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Research article

Identification of the expressed protein and the impact of change in ascorbate peroxidase activity related to endodormancy breaking in *Pyrus pyrifolia*

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ABSTRACT

Endodormancy is an important feature of perennial deciduous fruit trees that survive in the extreme climates brought about by seasonal variation. To acquire a comprehensive knowledge of the biochemical processes occurring just before endodormancy breaking, the buds collected in the pre-breaking period (PP) phase were used as samples to identify the proteins related to the breaking of endodormancy in the Japanese pear (*Pyrus pyrifolia* Nakai). Using nano-ESI-LC-MS/MS analysis, 96 proteins were overlapped by analyses of three times and identified as expressed proteins at the PP stage. Among these proteins, dehydrin, several classes of heat shock proteins (HSP), auxin-binding protein, and auxin-induced protein were identified in the floral bud in the PP stage. The majority of these proteins were involved primarily in the oxidation-reduction process. We focused on catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) as enzymes regulating the levels of hydrogen peroxide (H_2O_2) in the bud. From measurements taken during the deepest period (DP), PP, mid-breaking period (MP), and late-breaking period (LP) of endodormancy, CAT activity decreased gradually, while APX activity also decreased from DP to MP, but then increased rapidly during LP. Protein data for PP and the rapid increase in APX activity observed in LP provided knowledge of the biochemical processes that regulate the consecutive transition from endodormancy breaking to ecodormancy induction in the Japanese pear.

1. Introduction

Bud dormancy in temperate zone deciduous fruit trees is an adaptive mechanism for surviving unfavorable conditions during winter (Faust et al., 1997). The three stages of dormancy are para-dormancy, endodormancy, and ecodormancy (Lang, 1987). In autumn, buds enter endodormancy after defoliation and cessation of growth. In this state, trees cannot begin bud growth, even under favorable environmental conditions. Endodormancy breaks after a specified period of sufficiently low temperatures known as the chilling requirement, the required temperature and duration of which depends on the species and the cultivar (Westwood, 1978; Saure, 1985). However, if the chilling requirement is not met, such as in periods of climate change or global warming, endo-dormancy breaking does not occur, and the growth of new organs in spring does not occur (Sugiura et al., 2007). To address this issue, several studies have been conducted to examine endodormancy in various tree species.

The Japanese pear (*Pyrus pyrifolia* Nakai) is one of the most important fruits in Japan. Recently, in regions such as New Zealand (Kingston et al., 1990; Klinac and Geddes, 1995) and Brazil (Petri et al., 2002; Petri and Herter, 2002), low chilling in winter has resulted in defects in bud breaking in Japanese pears during the spring season. Several recent studies have focused on the breaking of dormancy in grapes (Or et al., 2000, 2002; Pang et al., 2007; Halaly et al., 2008), by using hydrogen cyanamide (HC) as a tool for modifying the breaking of endodormancy. However, the

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mechanism involved in this transition remains unclear. In grapes, treatment of buds with HC increased hydrogen peroxide (H_2O_2) concentration and inhibited catalase (CAT) activity (Pérez et al., 2008). Furthermore, H_2O_2 concentration in the buds of the Japanese pear decreased naturally after the period of endodormancy break (Kuroda et al., 2002). This is one of the step in a cascade that upregulates several signaling proteins, such as transcription factors, protein phosphatases, and protein kinases (Neill et al., 2002). Additionally, bud break is a phenomenon unambiguously specifying the interplay of environmentally favorable conditions with gene expression and protein synthesis and activation, causing changes in the physiological state (Rohde and Bhalerao, 2007). A recent study of *P. pyrifolia* focused on determining the molecular levels of MIKC-type dormancy-associated MADS-box genes as candidates for endodormancy-breaking genes (Ubi et al., 2010). In addition, a comparison of selected genes in 'Kosui' and the less dormant Taiwanese pear 'Hengshanli' (TP-85-119), identified two novel transcription factors (NAC and PRR), the expression levels of which varied concomitantly with dormancy phase changes (Nishitani et al., 2012). Furthermore, the gene encoding an auxin carrier component showed expression levels that increased following both HC treatment and during the transition from the deepest period to the breaking period of the endodormancy stage (Takemura et al., 2013). In a study that focused on the expressed proteins, the expression levels of dehydrin as protein known to be associated with cold hardiness were shown to increase during the endodormancy period in peach buds (Yamane et al., 2006). In the buds of the Japanese pear, a protein located at 19 kDa gradually disappeared during the interval between the deepest period to the breaking period of endodormancy (Tamura et al., 1998). The proteomes of the apical buds of *Pinus sylvestris* L. were characterized during the four critical stages that occur during the dormancy-to-growth transition; the majority of the proteins identified (57%) were found to be involved in metabolic and other cellular processes (Bi et al., 2011). In the peach, this has been shown through a quantitative assessment of changes in the bark proteome during cold acclimation and 57 protein spots identified by mass spectrometry (Renaut et al., 2008). Additionally, differences in proteome expression among four critical stages in Japanese apricot flower buds, from paradormancy before leaf fall to dormancy release, have been identified (Zhuang et al., 2013). However, to the best of our knowledge, there have been no reports regarding proteomic methods for studying the bud dormancy in *Pyrus* or *Malus* plants, and little is known regarding the comprehensive analysis of the proteins expressed immediately prior to the endodormancy breaking period in temperate zone deciduous fruit trees. Therefore, a comprehensive protein identification in *Pyrus* plants during endodormancy, for which there have been no previous case studies, is important for the elucidation of the mechanisms involved in endodormancy breaking in deciduous fruit trees belonging to the Rosaceae family. Additionally, the marker which able to assess the depth of endodormancy is needed in this family.

In our study, we investigated the development of the endodormancy stages in the floral buds of the Japanese pear and the proteins expressed in the pre-breaking period of endodormancy. The expressed proteins were identified using nano-LC-ESI-MS/MS combined with a database search. Additionally, to obtain knowledge of the biochemical processes involved in regulating the consecutive transition from endodormancy breaking to ecodormancy induction in the Japanese pear, we investigated (from among the identified proteins) the activity changes of the enzymes involved in the decomposition of H_2O_2 during the endodormancy stage.

2. Materials and methods

2.1. Changes of floral bud break in the Japanese pear (Experiment 1)

2.1.1. Plant materials and parentage of floral bud break

Samples were collected from 21-year-old Japanese pear trees (*P. pyrifolia* Nakai) 'Gold Nijisseiki' grafted onto *Pyrus betulaefolia* Bunge seedling in the orchard of Tottori University, Tottori, Japan (35°N, 133°E). Floral buds and 1-year-old branches were collected periodically from December 14, 2009 to January 20, 2010. Floral buds were frozen in liquid nitrogen immediately after collection and stored at −80 °C. The branches were shortened to approximately 30 cm in length; branches included five lateral floral buds that were used to determine the percentage of floral-bud break. The basal part of the cuttings was submerged in 0.03% (v/v) aluminum sulfate and 0.3% (v/v) 8-hydroxyquinoline. The cuttings were then maintained in a growth chamber at 23 ± 1 °C and 24-h photoperiod. Bud break is defined as a developmental stage of more than four phases characterized by swelling of the buds and the emergence of a green tip between scales (Fig. S1). The incidence of bud break was determined over 28 days on five single shoots having five buds. The period of endodormancy breaking is defined as the time required for the percentage of bud break to reach more than 70%.

On the basis of parentage of floral bud break, the buds were in the deepest period (DP) phase of endodormancy on December 14, 2009; the buds then transitioned to the pre-breaking period (PP) phase on December 24, 2009; the buds then transitioned to the mid-breaking period (MP) phase on January 7, 2010 and the buds then transitioned to the late-breaking period (LP) phase, as the ecodormancy stage on January 20, 2010 (Table 1).

2.2. Identification of expressed proteins on the pre-breaking period of endodormancy in the Japanese pear (Experiment 2)

2.2.1. Protein extraction and one-dimensional electrophoresis (1-D)

Total proteins were extracted from floral buds on December 24 and January 20 as described by Damerval et al. (1986). Floral buds (0.2 g fresh weight), frozen in liquid nitrogen, were ground using a mortar and pestle with a solution of 10% trichloroacetic acid (w/v) in acetone containing 0.07% (v/v) 2-mercaptoethanol (2-ME) and maintained at −18 °C for 45 min. The homogenates were centrifuged at 20,000× g and −9 °C for 15 min. The precipitates were washed with acetone containing 0.07% (v/v) 2-ME and maintained at −18 °C for 1 h, then centrifuged at 20,000× g and −9 °C for 15 min. The precipitates were dried under a vacuum and resuspended in 0.5 mL of a buffer containing 9 M urea, 2% (v/v) 2-ME, and 0.8 mM phenylmethylsulfonyl fluoride (PMSF). Protein content was estimated using a Bradford protein assay reagent (Bio-Rad, Hercules, CA).

The protein sample was analyzed by reducing 1-D SDS-PAGE, essentially using the method of Laemmli (1970). The extracted protein was diluted in an SDS-PAGE buffer containing 0.25 M

Table 1
Floral bud break (%) of 'Gold Nijisseiki' pear during 2009–2010 seasons.

Date	Bud break (%)[a]	Endodormancy stage
14 − Dec.	32.0 b[b]	Deepest period (DP)
24 − Dec.	40.0 b	Pre-breaking period (PP)
7 − Jan.	80.0 a	Mid-breaking period (MP)
20 − Jan.	96.0 a	Late-breaking period (LP)

[a] 28 days after forcing.
[b] Different letters within the same column show a significant difference at $P < 0.05$ by t-test.