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Title	High accumulation of soluble sugars in deep supercooling Japanese white birch xylem parenchyma cells.
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Citation	New Phytologist, 174(3), 569-579 <a href="https://doi.org/10.1111/j.1469-8137.2007.02025.x">https://doi.org/10.1111/j.1469-8137.2007.02025.x</a>
Issue Date	2007-05
Doc URL	<a href="https://hdl.handle.net/2115/22535">https://hdl.handle.net/2115/22535</a>
Rights	The definitive version is available at <a href="http://www.blackwell-synergy.com">www.blackwell-synergy.com</a> .
Type	journal article
File Information	NP174-3.pdf



High accumulation of soluble sugars in deep supercooling Japanese white birch xylem parenchyma cells

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Total word count: 7119 words

Summary: 199 words, Introduction: 1069 words, Materials and Methods: 1368 words, Result: 1197 words, Discussion: 1803 words, Acknowledgements: 66 words, References: 1417 words.

Seven figures and two tables

## Summary

· Seasonal changes in the accumulation of soluble sugars in extracellular freezing cortical parenchyma cells and deep supercooling xylem parenchyma cells in Japanese white birch were compared to identify the effects of soluble sugars on the mechanism of deep supercooling, which keeps liquid state of water in cells under extremely low

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### Abbreviations list:

Cryo-SEM	cryo-scanning electron microscopy
DTA	differential thermal analysis
DW	dry weight
FW	fresh weight
HPLC	high-performance liquid chromatography
LN <sub>2</sub>	liquid nitrogen

temperatures for long periods.

- Soluble sugars in both tissues were analyzed by HPLC, and the concentrations of sugars in cells were estimated by histological observation of occupancy rates of parenchyma cells in each tissue. Relative and equilibrium melting points of parenchyma cells were measured by differential thermal analysis and cryo-scanning electron microscopy, respectively.
- In both xylem and cortical parenchyma cells, amounts of sucrose, raffinose and stachyose increased in winter, but amounts of fructose and glucose exhibited little change throughout the entire year. In addition, no sugars were found to be specific for either tissue. Combined results of HPLC analyses, histological observation and melting point analyses confirmed that the concentration of sugars was much higher in xylem cells than in cortical cells.
- It is thought that the higher concentration of soluble sugars in xylem cells may contribute to facilitation of deep supercooling in xylem cells by depressing the nucleation temperature.

Key words: deep supercooling, cold acclimation, subfreezing temperature, Japanese white birch (*Betula platyphylla* var. *japonica*), xylem parenchyma cells, soluble sugars

## **Introduction**

Trees that are indigenous to cold regions have the highest level of winter freezing resistance within the plant kingdom (Sakai & Larcher, 1987). However, the mechanism of adaptation to freezing in such trees differs depending on the type of tissue. For example, cortical parenchyma cells adapt to subfreezing temperatures by extracellular freezing, whereas xylem parenchyma cells adapt to subfreezing temperatures by deep supercooling (Quamme *et al.*, 1982; Ashworth *et al.*, 1988; Malone & Ashworth, 1991). Extracellular freezing is the most common mechanism of adaptation to freezing, and adaptation by deep supercooling is unique to xylem parenchyma cells (Sakai & Larcher, 1987).

In the case of extracellular freezing adaptation, when extracellular water is frozen at relatively high subzero temperatures, the difference between vapor pressures of extracellular ice and intracellular water results in dehydration from living cells in direct

parallel to temperature reductions. Due to this equilibrium dehydration, cells are able to avoid the occurrence of lethal intracellular freezing. In extracellular freezing adaptation, survival of cells depends on the degree of dehydration tolerance, and the temperatures at which cells can survive range from just below zero to below the temperature of liquid nitrogen (LN<sub>2</sub>, -196°C) (Sakai, 1960; Sakai & Larcher, 1987). As a result of seasonal cold acclimation, cortical parenchyma cells in boreal trees, including Japanese white birch (*Betula platyphylla* var. *japonica*), obtain a very high level of dehydration tolerance in winter and can therefore tolerate freezing to the temperature of LN<sub>2</sub> during winter (Sakai and Larcher, 1987).

Xylem parenchyma cells in trees, on the other hand, are not dehydrated by freezing of apoplastic water, and intracellular water is maintained in a liquid state. Xylem cells are able to maintain this supercooled state in a metastable equilibrium for long periods of time which span more than several weeks under exposure to low temperatures. This adaptive mechanism is thus referred to as deep supercooling (Quamme, 1991). Xylem parenchyma cells usually survive freezing events in their supercooled state, but supercooling has a physical limit. When the temperature falls below the temperature limit for supercooling, the capacity for maintaining a metastable equilibrium is exceeded and lethal intracellular freezing occurs. Xylem cells in trees thus adapt to subfreezing temperatures by lowering the temperature limit for deep supercooling; an adaptation which depends upon environmental temperature changes experienced where trees are growing (George *et al.*, 1974; Fujikawa & Kuroda, 2000). The temperature limit for supercooling in xylem cells was found to be gradually lowered (from -10 to -70°C) in parallel with latitudinal temperature reduction from tropical zones toward cold zones (Fujikawa & Kuroda, 2000; Kuroda *et al.*, 2003). The temperature limit for supercooling also changes seasonally. The temperature limit for supercooling in xylem parenchyma cells of boreal trees, including Japanese white birch, is approximately -20°C in summer but is reduced to almost -60°C during winter (Kuroda *et al.*, 1999; Fujikawa & Kuroda, 2000; Kuroda *et al.*, 2003). The freezing resistance of xylem cells in boreal trees is thus lower than that of cortical cells (Quamme *et al.*, 1972; Quamme *et al.*, 1982) and has therefore become a critical factor that limits the distribution of trees to cold areas (Burke & Stushnoff, 1979; Sakai & Larcher, 1987).

The mechanism of deep supercooling in xylem parenchym cells has been explained solely by a physical phenomenon of water (Ashworth & Abeles, 1984) based on results

of experiments on supercooling of small isolated water droplets (Fletcher, 1970; MacKenzie, 1977). It has been suggested that protoplasts of xylem parenchyma cells do not contain heterogeneous ice nucleators and are isolated from the effects of extracellular ice crystals due to specific cell walls that inhibit intracellular dehydration and prevent the penetration of extracellular ice into intracellular spaces (George & Burke, 1977; Quamme *et al.*, 1982; George, 1983; Ashworth & Abeles, 1984). Thus, it is thought that xylem parenchyma cells can supercool to the homogeneous ice nucleation temperature of water ( $-40^{\circ}\text{C}$ ) and sometimes to lower temperatures by freezing temperature depression that results from the concentrations of solutions in the cells (Gusta *et al.*, 1983; Kuroda *et al.*, 2003).

The isolation of protoplasts from the effects of extracellular ice by the presence of specific cell walls is undoubtedly a prerequisite for achieving supercooling in xylem parenchyma cells. However, it is not clear whether deep supercooling of xylem cells in trees can be explained only by such a physical effect. It has been shown that the incidence of nucleation of isolated liquid droplets in a metastable equilibrium depends on the size of droplets and the time of cooling, smaller droplets and higher cooling rates yield lower nucleation temperatures (MacKenzie, 1977). In nature, however, the supercooling of xylem parenchyma cells, which correspond to water droplets of more than  $20\ \mu\text{m}$  in diameter, often continues for several weeks or more, a time frame that is far longer than the cooling of isolated water droplets under experimental conditions with more fair of nucleation of xylem cells during long time cooling in nature. It is also difficult to explain changes in supercooling capability due to seasonal cold acclimation and deacclimation (Wisniewski & Ashworth, 1986; Fujikawa & Kuroda, 2000) or due to artificial deacclimation (Hong & Sucoff, 1982) only by changes in the physical properties of cell walls.

A previous study demonstrated that the temperature limit for supercooling of xylem parenchyma cells is raised in thawed xylem cells after induction of intracellular freezing (Quamme *et al.*, 1973). It is thought that the thawing of xylem cells after intracellular freezing events might initiate the release of intracellular substances. Our preliminary study also showed that the release of intracellular substances resulted in a significant reduction in the supercooling ability of xylem parenchyma cells (Fujikawa *et al.*, 1994; Kasuga *et al.*, 2006). It is therefore reasonable to assume that particular intracellular substances function to facilitate or stabilize supercooling in xylem cells.

We focused on the effects of intracellular substances on the supercooling capability of xylem parenchyma cells. Among intracellular substances, in this study, we examined the role of intracellular soluble sugars in supercooling xylem parenchyma cells, in comparison with those in extracellular freezing cortical parenchyma cells using Japanese white birch trees. In Japanese white birch, deep supercooling of xylem parenchyma cells (Kuroda *et al.*, 2003) and extracellular freezing of cortical parenchyma cells (Sakai & Larcher, 1987) have been shown in previous studies. This study is the first study focusing on the role of soluble sugars in the deep supercooling ability of xylem parenchyma cells.

## **Materials and Methods**

### *Plant material*

Fresh twigs that were approximately 4 years old were cut from adult Japanese white birch (*Betula platyphylla* Sukat. var. *japonica* Hara) trees grown on the campus of Hokkaido University. Cut twigs were immediately placed on crushed ice and transported to the laboratory. The samples were divided into xylem and cortical tissues by peeling the bark off with a razor blade, and both tissues were processed for freezing resistance determination, soluble sugar extraction, differential thermal analysis (DTA) and microscopy.

### *Determination of freezing resistance ( $LT_{50}$ )*

Xylem tissues were sectioned (1 mm in the tangential direction x 5 mm in the radial direction x 15 mm in the axial direction) and three or four pieces were placed into a test tube with 0.5 ml of deionized water. These samples were maintained in a programmable freezer (ES-100P, Tajiri Co., Ltd., Sapporo, Japan) at  $-3^{\circ}\text{C}$  for 30 min. Freezing of samples in the test tubes was uniformly initiated by seeding with ice chips, and samples were maintained at  $-3^{\circ}\text{C}$  for an additional hour. Samples were subsequently cooled at a rate of  $0.2^{\circ}\text{C min}^{-1}$  to various temperatures, the final temperature being  $-60^{\circ}\text{C}$ . Shortly after reaching each given temperature, samples

were thawed overnight at 4°C in the dark. After adding 2.5 ml of deionized water to each test tube, the samples were gently agitated for 6 hours at room temperature in the dark to facilitate leakage of ions from the freeze-damaged samples. Electrolyte leakage was subsequently measured with a conductivity meter (B-173, Horiba Ltd., Kyoto, Japan). Samples were subsequently boiled for 10 min to induce complete injury (determined as 100% electrolyte leakage), and electrolyte leakage was measured again. The electrolyte leakage of fresh unfrozen samples was also measured and used as a baseline to estimate no injury (determined as 0% leakage). Based on these values, the survival (%) of the frozen samples was calculated (Fujikawa & Miura, 1986) and the minimum temperature that led to 50% survival (LT<sub>50</sub>) was identified as the freezing resistance temperature.

#### *Removal of apoplastic sugars*

Apoplastic sugars were removed to estimate intracellular soluble sugar contents. To facilitate the removal of apoplastic sugars, debarked xylem tissues (debarked twigs) were wrapped with parafilm along the debarked lateral faces and cut transversally into segments of 15 mm in length. A reservoir was connected to one side of the transverse plane of each segment and connected by parafilm. Each segment was fixed vertically with the transverse plane attached with the reservoir upside. Deionized water was placed in the reservoir to make water flow out to the other side of the transverse plane through the xylem. The washing of xylem tissue was continued for one hour with continuous addition of new deionized water.

On the other hand, cortical tissues were cut into small segments of 2 mm in length and placed in a sufficient volume of water for one hour with exchange of water three times.

Although it might not be complete removal, it was confirmed that these procedures for each tissue effectively removed the apoplastic sugars because further elution of sugars from both tissues was not detected after treatment for 5 hours by the anthron-sulfuric acid method (Yemm & Willis, 1954).

#### *Analysis of amino acids*

In order to estimate damage of cells caused by the above-described washing procedure, which might induce release of intracellular sugars, amino acid contents were measured in each tissue before and after washing. For measurement of amino acid contents (Inada *et al.*, 2006), samples before and after washing were cut into small segments and rinsed in water. Approximately 30 mg of segments were mixed with 2 ml of deionized water in a test tube. They were boiled for 10 min and cooled to 20°C for one hour in the dark. One hundred  $\mu$ l of boiled solution was mixed with 0.4 ml of 0.5 M citrate buffer (pH 5.0) containing 7 mM SnCl<sub>2</sub>. After addition of 1 ml of 4% (w/v) ninhydrin in 2-methoxyethanol, the solution was boiled for 20 min. The solution was then cooled and absorbance at 570 nm was measured. Calibration curves were prepared using glycine as a standard.

#### *Quantitative analysis of soluble sugars*

Xylem or cortical tissues were scraped by a razor blade to small tips. The weights of some of the samples before (fresh weight, FW) and after oven-drying at 80°C for 36 hours (dry weight, DW) were measured. Approximately 200 mg of these tissue tips were separately frozen in LN<sub>2</sub> and ground into fine pieces with a mortar and pestle. Five ml of 80% (v/v) ethanol was added to the fine pieces, and the samples were treated with ultrasonication for 10 min. Sonicated samples were maintained at room temperature overnight for extraction of soluble sugars, and lactose was added to the ethanol extract as an internal standard. Extracts were centrifuged at 14,000  $\times$ g for 5 min and the supernatant was collected. Ethanol in the supernatant was evaporated under a stream of nitrogen gas and the resultant aqueous solution was lyophilized. Lyophilized samples were redissolved in 0.3 ml distilled water and centrifuged at 14,000  $\times$ g for 5 min to remove insoluble materials. The sugar composition of the supernatant was analyzed by high-performance liquid chromatography (HPLC) with an NH2P-50 4E column (Shodex, Tokyo, Japan), and 75% (v/v) acetonitrile in water was used as the mobile phase. Eluate from the HPLC column was monitored by an RI detector (L-7490, Hitachi High-Technologies Corporation, Tokyo, Japan), and sugars were identified and quantified on the basis of retention times and peak areas relative to those of standard sugars.

### *Measurement of intracellular melting point by DTA*

Melting points of xylem and cortical parenchyma cells were determined by DTA. A block of fresh tissue (300 mg) from either bark or xylem tissue was connected to a thermocouple by wrapping it with parafilm. Samples were frozen by direct immersion in LN<sub>2</sub> and were rapidly warmed maintaining samples within a test tube kept at -20°C for one hour in an alcohol bath (F26, Julabo Labortechnik GmbH, Seelback, Germany). Samples were then warmed at a rate of 0.1°C min<sup>-1</sup>, and the differences between thermal responses of frozen samples and oven-dried reference samples were recorded.

### *Measurement of intracellular melting points by cryo-scanning electron microscopy (Cryo-SEM)*

Melting points of both tissue cell types were also analyzed with Cryo-SEM by ultrastructural observation of freezing and melting states of intracellular water. Small blocks of xylem (3 mm in the tangential direction x 4 mm in the radial direction x 3 mm in the axial direction) or cortical tissues (3 mm in the tangential direction x 1 mm in the radial direction x 4 mm in the axial direction) cut from fresh twigs were mounted in a specimen holder and were frozen by direct immersion in LN<sub>2</sub>. The frozen samples were warmed overnight in an alcohol bath by placing them in test tubes that were maintained at -8, -6, -4, -2, -1 and 0.5°C. Samples that had been warmed to each temperature were then cryofixed by direct immersion in LN<sub>2</sub>.

Cryofixed samples were transferred to a cold stage of a Cryo-scanning electron microscope (840A-SEM equipped with a cryo-unit, JEOL Co., Ltd., Tokyo, Japan). The samples were fractured, etched, and coated with platinum-carbon at -100°C in a specimen preparation chamber. The samples were then transferred to a cold stage in an SEM column that was kept at -160°C. Secondary emission images were observed and photographed at an accelerating voltage of 5 kV.

### *Light microscopy*

Small twig segments (5 mm in length) containing xylem and cortical tissues were fixed overnight in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.0)

at room temperature. Samples were subsequently washed with 0.1 M phosphate buffer solution, dehydrated using a graded ethanol series, and embedded in epoxy resin. Cross sections for cortical tissues and tangential sections for xylem tissues were cut to a thickness of 10  $\mu\text{m}$  by a steel blade mounted on a sliding microtome (Yamato Kohki Industrial Co., Ltd., Saitama, Japan). Sections were stained with a 1% aqueous solution of gentian violet and observed by a light microscope (Optiphot-2, Nikon Corporation, Tokyo, Japan), and light microscopic images were digitally recorded. Percentage of parenchyma cells occupying total sections were estimated by using scion image version beta 4.0.2 (National Institutes of Health, MD, USA).

## **Results**

### *Seasonal changes in freezing resistance in xylem parenchyma cells*

The freezing resistance of xylem parenchyma cells exhibited a dramatic seasonal change as determined by electrolyte leakage (Fig. 1). Temperatures for freezing resistance as determined by  $LT_{50}$  ranged between  $-10^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  in summer (June to October), decreased gradually from late October to November, reached  $-60^{\circ}\text{C}$  in mid-winter (December to March) and increased again in spring (mid-March to late May).

### *Seasonal changes in accumulation of soluble sugars in whole xylem tissues*

Seasonal changes in accumulation of soluble sugars in whole xylem tissues were examined by HPLC analysis. The sugars analyzed in this experiment represent a total fraction that originated from both extracellular (apoplast) spaces and xylem parenchyma cells. Analysis of HPLC profiles revealed 5 distinct peaks that corresponded to fructose, glucose, sucrose, raffinose and stachyose. Both the total amount of sugars and the amount of each individual sugar species increased significantly in winter and decreased in summer in total xylem tissues on an FW basis (Fig. 2). The water contents in xylem tissues of Japanese white birch fluctuated within narrow range between 44 and 54%, throughout all seasons with higher contents during summer than

during winter (see Table 1).

#### *Seasonal changes in accumulation of soluble sugars in xylem parenchyma cells*

In order to estimate the seasonal changes in accumulation of soluble sugars in xylem parenchyma cells, xylem tissues were washed with water to remove apoplastic sugars. As a result of washing, soluble sugars were decreased 20 to 50% from total xylem sugars. Although it is not clear whether apoplastic sugars were completely removed by this procedure, no additional removal occurred after washing for 5 hours. It should be noted that amino acid contents did not significantly change before and after washing (data not shown), and it was therefore concluded that the washing almost completely removed only apoplastic sugars. After washing, amounts of sucrose, raffinose and stachyose were found to clearly increase during winter, whereas amounts of fructose and glucose did not show any clear seasonal fluctuations (Fig. 3). The clear increase in amounts of fructose and glucose in winter and decrease in their amounts in summer in whole xylem tissues (Fig. 2) confirmed that these sugars change seasonally only in the apoplast and not within xylem parenchyma cells. Thus, an increase in the total amounts of soluble sugars in xylem parenchyma cells during winter, especially the specific accumulation of sucrose, raffinose and stachyose, directly correlated with the increase in freezing resistance of xylem parenchyma cells during winter (Fig. 1).

#### *Comparison of amounts of soluble sugars in xylem and cortical tissues before and after washing*

We compared the patterns of accumulation of soluble sugars in xylem and cortical tissues (Fig. 4). Patterns of seasonal accumulation were compared before (Fig. 4a) and after (Fig. 4b) washing out apoplastic sugars from both cortical and xylem tissues. In both whole xylem and cortical tissues (before washing) as well as in their parenchyma cells (after washing), the total amounts of soluble sugars increased in winter (Fig. 4). The major sugars that accumulated in both cortical and xylem parenchyma cells were similar. Fructose, glucose and sucrose accumulated in all seasons, whereas raffinose and stachyose accumulated only in winter.

### *Comparison of amounts of soluble sugars in xylem and cortical parenchyma cells*

A comparison of the accumulation of sugars showed that the amounts of sugars were much larger in cortical parenchyma cells than in xylem parenchyma cells in both winter and summer (Fig. 4). However, analysis of occupancy rates of parenchyma cells in both tissues revealed that xylem parenchyma cells occupied less than 6% of the total xylem areas in tangential sections (Fig. 5a), whereas cortical parenchyma cells occupied more than 66% of total cortical areas in cross sections (Fig. 5b). The areas observed for percentages of cell occupancy were the same areas of tissue that were sampled for HPLC analysis. Calculations based on two-dimensional occupancy rates showed that the concentration of sugars is much greater in xylem parenchyma cells than in cortical parenchyma cells both in winter (7.1-times and 6.1-times greater on a DW basis and an FW basis, respectively) and summer (4.4-times and 4.5-times greater on a DW basis and an FW basis, respectively) (Table 1).

### *Comparison of intracellular melting points of xylem and cortical parenchyma cells*

In order to confirm the difference between intracellular osmotic concentrations of xylem and cortical parenchyma cells, the melting points of these cells in tissues harvested in winter were compared by DTA. For DTA, intracellular water within fresh tissues was frozen by direct immersion in LN<sub>2</sub>. The frozen tissues were then slowly warmed at a rate of 0.1°C min<sup>-1</sup> and their endothermal profiles were recorded (Fig. 6). In both tissues, the warming profiles showed two clear endothermal peaks, a low temperature endotherm and a high temperature endotherm. It is thought that the low temperature endotherm is produced by the melting of intracellular water in parenchyma cells and the high temperature endotherm is produced by the melting of apoplastic water. The peak temperature for the low temperature endotherm of xylem tissues ( $-3.23 \pm 0.31^\circ\text{C}$ , n = 5) was lower than that of cortical tissues ( $-1.74 \pm 0.27^\circ\text{C}$ , n = 5), suggesting that the osmotic concentration in xylem cells was relatively higher than that in cortical cells. Although DTA revealed the difference between melting points of xylem and cortical parenchyma cells, we were unable to confirm the equilibrium melting points of the cells due to the fast warming rates that must be employed to optimize DTA analysis. In both tissues, the high temperature endotherm was far beyond 0°C to warmer

temperatures, suggesting that these cells did not undergo equilibrium warming.

In order to detect the equilibrium melting points of the cells, Cryo-SEM observation was performed. For Cryo-SEM observation, intracellular water within fresh tissues that were harvested in winter was frozen by direct immersion in LN<sub>2</sub>, and samples were rewarmed to subzero temperatures, equilibrated, and subsequently cryofixed by direct immersion in LN<sub>2</sub>. The differences among still frozen, partially melted and completely melted states were distinguished by the difference in sizes of intracellular ice crystals (Fig. 7). Direct immersion of fresh samples in LN<sub>2</sub> resulted in the production of numerous small ice crystals, which were almost undetectable by low-magnified Cryo-SEM images (Fig. 7a). These small and almost undetectable ice crystals are indicative of a liquid state of water in xylem parenchyma cells. After rewarming the samples, when intracellular water was still frozen, a few large intracellular ice crystals of more than 5 μm in diameter were produced (Fig. 7b, e). The formation of such large ice crystals is due to the recrystallization of numerous small ice crystals that were initially produced by freezing with LN<sub>2</sub>. When partial melting occurred by warming, intermediate-sized ice crystals (0.5 μm to 5 μm) were produced, probably due to the partial melting of large recrystallized ice crystals (Fig. 7c, f). Similar to the control sample (Fig. 7a), when complete melting occurred by rewarming, numerous undetectable small ice crystals were produced due to the cryofixation of liquid water (Fig. 7d). The results obtained by Cryo-SEM showed that the equilibrium melting points in winter were approximately -4°C and -2°C in xylem and cortical cells, respectively (Table 2).

## Discussion

Xylem parenchyma cells in most trees have characteristically thick and rigid cell walls. Due to the specific nature of their cell wall structure, xylem parenchyma cells are able to adapt to subfreezing temperatures by deep supercooling (Fujikawa & Kuroda, 2000). A previous Cryo-SEM study indicated that xylem parenchyma cells in boreal hardwood species, including Japanese white birch, adapt to subfreezing temperatures by deep supercooling that is accompanied by incomplete dehydration (Kuroda *et al.*, 2003). Other Cryo-SEM studies have also shown that the temperature limit for deep

supercooling in xylem cells of Japanese white birch changes from  $-60^{\circ}\text{C}$  in winter to  $-15^{\circ}\text{C}$  in summer (Kuroda *et al.*, 1999; Kuroda *et al.*, 2003). The temperature limit for supercooling in xylem parenchyma cells of Japanese white birch, therefore, corresponds to the survival of cells, which was determined by electrolyte leakage with  $\text{LT}_{50}$  in this study (Fig. 1). Many studies have also indicated that the temperature limit for supercooling corresponds to the temperature limit for survival of xylem cells in many hardwood species (Quamme *et al.*, 1972; George *et al.*, 1974; Hong *et al.*, 1980; Becwar *et al.*, 1981; Fujikawa & Kuroda, 2000).

In this study, we analyzed seasonal changes in accumulation of soluble sugars in deep supercooling xylem parenchyma cells in Japanese white birch. This approach was taken in an effort to determine effects of soluble sugars on the ability for cells to supercool. Our results showed that amounts of total soluble sugars not only in the whole xylem before washing out apoplastic sugars (Figs 2 and 4a) but also in xylem parenchyma cells after washing out apoplastic sugars (Figs 3 and 4b) increased during winter in parallel with increased supercooling ability (freezing resistance) of xylem parenchyma cells (Fig. 1). When we focused on intracellular soluble sugars after washing out apoplastic sugars, we found that sucrose, raffinose and stachyose increased in xylem parenchyma cells during winter (Figs 3 and 4b) with direct correlation to enhanced supercooling ability (freezing resistance) of xylem parenchyma cells (Fig. 1). Fructose and glucose also increased during winter but only in the apoplast of xylem tissues (compare Fig. 2 with Fig. 3).

Although the seasonal accumulation of soluble sugars in trees has been analyzed in many studies, almost all of those studies focused on sugars in cortical tissues with cortical parenchyma cells (Parker, 1962; Li *et al.*, 1965; Sakai & Larcher, 1987), which adapt to subfreezing temperatures by extracellular freezing (Sakai & Larcher, 1987; Malone & Ashworth, 1991). Seasonal accumulation of sugars in total stems that included both cortical and xylem tissues has been analyzed only in several studies (Nelson & Dickson, 1981; Fege & Brown, 1984; Bonicel *et al.*, 1987). There have also been a few studies on seasonal changes in accumulation of soluble sugars in xylem tissues in boreal hardwood species, including red osier dogwood (*Cornus sericea* L.) (Ashworth *et al.*, 1993), poplar (*Populus x canadensis* Moench) (Sauter & Kloth, 1987; Sauter *et al.*, 1996), willow (*Salix caprea* L.) (Sauter & Wellenkamp, 1998), and silver birch (*Betula pendula* Roth) (Piispanen & Saranpää, 2001).

However, none of those previous studies on seasonal accumulation of soluble sugars in xylem tissues focused on the relationship with deep supercooling capability of xylem parenchyma cells, because it has been thought for many years that xylem parenchyma cells of boreal hardwood species, such as willow, poplar, birch and red osier dogwood, adapt to subfreezing temperatures by extracellular freezing as do cortical parenchyma cells (Sakai & Larcher, 1987; Fujikawa & Kuroda, 2000). However, a recent Cryo-SEM study confirmed that xylem parenchyma cells in all of these boreal hardwood species respond to subfreezing temperatures by deep supercooling (Kuroda *et al.*, 2003).

Although the relationship between deep supercooling capability and accumulation of soluble sugars in xylem parenchyma cells was not examined in previous studies, the results of previous studies are similar to the results obtained in the present study for seasonal changes in soluble sugar accumulation in tissues of Japanese white birch. Greater accumulation of total soluble sugars in xylem parenchyma cells of boreal hardwood species during winter has been reported (Sauter *et al.*, 1996; Sauter & Wellenkamp, 1998). Significant accumulation of raffinose and stachyose was also observed during winter in the whole xylem of red osier dogwood (Ashworth *et al.*, 1993). Furthermore, it has been reported that a H<sup>+</sup>-hexose uptake carrier is inactivated during winter in xylem ray tissues in poplar (Himpkamp, 1988), which may result in accumulation of hexoses, such as fructose and glucose, in xylem apoplasts during winter (Figs 2 and 3). Collectively, results of these previous studies and the present study have indicated similar trends in seasonal accumulation patterns of soluble sugars in deep supercooling xylem parenchyma cells in boreal hardwood species.

The increased amounts of total soluble sugars, specifically intracellular accumulation of sucrose and/or its galactosides, in xylem parenchyma cells were associated with increase in supercooling capability (freezing resistance). However, it should be noted that such a seasonal change in soluble sugar accumulation in xylem parenchyma cells also occurs in cortical parenchyma cells in Japanese white birch, which adapt by extracellular freezing (Fig. 4). Previous studies have also shown that amounts of total soluble sugars increase during winter in cortical tissues of many boreal hardwood species with specific increases in amounts of sucrose, raffinose and stachyose (Parker, 1962; Sakai & Larcher, 1987). In a related study, a similar seasonal accumulation of soluble sugar species was found to occur both in xylem and cortical tissues of red osier

dogwood (Ashworth *et al.*, 1993). It has been suggested that these sugars protect a wide variety of biological materials, such as macromolecules (Crowe *et al.*, 1987; Caffrey *et al.*, 1988), cells (Santarius, 1973), tissues (Koster & Leopold, 1988; Blackman *et al.*, 1992; Brenac *et al.*, 1997; Bomal *et al.*, 2002) and whole plants (Taji *et al.*, 2002), from freezing-induced dehydration during extracellular freezing.

Thus, our findings in this study provide the first evidence that there is no specific difference between the accumulation of soluble sugars in xylem parenchyma cells and that in cortical parenchyma cells, which have different freezing adaptation mechanisms. However, we did find one clear difference concerning the accumulation of soluble sugars in xylem and cortical parenchyma cells with respect to their concentration. Our combined estimation, which was based on amounts of sugars in cells (after washing out of apoplastic sugars) and occupancy rates of parenchyma cells in both tissues, suggested that xylem parenchyma cells contain a much higher concentration of soluble sugars than do cortical parenchyma cells (Table 1). In red osier dogwood, the total sugar amount in cortical tissues on a DW basis was two-times higher than that recorded in xylem tissues (Ashworth *et al.*, 1993). Considering the difference in occupancy rates of parenchyma cells in tissues, however, xylem parenchyma cells of red osier dogwood may also contain a much higher concentration of soluble sugars than that in cortical parenchyma cells.

Our analysis of melting points in tissues provided evidence supporting the observation of higher accumulation of soluble sugars in xylem parenchyma cells of Japanese white birch. Profiles of DTA revealed lower melting points in xylem parenchyma cells than in cortical cells, suggesting relatively higher osmotic concentrations occur in xylem cells (Fig. 6). Cryo-SEM observation confirmed that the equilibrium melting points in winter were  $-4^{\circ}\text{C}$  in xylem parenchyma cells and  $-2^{\circ}\text{C}$  in cortical parenchyma cells (Table 2). These melting points correspond to intracellular osmotic concentrations of  $2.15 \text{ mol kg}^{-1}$  and  $1.08 \text{ mol kg}^{-1}$ , respectively (Cavender-Bares, 2005). We estimated that the concentration of soluble sugars in xylem parenchyma cells on an FW basis is 6.1-times higher than in cortical parenchyma cells during winter, whereas the difference between melting points of these cells was only  $2^{\circ}\text{C}$ . One possible explanation for this discrepancy, i.e., that large differences in the concentrations of soluble sugars were detected with only a minor difference in melting temperatures, may be due to the accumulation of osmolytes other than soluble

sugars in cortical parenchyma cells.

When solutes depress the equilibrium melting point of water, they also depress the nucleation temperature by nearly two-times for most solutes (Rasmussen & MacKenzie, 1972). Thus, it is possible that a high intracellular osmotic concentration in xylem parenchyma cells, which is mainly due to greater accumulation of soluble sugars, enhances the capacity for supercooling via melting point depression and consequent nucleation temperature depression. Furthermore, among the sugars that specifically accumulated in xylem parenchyma cells, sucrose has been shown to depress the homogeneous nucleation temperature of water by three-times (Charoenrein & Reid, 1989). These findings suggest that the supercooling ability of xylem parenchyma cells may be enhanced by a specific accumulation of sucrose.

On the other hand, it is possible that the accumulation of sugars serves another role to provide thermodynamic protection to some proteins in deep supercooling xylem cells. It has been suggested that protein structures in a non-frozen aqueous system are stabilized by sugars under the condition of temperature fluctuation as a “protein preferential hydration model” (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982). It is possible that the accumulation of sugars in xylem parenchyma cells during winter has a role in stabilization of cold-labile proteins under the condition of extremely low supercooling temperatures. The winter-specific accumulation of soluble sugars may also function to protect xylem parenchyma cells from dehydration stress. As already described, xylem parenchyma cells in boreal hardwood species, including Japanese white birch, are partially dehydrated during cooling (Kuroda *et al.*, 2003). Thus, the winter-specific accumulation of these sugars may confer dehydration tolerance to such xylem parenchyma cells, an event that occurs when the level of incomplete dehydration increases and results in a supercooling capability that reaches far below  $-40^{\circ}\text{C}$ .

While cold acclimation-induced changes related to the acquisition of freezing tolerance in plant cells that adapt to subfreezing temperatures by extracellular freezing have been investigated in many studies (Sakai & Larcher, 1987; Guy, 1990; Hughes & Dunn, 1996; Thomashow, 1999), few studies have focused on cold acclimation-induced changes that are associated with the acquisition of increased supercooling ability of xylem parenchyma cells in trees (Wisniewski & Ashworth, 1986; Arora *et al.*, 1992) despite the fact that the supercooling ability of xylem cells changes distinctly depending upon cold acclimation (Fujikawa & Kuroda, 2000). In this study, we showed that the

concentration of soluble sugars in xylem parenchyma cells fluctuates in association with change in supercooling ability (freezing resistance). Our recent study indicated that the supercooling ability of xylem parenchyma cells in beech trees (*Fagus crenata* Blume) was significantly reduced by disruption of protoplasts (Kasuga *et al.*, 2006). A significant reduction in supercooling ability may arise from dilution of intracellular solutions through mixing with extracellular solutions by disruption of protoplasts. Further studies focusing on the diverse cold acclimation-induced changes of xylem parenchyma cells in association with changes in their supercooling ability are necessary to fully understand the deep supercooling mechanisms of xylem parenchyma cells in trees.

### **Acknowledgements**

The authors wish to thank Dr. T. Suzuki and Mr. T. Ito (Graduate School of Agriculture, Hokkaido University) for their technical advice and support. This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Sports, Culture, Science and Technology of Japan (17380101 to S.F.) and a grant from the Japan Society for the Promotion of Science (17-9014 to J.K.).

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### **Figure legends**

**Fig. 1.** Seasonal changes in temperature limit for survival of xylem parenchyma cells as revealed by  $LT_{50}$ .

**Fig. 2.** Seasonal changes in soluble sugar contents in whole xylem tissues. Sugars were extracted from the whole xylem including apoplasts and xylem parenchyma cells. FW, fresh weight. Results are shown as mean values  $\pm$  S.D. (n = 3).

**Fig. 3.** Seasonal changes in intracellular soluble sugar contents in xylem parenchyma cells. Sugar contents were measured after washing out apoplastic sugars. FW, fresh weight. Results are shown as mean values  $\pm$  S.D. (n = 3).

**Fig. 4.** Comparison of sugars in xylem (X) and cortical (C) tissues. (a) Comparison of sugars in whole tissues containing sugars in both parenchyma cells and apoplasts (before washing). (b) Comparison of sugars in parenchyma cells (after washing). Samples were harvested in winter (January) and summer (June). FW, fresh weight. Results are shown as means  $\pm$  S.D. (n = 3).

**Fig. 5.** Light microscopic photographs showing distribution of parenchyma cells in xylem and cortical tissues. (a) A tangential section of xylem tissue. Xylem tissue was constructed of wood fibers (wf), vessel elements (ve) and xylem parenchyma cells. Arrows show some ray parenchyma cells. (b) A cross section of cortical tissue. For sugar analysis in cortical tissues, tissues including the area from the cambial zone (cz) to epidermis (ep) were used. Many living cells exist in this area except for regions occupied by epidermis and phloem fibers (pf). Bars = 100  $\mu$ m.

**Fig. 6.** Comparison of melting points in xylem and cortical parenchyma cells from twigs harvested in winter (January). Both tissues were frozen by direct immersion in

liquid nitrogen and warmed at a rate of  $0.1^{\circ}\text{C min}^{-1}$ . In both tissues during warming, differential thermal analysis revealed two endotherms by melting of intracellular water (low temperature endotherm: arrowheads) and melting of extracellular water (high temperature endotherm: arrows).

**Fig. 7.** Cryo-scanning electron microscopic photographs for determining equilibrium melting points of intracellular water in xylem parenchyma cells (a-d) and cortical parenchyma cells (e and f) from twigs harvested in winter (January). (a) Xylem tissues were frozen from room temperature by directly immersing them in liquid nitrogen ( $\text{LN}_2$ ). The intracellular ice crystals produced by this cryofixation technique are very small and difficult to detect. (b) Xylem tissues frozen by  $\text{LN}_2$  were rewarmed to  $-6^{\circ}\text{C}$  overnight and cryofixed again for analysis. The arrow shows a large intracellular ice crystal produced by recrystallization, suggesting that freezing was still occurring. (c) Xylem tissues frozen by  $\text{LN}_2$  were rewarmed to  $-4^{\circ}\text{C}$  overnight and cryofixed again for analysis. The size of intracellular ice (arrows) became smaller than that in the case of warming to  $-6^{\circ}\text{C}$ , suggesting the start of melting. (d) Xylem tissues frozen by  $\text{LN}_2$  were rewarmed to  $-2^{\circ}\text{C}$  overnight and cryofixed again for visualization. Ice crystals were very small and difficult to detect, and are indicative of melting. (e) Cortical tissues frozen by  $\text{LN}_2$  were rewarmed to  $-6^{\circ}\text{C}$  overnight and cryofixed again. The arrow shows a large intracellular ice crystal produced by recrystallization. (f) Cortical tissues frozen by  $\text{LN}_2$  were rewarmed to  $-2^{\circ}\text{C}$  overnight and cryofixed again. Arrows show ice crystals. Bars =  $10\ \mu\text{m}$ .

**Table 1.** Comparison of amounts of soluble sugars in xylem and cortical parenchyma cells in winter (January) and summer (June)

\*Comparison was made by dividing the dry weight (DW) or fresh weight (FW) of recovered sugars from total tissue after washing by the occupancy rate of parenchyma cells in tissues. The calculation for summer samples was done with occupancy rates of parenchyma cells in winter.

**Table 2.** Cryo-scanning electron microscopic results showing equilibrium melting points of xylem and cortical parenchyma cells as revealed by percentage of cells with different sizes of intracellular ice crystals in winter (January) samples

\*Values are means  $\pm$  S.D. Each value was obtained from 100 cells in total from 3 samples.

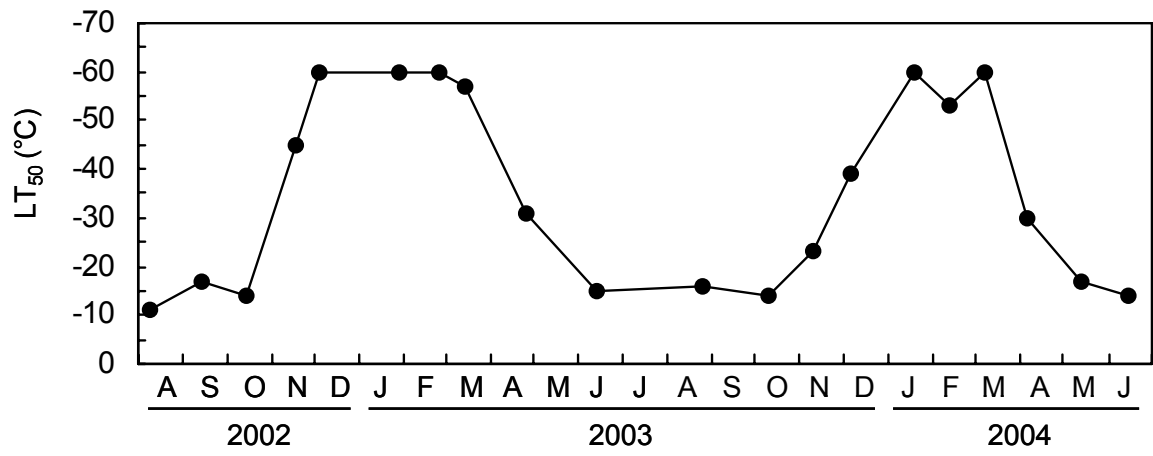


Fig. 1

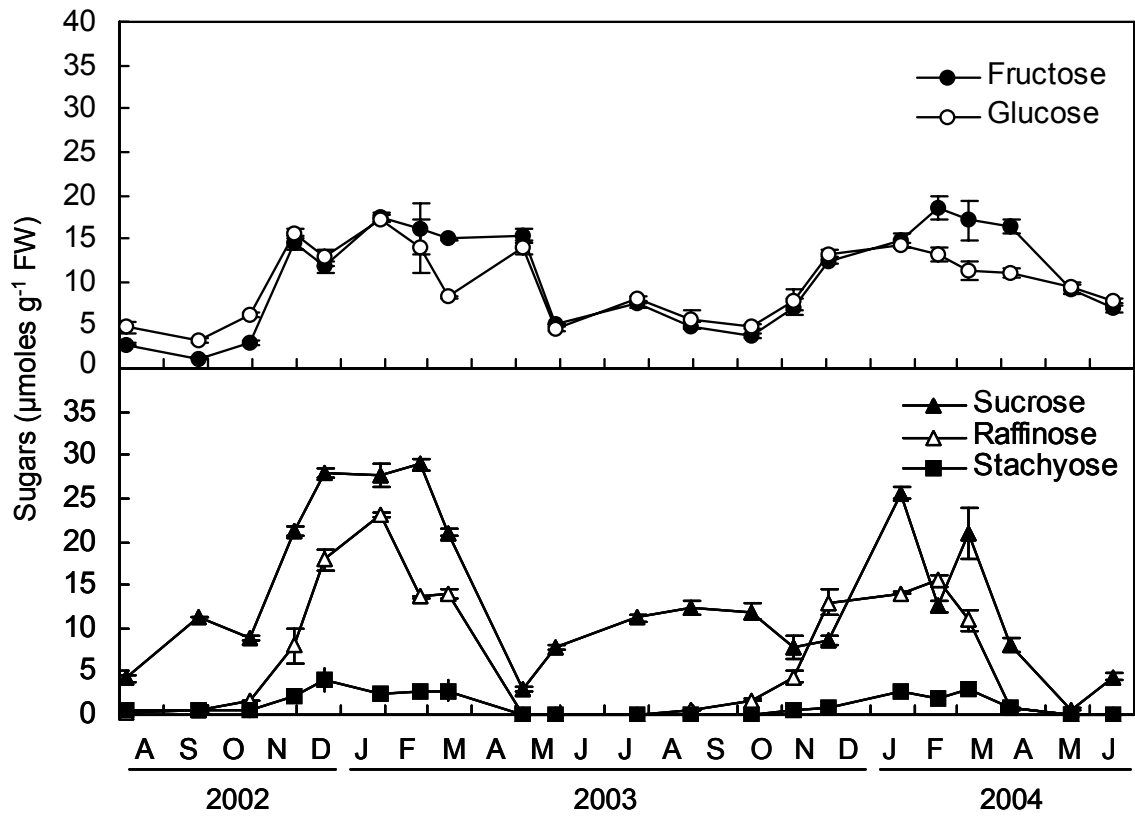


Fig. 2

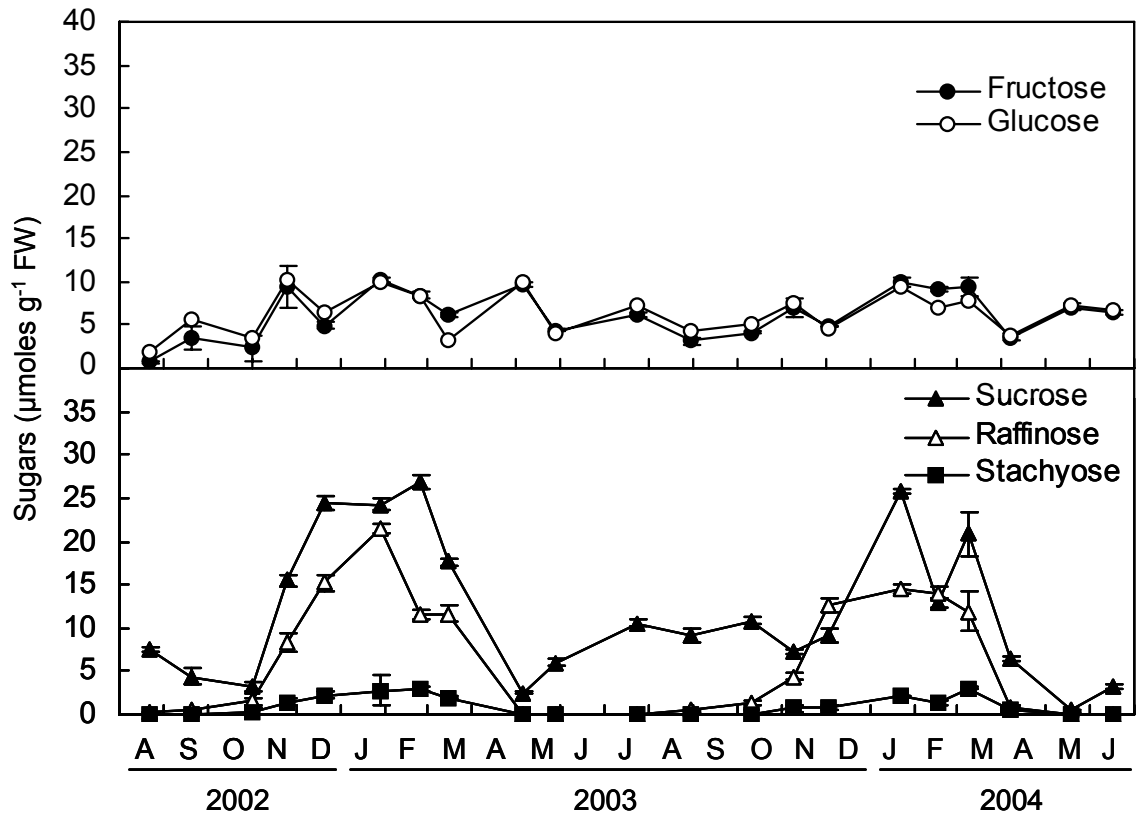
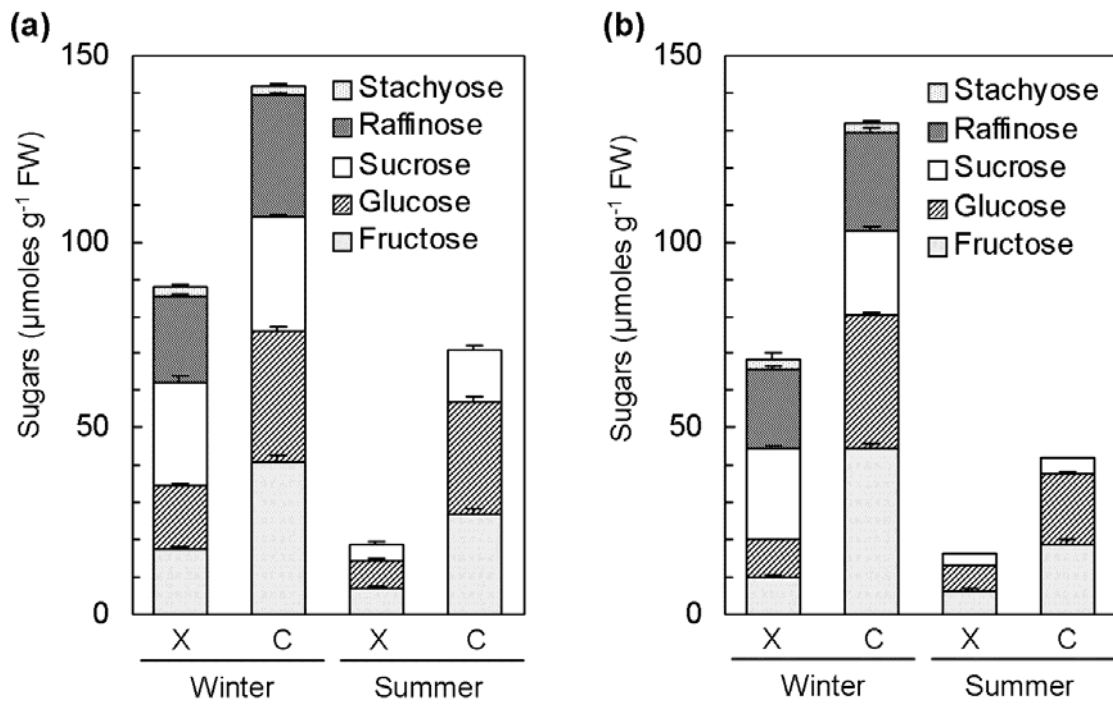
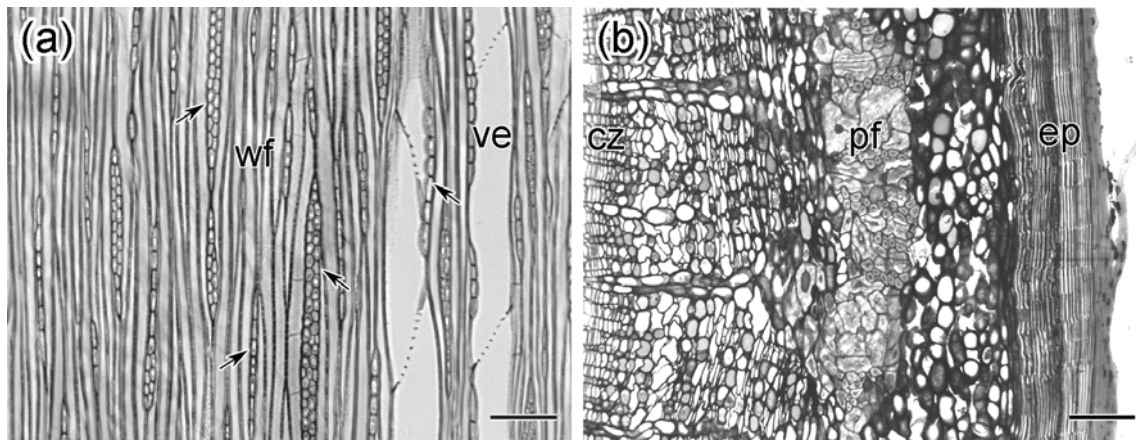


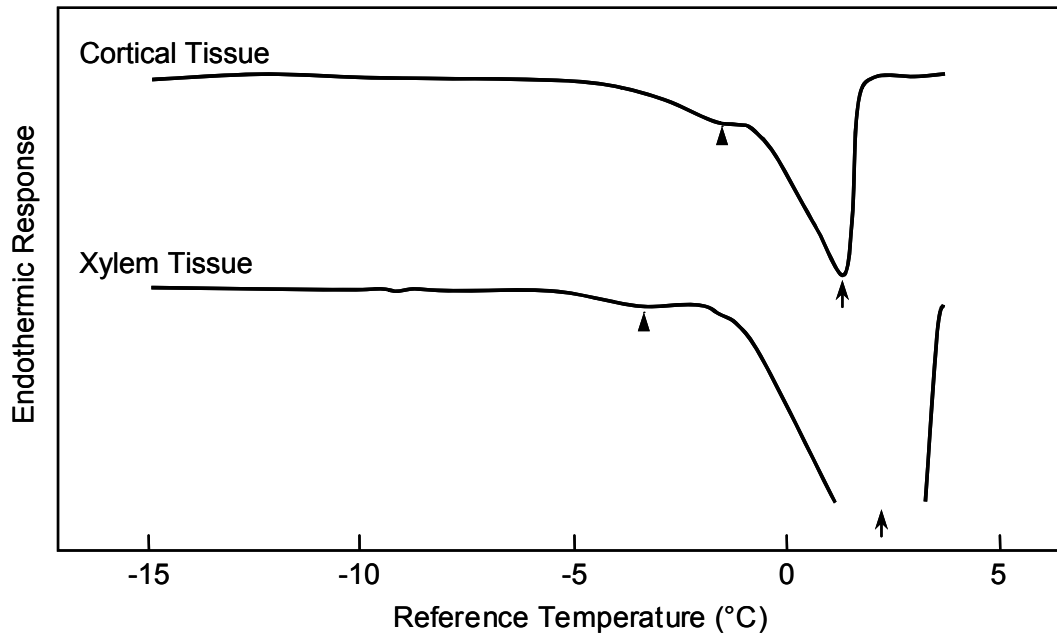
Fig. 3



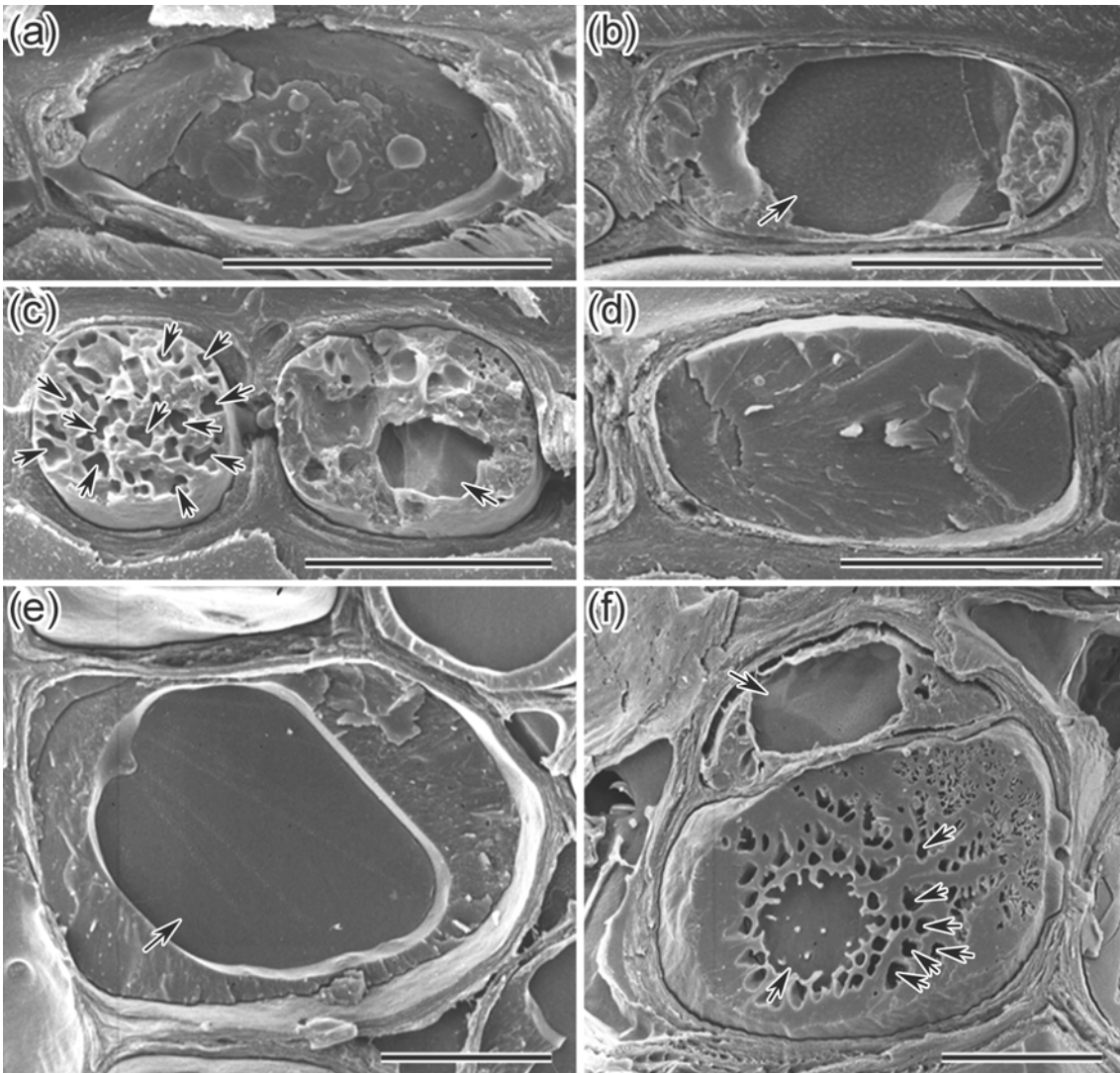
**Fig. 4**



**Fig. 5**



**Fig. 6**



**Fig. 7**

	Tissue	Water content (%)	A		B	A/B	
			Sugar content in total tissue ( $\mu\text{moles g}^{-1}$ DW) ( $\mu\text{moles g}^{-1}$ FW)		Occupancy rate of parenchyma cells (%)	Sugar content in parenchyma cells ( $\mu\text{moles g}^{-1}$ DW) ( $\mu\text{moles g}^{-1}$ FW)	
Winter	Xylem	45.7 $\pm$ 1.0	124.2 $\pm$ 3.9	68.7 $\pm$ 3.0	5.7 $\pm$ 1.8	2179	1205
	Cortex	35.6 $\pm$ 1.9	205.9 $\pm$ 2.5	131.8 $\pm$ 3.5	66.7 $\pm$ 4.9	309	198
Summer	Xylem	48.7 $\pm$ 1.5	31.6 $\pm$ 0.5	16.2 $\pm$ 0.3	-	554	284
	Cortex	50.2 $\pm$ 3.5	84.0 $\pm$ 3.6	41.8 $\pm$ 1.8	-	126	63

**Table 1**

Tissue	Size of ice crystals ( $\mu\text{m}$ )	Temperature ( $^{\circ}\text{C}$ )				
		-8	-6	-4	-2	-1
Xylem	>5	99 $\pm$ 1	87 $\pm$ 1	49 $\pm$ 10	0 $\pm$ 0	-
	5-0.5	0 $\pm$ 0	11 $\pm$ 2	50 $\pm$ 12	0 $\pm$ 0	-
	<0.5	2 $\pm$ 1	2 $\pm$ 2	2 $\pm$ 2	100 $\pm$ 0	-
Cortex	>5	-	100 $\pm$ 1	89 $\pm$ 5	55 $\pm$ 8	0 $\pm$ 0
	5-0.5	-	0 $\pm$ 0	2 $\pm$ 2	8 $\pm$ 5	1 $\pm$ 1
	<0.5	-	1 $\pm$ 1	9 $\pm$ 4	39 $\pm$ 8	99 $\pm$ 1

**Table 2**