Presence of a basic secretory protein in xylem sap and shoots of poplar in winter and its physicochemical activities against winter environmental conditions

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Abbreviations:

ABA, abscisic acid; AFP, antifreeze protein; BSA, bovine serum albumin; BSP, basic secretory protein; GLP, germin-like protein, IRI, ice recrystallization inhibition; LD, long-day; LDH, lactate dehydrogenase; LT, non-freezing low temperature; qRT-PCR, real-time quantitative reverse-transcription polymerase chain reaction; SD, short-day; SDS, sodium dodecyl sulfate; XSP, xylem sap protein

Abstract

XSP25, previously shown to be the most abundant hydrophilic protein in xylem sap of *Populus nigra* in winter, belongs to a secretory protein family in which the arrangement of basic and acidic amino acids is conserved between dicotyledonous and monocotyledonous species. Its gene expression was observed at the same level in roots and shoots under long-day conditions, but highly induced under short-day conditions and at low temperatures in roots, especially in endodermis and xylem parenchyma in the root hair region of *Populus trichocarpa*, and its protein level was high in dormant buds, but not in roots or branches. Addition of recombinant PtXSP25 protein mitigated the denaturation of lactate dehydrogenase by drying, but showed only a slight effect on that caused by freeze–thaw cycling. Recombinant PtXSP25 protein also showed ice recrystallization inhibition activity to reduce the size of ice crystals, but had no antifreezing activity. We suggest that PtXSP25 protein produced in shoots and/or in roots under short-day conditions and at non-freezing low temperatures followed by translocation via xylem sap to shoot apoplast may protect the integrity of the plasma membrane and cell wall functions from freezing and drying damage in winter environmental conditions.

Key words

Apoplast, Basic secretory protein, Dry, Freeze, Ice crystal, Short day, Winter, Xylem sap

Introduction

Due to their immobility, plants typically overcome abiotic stresses associated with seasonal climatic changes by altering their physiological and morphological status. In temperate and subarctic zones, perennial and winter annual plants must survive winter temperatures below 0 °C by overcoming freezing stress. Hence, deciduous trees form dormant buds in autumn and shed leaves in early winter as morphological adaptations, and they have also evolved a tolerance to freezing or acclimation to cold. In addition to the direct effects of freezing, plants cannot use soil water when the soil is frozen. Even under non-freezing low-temperature conditions, the supply of available water from roots decreases due to increased water viscosity and decreased water conductivity through water channels (aquaporins) in biological membranes (Wan et al. 2001). Moreover, aquaporin gene expression is suppressed under non-freezing low-temperature conditions (Aohara et al. 2016; Jang et al. 2004). Therefore, plants that are not covered by snow must overcome drying stress in addition to direct freezing stress in winter (Larcher 2001b).

The regulation of these adaptive physiological responses in shoots has been well studied in various plant species including trees (Larcher 2001a), and the regulatory functions of hormones and transcription factors, etc., have been largely clarified (Thomashow 1999; Welling and Palva 2006). In winter, when the temperature falls below the freezing point, ice is first formed extracellularly in the apoplast, including

xylem tracheary elements and intercellular spaces, and then intracellular freezing occurs, which damages the functions of biological membranes and the cytoplasm in plant tissues. Such extracellular freezing including extra-tissue and/or extra-organ freezing accompanied by the translocation of water from the cytosol to apoplasts leads to bud cytosol dehydration in some woody plant species (Ishikawa and Sakai 1982). Although intracellular freezing is suppressed by depression of the freezing point due to the rise in solute concentration within the cells, some plants produce antifreeze protein (AFP), which suppresses ice formation, thereby maintaining a supercooled state (Davies 2014; Wilson and Leader 1995; Wilson et al. 2010).

However, cell functions are adversely affected if solute concentration in the unfrozen cytosol becomes too high due to dehydration. Therefore, some plants have developed mechanisms to withstand dehydration stress associated with freezing by producing plasma membrane components that stabilize the membrane even at low temperatures (Uemura et al. 2006) and by accumulating soluble low molecular weight organic substances and stress proteins, including hydrophilic polypeptides, such as LEA protein and dehydrin, which stabilize biological membranes and cytoplasmic proteins (Goyal et al. 2005; Karlson et al. 2003). In addition, some plants produce proteins that suppress the enlargement of ice crystals, often called ice recrystallization inhibition (IRI) activity (Capicciotti et al. 2013). As ice crystals often develop at the surface of plasma membrane below the cell wall (Yamazaki et al. 2009), it is possible that IRI proteins present in the apoplast reduce damage to the plasma membrane caused by the formation of large ice crystals.

In contrast to the aboveground organs, annual changes in functions of the roots have yet to be fully clarified, although the roots play indispensable roles in the

absorption of minerals and water from soil, as well as the production of organic substances translocated to the inside and outside of the plant body. Xylem is one of the main components of the vascular system and functions as an extracellular route that systemically delivers root-absorbed inorganic and root-produced organic substances to aboveground organs (Satoh 2006).

To monitor the annual variation in root functions in deciduous trees under natural environmental conditions, we analyzed the components of the sap that flows in xylem (xylem sap) seasonally collected from the stumps of cut branches of field-grown poplar (*Populus nigra*) using a suction pump. Calcium, potassium, glucose, and protein levels were found to increase from winter to early spring, suggesting that these inorganic and organic substances may be involved in adaptation to the winter environment and/or in preparation for spring bud burst (Furukawa et al. 2011a).

Among the proteins present in poplar xylem sap, two highly abundant extracellular proteins, 25 kDa and 24 kDa xylem sap proteins (XSP25 and XSP24), have been shown to belong to the basic secretory protein (BSP) and germin-like protein (GLP) families, respectively, by mass spectrometry (Aohara et al. 2016; Furukawa et al. 2011b). The XSP25 and XSP24 genes were found to be abundantly expressed in the roots of winter poplar soil-grown in outdoor pots and in the roots of soil-grown plants under short-day and low-temperature conditions in the culture room.

As some GLPs have previously been reported to have oxalate oxidase and/or superoxide dismutase activities (Dunwell et al. 2008), these molecules have been suggested to be involved in stress tolerance. The BSPs, like XSP25, are hydrophilic extracellular proteins, some of which are inducible by abscisic acid (ABA) (Furukawa et al. 2011b; Kuwabara et al. 1999; Okushima et al. 2000), but their biological and

physicochemical functions have yet to be determined.

Here, we clarified the accumulation of PtXSP25 protein in the shoots of *Populus trichocarpa* in winter and its physicochemical activities against freezing and drying, which are major environmental stresses encountered in winter, using recombinant PtXSP25 protein.

Materials and methods

Plant materials and culture conditions

P. trichocarpa (Torr. & Gray) genotype Nisqually 1 and hybrid aspen T89 lines (*P. tremula* × *tremuloides*; Nilsson et al. 1992) kindly provided by Dr. C. J. Douglas, University of British Columbia, Canada, and Dr. B. Sundberg, Swedish University of Agricultural Sciences, Sweden, respectively, were aseptically cultured in pots as described previously (Ohtani et al. 2011) under long-day conditions (16 h light/8 h dark, 23 °C) with light intensity of 60 μmol m^{-2} s^{-1}. Plants aseptically cultured under long-day conditions for 3 weeks were sequentially transferred for culture under short-day conditions (8 h light/16 h dark, 23 °C) for 10 weeks, low-temperature conditions (8 h light/16 h dark, 4 °C) for 4 weeks, and long-day conditions (16 h light/8 h dark, 23 °C) for 3 weeks. Xylem sap was collected as previously described (Furukawa et al. 2011a) from the cut stumps of branches of *P. trichocarpa* grown for 4 years on the campus of Tsukuba University in February 2016. Stems and dormant apical buds were also sampled from the same plants.

Alignment of *P. trichocarpa* (Pt) XSP25 with the BSP family proteins

The amino acid sequence of PtXSP25, registered as XM_002300207.1 (Potri.001G299500) in the *P. trichocarpa* database, was aligned with tobacco NtPRp27 (BAA81904.1), *Arabidopsis* AT2G15220 (NP_565369.1), wheat WAS2 (AAD46133.1), and rice LOC_Os10g34930 (AAP54394.1) using Clustal W. The signal sequences for secretion and N-glycosylation sites were predicted using the SignalP (http://www.cbs.dtu.dk/services/SignalP/; Emanuelsson et al. 2007) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) programs, respectively.

Gene expression analysis

Whole roots and shoots sampled at various time points were ground in liquid nitrogen and then homogenized in the same volume of RLC buffer using the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol, and the resultant RNA was used to analyze *XSP25* expression by real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) with *ubiquitin* (gi566172648; Potri.005G198700) as an internal standard (= 1). cDNA was synthesized by ReverTra Ace (Toyobo, Tokyo, Japan) using oligo $(dT)_{20}$ primers at 42 °C for 60 min and 99 °C for 5 min, and real-time PCR was performed using the SYBR Premix Ex Taq II kit (TaKaRa Bio Inc., Shiga, Japan) under the following conditions: denaturation for 30 s at 95 °C, annealing for 10 s at 63 °C, and extension for 31 s at 72 °C, for a total of 50 cycles. PCR products were detected using the 7300 Real-Time PCR System (Thermo Fisher Scientific, Kanagawa, Japan). The following primers were used for qRT-PCR:

PtXSP25 forward 5′-ATGGACAAACACCAGGAGGA-3′, *PtXSP25* reverse 5′-ACCTTGATCCCACCTGTCAC-3′, *UBQ* forward 5′-TGAACCAAATGATACCATTGATAG-3′, and *UBQ* reverse 5′-GTAGTCGCGAGCTGTCTTG-3′.

Localization of *PtXSP25* promoter activity

To produce *PtXSP25promoter::GUS*-transgenic plants, the promoter (1970 bp) of *PtXSP25* was cloned into the pENTR-D-Topo vector (Thermo Fisher Scientific Inc., Tokyo, Japan) and used for transformation of the hybrid aspen T89 line according to the procedure described by Nilsson et al. (1992), because hybrid aspen T89 is a good plant material for genetic transformation. Roots of transgenic plants were embedded in Technovit 7100 resin (Kulzer, Hanau, Germany), and sections were prepared at the root hair region using an ultramicrotome, because the root hair region abundantly expresses genes that encode products delivered into xylem sap (Satoh 2006). Sections were subjected to immunohistochemistry using rabbit anti-GUS antibody and anti-rabbit IgG antibody coupled with fluorescein isothiocyanate as primary and secondary antibodies, respectively, according to the procedure described by Jasik et al. (2011), because the activity level was too low to identify β-glucuronidase-expressing tissues in the transformant lines. Immunofluorescence was visualized using a Leica DMRB fluorescence microscope (Leica, Wetzlar, Germany).

Production and purification of recombinant PtXSP25 protein

The coding region of *PtXSP25* without the signal peptide cloned into the pCold ProS2 vector (TaKaRa Bio Inc.) was expressed in *Escherichia coli*, and recombinant PtXSP25 protein was purified from the soluble fraction of *E. coli* lysate by Ni-chelate affinity resin, to release the protein from the resin, using Factor Xa protease and HRV3C protease for antigen and physicochemical assay, respectively, according to the manufacturer's instructions.

The primer sequences used to construct the recombinant PtXSP25 protein were as follows: rPtXSP25-HRV-F,
5'-CTGTTCCAGGGGCCCGTGGACTACACTGTCACCAACAGAG-3';
rPtXSP25-Xa-F,
5'-GGTATCGAAGGTAGGGTGGACTACACTGTCACCAACAGAG-3'; and
rPtXSP25-Inf-R (common to rPtXSP25-HRV and rPtXSP25-Xa),
5'-ACCGAGCTCCATATGCCTATTTTCCATACTTGGCCTTGTAGTC-3'

Detection of PtXSP25 protein in the tissues and xylem sap by immunoblotting

Proteins prepared from the xylem sap, branches, and dormant buds sampled from *P. trichocarpa* grown on the campus of Tsukuba University in February 2016 and whole roots and shoots sampled from aseptically cultured *P. trichocarpa* at various time points under artificial environmental conditions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Tissue samples were ground in liquid nitrogen and then homogenized in the same volume of 2× sample buffer for SDS–PAGE followed by heating at 95 °C for 5 min; the supernatant (equivalent to 3 mg tissue/lane) was then used for SDS–PAGE. Xylem sap protein was precipitated using

80% ethanol, dissolved in the sample buffer, and subjected to SDS–PAGE (equivalent to 1.25 mL xylem sap/lane) after heating at 95 °C for 5 min. Recombinant PtXSP25 protein was used as a standard (5 ng/lane). Total proteins and PtXSP25 protein were then detected by Coomassie Brilliant Blue or silver staining and immunoblotting using an antibody raised against recombinant PtXSP25 protein, respectively, as described previously by Oda et al. (2003) for xylem sap and Sakuta and Satoh (2000) for tissues. Recombinant PtXSP25 protein was used as the standard.

Drying and freeze–thaw treatments and measurement of lactate dehydrogenase (LDH) activity

To evaluate the effect of drying, a drop (50 μL) of water containing 200 ng of LDH from rabbit muscle (Roche, Basel, Switzerland) mixed with recombinant PtXSP25 protein or bovine serum albumin (BSA) at LDH:XSP25 or BSA molar ratio = 1:10 was placed on the wall of a polypropylene microtube, and left in a desiccator with phosphorus pentoxide for 3 or 12 h. Residual activity was measured after dissolution in 50 μL of 100 mM Na-phosphate buffer (pH 6.0). To evaluate the effects of freeze–thaw cycling, 50 μL of 100 mM Na-phosphate buffer (pH 6.0) containing 200 ng of LDH was mixed with recombinant PtXSP25 protein or BSA at LDH:XSP25 or BSA molar ratio = 1:10. The tube was immersed in liquid nitrogen to freeze and then thawed at room temperature. This freeze–thaw cycle was repeated three times. Initial and residual activities of LDH were measured by monitoring the decrease in absorbance at 340 nm at 27 °C in the reaction mixture (1 mL) containing 2 μL of the solution mentioned above, 100 mM Na-phosphate buffer (pH 6.0), 0.1 mM NADH, and 2 mM pyruvate.

Measurement of antifreeze and ice recrystallization inhibition activities

To measure antifreeze activity, droplets of recombinant PtXSP25 protein (5 mg mL^{-1}) and type III AFP (from *Zoarces elongatus Kner*, Notched-fin eelpout) (70 μg mL^{-1}) solutions in water were frozen at a cooling rate of 0.05 °C min^{-1}, and the morphology of ice crystals was observed as described previously (Nishimiya et al. 2005). To assess IRI activity, we used the "splat cooling" method (Capicciotti et al. 2015). A 10 μL droplet containing distilled water, a solution of recombinant PtXSP25 protein (5 mg mL^{-1} in water), or a solution of BSA (5 mg mL^{-1} in water) was dropped through a 2-m-high plastic tube onto a polished aluminum block cooled to approximately −80 °C. The wafer was separated from the surface of the block, transferred to a cryostage, stored at −6.4 °C for 30 min to allow annealing, and then photographed using a digital camera fitted to the microscope. We used ImageJ software to draw well-defined boundaries around the ice crystals within the image and to calculate the area of each crystal. The average ice crystal area (mean grain size) was compared with that of the control (distilled water).

Results

Structural characteristics of BSP family proteins

The amino acid sequence of PtXSP25, one of six BSP family proteins in the *P. trichocarpa* genome database, was aligned with BSPs from tobacco, Arabidopsis, wheat, and rice (Fig. 1; phylogenetic tree shown in Fig. S1). With the exception of that from

tobacco, these proteins all possess a signal peptide for protein secretion at the N-terminus. They are all hydrophilic with abundant charged amino acids, the positions of which are well conserved between dicotyledonous and monocotyledonous plants. *PtXSP25* has basic and acidic amino acids at 12.3% and 10.3%, respectively, with pI 7.9, and has a single putative N-glycosylation site.

Expression of *PtXSP25* under artificial environmental conditions

To analyze the effects of environmental factors on *PtXSP25* expression, qRT-PCR was performed on whole shoots and roots harvested at 23 °C under long-day conditions, after 6 weeks under short-day conditions, and after 2 weeks at 4 °C under short-day conditions. *PtXSP25* was moderately expressed both in roots and shoots under long-day conditions, but highly expressed in roots under short-day conditions, and this high expression was maintained at 4 °C (Fig. 2).

To analyze the tissue-specific expression of *PtXSP25*, the root hair region of hybrid aspen T89 transformed with the β-glucuronidase gene fused downstream of the *PtXSP25* promoter after 6 weeks of culture under short-day conditions was embedded in Technovit resin, followed by immunostaining of thin transverse sections using an antibody against β-glucuronidase. Expression was localized in the endodermis and xylem parenchyma, and not detected in the pericycle or other tissues (Fig. 3).

Purification of recombinant PtXSP25 protein

The recombinant protein containing the coding region of *PtXSP25* without the signal

sequence was expressed in *E. coli* using the pCold ProS2 vector and purified with Ni-chelate affinity resin using Factor Xa or HRV3C proteases for preparation of recombinant PtXSP25 protein used for antigen or physicochemical activity assays, respectively. The recombinant PtXSP25 protein was successfully purified from the soluble fraction of *E. coli* lysate as a single band in both cases (Fig. S2).

Immunological detection of PtXSP25 in poplar

For immunoblotting analysis, the proteins in xylem sap collected in February from the stumps of cut branches of *P. trichocarpa* grown on the campus of Tsukuba University using a suction pump and the proteins in branches and dormant buds collected from the same plants were separated by SDS-PAGE followed by immunological detection using anti-PtXSP25 antibody. Xylem sap contained a much lower total protein content compared with branches or buds; the amount and pattern of total protein was similar between branches and buds (Fig. 4a). However, a broad band representing the PtXSP25 protein was detected on the immunoblots, including a higher-molecular-weight region than that in the recombinant PtXSP25, probably due to N-glycosylation in buds and xylem sap, but not in branches (Fig. 4b). Figure 5 shows the results of immunological detection of PtXSP25 protein in plants grown aseptically under artificial environmental conditions. PtXSP25 protein was weakly detected at the start of short-day conditions, but it was abundant after 10 weeks under short-day conditions and after 2 weeks at 4 °C in shoots but not in roots. A weak slightly higher molecular weight band, probably due to N-glycosylation, was also detected.

Mitigation effects of PtXSP25 protein against denaturation of LDH by drying and freezing

As LDH is one of the fragile enzymes associated with drying and freezing damage, dry material from a drop of LDH solution mixed with the recombinant PtXSP25 protein was incubated with phosphorus pentoxide in a desiccator for 3 or 12 h and resolved with buffer. Residual LDH activity was measured using BSA as a positive control, because BSA is widely used as a protectant for various enzymes. LDH activity decreased to 55.1% of that at 0 h without the addition of any protectant after 3 h of desiccation, but decreased to only 80.1% of that at 0 h with the addition of recombinant PtXSP25 protein or BSA (Fig. 6). With the addition of PtXSP25 protein or BSA, 0.06% of LDH activity remained even after 12 h of desiccation, whereas no activity was detected at this time point without the addition of protectant.

As BSA protects various enzymes from denaturation induced by freeze–thaw cycling, we examined the mitigation effects of recombinant PtXSP25 protein against damage associated with repeated freeze–thaw cycles. As shown in Fig. 7, the addition of BSA strongly protected LDH from inactivation after three cycles of freeze–thaw treatment. Addition of recombinant PtXSP25 protein also significantly suppressed the inactivation of LDH, but its effect was much weaker than that of BSA.

Effects of PtXSP25 protein on ice formation

To evaluate the antifreeze activity of PtXSP25, recombinant PtXSP25 protein solution (5 mg mL^{-1}) was frozen and the shape of ice crystals was observed. A type III antifreeze

protein (AFP) (70 μg mL^{-1}) from the sea fish *Z. elongatus Kner* (notched-fin eelpout) showed the formation of bipyramidal crystals due to AFP binding as a positive control (Fig. 8c) (Nishimiya et al. 2005). However, the formation of round, dinner-plate-shaped, ice crystals, i.e., lacking bipyramidal ice crystals, was observed with recombinant PtXSP25 protein (Fig. 8a, b), indicating a lack of antifreeze activity. The relationship between hexagonal bipyramids and some thermal hysteresis (or antifreeze activity) is well documented (Wilson and Leader 1995), and the lack of bipyramidal ice in this experiment suggested the absence of any hysteresis. It must be noted that the presence of bipyramidal ice does not necessarily indicate measurable thermal hysteresis but does indicate inhibition of recrystallization. In all cases to date where an organism produces proteins that result in hexagonal bipyramidal ice, some inhibition of recrystallization has been observed. That is, if we measure thermal hysteresis or if we see hexagonal bipyramids there will be inhibition of recrystallization, but the manifestation of inhibition does not necessarily mean either bipyramids or hysteresis.

Next, to evaluate the IRI activity of PtXSP25, recombinant PtXSP25 protein solution (5 mg/mL) was subjected to splat-cooling assay to measure the mean grain size of ice crystals. The ice crystals were reduced in size to 64% of the size in the water controls that is similar to the data of BSA (Fig. 9). PtXSP25 activity was similar to that of TaIRI-2 (IRI protein) (Tremblay et al. 2005) and WCS120 (dehydrin) (Houde et al. 1995) in wheat measured previously in our laboratory (Chow-Shi-Yée et al. 2016).

Discussion

The functions of BSP family proteins including PtXSP25 are not yet known, but their

genes are widely distributed in dicotyledonous and monocotyledonous plants. The arrangement of basic and acidic amino acids is highly conserved among species (Fig. 1), suggesting that these abundant charged amino acids may have important functions in interactions with water molecules and other proteins.

Gene expression of *PtXSP25* was strongly induced in poplar roots under short-day conditions and at non-freezing low temperatures (Fig. 2). From fall to winter, boreal trees sequentially perceive and respond to changes in environmental signals (short days, non-freezing low temperatures, and freezing temperatures) to finally acquire maximum freezing resistance (Welling and Palva 2006). In late fall, plants may be exposed to mild freezing temperatures close to −5 °C before full acquisition of cold acclimation. Therefore, the induction of some level of cold tolerance in deciduous trees by short-day conditions may represent an adaptive response to such environmental changes. Leaves sense short-day conditions, resulting in cold acclimation through the production of ABA (Welling et al. 2002). Our previous study showed that *XSP25* expression in roots is induced by ABA application (Furukawa et al. 2011b). Therefore, it is possible that ABA synthesized in shoots under short-day conditions is translocated to the roots, and then induces *PtXSP25* expression in the roots.

PtXSP25 was highly expressed in roots under short-day conditions (Fig. 2), especially in the endodermis and xylem parenchyma within the central cylinder of the root hair region in roots (Fig. 3). As water absorption mainly occurs in the root hair region, the proteins produced and secreted in the central cylinder surrounded by endodermis in this region are thought to be efficiently transported to the shoots via xylem flow (Satoh 2006). In outdoor-cultivated poplar in winter, PtXSP25 protein was detected in xylem sap and dormant buds, but not in branches (Fig. 4). Xylem sap is

contained in branches, but at a low volume, and the concentration of XSP25 protein in xylem sap is low (approximately 50 ng mL^{-1}). Moreover, XSP25 proteins may flow through branches but do not accumulate in the branches. Therefore, XSP25 protein may not be detected in branches. In poplar aseptically grown in pots under artificial environmental conditions, PtXSP25 protein was not detected in roots, and its accumulation in shoots increased under short-day conditions and at non-freezing low temperatures (Fig. 5). These results may imply that the PtXSP25 protein produced in roots is transported through xylem to the shoots (especially to the dormant buds). However, because *PtXSP25* was also moderately expressed in shoots under short-day conditions (Fig. 2), PtXSP25 protein in buds may alternatively be produced within the buds themselves. Further analysis of *PtXSP25* gene expression at the tissue level and PtXSP25 protein translocation between organs and tissues will be required.

On the other hand, low temperatures in winter induce drying stress due to suppression of water movement in addition to freezing stress (Larcher 2001b). Therefore, the effects of PtXSP25 protein on denaturation of LDH by drying were investigated, and the results indicated that recombinant PtXSP25 protein significantly mitigated the decrease in LDH activity by drying (Fig. 6). Moreover, its effect was comparable to that of BSA, which is generally used as a protectant for various enzymes. This effect may have been because PtXSP25 protein has high hydrophilicity and is able to retain water molecules around the protein. PtXSP25 may function similarly to hydrophilic LEA proteins (Goyal et al. 2005). As PtXSP25 protein is present in xylem sap, a kind of apoplast (Furukawa et al. 2011b), it may prevent the inactivation of functional proteins, including plasma membrane aquaporins and hemicellulose/pectin-modifying enzymes on the surface of the plasma membrane and in

the cell wall.

Many enzymes are denatured by freeze–thaw cycling, and BSA is often used to suppress this denaturation. PtXSP25 protein showed only a slight protective effect against denaturation of LDH by freeze–thaw cycling compared with BSA (Fig. 7). On the other hand, some organisms living in cold environments produce AFP to prevent freezing of their cells and body fluid (Wilson et al. 2010). Therefore, we examined the shape of the ice crystals formed when the solution of PtXSP25 protein was frozen, but no antifreeze activity was observed as compared with fish Type III AFP (Fig. 8). Certain organisms also produce proteins that inhibit the growth of ice crystals (IRI activity) (Wilson et al. 2003). Therefore, PtXSP25 solution was subjected to splat-cooling assay to evaluate IRI activity. PtXSP25 showed IRI activity that is similar to the data of BSA (Fig. 9). As a serum protein, BSA lacks IRI activity under high ionic conditions such as in phosphate-buffered saline (PBS) containing 130 mM NaCl (Eniade et al. 2003); some non-IRI proteins also show IRI activity in water (Knight et al. 1995), perhaps due to the presence of these animal proteins in body fluids with high ionic concentrations. In contrast, PtXSP25 is present in xylem sap, a kind of apoplast, the ionic concentration of which is very low (nearly 5 mM) (Furukawa et al. 2011a). Since 5 mg mL^{-1} PtXSP25 was not soluble in PBS, we dissolved PtXSP25 and BSA in water for the IRI assay. Because PtXSP25 is generally present under low ionic conditions in the plant body, the IRI activity of PtXSP25 may be effective in plants. The IRI activity of PtXSP25 (Fig. 9) was comparable with those of wheat TaIRI (IRI protein) and WCS120 (dehydrin), which are involved in stress tolerance (Houde et al. 1995; Tremblay et al. 2005).

Extracellular freezing is an important process that depresses the freezing point by decreasing the water content of the cytosol to induce supercooling (Ishikawa and

Sakai 1982); however, the formation of large extracellular ice crystals can damage the cell surface. Therefore, PtXSP25, an extracellular protein, is thought to reduce the size of ice crystals, with consequent reduction in physical damage to the plasma membrane, especially at the boundary between the plasma membrane and the cell wall. As recombinant PtXSP25 protein was insolubilized when the solution was concentrated using a membrane filter at concentrations greater than 5 mg mL^{-1}, we could not determine the maximum IRI activity of PtXSP25 protein. N-glycosylation (Figs. 4, 5) may improve the solubility of PtXSP25 protein in plants.

Taken together, these observations indicate that PtXSP25 protein was synthesized in the central cylinder of polar roots from fall to early winter by perception of short-day conditions and non-freezing low temperatures. PtXSP25 protein production in shoots and/or in roots followed by its translocation via xylem sap and accumulation in shoot apoplast may protect the integrity of the plasma membrane and cell wall against freezing and drying damage in winter, thus helping the plant to overcome winter stresses and facilitate bud flush in spring.

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Figure Captions

Fig. 1 Amino acid sequence and alignment of PtXSP25 with other BSP family proteins, including tobacco NtPRp27, Arabidopsis AT2G15220, wheat WAS2, and rice LOC_Os10g34930. The characters in blue, red, green, and purple indicate the signal peptide, basic amino acids (R, K, H), acidic amino acids (D, E), and putative N-glycosylation sites, respectively. Identical amino acid residues are indicated by asterisks, strongly similar sequences by two dots, and weakly similar sequences by one dot.

Fig. 2 Gene expression of *PtXSP25* in *P. trichocarpa* cultured aseptically under artificial environmental conditions. The plants cultured under long-day conditions (16 h light/8 h dark, 23 °C) with light intensity of 60 μmol m^{-2} s^{-1} were sequentially transferred to culture under short-day conditions (8 h light/16 h dark, 23 °C) for 10 weeks, and low-temperature conditions (8 h light/16 h dark, 4 °C) for 4 weeks. Whole roots (white

column) and shoots (black column) were sampled from three plants each at the start of short-day conditions (SD0), after 6 weeks of short-day conditions (SD6), and after 2 weeks of low-temperature conditions (LT2), and used for qRT-PCR. Error bars indicate standard deviation ($n = 3$). Asterisks indicate statistically significant differences versus SD0 in roots or shoots (*$P < 0.05$, † $P < 0.1$; Student's t-test).

Fig. 3 Tissue-specific expression of *PtXSP25* in poplar roots. GUS protein was detected by anti-GUS antibody on Technovit sections of the root hair region of p*PtXSP25*::GUS-transgenic hybrid aspen roots cultured for 6 weeks under short-day conditions. The sections (**a**) were treated with (**b**, **c**, **d**) or without (**e**, **f**, **g**) anti-GUS antibody followed by treatment with FITC-coupled secondary antibody. UV images (blue: **b**, **e**) and fluorescence images showing GUS protein (green: **c**, **f**) were merged (**d**, **g**) indicating the localization of *PtXSP25* expression in endodermis and xylem parenchyma in stele but not in pericycle. Bars = 100 μm.

Fig. 4 Detection of PtXSP25 protein in winter poplar by immunoblotting. Xylem sap, branches, and dormant buds were sampled from *P. trichocarpa* grown on the campus of Tsukuba University in February 2016, and subjected to SDS–PAGE with recombinant PtXSP25 protein (P) followed by Coomassie Brilliant Blue staining (**a**) or immunoblotting using an antibody raised against recombinant PtXSP25 protein (**b**). The arrow indicates the position of PtXSP25 protein. M, molecular weight marker.

Fig. 5 Detection of PtXSP25 protein by immunoblotting in roots and shoots of poplar cultured under artificial environmental conditions. Whole roots and shoots were

sampled from *P. trichocarpa* cultured aseptically as described in Fig. 2 at the start of short-day conditions (SD0), after 10 weeks of short-day conditions (SD10), and after 2 weeks of low-temperature conditions (LT2), and subjected to SDS-PAGE with recombinant PtXSP25 protein (P) followed by silver staining (**a**) or immunoblotting using an antibody raised against recombinant PtXSP25 protein (**b**). The arrow indicates the position of PtXSP25 protein. M, molecular weight marker.

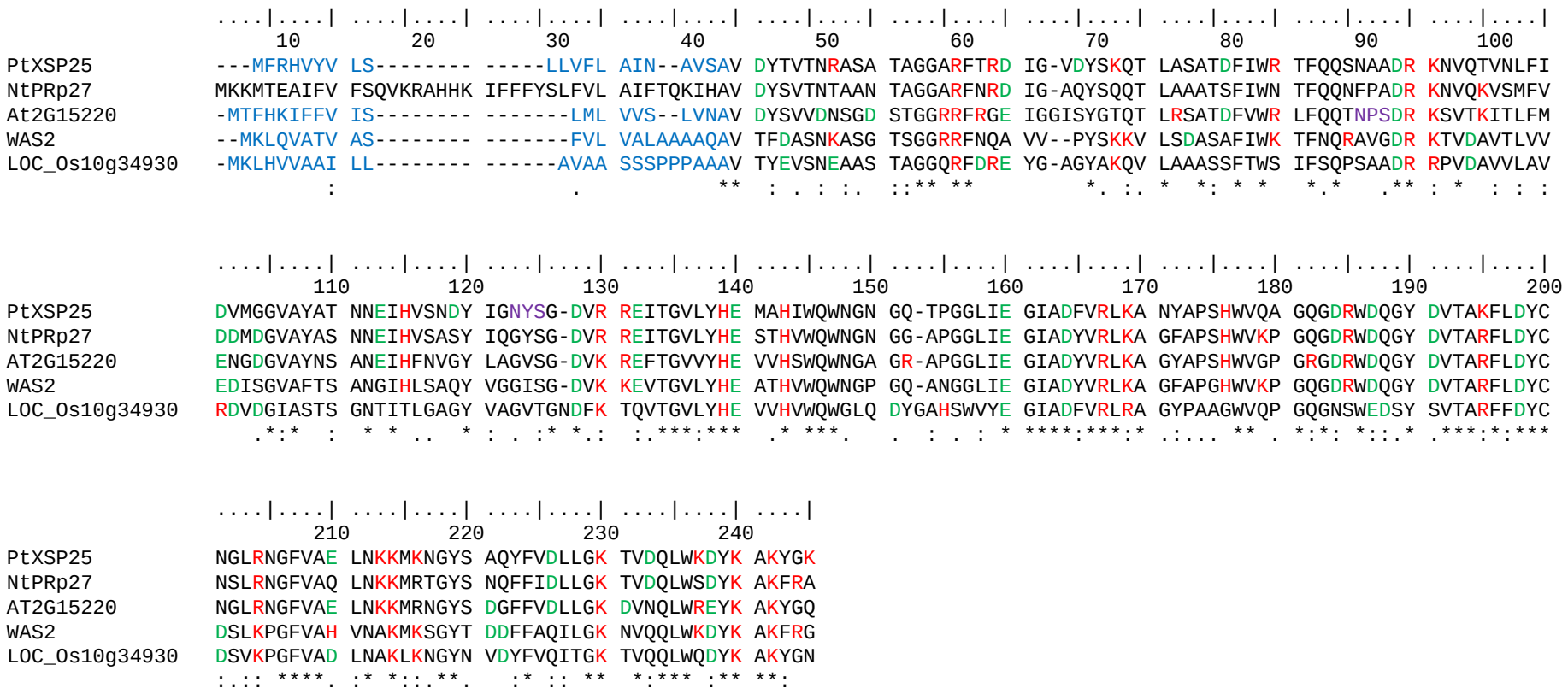
Fig. 6 Mitigation effect of PtXSP25 protein on the denaturation of LDH by drying. A drop (50 μL) of LDH solution mixed without (white bars) or with recombinant PtXSP25 protein (gray bars) or with BSA (black bars) at LDH:XSP25 or BSA molar ratio = 1:10 was left in the desiccator with phosphorus pentoxide for 3 or 12 h, and the residual activity was then measured after dissolution in 50 μL of buffer. Error bars indicate standard deviation (n = 3). ND, not detected. Asterisks indicate statistically significant differences versus without proteins at 0, 3, or 12 h (*P < 0.05; Student's t-test).

Fig. 7 Mitigation effect of PtXSP25 protein on denaturation of LDH by freeze–thaw cycling. LDH solution mixed without (Control) or with recombinant PtXSP25 protein (XSP25) or with BSA (BSA) at LDH:XSP25 or BSA molar ratio = 1:10 was subjected to freeze–thaw cycling (3 times), and the residual activity was then measured. Error bars indicate standard deviation (n = 3). Initial, without freeze–thaw cycling. Asterisks indicate statistically significant differences versus the control (*P < 0.05; Student's t-test).

Fig. 8 Photomicrographs of ice crystals in PtXSP25 protein solution. Droplets of

recombinant PtXSP25 protein (5 mg mL^{-1}) (**a**, **b**) and type III AFP (from *Zoarces elongatus Kner*, Notched-fin eelpout) (70 μg mL^{-1}) (**c**) solutions in water were frozen at a cooling rate of 0.05 °C min^{-1}, and the morphology of the ice crystals was examined. As a positive control, type III AFP showed formation of bipyramidal ice crystals due to AFP-binding (**c**), but PtXSP25 showed round-shaped crystals indicating no AFP activity (**a**, **b**). Scale bars = 500 μm (**a**, **b**), 50 μm (**c: just below the crystal**).

Fig. 9 Ice recrystallization inhibition activity of PtXSP25 protein. Distilled water (**a**), recombinant PtXSP25 protein solution (5 mg mL^{-1} in water) (**b**) or bovine serum albumin (BSA, 5 mg mL^{-1} in water) was subjected to a "splat-cooling" assay, and ice crystal images were taken after annealing at −6.4 °C for 30 min. PtXSP25 exhibited IRI activity with a mean grain size of 64% relative to water that is similar to the data of BSA (**c**). Error bars indicate the standard error of the mean. Each protein was assayed in triplicate ($n = 3$). Asterisks indicate statistically significant differences versus distilled water (*$P < 0.05$; Student's t-test).



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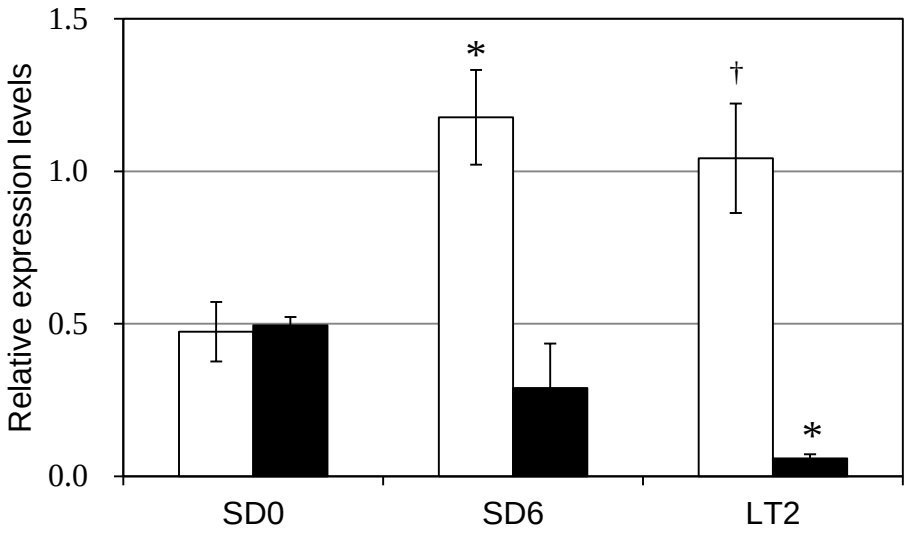

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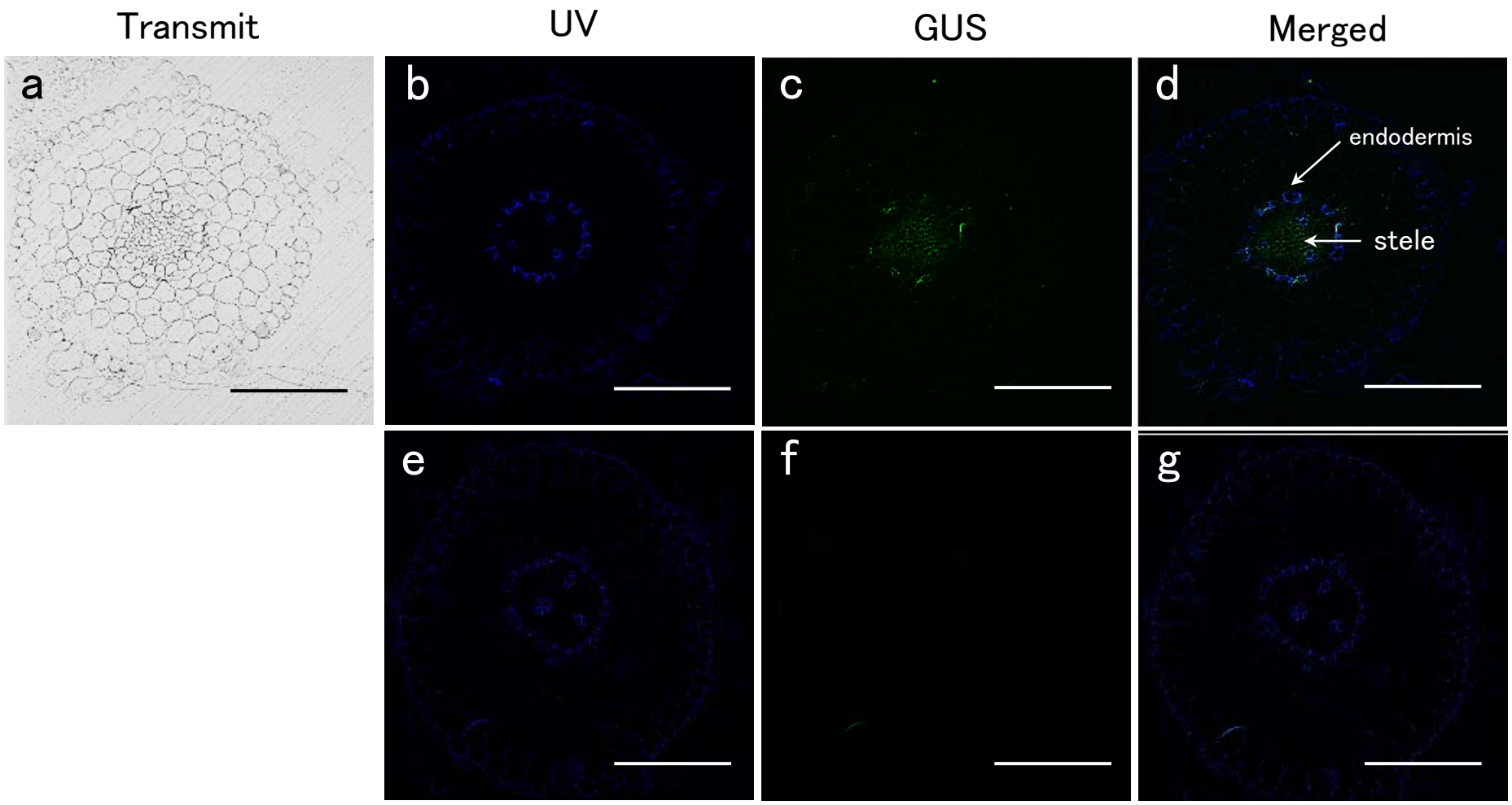

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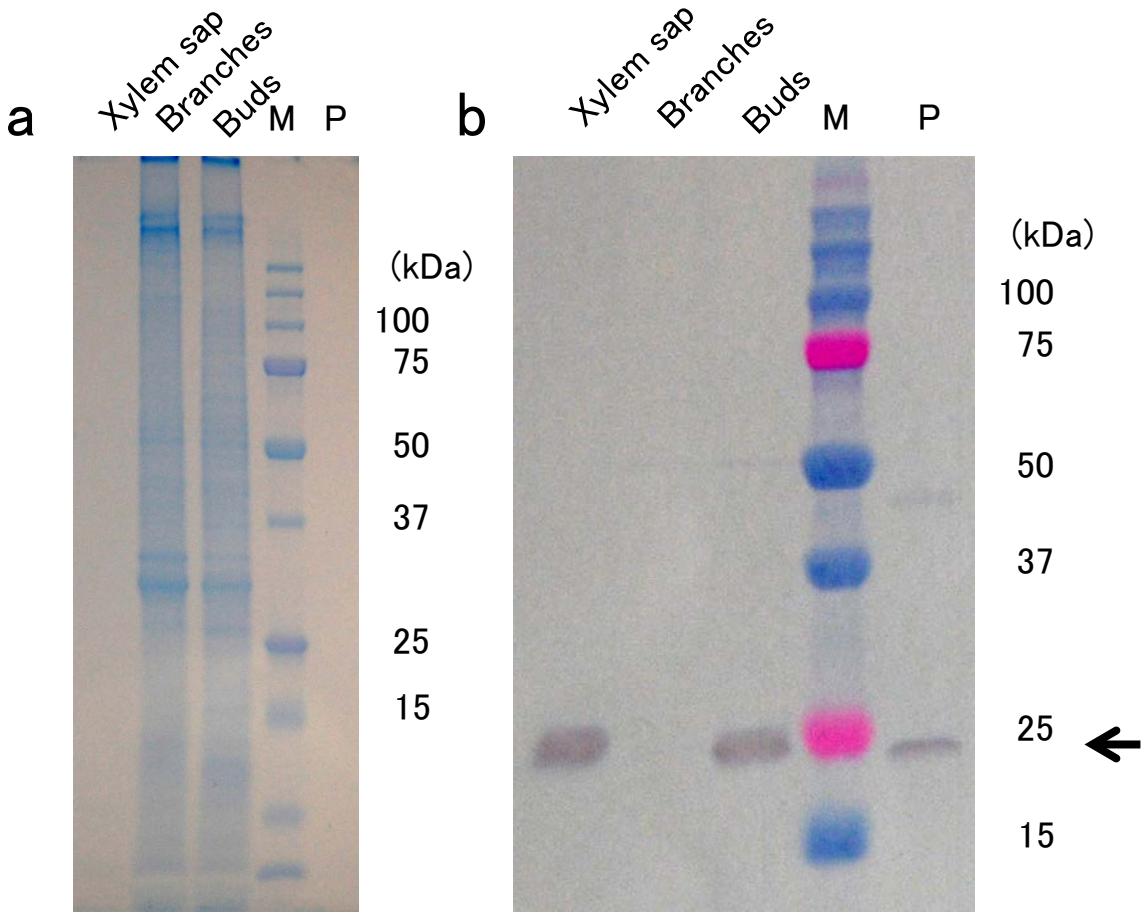

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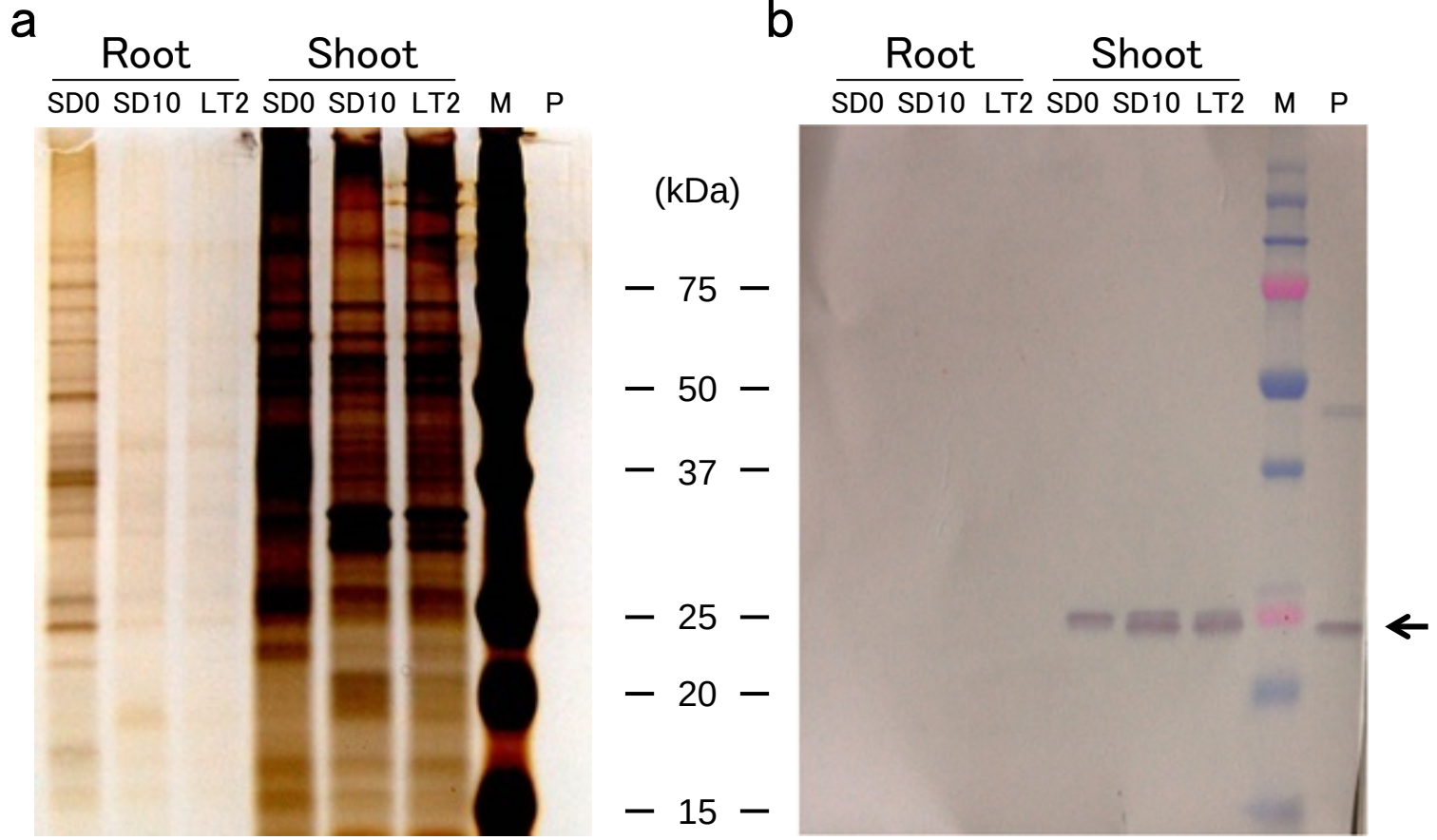

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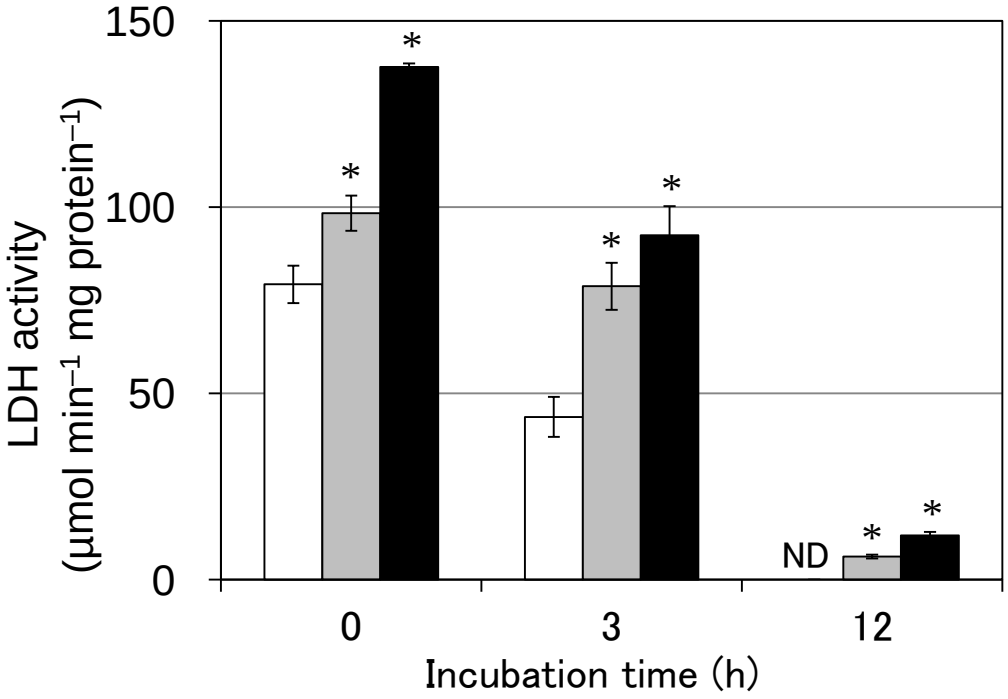

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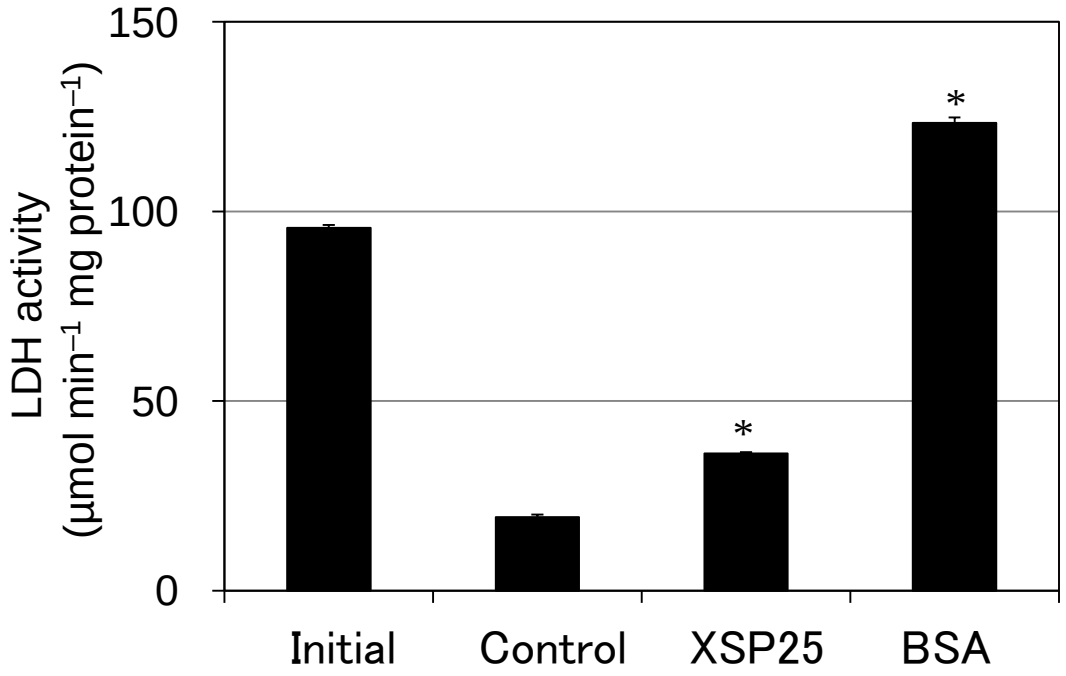

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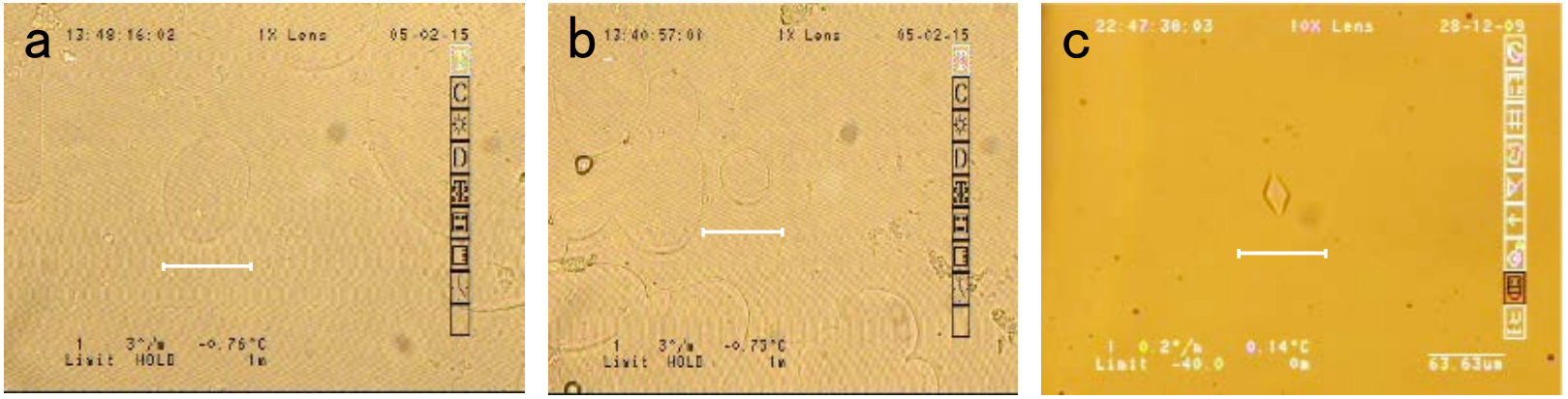

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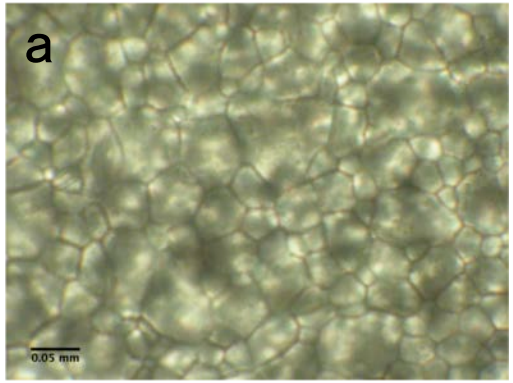

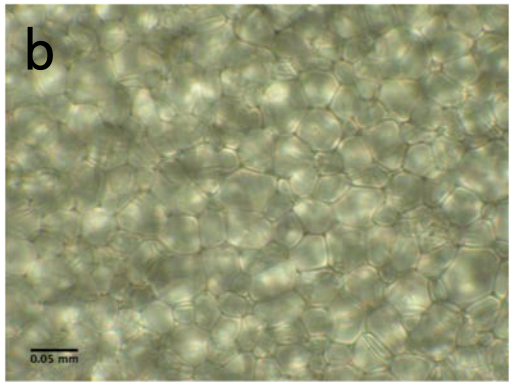

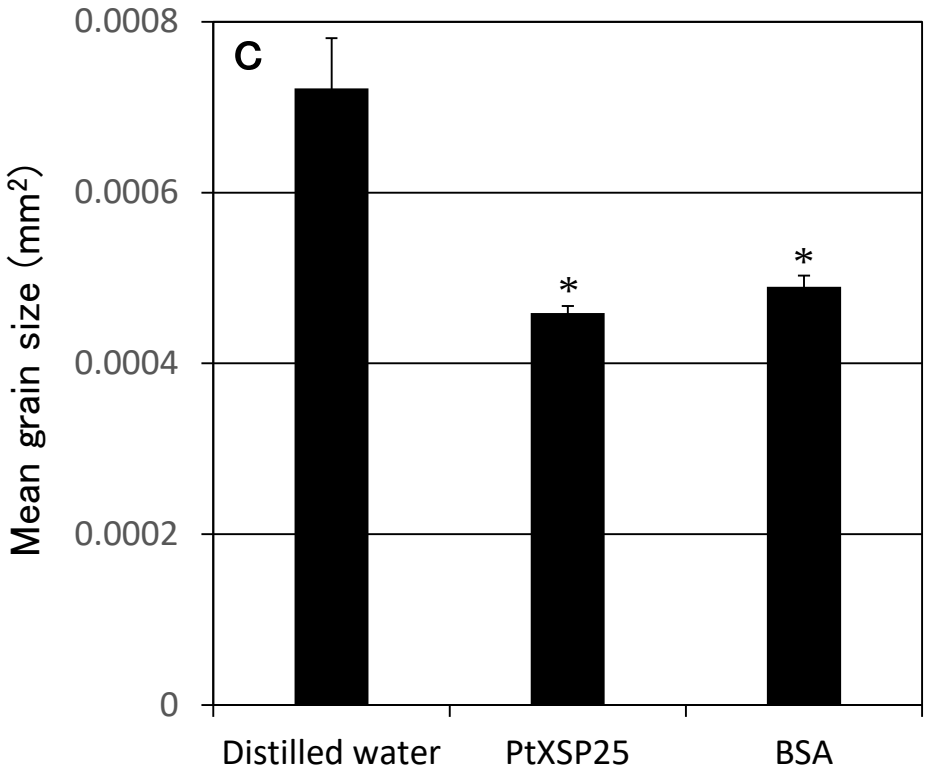

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Electronic supplementary materials

Title:

Presence of a basic secretory protein in xylem sap and shoots of poplar in winter and its physicochemical activities against winter environmental conditions

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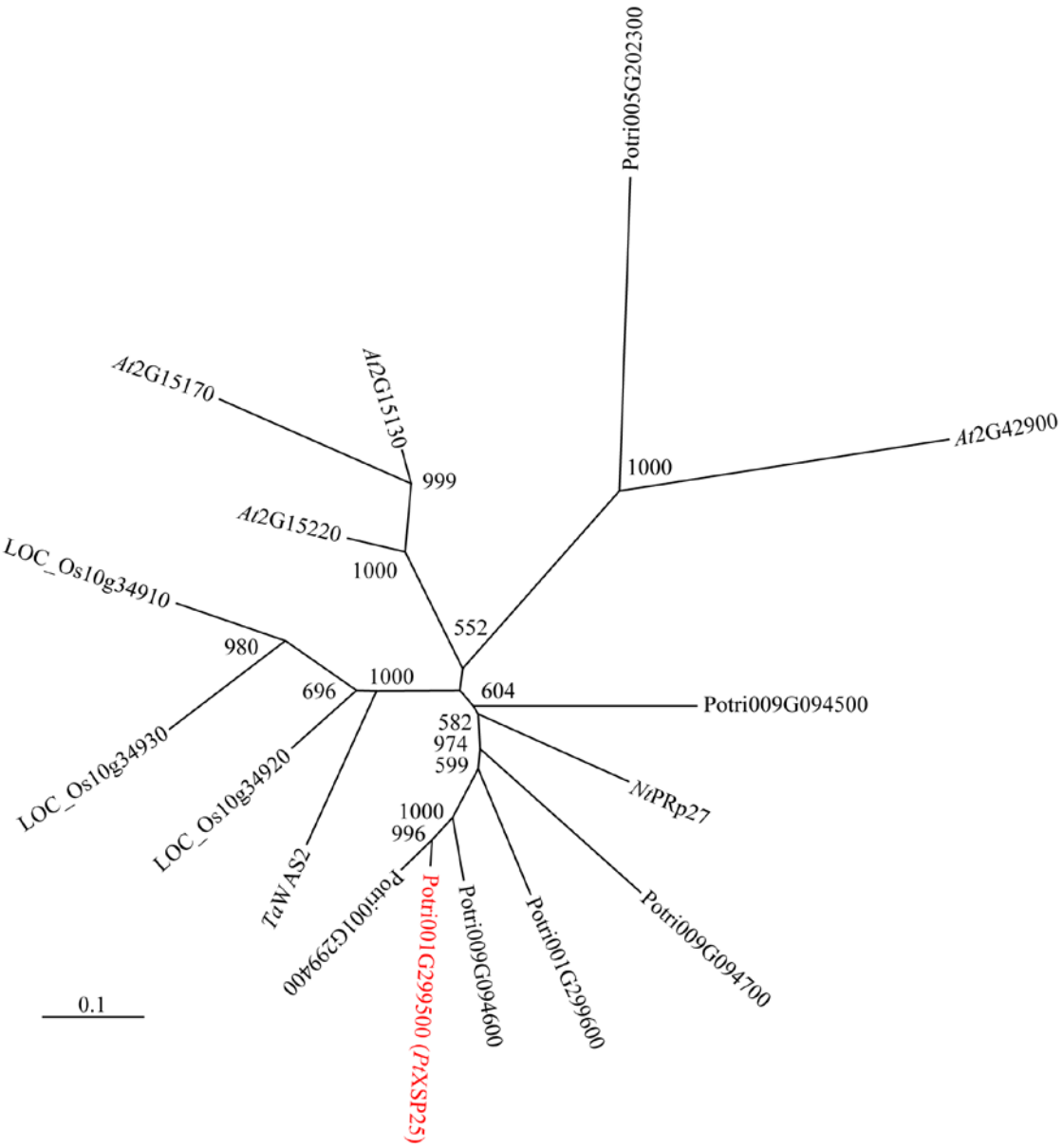

Fig. S1. Phylogenetic tree of PtXSP25 and its homologs in poplar (Potri), tobacco (NtPRp27), wheat (TaWAS2), Arabidopsis (At), and rice (LOC_Os), generated by the ClustalW program.

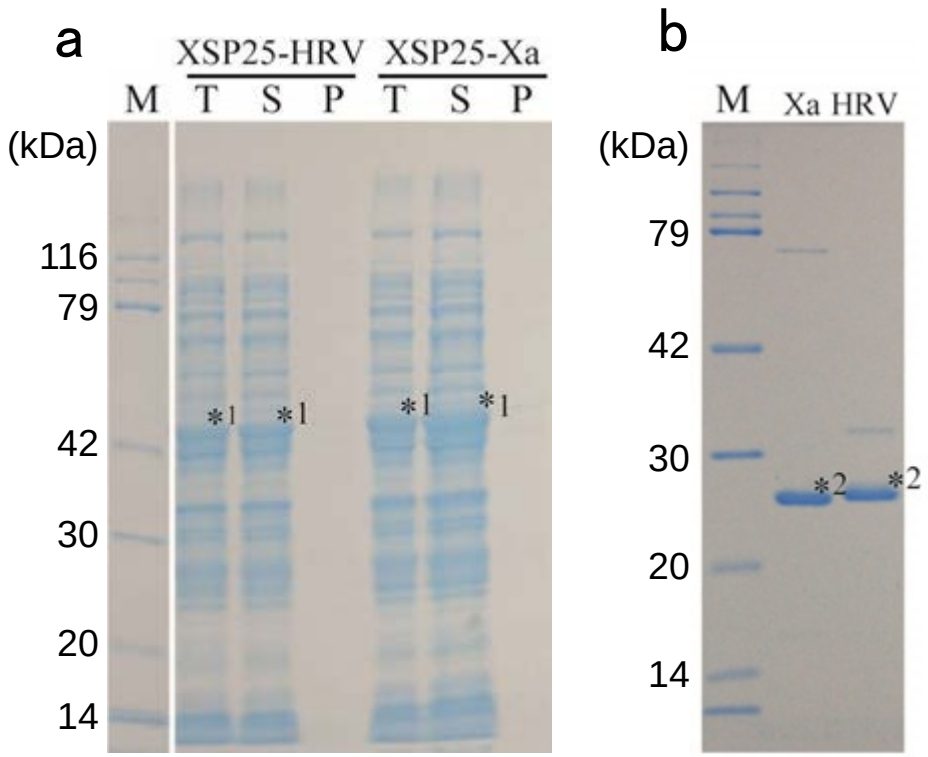

Fig. S2. Production of recombinant PtXSP25 protein and its purification by Ni-chelate affinity resin. (a) *PtXSP25* coding region without signal peptide cloned into the pCold ProS2 vector was expressed in *E. coli*, and total cell lysate (T) was separated into soluble (S) and insoluble (P) fractions. (b) Recombinant PtXSP25 protein was purified from the soluble fraction by Ni-chelate affinity resin using Factor Xa protease and HRV3C protease for antigen (Xa) and for physicochemical assay (HRV), respectively, to release the protein from the resin. *1, 6xHis-Tag:ProS2-Tag:protease-cleavage-sites:PtXSP25 fusion protein; *2, PtXSP25 protein purified by Ni-chelate affinity resin.