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Dormancy regulator *Prunus mume* DAM6 promotes ethylene-mediated leaf senescence and abscission

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Abstract

Leaf senescence and abscission in autumn are critical phenological events in deciduous woody perennials. After leaf fall, dormant buds remain on deciduous woody perennials, which then enter a winter dormancy phase. Thus, leaf fall is widely believed to be linked to the onset of dormancy. In Rosaceae fruit trees, DORMANCY-ASSOCIATED MADS-box (DAM) transcription factors control bud dormancy. However, apart from their regulatory effects on bud dormancy, the biological functions of DAMs have not been thoroughly characterized. In this study, we revealed a novel DAM function influencing leaf senescence and abscission in autumn. In *Prunus mume*, *PmDAM6* expression was gradually up-regulated in leaves during autumn toward leaf fall. Our comparative transcriptome analysis using two RNA-seq datasets for the leaves of transgenic plants overexpressing *PmDAM6* and peach (*Prunus persica*) *DAM6* (*PpeDAM6*) indicated *Prunus* DAM6 may up-regulate the expression of genes involved in ethylene biosynthesis and signaling as well as leaf abscission. Significant increases in 1-aminocyclopropane-1-carboxylate accumulation and ethylene emission in DEX-treated *35S:PmDAM6-GR* leaves reflect the inductive effect of PmDAM6 on ethylene biosynthesis. Additionally, ethephon treatments promoted autumn leaf senescence and abscission in apple and *P. mume*, mirroring the changes due to *PmDAM6* overexpression. Collectively, these findings suggest that PmDAM6 may induce ethylene emission from leaves, thereby promoting leaf senescence and abscission. This study clarified the effects of *Prunus* DAM6 on autumn leaf fall, which is associated with bud dormancy onset. Accordingly, in Rosaceae, DAMs may play multiple important roles affecting whole plant growth during the tree dormancy induction phase.

Keywords DORMANCY-ASSOCIATED MADS-box (DAM) · Prunus mume · Leaf senescence · Leaf abscission · Ethylene · AQUAPORIN (AQP)

Introduction

Temperate woody perennials enter dormancy in autumn, during which external growth is halted, with bud break subsequently initiated after an exposure to a specific period of chilling conditions. Dormancy prevents premature sprouting under unfavorable environmental conditions, thereby protecting the plant from potential damage (Faust et al. 1997).

In addition to bud dormancy, several phenological events typically occur during the tree dormancy induction phase. Saure (1985) reported that shoot growth cessation and leaf fall are closely linked to dormancy and cold acclimation in temperate deciduous woody perennials. To date, several studies generated evidence of the connection between leaf senescence/abscission and bud dormancy. For example, temperature increases in autumn delay leaf senescence and abscission, while also significantly affecting bud dormancy, resulting in delayed spring bud burst (Beil et al. 2021; Wang et al. 2022; Malyshev et al. 2024). An exogenous abscisic acid treatment significantly enhances shoot growth cessation and induces early dormancy, but also alters leaf senescence and cold acclimation in apple (*Malus* spp.) plants (Guak and Fuchigami 2001). However, the regulatory mechanisms underlying the relationship between leaf senescence/abscission and bud dormancy remain largely unclear.

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Rosaceae *DORMANCY-ASSOCIATED MADS-box* (*DAM*) genes encode the primary regulators of tree dormancy; their expression patterns in dormant buds are positively correlated with the timing of the dormancy phase transition (i.e., up-regulated expression during dormancy induction and down-regulated expression during dormancy release) (Bielenberg et al. 2008; Jiménez et al. 2009; Sasaki et al. 2011; Zhu et al. 2015; Tuan et al. 2017; Falavigna et al. 2019; Yamane et al. 2019; Lloret et al. 2021; Hsiang et al. 2024). *DAM* genes, which are phylogenetically closely related to *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-LIKE 24* (*AGL24*) in Arabidopsis, were initially identified as the genes associated with the failure of the peach (*Prunus persica*) *evergrowing* (*evg*) mutant to enter dormancy (Bielenberg et al. 2008; Li et al. 2009). *MdDAM1* RNA interference (RNAi) transgenic apple plants are reportedly unable to enter dormancy in winter, displaying evergrowing traits similar to those of the dormancy-insensitive peach *evg* mutant (Bielenberg et al. 2008; Moser et al. 2020). Additionally, simultaneously silencing several *DAM* and *SVP* genes in apple results in an evergrowing phenotype (Wu et al. 2021). A recent study showed that the *Prunus mume* gene *DAM6* (*PmDAM6*) encodes a transcription factor that modulates the expression of genes related to phytohormone and lipid body metabolism as well as the cell cycle in the dormant vegetative meristem, thereby controlling dormancy (Hsiang et al. 2024). However, other studies suggest Rosaceae DAMs affect tree growth in addition to dormancy. The expression of *PmDAM6* in leaves is up-regulated toward leaf fall (Sasaki et al. 2011). The overexpression of peach *PpeDAM6* and *P. mume PmDAM6* promotes bud set, while also repressing growth (Lloret et al. 2021; Yamane et al. 2019). Additionally, the overexpression of *PmDAM6* in poplar (*Populus tremula* × *Populus tremuloides*) and apple promotes growth cessation, whereas the RNAi-based silencing of *DAM* expression delays leaf shedding in transgenic plants (Bielenberg et al. 2008; Moser et al. 2020; Wu et al. 2021; Sasaki et al. 2011; Yamane et al. 2019). These results reflect the importance of DAMs on physiological processes other than bud dormancy.

The onset of leaf abscission and senescence in temperate woody perennials during autumn is a crucial indicator of water and nutrient remobilization within plants (Koyama 2014; Walde et al. 2024). Leaf senescence and abscission are influenced by auxin, ethylene, and jasmonic acid (JA) levels (He et al. 2002; Ferrante and Francini 2006; Koyama 2014; Hu et al. 2017; Li et al. 2018; Zhang et al. 2020). Specifically, leaf abscission is regulated by both auxin and ethylene (Ferrante and Francini 2006; Iqbal et al. 2017), with ethylene initiating senescence, especially in ethylene-sensitive species (Iqbal et al. 2017). In tomato and Arabidopsis, a prolonged exposure to ethylene results in starch and chlorophyll degradation, further suppressing photosynthesis and causing early leaf senescence (Zhang et al. 2022; Mohorović et al. 2023b). Furthermore, JA-induced leaf senescence depends on components of the ethylene signaling pathway, particularly the key components EIN2 and EIN3; *EIN3* overexpression and silencing accelerates and delays leaf senescence, respectively (Li et al. 2013). Therefore, ethylene regulates leaf senescence more than JA.

The objective of this study was to determine whether PmDAM6 affects leaf senescence and abscission. We used two mRNA-seq datasets to examine the leaves of *35S:PmDAM6-GR* transgenic apple (this study) and *35S:PpeDAM6* transgenic European plum (*Prunus domestica*) (Lloret et al. 2021). A comparative transcriptome analysis revealed the conserved mechanisms underlying the functions of DAM6 in *P. mume* and *P. persica*. Notably, this study provides the first evidence of the possible conserved regulatory effect of *Prunus* DAM6 on leaf senescence and abscission, suggesting it contributes to the regulation of bud dormancy as well as the seasonal tree growth/dormancy cycle during the dormancy induction phase.

Materials and methods

Plant materials

This study was completed using previously reported transgenic apple lines (*35S:PmDAM6-GR* and *35S:PmDAM6*) (Yamane et al. 2019). Specifically, two *35S:PmDAM6-GR* (chemical-inducible overexpression) transgenic apple lines (GR21 and GR22) were examined in the 2015–2016 (4-year-old) and the 2022–2023 (11-year-old) seasons, whereas two 12-year-old *35S:PmDAM6* (stable overexpression) transgenic apple 'JM2' lines (35S-2 and 35S-4) and WT plants were included for detecting ethylene and analyses of leaf abscission and senescence in the 2023–2024 season. All transgenic lines were cultivated under natural photoperiodic conditions in a sealed greenhouse in Kyoto, Japan. The greenhouse was cooled when temperatures exceeded 25 °C (May–September) or 15 °C (October–April), but was not heated during the experimental period.

Adult 'Nanko' (early leaf shedding, high-chill) and 'Ellching' (late leaf shedding, low-chill) *P. mume* trees (> 15 years old) from the Kyoto experimental farm of Kyoto University as well as adult 'Nanko' and 'SC' (late leaf shedding, low-chill) trees from the Kizu experimental farm of Kyoto University were also used. For seasonal analyses, leaves were collected from 'Nanko' and 'Ellching' trees twice per month from September to December 2023. They were immediately frozen in liquid nitrogen and stored at − 80 °C prior to extracting RNA. Leaf ethylene emission was measured using 'Nanko' and 'Ellching' leaves collected on November 23, 2023 at the Kyoto experimental farm as well as 'Nanko' and 'SC' leaves collected on November 27,

2023 at the Kizu experimental farm. Treatment analysis was performed using 'Nanko' and 'JM2' trees. The analyzed plant materials are listed in Supplementary Table S3.

DEX treatment of *35S:PmDAM6-GR* apple plants

A solution consisting of 100 µM DEX (Wako, Osaka, Japan) and 0.1% Tween 20 was thoroughly mixed and uniformly sprayed onto the leaf surface of *35S:PmDAM6-GR* transgenic apple lines and WT plants in October 2015 as well as on June 28 and October 31, 2023. Leaf samples collected at 0, 8, and 16 h after the DEX treatment were rapidly frozen in liquid nitrogen and stored at − 80 °C prior to extracting RNA. The leaf samples from the 2015 season were used for the mRNA-seq analysis, ACC measurement, and hormone (IAA and JA) extraction, whereas the leaf samples from the 2023 season were used for analyses of gene expression (via qRT-PCR) and ethylene emission.

RNA extraction

Total RNA was isolated from the collected leaves using a modified CTAB method (Yamane et al. 2008). Briefly, the frozen leaf tissue was ground to a powder, which was resuspended in 6 mL pre-heated extraction buffer containing 3% (v/v) 2-mercaptoethanol (65 °C). The sample was gently mixed, incubated at 65 °C for 30 min, and centrifuged (8000 rpm for 5 min). The supernatant was transferred to a tube and combined with an equivalent amount of chloroform:isoamyl alcohol [24:1 (v/v)]. The solution was gently shaken and subsequently centrifuged (8000 rpm for 5 min). These steps were repeated, after which the supernatant was transferred to a new tube. Nucleic acids were precipitated by adding the same volume of isopropanol and then incubating the solution at − 20 °C for 2 h. After centrifuging the solution (14,000 rpm for 30 min), the pellet was resuspended in 500 µL DEPC-treated H_2O and LiCl (one-third volume) was added. The solution was incubated overnight at − 20 °C and centrifuged (14,000 rpm for 30 min). The pellet was washed using 500 µL 70% ethanol and then centrifuged (14,000 rpm for 5 min). The pellet was air-dried and then resuspended in 30 µL DEPC-treated water.

mRNA-seq library preparation

To construct the mRNA-seq library, mRNA was isolated from the extracted total RNA (10–25 µg) using the Dynabeads® mRNA Purification Kit (Thermofisher scientific, MA, USA). Next, cDNA was synthesized from the isolated mRNA using Superscript III (Thermofisher scientific), with a double-stranded cDNA formed via nick translation. The KAPA HyperPlus Library Preparation Kit (NIPPON Genetics Co. Ltd., Tokyo, Japan) was employed to repair the cDNA ends, add adenine residues, and ligate indexing adapters for identifying samples. The cDNA fragments longer than 100 bp were purified after each step using AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA). A small amount of purified cDNA solution was used as a template for a PCR amplification using Bio-amp primers to determine the optimal number of amplification cycles. The remaining cDNA solution was used for the PCR amplification with the optimal cycle number. The concentration of the generated mRNA-seq library was measured using a Qubit® 2.0 Fluorometer (Thermofisher scientific). After adjusting the library amount (at least 20 ng), the mRNA-seq analysis was performed on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) to generate 100 bp paired-end reads. The sequencing analysis was conducted using two or three biological replicates per experimental time point.

Comparative transcriptome analysis

The following mRNA-seq datasets were analyzed: (1) mRNA-seq datasets for the DEX-treated and control leaves of *35S:PmDAM6-GR* transgenic apple (current study) and (2) mRNA-seq datasets for the leaves of *35S:PpeDAM6* transgenic European plum and WT available in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) (accession number PRJNA630876) (Lloret et al. 2021). For the analysis of apple mRNA-seq datasets, the GDDH13 genome (Daccord et al. 2017) served as the reference genome. Adapters and low-quality reads were removed using the Fastp software (Chen et al. 2018), after which clean reads were mapped to the reference genome using the STAR aligner (Dobin and Gingeras 2015). The European plum (*P. domestica*) mRNA-seq datasets were analyzed using the *Prunus domestica* Draft Genome Assembly v1.0.a1 (Callahan et al. 2021) as the reference genome. Because *P. domestica* is a hexaploid (2n = 6x = 48), the default settings of the cd-hit program (Li and Godzik 2006) were applied to exclude highly similar transcripts, while retaining longer and specific transcripts as references. Subsequently, Fastp (Chen et al. 2018) was used to eliminate adapters and low-quality reads. The clean reads were then mapped to unique transcripts in the *Prunus domestica* Draft Genome Assembly v1.0.a1 using the Salmon software (Patro et al. 2017).

Mapped reads were counted using featureCounts (Liao et al. 2014). Raw read counts were normalized to transcripts per million (TPM) values. The DESeq2 package in R (Love et al. 2014) and the following criteria were used

to identify DEGs: $|\log_2(\text{fold-change})| > 0.5$ and adjusted $p < 0.05$. Orthologs across species were classified using the OrthoFinder software (Emms and Kelly 2019). On the basis of orthogroups, conserved differentially expressed orthologs were identified and filtered out. A GO enrichment analysis was conducted using the default parameters of the ClusterProfiler package v3.16.1 (Yu et al. 2012). The criteria for identifying significantly enriched GO terms were as follows: pvalueCutoff = 0.01, pAdjustMethod = "BH", and qvalueCutoff = 0.05. The obtained data were visualized using the Revigo platform (http://revigo.irb.hr/).

Gene expression analysis via qRT-PCR

The cDNA synthesized from approximately 1 µg total RNA was used for the qRT-PCR analysis, which was performed using the SYBR Green Master mix (Roche), gene-specific primers, and the LightCycler 480 system (Roche, Basel, Switzerland). The apple *SAND* gene (MDP0000185470 and/or MDP0000202305) (Velasco et al. 2010) was selected as the reference control. The PCR program was as follows: 95 °C for 30 s and then 45 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 60 s. Gene-specific amplifications were confirmed by a dissociation curve analysis. Three biological replicates were analyzed, with three leaf samples per RNA extraction. Details regarding the qRT-PCR primers are provided in Supplementary Table S4.

Measurement of ethylene emission via gas chromatography

Ethylene emitted from leaf samples was detected according to an established protocol (Nakano et al. 2003) involving a gas chromatograph (GC8 CMPF, Shimadzu). Collected leaf samples were enclosed in 50 mL airtight tubes containing 20 mL water and kept at room temperature. Ethylene emission was quantified after 24 and 72 h for DEX-treated leaves or after 3, 5, and 7 days for *P. mume* leaves. More specifically, 1 mL headspace air was extracted from each tube; the minimum detectable ethylene concentration was 0.05 nL. At least three replicates were analyzed per time point.

Quantification of IAA, JA, and their derivatives in *35S:PmDAM6-GR* transgenic apple and WT leaves

Fresh leaves were stored at − 80 °C until they were ground to a fine powder using the Multi-beads shocker (Yasui Kikai Co., Osaka, Japan). Published solid phase extraction procedures were used to extract IAA, JA, and JA-isoleucine (JA-Ile) (Yamane et al. 2019). The IAA, JA and JA-Ile contents were quantified using a liquid chromatography–triple quadrupole mass spectrometry system (6410-LC/MS QQQ, Agilent).

Quantitative analysis of ACC, a precursor of ethylene in the ethylene biosynthesis pathway

In November 2015, control and DEX-treated GR22 leaves were collected at 0, 24, and 72 h post-treatment. Leaf tissues (three replicates with 40 mg per replicate) were frozen in liquid nitrogen and stored at − 80 °C. The primary metabolite profiles of the leaves were analyzed using a CE-MS system (G2201AA, Agilent) as described previously (Oikawa et al. 2011).

Evaluation of ethylene- and JA-induced leaf abscission and senescence in apple and *P. mume* plants

Wild-type apple 'JM2' and *P. mume* 'Nanko' trees were selected for an analysis of the effects of ethephon and JA treatments on leaf abscission and senescence. For the apple trees, 100 or 500 ppm ethephon solutions containing 0.1% Tween 20 were applied to the leaf surface using a hand sprayer on December 2, 2022. The leaf abscission rate was determined 2 weeks later. On October 26, 2017, samples were treated with 1 or 5 mM JA solutions containing 0.1% Triton X-100. They were then photographed 3 weeks later. For the 'Nanko' trees, 100 or 500 ppm ethephon solutions containing 0.1% Tween 20 were applied to the leaf surface on October 16, 2023, after which the leaves were photographed at 10 days post-treatment, whereas the leaf abscission rate was determined at 3 weeks post-treatment. On October 21, 2023, samples were treated with 1 or 5 mM JA solutions containing 0.1% Tween 20. They were photographed 3 weeks later.

Statistical analyses

Data were analyzed using Student's *t*-test, Fisher's exact test, and the Tukey Honestly Significant Difference test in R Studio. The threshold for determining statistically significant differences was $p < 0.05$.

Results

Comparative transcriptome analysis of transgenic apple and European plum leaves overexpressing *PmDAM6* and *PpeDAM6*, respectively

In *35S:PmDAM6-GR* transgenic apple leaves, 2862 and 1922 genes had up- and down-regulated expression levels,

respectively, following the dexamethasone (DEX) treatment. The 2,862 up-regulated genes included 2,079 orthogroups, whereas the 1,922 down-regulated genes included 1,465 orthogroups (Fig. 1a; Supplementary Fig. S1a). A total of 4,896 and 4,948 genes were expressed at significantly higher and lower levels, respectively, in the leaves of *35S:PpeDAM6* transgenic European plum lines TG1 and TG2 (Lloret et al. 2021) than in the wild-type (WT) leaves. The classification of these genes into orthogroups revealed that 3522 and 3702 orthogroups were expressed at higher and lower levels, respectively, in transgenic lines (Fig. 1a; Supplementary Fig. S1b). The expression levels of 713 and 491 orthogroups were higher and lower, respectively, in both DEX-treated *35S:PmDAM6-GR* transgenic apple and *35S:PpeDAM6* transgenic European plum than in the control and WT, respectively (Fig. 1a; Supplementary Fig. S1a). There was a significant overlap in the conserved orthogroups (Fisher's exact test, $p < 0.01$) in the *PmDAM6*- and *PpeDAM6*-overexpressing samples, suggesting the genes regulated by *Prunus* DAM6 are highly conserved among species. Moreover, *Prunus* DAM6 is likely a transcription factor that rapidly affects the expression of downstream genes (Fig. 1b; Supplementary Fig. S1b, Tables S1–S2).

Our Gene Ontology (GO) enrichment analysis revealed GO terms related to 'ethylene biosynthesis and signaling,' 'JA biosynthesis and signaling,' and 'leaf senescence and abscission' were enriched among the 1001 differentially expressed genes (DEGs) belonging to the 713 conserved orthogroups up-regulated by the overexpression of *Prunus DAM6* (Fig. 1b, Supplementary Table S1). Notably, previous studies did not indicate that DAM6 mediates ethylene biosynthesis and signaling. Thus, we conducted further analyses to address this possibility (described in the following section). The GO enrichment analysis also revealed photosynthesis-associated GO terms, such as 'photosynthetic electron transport in photosystem I,' 'photosystem II assembly,' 'photosystem II repair,' 'photosynthetic electron transport chain,' and 'chlorophyll biosynthetic process,' were enriched among the 605 DEGs belonging to the 491 conserved orthogroups down-regulated by the overexpression of *Prunus DAM6* (Supplementary Fig. S1, Table S2). Additionally, 'water transport' and 'response to water deprivation' were also enriched GO terms assigned to the genes down-regulated by *DAM6* overexpression (Supplementary Figs S1 and S2, Table S2). Of the genes annotated with these water-related GO terms, the expression levels of six *AQUAPORIN* (*AQP*) genes (MD01G1148400, MD03G1117900, MD01G1108500, MD01G1062800, MD11G1136200, and MD05G1122600) in *35S:PmDAM6-GR* transgenic apple leaves were strongly repressed by the DEX treatment (Supplementary Figs S1 and S2). In hormone-related pathways, the 'response to auxin' GO term was enriched among the genes down-regulated by *DAM6* overexpression (Supplementary Fig. S1). The expression levels of several genes encoding *AUXIN-RESPONSIVE PROTEINS* (MD04G1225100, MD12G1241800, MD02G1057200, and MD09G1202300) and *AUXIN RESPONSE FACTOR* (*ARF*) (MD00G1103900 and MD10G1192900) were down-regulated in the DEX-treated *35S:PmDAM6-GR* transgenic apple leaves (compared with the corresponding control expression levels). However, indole-3-acetic acid (IAA) levels in *35S:PmDAM6-GR* transgenic apple leaves treated with DEX did not differ from the IAA levels in the control and WT leaves (Supplementary Fig. S3; Table S2).

Overexpression of *Prunus DAM6* promotes ethylene and JA accumulation as well as leaf senescence and abscission in transgenic plants

The DEX treatment of *35S:PmDAM6-GR* transgenic apple plants in October promoted leaf abscission and senescence (Fig. 2a), which is in accordance with our GO enrichment analysis (Fig. 1b). The DEX treatment resulted in the following two distinct phenotypes: (1) leaves, which were brown and dehydrated, dropped within approximately 3–5 days; (2) leaves remained attached to branches, but were noticeably yellow (Fig. 2b).

Our GO enrichment analysis suggested peach and Japanese apricot DAM6 up-regulates the expression of genes associated with ethylene biosynthesis and signaling pathways (Fig. 1b). Ethylene promotes leaf senescence and abscission in model plants (Iqbal et al. 2017). Thus, we hypothesized that *Prunus* DAM6 may promote leaf senescence and abscission by regulating ethylene metabolism. To test this hypothesis, we examined the expression of genes involved in ethylene metabolic pathways. Consistent with our GO enrichment analysis, the expression levels of most genes involved in the ethylene biosynthesis pathway were significantly up-regulated from 8 h after the DEX treatment (Fig. 2c). Furthermore, our quantitative reverse transcription polymerase chain reaction (qRT-PCR) results confirmed the up-regulated expression of the ethylene biosynthesis-related genes in both *35S:PmDAM6-GR* transgenic lines (GR21 and GR22) at 8 h after the DEX treatment (Fig. 2d). Ethylene biosynthesis-related genes, namely *S-ADENOSYL-METHIONINE SYNTHETASE* (*MetK*) (MD16G1138300), *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE* (*ACS*) (MD06G1090600), and *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE* (*ACO*) (MD17G1106300 and MD09G1114800), had significantly up-regulated expression levels at 16 h after the DEX treatment (Fig. 2d). Similarly, in *35S:PpeDAM6* transgenic European plum lines TG1 and TG2, ethylene biosynthesis-related gene expression levels in leaves were

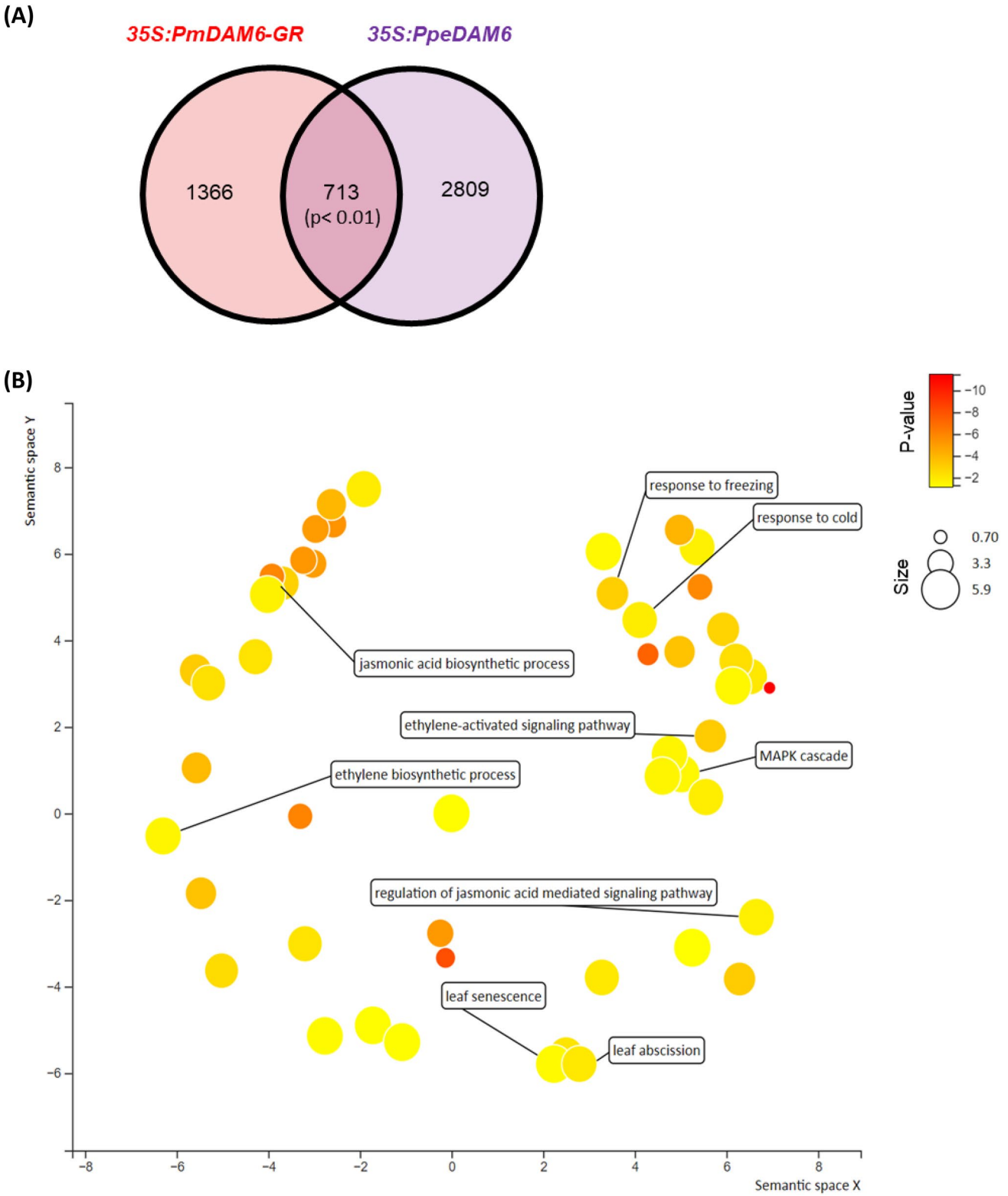


Fig. 1 Genes significantly up-regulated in DEX-treated *35S:PmDAM6-GR* apple and *35S:PpeDAM6* European plum leaves and the associated enriched GO terms (**A**) Venn diagram of the up-regulated orthogroups in DEX-treated *35S:PmDAM6-GR* transgenic apple and *35S:PpeDAM6* transgenic European plum leaves. (**B**) Visualization of the results of the GO enrichment analysis of commonly up-regulated DEGs by the Revigo platform

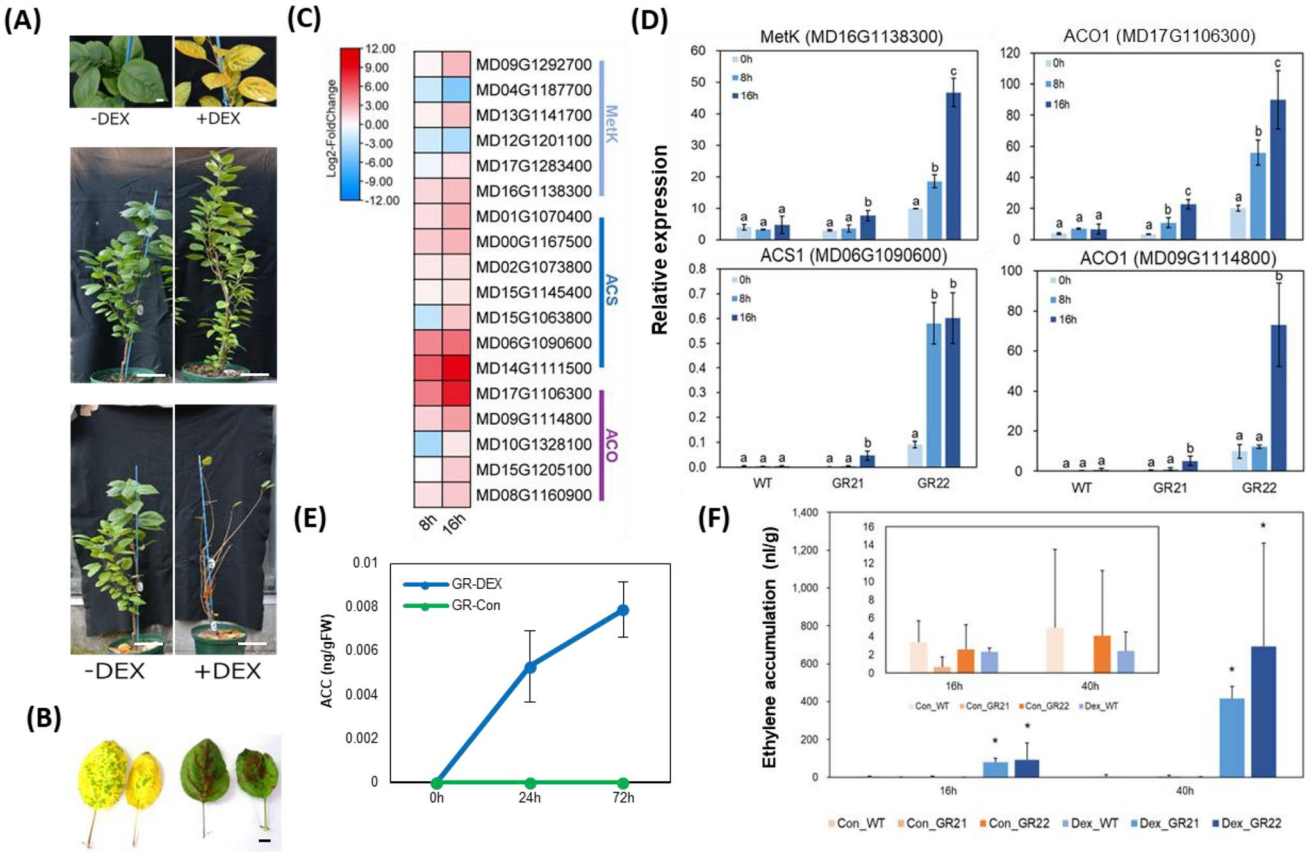


Fig. 2 DEX treatment up-regulated the expression of ethylene biosynthesis-related genes and increased ethylene emission from *35S:PmDAM6-GR* transgenic apple leaves. (**A**) DEX treatment induced *35S:PmDAM6-GR* transgenic apple leaf senescence and abscission. Scale bar = 1 cm (upper panel) and 30 cm (bottom panel). (**B**) DEX treatment induced leaf yellowing or browning at 3 weeks post-treatment. Scale bar = 1 cm. (**C**) Changes in ethylene biosynthesis-related gene expression at 8 and 16 h after *35S:PmDAM6-GR* transgenic apple leaves were treated with DEX (as determined by RNA-seq). The heat map presents $\log_2$(fold-change) values (expression levels in *35S:PmDAM6-GR* transgenic apple/control leaves; n = 2–3). (**D**) Changes in the expression of ethylene biosynthesis-related genes at 8 and 16 h after the leaves of two *35S:PmDAM6-GR* transgenic apple lines (GR21 and GR22) were treated with DEX (as determined by qRT-PCR); *S-ADENOSYL-METHIONINE SYNTHETASE* (*MetK*), *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE* (*ACS*), and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE* (*ACO*). (**E**) DEX treatment induced the accumulation of the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC) in leaves at 24 and 72 h after *35S:PmDAM6-GR* transgenic apple leaves were treated with DEX. (**F**) DEX treatment induced the emission of ethylene from *35S:PmDAM6-GR* transgenic apple leaves

consistently up-regulated (Supplementary Fig. S4a). In addition, there was a significant increase in the abundance of the ethylene biosynthesis precursor aminocyclopropane-1-carboxylate (ACC) in *35S:PmDAM6-GR* transgenic apple leaves following the DEX treatment (Fig. 2e). The leaves of both *35S:PmDAM6-GR* transgenic lines (GR21 and GR22) accumulated substantially more ethylene than the control and WT leaves at 16 h after the DEX treatment (Fig. 2f). In particular, DEX-treated GR22 leaves had an extremely high ethylene level, which corresponded with their relatively high ethylene biosynthesis-related gene expression levels (compared with the corresponding expression in GR21), suggestive of the positive effect of PmDAM6 on ethylene metabolic pathways (Fig. 2f). Finally, *35S:PmDAM6* transgenic apple lines 35S-2 and 35S-4 underwent earlier leaf abscission and senescence and accumulated more ethylene at 3 and 5 days after sampling than WT in December 2023 (Fig. 3).

In addition to the ethylene biosynthesis pathway genes, ethylene signaling pathway genes also had up-regulated expression levels in DEX-treated *35S:PmDAM6-GR* transgenic apple and *35S:PpeDAM6* transgenic European plum (Supplementary Figs S4b and S5). In *35S:PmDAM6-GR* transgenic apple lines GR21 and GR22, the expression levels of *ETHYLENE-INSENSITIVE 3* (*EIN3*) (MD07G1053800 and MD02G1266200) and *ETHYLENE RESPONSE FACTOR* (*ERF*) (MD05G1198700 and MD16G1216900) were significantly up-regulated after the DEX treatment (Supplementary Fig. S5a-b, Table S1). We also assessed the effects of exogenous ethylene on apple leaf senescence

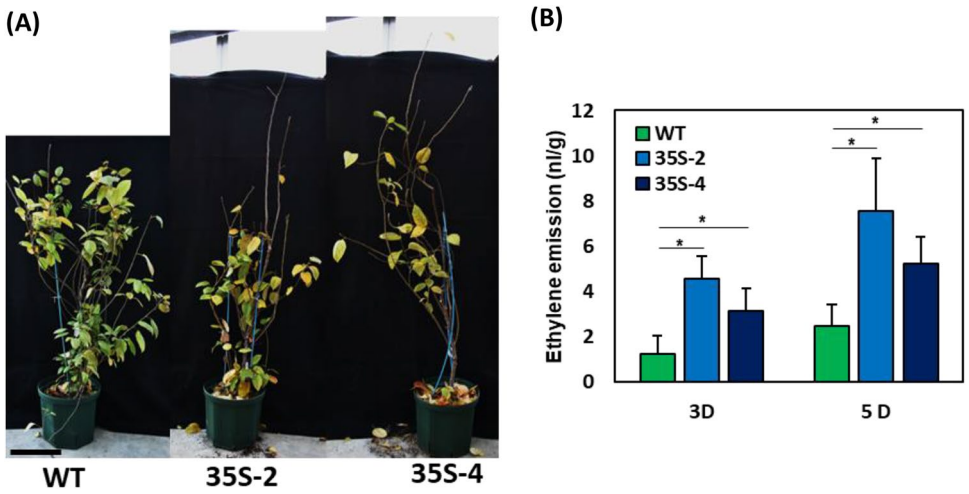


Fig. 3 Overexpression of *PmDAM6* resulted in earlier leaf senescence and abscission and increased ethylene emission from the leaves of transgenic apple plants (**A**) Leaf senescence and abscission occurred earlier for *PmDAM6*-overexpressing apple plants than for wild-type plants (photographed on December 13, 2020). Scale bar = 50 cm. (**B**) Ethylene emitted from the leaves of two apple transgenic lines overexpressing *PmDAM6* (35S-2 and 35S-4) and wild-type plants at 3 and 5 days after leaves were collected and incubated in a sealed tube. Leaf samples were collected on December 18, 2023. Data are presented as the mean ± standard error. An asterisk indicates significant differences (*t*-test, $p < 0.05$). Scale bar = 50 cm

and abscission. The application of 500 ppm ethephon significantly induced leaf abscission at 2 weeks post-treatment (Fig. 4).

Interestingly, the seasonal environment appeared to influence the ethylene-mediated leaf senescence and abscission phenotypes of the plants overexpressing *PmDAM6*. Applying DEX to *35S:PmDAM6-GR* transgenic apple leaves in summer resulted in leaf abscission, but not leaf senescence. Moreover, there were no significant differences in the total chlorophyll contents of the DEX-treated leaves and the control in summer (Supplementary Fig. S6a–b). We also investigated the changes in the expression of genes related to ethylene biosynthesis and signaling in leaf samples collected at 24 h after the DEX treatment in summer (relative to the corresponding control and WT gene expression). The expression levels of the ethylene biosynthesis-related genes *ACS* (MD06G1090600) and *ACO* (MD17G1106300 and MD09G1114800) were significantly higher in the DEX-treated leaves of *35S:PmDAM6-GR* transgenic lines GR21 and GR22 than in the control and WT leaves (Supplementary Fig. S6c). However, the expression of the ethylene signaling-related genes *EIN3* (MD07G1053800) and *ERF* (MD05G1198700 and MD16G1216900) was not up-regulated in GR21 and GR22, which was in contrast to the significantly up-regulated expression of these genes in autumn (Supplementary Fig. S6d). Collectively, these results suggest that the ethylene signaling pathway may need to be activated for PmDAM6-induced leaf senescence in autumn.

Because our comparative transcriptome analysis also revealed the increased expression of genes associated with

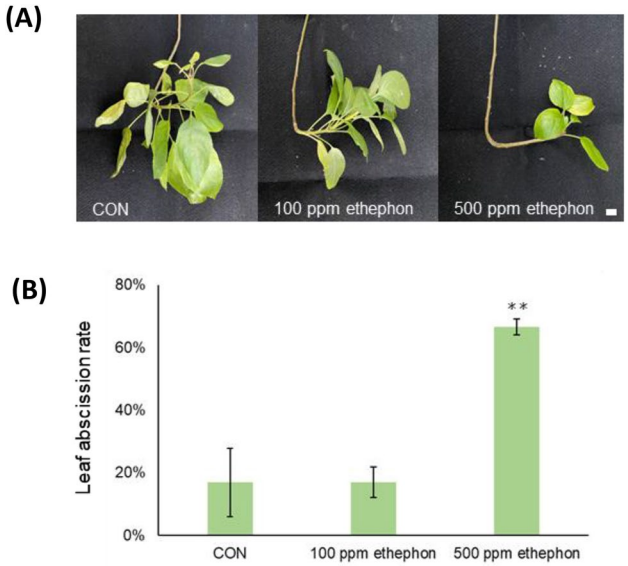


Fig. 4 Ethephon treatment induced leaf abscission of apple 'JM2' (**A**) Photographs of the shoot at 2 weeks after a treatment with 100 or 500 ppm ethephon on December 2, 2022. Scale bar = 1 cm. (**B**) Leaf abscission rate at 3 weeks post-treatment. Asterisks indicate significant differences (*t*-test, $p < 0.05$)

JA biosynthesis and signaling in transgenic plants overexpressing *Prunus DAM6* (Fig. 1b), we examined the expression of JA metabolism-related genes and JA accumulation. In *35S:PmDAM6-GR* transgenic apple leaves, the expression levels of genes related to JA biosynthesis and signaling were significantly up-regulated from 8 h after the DEX treatment

(Supplementary Fig. S7a–b). Additionally, the accumulation of JA and jasmonic acid-isoleucine (JA-Ile) peaked at 16 h after the DEX treatment (Supplementary Fig. S7c). Furthermore, JA biosynthesis-related gene expression levels were higher and more JA accumulated in *35S:PpeDAM6* transgenic European plum leaves than in WT leaves (Lloret et al. 2021). Similar to the ethephon treatment, the JA treatment induced leaf senescence and abscission, but only when the concentration was extremely high (5 mM), at 3 weeks after apple plants were treated (Supplementary Fig. S8a–b).

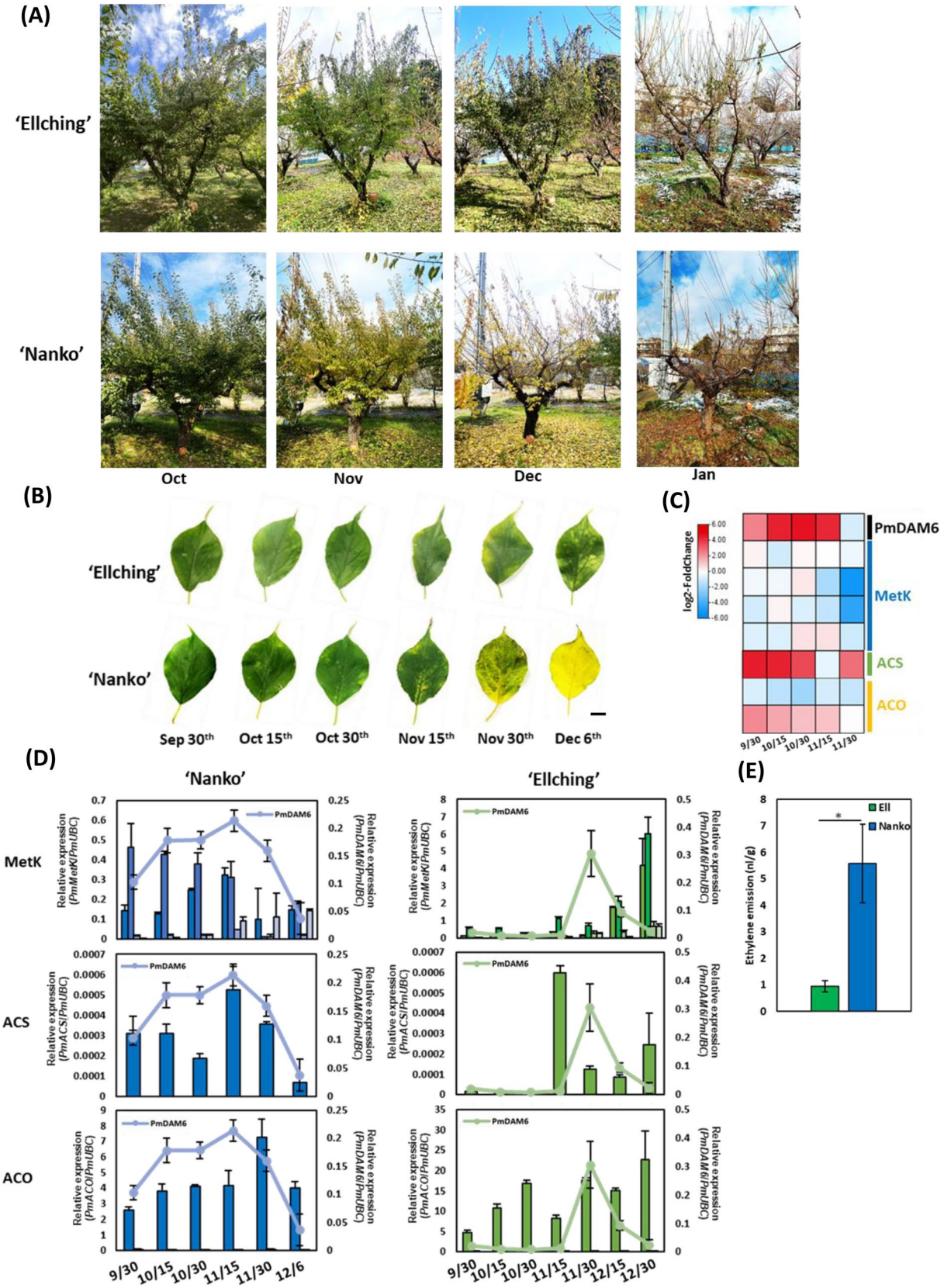
Correlation between PmDAM6 expression and ethylene biosynthesis-related gene expression and ethylene emission in *Prunus* mume leaves

We selected two *P. mume* cultivars with contrasting *PmDAM6* expression patterns and leaf shedding characteristics. 'Nanko' typically exhibits leaf senescence and shedding starting in mid-to-late November, with peak leaf shedding and yellowing in early December, whereas 'Ellching' does not exhibit leaf senescence and abscission by early December, but sheds leaves in early January, approximately 1 month later than 'Nanko' (Fig. 5a–b). Seasonal *PmDAM6* expression patterns in leaves also differed between the two cultivars, with significantly higher expression levels in 'Nanko' than in 'Ellching' before November 15 (Fig. 5c–d). The *PmDAM6* expression level in 'Nanko' leaves peaked on November 15, but then subsequently decreased (Fig. 5d). In contrast, in 'Ellching' leaves, *PmDAM6* expression increased significantly from November 15 to November 30 (peak expression) and then gradually decreased (Fig. 5d). From September 30 to November 15, *ACS* (LOC103337567) and *ACO* (LOC103329345) along with *PmDAM6* were more highly expressed in 'Nanko' leaves than in 'Ellching' leaves (Fig. 5c). The expression levels of ethylene biosynthesis-related genes, such as *MetK* (LOC103323276, LOC103327270, LOC103339824, and LOC103344495), *ACS* (LOC103337567), and *ACO* (LOC103329345), were significantly up-regulated following the *PmDAM6* expression peak on November 30 in 'Ellching' (Fig. 5d). Moreover, 'Nanko' emitted significantly more ethylene than 'Ellching' in late November (Fig. 5e). We also examined the seasonal changes in ethylene signaling-related gene expression (Supplementary Fig. S9a–d). The results revealed a gradual increase in *EIN3* (LOC103331049) expression before leaf senescence and shedding in 'Nanko' and 'Ellching' (Supplementary Fig. S9b). Similar results were obtained when we compared 'Nanko' with 'SC', which is another low-chill cultivar. Like 'Ellching', 'SC' did not exhibit leaf senescence and shedding from late November to December (i.e., leaf senescence and shedding occurred much later for 'SC' than for 'Nanko') and had lower *PmDAM6*, *MetK*, *ACS*, and *ACO* expression levels and ethylene emission rates than 'Nanko' (Supplementary Fig. S10). Finally, exogenous ethephon (ethylene-releasing compound) induced leaf senescence and abscission in *P. mume*, similar to its effects on apple (Fig. 6). We detected significant leaf shedding within 2 days of the 500 ppm ethephon treatment; the shed leaves had brown spots and appeared to be dehydrated. Furthermore, from 7 days after ethephon treatments (100 and 500 ppm), the leaves still attached to branches began to turn yellow. Notably, in *P. mume*, a visible abscission layer formed between the leaf petiole and the branch (Fig. 6d). In addition, the ethephon treatment significantly increased the leaf abscission rate, but JA did not substantially affect leaf abscission (Supplementary Fig. S8c).

Discussion

The regulatory role of *DAM* genes in controlling bud dormancy progression has been extensively documented in various Rosaceae species, including peach (*P. persica*) (Leida et al. 2012; Lloret et al. 2021), Japanese apricot (*P. mume*) (Yamane et al. 2019; Hsiang et al. 2024), apple (*Malus*×*domestica*) (Porto et al. 2016; Wu et al. 2021), and pear (*Pyrus pyrifolia* white pear group) (Niu et al. 2015; Tuan et al. 2017). However, although DAM mutants [*DAM* RNAi and *DAM*-overexpressing transgenic plants (Wang et al. 2002; Bielenberg et al. 2008; Wu et al. 2021)] reportedly show altered phenotypes in bud dormancy as well as other traits, such as leaf shedding, the regulatory effects of DAMs on phenological traits other than bud dormancy remain unclear. In this study, we used *DAM*-overexpressing transgenic lines to elucidate how *Prunus* DAM6 regulates the leaf traits of deciduous trees.

A previous study showed that the overexpression of peach *PpeDAM6* increases the expression of JA biosynthesis-related genes and JA accumulation in transgenic European plum leaves (Lloret et al. 2021). In the current study, DEX-treated *35S:PmDAM6-GR* transgenic apple leaves accumulated significantly more JA and JA-Ile than control leaves, which is consistent with the findings for *35S:PpeDAM6* transgenic European plum (Lloret et al. 2021). A treatment with an extremely high JA concentration promoted leaf senescence. In contrast to the JA treatment, the application of ethephon induced a rapid and significant response, which was characterized by substantial leaf yellowing, dehydration, and abscission within a short period. A comparative transcriptome analysis revealed the conserved regulatory effect of *Prunus* DAM6 on ethylene biosynthesis. Moreover, *35S:PmDAM6* and *35S:PmDAM6-GR* transgenic apple leaves emitted more ethylene than WT and control leaves, respectively. At the transcriptional level, the expression of


(A)
'Ellching'
'Nanko'
Oct
Nov
Dec
Jan
(B)
'Ellching'
'Nanko'
Sep 30th
Oct 15th
Oct 30th
Nov 15th
Nov 30th
Dec 6th
(C)
log2-FoldChange
PmDAM6
MetK
ACS
ACO
9/30
10/15
10/30
11/15
11/30
(D)
'Nanko'
'Ellching'
MetK
ACS
ACO
PmDAM6
Relative expression (PmMetK/PmUBC)
Relative expression (PmACS/PmUBC)
Relative expression (PmACO/PmUBC)
Relative expression (PmDAM6/PmUBC)
9/30
10/15
10/30
11/15
11/30
12/6
12/15
12/30
(E)
Ell
Nanko
Ethylene emission (nl/g)
*

◂**Fig. 5** Seasonal changes in leaf senescence, abscission, expression of ethylene biosynthesis-related genes, and ethylene emission in two *P. mume* cultivars with contrasting leaf shedding timing (**A**, **B**) Photographs of the whole tree (**A**) and leaves (**B**) of two *P. mume* cultivars with contrasting *PmDAM6* expression patterns and leaf abscission characteristics. Scale bar = 2 cm. (**C**) Heat map of the expression of ethylene biosynthesis-related genes in the *P. mume* v1.0 genome. The heat map presents $\log_2$(fold-change) values [expression levels in Nanko (high-chill)/Ellching (low-chill)]. (**D**) Seasonal changes in the expression of ethylene biosynthesis-related genes relative to the *PmDAM6* expression level in the leaves of Japanese apricot cultivars 'Nanko' and 'Ellching'; *S-ADENOSYL-METHIONINE SYNTHETASE* (*MetK*), *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE* (*ACS*), and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE* (*ACO*). Data are presented as the mean ± standard error (n = 3). Left and right Y-axes present the relative expression of ethylene biosynthesis-related genes and *PmDAM6*, respectively. (**E**) Ethylene emission from 'Nanko' and 'Ellching' leaves at 7 days after they were collected on November 23, 2023 and incubated in a sealed tube at room temperature. Data are presented as the mean ± standard error (n = 5). An asterisk indicates significant differences (*t*-test, $p < 0.05$)

genes related to ethylene biosynthesis and signaling was significantly up-regulated in the DEX-treated leaves of *35S:PmDAM6-GR* transgenic apple and *35S:PpeDAM6* transgenic European plum. In model plants, JA-induced leaf senescence depends on key factors associated with ethylene signaling, including EIN3 (Li et al. 2013). Earlier research indicated the overexpression of *EIN3* accelerates leaf senescence, whereas a loss-of-function mutation to this gene (e.g., in the *ein3* mutant) leads to delayed JA-induced leaf senescence (Li et al. 2013; Koyama and Sato 2018). On the basis of our results and those of reported studies, we propose that ethylene rather than JA plays a central regulatory role in *Prunus* DAM6-mediated leaf senescence and abscission. The seasonal environment significantly impacts ethylene-mediated leaf senescence in apples overexpressing *PmDAM6*. Previous studies show that ethylene signaling genes like *EIN2* and *EIN3* modulate leaf senescence sensitivity (Yu et al. 2021; Qiu et al. 2015). In our study, DEX treatment in summer did not activate ethylene signaling in *35S:PmDAM6-GR* transgenic apple leaves, leading to leaf abscission without senescence. However, in autumn, DEX treatment significantly increased ethylene signaling, resulting in pronounced senescence. This suggests that *PmDAM6*-induced senescence requires specific environmental cues or developmental stages, particularly in autumn (Fig. 7).

Our transcriptome analysis suggested *Prunus* DAM6 may have suppressive effects on 'photosystem I,' 'photosystem II assembly,' 'chlorophyll biosynthetic process,' 'water channel activity,' and 'water transport' in leaves. In several plant species, ethylene inhibits photosynthesis and water loss during the leaf and petal wilting process (Kays and Pallas 1980; Pallas et al. 1982; Mohorović et al. 2023a Qing et al. 2016). Previous studies indicated that a rose AQP protein, Rh-PIP2;1, is a crucial regulator of petal expansion; the inhibited expression of *Rh-PIP2;1* due

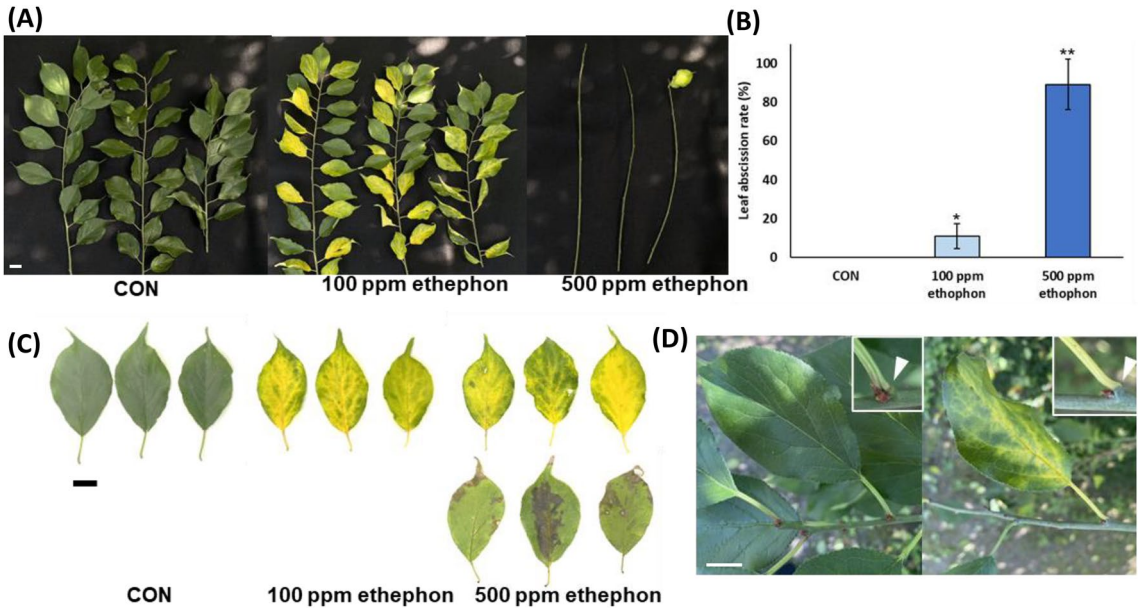
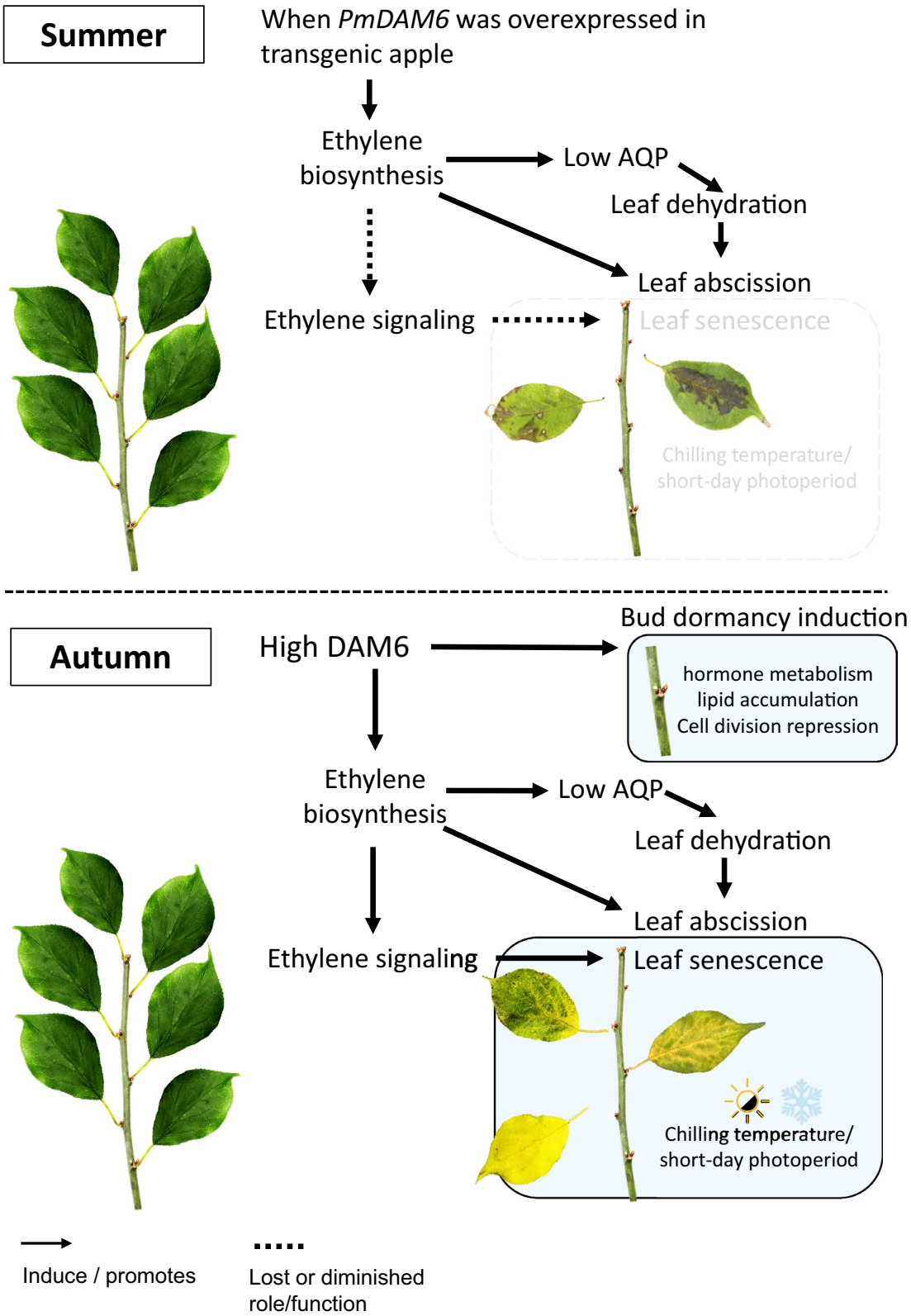


Fig. 6 Effects of an ethephon treatment on leaf senescence and abscission in *P. mume*. (**A**) Photographs of shoots treated with 100 or 500 ppm ethephon at 10 days post-treatment. Leaf senescence and abscission were promoted by 100 and 500 ppm ethephon on October 16, 2023. Scale bar = 2 cm. (**B**) Effects of ethephon on the leaf abscission rate. Data are presented as the mean ± standard error (n = 5). Asterisks indicate significant differences (** or *, $p < 0.01$ or 0.05; t-test). (**C**) Photographs of leaves treated with 100 or 500 ppm ethephon at 10 days post-treatment. Scale bar = 2 cm. (**D**) Ethephon stimulated the formation of a leaf abscission layer. Scale bar = 2 cm


Summer
When PmDAM6 was overexpressed in transgenic apple
Ethylene biosynthesis
Low AQP
Leaf dehydration
Leaf abscission
Ethylene signaling
Leaf senescence
Chilling temperature/ short-day photoperiod
Autumn
High DAM6
Bud dormancy induction
hormone metabolism
lipid accumulation
Cell division repression
Ethylene biosynthesis
Low AQP
Leaf dehydration
Leaf abscission
Ethylene signaling
Leaf senescence
Chilling temperature/ short-day photoperiod
Induce / promotes
Lost or diminished role/function

◂**Fig. 7** Proposed model of *PmDAM6* as a hub gene controlling leaf abscission/senescence and bud dormancy induction in *P. mume*. During summer, *PmDAM6* expression is low in *P. mume* leaves (Sasaki et al. 2011). In transgenic apple overexpressing *PmDAM6* in summer, *PmDAM6* promotes ethylene biosynthesis, initiating signals for leaf abscission and dehydration via decreased aquaporins (AQP) expressions. However, ethylene signaling for leaf abscission is reduced or inactive during summer. In contrast, during autumn, with the onset of chilling temperatures and shorter photoperiods, high *PmDAM6* expression is observed in *P. mume* leaves (Sasaki et al. 2011). In autumn, PmDAM6 stimulates not only ethylene biosynthesis but also ethylene signaling in both *P. mume* and transgenic apple, which led to significant leaf senescence and abscission. PmDAM6 concurrently inducing bud dormancy through changes in hormone metabolism, lipid accumulation, and repression of cell division (Hsiang et al. 2024)

to ethylene suppresses petal expansion (Ma et al. 2008). Another study showed that an ethylene treatment rapidly and significantly down-regulates *RhPIP1;1* expression and that silencing *RhPIP1;1* expression in rose significantly inhibits petal expansion, resulting in decreases in petal size and fresh weight (Chen et al. 2013). Similar to the growth of other plant organs, leaf growth involves irreversible cell expansion, which is dependent on the water potential. The expression of *AQP* genes in leaves is critical for maintaining cell growth and expansion because of the associated regulation of the leaf water potential (Uehleln et al. 2003; Volkov et al. 2007; Wei et al. 2007; Heinen et al. 2009; Fricke 2017). In tobacco, overexpressing *NtAQP1* leads to increases in leaf area and plant height (Uehleln et al. 2003; Siefritz et al. 2004; Kelly et al. 2014). The overexpression of *AtPIP1;2* increases plant growth and leaf transpiration rates as well as stomatal density and photosynthetic efficiency (Aharon et al. 2003). In antisense *NtAQP1* transgenic plants, net photosynthesis reportedly decreases, whereas photosynthetic efficiency increases in *NtAQP1*-overexpressing transgenic plants (Uehleln et al. 2003). Notably, the inhibition of photosynthesis is a necessary part of leaf senescence (Thakur et al. 2016; Krieger-Liszkay et al. 2019; Sakuraba 2021). In addition, water loss and rehydration promote leaf abscission (Agustí et al. 2007; Mahouachi et al. 2007; Dallstream and Piper 2021). For example, water stress enhances ethylene-mediated leaf abscission in cotton (Jordan et al. 1972). After considering these findings, we hypothesize that *Prunus* DAM6 may induce water stress by suppressing the expression of *AQP* genes through the activation of ethylene biosynthesis and signaling pathways, while also repressing photosynthesis in leaves, thereby facilitating ethylene-mediated leaf senescence and abscission.

To date, many studies have been conducted to clarify the molecular basis of the regulatory roles of Rosaceae DAMs in dormant buds. These studies revealed the regulatory functions of DAMs affecting phytohormone metabolism (Tuan et al. 2017; Busov 2019; Falavigna et al. 2019, 2021; Yamane et al. 2019; Yang et al. 2020; Lloret et al. 2021; Wu et al. 2021; Hsiang et al. 2024), cell division (Yang et al. 2020; Falavigna et al. 2021; Wu et al. 2021; Hsiang et al. 2024), and lipid catabolism (Hsiang et al. 2024), thereby control bud dormancy. However, the tree dormancy period is divided into several distinct phases, including dormancy induction (leaf abscission, terminal bud set, and bud development), dormancy establishment (complete developmental arrest), and dormancy release (release from bud break repression). It remains unclear which process is primarily affected by DAMs. In the present study, we determined that PmDAM6 regulates ethylene metabolism and promotes leaf senescence and abscission in autumn, which precedes the onset of bud dormancy (Fig. 7). This may imply that in Rosaceae, DAM may be involved in multiple metabolic pathways during the tree dormancy induction phase in autumn. Furthermore, *DAM* may be a hub gene linking leaf abscission with bud dormancy onset during the regulation of the tree growth/dormancy cycle.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11103-024-01497-y.

Author contributions HY: conceptualization; TFH, YYC, RN, AO, TM, and YI: analysis and investigation; HY: resources; TFH and HY: writing–original draft; TFH and YYC: visualization; HY and YI: writing–review & editing. All authors read and approved the final manuscript.

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Data availability All pertinent information is included within the manuscript and its accompanying materials. The RNA-seq data for the *35S:PmDAM6-GR* apple transformants and control produced in this study were archived in the Gene Expression Omnibus database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo) under the accession number PRJNA1072146.

Declarations

Conflict of interest No conflict of interest declared.

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