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Research paper

Association of the phenylpropanoid pathway with dormancy and adaptive trait variation in apricot (*Prunus armeniaca*)

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Trees use many mechanisms to adapt and respond to stressful conditions. The phenylpropanoid pathway in particular is known to be associated with a diverse suite of plant stress responses. In this study, we explored the relationship between the phenylpropanoid pathway metabolite production, gene expression and adaptive trait variation associated with floral bud reactivation during and following dormancy in *Prunus armeniaca* L. (apricot). Concentrations of eight phenylpropanoid metabolites were measured during chill accumulation and at developmental stages corresponding to the emergence of sepals and petals in floral buds of varieties that differ phenotypically in bloom date (BD). A significant interaction effect of chill hours and BD phenotype on the concentration of each of the compounds was observed (mixed analysis of variance, $P < 0.05$), with the concentration of most phenylpropanoid metabolites dropping precipitously when sepals and petals emerged. While phenylpropanoid biosynthetic gene expression patterns were more variable in general, expression changed over time and was impacted, although to a lesser degree, by BD phenotype. Furthermore, separation of BD phenotypic groups was most pronounced when early and late BD varieties were at different developmental stages, i.e., 800 chill hours. Taken together, these results suggest that the phenylpropanoid pathway is associated with floral bud reactivation in apricot. Furthermore, we show that the phenylpropanoid pathway is also impacted by phenological trait variation associated with dormancy. A better understanding of how apricot and other perennial tree species respond and adapt to environmental perturbations will be critical for improvement programs aimed at identifying and breeding trees more suitable for rapidly changing environments.

Keywords: bloom date, floral bud dormancy, phenology, phenylpropanoid metabolites and genes, Rosaceae.

Introduction

Trees are long-lived perennial organisms. Because of these life history characteristics, they encounter a diverse array of environmental conditions, and thus must respond and adapt to diverse stressors. From this perspective, phenological traits, such as dormancy, chilling requirement (CR) and timing of budbreak, can be viewed as ways in which trees have adapted to stressful conditions that are unfavorable for growth (reviewed in van Schaik et al. 1993, reviewed in Rohde and Bhalerao 2007, Ding and Nilsson 2016). Winter dormancy allows trees to survive freezing temperatures, shorter photoperiods and reduced water availability. It is composed of distinct phases including endodormancy—true rest, when intrinsic, physiological factors regulate growth; ecodormancy—when growth is regulated by environmental conditions; and paradormancy—when physiological factors within the plant, but away from the dormant tissue regulate growth (Perry 1971, Lang 1987, reviewed in Abbott et al. 2015). Understanding the underpinnings of dormancy and other adaptive phenological traits is crucial for developing strategies to rapidly develop genetically improved trees for target environments. This is of critical importance today, in light of the rapid and predicted long-term changes in the world's climate.

A myriad of defense mechanisms enable trees to survive when exposed to stressful conditions. Phenylpropanoids are one of the most structurally diverse and widespread families of plant secondary metabolites and are well known for their role in tree responses to biotic stress, such as plant pathogens and insect pests, and abiotic stress, such as drought, cold temperature and UV light (see reviews by Solecka 1997 and Vogt 2010). Phenylpropanoids and genes associated with their biosynthesis and/or transport, e.g., transparent testa (TT) genes, are also associated with plant growth and development particularly during dormancy. They are involved in seed dormancy regulation in *Arabidopsis* and are associated with bud dormancy induction and maintenance in woody species, including *Vitis* species (Debeaujon et al. 2001, Buer and Muday 2004, Buer et al. 2008, Fennell et al. 2015). In addition, concentrations of phenylpropanoids change as buds exit dormancy, either by natural mechanisms or artificial induction (e.g., via chemical treatment or exposure to heat), in woody plant species such as black currant (*Ribes nigra* L.) (Vagiri et al. 2015) and apple (*Malus* × *domestica* Borkh.) (Wang et al. 1991). Phenylpropanoid levels in buds of Norway spruce (*Picea abies* (L.) H. Karst.) and European silver fir (*Abies alba* Mill.) are also significantly correlated with bud development (i.e., bud opening score) during forced deacclimation (Dhuli et al. 2014), and higher levels of flavonoids were associated with earlier apical bud maturation in transgenic hybrid poplar (*Populus tremula* × *alba*) (Conde et al. 2017).

While the exact function of phenylpropanoids in growth and development is not fully understood in woody perennials, some phenylpropanoid classes (e.g., flavonoids) may directly or indirectly impact the transport and/or biosynthesis of plant hormones, such as auxin (Brown et al. 2001, Buer and Muday 2004, Silva-Navas et al. 2016). The role of plant hormones in growth and development and in induction, maintenance and release of dormancy is well established (Kumar et al. 2017). As a result, the flux of phenylpropanoids in response to environmental cues may be integral for arresting or releasing meristem growth during winter dormancy and the resumption of growth in the spring, respectively.

Plant germplasm exhibiting genetically based variation in dormancy response are essential for connecting the important physiological products (e.g., phenylpropanoids) to the regulated gene networks associated with the onset and duration of the winter dormant state. Deciduous fruiting trees of the Rosaceae, including peach (*Prunus persica* L. Batsch), apricot (*Prunus armeniaca L.*) and others, are ideally suited for such analyses due to the traditional selective breeding of varieties suited to different climatic regimes. Furthermore, apricot is an ideal fruiting tree for studying this phenomenon because a wealth of information is available on the genetic underpinnings of adaptive traits associated with dormancy, including CR and bloom date (BD) (e.g., Olukolu et al. 2009, Dirlewanger et al. 2012). Furthermore, there is a high-quality draft genome for peach, a closely related species in the Rosaceae family, which is publicly available and includes putative annotations for genes within the phenylpropanoid pathway (Jung et al. 2004, 2014, Verde et al. 2013). Thus, apricot serves as an ideal species to examine the relationship between variation in BD and the production of metabolites and expression of genes within the phenylpropanoid pathway.

The goal of this study was to examine the association of specific phenylpropanoid pathway genes and their products within particular stages of floral bud dormancy and post-dormancy development in early BD and late BD varieties of apricot. The phenylpropanoid metabolites analyzed were selected based on their association with TT genes, which are known in the model species *Arabidopsis* to be essential for the biosynthesis of phenylpropanoids, including flavonols and proanthocyanidins (Zhao et al. 2010), and based on previous studies, involved in determining the stratification requirements of the seed (Debeaujon et al. 2001). The goal of the current work is to assess phenotypic variation in the concentration of specific phenylpropanoid metabolites during dormancy and its release, and to correlate this with phenotypic-based variation in the expression levels of genes associated with phenylpropanoid biosynthesis. Our results show that in apricot floral buds, concentrations of specific metabolites and the expression of genes predicted to be associated with phenylpropanoid biosynthesis change during floral bud reactivation. Furthermore, we show that there appears to be a lag in the timing of changes between early and late BD apricot varieties. To our knowledge,

Table 1. Sampling time points and average BD for each apricot variety. Note that in early BD varieties, 800 chill hours occurred chronologically after C2 and D1.

Phenotype	Early BD			Late BD	
Variety	A2312[1]	A2137[1,2]	A1956[1,2]	A660[1,2]	A1267[1,2]
BD (Julian days)[3]	56.5	60.0	61.2	73.6	84.6
Time point	Sampling date				
0	29-Oct-15	29-Oct-15	29-Oct-15	29-Oct-15	29-Oct-15
100	25-Nov-15	25-Nov-15	25-Nov-15	25-Nov-15	25-Nov-15
400	18-Jan-16	18-Jan-16	18-Jan-16	18-Jan-16	18-Jan-16
800	1-Mar-16	1-Mar-16	1-Mar-16	1-Mar-16	1-Mar-16
C2[4]	8-Feb-16	1-Feb-16	8-Feb-16	7-Mar-16	7-Apr-16
D1[5]	18-Feb-16	8-Feb-16	18-Feb-16	21-Mar-16	7-Apr-16

[1]Variety included in metabolite analysis.
[2]Variety included in RNA-sequencing analysis.
[3]Average BD over a 5–11 year period, where 50% of blooms have emerged on the tree.
[4]C2: sepals visible.
[5]D1: petals visible.

this is the first report of timing changes in gene expression and metabolite levels between early and late flowering varieties.

Materials and methods

Plant material

Apricot floral buds were collected at various stages from the same individual trees throughout dormancy and just before bloom. One to three trees were sampled from each of five varieties, including three early and two late flowering (i.e., BD) varieties (Table 1). Time points included 0, 100, 400 and 800 chill hours, and at developmental stages where sepals (C2) and petals (D1) were visible. Early BD varieties sampled included: Flamingold (A2312; $n = 1$, but sampled twice at each time point and only included in the metabolite analysis), Bakour (A2137; $n = 2$) and Palsteyn (A1956; $n = 2$). Late BD varieties sampled included: Bergeron (A660; $n = 3$) and Badami (A1267; $n = 3$). All trees were sampled at each of the six time points. Trees were grown at the Institut National de la Recherche Agronomique (INRA) domaine des Pins de l'Amarine field site in Bellegarde (France). Accumulation of chill hours was calculated according to the Weinberger method (Weinberger 1950) using weather data from the nearby Ctifl Balandran station, which is representative of the environmental conditions encountered at the Amarine site. On each sampling date, one or two branches were collected from each of the 11 trees. Samples were transported from the field to the laboratory (INRA-GAFL Avignon) in a cooler at 4 °C to prevent buds from damage. Once in the laboratory, buds were removed, flash frozen in liquid nitrogen and then stored at −80 °C. Frozen buds were then transported on dry ice to the UMR Biologie du Fruit et Pathologie (INRA-Villenave d'Ornon) for further processing and metabolite extraction.

Extraction and quantification of phenylpropanoid metabolites

Bud tissue was finely ground and homogenized in liquid nitrogen using a mortar and pestle. Phenylpropanoids were extracted from finely ground tissue using a modified soluble phenolics extraction method from Bonello and Blodgett (2003). In brief, ∼100 mg (samples range from 75–208 mg) of finely ground tissue was extracted for ∼24 h at 4 °C with 1 ml HPLC-grade methanol twice. Samples were stored at −80 °C following extraction. Then, methanol from apricot extracts was evaporated using a Savant SpeedVac (Thermo Scientific, Asheville, NC, USA). Dried down extracts were shipped on dry ice to the University of Kentucky (Lexington, KY, USA) where upon arrival they were stored at −70 °C. Extracts were then shipped on dry ice to the Analytical Sciences Laboratory at the David H. Murdock Research Institute (Kannapolis, NC, USA) for targeted metabolomics analysis. Upon arrival, all extracts were stored at −80 °C. Eight phenylpropanoid metabolites were targeted for analysis. These were three flavonoid aglycones (apigenin, kaempferol and quercetin), four flavan-3-ols (epicatechin-3′-*O*-glucoside, procyanidin B1, procyanidin B2 and procyanidin B3) and the stilbenoid, resveratrol. In order to quantify these compounds, dried extracts were re-suspended in 1 ml of methanol spiked with 0.5 µM 4-chlorophenylalanine, an internal standard. Re-suspended samples were then vortexed until the pellet was dissolved, and extracts were centrifuged at 5000 × *g* for 10 m at 4 °C.

An Acquity UPLC-Quattro Premier XE MS (Waters Corporation, Milford, MA, USA) in negative ion mode with electrospray ionization was used to measure the eight pre-selected phenylpropanoid metabolites using multiple reaction monitoring. Five microliters of each sample were injected into an Acquity UPLC BEH C18 1.7 µM column (2.1 × 100 mm) column held at 40 ± 0.5 °C. Compounds were separated using a 0.1% formic acid/water (A) and acetonitrile/0.1% formic acid

(B) gradient (the following percentages correspond to solvent B): 0–1.0 min (2%), 1.0–5.2 min (2–52.4%), 5.21–6.2 min (99%) and 6.21–7.0 min (2%). The flow rate was 0.4 ml min^{-1}, and samples were held at 4 ± 0.5 °C during analysis. The parameters for the Quattro Premier MS were as follows: capillary voltage was 2.5 kV; sampling cone was 30 V; extraction cone was 4.0 V; source temperature was 120 °C; desolvation temperature was 350 °C; desolvation gas flow was 800 l h^{-1}; and cone gas was 0 l $^{-1}$.

Raw data were processed and compounds were quantified using the TargetLynx application manager (Waters Corporation). The concentration of each compound was determined relative to the fresh weight of each individual sample using a 15-point second order polynomial regression standard curve, with two exceptions. No commercially available standard was available for epicatechin-3′-*O*-glucoside, so cynaroside (Chromadex, Irvine, CA, USA), a structurally similar compound, was used for relative quantification. Additionally, procyanidins B1 and B2 could not be separated using the analytical method stated above, so procyanidin B1 and B2 were quantified together. Standards for resveratrol, apigenin, kaempferol and quercetin were purchased from Sigma-Aldrich (St Louis, MO, USA), and procyanidins B1, B2 and B3 were purchased from EMMX Performance Materials Inc. (Center Valley, PA, USA). The concentrations (in µM) used for each compound in the standard curve were 0, 0.012207, 0.024414, 0.048828, 0.097656, 0.195313, 0.390625, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 and 200. All standards were dissolved in methanol. The mass spectrum (m/z) and retention time of each sample signal compared with the analytical standard, when available, were used to identify each of the eight phenylpropanoid metabolites. Approximate retention times and mass fragmentation patterns for each compound can be found in Table S1 available as Supplementary Data at *Tree Physiology* Online. A representative chromatogram for apricot floral bud metabolites can be found in the Supplementary Data (Figure S1 Supplementary Data at *Tree Physiology* Online).

Extraction of total RNA and RNA sequencing analysis

Total RNA was extracted from a subset of apricot floral bud samples, from four of the five varieties included in the metabolite analysis (Table 1 and Table S2 Supplementary Data at *Tree Physiology* Online). Tissue was finely ground in liquid nitrogen, and for each bud sample, 100 mg of frozen, finely ground tissue was extracted according to standard protocols included with the NucleoSpin RNA XS Isolation kit (Macherey-Nagel, France). RNA samples were further purified by phenol:chloroform extraction twice, quantified, analyzed on a gel for RNA integrity, and ~ 5 µg of each sample was submitted to MOgene, LLC (St Louis, MO, USA) for sequencing using Illumina Hi-Seq.

Raw 50 bp, single end reads were quality checked using FastQC (Andrews 2010) and trimmed with Skewer (Jiang et al. 2014). STAR (Dobin et al. 2013) was used to map reads to the *P. persica* (peach) genome v2.0.a1 (Verde et al. 2013, 2017) and then HTSeq-count (Anders et al. 2015) was used to count reads. Counts were imported to the R package DESeq2 (Love et al. 2014) and normalized gene counts were output for further analysis. Functional gene annotations were downloaded from Phytozome (Goodstein et al. 2012).

A list of approximately 400 phenylpropanoid biosynthesis genes (including transcript variants) of interest were compiled to examine changes in phenylpropanoid gene expression patterns. *Prunus persica* v2.0.a1 annotated genes mapped to KEGG pathways 00940 (phenylpropanoid biosynthesis), 00941 (flavonoid biosynthesis), 00942 (anthocyanin biosynthesis), 00944 (flavone and flavonol biosynthesis) and 00945 (stilbenoid, diarylheptanoid and gingerol biosynthesis) were included (Genome Database Rosaceae, GDR, https://www.rosaceae.org) (Jung et al. 2004, 2014). In addition, *P. persica* v1.0 genes predicted to be associated with phenylpropanoid biosynthesis (i.e., flavonoid biosynthesis, flavonol biosynthesis, phenylpropanoid biosynthesis initial reactions, phenylpropanoid biosynthesis, proanthocyanidins biosynthesis from flavonols, resveratrol biosynthesis and leucopelargonidin and leucocyanidin biosynthesis) available on PeachCyc (http://pathways.rosaceae.org/; Jung et al. 2014) were also included, unless no v2.0.a1 gene annotation match was available. *Prunus persica* v1.0 gene names were converted to v2.0.a1 gene predictions using a conversion table (available at https://www.rosaceae.org/species/prunus_persica/genome_v2.0.a1). KEGG and PeachCyc predicted phenylpropanoid biosynthesis gene lists were combined and curated to exclude duplicate genes and to remove transcript variants that were not included as part of our gene annotation process. The final curated list contained 306 phenylpropanoid biosynthesis genes. PeachCyc was also used to identify a list of predicted TT genes. In total, 47 TT genes with *P. persica* v2.0.a1 gene annotations were identified.

Statistical analysis

Effects of adaptive phenology on the concentration of each phenylpropanoid metabolite, and the expression of two genes predicted to be associated with phenylpropanoid biosynthesis, were assessed using mixed model analyses of variance (ANOVAs) (repeated measures general linear model) with type III sums of squares (package: 'car') (Fox and Weisberg 2011) for chill hours (0–800 h) and developmental stages (C2 and D1), respectively. In each model, time point was the within-subjects factor and phenotype (early versus late BD) was the between-subjects factor. Subjects were individual trees. The statistical analysis was performed on individuals grouped by early or late BD phenotype due to the limited number of biological replicates for each variety. Normality was assessed using the Shapiro–Wilks test. Homogeneity of variance and

sphericity were assessed using Levene's test and Mauchly's Test of Sphericity, respectively. Metabolite data that violated the assumption of homogeneity of variance were square root transformed, and normalized gene count data that violated the assumption of homogeneity of variance or normality were natural log transformed prior to analysis. Data that violated the assumption of sphericity were analyzed using the Greenhouse–Geisser correction. In order to evaluate simple main effects for metabolite data, when the interaction term was significant, separate tests for time (one-way repeated measures ANOVA, package: 'car') and phenotype (two samples *t*-test) were performed (Fox and Weisberg 2011). For the two-sample *t*-test, data with significant Levene's test results were square root transformed prior to analysis. For one-way repeated measures ANOVAs with significant time effects, pairwise comparisons were made using the Bonferroni multiple test correction for *P*-values. Log2 fold change (log2FC) values were also calculated for candidate genes to compare changes in expression patterns in early and late BD varieties over time.

Principal components analysis (PCA) with scaling was used on phenylpropanoid metabolite concentration data to visualize natural groupings between early and late BD trees at different time points, and to identify phenylpropanoid metabolites that may be important for driving the separation between groups based on loading values (package 'stats::prcomp', R Development Core Team 2018). Principal components analysis without scaling was also used to visualize natural groupings based on normalized counts data for 306 predicted phenylpropanoid biosynthesis genes of interest.

Finally, log2FC phenylpropanoid metabolite and transcriptomic data were integrated using the program Pathview (https://pathview.uncc.edu/) to visualize changes across the phenylpropanoid biosynthetic pathway over time between early and late BD varieties (Luo and Brouwer 2013, Luo et al. 2017). Data from only those individuals where both metabolite and transcriptomic data were available were included in the integrated analysis. One hundred and thirty-six predicted phenylpropanoid biosynthesis genes and five phenylpropanoid metabolites (resveratrol, apigenin, kaempferol, quercetin and procyanidin B2) with KEGG identifiers were included in the analysis (http://www.genome.jp/kegg/pathway.html).

All statistical analyses were performed in R (R Development Core Team 2018), unless otherwise stated.

Results

Effect of early vs late BD phenotype on timing of resumption of growth post-dormancy

Apricot floral bud tissue was collected at six time points: 0, 100, 400 and 800 h of chill accumulation, and upon the emergence of sepals (C2) and petals (D1). Early BD varieties began to progress towards blooming (stages C2 and D1) approximately 1 month or more before late BD varieties (Table 1). Differences in the date of bud sepal emergence were also observed between late BD varieties, which were previously known to differ in BD based on 11-year averages (Table 1) (Audergon 1993, Dirlewanger et al. 2012, Andreini et al. 2014, Quero-Garcia et al. 2015). There was also variation in the timing of sepal and petal emergence within early BD varieties (Table 1). A2137 had buds in stages C2 and D1 approximately 1 week prior to A2312 and A1956. Of note, early BD varieties had already passed developmental stage D1 by the time buds were collected at 800 chill hours, whereas buds collected from late BD varieties had not yet progressed to developmental stage C2 (Figure S2 available as Supplementary Data at *Tree Physiology* Online).

Effect of early vs late BD phenotype and time on concentration of phenylpropanoid metabolites

In addition to visually observed differences in the progression of bud development during dormancy, there was a significant interaction effect of BD phenotype and time point on the concentration of each of the seven phenylpropanoid metabolites measured between 0 and 800 chill hours (Table 2). Resveratrol was the only metabolite significantly impacted by the interaction of BD phenotype and time point between stages C2 and D1 (Table 2). For compounds with significant interaction effects, simple main effects for time and phenotype were examined independently using one-way repeated measures ANOVA and two sample *t*-tests, respectively (Tables S3–S5 available as Supplementary Data at *Tree Physiology* Online).

In general, concentrations of phenylpropanoid metabolites in early BD varieties decreased as chill hours accumulated with a continuous drop in concentration after the 100 chill hours sampling and leading up to the emergence of sepals (C2) and petals (D1) (Figure 1). In contrast, concentrations of phenylpropanoid metabolites in late BD varieties increased or stayed relatively unchanged, with only slight decreases in concentration as chill accumulated, up to 800 h of chill, after which concentrations of phenylpropanoid metabolites dropped precipitously when sepals (C2) and petals (D1) became visible (Figure 1). The decline in the concentration of phenylpropanoid metabolites was generally more gradual in early BD varieties compared to late BD varieties. However, concentrations of the measured phenylpropanoid metabolites dropped chronologically earlier, i.e., buds progressed to stages C2 and D1 earlier in early BD versus late BD varieties, which in both instances was accompanied by a drop in the concentration of phenylpropanoid metabolites (Figure 1).

Prior to (0 chill hours) and at the beginning of chill accumulation (100 chill hours), concentrations of most phenylpropanoid metabolites did not differ significantly between early and late BD varieties ($P > 0.05$), except for quercetin at 100 chill hours

Table 2. Results of mixed model ANOVAs examining the effect of phenotype (P)—early vs late BD—and time point (T) on the concentration of phenylpropanoid metabolites in apricot floral buds from 0 to 800 chill hours and between stages C2 and D1. Bolded *P*-values indicate significant effects. The mean untransformed concentration of these compounds as a factor of time point and phenotype can be found in Figure 1.

Model	Compound	F_P	P_P	F_T	P_T	F_{P*T}	P_{P*T}
0–800 chill hours	Resveratrol	3.36	0.1001	2.82	0.0576	8.10	**0.0005**
	Apigenin	11.64	**0.0077**	0.60	0.6186	16.46	**<0.0001**
	Kaempferol	3.29	0.1033	8.78	**0.0003**	7.73	**0.0007**
	Quercetin[1]	27.92	**0.0005**	0.51	0.3164	0.51	**0.0002**
	Epicatechin-3′-*O*-glucoside	11.03	**0.0089**	8.69	**0.0003**	8.24	**0.0005**
	Procyanidins B1 and B2	6.84	**0.0280**	18.74	**<0.0001**	6.10	**0.0026**
	Procyanidin B3	18.69	**0.0019**	1.78	0.1754	7.86	**0.0006**
	Degrees of freedom	1,9		3,27		3,27	
Stages C2 and D1[3]	Resveratrol[2]	2.68	0.1360	13.79	**0.0048**	8.14	**0.0190**
	Apigenin[2]	0.05	0.8296	19.63	**0.0016**	0.69	0.4291
	Kaempferol	1.57	0.2424	0.92	0.3629	1.40	0.2669
	Quercetin	2.85	0.1258	35.07	**0.0002**	0.41	0.5386
	Epicatechin-3′-*O*-glucoside	0.64	0.4432	23.30	**0.0009**	1.62	0.2354
	Procyanidins B1 and B2	2.56	0.1442	16.61	**0.0028**	0.05	0.8344
	Procyanidin B3[2]	5.65	**0.0414**	11.12	**0.0087**	0.35	0.5670
	Degrees of freedom	1,9		1,9		1,9	

[1]Greenhouse–Geisser correction used to account for lack of sphericity on Time and P*T.
[2]Raw data square root transformed to satisfy the assumption of homogeneity of variance.
[3]Sphericity could not be tested for stages models since test df = 0.

($t = -4.10$, $df = 9$, $P = 0.0027$) (Figure 1 and Table S5 available as Supplementary Data at *Tree Physiology* Online). Concentrations of most metabolites were also similar in early and late BD varieties during stages C2 (sepals visible) and D1 (petals visible), except for resveratrol at C2 ($t = -2.51$, $df = 9$, $P = 0.0333$) and procyanidin B3 (significant effect of phenotype, mixed ANOVA, $F_{P1,9} = 5.65$, $P = 0.0414$), although the mean difference was relatively small in this instance (Figure 1 and Table 2). In contrast, concentrations of the three flavonoids and procyanidin B3 were significantly higher in late BD varieties at 400 chill hours and concentrations of all metabolites were significantly higher in late BD varieties at 800 chill hours (of note, at this point early BD varieties had already passed developmental stage D1) (two sample *t*-test, $P < 0.05$) (Figure 1, Table S5 available as Supplementary Data at *Tree Physiology* Online).

Principal components analysis to examine natural groupings of samples

Principal components analysis with data scaled was used to evaluate the natural groupings of individual trees during dormancy based on the concentration of phenylpropanoid metabolites in floral bud tissue. The first two PCs explained 88.8% of the variance (Figure 2). Visually, the most pronounced separation between early and late BD varieties was at 400 and 800 chill hours; observed developmental differences between early and late BD varieties were also most pronounced at these times. In contrast, there was no clear separation of BD phenotype groups at C2 and D1.

Principal components analysis on normalized gene count data was used to evaluate natural groupings based on the expression of genes predicted to be associated with phenylpropanoid biosynthesis. The first two PCs explained 69.0% of the variance (Figure 3). While not identical to the groupings observed based on the PCA of phenylpropanoid metabolite concentrations, samples at developmental stages C2 and D1 for both early and late BD varieties, and 800 chill hours for early BD varieties, were visually separated from the other groups. In contrast, samples collected from earlier time points, particularly around the time that chill began to accumulate, had a tendency to group together.

Effect of early vs late BD phenotype and time on the expression of predicted phenylpropanoid biosynthesis genes

Expression of predicted genes associated with phenylpropanoid biosynthesis, including resveratrol synthase 2 (the gene for resveratrol biosynthesis), six genes associated with naringenin biosynthesis (a precursor of apigenin, kaempferol and quercetin, as well as other flavonols) and TT genes, were examined (Figure S3 and Table S6 available as Supplementary Data at *Tree Physiology* Online). In general, gene expression changed over time. Specifically, Prupe.3G292900—a gene predicted to be associated with naringenin biosynthesis—and Prupe.4G200500—a predicted TT3 protein—showed expression patterns that were consistent with BD phenotype. Furthermore, the interaction between phenotype and time had a significant effect on the expression of Prupe.3G292900 from 0 to 800 chill hours and between stages C2 and D1 and on the expression of Prupe.4G200500 from 0 to 800 chill hours (Table 3).

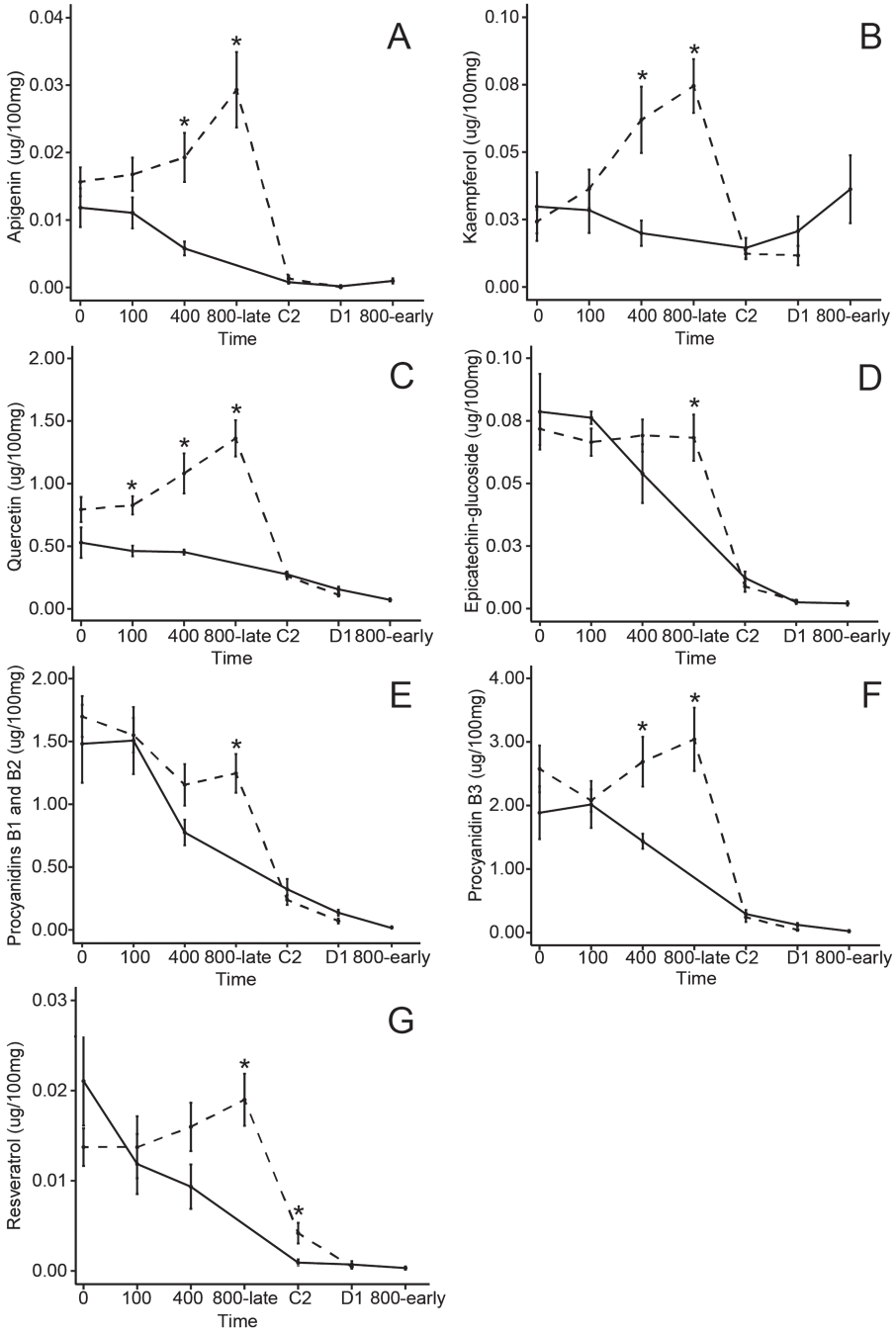

Figure 1. Mean concentration ± standard error (in µg 100 mg^{-1}) of (A) apigenin, (B) kaempferol, (C) quercetin, (D) epicatechin-3′-*O*-glucoside, (E) procyanidins B1 and B2, (F) procyanidin B3 and (G) resveratrol in apricot floral buds in early (solid line) and late (dashed line) BD varieties at 0 chill hours (0), 100 chill hours (100), 400 chill hours (400), 800 chill hours (800), stage C2 (C2) and stage D1 (D1). Note that at 800 chill hours, early (800-early) and late (800-late) BD varieties were at different developmental stages. Asterisks indicate significant difference between early and late BD varieties at a given time point (Table S5 available as Supplementary Data at *Tree Physiology* Online). Note differences in scales of y-axes.

Integrated metabolite and gene expression analysis

Using the program Pathview (https://pathview.uncc.edu/; Jung et al. 2004, 2014), phenylpropanoid log2FC metabolite and gene expression data were integrated on KEGG pathway maps for phenylpropanoid biosynthesis—00940, flavonoid biosynthesis—00941 and stilbenoid, diarylheptanoid and gingerol biosynthesis—00945, to visualize and highlight changes in gene expression and metabolite production over time between early and late BD varieties. Negative log2FC values represent higher expression or metabolite concentrations in late versus early BD varieties. Where applicable, multiple genes at each node were averaged. Forty-seven nodes were mapped to KEGG pathway 00940 (Figure S4 available as Supplementary Data

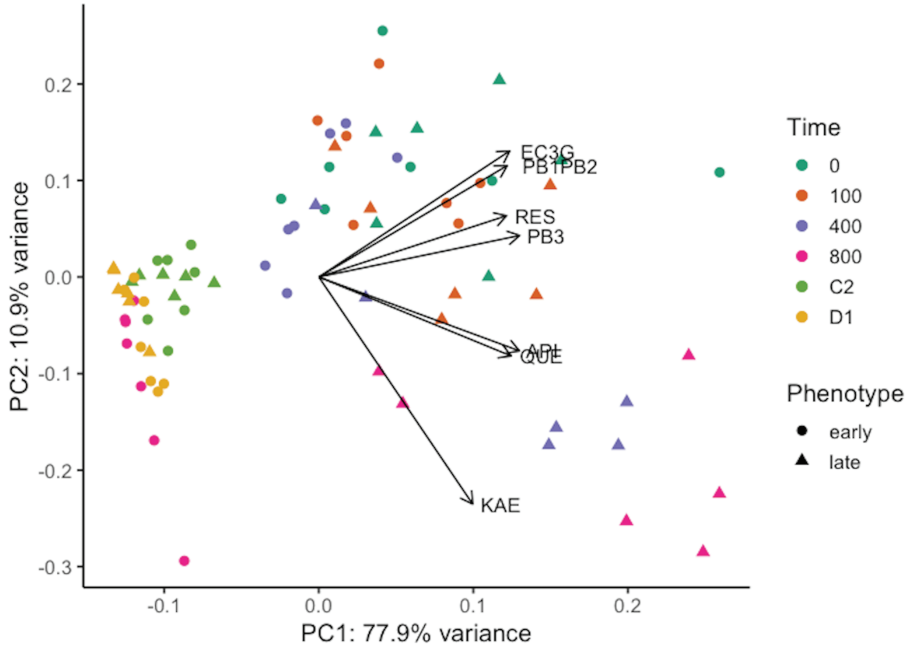

Figure 2. Score plot from PCA (with scaling) of phenylpropanoid metabolite concentrations in early and late BD apricot varieties (phenotype) at the following time points: 0 chill hours (0), 100 chill hours (100), 400 chill hours (400), 800 chill hours (800), stage C2 (C2) and stage D1 (D1). Black arrows indicate loadings for each metabolite: procyanidin B3 (PB3), procyanidin B1 and B2 (PB1PB2), epicatechin-3′-O-glucoside (EC3G), resveratrol (RES), quercetin (QUE), apigenin (API) and kaempferol (KAE). Note that early and late BD varieties were at different developmental stages at 800 chill hours.

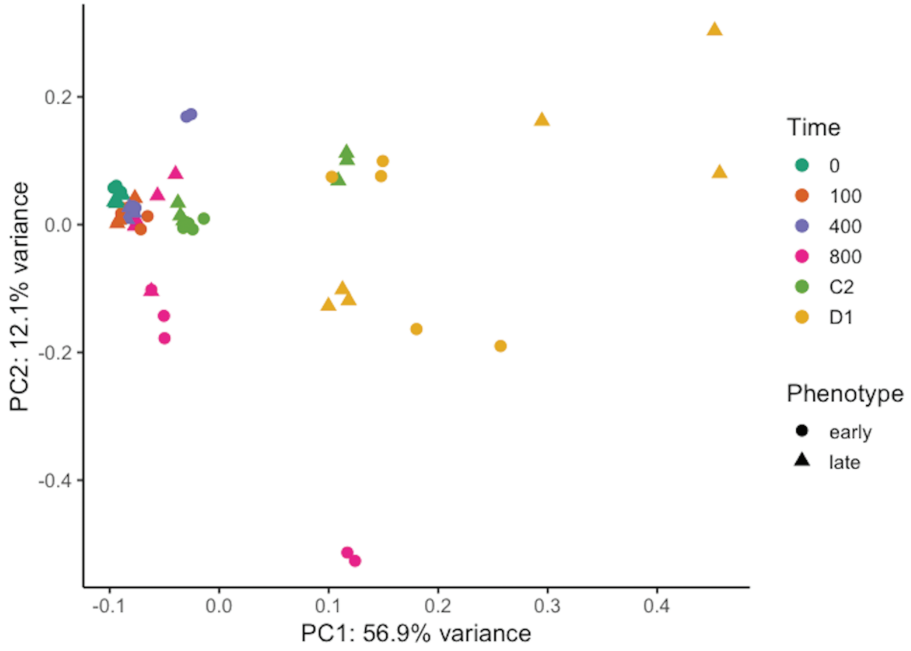

Figure 3. Score plot from PCA of normalized expression (counts) of 306 predicted phenylpropanoid biosynthesis genes in early and late BD apricot varieties (phenotype) at the following time points: 0 chill hours (0), 100 chill hours (100), 400 chill hours (400), 800 chill hours (800), stage C2 (C2) and stage D1 (D1). Note that early and late BD varieties were at different developmental stages at 800 chill hours.

at *Tree Physiology* Online). Twenty-four nodes were mapped to KEGG pathway 00941 (Figure S5 available as Supplementary Data at *Tree Physiology* Online), and eight nodes were mapped to KEGG pathway 00945 (Figure 4). These pathways are upstream of or directly associated with the biosynthesis of the phenylpropanoid metabolites measured in this study (00940—upstream; 00941—quercetin, kaempferol, apigenin; 00945—resveratrol). Genes with the highest absolute log2FC values at each time point, for each pathway, are listed in Table 4.

Table 3. Results of mixed model ANOVA examining the effect of phenotype (P) and time (T) on the expression of candidate phenylpropanoid biosynthesis genes in apricot floral buds. Bolded *P*-values indicate significant effects at $P < 0.05$. Mean normalized expression (counts) can be found in Figure S3 available as Supplementary Data at *Tree Physiology* Online.

Model	Predicted gene	F_P	P_P	F_T	P_T	F_{P*T}	P_{P*T}
0–800 chill hours	Prupe.3G292900[1]	23.09	**0.0049**	0.36	**0.0007**	0.36	**0.0023**
	Prupe.4G200500[1,2]	50.23	**0.0009**	0.37	**0.0379**	0.37	**0.0351**
	Degrees of freedom	1,5		3,15		3,15	
Stages C2 and D1[3]	Prupe.3G292900	24.41	**0.0008**	89.90	**<0.0001**	8.83	**0.0157**
	Prupe.4G200500	2.84	0.1263	9.75	**0.0123**	0.30	0.5950
	Degrees of freedom	1,9		1,9		1,9	

[1]Greenhouse–Geisser correction used to account for lack of sphericity on Time and P*T.
[2]Raw data natural log transformed to correct for a lack of homogeneity of variance.
[3]Sphericity could not be tested for stage models since test *df* = 0.

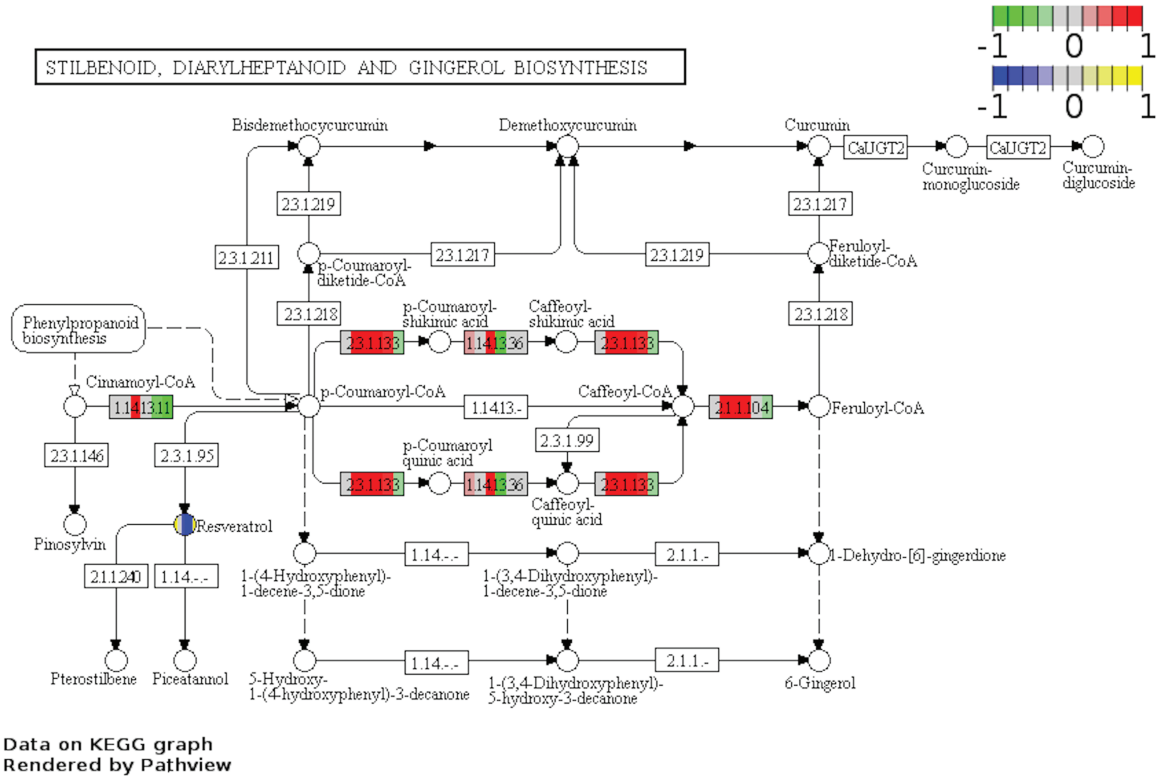

Figure 4. Log2FC between early and late BD apricot varieties in predicted stilbenoid biosynthesis gene expression (green to red scale) and concentration of resveratrol (blue to yellow scale) in floral buds at six time points: 0 chill hours, 100 chill hours, 400 chill hours, 800 chill hours, stage C2 and stage D1. Multiple genes at each node are averaged, when applicable. Negative log2FC values represent higher expression/concentration in late versus early BD varieties. Note that early and late BD buds at 800 chill hours were collected at different developmental stages.

Discussion

The phenylpropanoid pathway is known to be associated with environmental stress response and with dormancy and growth regulation in some herbaceous and woody plant species (Solecka 1997, Debeaujon et al. 2001, Debeaujon et al. 2001, Buer et al. 2008, Buer and Muday 2004, Fennell et al. 2015). For this reason, we examined the association between the phenylpropanoid pathway and floral bud dormancy and reactivation in phenologically different apricot varieties.

Concentrations of phenylpropanoid metabolites in apricot floral buds differed depending on BD phenotype (the effect of variety, within BD phenotype group, was not assessed in this study since the number of biological replicates per variety was low, with < 3 replicates in some instances) and also by the time, although gene expression patterns were highly variable within phenotypes (between varieties within phenotypes) and even within varieties (between trees within varieties). Still, the observed association between phenylpropanoids, adaptive trait variation (early versus late BD) and timing (chill hours/

Table 4. Predicted phenylpropanoid biosynthesis genes in apricot floral buds with highest absolute log2FC values from integrated pathways analysis.

Pathway	Time	Gene with highest (absolute) log2FC	abs(log2FC)
00940 (47 nodes mapped)	0	Cytochrome P450 84A1	0.88
	100	Shikimate O-hydroxycinnamoyltransferase	2.15
	400	4-coumarate—CoA ligase-like 7	3.52
	800	Cinnamoyl-CoA reductase 1	4.92
	C2	Anthocyanidin 3-O-glucosyltransferase 5	2.84
	D1	Cinnamoyl-CoA reductase 1	2.80
00941 (24 nodes mapped)	0	Flavonol synthase/flavanone 3-hydroxylase	0.62
	100	Shikimate O-hydroxycinnamoyltransferase	2.15
	400	Flavonol synthase/flavanone 3-hydroxylase	2.30
	800	Leucoanthocyanidin reductase	2.31
	C2	Polyketide synthase 5	1.08
	D1	Flavonol synthase/flavanone 3-hydroxylase	0.84
00945 (8 nodes mapped)	0	Cytochrome P450 98A2	0.26
	100	Shikimate O-hydroxycinnamoyltransferase	2.15
	400	Shikimate O-hydroxycinnamoyltransferase	1.99
	800	Caffeoyl-CoA O-methyltransferase 5, flavonoid 3′,5′-methyltransferase	1.33
	C2	Shikimate O-hydroxycinnamoyltransferase	0.72
	D1	Trans-cinnamate 4-monooxygenase	0.75

developmental stage) was not unexpected. TT genes, which are essential for phenylpropanoid biosynthesis and transport in *Arabidopsis*, also impact seed dormancy duration (Buer and Muday 2004, Debeaujon et al. 2001, Zhao and Dixon 2009, Zhao et al. 2010) and root growth (Silva-Navas et al. 2016). We observed the expression of TT genes, in particular Prupe.4G200500, a predicted TT3 protein involved in the biosynthesis of leucoanthocyanidins from dihydroflavonols, change during floral bud reactivation (Zhao et al. 2010). Specifically, Prupe.4G200500, which is located on scaffold four in peach, spiked in expression at 800 chill hours only in late BD varieties; this time point preceded the appearance of sepals and petals in late BD trees. From previous genetic mapping in *Prunus* species, including peach, sweet cherry, almond and apricot, linkage group four is known to contain quantitative trait loci associated with CR, flowering date and/or heat requirement (Fan et al. 2010, Dirlewanger et al. 2012, Castède et al. 2014, Sánchez-Pérez et al. 2014, Bielenberg et al. 2015). Moreover, changes in concentrations of phenylpropanoids have been observed in the buds of other tree species, including *M. domestica*, *A. alba* and *P. abies*, following forced dormancy break (Wang et al. 1991, Dhuli et al. 2014).

In these studies, concentrations of phenylpropanoids, including compounds closely related to those measured in this study, e.g., catechin, epicatechin, rutin and naringin, changed as buds left a dormant state, began to swell and then opened and formed new tissue (Dhuli et al. 2014, Wang et al. 1991). In the present study, concentrations of most phenylpropanoid metabolites examined decreased over time, with some compounds increasing in concentration (most noticeable in late BD trees) before dropping precipitously once buds began to flower. A general trend of decreasing levels of phenylpropanoids in floral buds was observed previously in apricot, where chill hour accumulation was associated with decreasing concentrations of total phenolics (Laslo and Vicas 2012). An increase followed by decrease at the end of dormancy in total phenol amount was also observed in pistachio floral buds (Zahra et al. 2009). It is possible that the pronounced drop in phenylpropanoid concentrations could be due to a dilution effect, i.e., buds fill with water and swell as growth resumes, diluting the concentration of phenylpropanoids per fresh weight of tissue. However, this trend was observed in other species and studies, and was less pronounced or absent in early BD varieties, suggesting that the decline is due to modifications in the biosynthesis and/or transport of phenylpropanoids within the developing floral bud, and not solely attributed to a dilution effect. This decline is consistent with dormancy progress/transition from an endodormant to an ecodormant state, suggesting that phenylpropanoid production is relevant to dormancy regulation. Whether such declines are a direct cause of dormancy stage transition or a consequence of the transition remains to be investigated.

Furthermore, we observed changes in the expression of phenylpropanoid biosynthesis genes, not only TT genes, over time; this change was impacted in some instances by BD phenotype. Similarly, studies in grapevine (*Vitis* species) and pear (*Pyrus pyrifolia*) found that phenylpropanoid pathway gene expression was associated with bud dormancy (Deluc et al. 2011, Fennell et al. 2015, Liu et al. 2012). In this study, phenotypic effects on phenylpropanoids were generally most pronounced around 400 and 800 chill hours—at 800 chill hours, early BD varieties were far more developmentally

advanced compared to late BD varieties. Since 400 and 800 chill hour points occurred prior to developmental changes associated with flowering in early and late BD varieties, respectively, this may indicate a lag in the timing of changes in phenylpropanoid pathway activity in early versus late BD varieties.

Potential role of the phenylpropanoid pathway in regulating hormones associated with growth and development

Phenylpropanoids are known to change in response to environmental variation, and are known to affect the regulation, biosynthesis and transport of plant hormones associated with plant growth and development. While we could not directly link changes in phenylpropanoid concentrations and gene expression patterns to environmental variation in this study, these changes were associated with chill accumulation, and were impacted by phenological trait variation associated with the timing of growth post-dormancy. Temperature is known to affect the concentration of procyanidins and quercetins in *Arabidopsis* seeds, and procyanidin concentration is known to be associated with shorter seed stratification times (Debeaujon et al. 2001, MacGregor et al. 2015). Furthermore, flavonoids such as kaempferol and quercetin are known negative regulators of auxin transport, and scavengers of reactive oxygen species produced as a product of auxin catabolism (Brown et al. 2001, Peer et al. 2001, Buer and Muday 2004, reviewed in Peer and Murphy 2007, Santelia et al. 2008).

In addition to procyanidins and flavonoids, stilbenes, such as resveratrol, have been associated with the induction and maintenance of dormancy, and may be involved in regulating abscisic acid (ABA) biosynthesis (Fennell et al. 2015, Lee et al. 2014). In this study, concentrations of resveratrol in early BD varieties started to decline shortly after chill began to accumulate, and reached minimum concentrations once buds begin to show signs of bud break. In late BD varieties, the decline was postponed and occurred only after additional chill accumulation. Changes in the expression of Prupe.2G122600 (Figure S3 available as Supplementary Data at Tree Physiology Online), which codes for the chalcone and stilbene synthase family protein resveratrol synthase 2—the enzyme associated with the biosynthesis of *trans*-resveratrol from 4-coumaroyl-CoA in peach (http://pathways.rosaceae.org/; Jung et al. 2014)—were also observed. Biosynthesis of resveratrol is also known to increase in response to water deficit in grape berries, and to do so differentially depending on the variety (Deluc et al. 2011). Thus, resveratrol concentrations in floral buds may be impacted by changes in the availability of water in buds or elsewhere in the tree during dormancy and upon resumption of growth post-dormancy (Erez et al. 1998). Changes in resveratrol levels may in turn impact ABA, and subsequently dormancy maintenance and release (Fennell et al. 2015, Zheng et al. 2015).

Conclusions

Phenotypic variation in BD, an adaptive trait associated with dormancy, was associated with changes in phenylpropanoid pathway activity—including the concentration of phenylpropanoid intermediates and the expression of phenylpropanoid biosynthetic genes—in apricot floral buds. The association of phenylpropanoid metabolites and genes with floral bud reactivation suggests a potential role of phenylpropanoids in regulating and/or mediating growth and development in apricot. We hypothesize that phenylpropanoid concentrations change in response to environmental cues (e.g., light, temperature, water), and influence dormancy progression and the timing of growth post-dormancy indirectly, by regulating and/or modifying the biosynthesis and/or transport of plant hormones. Future work should therefore focus on examining the impact of environmental cues on phenylpropanoid levels, as well as the impact of over- or under-expression of phenylpropanoid biosynthesis genes on phenylpropanoid levels in buds, dormancy timing and hormone production. If this relationship proves to be correct, then phenylpropanoids (e.g., resveratrol) and/or genes associated with their biosynthesis (e.g., TT3) may be useful indicators or biomarkers for tracking developmental progression and the introgression of adaptive traits associated with dormancy in tree breeding programs. Moreover, intraspecific variability in phenylpropanoid biosynthesis may be crucial for buffering against variable environmental conditions, associated with rapid and long-term changes in climate.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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