Study on Proteins Related to Deep Supercooling Ability of Xylem Tissues of *Fagus crenata* L.

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Change in the deep supercooling (DSC) ability of xylem tissues of beech (*Fagus crenata* L.) twigs was detected by a differential thermal analysis (DTA). DSC abilities of xylem tissue shown as peaks of low temperature exotherms in DTA were about -30°C in summer and -40°C in winter. DSC ability of beech twigs in winter was lowered to about -30°C by heat treatment at 60°C for 10 min but was not lowered by heat treatment at 50°C for 10 min. In order to find protein factors related to DSC ability, changes in the protein composition of xylem tissues caused by seasonal cold acclimation and heat treatments were analyzed by two-dimensional gel electrophoresis. About 350 protein spots were detected in the crude soluble fraction of xylem tissues in winter. Seventy-five protein spots were induced during seasonal cold acclimation. Fifty-six of the cold acclimation-induced proteins remained after heat treatment of xylem tissues at 50°C for 10 min, and 12 of the 56 proteins were decreased by heat treatment of xylem tissues at 60°C for 10 min.

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INTRODUCTION

Boreal woody plants possess high freezing resistance in winter. Freezing behavior of a boreal woody trunk differs depending on the tissue. Cambial and cortical parenchyma cells respond to subzero temperature by extracellular freezing, and xylem ray parenchyma cells (XRPCs) respond by deep supercooling (DSC). In general, freezing resistance of cambial and cortical parenchyma cells of boreal woody plants is much higher than that of XRPCs. Since XRPCs are essential for growth of the whole tree, freezing resistance of XRPCs may be one of the important factors for survival of trees during winter in cold climate regions.

It is known that DSC ability of xylem parenchyma cells is dependent on the physical features of cell walls. It seems that the thick and rigid cell walls of xylem cells might function as a physical barrier against dehydration of the protoplast and ice nucleation of cell sap by extracellular ice. However, we have recently shown that pore size of cell walls of several boreal woods was not small enough to protect cells from ice nucleation by extracellular ice crystals. In addition, destruction of cell membranes by freeze-thawing using liquid nitrogen resulted in a decrease in the DSC ability of cell sap surrounded by cell walls. Thus, we hypothesized that the intracellular factors other...
than the physical properties of cell walls contribute to the DSC ability of xylem parenchyma cells in winter. In order to study the molecular mechanism of DSC in XRPCs of boreal woody plants, we attempted to detect and identify the intracellular factors related to DSC ability. Cold acclimation-induced genes have been isolated from XRPCs of larch (Larix kaempferi L.) by differential display and filter alley screening and characterized. Furthermore, anti-ice-nucleating activity has been detected in xylem tissues of several boreal woody plants (Kasuga et al. in preparation). However, the mechanism of DSC in xylem tissue of boreal woody plants has not been clarified yet.

Differential thermal analysis (DTA) is a convenient method for studying DSC ability. Since intracellular freezing of sap of parenchyma cells resulted in the generation of a peak of low temperature exotherm (LTE) in the DTA profile, temperature for the LTE peak is an index of the limit of supercooling ability. However, xylem tissues in several boreal woods showed no peaks of LTE because of the low rate of occupation of parenchyma cells in xylem tissues and/or low water content.

In twigs of beech (Fagus crenata L.), DSC ability of xylem tissues was estimated by DTA. A preliminary study showed that DSC ability of beech twigs was lowered by heat treatments. Therefore, in this study we tried to detect proteins that respond to the change in DSC ability by heat treatments or seasonal cold acclimation.

**MATERIALS AND METHODS**

**Plant materials**

Twigs of beech (Fagus crenata L.) were seasonally harvested in the Sapporo Experimental Nursery of Hokkaido University. Twigs collected in winter were stored at about -10°C until use for analyses.

**DTA**

Debarked tissues (2 cm long) were axially sliced in half and the pith was removed. A thermocouple was placed on the sliced face, sandwiched in the set of the tissues, fixed, and covered with Parafilm (Pechiney Plastic Packing, Inc., USA). As a reference, tissue dried overnight at 105°C was used. Samples and references were separately placed into sample tubes and set in a programmable freezer. After equilibration at 5°C for 1 h, they were cooled to -55°C at a rate of 0.2°C/min. Difference in thermal responses was recorded.

DSC ability of a sample was estimated by the temperature of an LTE peak in the DTA profile for evaluation of freezing resistance of the tissue.

**Protein analysis**

Soluble proteins were extracted from debarked tissues of beech. All procedures were fundamentally performed at 4°C. After tissues had been shredded and ground in liquid nitrogen using a mortar and a pestle, tissue flakes were homogenized in 5 volumes of extraction buffer [50 mM Tris-HCl (pH 7.5), 0.2 M sucrose, 5 mM ethylenediaminetetraacetic acid, 5 mM potassium metabisulfite, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) polyvinylpolypyrrolidone] using a Polytron homogenizer (Kinematica, Switzerland). The extract was filtrated by 4 layers of gauzes, and the filtrate was centrifuged at 10,000xg for 15 min. The supernatant was further centrifuged at 156,000xg for 20 min, and the resultant supernatant was collected as a crude soluble fraction. For protein analyses, the crude soluble fraction was concentrated and desalted.

Protein concentration was determined using a protein assay kit (BioRad, USA).

In two-dimensional gel electrophoresis (2GE), isoelectric focusing (IEF) and SDS-polyacrylamide gel electrophoresis
(SDS-PAGE) were done as the first and second dimensions, respectively. For IEF, an immobilized pH-gradient gel system (Amersham Pharmacia Biotech, Sweden) was used according to the attached manual. SDS-PAGE for 2GE and detection of proteins in the gels by silver-staining were done as described previously\(^{10}\).

**RESULTS AND DISCUSSION**

Seasonal change in the freezing resistance of xylem tissues of beech twigs was analyzed by DTA (Table 1).

DSC ability as an index of freezing resistance of beech twigs was increased during seasonal cold acclimation. DSC ability was about -30°C in late August, increased gradually from mid-October, reached to maximal level (-40°C) in late December, and remained at that level in February. Since our preliminary study showed that an LTE peak in DTA of beech xylem was shifted to a higher temperature by heat treatment, we analyzed the response of beech xylem tissue to heat treatments.

Table 1. Changes in DSC ability of xylem tissue of beech twigs during seasonal cold acclimation. DSC ability is shown as temperature of the LTE peak in DTA.

<table>
<thead>
<tr>
<th>Month</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>Feb</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTE (°C)</td>
<td>-30</td>
<td>-27</td>
<td>-36</td>
<td>-37</td>
<td>-39</td>
<td>-40</td>
</tr>
</tbody>
</table>

When debarked tissues of beech twigs in winter were treated by heating at various temperatures for 10 min, the LTE peak was shifted to a higher temperature by heat treatment at more than 60°C but was not shifted by heat treatment at 50°C (Table 2). However, the LTE peak was shifted by heating at 50°C for more than 30 min (data not shown). Electrolyte leakage of the tissue was not increased by heat treatment at 50°C for 10 min (data not shown). DSC ability of xylem tissue of beech twigs in winter may be responsive to heat treatments and sensitive to heating at a temperature higher than 50°C. Thus, in order to identify proteins that may be related to DSC ability, we analyzed cold acclimation-induced proteins that responded to heat treatments of xylem with the change in DSC ability of xylem tissue of beech twigs in winter.

Table 2. Changes in DSC ability of xylem tissue of beech twig in winter caused by heat treatment. DSC ability of xylem tissue was measured after heat treatment at 50°C, 60°C or 100°C for 10 min using a drying oven. DSC ability is shown as temperature of the LTE peak in DTA.

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>None</th>
<th>50°C</th>
<th>60°C</th>
<th>100°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTE (°C)</td>
<td>-40</td>
<td>-40</td>
<td>-30</td>
<td>-27</td>
</tr>
</tbody>
</table>

Crude soluble fractions were prepared from xylem tissues, and soluble protein composition of xylem tissue was analyzed by 2GE. About 350 protein spots were detected in the crude soluble fraction in winter. A comparison of protein compositions in summer and winter samples showed that 75 protein spots were induced during seasonal cold acclimation. In particular, 25 of the 75 spots were induced with close relation to the increase in DSC ability during seasonal cold acclimation (data not shown).

Next, compositional change in the winter-accumulating proteins after heat treatment of xylem tissues at 50°C for 10 min was compared with that after heat treatment at 60°C for 10 min (Fig. 1). A few winter-accumulating proteins disappeared, and 56 of the 75 winter-accumulating protein spots remained after heating at 50°C for 10 min. Thirty-five of the 56 spots were decreased by heat treatment at 60°C for 10 min. From these results, 12 protein spots were finally selected as winter-accumulating proteins that responded to heat treatments with close relation to the change in DSC ability of xylem tissue in winter.

Although cold-induced genes have been characterized in many plant cells that respond to subzero temperature by extracellular freezing\(^{11}\),
Fig. 1. Compositional changes in soluble proteins of xylem tissues of beech twigs in winter in response to heat treatment at 50°C or 60°C for 10 min. Arrows indicates the winter-accumulating proteins that were related to changes in DSC ability.

Few cold-induced gene products have been physiologically characterized in xylem tissues of woody plants. However, recent studies showed that the accumulation of 24-kDa dehydrin-like protein in xylem tissues correlated to the increase in freezing resistance of xylem tissues of Cornus sericea L. during seasonal cold acclimation. But, role of this protein on DSC mechanism is still unclear. In this study, we detected 12 proteins that might correlate to the DSC ability of xylem tissues of beech. For further analysis, identification of these proteins should be done in order to clarify their function in DSC ability of xylem tissue of beech twigs in winter.

REFERENCES
