Effects of dormancy progression and low-temperature response on changes in the sorbitol concentration in xylem sap of Japanese pear during winter season

Akiko Ito¹, Toshihiko Sugiura, Daisuke Sakamoto and Takaya Moriguchi

Plant Physiology and Fruit Chemistry Division, NARO Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan; ¹Corresponding author (akiko@affrc.go.jp)

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In order to elucidate which physiological event(s) are involved in the seasonal changes of carbohydrate dynamics during winter, we examined the effects of different low temperatures on the carbohydrate concentrations of Japanese pear (Pyrus pyrifolia (Burm.) Nakai). For four winter seasons, large increases in the sorbitol concentration of shoot xylem sap occurred during mid- to late December, possibly due to the endodormancy completion and low-temperature responses. When trees were kept at 15 °C from 3 November to 3 December in order to postpone the initiation and completion of chilling accumulation that would break endodormancy, sorbitol accumulation in xylem sap was always higher from trees with sufficient chilling accumulation than from trees that received insufficient chilling. However, an additional increase in xylem sap sorbitol occurred around late December in trees regardless of whether their chilling accumulation naturally progressed or was postponed. To examine different temperature effects more closely, we compared the carbohydrate concentrations of trees subjected to either 6 or 0 °C treatment. The sorbitol concentration in xylem sap tremendously increased at 0 °C treatment compared with 6 °C treatment. However, an additional increase in xylem sap sorbitol occurred at both the temperatures when sufficient chilling accumulated with a peak coinciding with the peak expression in shoots of the sorbitol transporter gene (PpSOT2). Interestingly, the total carbohydrate concentration of shoots tremendously increased with exposure to 0 °C compared with exposure to 6 °C, but was not affected by the amount of accumulated chilling. Instead, as chilling accumulated the ratio of sorbitol to total soluble sugars in shoots increased. We presumed that carbohydrates in the shoot tissues may be converted to sorbitol and loaded into the xylem sap so that the sorbitol accumulation patterns were synchronized with the progression of dormancy, whereas the total carbohydrate transported into shoots from other storage organs may be related to freezing tolerance acquisition independent of dormancy progression. We thus propose that there are different effects of dormancy progression and low-temperature responses on carbohydrate dynamics in Japanese pear.

Keywords: freezing tolerance, hardening, carbohydrate metabolism, endodormancy, Pyrus pyrifolia (Burm.) Nakai.

Introduction

Survival and the competitive success of woody and perennial plants in the temperate and boreal zones depend on precise timing of growth, winter dormancy and frost hardiness in synchrony with seasonal changes in temperature (Olsen 2010). Generally, these processes take place in harmony with the seasonal transition, making it difficult to evaluate each step independently of environmental conditions (Olsen 2010, Arora and Rowland 2011).

Dynamics of carbohydrates in deciduous trees during the winter season are of great interest because carbohydrates are essential for the winter survival of trees in diverse roles, such...
as in osmoregulation and cryoprotection, and as energy sources (e.g., reviewed in Welling and Palva 2006). Sugars are one of the most important factors for freezing tolerance; sugar accumulation correlates with an increase in freezing tolerance in woody plants (Sauter et al. 1996). Short day lengths during autumn trigger the accumulation of sugars stored as starch in stems and buds after growth cessation (Kuroda and Sagisaka 1993, Rinne et al. 1994). Stored starch is converted to soluble sugars in response to low and freezing temperatures (Sauter et al. 1996, Rinne and van der Schoot 2003). Large amounts of the deposited starch, thus, provide trees with sugars at the time of the year when deciduous trees have already shed their leaves (Sauter and Wellenkamp 1998). Many plants are known to show an increase in freezing tolerance upon exposure to low non-freezing temperatures (Thomasow 1999), and the freezing tolerance develops differently in response to different temperature regimes (Junttila and Kaurin 1990, Rom 2003).

On the other hand, carbohydrate metabolism changes with endodormancy progression, possibly for meeting the carbohydrate demands in buds for growth resumption after breaking endodormancy (Yoshioka et al. 1988, Maurel et al. 2004a, Ben Mohamed et al. 2010, Marafon et al. 2011). In our previous study, we showed that the concentration of sorbitol, the main translocated sugar in Rosaceae plants, notably increased in the xylem sap of Japanese pear (Pyrus pyrifolia (Burm.) Nakai) ‘Kosui’ in late December. This time period was coinciding with the timing of breaking of endodormancy in an experimental orchard in Tsukuba, Japan (Ito et al. 2012). In contrast, pear plants near the Tsukuba area encounter several periods of subfreezing temperatures and largely increase their freezing tolerance from mid- to late December (Honjo and Omura 1987). Thus, it remains unclear how and to what extent these two physiological events affect sugar accumulation.

Xylem sugar transport in woody plants is important during winter because in this period phloem transport is greatly reduced and the xylem stream functions as a primary carbon source (Decourteix et al. 2008). In addition, carbohydrates in xylem during winter may have multiple functions, such as prevention of freezing and repair of xylem embolism (Ameglio et al. 2004, Decourteix et al. 2008). Recent studies revealed that the sucrose concentration in xylem sap during winter is regulated by the balance of two opposite movements: (i) a loading of sugars from xylem parenchyma cells into the xylem vessels (Sauter 1982, Decourteix et al. 2006), presumably mediated by a facilitated diffusion mechanism (Ameglio et al. 2004); and (ii) an influx of sugars from the xylem sap into xylem parenchyma cells, mediated by an H+/sugar symport (Sauter 1982) coupled with a plasma membrane H+-APTase located in vessel-associated cells (Alves et al. 2007).

Rosaceae plants such as pears, apples and peaches are unique in the sense that sorbitol is their main translocatable carbohydrate, whereas many other plants generally translocate carbohydrates in the form of sucrose (Loescher and Everard 1996). Almost 70% of the translocated carbon in apple (Klages et al. 2001) or 65–75% in apricot (Bieleski and Redgwell 1985) exists in the form of sorbitol. The ratio of sorbitol to sucrose even increases in response to water and salt stresses in apple (Wang et al. 1995), cherry (Ranneby et al. 1991) and peach (Escobar-Gutierrez et al. 1998). However, unlike sucrose transporters, little is known about sorbitol transporters in either sink or source tissues in Rosaceae plants. Recently, the transporters for sorbitol were identified in higher plants, including sour cherry, PcSOT1 and PcSOT2 (Gao et al. 2003), apple, MdSOT3–MdSOT5 (Watari et al. 2004), MdSOT1 and MdSOT2 (Gao et al. 2005), and MdSOT6 (Fan et al. 2009). In our previous study, we isolated a partial fragment of a sorbitol transporter gene from the winter shoots of Japanese pear, named PpSOT2 (AB719045, Ito et al. 2012), and found that PpSOT2 was highly expressed during late December concomitant with the increase in sorbitol concentration in xylem sap. Because the PpSOT2 sequence shows high similarity to MdSOT5 (91%) whose expression is largely localized in apple source leaves, we hypothesized that PpSOT2 may mainly participate in sorbitol loading from source to sink organs in winter.

It is of a great interest to know which physiological event(s) are involved in the seasonal changes of carbohydrate dynamics during winter. Our goal in this study is to distinguish the effects of endodormancy progression and low-temperature responses on the regulation of carbohydrate dynamics. In order to elucidate these processes, we examined the effects of different temperatures on changes in the concentrations of carbohydrates, especially of sorbitol, during the winter season with field experiments over 4 years and with exposing trees to different temperature conditions. In Japanese pear ‘Kosui’, temperatures between −6 and 12 °C have positive effects on dormancy progression. The most effective temperature range is between 0 and 6 °C with a 750-h exposure to these temperatures regimes required for breaking endodormancy sufficiently (Sugiura and Honjo 1997). Depending on different responses of sugar metabolism to different temperatures, we discuss the possible involvement of endodormancy progression and low-temperature responses in the regulation of carbohydrate dynamics in Japanese pear during the winter season.

Materials and methods

Plant materials

For the field experiment, mature trees of Japanese pear ‘Kosui’ grafted on the rootstocks Pyrus betulaefolia Bunge (35–40 years old), growing in an experimental orchard at the NARO Institute of Fruit Tree Science (Ibaraki, Japan), were used. The experiment was conducted during the four winter seasons of 2008–09, 2009–10, 2010–11 and 2011–12. To analyze changes in soluble sugar concentrations in xylem sap under
field conditions, we sampled three uniform shoots (70–120 cm in length) from October to March at arbitrary intervals.

For the environmentally controlled experiment, seedlings of ‘Kosui’ were purchased from a commercial nursery on December 2009, transplanted in 20-l pots in March 2010 and grown in the open field until used. The experiment for changing the chilling accumulation period was conducted during 2010–11 and that for comparing 6 and 0 °C temperature effects was conducted during 2011–12. All temperature treatments were conducted under dark conditions because low temperature, but not photoperiod, is a prerequisite in pear plants for the progression of endodormancy (Heide and Prestrud 2005, Takemura et al. 2011), which may be different from other woody plants (e.g., reviewed by Horvath 2009).

Dormancy evaluation

In this study, endodormancy breaking was defined as occurring when ≥60% of the lateral flower buds of a whole plant opened during 2 months of forcing treatment (15 °C, natural day length). Dormancy status was evaluated either by forcing whole trees or by calculating the DVI value. The DVI is one parameter of a dynamic model, and the accumulated developmental rate (DVR; DVI = ΣDVR) (Nakagawa Horie 1995), where DVR is a function of hourly mean temperature that characterizes the different effects of the temperature range on dormancy progression. DVI is defined as 0 at the onset of chilling and 1.0 at sufficient chilling accumulation; therefore, DVI < 1.0 indicates insufficient chilling accumulation, whereas DVI ≥ 1.0 indicates sufficient chilling accumulation. The DVI value was calculated according to a previously reported method (Sugiura and Honjo 1997). The air temperatures in the controlled environments were monitored with Thermo recorders (TR-71U, T&D Corporation, Tokyo, Japan), and the recorded temperature data (hourly mean temperature) were obtained from the nearest weather station (Japan Meteorological Agency) located about 1.5 km west of the experimental orchard.

Effect of the amounts and timing of the initiation of chilling accumulation

To stagger the timings of endodormancy progression and encountering low temperatures in the field, potted ‘Kosui’ trees (2 years old) were subjected to two different temperature conditions; one set of trees was subjected to field conditions throughout the period of chilling accumulation, and the other set of trees was first subjected to 15 °C in the greenhouse with natural sunlight for 1 month (from 3 November to 3 December in 2010) and then transferred to field conditions, in order to postpone the initiation and completion of sufficient chilling. Before transferring the trees from the 1 month incubation at 15 °C to field conditions, trees were subjected to 6 °C (dark) for 7 days for chilling adaptation. The DVI progression of each treatment is presented in Figure 1. Twenty of these trees

![Figure 1. DVI progressions during chilling accumulation in 2010–11 for trees held under field conditions and for trees treated at 15 °C between 3 November and 3 December 2010. Trees treated at 15 °C were exposed to 6 °C for 7 days for cold adaptation before being transferred to field conditions. The DVI is defined as 0 at the onset of chilling accumulation and 1.0 at the chilling satisfaction point to break endodormancy.](image)

Effect of 6 or 0 °C treatment on sugar metabolism

Potted trees were exposed to either 6 °C (dark) or 0 °C (dark) temperatures (three trees per temperature treatment) from 30 November (at DVI = 0.35) in 2011. When trees were subjected to these temperature regimes until the DVI reached 0.6 (7 December), 0.8 (14 December), 1.0 (20 December), 1.2 (26 December) and 1.5 (4 January), one shoot was sampled from each tree (three shoots per treatment). Internodes were excised from a central part of the sampled shoots, cut into pieces and frozen until the analyses were conducted (referred to as ‘shoots’ hereafter). Xylem sap was also collected from the shoots as per the method described below. Shoots and xylem sap samples were used to determine the carbohydrate concentration, and shoot samples were used to analyze the expression of the sorbitol transporter (SOT) gene.
Data were statistically analyzed first with a one-way analysis of variance (ANOVA) and least significant differences (LSD) to show the periodic change from DVI = 0.35 to 1.5 and additionally with a two-way ANOVA to show the independent and interactive effects of the two factors, ‘temperature’ and ‘DVI’, respectively, when DVI = 0.6–1.5.

In the 2009–10 season, sorbitol concentrations of the shoot xylem sap and those of shoot cells (symplast, Tsurusaki et al. 1997) were compared on the basis of milligrams of sorbitol per gram of xylem sap or cellular water. For the xylem sap, sorbitol concentrations obtained as mg ml⁻¹ were calibrated with the specific gravities of the respective sap samples, and expressed as the sorbitol weight (mg) per xylem sap weight (g). For the shoot cells, the remaining internode segments after collection of the xylem sap were cut into pieces, and dried at 70 °C for 48 h. The lost weights (differences between before and after drying) were assumed to be the weights of water contained in the shoot cells. The dried shoot samples were used for sugar analyses. Sorbitol concentrations of the shoot cells were expressed as milligrams of sorbitol per gram of water contained in the shoot cells.

**Xylem sap collection**

Each sampled shoot was divided into three parts (distal, central and proximal sections of shoots), and internodes (10 cm in length) were excised from the central section. Xylem sap was collected from internodes by the centrifugation method (Tsurusaki et al. 1997). Briefly, internode segments were placed in a 50-ml Falcon tube and centrifuged for 10 min at 3000 × g at 4 °C to collect xylem sap. The xylem sap solutions were immediately frozen in liquid N₂, stored at −80 °C and used to determine soluble sugar concentrations. From 100 g of shoot segments, 7–10 g of xylem sap was recovered by this method.

**Determination of sugar and starch concentrations**

Shoot samples were used for extraction and analyses of soluble sugars (sorbitol, sucrose, fructose and glucose) and starch, and xylem sap was used for the analyses of soluble sugars as described by Ito et al. (2012). Briefly, ~1 g of the frozen sample was extracted three times with 10 ml of 80% methanol at 80 °C. The samples were evaporated to dryness, the residue was re-dissolved in water and 2 mg of mannitol was added as an internal standard. The solution was then passed through a StrataSax cartridge (Shimadzu GLC Ltd, Tokyo, Japan) to remove weakly acidic compounds. An aliquot of the eluate was identified and quantified by high-performance liquid chromatography (HPLC) equipped with a Rezex RCM-Monosaccharide Ca column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) and a refractive index detector. The column was maintained at 80 °C and eluted with water at a flow rate of 0.8 ml min⁻¹. For the xylem sap analyses, 1 ml of sap was first heated in boiling water for 3 min, 2 mg of mannitol was added and the sap was passed through a StrataSax cartridge. Soluble sugars were determined by HPLC as described above. Since the concentrations of fructose and glucose were quite low (ca. 0.1–5.0 and 1.9–6.5% of the total soluble sugars in xylem sap and shoot extract, respectively) in all samples, we did not report these results; however, these values were needed for calculations of total soluble sugars.

Starch was extracted three times from the methanol-insoluble residue with 10 ml of dimethyl sulfoxide (DMSO) in a boiling water bath. The amount of starch was determined from aliquots (200 µl) of DMSO extracts by measuring glucose following digestion with amylglucosidase (EC 3.2.1.3) at pH 4.5 for 3 h at 37 °C, using a glucose assay kit (EnzyPlus Sucrose/D-Glucose, BioControl Systems, Hong Kong).

**Total RNA extraction and quantitative reverse transcriptase polymerase chain reaction**

The expression of the SOT gene (PpSOT2, AB719045) was analyzed by quantitative reverse transcriptase polymerase chain reaction (qPCR). Total RNA was extracted from shoot samples and first-strand cDNA was synthesized by the method of Ubi et al. (2010). A 5-µg aliquot of total RNA used in the reaction was first treated with RNase-free DNase I (Promega, Madison, WI, USA) and reverse-transcribed using SuperScript III oligo (dT) 20 primers according to the manufacturer’s instructions (Invitrogen). Quantitative PCR was performed in a 7500 Real-Time PCR System (Applied Biosystems, Tokyo, Japan) using a SYBR Premix Ex Taq kit (TaKaRa, Kyoto, Japan) as described in the manufacturer’s protocol and using specific primers as reported in Ito et al. (2012). We have compared the expression of several reference genes in Japanese pear and confirmed the stability of HistoneH3 during the winter. Thus, HistoneH3 was used as an internal standard in this study. Quantitative reverse transcriptase PCR was performed three times for each gene using aliquots of the same purified total RNA. One data set was used to produce the figures. The mean of at least two individual PCR experiments was determined from separate RNA samples but concurrent reactions.

**Results**

**Field experiment**

Based on the recorded hourly mean temperatures, the values for the DVI started increasing in late October and reached a value of 1.0 on 26, 25, 27 and 25 December in 2008, 2009, 2010 and 2011, respectively.

Throughout these four seasons, sorbitol increased the most in xylem sap among the sugars examined (see File S1 available as Supplementary Data at Tree Physiology Online); for example, the concentrations of glucose, fructose, sucrose and sorbitol in xylem sap were 0.03–0.17, 0.04–0.21, 0.45–0.83 and
O.62–27.4 (mg ml\(^{-1}\)) respectively, throughout the 2011–12 season. Sorbitol in xylem sap began to increase in early to mid-December, and showed a large incremental change (\(\Delta\) sorbitol in mg/\(\Delta\) day) in late December for all the four observed seasons (Figure 2).

The patterns of sorbitol accumulation showed yearly variation. In detail, a large increase in sorbitol accumulation was clearly found at approximately DVI = 1.0 in 2010–11. In contrast, sorbitol accumulation began at approximately DVI = 0.6 in 2011–12, an earlier time than the other seasons. A significant increase in sorbitol accumulation was observed between DVI = 1.0 and 1.5 in 2009–10 and between DVI = 0.6 and 1.0 in 2008–09, respectively, although it is difficult to know the actual timing of sorbitol increase due to the less frequent measurements in these two seasons.

In 2009–10, the sorbitol concentration in the shoot cells continuously increased during December but showed a temporary decrease on 13 January, and then remained at a higher level from late January to early March (Figure 3). The sorbitol concentration in the xylem sap always remained at a lower level than that in the shoot cells.

**Effect of the amount and timing of initiation of chilling accumulation**

By subjecting the potted trees to 15 °C for 1 month from 3 November to 3 December, the initiation and completion of chilling accumulation for endodormancy breaking were postponed as expected. The trees under field conditions throughout the chilling accumulation period reached DVI = 0.8 and 1.2 on 17 December and 4 January, respectively, whereas trees maintained at 15 °C showed about a half-month delay in reaching DVI = 0.8 (4 January) and 1.2 (19 January) (Figure 1). At DVI = 1.2, >60% of the lateral flower buds flowered in both the field and 15 °C treatments, whereas flowering percentages were <15% at DVI = 0.8 regardless of treatments (Figure 4a). These results show that plants that received sufficient chilling hours (DVI = 1.2) actually broke dormancy, but those with insufficient chilling (DVI = 0.8) were still in an endodormant state regardless of the timing of initiation and completion of chilling.

In trees held under field conditions, the sorbitol concentration in the shoot cells reached its maximum at DVI = 1.2 (4 January), whereas in trees from the 15 °C treatment, the sorbitol concentration continued to increase until the end of the sampling period with a slower rate of accumulation from DVI = 1.0 to 1.2 (Figure 4b). Sorbitol accumulation in xylem sap from trees with insufficient chilling (DVI < 1.0) was always lower than that from trees that received sufficient chilling (DVI ≥ 1.0), suggesting a correlation between the breaking of endodormancy and sorbitol increase in the xylem sap. However, a steep increase in sorbitol accumulation in xylem sap occurred between late December and early January irrespective of the temperature treatments.

**Effect of 6 or 0 °C treatments**

Temperature treatments at 6 and 0 °C equally influence dormancy progression of Japanese pear ‘Kosui’ (Sugiura and Honjo 1997). Therefore, to know whether the increase in
sorbitol concentration was ascribed to the effects of endodormancy progression, low-temperature response or both endodormancy and low temperature, we compared the effects of 6 and 0 °C treatments on the xylem sap sugar concentration. Similar to the results from field-grown trees, the primary sugar in xylem sap was sorbitol (data not shown). The sorbitol concentration in xylem sap from trees held at 0 °C tremendously increased compared with trees held at 6 °C (Figure 5a). Accompanying the DVI progression from DVI = 0.35, a slight but significant increase in sorbitol concentration was observed in the sap of trees held at 6 °C with its peak at DVI = 1.2. By contrast, the sorbitol concentration largely increased from DVI = 0.35 to 0.6 in the sap of trees held at 0 °C, and continued to increase until DVI = 1.0. Thus, temperature significantly contributed to large increases in the sorbitol concentration of xylem sap, although sorbitol concentrations also changed along with dormancy progression (DVI). The patterns of

Figure 4. Effects of the amount and initiation timing of chilling accumulation on the flowering rate (a) and the sorbitol concentration in xylem sap (b) of potted trees of Japanese pear ‘Kosui’ in 2010–11. The initiation of chilling accumulation was postponed with a 1-month treatment at 15 °C compared with continuous field conditions. The flowering rate was estimated when trees accumulated chilling until DVI = 0.8 (insufficient chilling) and 1.2 (sufficient chilling), and xylem sap analyses were conducted when chilling accumulated until DVI = 0.6, 0.8, 1.0, 1.2 and 1.5.

Figure 5. Effects of 6 and 0 °C temperature treatments on the sorbitol and sucrose concentrations in xylem sap (a), and the sorbitol and sucrose concentrations in shoot tissue (b) and the starch concentration in shoot tissue (c) of Japanese pear ‘Kosui’ in 2011–12. Fructose and glucose data are not shown because their concentrations were very low in comparison with the sorbitol and sucrose concentrations. The ratios of sorbitol to the sum of these soluble sugars (w/w, %) were also calculated. Temperature treatment was initiated on 31 November 2011 when chilling accumulated to DVI = 0.35 under field conditions. Sampling occurred when the accumulated chilling reached DVI = 0.6, 0.8, 1.0, 1.2 and 1.5 under each temperature regime. Different letters indicate a significant difference at $P \leq 0.05$ with LSD. Differences in the starch concentration (c) were insignificant.
sorbitol accumulation in the xylem sap with their peaks between DVI = 1.0 and 1.2 irrespective of the temperature were similar to those from the orchard trees for all 4 years (Figure 2). Sucrose concentrations increased in the xylem sap of trees held at 0 °C, but the increase in sucrose concentration was much smaller than the increase in sorbitol concentration (Figure 5a). As a consequence, the portion of sorbitol (%) to the total soluble sugars largely increased in the xylem sap of trees held at 0 °C compared with trees held at 6 °C. The portion of sorbitol relative to the total sugar concentration increased to 96.7% at DVI = 1.0 in the sap of trees held at 0 °C but did not show significant changes during endormancy progression (Figure 5a), probably because the effect of temperature (0 °C) was too large and masked the effect of the DVI.

Next, when we calculated a two-way ANOVA on sorbitol and sucrose concentrations in xylem sap, we found that the sorbitol concentration was affected both by temperature (P < 0.001) and by DVI (P < 0.01), and the interaction (temperature × DVI) was also significant (P < 0.01), but the sucrose concentration was affected only by temperature (P < 0.001) (Table 1). This result means that the increase in sorbitol concentration in xylem sap was ascribed to both dormancy progression (DVI) and low temperature, with low temperature having a larger effect than DVI. The increase in sucrose concentration was exclusively due to the effect of low temperature on xylem sap.

To determine the origin of the sorbitol that accumulated in xylem sap upon DVI progression and low temperature, we analyzed the sorbitol, sucrose and starch concentrations in the shoots that included xylem tissues. Differences in the concentration of sorbitol in samples from the trees held between 6 and 0 °C were relatively small in shoots (Figure 5b) compared with xylem sap (Figure 5a). A significantly greater amount of sucrose relative to sorbitol accumulated in shoots than in xylem sap both at 6 and 0 °C (Figure 5b). The temperature effect on starch concentration in shoots was insignificant throughout the period of measurement as determined by a one-way ANOVA (Figure 5c). The portion of sorbitol relative to total soluble sugars changed responding to the DVI and decreased once at DVI = 0.8 and increased again thereafter and became highest at DVI = 1.5 for both temperature regimes (Figure 5b).

A two-way ANOVA and subsequent LSD analyses indicated that sorbitol and sucrose concentrations, but not starch, significantly increased in the shoots of trees held at 0 °C in comparison with those held at 6 °C (Table 2). The degree of increase was larger in sucrose than in sorbitol, and sorbitol + sucrose composed >90% of the total soluble sugars in shoots (data not shown); therefore, the proportion of sorbitol to total soluble sugars became larger in shoots held at 6 °C compared with shoots subjected to 0 °C (Figure 5b). DVI progression affected the sorbitol, sucrose and starch concentrations in shoots. The total carbohydrate concentration increased significantly in shoots of trees held at 0 °C compared with 6 °C, but did not change with DVI progression. This result means that both dormancy progression (DVI) and temperature difference (6 °C vs. 0 °C) independently affected sorbitol and sucrose concentrations in shoots, but starch and total soluble sugars were affected by dormancy progression (DVI) and temperature difference, respectively.

To infer the movement of sorbitol, the expression of PpSOT2 was analyzed in shoots. PpSOT2 expression was significantly higher in the shoots of trees held at 0 °C compared with those held at 6 °C (Figure 6). In the shoots of trees held at 6 °C, PpSOT2 expression showed a generally decreasing trend with DVI progression with a temporal peak at DVI = 1.2, whereas in the shoots of trees held at 0 °C, PpSOT2 expression showed a peak at DVI = 1.0. These peaks of PpSOT2 expression roughly corresponded to the sorbitol concentration peaks in the xylem sap of the respective temperature treatments (Figure 5a).

**Table 1.** Statistical analyses of the effects of 6 and 0 °C temperature treatments and their durations on the sorbitol and sucrose concentrations, and the ratio of sorbitol to the total soluble sugar concentration (%) in xylem sap of Japanese pear ‘Kosui’ shoots with a two-way ANOVA (factors: temperature and DVI).

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<th>Concentration (mg/g FW)</th>
<th>Sorbitol (%) a</th>
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<td>Sorbitol</td>
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†The ratios of sorbitol to the sum of soluble sugars (sorbitol, sucrose, fructose and glucose, w/w, %) were analyzed and calculated.
‡Data were first analyzed with the two-way ANOVA (factors: temperature and DVI). When the interaction (temperature × DVI) was insignificant, analyses of the mean separation with LSD were conducted based on the significant factor(s) (temperature and/or DVI). ns, **, *** indicate non-significant or significant at P < 0.01 and 0.001, respectively, with the ANOVA test. Different letters indicate a significant difference at P < 0.05 with LSD.

**Discussion**

*Seasonal changes in the sorbitol concentration of xylem sap in the field and in temperature-controlled conditions*

The main sugar component of xylem sap in Japanese pear was identified as sorbitol, the concentration of which sharply increased in late December in field investigations. The sharp increase always seemed to take place at approximately DVI = 1.0 in all 4 years, although the patterns of sorbitol accumulation varied yearly (Figure 2). On the other hand, experiments with potted trees where the environmental conditions...
were controlled revealed that endodormancy broke only after trees received sufficient chilling regardless of the timing of chilling initiation and completion (Figures 1 and 4). In these experiments, endormant trees had a lower xylem sap sorbitol concentration than those after endodormancy had broken (Figure 4). In addition, again in both treatments, a steep increase in sorbitol accumulation in xylem sap occurred between late December and early January as observed in the field investigations. These observations with field and environmentally controlled investigations suggested that endodormancy progression may be one of the factors regulating sorbitol accumulation in xylem sap, but other factor(s) also activate sorbitol accumulation around late December.

The most plausible candidate factor for this activation may be low temperature. Sorbitol in apple xylem sap increases in response to exposure to subfreezing temperatures (Williams Raese 1974), and the temperature in Tsukuba frequently drops to ~0 °C during late December in typical winter seasons (see File S2 available as Supplementary Data at Tree Physiology Online). Interestingly, a temporal decrease in sorbitol concentration in xylem sap occurred between DVI = 0.87 (20 December) and 0.92 (24 December) in the 2010–11 season (Figure 2), corresponding to unusually warm temperatures for this season in Tsukuba (see File S2 available as Supplementary Data at Tree Physiology Online); the average hourly mean temperature for each of the 4 days from December 16 to December 20, from December 20 to December 24 and from December 24 to December 28 2009 was 4.0, 9.0 and 2.8 °C, respectively. This observation suggests that sorbitol values fluctuated in relation to changes in temperature; when temperatures were high, the sorbitol concentrations were lower and vice versa, a pattern confirmed by the temperature treatments of 6 and 0 °C (Table 1). A similar result was reported in apple where sorbitol increased in the xylem sap during subfreezing temperatures and decreased during warm periods throughout the dormant season (Williams Raese 1974). Earlier accumulation of xylem sap sorbitol was recorded during 2011–12 than in the other seasons (Figure 2). The average

### Table 2. Statistical analyses of the effect of 6 and 0 °C temperature treatments and their durations on the concentrations of sorbitol, sucrose, starch and total carbohydrates, and the ratio of sorbitol to the total soluble sugar concentration (%) in Japanese pear ‘Kosui’ shoot by a two-way ANOVA (factors: temperature and DVI).

<table>
<thead>
<tr>
<th>Concentration (mg/gFW)</th>
<th>Sorbitol</th>
<th>Sucrose</th>
<th>Starch</th>
<th>Total</th>
<th>Sorbitol (%)*</th>
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<td>12.4 b</td>
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<td>59.1</td>
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*The ratios of sorbitol to the sum of soluble sugars (sorbitol, sucrose, fructose and glucose, w/w, %) were analyzed and calculated.

b Data were first analyzed with the two-way ANOVA (factors: temperature and DVI). When the interaction (temperature × DVI) was insignificant, analyses of the mean separation with LSD were conducted based on the significant factor (temperature and/or DVI). ns, *, **, *** indicate non-significant or significant at $P < 0.05$, 0.01 and 0.001, respectively, with the ANOVA test. Different letters indicate a significant difference at $P < 0.05$ with LSD.

**Figure 6. Effects of 6 and 0 °C treatments on the expression of PpSOT2 in Japanese pear ‘Kosui’ shoot tissue in 2011–12. Sampling conditions and data analysis were the same as described in the caption of Figure 5.**
temperature during December 2011 was lower than in the other winter seasons; the average temperatures in December of 2008, 2009, 2010 and 2011 were 6.7, 6.0, 6.8 and 4.4 °C with 50, 84, 69 and 158 h exposures to temperatures below 0 °C, respectively. Similarly, in the controlled temperature experiment, large increases in xylem sap sorbitol were observed in late December in trees continuously maintained under field conditions and those held for 1 month at 15 °C. In this experiment, temperatures decreased to below 0 °C for 16 h until 17 December, and the duration continued to increase up to 97 h below 0 °C until 4 January (data not shown). These changes in xylem sap sorbitol indicate that trees may quickly respond to low temperature and accumulate a large amount of sorbitol in the xylem sap.

There is a possibility that sorbitol accumulation in xylem sap may be responding to the shortest photoperiod of the year in late December, but the magnitude may be much smaller than the temperature effect, since subjecting trees to dark conditions at 6 °C for 7 days only slightly increased the sorbitol accumulation but exposing trees to the dark at 0 °C strongly influenced sorbitol accumulation (Figure 5a).

Possible mechanism of sorbitol loading into xylem sap in winter

To our knowledge, there have been no reports of studies on how sorbitol is loaded from living shoot cells to the xylem sap during winter, whereas an extensive body of work in sucrose-transporting plants has reported that sucrose loading from xylem parenchyma cells into the xylem vessels during winter occurs by a facilitated diffusion mechanism (Sauter 1982, Decourteix et al. 2006) mediated by H+/sucrose symporters (Ameglio et al. 2004). We also assumed that the sorbitol detected in xylem sap was due to loading from xylem cells in a manner similar to sucrose-transporting plants. The sorbitol concentration in shoot cells was always higher than that in xylem sap for newly grown shoots (Figure 3), which may have facilitated diffusive unloading of sorbitol by its gradient as occurs in the sucrose-transporting plants. PpSOT2 in this experiment had the highest homology to MdSOT5 (91%) (Ito et al. 2012) and had conserved consensus sequences for the sugar transporter subfamily (Watari et al. 2004, Ito et al. 2012), which allows us to infer that PpSOT2 is an H+/sorbitol symporter. MdSOT5 is expressed mainly in phloem tissues in apple source leaves, but is also expressed in the xylem tissues (major veins) of source leaves, and in young leaves and in dormant buds whose phloem transport systems are not fully developed or functional (Watari et al. 2004). With these findings, we speculate that PpSOT2 may mediate sorbitol loading into xylem sap during winter at least when the phloem loading function is arrested. Further studies on the properties and localization of sorbitol transporters need to be carried out in the future.

Different effects of 6 and 0 °C treatment on sugar dynamics in xylem sap and shoots

The total amount of carbohydrate in shoots increased in response to low temperature, being higher with the 0 °C treatment than with the 6 °C treatment (Table 2). This increase may be due to increased import from other larger carbohydrate pools such as older (perennial) branches and roots (Pallardy 2007) because (i) shoots do not synthesize carbohydrates in the season when leaves are absent and (ii) the translocating sugar (sorbitol) increased significantly more with the 0 °C treatment than with the 6 °C treatment in xylem sap, the principal route of carbohydrate transport during winter (Loescher et al. 1990, Maurel et al. 2004a, Alves et al. 2007), indicating that sugar transport is activated more at 0 °C treatment than at 6 °C treatment.

Temperatures below or near 0 °C induce starch hydrolysis at storage sites, which is induced by exposure to 0 and −2 °C but not by exposure to 5 and 10 °C in 5-year-old stems of Populus × canadensis Moench (Sauter 1988). Starch hydrolysis is also induced in walnut twigs by exposure to 1 °C but not by exposure to 15 °C (Ameglio et al. 2004). Therefore, we assumed that more starch degradation would occur in the storage sites at 0 °C than at 6 °C in our experiment. Although we did not analyze PpSOT2 expression in branches and roots, if branch and root expression of PpSOT2 is similar to the expression in annual shoots, PpSOT2 expression may be induced in these carbohydrate-accumulating organs and, therefore, would be another contributor to the larger sorbitol accumulation in the xylem sap at 0 °C.

Degraded starch in source tissues, here older branches and roots, may be metabolized to sorbitol, and transported to shoots via xylem sap and accumulated there. The larger increase in the ratio of sucrose to sorbitol at 0 °C treatment compared with 6 °C treatment in the shoots (Figure 5b, Tables 2) indicated that the transported sorbitol may be subsequently converted into sucrose in shoots better at 0 °C than at 6 °C. Sorbitol unloading from xylem sap into sink tissues occurs diffusively (passively) (Yamaki 2010) and energetically (actively) (Marquat et al. 1996, Maurel et al. 2004b). The conversion of sorbitol into sucrose in shoots may drive the diffusive unloading of sorbitol from xylem sap into shoots by reducing the sorbitol concentration in shoot tissues and thereby enhancing the accumulation of carbohydrates in shoots.

Exposure to low and non-freezing temperatures after growth cessation in autumn enhances freezing tolerance of woody plants rapidly, and the process of developing freezing tolerance influences sugar metabolism and allocation (e.g., reviewed in Welling and Palva 2006). In addition, plants generally develop freezing tolerance more effectively at sub- or near-freezing temperatures than at temperatures within the normal (higher) range (Junttila and Kaurin 1990, Rom 2003). From these findings, we propose that these different responses to 6
and 0 °C in carbohydrate dynamics may be related to the different effects of these low temperatures on acquiring freezing tolerance. However, further studies are needed in the future to substantiate these points.

Effect of DVI on sugar dynamics in xylem sap and shoots

In xylem sap, the sorbitol concentration peaked at DVI = 1.2 and 1.0 in trees treated at 6 and 0 °C, respectively (Figure 5a), and these peaks of sorbitol concentration were coinciding with the peaks of PpSOT2 expression for the respective temperature treatments (Figure 6). Although the maximum concentration of xylem sorbitol at 6 °C was much lower than that at 0 °C, xylem sorbitol concentration ceased to increase before the end of the treatment. These observations were consistent throughout the four seasons of this experiment (Figure 2), suggesting that the response to low temperature may not have been the sole factor that caused peaks of sorbitol concentration both at DVI = 1.0 (6 °C) and 1.2 (0 °C) and may be related to the transition from endodormancy to its breaking for the respective temperature regimes. Differences in the timing of the peak in sorbitol concentration between the 6 and 0 °C treatments may be due to different additive effects of freezing tolerance on sorbitol accumulation.

In the shoot tissues, the ratio of sorbitol (%) to total carbohydrate started to increase from DVI = 1.0 (Figure 5b), but the total amount of carbohydrate was not affected by DVI progression irrespective of the temperature treatments (Table 2). We propose that as DVI progresses, (i) sorbitol conversion from other carbohydrate molecules is enhanced in shoots and (ii) PpSOT2 expression increases in the shoot tissues and then (iii) the accumulated sorbitol largely loads into the xylem sap with concentration peaks at DVI = 1.0 or 1.2 in the 6 or 0 °C treatments, respectively. A temporal decrease of sorbitol concentration in the shoot cells between DVI = 1.2 and 1.5 (Figure 3) may also suggest that a greater extent of transport of sorbitol from shoot cells into the xylem sap occurs at that time.

Conclusions

The increase in sorbitol concentration in xylem sap of Japanese pear during winter was due to both endodormancy progression and exposure to low temperatures. The ratio of sorbitol to total soluble sugars in shoots changed as dormancy progressed, whereas the shoots of trees exposed to 0 °C had a higher concentration of total carbohydrates than those exposed to 6 °C. We propose that the effects of dormancy progression and exposure to low temperature on the carbohydrate dynamics of Japanese pear were different from each other. Carbohydrate molecules in shoot tissues may be converted into sorbitol with dormancy progression, and the resulting sorbitol may be loaded into the xylem sap when dormancy breaks, whereas carbohydrate transport into shoots from other organs may be stimulated by low temperature that may be due mostly to the development of freezing tolerance. Additional studies need to be carried out in the future to test this hypothesis.

Supplementary data

Supplementary data for this article are available at Tree Physiology Online.

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Conflict of interest

None declared.

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References


