Effects of Fluridone Treatment on Seed Germination and Dormancy-associated Gene Expression in an Ornamental Peach (*Prunus persica* (L.) Batsch)

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Fluridone, an inhibitor of carotenoid biosynthesis, is known to prevent abscisic acid (ABA) biosynthesis and to affect the germination and dormancy of seeds in several plant species. This study investigated the effects of fluridone on seed germination of the ornamental peach ‘Yaguchi’, and on the transcript levels of genes related to seed dormancy in this plant. Seeds were rinsed for 2 days under running tap water (RS), then soaked for 1 day in 100 μM fluridone (F), and kept at 5°C for 2 weeks (2W). The germination rate significantly increased from 0% to 9.5% in the RS+F treatment and to 71.4% in the RS+F+2W treatment, while no germination occurred in the RS and RS+2W treatments. Seedlings in RS+F and RS+F+2W treatments formed dwarf shoots, i.e. about 10 cm, and morphological lesions on the leaves. The ABA content in embryonic axes decreased after RS and increased with RS+2W. It was decreased by the RS+F+2W treatment. RS+F+2W downregulated *ABA-hy3*, which encodes enzymes with key roles in ABA catabolism, while its effects on 9-cis-epoxy-carotenoid dioxygenase (*NCED1*) in relation to ABA synthesis fluctuated. In contrast, *Empfindlicherim Dunkelroten Licht 1* (*EID1*) was upregulated after fluridone treatment, suggesting that fluridone may activate positive ABA signaling pathways. Expression of *GA2-oxidase8* (*GA2-ox8*) was not affected by fluridone. *MADS-box protein JOINTLESS* (*LeMADS*) and *Late embryogenesis abundant protein D-34* (*LEA D-34*) were downregulated in the RS+F+2W treatment, suggesting that the expression of these genes are controlled by low temperature and the ABA inhibitor, and are involved in seed dormancy. These results suggest that ABA inhibitor treatments can be an alternative method to promote germination by controlling ABA content and its metabolism, and consequently changing expression of certain ABA- and dormancy-related genes including *ABA-hy3*, *EID1*, *LeMADS*, and *LEA D-34*, even under insufficient chilling conditions.

**Key Words:** ABA metabolism, *EID1*, insufficient chilling, *LEA D-34*, *LeMADS*.
plants whose growth is arrested until environmental conditions are optimal for survival. In general, dry seeds start to germinate with the uptake of water by imbibition, followed by embryo expansion. Seeds of most Prunus species require a period of chilling to break seed dormancy and allow normal growth (Chang and Werner, 1983; Leida et al., 2012; Suszka et al., 1996). In peaches, when the cold treatment is insufficient, seedlings show physiological dwarfing which is considered a special case of embryo dormancy (Bewley and Black, 1982).

Phytohormone abscisic acid (ABA) plays an important role in many physiological processes in plants, including the regulation of seed dormancy. In our previous study (Pawasut et al., 2010), seeds rinsed in running tap water for 2 days and then chilled at 5°C for more than 8 weeks showed decreased ABA contents in the embryonic axis and seed coat and an increased germination rate to 100%. The ‘Yaguchi’ peach seeds treated in this way showed uniform germination and normal growth.

Our previous transcriptomic study, based on RNA-seq analysis, revealed a set of differentially expressed genes during seed dormancy in peaches (Worarad et al., 2016). We focused on genes related to the phytohormone ABA and transcription factors by using Swissprot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene ontology (GO) data. Among these genes were those encoding 9-cis-epoxycarotenoid dioxygenase (NCED), which is the key enzyme in ABA biosynthesis in higher plants (Tan et al., 1997), and CYP707A (ABA 8'-hydroxylase), which inactivates ABA by catalyzing hydroxylation at the 8' position (Cutler, 2007; Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005; Nambara et al., 2010). Other important ABA-related genes include Empfindlicherim Dunkelroten Licht 1 (EID1), which encodes an F-box protein that positively regulates the ABA-dependent signaling cascades controlling seed germination, root growth, greening of etiolated seedlings, and transition to flowering in Arabidopsis thaliana (Büche et al., 2000; Dieterle et al., 2001; Marrocco et al., 2006; Zhou et al., 2002). Negative regulators of ABA signaling include protein phosphatases type-2C (PP2C) like ABI1 and ABI2 in Arabidopsis and FsPP2C1 in Fagus sylvatica (González-Garcia et al., 2003; Merlot et al., 2001).

Several gibberellin (GA) catabolic genes including GA2-oxidase (GA2-ox) are important for controlling GA levels (Reid et al., 1992; Ross et al., 1995; Sakamoto et al., 2001; Thomas et al., 1999). GA3 promoted germination in Prunus campanulata (Chen et al., 2007) and American ginseng seeds (Qi et al., 2015).

MADS-box transcription factors are involved in regulating bud dormancy in peaches and other species (Horvath et al., 2008, 2010; Li et al., 2009; Sasaki et al., 2011). In the Japanese pear, PpMADS13-1 and -2 in the leaf bud were shown to be upregulated during endodormancy establishment and downregulated during endodormancy release (Nishitani et al., 2012; Saito et al., 2013). In a previous study, LeMADS were also significantly repressed during chilling treatment of peach embryos (Worarad et al., 2016). Similarly, the expressions of DAM1 and DAM6 that are in the DORMANCY ASSOCIATED MADS-box (DAM) group were decreased significantly by chilling treatment in peach seeds (Leida et al., 2012), suggesting that MADS box genes are associated with release of seed dormancy by stratification in mechanisms of transcriptional regulation. Furthermore, most of the genes encoding LEA proteins have been shown to contain ABA response (ABRE) and/or low temperature response (LTRE) elements in their promoters, and many of these genes are induced by ABA, cold, or drought stress (Hundertmark and Hincha, 2008).

The herbicide fluridone [1-methyl-3-phenyl-5-(3-(trifluoromethyl)phenyl)-4-(1H)-pyridinone] blocks phytoene desaturase, which catalyzes the desaturation of phytoene to phytofluene in the carotenoid biosynthesis pathway (Bartels and Watson, 1978; Sandmann and Böger, 1997). It also inhibits ABA biosynthesis in plants (Popova and Riddle, 1996; Stewart and Voetberg, 1987; Xu and Bewley, 1995; Yoshioka et al., 1998), and it has been shown to break seed dormancy in several plant species (Ali-Rachedi et al., 2004; Chen et al., 2007; Grappin et al., 2000; Kusumoto et al., 2006). For example, in Prunus campanulata, 50 μM fluridone increased the germination rate from 21% to 94.7% for true seeds (without an endocarp) (Chen et al., 2007). However, there are few reports on the effects of fluridone on peaches.

The aim of this study was to investigate the effects of fluridone on the germination, seedling development, ABA content, and expression of key seed dormancy-related genes in embryonic axes of the peach ‘Yaguchi’ under deficient chilling conditions.

**Materials and Methods**

**Plant materials**

Fully ripened ‘Yaguchi’ peaches were collected from the peach flower garden of Koga Park in Koga city (36°10’N, 139°42’E), Ibaraki Prefecture, Japan, in mid-October 2015. After the skin and flesh were removed, endocarps were carefully cracked in a vice to remove the seeds. Seeds were rinsed for 2 days under running tap water (RS) and soaked for 24 h in 100 μM fluridone (F) in a flask. Seeds were transferred into Petri dishes containing two-layers of filter paper moistened with sterile water. The plastic Petri dishes were sealed with parafilm and kept in a refrigerator at 5°C for 2 weeks (2W) (Zigas and Coombe, 1977). Three biological replicates of 10 embryonic axes of dry seeds before rinsing (BR), 2 days rinsing (RS), 2 days rinsing combined with fluridone (RS+F), 2 days rinsing combined with chilling 2 weeks (RS+2W), and 2 days rinsing com-
bined with 100 μM fluridone and chilling 2 weeks (RS +F+2W) were immediately frozen in liquid nitrogen and stored at −80°C until use. The germination percentage, lesions, and height of seedlings were noted. Total RNA was extracted from the embryonic axes of seeds before and after the treatments.

**Seed germination and seedling development**

The seeds were transferred from the refrigerator and rinsed three-times with sterile water before being transferred into plastic boxes containing a 1:1 (v/v) mix of vermiculite: peat moss. The boxes were maintained at 23°C under cool white fluorescent lights with a 12-h light/12-h dark photoperiod. Seed germination percentages and seedling height were recorded once per week. After 30 days, the percentage of leaves at node 3 and above on the epicotyledonary stem showing symptoms was calculated. The severity of lesions was assessed against a scale of morphological lesions (Zigas and Coombe, 1977).

**Quantification of 2-cis,4-trans-abscisic acid (ABA) by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).**

For quantitative measurement of endogenous plant hormones in crude plant extracts, LC–MS/MS provides high sensitivity, specificity, accuracy, and reproducibility (Pan et al., 2010). ABA was extracted from embryonic axes and detected by an LC–MS/MS system. Embryonic axes samples (≥1 mg) were transferred to microfuge tubes and stored at −80°C until use. To extract ABA, ≥1 mg of five replications of embryonic axes collected at designated times were transferred into a 3-mL vial containing 0.5 mL of 80% aqueous acetone solution of [13C7] ABA (1 ng·mL⁻¹). The vials were sealed and sonicated in an ultrasonic cleaner below 5°C for 20 min. These vials were stored in a dark cold room at 4°C for at least 10 days. The resulting solutions were filtered (Disk filter; Merck, Tokyo, Japan) and aliquots of 5 μL were injected into an ultra-performance liquid chromatography (UPLC)-connected tandem mass spectrometer. UPLC separation was performed using a Nexera X2 instrument (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved on a C18 column (Kinetex F5, φ 2.1 × 150 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). Elution of the samples was carried out using acetonitrile (solvent A) and water (solvent B), both of which contained 0.1% (v/v) acetic acid with the following gradient (v/v): initially 20% A, rising to 90% A at 9 min, followed by a 0.5 min gradient to 100% A. Finally, the column was equilibrated for 3 min using this solvent composition. The column was operated at 30°C with a flow rate of 0.2 mL·min⁻¹. Mass spectrometry was performed using a triple quadrupole/linear ion trap instrument (LIT) (QTRAP 5500; AB Sciex, Framingham, MA, USA) with an electrospray ionization (ESI) source system. For the analysis of ABA, MS/MS spectra were recorded in product ion scan mode using LIT. The ion source was maintained at 400°C with curtain gas at 20, collisional activated dissociation (CAD) gas at 7 psi (12 psi for LIT), ion source gas at 80 psi, and ion source gas2 at 70 psi. The ionspray voltage was set at −45000 V in negative mode. Declustering, entrance, and collision cell exit potentials were maintained at −15 V. For the quantification of ABA, multiple reactions monitoring (MRM) was employed with monitoring transitions of m/z 263 to 153 for ABA and 265 to 153 for the internal standard, [13C7] ABA. The quantification limit for ABA was approximately 0.01 pg. Data acquisition and analyses were performed with Analyst and Multi Quant software (ver. 3.01). All the quantifications were performed with triplicate samples of each treatment.

**RNA isolation**

Frozen samples (10 mg/sample) of dry seeds and those in the RS, RS+F, and RS+F+2W treatments were ground in liquid nitrogen to a fine powder. Total RNA was extracted from these tissues as described by Chang et al. (1993). Ten embryonic axes were extracted with three replications. The integrity of the extracted RNA was evaluated by electrophoresis on a 1.0% agarose gel and staining with ethidium bromide. Then, total RNA was quantified and examined for protein contamination. Total RNA was isolated from the embryonic axes samples (≥1 mg) of five replications of embryonic axes and detected by an LC–MS/MS system. Total RNA was isolated from embryonic axes and cDNAs were generated from the same RNA samples. For each sample, 1 μg total RNA was used with the QuantiTect® SYBR® Green RT-PCR Kit, according to the manufacturer’s protocol (Agilent, Santa Clara, CA, USA). The cDNA was stored at −80°C until use in the qRT-PCR analysis. The qRT-PCR assay was performed with three technical replicates using the Fast SYBR Green Master Mix (Life Technologies, Carlsbad, CA, USA) on a Lightcycler 96 Real Time PCR system in a total reaction volume of 15 μL. The PCR cycle comprised one 600 s cycle at 95°C, followed by 45 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All amplified products were subjected to a melt curve analysis. A negative control without a cDNA template was run with all analyses to evaluate the overall specificity. The reference gene ubiquitin was used to normalize the total amount of cDNA in each reaction (Yamane et al., 2011b). Amplification efficiency and relative gene expression levels were calculated using the ΔΔCT and 2⁻ΔΔCT methods (Ct; cycle threshold). The ΔCt value of each gene was calculated by subtracting the Ct value of the endogenous control from the Ct value of the target gene. Gene-specific primers were designed using primer-BLAST (Ye et al., 2012) (Table 1).
Statistical analysis

The significance of differences in germination, plant height, and gene expression among treatments was tested by one-way analysis of variance using the software package (StatView Ver.5.0; SAS Institute Inc. Cary, NC, USA). Percentage data were arc-sin transformed. Multiple range tests were performed using Fisher’s least significant difference method with a confidence level of 95%.

Results and Discussion

Effects of fluridone and chilling on germination

Fluridone significantly increased the germination rate of ‘Yaguchi’ seeds from 0% to 9.5% by RS+F and 71.4% by RS+F+2W, respectively (Fig. 1A, 2). Rinsing only (RS) or chilling treatment for 2 weeks (RS+2W) was insufficient for germination and shoot emergence in peaches (Pawasut et al., 2010), while a synergetic effect of F and 2W was found. Fluridone induced the seed germination of Arabidopsis thaliana, Nicotiana plumbaginifolia, and Striga asiatica (Ali-Rachedi et al., 2004; Grappin et al., 2000; Kusumoto et al., 2006). In contrast, the germination rate of Prunus campanulata, native to Taiwan, was more effectively increased from 21.3% to 94.7% after treated with 50 μM fluridone without chilling (Chen et al., 2007), probably due to its lower chilling requirement.

All seedlings in both of the fluridone treatments showed morphological lesions on all of the leaves, with grade 2 lesions the most abundant (Fig. 1D). In the previous research, stem length of seedling from seeds with chilled for 6–8 weeks were 15–18 cm (Worarad et al., 2016), while RS+F and RS+F+2W promoted dwarfing; seedlings were 8–12 cm height (Fig. 1B) with 13–17 nodes (Fig. 1C). Physiological dwarves are characterized by a temperature-dependent rosette-type growth habit, with short internodes, and deformed leaves in peach (Pollock, 1962). Short chilling treatments also induce physiological dwarfing of seedlings in almonds (Hartmann et al., 2011). Zigas and Coombe

<table>
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<tr>
<th>ID</th>
<th>Genes</th>
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<tr>
<td>TR24454</td>
<td>c0_g1_i1</td>
<td>EID1-like F-box protein 3 ATGTTCTTTTGCTGCGGCTG</td>
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<tr>
<td>TR8743</td>
<td>c0_g2_i1</td>
<td>NCED1 GCCAGTACCCCAGCAAATGGA</td>
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<tr>
<td>TR1393</td>
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<td>P22CA GGTGCTCTCATGTGGCTT</td>
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<tr>
<td>TR10071</td>
<td>c0_g2_i1</td>
<td>LEA D-34 CCGAGGAGACATCTGAGG</td>
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<tr>
<td>TR13511</td>
<td>c0_g2_i1</td>
<td>ABA 8′-hydroxylase 3 GGTGTGTCACAAAATGCGA</td>
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<tr>
<td>TR9163</td>
<td>c0_g2_i1</td>
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</tr>
<tr>
<td>TR15408</td>
<td>c4_g2_i5</td>
<td>LeMADS GCTGACGTGGTAGAAGCCAA</td>
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Ubiquitin was selected as a reference and its primers were designed according to Yamane et al. (2011b).

Fig. 1. Germination rate (A), seedling height (B), node number (C), and lesion severity on leaves (D) in the ‘Yaguchi’ seedlings treated with rinsing, chilling, and fluridone. RS: rinsed for 2 days, F: 100 μM fluridone, 2W: chilled for 2 weeks. Vertical bar indicates SE (n = 7). NG: No germination occurred. Different letters indicate significant differences at $P < 0.05$. Z
(1977) found that short stratification promoted the production of morphological lesions on peach leaves. An in vitro culture experiment on peach seeds showed that seedlings grew with a dwarf phenotype when the chilling time was insufficient (Leida et al., 2012). These results suggest that chilling seeds at 5°C for 2 weeks was not enough to avoid dwarfism and lesions in this study.

On the other hand, fluridone, a type of herbicide, inhibited all carotenoids and carotenoid-derived metabolites and could cause plants lethal damage because carotenoids play an important role in protecting photosynthetic organisms against damage by photooxidation and absorb light energy in plants (Kitahata et al., 2006). Therefore, fluridone could influence lesion syndrome even in treated seeds in this study. Fluridone can break seed dormancy, but could not complement the lack of chilling to alleviate other disorders.

Effects of fluridone and chilling on ABA accumulation

The ABA content in peach embryonic axes significantly decreased from 0.028 ng·mg⁻¹ (BR) to less than 0.01 ng·mg⁻¹ rinsing (after RS), then increased to 0.025 ng·mg⁻¹ after 2 week’s chilling (RS+2W; Fig. 4). The ABA content was significantly decreased by the interaction of fluridone and a 2-week chilling treatment (RS+F+2W) compared with the control (RS+2W; Fig. 4); this correlated with the germination by RS+F+2W (Fig. 1A).

In many plants, carotenoid biosynthesis inhibitors are hypothesized to affect seed germination by reducing the ABA concentration (Yoshioka et al., 1998). For example, the caryopses and embryos of fluridone-treated wheat accumulated a lower ABA content (Russell et al., 1997). Zhang et al. (2009) found that peach and grape fruits treated with fluridone showed a clear decrease in endogenous ABA content. Seeds rinsed for 2 days and chilled at 5°C for more than 8 weeks showed low ABA contents in the embryonic axis and seed coat (Pawasut et al., 2010). In this study, the interaction between fluridone and 2 weeks of chilling significantly decreased the ABA contents and shortened the stratification period necessary to break dormancy and trigger germination.

However, no germination occurred in RS (Fig. 1A), even though the ABA content was as low as that in RS+F+2W (Fig. 4). The seeds in RS contained low levels of ABA, suggesting that ABA in the embryonic axes was leached away by rinsing. ABA could increase again after sowing by de novo ABA synthesis to a level inhibiting germination, which is inferred by the increment of ABA in RS+F (i.e., after 1 day) and RS+2W. On the other hand, ABA synthesis after RS+F may have been inhibited, leading to a slight increase in germination (Fig. 1A). Chen et al. (2007) reported that combined treatments of 50 μM ABA and 50 μM fluridone delayed the germination, but finally promoted it to 95.4% without chilling in Prunus campanulata, suggesting that the influence of the initial ABA content is
limited temporarily because of its catabolism, while that of ABA synthesis is more critical for the final germination rate.

Moreover, seed coat properties could affect the differences in germination rate between RS+F and RS+F+2W. The seed coat is known to inhibit germination both physically and chemically (Debeaujon et al., 2000; Martínez-Gómez and Dicenta, 2001; Pawasut et al., 2010). Moist chilling for 2 weeks could alter mechanical strength and the ABA content in the seed coats, resulting in higher germination in RS+F+2W.

Effects of fluridone and chilling on expression of genes for ABA synthesis and catabolism in seed embryonic axes

In higher plants, NCED1 and ABA 8’-hydroxylase 3 encode key enzymes in ABA biosynthesis and catabolism, respectively (Cutler, 2007; Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005; Nambara et al., 2010; Tan et al., 1997). In the peach and the grape, PpNCED1 and VvNCED1 were shown to be highly expressed at the beginning of ripening when the ABA content was high (Zhang et al., 2009). In this study, the NCED1 gene expression was lower in RS (Fig. 5A) similar to ABA levels (Fig. 4), but this treatment could not induce germination (Fig. 1A). The transcript levels of NCED1 were highest in RS+F and there were no significant differences between RS+2W and RS+F+2W (Fig. 5A). These results indicated that expression of NCED1 did not correspond to the germination or ABA content.

Fluridone blocks phytoene desaturase, which catalyzes the desaturation of phytoene to phytofluene in the carotenoid biosynthesis pathway (Bartels and Watson, 1978; Sandmann and Böger, 1997). The inhibition of phytofluene by fluridone directly leads to a shortage of xanthophylls such as violaxanthin and neoxanthin, which are the substrates of NCED1 (Ren et al., 2007). NCED cleaves 9-cis xanthophylls to xanthoxin, an ABA precursor (Schwartz et al., 1997). Therefore, we assumed that the expression of NCED1 and its enzyme activity were not correlated to the ABA level because fluridone decreased the substrates of NCED1, which is the key factor that decides ABA synthesis in treated seeds.

ABA catabolic genes are induced during seed development to inactivate ABA, and are associated with dormancy release in some higher plants (Corbineau et al., 2002; Feurtado et al., 2004; Jacobsen et al., 2002). In Arabidopsis, AtCYP707A2 was associated with the rapid decline of ABA in germinating seeds (Kushiro et al., 2004) and CYP707A3 plays an important role in degrading endogenous ABA during dehydration (Umezawa et al., 2006). In the peach, PpCYP707A2 and PpCYP707A3 were shown to be significantly upregulated during dormancy release in seeds and buds (Wang et al., 2016). In this study, ABA 8’-hydroxylase 3 (CYP707A3) was highly upregulated in the RS, RS+F, and RS+F+2W treatments, and significantly downregulated in the RS+F+2W treatment (Fig. 5B). This lower expression of ABA 8’-hydroxylase 3 could not explain the lowest ABA content in RS+F+2W (Fig. 4). It is thought that the fluridone lowered the level of ABA, the substrate of ABA 8’-hydroxylase 3, for 2 weeks and consequently suppressed the expression of ABA 8’-hydroxylase 3.

Effects of fluridone and chilling on expression of ABA signaling genes in seed embryonic axes

Previous studies have shown that seed dormancy in the peach is associated with ABA signaling in the seeds (Worarad et al., 2016). ABA signaling is important for stress responses, seed development, and dormancy (Hubbard et al., 2010; Nakashima et al., 2009). The transcript level of EID1 was significantly increased by fluridone treatment (Fig. 5C). EID1 is an F-box protein that negatively regulates phytochrome A-specific light signaling and positively regulates the ABA-dependent signaling cascades that control seed germination, root growth, greening of etiolated seedlings, and transition to flowering in Arabidopsis thaliana (Koops et al., 2011; Marrocco et al., 2006). In our previous study (Worarad et al., 2016), longer rinsing (7 days) of seeds before chilling treatment decreased the transcript levels of EID1. However, the EID1 transcript levels were unexpectedly increased by fluridone treatments in this study (Fig. 5C), suggesting that EID1 may be still active at the transcriptional level. This may contribute to maintaining the ABA signaling pathway for growth and development under low ABA conditions. However, the functions of EID1 genes are not clear in the seed dormancy mechanism and further study is needed to clarify their roles.

Negative regulators of ABA signaling include PP2C in Arabidopsis and FsPP2C1 in Fagus sylvatica (González-García et al., 2003; Merlot et al., 2001). In buds of Japanese pears, 21 differentially expressed genes were annotated as probable PP2Cs and were upregulated during endodormancy (Takemura et al., 2015). In this study, however, PP2C was upregulated by fluridone without chilling and maintained after two week’s chilling (Fig. 5D), suggesting that the ABA signaling pathway was still active after fluridone and chilling treatment. It is thought that ABA signaling is important for stress responses and seedling development.

Effects of fluridone and chilling on GA2-oxidase transcript levels in seed embryonic axes

In plants, ABA and GAs share the initial steps of the terpenoid pathway for their biosynthesis (Crozier et al., 2000). Therefore, the inhibition of ABA biosynthesis by fluridone may not only decrease the ABA content, but also increase the GA content, thereby resulting in
germination (Chen et al., 2007). However, in this study, the transcript levels of the gene encoding GA2-ox 8, which inactivates GA1, were not significantly different among the treatments (Fig. 5E). We assumed that the GA inactivating genes were not directly involved in the germination process in this study.

In our preliminary experiment, GA3 peach treatment decreased the germination rate in the ‘Hokimomo’ peach (Yamane et al., 2011a) and ‘Yaguchi’ (data not shown). On the other hand, GA3 treatment was effective in breaking dormancy and promoting germination. In addition, endogenous GAs, especially GA4, increased after chilling in Prunus campanulata (Chen et al., 2007). Treatment with paclobutrazol, an inhibitor of GA synthesis, completely suppressed the germination in Prunus campanulata (Chen et al., 2007), implying that an increase in endogenous GAs is necessary for its germination. Further study is needed to clarify the relationship between GAs and germination in peach seeds.
Effects of fluridone on expression of MADS box and stress responsive genes in embryonic axes of seeds

A fluridone treatment combined with chilling for 2 weeks resulted in decreased transcript levels of LeMADS (Fig. 5F). Similarly, the transcript levels of DAM1 and DAM6 were downregulated during dormancy release in seeds of two different peach cultivars (Leida et al., 2012). Chilling accumulation has been postulated as the major input conditioning seasonal fluctuations in the expression of MADS-box genes in peach buds (Li et al., 2009). In peach buds, prolonged low temperature and the dormancy-breaking reagent cy-anamide were shown to downregulate the expression of DAM5 and DAM6, concomitant with dormancy release (Yamane et al., 2011b). These results indicated that fluridone combined with chilling lowered ABA level and thereby downregulated LeMADS, and finally contributed to breaking seed dormancy.

The transcript levels of LEA D-34, which encodes the LEA D-34 protein or dehydrin (related to drought stress), were relatively higher in the beginning and then decreased sharply in RS+F+2W (Fig. 5G). In dormant Japanese pears buds, LEA genes were shown to be expressed at significantly higher levels during the deepest endodormancy period (Takemura et al., 2015). De-hydrins are synthesized by cells in response to ABA (Chandler and Robertson, 1994) and an ABA treatment was shown to upregulate most PmLEA genes (Du et al., 2013). These results indicated that fluridone combined with chilling was highly effective to decrease the transcript levels of LEA D-34, whose gene product is required to maintain dormancy. The lower transcript levels of LEA D-34, therefore, were associated with germination.

How did fluridone and chilling treatments break dormancy and promote germination?

As described above, dry seeds started to germinate with the uptake of water by imbibition and seeds of most Prunus species require a period of low temperature to break seed dormancy and allow normal growth (Chang and Werner, 1983; Leida et al., 2012; Suszka et al., 1996). In this study, we applied fluridone and moist chilling to peach seeds and determined the germination, ABA content, and expressions of the seven ABA-related genes. Among these parameters, ABA content in embryos appears to be the most important factor in the regulation of dormancy and de novo synthesis.

Fluridone specifically blocks the supply of substrates and thus inhibits the biosynthesis of ABA. The expression of NCED1 was not correlated to ABA level because a shortage of its NCED1 substrates seemed more critical to ABA synthesis in the fluridone-treated seeds. Fluridone could suppress the expression of ABA 8'-hydroxylase 3 by lowering the substrate for 2 weeks. Nevertheless, ABA signaling-related genes, PP2C and EID1, were still active in RS+F+2W and were not correlated with ABA level and germination. The GA catabolism gene, GA2-ox 8, was not influenced by fluridone or chilling treatments. The interaction of fluridone and moist chilling sharply downregulated LeMADS and LEA-34 genes, suggesting that these genes are involved in seed dormancy and possibly regulated by the ABA level.

In conclusion, the results suggest that ABA inhibitor treatments can be an alternative method to promote germination by controlling ABA content and metabolism, and consequently alternating the transcription of ABA-related genes (NCED1, ABA 8'-hydroxylase 3, LeMADS, and LEA D-34) even under insufficient chilling conditions. Further studies will be needed to clarify the role of ABA-related genes in seed dormancy.

Literature Cited


Crozier, A., Y. Kamiya, G. Bishop and T. Yokota. 2000. Biosyn-


