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Author(s)	Kasuga, Jun; Mizuno, Kaoru; Miyaji, Natsuko; Arakawa, Keita; Fujikawa, Seizo
Citation	CryoLetters, 27(5), 305-310
Issue Date	2006-09
Doc URL	<a href="http://hdl.handle.net/2115/30142">http://hdl.handle.net/2115/30142</a>
Type	article (author version)
File Information	CRL27-5.pdf



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# **ROLE OF INTRACELLULAR CONTENTS TO FACILITATE SUPERCOOLING CAPABILITY IN BEECH (*Fagus crenata*) XYLEM PARENCHYMA CELLS**

Jun Kasuga, Kaoru Mizuno, Natsuko Miyaji, Keita Arakawa and Seizo Fujikawa\*  
Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan.

\*Corresponding author – [sfuji@for.agr.hokudai.ac.jp](mailto:sfuji@for.agr.hokudai.ac.jp)

## **Abstract**

In order to find the possible role of intracellular contents in facilitating the supercooling capability of xylem parenchyma cells, changes in the temperature of supercooling levels were compared before and after the release of intracellular substances from beech xylem parenchyma cells by DTA. Various methods were employed to release intracellular substances from xylem parenchyma cells and all resulted in a reduction of supercooling ability. It was concluded that the reduction of supercooling ability primarily resulted from changes of intracellular conditions, including the release of intracellular contents or their mixing with extracellular solutions, rather than due to changes of cell wall structures. It is therefore suggested that any unidentified intracellular contents may function to facilitate supercooling capability in xylem parenchyma cells.

**Keywords:** Deep supercooling, xylem parenchyma cells, cold acclimation, intracellular freezing

## **INTRODUCTION**

Xylem parenchyma cells of trees adapt to subfreezing temperature by deep supercooling (15, 20). Until now, the mechanism which maintains water in a supercooled liquid state in xylem parenchyma cells for long periods of time at low temperatures has only been explained by a physical phenomenon (1). It was 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 the presence of barriers by specific cell walls that neither allow for dehydration of protoplasts nor intracellular penetration of extracellular ice to protoplasts (1, 10, 18). Thus, xylem parenchyma cells can act as an isolated droplet and their intracellular water can supercool to the homogenous ice nucleation temperature which is approximately  $-40^{\circ}\text{C}$  (2, 16).

Undoubtedly, the isolation of protoplasts from the effects of extracellular ice crystals by

the presence of specific cell walls is a prerequisite for achieving supercooling in xylem parenchyma cells. However, a question arises whether the mechanism for deep supercooling of xylem parenchyma cells can be explained only by such a physical effect due to cell walls. The limiting temperature of supercooling demonstrates distinct seasonal changes that are a direct result of seasonal cold acclimation and deacclimation (7, 12, 21). Furthermore, the capability for supercooling is rapidly reduced by exposure to warming temperatures (11). The incidence of nucleation of isolated water droplets in a metastable equilibrium depends upon a function of the size and the time of cooling, where smaller droplets and higher cooling rates yield lower nucleation temperatures (16). However, the supercooling of xylem parenchyma cells often spans periods that are greater than several weeks or more under natural conditions. This period of cooling time is far greater than the cooling of isolated water droplets that has been performed under experimental conditions. In addition, there is no evidence to suggest that xylem parenchyma cells lack heterogeneous ice nucleating substances.

The aforementioned evidences support the notion that not only the physical state of protoplasts that is due to the cell wall properties, but also intracellular contents might function to stabilize or facilitate supercooling ability in xylem parenchyma cells. In the present study, therefore, we performed preliminary examinations on the effect of intracellular conditions in relation to changes of supercooling ability in beech xylem parenchyma cells. We compared the change of supercooling temperatures in xylem parenchyma cells of beech by differential thermal analysis (DTA) before and after several treatments of xylem tissue. These treatments were performed to initiate the release of intracellular substances from the cells or to produce mixing of them with extracellular solutions. From these results, it was concluded that intracellular contents might greatly affect the supercooling capability in xylem parenchyma cells.

## **MATERIALS AND METHODS**

### *Materials*

Five year-old twigs were cut from mature beech (*Fagus crenata*) trees that were growing on the campus of Hokkaido University in winter (early to late February). Bark and pith tissues were removed from the twigs and the xylem tissues were further cut into small blocks (5 x 5 x 5 mm) at 4°C.

### *Pretreatments of xylem*

For fresh control samples, xylem blocks that were maintained at 4°C for 1 h were used

for DTA. For boiled samples, xylem blocks were heated in boiling water for 1 h, washed and placed in distilled water at 4°C for an additional 1 h. For freezing treatments at different cooling rates, fresh xylem blocks were frozen from 4°C to –60°C at a cooling rate of 0.2°C/min. These samples were then thawed to 4°C shortly thereafter at a warming rate of 0.2°C/min and equilibrated at 4°C for 1 h. For alternative cooling methods, fresh xylem blocks were frozen from 4°C by abrupt immersion into either liquid nitrogen (cooling rate of about 1,000°C/min to –196°C) (3) or into partially frozen Freon 22 (cooling rate of about 10,000°C/min to –150°C) (3). After both of these freezing treatments, samples were soon thawed at 4°C for 1 h.

#### *Differential Thermal Analysis*

All of the samples described above were connected to a thermocouple, equilibrated at 4°C and were then cooled at a rate of 0.2°C/min to –60°C and the changes in temperature (exotherms) were recorded. Analyses were performed on three independent examinations in each case and typical exothermic profiles are illustrated in the figure.

#### *Cryo-scanning electron microscope (cryo-SEM) observation*

The boiled samples were placed in SEM specimen holders and cooled from 4°C to the desired temperatures at a cooling of 0.2°C/min, similar to cooling by DTA. Soon after reaching the desired temperatures, samples were cryofixed by direct immersion in liquid nitrogen and stored in liquid nitrogen until used.

Cryofixed samples were transferred to a cold stage on a cryo-SEM (840A-SEM equipped with a cryo-unit, JEOL Co., Ltd., Tokyo) (9). The samples were freeze-fractured, etched and coated with platinum-carbon at –100°C in a specimen preparation chamber of the SEM. The samples were then transferred to a cold-stage in a SEM column that was kept at –160°C. Secondary emission images were observed and photographed at an accelerating voltage of 5 kV.

## **RESULTS AND DISCUSSION**

The DTA profiles in fresh xylem produced both a high temperature exotherm (HTE) and a low temperature exotherm (LTE) (Fig. 1A). The LTE in fresh xylem showed the maximum peak at  $-42.0 \pm 0.7^\circ\text{C}$  ( $n=3$ ), with initiation and termination of the LTE at  $-35.1 \pm 0.8^\circ\text{C}$  and at  $-44.9 \pm 0.7^\circ\text{C}$ , respectively. It is believed that while the HTE is produced by the freezing of apoplast water, the LTE is produced by intracellular freezing of xylem parenchyma cells when reductions of temperature exceed the capacity for supercooling (10,

19). Previous cryo-SEM studies confirmed that the LTE corresponded to temperatures of intracellular freezing in xylem parenchyma cells by the breakdown of supercooling in many tree species including beech (14).

In order to test the effects of intracellular contents that may function in relation to the supercooling ability of xylem parenchyma cells in beech, xylem was boiled. As a result of boiling, it is expected that structures of protoplasts, including plasma membranes, were completely broken and the majority of the intracellular substances were released from the xylem parenchyma cells and mixed with boiling water. After boiling treatments, the DTA of these samples still produced a distinct LTE peak, but the maximum peak was significantly reduced to  $-20.8 \pm 0.7^{\circ}\text{C}$  (Fig. 1B). Cryo-SEM observation in boiled samples showed that cooling to  $-15^{\circ}\text{C}$ , the temperature above the peak of LTE, resulted in small intracellular ice crystals (smaller than  $0.3 \mu\text{m}$ ,  $n=100$ ) in the majority (more than 80%,  $n = 100$ ) of xylem parenchyma cells (Fig. 2A), whereas cooling to  $-30^{\circ}\text{C}$ , the temperature below LTE, resulted in very large intracellular ice crystals (larger than  $1 \mu\text{m}$ ,  $n = 100$ ) in the majority (more than 60%,  $n = 100$ ) of cells (Fig. 2B). It is suggested that small intracellular ice crystals were produced by cryofixation of supercooled liquid, whereas large intracellular ice crystals were produced by breakdown of supercooling during slow cooling of samples at  $0.2^{\circ}\text{C}/\text{min}$  before cryofixation (8). Neither shrinkage nor any other detectable ultrastructural change in the cell wall was detected by cooling of boiled samples (Fig. 2A and B). Thus, these results confirmed that even after boiling, cell walls in xylem parenchyma cells kept function as a barrier from effects of extracellular ice crystals, and that supercooling ability of such xylem parenchyma cells was reduced by release of intracellular contents and/or mixture of intracellular contents with extracellular solutions.

Although the examination mentioned above suggested a strong relation of intracellular conditions to supercooling of xylem parenchyma cells, the possibility for a reduction in supercooling capability that might result from changes in cell wall properties by boiling cannot be ignored. In order to test this possibility, we subsequently attempted rapid freezing (and thawing) as a method to release intracellular substances with minor effects to cell wall structures. We froze fresh xylem by abrupt immersion into partially frozen Freon 22 ( $-150^{\circ}\text{C}$ ), which is usually employed for cryofixation of biological samples for ultrastructural preservation. In such rapid freezing, ultrastructural changes of cell walls have not been reported in previous studies (4, 5, 14). On the other hand, such rapid freezing still produces detectable intracellular ice crystals that promote serious damage of protoplasts, including plasma membranes (6, 8), and result in the leakage and/or mixture of intracellular substances with extracellular solutions after thawing. The DTA in xylem after such rapid freezing (and thawing) exhibited a LTE with two distinct peaks (Fig. 1C) that were different

to that of control (Fig. 1A), as well as that of boiled samples (Fig. 1B). In comparison to fresh control samples, however, DTA indicated a total reduction of supercooling ability in rapidly frozen (and thawed) samples. Although a small peak was still detected at  $-40.3 \pm 0.4^\circ\text{C}$  in rapidly frozen samples, the maximum peak of LTE was to  $-31.4 \pm 1.0^\circ\text{C}$ , with initiation and termination of the LTE at  $-26.9 \pm 0.9^\circ\text{C}$  and  $-43.5 \pm 0.9^\circ\text{C}$ , respectively. From these results, it is suggested that the reduction of supercooling ability by rapid cooling (and thawing) may be predominantly due to the release and/or mixture of intracellular substances rather than changes in cell wall properties. The freezing (and thawing) of fresh xylem samples by intermediate (Fig. 1D) or very slow cooling rates (Fig. 1E) also showed similar LTE profiles compared to those of rapid freezing (Fig. 1C). Although the effects exerted on cell wall ultrastructures as a result of different cooling rates are unknown (4, 5, 14), it is possible that they might produce different stresses to the cell walls. However, the similarity of DTA profiles by such different cooling rates (and thawing) supports the notion that intracellular contents that are released by intracellular freezing have greater impact on supercooling rather than any changes of cell wall ultrastructures that may occur by freezing.

The reasons why samples after freezing (and thawing) produced two LTE peaks are unclear. It is possible that the formation of two LTE peaks in samples after freezing (and thawing) reflects a change in the amounts of released intracellular substances from the damaged xylem parenchyma cells. Beech xylem has complex parenchyma cells which includes uniseriate ray, multiseriate ray, and axial parenchyma (14). For example, in multiseriate rays it would be easier for ray parenchyma cells that are in contact to vessels (contact cells) to release intracellular substances to extracellular solutions rather than cells that are surrounded by parenchyma cells (isolate cells). Due to our intention to inhibit the change of cell walls after thawing by the activation of endogeneous degradative enzymes (22), the thawing time was very short (1 h at  $4^\circ\text{C}$  without washing) prior to the start of DTA. Such treatments may result in different amounts of intracellular substances being released from parenchyma cells in different locations, and may result in plural peaks. The clear changes in supercooling ability between boiled cells and frozen cells may also relate to different amounts of released intracellular substances.

One possible cause for reduction of supercooling ability by the release and/or mixture of intracellular contents may simply be due to a dilution of intracellular solutions by the breakdown of the plasma membrane, allowing mixing with extracellular solutions. Reduction of supercooling capability may also result from intracellular penetration of extracellular ice nucleation substances. Furthermore, it is also possible that some specific substances that may facilitate or stabilize supercooling ability are released from the damaged cells and consequently result in reduced supercooling ability in xylem parenchyma

cells. Our previous studies showed that crude extracts from supercooling xylem parenchyma cells in softwood (17) and hardwood (13) had a strong ability to promote supercooling of water droplets.

At the present time, we know that many diverse cold acclimation-induced changes are associated with the acquisition of freezing tolerance in plant cells that adapt to subfreezing temperature by extracellular freezing (20). On the contrary, we currently have little knowledge about cold acclimation-induced changes in xylem parenchyma cells of trees that adapt to subfreezing temperature by deep supercooling, although changes in the supercooling capability in xylem parenchyma cells are distinctly dependent upon seasonal cold acclimation and deacclimation (7, 12, 21). The present study indicated the possibility that intracellular contents may function to alter the supercooling capability in xylem parenchyma cells. We are currently attempting to correlate the diverse cold acclimation-induced changes in xylem parenchyma cells to changes in their supercooling ability.

**Acknowledgements:** This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Culture, Science and Technology of Japan (17380101 to S.F.) and by a grant from the Japan Society for the Promotion of Science (17-9014 to J.K.).

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#### FIGURE LEGENDS

**Figure 1.** DTA of xylem from winter beech samples. All profiles were obtained by cooling xylem, with treatments indicated by (A) to (E), at 0.2°C/min from 4°C to –60°C. (A) Profile of fresh xylem maintained at 4°C for 1 h. (B) Profile of xylem that was boiled for 1 h and then kept at 4°C for 1 h. (C) Profile of xylem frozen by Freon 22 (10,000°C/min) and thawed at 4°C for 1 h. (D) Profile of xylem frozen by liquid nitrogen (1,000°C/min) and thawed at 4°C for 1 h. (E) Profile of xylem frozen at 0.2°C/min from 4°C to –60°C, thawed at 0.2°C/min to 4°C and then kept at 4°C for 1 h. The peak produced at around –10°C is HTE, and the peak (s) produced at lower temperatures is LTE.

**Figure 2.** Cryo-SEM photographs of xylem parenchyma cells from boiled winter beech xylem. Boiled samples were cooled from 4°C to indicated temperatures at 0.2°C/min and soon cryofixed. (A) Xylem parenchyma cells cooled to –15°C, showing small intracellular ice (arrows) produced by cryofixation of supercooled water. (B) Xylem parenchyma cells cooled to –30°C, showing large intracellular ice crystals (arrows) produced during slow cooling by breakdown of supercooling before cryofixation. Bars=10 µm.



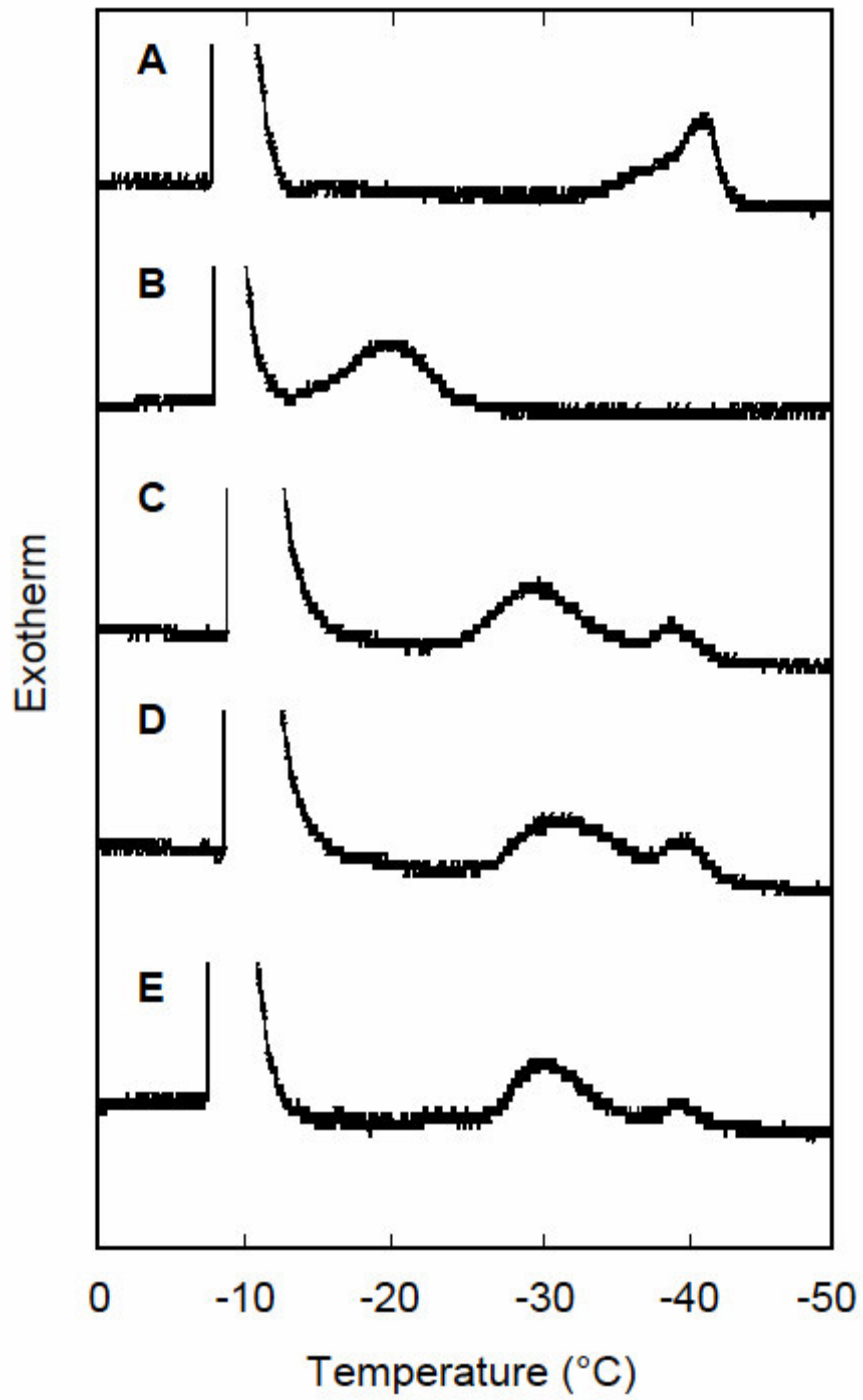


Figure 1

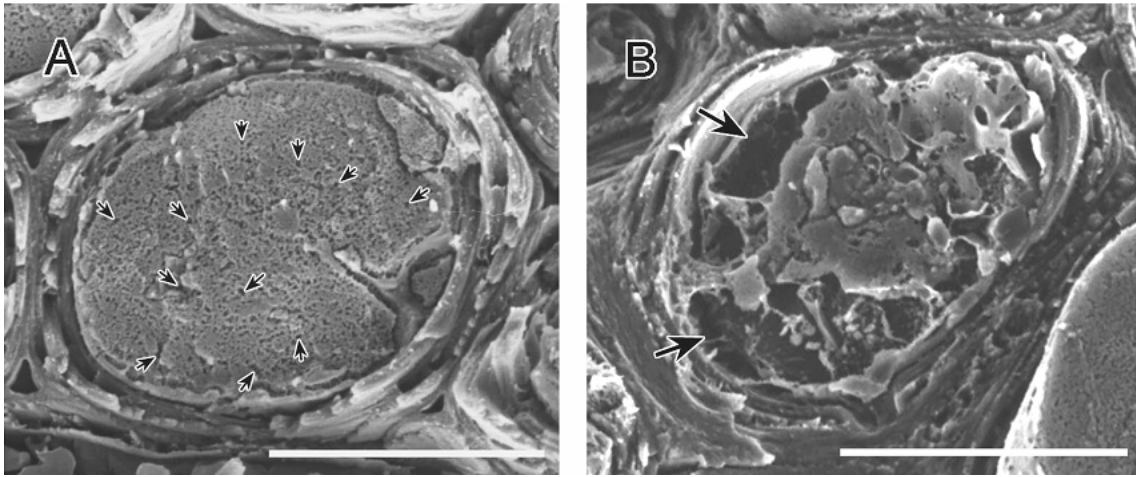


Figure 2