Proline Accumulates in Response to Higher Temperatures during Dehardening in Peach Shoot Tissues

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Changes in cold hardiness, proline (Pro) content, and related gene expression were confirmed in the shoots of two peach cultivars (*Prunus persica* ‘Janghowon Hwangdo’ and ‘Odoroki’) during natural cold hardening (CH) and dehardening (DH), and 10 supplemental peach cultivars during experimental DH. Specifically, transcript accumulations of *P5CS* (Δ¹-pyrroline-5-carboxylate synthase) and *P5CR* (Δ¹-pyrroline-5-carboxylate reductase) were examined using quantitative real-time RT-PCR. The cold hardiness of the shoots of the two cultivars differed significantly during the entire experimental period. The cold hardiness of both cultivars increased gradually until December 2012, and then decreased until April 2013, whereas their Pro contents decreased from the beginning of the experiment until February 2013 and then increased in the spring. While the cold hardiness of the 10 peach cultivars decreased, their Pro contents increased during DH. Interestingly, the expression of the *P5CS* gene encoding an enzyme that catalyzes the conversion from glutamate (Glu) to glutamic-γ-semialdehyde (GSA) in the first step of the Pro pathway showed patterns contrasting with the Pro contents in both experiments. In contrast, the expression of the *P5CR* gene encoding an enzyme that catalyzes the conversion from Δ¹-pyrroline-5-carboxylate (P5C) to Pro in the final step of the Pro pathway showed patterns similar to the Pro contents in both experiments. Our results demonstrate that Pro accumulation responds positively to higher temperatures in the shoots of different peach cultivars, including the 10 supplemental peach cultivars, and the expression of both *P5CS* and *P5CR* genes showed contrasting patterns. Furthermore, notably, during the experimental DH, the expression of *OAT* (ornithine-δ-aminotransferase) increased. Our results suggest that the ornithine pathway could serve as an alternative pathway in the Pro synthesis process during DH in peach.

Key Words: *OAT*, ornithine pathway, *P5CR*, *P5CS*, *Prunus persica*.

Introduction

Freezing temperature is a primary environmental stress, resulting in economic damage and limiting the distribution of horticultural crops (Parker, 1963). Freezing injury in sensitive species occurs when plants cannot prevent the nucleation and growth of ice in extracellular spaces, which results in cellular dehydration, a primary cause of freezing injury (Pearce, 2001).
The peach *Prunus persica* possesses insufficient tolerance to freezing temperatures compared with the other *Prunus* species (Flore, 1994). Freezing injury in peaches has increased with the recent abnormal climate. In our previous studies, peach shoot tissues responded sensitively to seasonal or abrupt changes of temperatures, accompanying changes of soluble sugars, dehydrins and related gene expression depending on cultivar-specific differences in cold hardness (Shin et al., 2015a, b, c).

Proline (Pro), one of the compatible solutes, has been widely reported to accumulate in response to abiotic stresses, such as water, salt, drought, high temperature, low temperature, and UV irradiation (Handa et al., 1986; Hare and Cress, 1997; Lee et al., 2012; Liu and Zhu, 1997; Saradhi et al., 1995). Pro plays important roles as a mediator of osmotic adjustment, chaperones in the cell, and a major component of cell wall structural proteins (Handa et al., 1986; Nanjo et al., 1999). In plants, Pro is synthesized mainly via the glutamate (Glu) pathway (Kishor et al., 1995; Nanjo et al., 1999; Szabados and Savouré, 2009; Yoshiba et al., 1997). Pro biosynthesis is related to two enzymes, P5C synthase (P5CS) and P5C reductase (P5CR), which catalyze conversions from Glu into glutamic-γ-semialdehyde (GSA) and from P5C into Pro, respectively (Nakashima et al., 1998). Pro can alternatively be synthesized from ornithine, which is transaminated by ornithine-δ-amino transferase (OAT) producing GSA and P5C (Delauney et al., 1993; Roosens et al., 1998). Yoshiba et al. (1997) reported the relationship between the expression of P5CS and P5CR genes and the accumulation of Pro under dehydration stress in mothbean and *Arabidopsis thaliana*. Whereas Pro accumulation is known to confer tolerance in response to salt or temperature stress in *Oryza sativa*, barley, and radish (Bagdi and Shaw, 2013; Chu et al., 1974), some studies reported that Pro accumulation did not necessarily lead to higher temperatures during natural and experimental DH in the shoots of different peach cultivars, and that the expression of both P5CS and P5CR genes can show contrasting patterns. Thus, our results suggest that identification of the expression of both P5CS and P5CR genes is required for accurate analysis of Pro biosynthesis because Pro accumulation can be affected more by the expression of the P5CR gene. Furthermore, our results suggest that the ornithine pathway could serve as an alternative pathway for the production of GSA or P5C instead of P5CS in the Pro synthesis process during DH in peach shoot tissues.

### Materials and Methods

#### Plant materials

Current-year shoots from seven- to eight-year-old deciduous peach trees of two cultivars (the relatively cold-tolerant ‘Janghowon Hwangdo’ and the relatively cold-susceptible ‘Odoroki’), grafted on wild peach seedlings, were collected near the end of each month from November 30, 2012, to April 24, 2013, at Cheongdo Peach Experiment Station, Cheongdo, Korea (latitude 35°38'50.3"N, longitude 128°39'03.3"E), for natural CH and DH. The samples were randomly collected from 15 trees of each cultivar.

As a supplemental experiment to reconfirm rapid changes of Pro content and gene expression in response to higher temperatures, one-year-old peach trees of 10 cultivars (‘Aikawanakajima’, ‘Chiyomaru’, ‘Daewol’, ‘Janghowon Hwangdo’, ‘Kiraranokiwami’, ‘Mihong’, ‘Misschong’, ‘Soomee’, ‘Suhong’, and ‘Sun Gold’—we could not purchase ‘Odoroki’ because peach nurseries prefer not to grow it due to its poor cold hardiness), commercially grafted on wild peach seedlings, were used for an experimental DH treatment. Sixteen plants of each cultivar, which had been cultivated in natural field conditions, were planted in 2-L pots containing sandy loam soil on February 2, 2014. On February 6, all trees were moved into a storeroom (kept in a storeroom at 4°C with 35 μmol·m⁻²·s⁻¹ fluorescent light for 10.5 h·day⁻¹). After 4 days, eight cold-hardened plants of each cultivar were used for estimation of the starting point of cold hardness and respective experiments. The remaining plants of each cultivar underwent the experimental DH in a greenhouse [kept under a 10.5-h photoperiod at 105 μmol·m⁻²·s⁻¹ and 23.2/18.5°C (day/night)] for 14 days. After that, the upper shoots were collected from eight trees of each cultivar.

The collected samples from both treatments were packed on ice, brought to the laboratory, and appropriately processed for Pro analysis and relative gene expression analysis.

#### Determination of cold hardness

Cold hardness was determined by the relative electrolyte leakage (REL) method described by Arora et al. (1992) and Shin et al. (2015b), with slight modification. Twenty-seven 5-cm-long shoot segments of similar diameter from 15 trees of each cultivar were randomly collected from the upper part of the current-year shoots.
The shoot segments were rinsed under cold running tap water for 15 s and then placed in 50-mL conical tubes containing 1 mL of distilled water to initiate ice formation. The tubes were incubated in a refrigerated circulating bath (RW-2040G; Jeio Tech, Daejeon, Korea) equipped with a temperature controller and were cooled by 5°C·h⁻¹ from 5°C to −40°C until 10 target temperatures at −5°C intervals were reached. The target temperatures were maintained for 2 h, after which the tubes were removed from the bath at 0°C, −10°C, −20°C, −30°C, and −40°C and thawed at 4°C. After the freezing treatment, shoots were cut into 1-cm-long sections and placed in 15-mL conical tubes containing 8 mL of distilled water. The tubes were shaken at 125 rpm on a shaker (Green Sseriker VS-202D; Vision Scientific Co. Ltd., Daejeon, Korea) at room temperature for 20 h and the electrical conductivity (EC) of the aliquots was then measured using an EC meter (TetraCon 325; WTW GmbH, Weilheim, Germany). After samples had been autoclaved at 120°C for 30 min, EC was measured again and percent injury was then calculated according to the method of Pagter et al. (2008).

Pro analysis

Pro content was determined using the ninhydrin method with slight modification (Patton et al., 2007). Seven 5 cm-long shoot segments collected from each cultivar were immediately frozen in liquid N₂, ground using a mixer mill with grinding jars and balls (TissueLyser II; Qiagen, Dusseldorf, Germany) for 1 min, and stored as powder at −80°C until use. The ground powder (50 mg) was put in a 1.5-mL sample tube containing 1 mL of 3% sulfosalicylic acid, vortexed for 15 s, incubated at 80°C for 15 min, and then shaken for 15 min on a shaker (Green Sseriker VS-202D; Vision Scientific Co. Ltd.). The supernatants were collected by centrifugation at 16000 g and room temperature for 10 min and diluted with 900 μL of distilled water. One mL of each of acid ninhydrin and glacial acetic acid were added to 1 mL of diluted supernatants in a 15-mL conical tube. The mixtures were heated at 100°C for 1 h in a boiling water bath. After cooling to room temperature, 4 mL of toluene was added and vortexed for 20 s. After 5 min, the absorbance at 520 nm was measured using a spectrophotometer (UV-1650PC; Shimadzu Co., Kyoto, Japan).

RNA extraction and relative gene expression analysis

Seven shoot segments of 5 cm in length from each cultivar were immediately frozen in liquid N₂, ground using the mixer mill with the grinding jars and balls (Qiagen) for 1 min, and stored at −80°C until use. Total RNA was extracted according to the protocol described by Chang et al. (1993) and Gasic et al. (2004). Finely ground samples were placed into 2.0-mL sample tubes. Then, 900 μL of RNA extraction buffer [2% cetyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP, K-30), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 0.05% spermidine, 2% β-mercaptoethanol], and an equal volume of chloroform:isoamylalcohol (24:1) were added and vortexed. The mixtures were inverted thoroughly every 3 min while they were incubated in a water bath at 65°C for 30 min. Then, the sample tubes were centrifuged at 13000 g and room temperature for 20 min. The supernatants were moved to new 2.0-mL sample tubes, 900 μL of chloroform:isoamylalcohol (24:1) was added, and the mixtures were centrifuged at 13000 g and room temperature for 20 min. The supernatants were then moved to new 1.5-mL sample tubes and 225 μL of 8 M LiCl was added to them and mixed. RNA was precipitated overnight at 4°C and harvested by centrifugation at 13000 g and 4°C for 30 min. After the presence of a settled pellet had been confirmed, the supernatant was discarded. Next, after the pellet had been dissolved in 500 μL of SSTE [1 M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)], the mixture was vortexed and incubated at 37°C for 5 min. An equal volume of chloroform:isoamylalcohol (24:1) was added and the mixture was vortexed. Then, the sample tubes were centrifuged at 13000 g and room temperature for 20 min and the supernatants were moved to new 1.5-mL sample tubes. Two volumes of ethanol were added to the supernatants and the sample tubes were precipitated at −80°C for at least 40 min. Then, the sample tubes were centrifuged at 13000 g and 4°C for 30 min. The supernatant was discarded after the presence of a settled pellet had been confirmed. Finally, the pellet was dried and dissolved in 30 μL of RNase-Free Water (Qiagen).

Complementary DNA (cDNA) synthesis of 1 μg of total RNA was performed using a QuantiTect® Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed with 2× QuantiSpeed SYBR Kit (PhileKorea Inc., Seoul, Korea) with a Rotor-Gene 6000 Real-Time Cycler (Corbett Research, Mortlake, Australia). PCR conditions were a pre-denaturing step at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s. Relative expression of RNA was calculated using Rotor-Gene™ 6000 Real-Time Rotary Analyzer 1.7 software (Corbett Research). Relative gene expression value was calculated in comparison with gene expression levels, expressed as 1 measured in December 2012 for both cultivars during natural CH and DH, and in ‘Daewol’ during an experimental DH. Three technical replicates were performed.

Information on the target genes, reference genes, and their primers for quantitative real-time RT-PCR is listed in Table 1. A BLAST search against the GenBank EST database enabled the nucleotide sequences of peach corresponding to the P5CS, P5CR, and OAT genes to be found. The similarity levels between Prunus mume and the peach EST were 89% for 439 nucleotides in P5CS, 85% for 206 nucleotides in P5CR and 99% for 390
nucleotides in OAT. RNA polymerase II (RP II) (Tong et al., 2009) was used as a reference gene for the natural CH and DH experiment and RP II and 26S rRNA (Bassett et al., 2009) were used as reference genes for the supplemental DH experiment.

**Statistical analysis**

The significance of differences was assessed via analysis of variance (ANOVA) with the SAS 9.1 software package (SAS Institute Inc., Cary, NC, USA).

**Results**

**Changes in air temperature and cold hardness**

Daily air temperatures at the experimental site at Cheongdo Peach Experiment Station, Cheongdo, Korea (latitude 35°38’50.3”N, longitude 128°39’03.3”E), decreased steadily from November, and reached a minimum temperature (−12.9°C) at the beginning of January (Fig. 1). The daily temperature range from February to March 2013 was greater than usual.

The REL in the shoots of the two cultivars differed significantly during the entire experimental period (Fig. 2). The maximum cold hardiness of both cultivars was reached at the end of December 2012. During DH, from the end of January to April 2013, the cold hardness of both cultivars gradually decreased. The REL of ‘Janghowon Hwangdo’, known to be relatively cold-tolerant, was lower than that of ‘Odoroki’ upon −30°C and −40°C treatments in February to March 2013. As expected, cold hardness, estimated as the temperature resulting in 50% injury (LT_{50}), in the 10 supplemental peach cultivars decreased rapidly during experimental DH—LT_{50} values of ‘Chiyomaru’ were lower than

| Gene abbreviation | Primer sequence (5′-3′) (forward/reverse) | Product size (bp) | Origin | GenBank accession No. |
|-------------------|------------------------------------------|-------------------|--------|-----------------------|
| P5CS              | F: CCA AGG GGC AGC AAT AAA CTG  
R: CTT CTG GGT CCT CGT CGA TAA | 439 | Prunus mume | XP008233492.1 |
| P5CR              | F: GCA TCC AGG TCA GCT AAA GG  
R: CCA GCG CTA TGA AAA GGA AG | 206 | Prunus mume | XP008240728.1 |
| OAT               | F: GTC AGA GAG CTG TGC TCA AA  
R: ACC TCT TCC TCG AAC TTC CT | 389 | Prunus mume | XM008227284.1 |
| RP II             | F: TGA AGC ATA CAC TGA TGA TGA AG  
R: CTT TGA CAG CAC CAG TAG ATT CC | 128 | Arabidopsis thaliana | AT2G15430 |
| 26S rRNA          | F: GCA GCC AAG CCT TCA TAG CC  
R: GTG CGA ATC AAC GGT TCC TG | | Prunus persica | |

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Fig. 1. Maximum and minimum daily air temperatures at the experimental site (latitude 35°38’50.3”N, longitude 128°39’03.3”E) from November 2012 to April 2013.

Fig. 2. Relative electrolyte leakage in shoots of ‘Janghowon Hwangdo’ (A, cold-tolerant) and ‘Odoroki’ (B, cold-susceptible) peach trees from November 2012 to April 2013. Data are presented as means ± SE (n = 3). Different letters of each cultivar indicate significant differences within the same month (P ≤ 0.05).
−40°C, but could not be precisely determined as the lowest temperature reached by the freezer was −40°C (Fig. 3). However, the temperature of LT$_{50}$ after DH in ‘Janghowon Hwangdo’ was the lowest among the 10 cultivars, indicating that ‘Janghowon Hwangdo’ was less freezing-tolerant.

**Pro analysis**

Pro content had an inverse seasonal trend in comparison with cold hardiness in the shoots of both cultivars (Fig. 4). The Pro contents of ‘Janghowon Hwangdo’ and ‘Odoroki’ declined from the beginning of the experiment to February 2013 and then increased in the spring. The Pro contents of the two cultivars ranged from approximately 11 to 25 mg·g$^{-1}$ throughout the experimental period and were not significantly different in the same month. Although the Pro contents of the 10 supplemental cultivars increased during experimental DH, there were no significant differences in ‘Chiyomaru’, ‘Daewol’, ‘Misshong’, and ‘Sun Gold’ (Fig. 5).

**Relative expression of P5CS, P5CR, and OAT**

The relative expression of P5CS in the shoots of ‘Janghowon Hwangdo’ and ‘Odoroki’ significantly increased during CH, achieving a maximum in January 2013 and decreasing sharply during DH (Fig. 6A, C). The expression of P5CR gradually decreased and reached a minimum in February 2013 (Fig. 6B, D). After that, the expression of P5CR increased in March 2013 in both cultivars. Finally, the expression of P5CR decreased temporarily in April 2013 (Fig. 6B, D). In addition, while the expression of P5CS declined during experimental DH in 10 peach cultivars, P5CR increased...
during the same time. Interestingly, $P5CS$ exhibited contrasting fluctuations in the Pro contents during natural CH and DH, and an experimental DH (Figs. 4–7). In contrast, the expression of $P5CR$ had a similar pattern to the Pro contents during natural CH and DH, and experimental DH (Figs. 4–7). Furthermore, although it was only investigated during the experimental DH, the expression of $OAT$ increased similarly to that of Pro and $P5CR$ in the 10 peach cultivars (Fig. 7). The results of gene expression differed depending on the reference genes.

**Discussion**

Changes in cold hardiness reached a maximum in midwinter in the two cultivars. Then, both cultivars started to deharden, starting at the end of January 2013 (Fig. 2). These patterns of cold hardiness have been reported in many woody plants, including the peach (Arora et al., 1992; Kuroda et al., 1990; Mattheis and Ketchie, 1990; Parker, 1962; Shin et al., 2015a, c; Siminovitch and Briggs, 1949). Notably, our results indicated that the rate of DH was later in ‘Janghowon Hwangdo’, which is known to be relatively cold-tolerant, than in ‘Odoroki’, which is known to be relatively cold-susceptible. This result indicates that ‘Janghowon Hwangdo’, which had a lower percent of REL, had higher tolerance against DH when there was a wide daily temperature range from February to March 2013 (Figs. 1 and 2). Kalberer et al. (2007) observed a similar result that less frost-hardy *Rhododendron* genotypes exhibited a pattern of low DH tolerance to warm temperatures. Pagter et al. (2011) also reported that *Hydrangea paniculata*, which is a cold-hardy species, had higher DH tolerance.

Pro is synthesized in response to environmental stresses such as drought, high salinity, and low temperatures (Hare et al., 1998). Low temperatures without the dehydration of plant tissues trigger Pro accumulation, which may play a role in establishing cold resistance in some herbaceous plants (Chu et al., 1974). However, the Pro contents of ‘Janghowon Hwangdo’ and ‘Odoroki’ decreased from the beginning of the experiment to February 2013 and then increased in the spring (Fig. 4). We performed another supplemental experiment in February 2014 to reconfirm the correlation between Pro and cold hardiness in 10 peach cultivars. Their cold hardiness decreased dramatically during DH, whereas their Pro contents increased significantly at the same time (Figs. 3 and 5). These results suggest that there is a negative correlation between cold hardiness and Pro contents in peach. In other words, Pro accumulation is positively correlated to higher temperatures during natural and experimental DH in the shoots of different peach cultivars. Szabados and Savouré (2009)

![Fig. 6. Relative gene expression of $P5CS$ and $P5CR$ in shoots of ‘Janghowon Hwangdo’ (A and B) and ‘Odoroki’ (C and D) peach trees from November 2012 to April 2013. Data are presented as means ± SE (n = 3). Different letters of each cultivar indicate significant differences among the sampling dates ($P \leq 0.05$).](image-url)
reported that the correlation between Pro accumulation and abiotic stress tolerance in plants is not always positive. For instance, high levels of Pro and P5CS gene expression may be a characteristic of salt-hypersensitive Arabidopsis mutants (sos1 mutant plants) (Liu and Zhu, 1997).

Many researchers have identified the biosynthetic pathway of Pro and have confirmed the expression of genes, such as P5CS and P5CR, involved in the pathway from various species (Gleeson et al., 2005; Hur et al., 2004; Kishor et al., 1995; Nanjo et al., 1999). Interestingly, we also found that the expression of P5CS, which catalyzes the conversion of Glu into GSA, significantly increased during the CH (low temperature) in both cultivars of the first experiment (Fig. 6) and decreased sharply during DH (higher temperature) in both experiments (Figs. 4–7) and P5CS exhibited contrasting patterns compared with the Pro contents during natural CH and DH, and experimental DH (Figs. 4–7). However, the expression of the P5CR gene encoding an enzyme that catalyzes the conversion of P5C into Pro in the final step of Pro pathway exhibited similar patterns, namely, a decrease during CH and an increase during DH, compared with the Pro contents during natural CH and DH, and experimental DH (Figs. 4–7). Most studies on Pro accumulation and the expression of P5CS and
P5CR genes in response to abiotic stresses have identified either P5CS or P5CR. Thus, our results suggest that the identification of both P5CS and P5CR might be required for accurate analysis of Pro biosynthesis. Furthermore, although it was only investigated during the experimental DH, OAT increased similarly to Pro and P5CR in the 10 peach cultivars (Figs. 5 and 7). Our results suggest that the ornithine pathway, which has been suggested to be important during seedling development and for proline accumulation against abiotic stresses in some plants (Armengaud et al., 2004; Roosens et al., 1998; Xue et al., 2009), could serve as an alternative pathway for the production of GSA or P5C (Delauney et al., 1993; Roosens et al., 1998) in the Pro synthesis process during DH in peach. Further research is required to clarify whether the enhancement of Pro synthesis or a high-temperature-stressed response. Furthermore, a tendency for this Pro accumulation might be a characteristic of plants belonging to Prunus spp. or Rosaceae.

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