *MdGolS1*, *3* e *4* foram majoritariamente expressos em sementes de frutos maduros, coincidindo com outros relatos em ervilha, tomate e milho (DOWNIE *et al.*, 2003; ZHAO *et al.*, 2004; BLÖCHL *et al*., 2007; PETERBAUER & RICHTER, 2007). *MdGolS2* apresentou os maiores níveis de expressão em gemas dormentes. Estudos em pêssego demonstraram rotas comuns entre a dormência de gemas e de sementes, principalmente para metabólitos envolvidos em respostas adaptativas (LEIDA *et al.*, 2012). Complementariamente, galactinol, rafinose, *MdGolS1* e *MdGolS2* apresentaram um perfil sazonal de acúmulo ao longo do ano, atingindo seus picos durante os meses de inverno e apresentando uma forte correlação entre os níveis de expressão e de carboidratos. Padrões similares de acúmulo de transcritos e de carboidratos já foram descritos na literatura durante a dormência (COX & STUSHNOFF, 2001; DERORY *et al.*, 2006; SANTAMARÍA *et al.*, 2011; LIU *et al.*, 2012; UNDA *et al.*, 2012; IBÁÑEZ *et al.*, 2013).

Os resultados apresentados no Capítulo IV permitem sugerir que galactinol e rafinose integram uma série de mecanismos que agem em conjunto durante a dormência a fim de proteger a integridade da gema durante condições ambientais adversas. A provável função de galactinol e RFOs é baseada na sua capacidade de sequestrar radicais hidroxila, bem como funcionar como osmólitos para manter o turgor celular e estabilizar proteínas (NISHIZAWA *et al*., 2008; ELSAYED *et al*., 2014). Adicionalmente, a drástica redução dos níveis destes carboidratos antes da brotação sugere a sua utilização como fonte de energia. Ao longo da evolução, o aparecimento de novas estruturas e programas de desenvolvimento, tais como a gema e a dormência, requereram a adaptação de vias

moleculares já estabelecidas. Finalmente, os nossos resultados permitem sugerir que o gene *MdGolS2* é o mais indicado a ser utilizado na geração de plantas melhor adaptadas à exposição a estresses abióticos. A geração de plantas transgênicas de *A. thaliana* capazes de realizar expressão ectópica de *MdGolS2* está em andamento e possivelmente permitirá incrementar os resultados apresentados no Capítulo IV da presente Tese.

7.3 PERSPECTIVAS DE PESQUISA

A identificação de genes com potencial utilização na geração de plantas geneticamente modificadas melhor adaptadas a condições adversas de cultivo é essencial sob a perspectiva das mudanças climáticas. O presente trabalho envolveu a caracterização dos genes *DHN* e *GolS* de macieira, bem como a identificação de quais membros de cada uma dessas classes gênicas são os mais indicados visando obter plantas transgênicas mais tolerantes a exposição ao déficit hídrico. Estudos anteriores demonstraram que, apesar de pertencerem a classes gênicas distintas, os seus genes possuem perfis similares de acúmulo de transcrito durante estádios específicos do desenvolvimento vegetal, tais como a maturação de sementes, além de as proteínas DHN, o galactinol e os RFOs desempenharem papéis protetores similares na célula, principalmente atuando como osmoprotetores e na estabilização de membranas e proteínas (HANIN *et al*., 2011; ELSAYED *et al*., 2014; GRAETHER & BODDINGTON, 2014; SENGUPTA *et al.*, 2015). Os dados reunidos nos Capítulos II e IV da presente Tese fornecem evidências de que os genes *DHN* e *GolS* de macieira possuem funções divergentes e com níveis de sobreposição, sendo que alguns de seus membros estão envolvidos no processo de dormência de gemas ou de sementes. Entretanto, devido a essa pluralidade de funções, fez-se necessária a identificação de quais genes podem ser empregados em estudos visando incrementar a tolerância de plantas ao estresse hídrico.

Análises genômicas comparativas e de acúmulo de transcrito permitiram identificar os genes *MdDHN11* e *MdGolS2* como possíveis responsáveis por conferir tolerância à dessecação de sementes de maçã e de proteção à integridade da gema de macieira durante o inverno, respectivamente (Capítulos II e IV). A caracterização funcional de *MdDHN11*

permitiu demonstrar que a sua expressão em *A. thaliana* é capaz de conferir uma maior tolerância ao déficit hídrico (Capítulo III). Um maior número de plantas de *A. thaliana* carregando a expressão ectópica de *MdDHN11* está em andamento de modo a confirmar os resultados obtidos. Neste contexto, as DHNs de macieira surgem como um novo recurso na geração de plantas melhor adaptadas a condições adversas de cultivo, principalmente aquelas envolvendo limitada disponibilidade de água. Complementariamente, estudos demonstraram que a expressão ectópica de *GolS* de *A. thaliana*, *Ammopiptanthus mongolicus* e *Thellungiella salsuginea* foram capazes de gerar plantas mais tolerantes à seca e ao congelamento, evidenciando a aplicabilidade desses genes (TAJI *et al*., 2002; SONG *et al.*, 2013; SUN *et al.*, 2013). De modo a confirmar o potencial biotecnológico de genes *GolS* de macieira, plantas de *A. thaliana* superexpressando *MdGolS2* foram geradas e ensaios de simulação de seca similares aos realizados para o gene *MdDHN11* estão em andamento. Paralelamente, uma análise de patenteabilidade dos genes *MdDHN11* e *MdGolS2* está em curso no Comitê Local de Propriedade Intelectual da Embrapa Uva e Vinho. Adicionalmente, pretende-se realizar experimentos de simulação de seca com as linhagens T3 e T4 que superexpressam independentemente ambos os genes, de modo a avaliar o efeito aditivo da expressão ectópica no fenótipo dessas plantas. Finalmente, está previsto a realização de ensaios de congelamento, onde as plantas transgênicas serão expostas a 10ºC negativos por oito horas consecutivas. Este ensaio visa simular o efeito de geadas tardias, o qual é um fenômeno comum na região Sul do Brasil e que é responsável por causar severas perdas de produtividade nas mais diversas culturas vegetais.

Outra estratégia a ser abordada é combinar a expressão ectópica do gene *MdDHN11* juntamente com o gene *MdGolS2* em *A. thaliana*. Desta forma, duas rotas metabólicas diferentes, atuando tanto em nível de proteína quanto em de carboidrato, seriam utilizadas de modo a incrementar a proteção de tecidos e órgãos ao estresse hídrico. Esta abordagem mimetiza o que naturalmente acontece em órgãos dormentes, podendo levar a geração de plantas capazes de uma melhor utilização dos recursos hídricos disponíveis, tornando-as melhor adaptadas a crescer sob escassez de água e capazes de tolerar geadas tardias. Portanto, este trabalho contibuiu para o avanço da fronteira do conhecimento na área de dormência de gemas e também gerou conhecimentos com alto potencial de aplicação em biotecnologia vegetal.

8 REFERÊNCIAS BIBLIOGRÁFICAS

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YANG, Y.; SUN, X.; YANG, S.; LI, X. & YANG, Y. Molecular cloning and characterization of a novel SK3-type dehydrin gene from *Stipa purpurea*. *Biochem Biophys Res Commun*, 448:145–150, 2014.

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9 CURRICULUM VITÆ

FALAVIGNA, V.S.

DADOS PESSOAIS

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FORMAÇÃO

2012 – 2016

Doutorado em Biologia Celular e Molecular, PPGBCM, Universidade Federal do Rio Grande do Sul, UFRGS, Brasil. Análise funcional e potencial biotecnológico de desidrinas e galactinol sintases de macieira. Orientadores: Dr. Giancarlo Pasquali e Dra. Márcia Pinheiro-Margis.

2011 – 2012

Mestrado em Biologia Celular e Molecular, PPGBCM, Universidade Federal do Rio Grande do Sul, UFRGS, Brasil. Perfil transcricional de genes relacionados à dormência em gemas de macieira. Orientadores: Dr. Giancarlo Pasquali e Dra. Márcia Pinheiro-Margis.

2006 – 2010

Bacharelado em Engenharia de Bioprocessos e Biotecnologia. Universidade Estadual do Rio Grande do Sul, UERGS, Brasil. Expressão gênica diferencial entre duas cultivares de macieira com requerimento de frio hibernal contrastante. Orientadores: Dr. Fábio Luís Maciel, Dr. Diogo Denardi Porto e Dr. Luís Fernando Revers.

FORMAÇÃO COMPLEMENTAR

2010 – 2011

Estágio de Iniciação Científica no **Laboratório de Genética Molecular Vegetal**. Embrapa Uva e Vinho, Bento Gonçalves/RS.

Orientação: Dr. Luís Fernando Revers.

Pesquisa inserida no projeto: "Melhoramento genético de maçã: estratégias inovadoras no desenvolvimento de cultivares adaptadas às condições climáticas sul-brasileiras".

2009 – 2009

Bolsista de ITI no **Laboratório de Biologia Molecular Vegetal**. Embrapa Uva e Vinho, Bento Gonçalves/RS.

Orientação: Dr. Luís Fernando Revers.

Atuando nos seguintes temas: Caracterização da dormência de gemas em macieira: obtenção de bibliotecas supressivas subtrativas para identificação de genes associados ao requerimento de frio hibernal; caracterização do nível de oxidação de glutationa durante a dormência hibernal em gemas de macieira; e busca por genes candidatos de macieira através de ferramentas da bioinformática.

2008 – 2009

Estágio de Iniciação Científica no **Laboratório de Enoquímica**. Embrapa Uva e Vinho, Bento Gonçalves/RS.

Orientação: Dr. Alberto Miele.

Atuando nos seguintes temas: Inovação tecnológica e rastreabilidade da produção de uvas e da qualidade dos sucos de uva e dos vinhos produzidos pelo sistema orgânico; avaliação do estado nutricional pelo método DRIS para culturas da macieira e videira no sul do Brasil; e efeito das mudanças climáticas na composição físico-química e nas características sensoriais do vinho fino.

PRÊMIOS E TÍTULOS

2015

Proficiência em leitura da Língua Espanhola, Universidade Federal do Rio Grande do Sul.

2013

Menção honrosa - Prêmio Painel Iniciação Científica, apresentado por Yohanna Evelyn Miotto, IV Simpósio de Genética Molecular de Plantas, Sociedade Brasileira de Genética.

Prêmio de melhor trabalho de Graduação, apresentado por Yohanna Evelyn Miotto, no 11º Encontro de Iniciação Científica e 7º Encontro de Pós-Graduandos da Embrapa Uva e Vinho, Embrapa Uva e Vinho.

2011

Proficiência em leitura da Língua Inglesa, Universidade Federal do Rio Grande do Sul.

ARTIGOS COMPLETOS PUBLICADOS

DA SILVA, D.C.; FALAVIGNA, V.S.; FASOLI, M.; BUFFON, V.; PORTO, D.D.; PAPPAS JR, G.J.; PEZZOTTI, M.; PASQUALI, G.; REVERS, L.F. Transcriptome analyses of the Dof-like gene family in grapevine reveal its involvement in berry, flower and seed development. *Horticulture Research*, 3: 16042, 2016.

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FALAVIGNA, V.S.; MIOTTO, Y.E.; PORTO, D.D.; ANZANELLO, R.; SANTOS, H.P.; FIALHO, F.B.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L.F. Functional diversification of the dehydrin gene family in apple and its contribution to cold acclimation during dormancy. *Physiologia Plantarum* (Kobenhavn. 1948), 155: 315-329, 2015.

FALAVIGNA*, V.S.; PORTO*, D.D.; BUFFON, V.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L.F. Differential transcriptional profiles of dormancy-related genes in apple buds. *Plant Molecular Biology Reporter*, 32: 796-813, 2014.
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CAPÍTULOS DE LIVROS PUBLICADOS

FALAVIGNA*, V.S.; PORTO*, D.D.; SILVEIRA, C.P.; REVERS, L.F. Recent advances in genetics and molecular control of bud dormancy in pipfruits. In: Anderson, J.V. *Advances in Plant Dormancy*. New York: Springer International Publishing, Cap. 5, p. 107-122, 2015.
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RESUMOS PUBLICADOS EM ANAIS DE CONGRESSOS

FALAVIGNA, V.S.; COSTENARO-DA-SILVA, D.; PASQUALI, G.; REVERS, L.F. Genome-wide identification of genes encoding Dof transcription factors in grapevine. In: IV Simpósio Brasileiro de Genética Molecular de Plantas, Bento Gonçalves. IV SBGMP, 2013.

MIOTTO, Y.E.; FALAVIGNA, V.S.; PORTO, D.D.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L.F. Dormancy-related transcript accumulation of the dehydrin gene family in apple buds. In: IV Simpósio Brasileiro de Genética Molecular de Plantas, Bento Gonçalves. IV SBGMP, 2013.

DALMEIDA, G.S.; BRETON, M.C.; MARTINS, P.D.; FALAVIGNA, V.S.; TURCHETTO-ZOLET, A.C.; REVERS, L.F.; PASQUALI, G. Characterization of

Eucalyptus grandis Dof gene family of transcription factors. In: IV Simpósio Brasileiro de Genética Molecular de Plantas, Bento Gonçalves. IV SBGMP, 2013.

REVERS, L.F.; PORTO, D.D.; FALAVIGNA, V.S.; BRUNEAU, M.; ANZANELLO, R.; TESSELE, C.; ALENCAR, S.A.; CZERMAINSKI, A.B.C.; PAPPAS JR, G.J.; RENOU, J.; FIALHO, F.B.; SANTOS, H.P.; DENARDI, F.; KVITSCHAL, M.V.; PASQUALI, G.; MARGIS-PINHEIRO, M; DELATORRE, C.A; OLIVEIRA, P.R.D. Integrating genetics, genomics and modeling of bud dormancy to improve apple adaptation to climatic changes. In: Plant Dormancy Symposium 2013, Auckland. PDS 2013, 2013.

MIOTTO, Y.E.; FALAVIGNA, V.S.; PORTO, D.D.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L.F. Identificação, classificação e caracterização transcricional das desidrinas de macieira. In: XXII Congresso Brasileiro de Fruticultura, Bento Gonçalves. XXII CBF, 2012.

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FALAVIGNA, V.S.; PERINI, P.; REVERS, L.F. Identification of dormancy-associated mads-box genes in apple. In: III Simpósio Brasileiro de Genética Molecular de Plantas, Ilhéus. III SBGMP, 2011.

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contrasting chilling requeriment. In: 5th International Rosaceae Genomics Conference, Cape Town. RGC5, 2010.

PARTICIPAÇÃO EM CURSOS

REVERS, L.F.; PORTO, D.D.; FALAVIGNA, V.S.; BUFFON, V.; MALABARBA, J. Genética Molecular Vegetal: mapeamento genético, identificação de QTLs, genes candidatos e suas aplicações tecnológicas. UNISINOS, 43h, 2012. (Curso de curta duração ministrado/Extensão).

ORIENTAÇÕES E SUPERVISÕES CONCLUÍDAS

YOHANNA EVELYN MIOTTO. Identificação e caracterização espaço temporal da família de genes codificadores de galactinol sintases em macieira (*Malus* x *domestica* Borkh.). 2013. Trabalho de Conclusão de Curso (Bacheralado em Engenharia de Bioprocessos e Biotecnologia), Universidade Estadual do Rio Grande do Sul. Orientador: Dr. Fábio Luís Maciel. Co-orientador: MSc. Vítor da Silveira Falavigna.