Application of ABA and ACC in Autumn Affects Dormancy and Cold Hardiness of Pear (*Pyrus communis*)

Kelsey D. Galimba[1], Mackenzie Denew[1], and Jessica M. Waite[2]

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Abstract. **Dormancy in fruit trees is a crucial process that involves many molecular and physiological mechanisms to protect plant organs from abiotic stresses throughout the winter months. This process has become an increasingly important focus of study in light of a changing and more volatile climate. Two plant hormones, abscisic acid (ABA) and ethylene, have been shown to play a role in the regulation of dormancy. Both ABA and ethylene-related compounds have been applied previously to fruit trees to alter the onset and duration of endodormancy, which is the phase of dormancy during which cold hardiness is greatest and buds are protected until a specific number of cold hours are met. We aimed to investigate pear (*Pyrus communis* cv. d'Anjou) endodormancy responses to the application of both compounds simultaneously using S-ABA and 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor. Treatment initially resulted in earlier leaf abscission relative to untreated trees. Using freeze tests, we found that cold hardiness was generally higher for treated vegetative shoots in the weeks and months after application following an immediate temporary decrease in cold hardiness. Gene expression of an ABA catabolism gene related to *CYP707A* genes was significantly lowered in reproductive buds at two timepoints corresponding to higher cold tolerance of treated vegetative shoots. However, expression of other dormancy-related genes tested was not significantly different between buds of treated and untreated trees on these dates. Overall, this research indicates that pear dormancy and cold hardiness can be at least partially altered by hormone plant growth regulator application.**

Winter dormancy is an important adaptation for all temperate fruit tree species, including European pear (*Pyrus communis*), because it allows for tree survival in cold climates. For pear, dormancy is triggered by low temperatures (<12 °C) in the autumn (Heide and Prestrud 2005), when cessation of vegetative growth, formation of protective bud scales around vegetative and reproductive meristems, and leaf senescence and abscission occur. As physiological changes increase cold hardiness in the branches and vegetative and reproductive buds, trees reduce their need for water and nutrients. This marks the transition from late summer/autumn paradormancy to deep winter endodormancy (Lang et al. 1987; Nilsson 2022). Trees remain in endodormancy until a specific number of chilling hours are completed; while in this state, they are the most protected from cold damage. A better understanding of how pear trees enter endodormancy may allow for control over this transition, which could protect trees from sudden drops in temperature in the autumn when trees are still susceptible to freeze damage.

Chemical applications have been explored in many tree fruit species as a means to manipulate the progression of dormancy or cause physiological effects that are tied to dormancy, such as leaf abscission or flowering time. The use of abscisic acid (ABA) applications to induce dormancy has been explored in apples and appears to be dependent on factors including cultivar, tree age, and timing of application (Allderman et al. 2011; Guak and Fuchigami 2001). Young fruit trees in nurseries have been treated with a variety of defoliants to hasten the propagation process, including urea, copper, iron, and compounds that promote the hormone ethylene. This causes leaves to senesce and abscise earlier in the season compared to that which could occur naturally, allowing for more efficient removal from the ground (Jones et al. 1973, 1974; Knight 1983; Larsen et al. 1984). Leaf defoliation using urea and zinc sulfate has also been investigated in fruit trees as a means to control plant diseases (Sallato and Whiting 2022). Furthermore, chemical applications have been studied to delay flowering of fruit trees in the spring; for example, ethylene-promoting compounds and synthetic auxins have been used to delay flowering in apricot (Nečas et al. 2023). Although many of the compounds studied are element-based plant growth regulators (PGRs) that affect hormone metabolism and homeostasis, ABA and ethylene are two of the most widely studied and used.

Many aspects of plant growth and development, including dormancy transitions, are controlled by ABA, which is a plant hormone (Finkelstein 2013). The involvement of ABA in different stages of dormancy differs from plant to plant (Yang et al. 2021). In poplar, ABA is central to bud endodormancy because it is involved in mediating the callose deposition and closure of plasmodesmata that is key for closing the buds and preparing them for winter (Tylewicz et al. 2018). Additionally, ABA induces *SHORT VEGETATIVE PHASE LIKE* (*SVL*), a MADS-box gene important for promoting bud endodormancy in hybrid aspen (Singh et al. 2018). During endodormancy induction, ABA increases; however, in both grapes and pears, ABA decreases toward release (Li et al. 2018; Or et al. 2000). In *Pyrus pyrifolia*, ABA biosynthesis genes *NCED-2* and *NCED-3* were highly expressed and *CYP707A* catabolism genes were low during transition to endodormancy and exogenous application of ABA to reproductive buds accelerated this transition (Li et al. 2018). In the same species, *DORMANCY ASSOCIATED MADS-BOX 1* (*PpDAM1*) bound to and upregulated *PpNCED3* during endodormancy (Tuan et al. 2017).

1-Aminocyclopropane-1-carboxylic acid (ACC) is a precursor to the plant

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[1]Mid-Columbia Agricultural Research and Extension Center, Oregon State University, 3005 Experiment Station Drive, Hood River, OR 97031, USA

[2]United States Department of Agriculture Tree Fruit Research Laboratory, 1104 North Western Avenue, Wenatchee, WA 98801, USA

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K.D.G. is the corresponding author. E-mail: kelsey.galimba@oregonstate.edu.

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hormone ethylene and is used as a PGR to increase endogenous ethylene levels. Ethylene mediates many processes, including the abscission of leaves and fruits, and it may play roles in both endodormancy induction and release (Chang 2016; Kende 1993; Yang et al. 2021). In poplar, many ethylene biosynthesis and signaling genes are upregulated in dormancy induction conditions (Ruttink et al. 2007). The application of ethylene and related compounds has been used as a way to delay spring bloom in fruit trees to avoid damaging frosts (Liu and Sherif 2019). Interestingly, in grapes, a transient increase in ethylene followed by a sharp decrease have been suggested to activate release from dormancy (Shi et al. 2018). Autumn application of ethephon, a compound that degrades into ethylene inside plant cells, increased bud survival, leave abscission, and cold hardiness in *Prunus* species as well as delayed bloom in the spring (Liu and Sherif 2019; Liu et al. 2021).

We tested whether the application of both ABA and ACC can simultaneously alter pear (*P. communis* cv. d'Anjou) transition into dormancy. We report the effects on leaf abscission, flowering time, cold hardiness, and gene expression of dormancy-related genes.

Materials and methods

Spray applications. A mixture of 500 ppm Accede® (ACC), 500 ppm ProTone® [S-abscisic acid (S-ABA)] (Valent USA, San Ramon, CA, USA), and 0.25% v/v SYL-COAT® (silicone surfactant) (Wilber-Ellis, Helm, CA, USA) in water was applied to 8-year-old 'd'Anjou' pear trees grafted to 'Old Home × Farmingdale 87' located at the Mid-Columbia Agricultural Research and Extension Center in Hood River, OR, USA. Applications were made on 22 Oct 2021, using a backpack sprayer (Stihl Incorporated, Virginia Beach, VA, USA). Five individual trees trained to a biaxis were treated and five were left untreated; trees were assigned treatment using a randomized block design with five blocks.

Weather and phenology observations. Daily maximum and minimum temperatures were recorded by the Agrimet HOXO station located at Mid-Columbia Agricultural Research and Extension Center. Trees were observed for leaf necrosis and abscission for 2 weeks following spray application. Trees were monitored daily the following spring, starting at first white, to determine the date of first bloom.

Testing cold hardiness of vegetative shoots. Three 1-year-old shoots were collected from five untreated and five treated trees. Collections were made 2 d before spray applications and at 3, 10, 17, 31, 45, and 73 d postapplication (DPA). Shoots were sectioned into 1-in. segments and placed in foil packets. Packets were frozen in a programmable freeze chamber (Tenney Environmental, New Columbia, PA, USA) programmed to drop at a rate of 4 °C per hour, to a final temperature of −40 °C. Packets were removed from the chamber at 0 °C, −10 °C, −20 °C, −30 °C, and −40 °C after a 1-h soak at the target temperature. Samples were incubated at room temperature for 24 h following removal and cut transversely at the midway point of each stem section; then, a stereomicroscope was used to assess oxidative browning of phloem, cambium, and xylem. A 5-point scale was used to rate freeze damage, with 1 indicating no damage, 2 indicating ≤25% area darkened, 3 indicating ≤50% area darkened, 4 indicating ≤75% area darkened, and 5 indicating 100% completely oxidized (Fig. 2A). Average damage ratings of untreated control and ABA/ACC-treated samples at each temperature were compared using an unpaired Student's *t* test for each date.

Development of primers for assessing dormancy-related gene expression. After choosing dormancy-related genes from the literature, sequences were entered into the Genome Database for Rosaceae (Jung et al. 2019) BLAST+ tool, and the *Pyrus communis* cv. d'Anjou genome was selected to find best matches. Then, sequences were compared with publicly available transcriptome data to confirm that the intron/exon structure was correct. Primers were manually designed and the following parameters were maintained: mismatches of at least 2 to 5 bp from sequences of duplicate or similar genes in gene family; length of 18 to 23 bp; GC% between 50 and 60; Tm between 59 and 62; product spans intron whenever possible; and product between 70 and 200. After developing primers, sequences were entered into BLAST+ again with the word size decreased to seven to confirm that sequences did not match other locations in the genome. Housekeeping genes were chosen from the work of Liu et al. (2018). Sequences and parameters of primers can be found in Supplemental Table 1.

Characterizing expression of dormancy-related genes in floral buds. Fifteen to 20 floral buds were collected from treated and untreated trees at −2, 17, and 45 DPA. Buds were frozen in liquid nitrogen. Bud tissue was ground using a Spex® SamplePrep 6875D Freezer/Mill® (now Cole-Parmer CG-500). The RNA was extracted from ground tissue using a modified CTAB protocol (Honaas and Kahn 2017), quantity and quality were assessed using an Epoch Microplate Spectrophotometer, and 20 ng/uL dilutions of each sample were made. Quantitative reverse-transcription polymerase chain reactions were performed using the Bio-Rad iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA) and 12-uL reactions including 2 uL of 25 ng/uL RNA from buds. Reactions were run on a Bio-Rad CFX-384 machine using standard protocols for SYBR Green. Standard curves were run for each primer set, and the Agilent slope efficiency calculator (https://www.agilent.com/store/biocalculators/calcSlopeEfficiency.jsp?_requestid=1161610) was used to determine primer efficiency and amplification factors. Results were analyzed using the Pfaffl method (Pfaffl 2001) and normalized to two separate housekeeping genes, ACTIN2 (ACT2) and ARMADILLO (ARM) (Jung et al. 2019; Liu et al. 2018).

Results

Effects on phenology. A difference in leaf abscission was observed as early as 3 DPA of ABA/ACC, and treated trees shed leaves earlier (Fig. 1). No necrosis beyond what is typical in senescing leaves was observed. No difference was seen in bloom time the following spring, with all treated and untreated trees exhibiting first bloom on 30 Mar 2022.

Effect of ABA/ACC applications on cold hardiness of vegetative shoots. Cold hardiness measurements were performed before ABA/ACC application (−2 DPA) and at increasing timepoints after that application (3, 10, 17, 31, 45, and 73 DPA) by characterizing freeze damage (Fig. 2). After application, significant differences were seen 3 DPA at −40 °C, 17 DPA at

Fig. 1. Effects of abscisic acid (ABA)/1-aminocyclopropane-1-carboxylic acid (ACC) on leaf abscission. Treated and untreated control trees 3 d postapplication showing rapid advance of abscission following ABA/ACC application.

−20 °C and −40 °C, 31 DPA at −40 °C, and 73 DPA at −40 °C. All differences except for those at 3 DPA at −40 °C represent more severe freeze damage in the untreated control shoots. A significant difference was also observed before treatment, with more severe damage observed in the control shoots at −10 °C.

Effect of ABA/ACC applications on gene expression in reproductive buds. To address whether ABA/ACC application affected molecular signatures of dormancy, we measured gene expression of dormancy-related genes in reproductive buds, including those related to the involvement of ABA and ethylene. Gene expression was measured at 0, 17, and 45 DPA to include times that corresponded with improved cold hardiness in stems as well as times when the two treatments were similar (Fig. 3). *DAM* genes identified in multiple fruit species play a role in dormancy (Falavigna et al. 2019). Reports of expression of apple, *Prunus avium*, *P. persica*, and *P. mume* suggest that *DAM1*, *DAM2*, and *DAM3* play roles in the onset and maintenance of endodormancy, with increased expression during bud set and into autumn (Falavigna et al. 2019; Yang et al. 2021). We found that two 'd'Anjou' genes related to *DAM1* (*Pyrco.da.v2a1.chr8A.397760* and *Pyrco.da.v2a1.chr8A.397720*) increased at 17 DPA and decreased at 45 DPA, but they did not show a significant difference between treated and untreated buds. A *DAM2* homolog (*Pyrco.da.v2a1.chr8A.397680*) showed higher expression at 0 DPA and decreased steadily through the winter (Fig. 3). A small but significant difference was seen at 45 DPA, with treated buds showing lower expression of this gene.

ABSCISIC ACID INSENSITIVE (*ABI*) genes are important for ABA responses and have also been associated with dormancy. In poplar, the expression of a dominant negative allele of *ABI3* resulted in the failure to close plasmodesmata at the onset of endodormancy (Tylewicz et al. 2018). ABA-responsive *PavABI5-like* in sweet cherries interacts with and may regulate expression of *CBF/DREB* genes, which are important for cold hardiness, leaf senescence, and bud set and break (Wang et al. 2021; Wisniewski et al. 2015). While 'd'Anjou' *ABI5-Like* homolog *Pyrco.da.v2a1.chr15A.007080* increased from October to December, as would be expected, no significant differences between treated and untreated buds were observed.

CYP707A genes, which are involved in ABA catabolism, exhibit low expression at the onset of endodormancy in *Pyrus pyrifolia*, and the overexpression of a *CYP707A4* gene in grapes enhanced budbreak (Li et al. 2018; Zheng et al. 2018). Two 'd'Anjou' homologs of the *CYP707A4* gene used in grapes (*Pyrco.da.v2a1.chr8A.389110* and *Pyrco.da.v2a1.chr15A.007150*), and both showed increasing expression throughout the winter (Fig. 3). However, treatment with ABA/ACC appeared to suppress or delay the increase of *Pyrco.da.v2a1.chr8A.389110* relative to untreated buds.

Discussion

The combined application of ABA and ACC had a number of physiological effects that are typically associated with the onset of dormancy in pear and other fruit trees. Leaf abscission occurred almost immediately, with a large observable difference in the amount of fallen leaves by 3 DPA (Fig. 1). Under typical conditions, this leaf drop would be a key event in the transition to dormancy, allowing trees to slow metabolism, conserve water, and avoid significant freeze

A

B

	-2 DPA		3 DPA		10 DPA		17 DPA		31 DPA		45 DPA		73 DPA	
	ABA ACC	Control	ABA ACC	Control	ABA ACC	Control	ABA ACC	Control	ABA ACC	Control	ABA ACC	Control	ABA ACC	Control
0°C	1	1.13	1.2	1.13	1.13	1	1	1	1.07	1	1	1	1	1
-10°C	1.00*	1.40*	1.47	1.07	1	1.07	1	1.13	1.07	1.13	1.13	1.07	1	1
-20°C	1.87	2.2	3.13	2.6	2.07	2.07	2.13*	2.53*	2.07	2.2	1.73	2	2.13	2.2
-30°C	4.4	4.47	4.47	4.27	3.47	3.33	3.83	3.87	4	4	3.4	3.4	2.80*	3.27*
-40°C	5	4.8	4.87*	4.40*	4.33	4.13	4.47*	4.93*	4.33*	4.87*	4.4	4.67	4.33	4.53

C

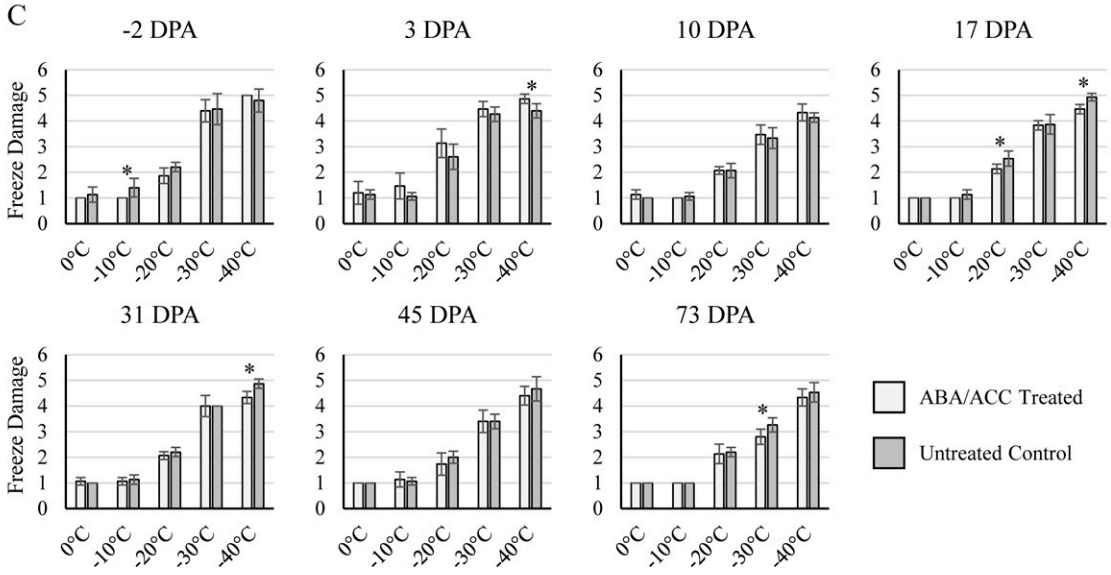

Fig. 2. Characterization of cold hardiness of vegetative shoots following abscisic acid (ABA)/1-aminocyclopropane-1-carboxylic acid (ACC) application. (A) A 5-point scale used to rate freeze damage with representative stem cross-sections: 1, no damage; 2, ≤25% area darkened; 3, ≤50% area darkened; 4, ≤75% area darkened; and 5, 100% completely oxidized (black). (B) Average freeze damage ratings at five decreasing temperatures for ABA/ACC and untreated control shoots. Comparisons began 2 d before application in mid-October and progressed until January. Asterisks indicate significant differences, with yellow color indicating significantly higher damage in untreated control shoots and blue indicating significantly higher damage in ABA/ACC-treated shoots. (C) Average freeze damage ratings seen in (B) graphed by date. Bars indicate standard deviation. Asterisks indicate significant differences.

damage of delicate tissues. This should normally coincide with an increase in cold hardiness of the shoots and buds that remain. However, 3 DPA was the only timepoint at which the ABA/ACC-treated trees were less cold-hardy than the untreated controls (Fig. 2). This could be an effect of the ABA/ACC application itself, or it could be caused by the defoliation of the leaves. Early defoliation in late summer/early autumn has been shown to alter hormone metabolism in *Pyrus pyrifolia*, causing out-of-season bloom (Wei et al. 2022); it is possible that defoliation in this case had the unanticipated effect of making shoot tissues temporarily less cold-hardy.

An increase in cold hardiness was observed in ABA/ACC-treated shoots for multiple timepoints after 3 DPA, including at 17, 31, and 73 DPA. This increase in cold hardiness was not large; however, it was statistically significant. It is possible that a greater improvement could be achieved with different concentrations or combinations of the PGRs applied. It is interesting to note that the two timepoints at which differences in cold hardiness were not observed, 10 DPA and 45 DPA, followed closely after warm periods (Fig. 4). Deacclimation, whereby cold hardiness is lost because of warm temperatures, has been shown to occur in a number of fruit tree species, but the process is complicated and dependent on many factors (Kalberer et al. 2006). More research of acclimation and deacclimation of *Pyrus communis* is necessary. Interestingly, we did not observe a difference in bloom time in the spring following application, unlike what was shown by studies of ethylene-promoting applications in *Prunus* (Liu and Sherif 2019; Liu et al. 2021; Nečas et al. 2023). Whether this is a result of our specific application protocols or whether this is related to the difference in species is unknown.

CYP707A genes across species are involved with endodormancy onset

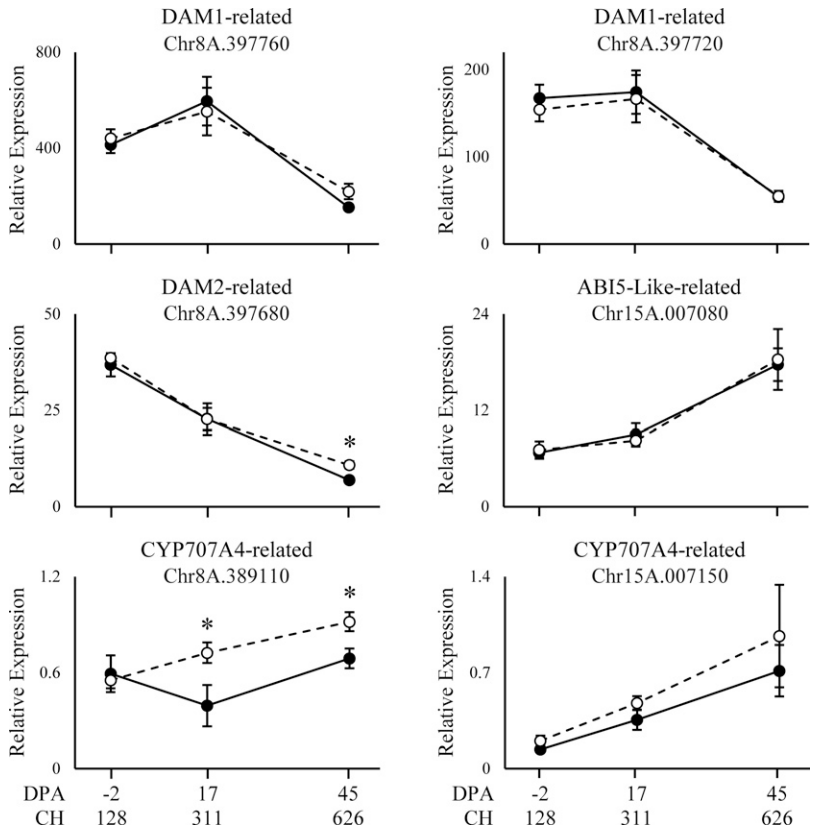

Fig. 3. Gene expression of dormancy-related genes in treated and untreated trees. Quantitative reverse-transcription polymerase chain reaction of six dormancy-related genes in the 'd'Anjou' genome. Expression was measured in dormant reproductive buds at the time of treatment, as well as 17 and 31 d postapplication. Gene expression was normalized to a housekeeping gene identified by Liu et al. (2018): ARMADILLO (*ARM*, *Pyrco.da.v2a1.chr4A.416680*). The number of chilling hours for the year at each timepoint is also included along the x-axis. Results of treated trees are represented by a solid line. Results of untreated trees are represented by a dashed line. Asterisks indicate significant differences between treated and control buds. $P < 0.05$ according to Student's *t* test.

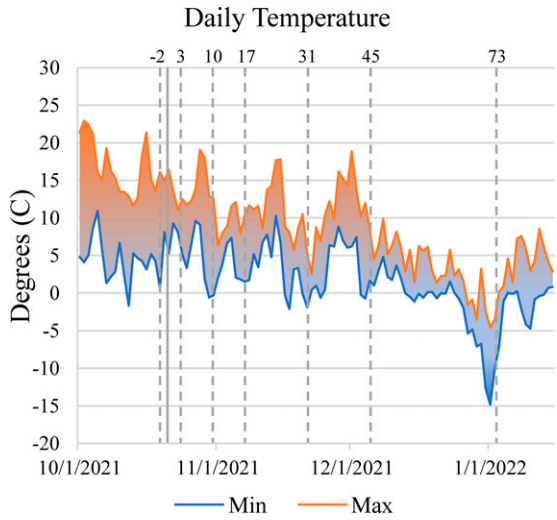

Fig. 4. Temperatures during abscisic acid (ABA)/1-aminocyclopropane-1-carboxylic acid (ACC) application and sample collections. Maximum and minimum daily temperatures (°C) from 1 Oct through 15 Jan. The ABA/ACC application date is indicated with a solid gray line. Collection dates for both vegetative and floral tissues are indicated with dashed gray lines. Days postapplication are indicated above the dashed lines.

and release. In *Pyrus pyrifolia*, trees show low *CYP707A3* gene expression throughout the fall, drastically increase, and peak near the transition of endodormancy to ecodormancy (Li et al. 2018; Yang et al. 2020). Another *P. pyrifolia CYP707A* gene, which the authors refer to as *PpABA8'OH*, shows a peak of expression just after release from endodormancy, and this peak is shifted forward when trees are treated with hydrogen cyanamide, coinciding with a shift in budbreak (Tuan et al. 2017). Transgenic pear calli overexpressing *CYP707A3* not only have lower ABA content but also show decreased *DAM3* expression levels (Yang et al. 2020). Further experimentation showed that *PpyABF3*, an *ABRE-BINDING FACTOR* gene, binds to the *DAM3* to regulate its expression (Yang et al. 2020). In grapes, overexpression of a *CYP707A4* gene leads to significantly enhanced budbreak in the spring (Zheng et al. 2018). The delayed increase in *CYP707A*-related *Pyrco.da.v2a1.chr8A.389110* gene expression in treated 'd'Anjou' trees may represent a shift in peak expression, suggesting that treatment might have led to an earlier release from endodormancy to ecodormancy. However, analysis of gene expression later in the winter with finer timepoints, as well as bud forcing tests, would be needed to confirm this. Furthermore, whether a shift in dormancy-related *CYP707A* gene expression represents a shift in the number of chilling hours required for release from dormancy is unknown because the mechanistic relationship between ABA catabolism and completion of chill hours is still unclear. 'd'Anjou' trees have been suggested to need approximately 800 chill hours; however, research is needed to verify this requirement.

We saw little difference in the expression of other dormancy-related genes at the timepoints assayed (17 DPA and 45 DPA). This may suggest no major changes in dormancy status between treatments. However, differences may be more dynamic and may occur earlier than 17 DPA. Furthermore, the amount of product applied and the timing of PGR applications have been shown to be important for their effects on dormancy. For example, application of ethephon in peaches altered bloom time and was dependent on both the concentration and whether it was applied at 10%, 50%, or 100% leaf

fall in the autumn (Liu et al. 2021). Future work with finer time scales and treatment variations are needed to determine the usefulness of these applications as a measure used to induce dormancy.

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