Transcriptomic Study of Early Responses to the Bud Dormancy-breaking Agent Hydrogen Cyanamide in ‘TropicBeauty’ Peach

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ABSTRACT. To determine how the dormancy-breaking agent hydrogen cyanamide (HC) advances budbreak in peach (*Prunus persica*), this study compared the transcriptome of buds of low-chill ‘TropicBeauty’ peach trees treated with 1% (v/v) HC and that of nontreated trees at 3 and 7 days after treatment (DAT), respectively, using an RNA sequencing analysis. The peak of total budbreak occurred 6 weeks earlier in the HC-treated trees (at 32 DAT) than the nontreated trees (at 74 DAT). There were 1312 and 1095 differentially expressed genes (DEGs) at 3 and 7 DAT, respectively. At 3 DAT, DEGs related to oxidative stress, including the response to hypoxia, lipid oxidation, and reactive oxygen species (ROS) metabolic process, were upregulated in HC-treated buds. Additionally, DEGs encoding enzymes for ROS scavenging and the pentose phosphate pathway were upregulated at 3 DAT but they were not differently expressed at 7 DAT, indicating a temporary demand for defense mechanisms against HC-triggered oxidative stress. Upregulation of DEGs for cell division and development at 7 DAT, which were downregulated at 3 DAT, suggests that cell activity was initially suppressed but was enhanced within 7 DAT. At 7 DAT, DEGs related to cell wall degradation and modification were upregulated, which was possibly responsible for the burst of buds. The results of this study strongly suggest that HC induces transient oxidative stress shortly after application, leading to the release of bud dormancy and, subsequently, causing an increase in cell activity and cell wall loosening, thereby accelerating budbreak in peach.

During the fall in temperate climates, buds of deciduous woody perennials, including peach, enter dormancy, which is a state in which the visible growth of a plant structure containing a meristem is absent (Lang et al., 1987). Bud dormancy has two steps, endodormancy and ecodormancy, which are caused by internal tree factors and controlled by environmental conditions, respectively (Considine and Considine, 2016; Lang et al., 1987). When buds are in endodormancy, or “true dormancy,” the meristem remains resting, even if the environmental conditions are optimal for growth resumption (Considine and Considine, 2016; Rohde and Bhalerao, 2007). The release of endodormancy requires cumulative exposure to significant low temperatures during winter; this required duration of low temperatures for dormancy-breaking is termed the chilling requirement and is specific to plant species and cultivars (Considine and Considine, 2016). Dormancy release occurs only when the chilling requirement is fulfilled; as a result, vegetative and flower buds start to swell and burst (“bud-break”), followed by vegetative growth or flowering and subsequent fruit development (Beauvieux et al., 2018; Dennis, 2003). Therefore, the timing of dormancy release and budbreak is critical for the onset of fruit production and the market window.

For peach production in subtropical climates such as the southeastern United States, low-chill cultivars, which require less chill accumulation than those originating from temperate climates, have been developed (Okie, 1998; Olmstead et al., 2016). Nevertheless, the mild winter temperatures in subtropical areas are often insufficient to fulfill the chilling requirement of low-chill cultivars, thereby resulting in nonuniform budbreak and poor fruit sets (George et al., 1992; Olmstead, 2014). The use of plant growth regulators containing the active ingredient of hydrogen cyanamide (HC) to break bud dormancy and to achieve uniform budbreak has been documented for peach (George et al., 1992; Olmstead, 2014; Yamane et al., 2011) and a number of deciduous fruit crops, such as apple (*Malus domestica*), grapevine (*Vitis vinifera*), kiwifruit (*Actinidia deliciosa*), sweet cherry (*Prunus avium*), and both highbush blueberry (*Vaccinium corymbosum* hybrid) and rabbiteye blueberry (*V. ashei*) (Bound and Jones, 2004; Godini et al., 2008; Henzell et al., 1992; Ionescu et al., 2017b; Jackson and Bepete, 1995; Shulman et al., 1986; Williamson et al., 2002).

Although the use of HC is well-documented, the underlying mechanism of its effects on budbreak is not completely understood. For grapevine, a reduction in catalase transcript levels and activity and the accumulation of the reactive oxygen species (ROS), including hydrogen peroxide, superoxide, and nitric oxide, were observed in buds soon after exogenous HC

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application, with a higher budbreak rate compared with that of the nontreated control (Halaly et al., 2008; Nir et al., 1986; Or et al., 2002; Sudawan et al., 2016). Furthermore, HC resulted in a rapid decrease in the consumption of oxygen in mitochondria and the temporary induction of alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDH) expressions in buds of grapevine, indicating the disturbance of aerobic respiration by HC (Halaly et al., 2008; Or et al., 2000; Pérez et al., 2009). The application of HC also reduced the expression of dormancy-associated MADS-box (*DAM*) genes *DAM5* and *DAM6*, which are two of six *DAM* genes isolated in the nondormant peach mutant with a role in dormancy maintenance (Bielenberg et al., 2008; Jiménez et al., 2010; Rodriguez-A et al., 1994), in peach buds at 2 and 4 weeks after treatment (Yamane et al., 2011). These results led to a generally accepted hypothesis that exogenous HC application results in transient oxidative stress that, at least in part, induces the release of bud dormancy and budbreak in deciduous crops (Beauvieux et al., 2018; Halaly et al., 2008; Or et al., 2000). However, this proposed mode of action has not yet been studied in peach.

In recent years, the peach industry in subtropical Florida has grown substantially with the development of low-chill cultivars as an alternative crop to *Citrus* species (Morgan and Olmstead 2013; Singerman et al., 2017), but the effects of exogenously applied HC have never been investigated in low-chill peach cultivars at transcript levels. Better knowledge of the mechanism of HC breaking bud dormancy is fundamental to develop strategies to efficiently manipulate the time of budbreak using plant growth regulators, thereby promoting uniform flowering and fruiting for commercial peach production. Therefore, the goal of this research was to determine the effect of HC treatment at the transcriptome level and the underlying mechanism that leads to early budbreak in peach. The responses of exogenous HC application were assessed by comparing the transcriptome using RNA sequencing in buds of trees treated with 1% (v/v) HC and those of nontreated trees of peach cultivar TropicBeauty, which has a chilling requirement of 150 chill hours. The results provide evidence that HC resulted in oxidative stress in buds shortly after treatment, followed by the induction of cell growth and development and advanced budbreak for the low-chill peach cultivar grown in the subtropical climate.

Materials and Methods

PLANT MATERIALS AND HC TREATMENT. Five-year-old ‘TropicBeauty’ peach, which have a chilling requirement of 150 chill hours, on ‘Flordaguard’ rootstock in an experimental orchard (Citra, FL) were used for this research. Chill hours for the duration of the experiment were calculated based on the Weinberger model (Weinberger, 1950), starting from 1 Oct. 2015 ($\approx$ 2 months before the experiment). The experiment had a complete randomized design, with two treatments replicated on four individual trees per treatment ($n = 4$). In Florida, commercial peach growers usually apply HC around mid-December to promote uniform and early fruit sets. Therefore, on 16 Dec. 2015 (11 chill hours), one set of trees was sprayed with 1% (v/v) HC (BudPro; Green Trees & Plants II, Woodstock, GA) containing 0.15% (v/v) nonionic surfactant (Silwet L-77; Helena Agri-Enterprises, Collierville, TN) with a backpack sprayer until dripping; the other set of trees was not treated with HC (they were sprayed with water with surfactant) and was referred to as the control trees during this research.

Ten buds of each of the four control and HC-treated trees (as four biological replicates) were collected at 0 (pretreatment), 1, 3, and 7 DAT, respectively. Upon collection, bud samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

BUDBREAK EVALUATION. Starting on 7 Jan. 2016 (21 DAT), the number of vegetative and flower buds that were swollen (beginning to open) or open on the four branches of uniform size per tree was quantified every 4 to 7 d until 15 Feb. 2016 (60 DAT) for HC-treated trees and until 7 Mar. 2016 (81 DAT) for control trees. The budbreak rate was expressed as the average number of swollen or open buds per branch per tree for the individual trees (replications) per treatment.

RNA EXTRACTION AND RNA SEQUENCING ANALYSIS. Total RNA was extracted using an RNA isolation kit (Plant/Fungi Total RNA Purification Kit; Norgen Biotek, Thorold, ON, Canada). The quality and quantity of RNA were evaluated using a spectrophotometer (Epoch 2 Microplate; BioTek Instruments, Winooski, VT) and denaturing formaldehyde 1.2% agarose gels (Rio, 2015).

To determine the early response to HC in peach buds at a transcriptomic level, RNA samples of control and HC-treated trees at 3 and 7 DAT ($n = 4$) were sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL) for RNA sequencing (RNA-seq), alignment, and differential expression analysis. The Illumina TruSeq mRNA protocol (Illumina, San Diego, CA) was performed for transcript sequencing. The sequenced transcripts were annotated using the *Prunus persica* v2.1 genome from the Phytozome database (Verde et al., 2017). The RNA-seq read data are summarized in Supplemental Table 1. Differential gene expression was analyzed using RSEM v1.2.31 (Li and Dewey, 2011). An absolute value of the $\log_2$ fold change greater than 1 and a false discovery rate (FDR)-corrected $P < 0.05$ were used as the thresholds to identify the significantly differentially expressed genes (DEGs) of trees (HC-treated vs. control trees) at 3 and 7 DAT, respectively. The data discussed in this publication were deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO) (Barrett et al., 2013; Edgar et al., 2002) and are accessible through GEO Series accession number GSE128158.

GENE ONTOLOGY ENRICHMENT ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES. To investigate the biological significance of the results of RNA-seq, DEGs at 3 and 7 DAT, respectively, were subjected to functional analyses. MapMan software (version 3.5.1.R2) (Thimm et al., 2004; Usadel et al., 2005) was used to identify the physiological or biochemical processes represented by the DEGs; only the statistically significant functional categories (BINs) based on the Wilcoxon rank sum test ($P < 0.05$) are reported here. A gene ontology (GO) enrichment analysis was performed using AgriGO, which uses Fisher’s exact test (Tian et al., 2017). To obtain the results of the GO analysis, the GO terms and FDR according to multi-test adjustments more than 0.05 were discarded, leaving only statistically significant GO terms ($P < 0.05$). Redundant GO terms were further removed using REVIGO (Supek et al., 2011). The enrichment values and the absolute value of the $\log_{10}$ FDR-adjusted P value (Huang et al., 2009) of each significant GO term for the biological process, molecular function, and cellular component were plotted on heatmaps in which upregulated and downregulated DEGs were expressed as positive and negative, respectively, using Morpheus software

(Broad Institute, Cambridge, MA). Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper tool (Kanehisa et al., 2017) was used to determine the metabolic pathways that involve the enzymes encoded by DEGs.

ANALYSIS OF GENE EXPRESSION USING REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION. To validate the results of RNA-seq analysis, the relative expressions of *formate dehydrogenase* [*FDH* (Prupe.3G284000)], *manganese superoxide dismutase1* [*MSD1* (Prupe.2G262400)], *glutathione S-transferase tau8* [*GSTU8* (Prupe.6G264900)], *glutathione S-transferase tau19* [*GSTU19* (Prupe.4G147400)], *peptide methionine sulfoxide reductase1* [*PMSR1* (Prupe.5G161000)], and *nadph:quinone oxidoreductase* [*NQR* (Prupe.2G038400)] selected from the DEGs were determined using real-time quantitative polymerase chain reaction (qPCR). In addition, *DAM1*, *DAM5*, *DAM6*, *apetalal* (*API*), *pistillata* (*PI*), *agamous* (*AG*), and *floral binding protein 9* (*FBP9*) were analyzed because their expressions were strongly related to dormancy or flowering in peach (Bielenberg et al., 2008; Jiménez et al., 2010; Tani et al., 2009; Zhang et al., 2008). Gene-specific primers were designed using Primer BLAST (National Center for Biotechnology Information, 2018). The sequences of the primers are listed in Supplemental Table 2.

For cDNA synthesis, 1 µg of total RNA of the buds collected at 0, 1, 3, and 7 DAT, respectively, was first treated with DNase I (RQ1; Promega, Madison, WI) and used for first-strand synthesis using oligo (dT)₁₅ primer, dNTP mix, and reverse transcriptase (ImProm-II; Promega) in a 20-µL reaction according to the manufacturer's protocol.

A real-time PCR system (7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA) was used to perform qPCR in a 10-µL reaction system containing 10 ng of cDNA (calculated from RNA), 300 nM forward and reverse primers, and SYBR green reagent mix [PowerUp SYBR Green Master Mix (2X); Applied Biosystems]. Each reaction was performed at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Dissociation curve analysis ranging from 60 to 95 °C was performed at the end of each qPCR to confirm that nonspecific products were not formed.

Using the quantification cycle (Ct), the levels of relative expression (fold change) of genes of interest were calculated using the Pfaffl method (Hellemans et al., 2007; Pfaffl, 2001) with *translation elongation factor 2* (*TEF2*) and *ubiquitin* (*UBQ*) as the reference genes (Tong et al., 2009; Yamane et al., 2011). The two reference genes were selected among other housekeeping genes tested preliminarily (*translation elongation factor 1-alpha*, *18s ribosomal RNA*, *RNA polymerase II*, *α-tubulin*, *β-tubulin*, and *actin 2/7*) based on their stability across bud samples collected on different days. Gene expression data for each sampling date were the mean of four biological replicates; each biological replicate was the mean of two qPCR (technical) replicates.

STATISTICAL ANALYSIS. Student's *t* test was used to test for the treatment effect of HC on the budbreak rate and relative gene expression levels (qPCR results) using SAS (version 9.3; SAS Institute, Cary, NC). An analysis of variance (ANOVA) was used to determine the changes in the budbreak rate over time using the General Linear Model procedure of SAS; when ANOVA testing indicated significant differences, post hoc comparisons were performed using Tukey's honestly significant difference (HSD) procedure with a family error rate of $\alpha \leq 0.05$. Pearson's correlation coefficients were calculated to

identify significant relationships ($r > 0.5$; $P \leq 0.05$) between relative gene expression quantified with qPCR and fold change from the RNA-seq analysis.

Results

EFFECTS OF HC ON BUDBREAK. For nontreated control trees, only a few buds (<3 buds/branch) had emerged within 60 DAT (Fig. 1). The maximum budbreak rate over time occurred at 74 and 81 DAT for the control trees ($P < 0.001$). In contrast, a substantial number of swollen or open buds (≈ 10 buds/branch) were observed in HC-treated trees starting at 21 DAT and the budbreak rate reached its maximum at 32 and 39 DAT ($P < 0.001$), which was 6 weeks earlier than that of the nontreated trees. For HC-treated trees, the reduction in the budbreak rate after 39 DAT was due to the reduction in flower buds as a result of fruit setting following bloom. There was no significant difference in the number of vegetative buds, flower buds, or total buds on the day when the maximum budbreak occurred (39 DAT for HC-treated trees and 81 DAT for control) for HC-treated and control trees, indicating that HC treatment resulted in early budbreak without suppressing the budbreak rate.

ANALYSIS OF DIFFERENTIALLY EXPRESSION GENES. Differences in the transcriptome of the buds of HC-treated trees compared with the nontreated control at 3 and 7 DAT were obtained using RNA-seq analysis. There were 1312 and 1095 DEGs at 3 and 7 DAT, respectively, with absolute values of fold change greater than 1 and FDR less than 0.05. For DEGs at 3 DAT, 610 were upregulated and 702 were downregulated in buds of HC-treated trees compared with nontreated trees, whereas there were 689 and 406 upregulated and downregulated, respectively, DEGs at 7 DAT of HC-treated trees. Only 88 DEGs were common between the two sampling days; among them, 40 were upregulated and 48 were downregulated in buds of treated trees in comparison with those of nontreated trees. The extracellular region (GO:0005576) was the only significant GO term for the 40 common upregulated DEGs. For the DEGs that were commonly downregulated at both 3 and 7 DAT, significant GO terms were related to stress responses, including the response to abiotic stimulus (GO:0009628), response to stimulus (GO:0050896), response to chemical stimulus (GO:0042221), secondary metabolic process (GO:0019748), and response to temperature stimulus (GO:0009266).

ENRICHMENT ANALYSIS OF MAPMAN FUNCTIONAL CATEGORIES. The enrichment analysis of gene categories using MapMan software provided an overview of the physiological and biochemical processes of DEGs at 3 and 7 DAT, respectively. The results of the MapMan analysis demonstrated that most or all DEGs in the metabolic pathways involving nitrile lyases and nitrilases [BIN 26.8 ($P = 0.010$)] and glutathione S-transferases (GST) [BIN 26.9 ($P = 0.005$)] were upregulated at 3 DAT (Table 1). The majority of DEGs for regulating DNA [BIN 28 ($P < 0.001$)], DNA synthesis and chromatin structure [BIN 28.1 ($P < 0.001$)], and kinase-involved post-translational modifications [BIN29.4.1 ($P = 0.048$)] were downregulated at 3 DAT. Interestingly, DEGs involved in the photosynthesis pathway [BIN 1 ($P = 0.018$)] were upregulated at 3 DAT; nevertheless, this result was unexpected because no visible green photosynthetic tissues had emerged from the bud scales at 3 DAT for HC-treated trees. At 7 DAT, most DEGs involving the regulation of DNA [BIN 28 ($P = 0.002$)], DNA synthesis and

chromatin structure [BIN 28.1 ($P = 0.002$)], kinase-involved post-translational modifications [BIN29.4.1 ($P = 0.019$)], and cell organization [BIN 31.1 ($P = 0.029$)] were upregulated, as opposed to the results at 3 DAT. There were several upregulated DEGs assigned to cell wall degradation [BIN 10.6 ($P = 0.031$)].

ENRICHMENT ANALYSIS OF GENE ONTOLOGY. To obtain an interpretation of the more recent assignment of gene function, a GO enrichment analysis was performed with upregulated and downregulated DEGs, respectively, at 3 and 7 DAT. The significant GO terms analyzed using AgriGO and REVIGO were visualized as heatmaps for biological processes, molecular functions, and cellular components (Supplemental Fig. 1). Regarding biological processes, 238 significant GO terms were

further categorized into two major groups (groups 1 and 2) based on the patterns of DEGs on the two sampling days (Table 2). Group 1 included the two subgroups of GO terms in which DEGs had decreasing expression patterns from 3 to 7 DAT. For the GO terms of subgroup 1A, DEGs were upregulated at 3 DAT; however, these GO terms were not statistically significant at 7 DAT. The GO terms of subgroup 1B were not significant at 3 DAT; however, DEGs were downregulated at 7 DAT. In contrast to group 1, the expression of DEGs in the GO terms assigned to group 2 increased over time. Group 2 was further divided into three subgroups of GO terms: GO terms that consisted of downregulated and upregulated DEGs at 3 and 7 DAT, respectively (subgroup 2A); GO terms with downregulated DEGs at 3 DAT but that were not significant at 7 DAT (subgroup 2B); and GO terms that were nonsignificant at 3 DAT but contained upregulated DEGs at 7 DAT.

The GO terms for which DEGs had decreasing patterns (group 1) included response to oxidative stress (GO:0006979), response to hypoxia (GO:0001666), respiratory burst (GO:0045730), oxygen and ROS metabolic process (GO:0006800), hydrogen peroxide metabolic process (GO:0042743), and lipid oxidation (GO:0034440) (Table 2). In addition, a number of DEGs related to antioxidant activity were upregulated at 3 DAT (Table 3), suggesting the activation of defense mechanisms against oxidative stress. Death (GO:0016265) and cell death (GO:0008219) were also included in group 1. Consistently, group 2 included the GO terms related to growth and development,

Fig. 1. Budbreak rate expressed as the number of swollen (about to open) or open flower and vegetative buds for nontreated ‘TropicBeauty’ peach control trees and ‘TropicBeauty’ peach trees treated with 1% (v/v) hydrogen cyanamide (HC) 21 to 81 d after treatment (DAT) and 21 to 60 DAT, respectively, and the accumulation of chill hours (Weinberger, 1950) in Citra, FL, since 1 Oct. 2015. Data are means $\pm$ SE of four biological replicates. *Significant differences based on Student’s *t* test for total budbreak rate between control and HC-treated trees on the same day.

Table 1. Selected significant categories (BINs) of MapMan enrichment analysis (Thimm et al., 2004; Usadel et al., 2005) for differentially expressed genes (DEGs) in buds of ‘TropicBeauty’ peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated control trees at 3 and 7 d after treatment (DAT).

DAT	BIN	Function	Upregulated DEGs (no.)	Downregulated DEGs (no.)	<i>P</i>
3	1	Photosynthesis	20	2	0.018
3	26	Misc.	54	48	0.040
3	26.8	Misc. nitrilases, nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	6	1	0.010
3	26.9	Misc. glutathione s-transferases	6	0	0.005
3	26.24	Misc. GCN5-related n-acetyltransferase	4	0	0.037
3	26.28	Misc. GDSL-motif lipase	1	6	0.042
3	27	RNA	29	74	0.020
3	27.3	RNA regulation of transcription	29	61	0.002
3	28	DNA	5	23	0.001
3	28.1	DNA synthesis/chromatin structure	1	21	<0.001
3	29.4.1	Protein post-translational modification kinase	0	3	0.048
7	10	Cell wall	25	5	0.050
7	10.6	Cell wall degradation	10	2	0.031
7	28	DNA	27	3	0.002
7	28.1	DNA synthesis/chromatin structure	22	2	0.002
7	29.4.1	Protein post-translational modification kinase	7	0	0.019
7	31.1	Cell organization	23	2	0.029

Table 2. Selected significant gene ontology (GO) terms of differentially expressed genes (DEGs) with enrichment values (EV) in buds of ‘TropicBeauty’ peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated control trees at 3 and 7 d after treatment (DAT) in the aspect of GO biological process.

GO term	3 DAT		7 DAT	
	EV	DEGs (no.)	EV	DEGs (no.)
Group 1				
Subgroup 1A				
GO:0006952 defense response	10.7 ^z	76	0.0	0
GO:0055114 oxidation reduction	10.7	68	0.0	0
GO:0006800 oxygen and reactive oxygen species metabolic process	5.4	24	0.0	0
GO:0042743 hydrogen peroxide metabolic process	5.1	23	0.0	0
GO:0034641 cellular nitrogen compound metabolic process	5.0	53	0.0	0
GO:0010310 regulation of hydrogen peroxide metabolic process	4.4	16	0.0	0
GO:0080010 regulation of oxygen and reactive oxygen species metabolic process	4.3	16	0.0	0
GO:0045730 respiratory burst	4.3	13	0.0	0
GO:0044255 cellular lipid metabolic process	4.2	51	0.0	0
GO:0008219 cell death	3.6	25	0.0	0
GO:0009626 plant-type hypersensitive response	3.6	22	0.0	0
GO:0016265 death	3.6	25	0.0	0
GO:0012501 programmed cell death	3.3	23	0.0	0
GO:0070482 response to oxygen levels	2.4	9	0.0	0
GO:0048583 regulation of response to stimulus	2.3	26	0.0	0
GO:0006979 response to oxidative stress	2.1	23	-2.3	20
GO:0034440 lipid oxidation	1.4	10	0.0	0
GO:0001666 response to hypoxia	1.3	7	0.0	0
Subgroup 1B				
GO:0009266 response to temperature stimulus	0.0	0	-3.0	30
GO:0009642 response to light intensity	0.0	0	-2.6	14
GO:0009644 response to high light intensity	0.0	0	-2.3	12
GO:0031326 regulation of cellular biosynthetic process	0.0	0	-1.9	53
Group 2				
Subgroup 2A				
GO:0051301 cell division	-1.4	18	7.4	33
GO:0009825 multidimensional cell growth	-1.5	9	3.0	12
GO:0000280 nuclear division	-1.6	9	3.8	13
GO:0022403 cell cycle phase	-1.8	19	5.3	28
GO:0007000 nucleolus organization	-1.9	5	1.8	5
GO:0006275 regulation of DNA replication	-2.5	12	6.4	19
GO:0000226 microtubule cytoskeleton organization	-2.6	17	5.3	23
GO:0022402 cell cycle process	-2.9	30	8.7	46
GO:0000911 cytokinesis by cell plate formation	-3.0	16	7.4	25
GO:0040029 regulation of gene expression, epigenetic	-3.5	29	3.3	29
GO:0048589 developmental growth	-3.6	39	3.7	40
GO:0007017 microtubule-based process	-3.6	22	8.1	32
GO:0006996 organelle organization	-3.9	78	8.0	96
GO:0007049 cell cycle	-4.0	41	9.7	58
GO:0006260 DNA replication	-4.4	25	6.1	29
GO:0048507 meristem development	-4.5	32	4.5	32
GO:0006261 DNA-dependent DNA replication	-5.0	23	6.1	25
GO:0006325 chromatin organization	-5.8	38	8.7	45
GO:0006270 DNA replication initiation	-6.1	14	2.7	9
GO:0051276 chromosome organization	-6.7	48	9.5	55
GO:0008283 cell proliferation	-8.0	30	6.2	26
Subgroup 2B				
GO:0048522 positive regulation of cellular process	-1.6	32	0.0	0
GO:0048518 positive regulation of biological process	-2.1	37	0.0	0
GO:0006310 DNA recombination	-2.5	17	0.0	0

Continued next page

Table 2. Continued.

GO term	3 DAT		7 DAT	
	EV	DEGs (no.)	EV	DEGs (no.)
GO:0032774 RNA biosynthetic process	-3.1	90	0.0	0
GO:0019219 regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	-4.5	94	0.0	0
GO:0016570 histone modification	-4.9	28	0.0	0
Subgroup 2C				
GO:0033043 regulation of organelle organization	0.0	0	1.5	12
GO:0042547 cell wall modification during multidimensional cell growth	0.0	0	1.6	5
GO:0071669 plant-type cell wall organization or biogenesis	0.0	0	2.2	25
GO:0071554 cell wall organization or biogenesis	0.0	0	2.3	41
GO:0009832 plant-type cell wall biogenesis	0.0	0	2.6	13
GO:0044238 primary metabolic process	0.0	0	3.4	317
GO:0035266 meristem growth	0.0	0	4.5	18
GO:0010389 regulation of G2/M transition of mitotic cell cycle	0.0	0	4.6	12
GO:0051567 histone H3-K9 methylation	0.0	0	6.1	21
GO:0009888 tissue development	0.0	0	6.3	56
GO:0043687 post-translational protein modification	0.0	0	6.4	91

^zEnrichment values were the absolute values of $\log_{10}$ false discovery rate-corrected *P* values based on Fisher's exact test for individual GO terms, where up and downregulated DEGs are indicated as positive and negative values, respectively.

such as cell division (GO:0051301), cell cycle (GO:0007049), cell proliferation (GO:0008283), and DNA replication (GO:0006996) (Table 2).

EFFECTS OF HC ON CYANIDE METABOLISM. To investigate the cyanide-related metabolic pathways in which DEGs were involved, KEGG Mapper was used. At 3 DAT, three DEGs encoding the enzymes for the last step of hydrogen cyanide synthesis, including mandelonitrile lyase [*MLD* (Prupe.1G093600 and Prupe.1G093000)] and catalase [*CAT2* (Prupe.5G011400)], were upregulated (Fig. 2), indicating the production of hydrogen cyanide in buds of peach trees treated with HC; however, upstream of mandelonitrile was downregulated. Nevertheless, DEG for a β -glucosidase [*BGLU15* (Prupe.3G111000)], which converts cyanogenic glycosides prunasin and amygdalin to mandelonitrile, the precursor of cyanide, was downregulated at 3 DAT. In addition, on the same day, a nitrilase [*NIT4* (Prupe.6G137600)] that catalyzes the production of aspartate and asparagine from L-3-cyanoalaine, which is the intermediate product of cyanide detoxification by L-3-cyanoalaine synthase, was upregulated.

EFFECTS OF HC ON CELLULAR RESPIRATION. Differentially expressed genes that were involved in respiration, including glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (PPP), are presented in Fig. 3. At 3 DAT, DEGs encoding fructose-1,6-bisphosphatase (Prupe.8G028300), triosephosphate isomerase (Prupe.6G076300), 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Prupe.6G300000), and enolase (Prupe.2G281900) were upregulated; the DEG encoding pyruvate kinase (Prupe.8G151100), during the last step of glycolysis and during which ATP is produced, was downregulated in buds of HC-treated trees (Fig. 3A). Interestingly, DEGs encoding enzymes in the PPP, such as glucose-6-phosphate dehydrogenase [*G6PD* (Prupe.6G307600)] and transketolase (Prupe.2G143600), were upregulated at 3 DAT, indicating an increase in NADPH in the buds of trees treated with HC. At 7 DAT, the DEG encoding a pyruvate kinase (Prupe.7G120700) for the ATP-producing step of glycolysis was upregulated

(Fig. 3B). Moreover, DEGs that encode enzymes for the TCA cycle, including pyruvate dehydrogenase (Prupe.1G262100), dihydrolipoamide acetyltransferase (Prupe.2G204600), isocitrate dehydrogenase (Prupe.3G288200), and phosphoenolpyruvate carboxykinase (Prupe.6G210900), were upregulated at 7 DAT.

WRKY AND EXPANSIN TRANSCRIPTION FACTOR FAMILIES. Several genes encoding transcription factors (TFs) were differentially expressed as a result of HC application at both 3 and 7 DAT (Supplemental Table 3). WRKY is a TF family of particular interest because many members of this family have been reported to be related to not only pathogen responses but also stress responses (Babitha et al., 2013; Chen et al., 2012). At 3 DAT, DEGs encoding WRKY6, WRKY28, WRKY40, and WRKY75 were upregulated, whereas that encoding WRKY12 was downregulated (Table 4). At 7 DAT, DEGs encoding WRKY7 and WRKY57 were upregulated and DEGs encoding WRKY22 were downregulated. The application of HC also had effects on the expression of the genes encoding expansins (EXPs), a family of nonenzymatic proteins in plant cell walls (Cosgrove, 2000). At 3 DAT, eight DEGs encoding EXPs were downregulated and two were upregulated (Table 5). Nonetheless, DEGs for EXP3, EXPA1, EXPA8, EXPA15, and EXPB2 (5 out of 6 DEGs) were upregulated in buds of HC-treated trees at 7 DAT.

RELATIVE GENE EXPRESSION ANALYSIS. Despite being differentially expressed at 3 DAT based on the RNA-seq analysis, the levels of *FDH*, *MSD1*, *GSTU8*, *GSTU19*, *PMSR1*, and *NQR* expression quantified using qPCR were not significantly greater in buds of HC-treated trees compared with nontreated controls on any sampling day (Supplemental Fig. 2). This discrepancy between the results of qPCR and RNA-seq analyses could be attributed to the different principles of the two technologies. Nevertheless, there was a positive and significant correlation between relative expression levels (obtained by qPCR analysis) and fold changes (obtained by RNA-seq analysis) for these six genes ($r = 0.80$; $P = 0.050$), indicating that the RNA-seq data are valid.

Table 3. Antioxidants and related enzymes encoded by differentially expressed genes (DEGs) with log₂ fold changes (LFC) in buds of ‘TropicBeauty’ peach trees treated with 1% (v/v) hydrogen cyanamide (HC) compared with nontreated control trees at 3 d after treatment.

DEG	<i>Arabidopsis thaliana</i> ortholog	Description	LFC
Superoxide dismutase (superoxide + 2 H ⁺ → H ₂ O ₂ + O ₂)			
Prupe.2G262400	AT3G10920	Manganese superoxide dismutase 1	1.0
Prupe.2G269400	AT5G51100	Iron superoxide dismutase 2	-1.8
Catalase (2 H ₂ O ₂ → 2 H ₂ O + O ₂)			
Prupe.5G011400	AT4G35090	Catalase 2	1.9
Peroxidase (2 H ₂ O ₂ + RH ₂ → 2 H ₂ O + R)			
Prupe.6G289400	AT5G05340	Peroxidase superfamily protein	3.2
Prupe.8G038900	AT1G71695	Peroxidase superfamily protein	1.5
Prupe.4G021100	AT4G21960	Peroxidase superfamily protein	-2.0
Prupe.1G397400	AT2G24800	Peroxidase superfamily protein	-2.6
Prupe.6G091600	AT1G07890	Ascorbate peroxidase 1	1.0
Prupe.6G155100	AT5G21105	Plant L-ascorbate oxidase	-3.4
Thioredoxin (R-S-S-R → 2 R-SH)			
Prupe.1G140400	AT5G18600	Thioredoxin superfamily protein	3.7
Prupe.8G126900	AT2G15570	Thioredoxin superfamily protein	2.1
Prupe.5G170800	AT5G63030	Thioredoxin superfamily protein	-1.6
Prupe.1G265600	AT5G39950	Thioredoxin 2	-2.1
Enzymes maintaining the scavenger function of ascorbate			
Prupe.3G286700	AT3G27820	Monodehydroascorbate reductase 4	1.3
Prupe.2G227100	AT1G75270	Dehydroascorbate reductase 2	-1.2
Enzymes maintaining the scavenger function of glutathione			
Prupe.6G244700	AT5G02790	Glutathione S-transferase family protein	2.0
Prupe.1G264700	AT1G10370	Glutathione S-transferase family protein	-1.4
Prupe.8G256900	AT2G47730	Glutathione S-transferase PHI 8	4.4
Prupe.8G210800	AT2G29420	Glutathione S-transferase TAU 7	3.8
Prupe.1G526000	AT2G29420	Glutathione S-transferase TAU 7	2.9
Prupe.2G101600	AT2G29420	Glutathione S-transferase TAU 7	2.5
Prupe.1G055000	AT2G29420	Glutathione S-transferase TAU 7	1.7
Prupe.8G210700	AT3G09270	Glutathione S-transferase TAU 8	3.8
Prupe.6G264900	AT3G09270	Glutathione S-transferase TAU 8	3.5
Prupe.5G228000	AT3G09270	Glutathione S-transferase TAU 8	2.9
Prupe.4G147400	AT1G78380	Glutathione S-transferase TAU 19	5.7
Prupe.4G146800	AT1G78380	Glutathione S-transferase TAU 19	2.9
Prupe.4G146400	AT1G78380	Glutathione S-transferase TAU 19	2.3
Prupe.4G147000	AT1G78380	Glutathione S-transferase TAU 19	2.1
Prupe.4G147200	AT1G78380	Glutathione S-transferase TAU 19	1.1
Prupe.4G147100	AT1G78380	Glutathione S-transferase TAU 19	1.1
Prupe.4G146000	AT1G78380	Glutathione S-transferase TAU 19	-2.3
Prupe.3G165500	AT1G65820	Microsomal glutathione s-transferase	1.1
Other enzymes with reducing activity			
Prupe.5G161000	AT5G61640	Peptide methionine sulfoxide reductase	1.3
Prupe.2G038400	AT3G27890	NADPH:quinone oxidoreductase	2.3

The expressions of *DAM1*, *DAM5*, and *DAM6* did not change over the sampling time in buds of nontreated trees (Supplemental Fig. 3). The application of HC, despite advancing budbreak by 6 weeks, did not result in significant differences in the levels of *DAM1*, *DAM5*, and *DAM6* expression within 7 d following the treatment compared with the nontreated control. There was no significant difference in the expression levels of floral organ identity genes *API*, *PI*, *AG*, and *FBP9* on any sampling day in HC-treated and nontreated trees (Supplemental Fig. 3). It should also be noted that none of these seven dormancy-related or flowering-related genes was differentially expressed in HC-treated and nontreated trees at either 3 or 7 DAT based on RNA-seq analysis.

Discussion

For ‘TropicBeauty’ peach, maximum budbreak occurred at 32 DAT in trees treated with HC and at 74 DAT in nontreated trees (Fig. 1). The results reported herein demonstrated that the application of HC in mid-December advanced budbreak by 6 weeks, consistent with results reported previously (George et al., 1992; Olmstead, 2014; Yamane et al., 2011). Although the levels of *DAM1*, *DAM5*, and *DAM6* transcripts in buds were not significantly affected within 7 d after HC application (Supplemental Fig. 3), the results cannot exclude the possibility that the repression of *DAM* expression by HC may occur after 7 d. This possibility is supported by the results demonstrating a decrease in *DAM5* and *DAM6* expression levels in vegetative peach buds in response to cyanamide at 2 and 4 weeks after treatment, respectively (Yamane et al., 2011).

At 3 DAT, 4 weeks before maximum budbreak, metabolic processes involving nitrile lyases and nitrilases were upregulated (Table 1). In plants, hydrogen cyanide can be generated through cyanogenesis, which is the hydrolysis of cyanogenic glycosides that requires cyanogenic β-glucosidase and nitrile lyase (Gleadon and Møller, 2014; Morant et al., 2008). For sweet cherry, genes for both enzymes had greater transcript levels in buds from the branches sprayed with HC (followed by accelerated budbreak) than those from the nontreated branches (Ionescu et al., 2017b). Consistently, for buds of HC-treated trees in this study, in addition to *MLD*, the DEG encoding CAT2, which is involved in cyanide synthesis via a different pathway (Ionescu et al., 2017a; Shirota et al., 1987), was upregulated at 3 DAT (Fig. 2), indicating that exogenous HC application caused an ostensible increase in hydrogen cyanide in buds at 3 DAT. In plants, toxic cyanide produced endogenously is eradicated primarily by β-cyanoalanine synthase (β-CAS), resulting in L-3-cyanoalaine, which can be further hydrolyzed to aspartate and asparagine by NIT4 as a part of the detoxification system (Piotrowski et al., 2001; Siegień and Bogatek, 2006). In the current study, although β-CAS was not differentially expressed, *NIT4* was upregulated in buds of HC-treated trees at 3 DAT (Fig. 2), suggesting the need for hydrogen cyanide detoxification in response to its production in buds triggered by exogenous HC application.

Hydrogen cyanide interrupts cellular respiration by disrupting the electron transport pathway through binding to cyto-

Fig. 2. Overview of hydrogen cyanide-related metabolic pathways in which enzymes were encoded by the differentially expressed genes (DEGs), with red and blue indicating upregulated and downregulated DEGs, respectively, in buds of 'TropicBeauty' peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated control trees at 3 d after treatment (DAT) (A). A list of these enzymes, DEGs, putative *Arabidopsis thaliana* orthologs, and log₂ fold change (LFC) at 3 DAT (B) is provided.

chrome c oxidase (COX) (Antonini et al., 1971; Bendall and Bonner, 1971; Cooper and Brown, 2008). In grape, exogenous HC application, which accelerated budbreak, also inhibited the uptake of oxygen in mitochondria (Pérez et al., 2009). Interestingly, DEGs encoding an alternative oxidase, *AOX1a* (Prupe.5G018700), which has a similar function as COX in the electron transport chain of respiration but is insensitive to cyanide toxicity (Vanlerberghe and McIntosh, 1997), and an alcohol dehydrogenase, Prupe.8G018300, which is involved in fermentation (anaerobic respiration) (Or et al., 2000; Perata and Alpi, 1993), were upregulated in buds at 3 DAT (Fig. 3C). Taken together, the results of the current research suggested that in peach buds, the application of HC and HC-induced endogenous hydrogen cyanide triggered similar metabolic responses to hypoxia as a result of dysfunctional mitochondrial respiration.

Under hypoxia, the production of ROS increases (Blokhina and Fagerstedt, 2010; Turrens, 2003); furthermore, accumulations of hydrogen peroxide, superoxide, and nitric oxide were observed in grapevine buds after 12 h of HC treatment that induced budbreak in the summer (Sudawan et al., 2016). For peach, the ROS content in buds in relation to the release of endodormancy by either natural chill hours or exogenous treatments has never been investigated. Nonetheless, in this study, DEGs for oxidative stress (GO:0006979), response to hypoxia (GO:0001666), response to oxygen levels

(GO:0070482), ROS metabolic process (GO:0006800), and regulation of ROS metabolic process (GO:0080010) were upregulated in buds of HC-treated trees at 3 DAT (Table 2), suggesting an increase in ROS generation under the hypoxic conditions imposed by HC. In addition, on the same day, *WRKY6*, *WRKY28*, *WRKY40*, and *WRKY75*, which belong to the WRKY TF family and have a role in oxidative stress (Bakshi and Oelmüller, 2014; Chen et al., 2012; Scarpeci et al., 2008), were upregulated (Table 4). Among them, expressions of *WRKY6* and *WRKY75* have been demonstrated to be increased in response to hydrogen peroxide in *Arabidopsis thaliana* (Bakshi and Oelmüller, 2014), lending further support to the possibility that HC-triggered oxidative stress leads to the accumulation of ROS in peach buds.

The DEGs for lipid oxidation (GO:0034440) were upregulated in buds of HC-treated trees at 3 DAT (Table 2). It is of relevance that oxidation or peroxidation of lipids, which are generally accepted as a hallmark of oxidative stress, causes the loss of membrane integrity and cell damage (Demidchik, 2015; Farmer and Mueller, 2013). The results of the current research demonstrated that DEGs for the GO terms

related to cell cycle, growth, and development were down-regulated in buds of HC-treated trees at 3 DAT (Tables 1 and 2), thus providing evidence that normal cell activities in buds were repressed by HC-induced oxidative stress within only 3 d after application. For HC-treated trees, bud *FDH* was differentially expressed at 3 DAT according to the RNA-seq analysis. In *Arabidopsis*, *FDH* transcripts were increased under stress conditions such as hypoxia (Li et al., 2001), and this gene was reported to have a role in cell death and defense responses to bacterial pathogens in pepper [*Capsicum annuum* (Choi et al., 2014)]. Consistently, the current results indicating the upregulation of programmed cell death (GO:0012501), cell death (GO:0008219), and death (GO:0016265) at 3 DAT (Table 2) further suggest a detrimental effect of HC on cell proliferation. Nevertheless, in many plants, layers of dead cells shield the living tissues from harmful factors under stress conditions (Demidchik et al., 2014; Demidchik, 2015), suggesting that a strategy for survival under oxidative stress may be triggered by HC in buds of peach trees.

Glucose 6-phosphate dehydrogenase is the first rate-limiting enzyme of the PPP, and its expression has been reported to be related to the defense against oxidative stress (Boada et al., 2000; Couée et al., 2006; Salvemini et al., 1999). In grapevine, an increase in *G6PD* transcripts was observed at 1, 2, and 4 d after HC treatment (Pérez et al., 2009). It is of interest that *G6PD* and the DEG encoding a transketolase were upregulated in the buds of HC-treated peach trees at 3 DAT, but not at 7

Fig. 3. Overview of glycolysis (highlighted in orange), tricarboxylic acid cycle (highlighted in yellow), and pentose phosphate pathway (highlighted in green) in which enzymes were encoded by the differentially expressed genes (DEGs), with red and blue indicating upregulated and downregulated DEGs, respectively, in buds of 'TropicBeauty' peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated control trees at 3 d after treatment (DAT) (A) and 7 DAT (B). A list of these enzymes, DEGs, putative *Arabidopsis thaliana* orthologs, and log₂ fold change (LFC) at 3 and 7 DAT (C) is provided.

Table 4. Members of WRKY transcription factor family encoded by differentially expressed genes (DEGs) with log₂ fold changes (LFC) in buds of 'TropicBeauty' peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated trees at 3 and 7 d after treatment (DAT).

WRKY member	DEG	<i>Arabidopsis thaliana</i> ortholog	LFC at 3 DAT	LFC at 7 DAT
WRKY6	Prupe.4G017600	AT1G62300	1.3	—
WRKY7	Prupe.1G431100	AT4G24240	—	1.7
WRKY12	Prupe.2G133800	AT2G44745	-2.0	—
WRKY22	Prupe.2G177400	AT4G01250	—	-1.7
WRKY28	Prupe.3G174300	AT4G18170	2.7	—
WRKY40	Prupe.3G098100	AT1G80840	1.9	—
WRKY57	Prupe.1G283500	AT1G69310	—	2.5
WRKY75	Prupe.3G308200	AT5G13080	4.2	—

DAT (Fig. 3), indicating a temporary induction of the PPP, which is a response to oxidative stress documented in *Arabidopsis* (Baxter et al., 2007). Cytosolic PPP generates NADPH, which has a pivotal role in removing ROS in the scavenging systems (Beauvieux et al., 2018; May et al., 1998; Mittler, 2002; Pandolfi et al., 1995). Therefore, for buds of peach trees treated with HC in this study, it is likely that, after the induction of the PPP, the reducing power was enhanced in the form of NADPH to surmount oxidative stress.

At 3 DAT, 26 DEGs encoding the antioxidants and enzymes maintaining their scavenging activities were upregulated in buds of HC-treated trees according to the RNA-seq results

(Table 3). Among them were superoxide dismutase, catalase, peroxidase superfamily protein, ascorbate peroxidase, thioredoxin, dehydroascorbate reductase, and GST, of which the transcript levels and enzymatic activity have been reported to be correlated with the release of bud dormancy in response to dormancy-breaking treatments for apricot [*Prunus armeniaca* (Scalabrelli et al., 1991; Viti et al., 2012)], grapevines (Halaly et al., 2008), Japanese apricot [*Prunus mume* (Zhuang et al., 2013)], and Japanese pear [*Pyrus pyrifolia* (Bai et al., 2013)]. As many as 16 DEGs for GST were upregulated at 3 DAT (Table 3), consistent with the results of the MapMan analysis indicating the upregulation of the metabolic process involving GST (BIN 26.9) at 3 DAT (Table 1). For grapevine, HC treatment used to break bud dormancy resulted in an increase in GST transcript levels at 2 and 4 DAT, respectively, compared with the nontreated control (Halaly et al., 2008; Keilin et al., 2007), in agreement with the results presented here. Taken together, the results of the current research suggest that within 3 d after the HC application, the detoxification mechanism against ROS

Table 5. Members of expansin (EXP) transcription factor family encoded by differentially expressed genes (DEGs) with log₂ fold changes (LFC) in buds of ‘TropicBeauty’ peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated trees at 3 and 7 d after treatment (DAT).

EXP member	DEG	<i>Arabidopsis thaliana</i> ortholog	LFC at 3 DAT	LFC at 7 DAT
EXP3	Prupe.6G256500	AT2G37640	-1.1	2.3
EXPA1	Prupe.1G276700	AT1G69530	-2.1	1.9
EXPA4	Prupe.6G075100	AT2G39700	-1.3	—
EXPA8	Prupe.2G263600	AT2G40610	-2.6	2.7
EXPA8	Prupe.6G042000	AT2G40610	-3.0	—
EXPA15	Prupe.3G265800	AT2G03090	-1.7	2.0
EXPB2	Prupe.2G136500	AT1G65680	-1.4	—
EXPB2	Prupe.2G274400	AT1G65680	—	1.7
EXPB3	Prupe.3G155600	AT4G28250	-2.3	—
EXP-like B1	Prupe.5G057900	AT4G17030	4.9	-2.1
EXP-like B1	Prupe.5G047300	AT4G17030	1.6	—

triggered by exogenous HC application has already been activated in peach buds.

At 7 DAT, most of the GO terms associated with stress responses were no longer statistically significant, and DEGs for the response to oxidative stress (GO:0006979) were down-regulated in buds of HC-treated trees (group 1 in Table 2), indicating that HC-induced oxidative stress was transient and offset within 7 d following the application, consistent with previous studies of grapevine (Or et al., 2000; Pérez et al., 2009; Sudawan et al., 2016). The results presented in this report demonstrated that DEGs encoding enzymes during the last step in glycolysis and those for TCA cycle were upregulated, and that *G6PD* for PPP was not differentially expressed in buds of HC-treated trees at 7 DAT (Fig. 3), suggesting that the carbon substrate is oxidized via glycolysis and subsequently enters the TCA cycle for efficient energy production. Based on the MapMan enrichment analysis, DNA synthesis (BIN 29.4.1) and cell organization (BIN 31.1) were upregulated at 7 DAT (Table 1). Similarly, for DEGs of the GO terms for cell development and proliferation, there was a shift from downregulation at 3 DAT to upregulation at 7 DAT (group 2A in Table 2). These results provided evidence that in peach buds, growth and development did not resume after the initial inhibition by HC; instead, they were markedly enhanced within 1 week after HC application. Accordingly, it is speculated that the increased ATP production as a result of increased aerobic respiration is used to supply the metabolic and physiological processes for cell growth and tissue development in preparation for budbreak. In support of this possibility, Bonhomme et al. (2000) reported that in peach buds, the ATP concentration was very low during the dormancy phase and increased rapidly when dormancy was released following the fulfillment of the chilling requirement.

Because cell walls loosen to allow the expansion of flower buds of rose (*Rosa hybrida*) upon opening (Yamada et al., 2009), the gene sets associated with cell wall loosening were of particular interest in this study. The results of both MapMan and GO enrichment analyses demonstrated the upregulation of processes involving the degradation and modification of the cell wall at 7 DAT (Tables 1 and 2). On the same day, DEGs encoding five EXPs, which are proteins responsible for acid-induced wall extension without any enzymatic activity (Cosgrove, 2000), were also upregulated, including *EXP3*, *EXPA1*, *EXPA8*, *EXPA15*, and *EXLB1* (Table 5). Given that bud *EXP* transcript levels were positively correlated with the

degree of the opening of flower buds of four o'clock flower [*Mirabilis jalapa* (Gookin et al., 2003)], it is possible that the upregulation of bud *EXP* expression by HC at 7 DAT resulted in the swelling and burst of buds 3 weeks later (at 32 DAT) in peach. For HC-treated grapevine that had a greater budbreak rate than the nontreated control, ROS were accumulated in buds at 12 h after treatment before the upregulation of *EXP* genes (Sudawan et al., 2016). It is still unclear if ROS induce the modification of cell wall structures that results in budbreak in deciduous trees. Nevertheless, it has been reported that ROS have a role in

wall loosening of growing tissues in the roots of maize [*Zea mays* (Liszkey et al., 2004)], roots of arabidopsis (Joo et al., 2001), and seeds of cress (*Lepidium sativum*) during germination (Müller et al., 2009).

The results of this study provide evidence that, for low-chill ‘TropicBeauty’ peach, exogenous HC application causes transient oxidative stress that likely induces the release of endodormancy, in agreement with the currently hypothesized mode of HC action for other fruit crops. The results also strongly suggest that HC-triggered oxidative stress enhances cell development and bud enlargement, ultimately leading to earlier budbreak than that in nontreated peach trees. Nonetheless, the underlying mechanism of the promotion of dormancy release by oxidative stress remains to be determined. Recently, ROS have been shown to serve as signal molecules that are essential for proper cellular function and normal physiological processes, which is in contrast to their traditional association with cell damage and death in relation to stress (Mittler, 2017; Mittler et al., 2004, 2011; Reczek and Chandel, 2015). Therefore, the results of the current research suggesting the generation of ROS under oxidative stress in buds of HC-treated peach trees at 3 DAT, along with the increased ROS levels in japanese pear and grapevine buds before budbreak in response to HC application (Kuroda et al., 2002; Sudawan et al., 2016), indicate the possibility that HC advances budbreak in peach by inducing the production and accumulation of ROS that mediate the regulation of physiological events such as differentiation of the meristem and cell wall expansion. To address this, further studies of HC-treated peach trees including an assessment of the expression and activity of the enzymes involved in ROS generation and turnover, such as respiratory burst oxidase homologs, glutaredoxins thioredoxins, and peroxiredoxins (Kobayashi et al., 2007; Mittler, 2017; Ogasawara et al., 2008; Rouhier, 2010), are necessary, as is close monitoring of ROS contents in buds.

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Supplemental Fig. 1. Significant gene ontology (GO) terms of upregulated and downregulated differentially expressed genes (DEGs), indicated by red and blue, respectively, in buds of 'TropicBeauty' peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated control trees at 3 and 7 d after treatment (DAT) for the biological process (A), molecular function (B), and cellular component (C). The intensity of the color indicates the enrichment values [absolute values of the logarithm (to the base of 10) of false discovery rate-corrected *P* values based on Fisher's exact test for GO enrichment analysis] of individual GO terms.

Supplemental Fig. 2. Relative expressions of formate dehydrogenase (FDH), manganese superoxide dismutase1 (MSD1), glutathione s-transferase tau8 (GSTU8), glutathione s-transferase tau19 (GSTU19), peptide methionine sulfoxide reductase1 (PMSR1), and nadph:quinone oxidoreductase (NQR) in buds of nontreated control ‘TropicBeauty’ peach trees (solid lines with white circles) and trees treated with 1% (v/v) hydrogen cyanamide [HC (broken lines with black circles)] at 0, 1, 3, and 7 d after treatment (DAT) analyzed using real-time quantitative polymerase chain reaction (qPCR); data are means $\pm$ SE of four biological replicates; asterisks indicate significant differences based on Student’s *t* test between control and HC-treated trees on the same day; NS = no significant difference.

Supplemental Fig. 3. Relative expression of dormancy-associated MADS-box 1 (DAM1), DAM5, and DAM6, apetala1 (AP1), pistillata (PI), agamous (AG), and floral binding protein9 (FBP9) in buds of nontreated control 'TropicBeauty' peach trees (solid lines with white circles) and trees treated with 1% (v/v) hydrogen cyanamide [HC (broken lines with black circles)] at 0, 1, 3, and 7 d after treatment (DAT) analyzed using real-time quantitative polymerase chain reaction (qPCR); data are means $\pm$ SE of four biological replicates; NS = no significant difference based on Student's *t* test between control and HC-treated trees on the same day.

Supplemental Table 1. Summary of RNA sequencing read information for ‘TropicBeauty’ peach bud samples of nontreated control trees and trees treated with 1% (v/v) hydrogen cyanamide (HC) at 3 and 7 d after treatment (DAT).

Treatment	DAT	Biological replicate	Raw reads	Trimmed reads	Transcriptome alignments
Control	3	1	36276808	29884999	12009560
Control	3	2	37238081	30307565	4741934
Control	3	3	39282984	33706513	37164310
Control	3	4	38817678	33371934	45529258
HC-treated	3	1	45601653	40573824	51555728
HC-treated	3	2	37073677	32270752	21196510
HC-treated	3	3	45042543	39242321	73268566
HC-treated	3	4	33841769	29584090	19589144
Control	7	1	46945094	38456093	1883158
Control	7	2	48016978	39702896	12044624
Control	7	3	35387093	27174001	7074268
Control	7	4	36725383	31801548	31182584
HC-treated	7	1	39365521	34862495	57544194
HC-treated	7	2	45332061	39025325	12125968
HC-treated	7	3	43556959	36183179	8804520
HC-treated	7	4	38107726	33323015	11618240

Supplemental Table 2. Gene-specific primer sequences for relative gene expression analysis of ‘TropicBeauty’ peach with quantitative real-time polymerase chain reaction.

Gene	Description	Accession no.	Forward and reverse primer* sequences (5' to 3')	Amplicon size (bp)
<i>FDH</i>	Formate dehydrogenase	XM_020559076.1	AATGCTCGAGGGGCAATCAT TCGGAGCTGGTTGTGGATTC	106
<i>MSD1</i>	Manganese superoxide dismutase	XM_007218235.2	AGATCAATGCAGAAGGCGCT TGGGTCCTGATTTGCAGTGG	107
<i>GSTU8</i>	Glutathione S-transferase TAU8	XM_007208120.2	ATGAGACTTGGAAGCAGGGTTT CATGTTCTTCACTCCCCCAGC	138
<i>GSTU19</i>	Glutathione S-transferase TAU19	XM_020561747.1	AGTCCCTCATTGCGGTTTCAG GTTGTCCACACCTTCCTCCC	151
<i>PMSR1</i>	Peptide methionine sulfoxide reductase	XM_007209566.2	TCTCTGTCTTTTGGGCTCGT TTCAACTCCACTTGCTTTGCC	151
<i>NQR</i>	NADPH:quinone oxidoreductase	XM_007218384.2	CGGTCGTTGAAGCATTTTCGT GGCAGCCTTATCACCCAAA	149
<i>DAM1</i>	Dormancy-associated MADS-box gene	XM_020563603.1	GCGCACATAAATGGTGCTGA GCGGCTCTTCTCCTCAAGTT	105
<i>DAM5</i>	Dormancy-associated MADS-box gene	XM_007223804.2	TGTGTGAATCTGAGGTGGCA TCGACACCATTATGTCTGCG	118
<i>DAM6</i>	Dormancy-associated MADS-box gene	XM_007227500.2	GATGACTGCTCCGATGTCCT CACTACTGATGCGTCTCACA	139
<i>AP1</i>	Class A floral organ identity gene <i>APETALA1</i>	XM_007223759.2	AGAAGATCAAGGAGAAGGAGAAG TTAGACATGGAAGCGGCTGT	102
<i>PI</i>	Class B floral organ identity gene <i>PISTILLATA</i>	XM_020554792.1	GAGCACTGGAAGAGGAGCAT GGCTGAACACGAAAGGCAAAA	151
<i>AG</i>	Class C floral organ identity gene <i>AGAMOUS</i>	XM_007211863.2	TTCACGCCAAGATCCGATGG CTTGACAAAGTGCCCGTAGC	130
<i>FBP9</i>	Class E floral organ identity gene <i>FLORAL BINDING PROTEIN9</i>	XM_007209443.2	TCCAGCCGTGGCAAACCTTTA ACTGGTCTGTTGGCTTCCAG	107
<i>TEF2</i>	Translation elongation factor 2	XM_007214747.2	TGTAGTCAGCATCCTGTGCC ACCAGCAATGGCAGAAATGG	108
<i>UBQ</i>	Ubiquitin	XM_020559001.1	ACTACTGTGGTAAGTGCGGC AACCAACTGTTACAGACCCT	119

Supplemental Table 3. Numbers of differentially expressed genes (DEGs) encoding the members of transcription factor (TF) families in buds of ‘TropicBeauty’ peach trees treated with 1% (v/v) hydrogen cyanamide compared with nontreated control trees at 3 and 7 d after treatment (DAT).

TF family	Upregulated DEGs at 3 DAT (no.)	Downregulated DEGs at 3 DAT (no.)	Upregulated DEGs at 7 DAT (no.)	Downregulated DEGs at 7 DAT (no.)
AP2	0	0	1	0
ARF	0	0	2	1
bHLH	1	8	2	3
C2H2	0	4	0	0
ERF	1	9	3	3
GATA	0	1	0	1
GRF	0	2	0	0
HSF	1	1	0	1
LBD	1	3	0	0
MIKC_MADS	0	3	1	2
MYB	4	6	3	2
MYB-related	1	3	0	5
NAC	3	1	2	3
WRKY	4	1	2	1
YABBY	0	1	2	0