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

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1 **Abbreviations:**

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

7
8 **Abstract**

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

1

2 **Key words**

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

4

5 **Introduction**

6 Due to their immobility, plants typically overcome abiotic stresses associated
7 with seasonal climatic changes by altering their physiological and morphological status.
8 In temperate and subarctic zones, perennial and winter annual plants must survive
9 winter temperatures below 0 °C by overcoming freezing stress. Hence, deciduous trees
10 form dormant buds in autumn and shed leaves in early winter as morphological
11 adaptations, and they have also evolved a tolerance to freezing or acclimation to cold. In
12 addition to the direct effects of freezing, plants cannot use soil water when the soil is
13 frozen. Even under non-freezing low-temperature conditions, the supply of available
14 water from roots decreases due to increased water viscosity and decreased water
15 conductivity through water channels (aquaporins) in biological membranes (Wan et al.
16 2001). Moreover, aquaporin gene expression is suppressed under non-freezing
17 low-temperature conditions (Aohara et al. 2016; Jang et al. 2004). Therefore, plants that
18 are not covered by snow must overcome drying stress in addition to direct freezing
19 stress in winter (Larcher 2001b).

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

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

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

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

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

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

13 Among the proteins present in poplar xylem sap, two highly abundant
14 extracellular proteins, 25 kDa and 24 kDa xylem sap proteins (XSP25 and XSP24),
15 have been shown to belong to the basic secretory protein (BSP) and germin-like protein
16 (GLP) families, respectively, by mass spectrometry (Aohara et al. 2016; Furukawa et al.
17 2011b). The XSP25 and XSP24 genes were found to be abundantly expressed in the
18 roots of winter poplar soil-grown in outdoor pots and in the roots of soil-grown plants
19 under short-day and low-temperature conditions in the culture room.

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

1 physicochemical functions have yet to be determined.

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

6

7 **Materials and methods**

8

9 **Plant materials and culture conditions**

10

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

24

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

2
3 The amino acid sequence of PtXSP25, registered as XM_002300207.1
4 (Potri.001G299500) in the *P. trichocarpa* database, was aligned with tobacco NtPRp27
5 (BAA81904.1), *Arabidopsis* AT2G15220 (NP_565369.1), wheat WAS2 (AAD46133.1),
6 and rice LOC_Os10g34930 (AAP54394.1) using Clustal W. The signal sequences for
7 secretion and N-glycosylation sites were predicted using the SignalP
8 (<http://www.cbs.dtu.dk/services/SignalP/>; Emanuelsson et al. 2007) and NetNGlyc 1.0
9 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) programs, respectively.

10

11 **Gene expression analysis**

12

13 Whole roots and shoots sampled at various time points were ground in liquid nitrogen
14 and then homogenized in the same volume of RLC buffer using the RNeasy Plant Mini
15 Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol, and the resultant
16 RNA was used to analyze *XSP25* expression by real-time quantitative
17 reverse-transcription polymerase chain reaction (qRT-PCR) with *ubiquitin*
18 (gi566172648; Potri.005G198700) as an internal standard (= 1). cDNA was synthesized
19 by ReverTra Ace (Toyobo, Tokyo, Japan) using oligo (dT)₂₀ primers at 42 °C for 60 min
20 and 99 °C for 5 min, and real-time PCR was performed using the SYBR Premix Ex Taq
21 II kit (TaKaRa Bio Inc., Shiga, Japan) under the following conditions: denaturation for
22 30 s at 95 °C, annealing for 10 s at 63 °C, and extension for 31 s at 72 °C, for a total of
23 50 cycles. PCR products were detected using the 7300 Real-Time PCR System (Thermo
24 Fisher Scientific, Kanagawa, Japan). The following primers were used for qRT-PCR:

1 *PtXSP25* forward 5'-ATGGACAAACACCAGGAGGA-3', *PtXSP25* reverse

2 5'-ACCTTGATCCCACCTGTAC-3', *UBQ* forward

3 5'-TGAACCAAATGATACCATTGATAG-3', and *UBQ* reverse

4 5'-GTAGTCGCGAGCTGTCTTG-3'.

5

6 **Localization of *PtXSP25* promoter activity**

7

8 To produce *PtXSP25promoter::GUS*-transgenic plants, the promoter (1970 bp) of

9 *PtXSP25* was cloned into the pENTR-D-Topo vector (Thermo Fisher Scientific Inc.,

10 Tokyo, Japan) and used for transformation of the hybrid aspen T89 line according to the

11 procedure described by Nilsson et al. (1992), because hybrid aspen T89 is a good plant

12 material for genetic transformation. Roots of transgenic plants were embedded in

13 Technovit 7100 resin (Kulzer, Hanau, Germany), and sections were prepared at the root

14 hair region using an ultramicrotome, because the root hair region abundantly expresses

15 genes that encode products delivered into xylem sap (Satoh 2006). Sections were

16 subjected to immunohistochemistry using rabbit anti-GUS antibody and anti-rabbit IgG

17 antibody coupled with fluorescein isothiocyanate as primary and secondary antibodies,

18 respectively, according to the procedure described by Jasik et al. (2011), because the

19 activity level was too low to identify β -glucuronidase-expressing tissues in the

20 transformant lines. Immunofluorescence was visualized using a Leica DMRB

21 fluorescence microscope (Leica, Wetzlar, Germany).

22

23 **Production and purification of recombinant *PtXSP25* protein**

24

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

7 The primer sequences used to construct the recombinant PtXSP25 protein were
8 as follows: rPtXSP25-HRV-F,

9 5'-CTGTTCCAGGGGCCCGTGGACTACTGTCACCAACAGAG-3';

10 rPtXSP25-Xa-F,

11 5'-GGTATCGAAGGTAGGGTGGACTACTGTCACCAACAGAG-3'; and

12 rPtXSP25-Inf-R (common to rPtXSP25-HRV and rPtXSP25-Xa),

13 5'-ACCGAGCTCCATATGCCTATTTCCATACTTGGCCTTG TAGTC-3'

14

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

16

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

1 80% ethanol, dissolved in the sample buffer, and subjected to SDS–PAGE (equivalent to
2 1.25 mL xylem sap/lane) after heating at 95 °C for 5 min. Recombinant PtXSP25
3 protein was used as a standard (5 ng/lane). Total proteins and PtXSP25 protein were
4 then detected by Coomassie Brilliant Blue or silver staining and immunoblotting using
5 an antibody raised against recombinant PtXSP25 protein, respectively, as described
6 previously by Oda et al. (2003) for xylem sap and Sakuta and Satoh (2000) for tissues.
7 Recombinant PtXSP25 protein was used as the standard.

8

9 **Drying and freeze–thaw treatments and measurement of lactate dehydrogenase**

10 **(LDH) activity**

11

12 To evaluate the effect of drying, a drop (50 µL) of water containing 200 ng of LDH
13 from rabbit muscle (Roche, Basel, Switzerland) mixed with recombinant PtXSP25
14 protein or bovine serum albumin (BSA) at LDH:XSP25 or BSA molar ratio = 1:10 was
15 placed on the wall of a polypropylene microtube, and left in a desiccator with
16 phosphorus pentoxide for 3 or 12 h. Residual activity was measured after dissolution in
17 50 µL of 100 mM Na-phosphate buffer (pH 6.0). To evaluate the effects of freeze–thaw
18 cycling, 50 µL of 100 mM Na-phosphate buffer (pH 6.0) containing 200 ng of LDH was
19 mixed with recombinant PtXSP25 protein or BSA at LDH:XSP25 or BSA molar ratio =
20 1:10. The tube was immersed in liquid nitrogen to freeze and then thawed at room
21 temperature. This freeze–thaw cycle was repeated three times. Initial and residual
22 activities of LDH were measured by monitoring the decrease in absorbance at 340 nm at
23 27 °C in the reaction mixture (1 mL) containing 2 µL of the solution mentioned above,
24 100 mM Na-phosphate buffer (pH 6.0), 0.1 mM NADH, and 2 mM pyruvate.

1

2 **Measurement of antifreeze and ice recrystallization inhibition activities**

3

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

17

18 **Results**

19

20 **Structural characteristics of BSP family proteins**

21

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

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

6

7 **Expression of *PtXSP25* under artificial environmental conditions**

8

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

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

21

22 **Purification of recombinant PtXSP25 protein**

23

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

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

6

7 **Immunological detection of PtXSP25 in poplar**

8

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

24

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

3

4 As LDH is one of the fragile enzymes associated with drying and freezing damage, dry
5 material from a drop of LDH solution mixed with the recombinant PtXSP25 protein was
6 incubated with phosphorus pentoxide in a desiccator for 3 or 12 h and resolved with
7 buffer. Residual LDH activity was measured using BSA as a positive control, because
8 BSA is widely used as a protectant for various enzymes. LDH activity decreased to
9 55.1% of that at 0 h without the addition of any protectant after 3 h of desiccation, but
10 decreased to only 80.1% of that at 0 h with the addition of recombinant PtXSP25
11 protein or BSA (Fig. 6). With the addition of PtXSP25 protein or BSA, 0.06% of LDH
12 activity remained even after 12 h of desiccation, whereas no activity was detected at this
13 time point without the addition of protectant.

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

20

21 **Effects of PtXSP25 protein on ice formation**

22

23 To evaluate the antifreeze activity of PtXSP25, recombinant PtXSP25 protein solution
24 (5 mg mL⁻¹) was frozen and the shape of ice crystals was observed. A type III antifreeze

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

15 Next, to evaluate the IRI activity of PtXSP25, recombinant PtXSP25 protein
16 solution (5 mg/mL) was subjected to splot-cooling assay to measure the mean grain size
17 of ice crystals. The ice crystals were reduced in size to 64% of the size in the water
18 controls that is similar to the data of BSA (Fig. 9). PtXSP25 activity was similar to that
19 of TaIRI-2 (IRI protein) (Tremblay et al. 2005) and WCS120 (dehydrin) (Houde et al.
20 1995) in wheat measured previously in our laboratory (Chow-Shi-Yée et al. 2016).

21

22 **Discussion**

23

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

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

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

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

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

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

1 the cell wall.

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

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

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

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

16

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18

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20 Technology for their help regarding aseptic culture and genetic transformation of poplar.
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23

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9

10 Figure Captions

11

12 **Fig. 1** Amino acid sequence and alignment of PtXSP25 with other BSP family proteins,

13 including tobacco NtPRp27, Arabidopsis AT2G15220, wheat WAS2, and rice

14 LOC_Os10g34930. The characters in blue, red, green, and purple indicate the signal

15 peptide, basic amino acids (R, K, H), acidic amino acids (D, E), and putative

16 N-glycosylation sites, respectively. Identical amino acid residues are indicated by

17 asterisks, strongly similar sequences by two dots, and weakly similar sequences by one

18 dot.

19

20 **Fig. 2** Gene expression of *PtXSP25* in *P. trichocarpa* cultured aseptically under artificial

21 environmental conditions. The plants cultured under long-day conditions (16 h light/8 h

22 dark, 23 °C) with light intensity of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ were sequentially transferred to

23 culture under short-day conditions (8 h light/16 h dark, 23 °C) for 10 weeks, and

24 low-temperature conditions (8 h light/16 h dark, 4 °C) for 4 weeks. Whole roots (white

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

6
7 **Fig. 3** Tissue-specific expression of *PtXSP25* in poplar roots. GUS protein was detected
8 by anti-GUS antibody on Technovit sections of the root hair region of
9 *pPtXSP25::GUS*-transgenic hybrid aspen roots cultured for 6 weeks under short-day
10 conditions. The sections (**a**) were treated with (**b, c, d**) or without (**e, f, g**) anti-GUS
11 antibody followed by treatment with FITC-coupled secondary antibody. UV images
12 (blue: **b, e**) and fluorescence images showing GUS protein (green: **c, f**) were merged (**d,**
13 **g**) indicating the localization of *PtXSP25* expression in endodermis and xylem
14 parenchyma in stele but not in pericycle. Bars = 100 μm .

15
16 **Fig. 4** Detection of PtXSP25 protein in winter poplar by immunoblotting. Xylem sap,
17 branches, and dormant buds were sampled from *P. trichocarpa* grown on the campus of
18 Tsukuba University in February 2016, and subjected to SDS-PAGE with recombinant
19 PtXSP25 protein (P) followed by Coomassie Brilliant Blue staining (**a**) or
20 immunoblotting using an antibody raised against recombinant PtXSP25 protein (**b**). The
21 arrow indicates the position of PtXSP25 protein. M, molecular weight marker.

22
23 **Fig. 5** Detection of PtXSP25 protein by immunoblotting in roots and shoots of poplar
24 cultured under artificial environmental conditions. Whole roots and shoots were

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

7

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

15

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

23

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

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

7
8 **Fig. 9** Ice recrystallization inhibition activity of PtXSP25 protein. Distilled water (**a**),
9 recombinant PtXSP25 protein solution (5 mg mL⁻¹ in water) (**b**) or bovine serum
10 albumin (BSA, 5 mg mL⁻¹ in water) was subjected to a “splat-cooling” assay, and ice
11 crystal images were taken after annealing at -6.4 °C for 30 min. PtXSP25 exhibited IRI
12 activity with a mean grain size of 64% relative to water that is similar to the data of
13 BSA (**c**). Error bars indicate the standard error of the mean. Each protein was assayed in
14 triplicate ($n = 3$). Asterisks indicate statistically significant differences versus distilled
15 water ($*P < 0.05$; Student’s t-test).

16
17



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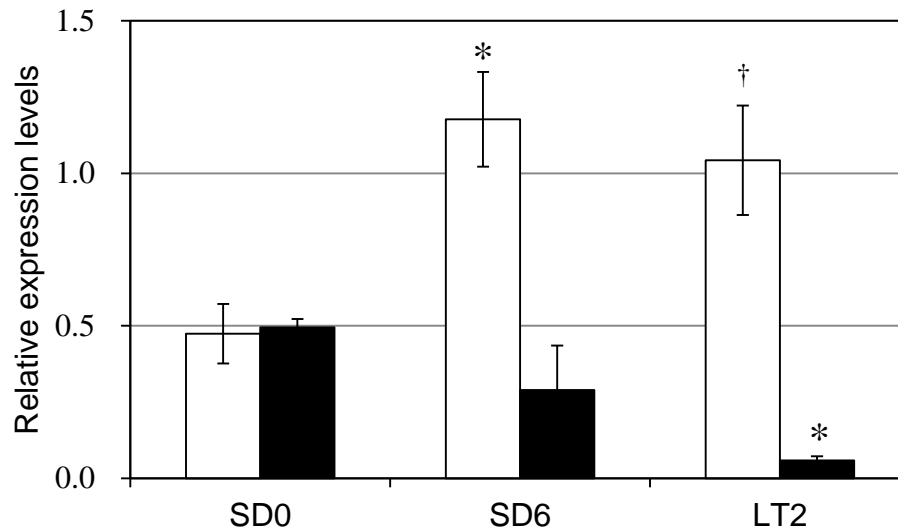


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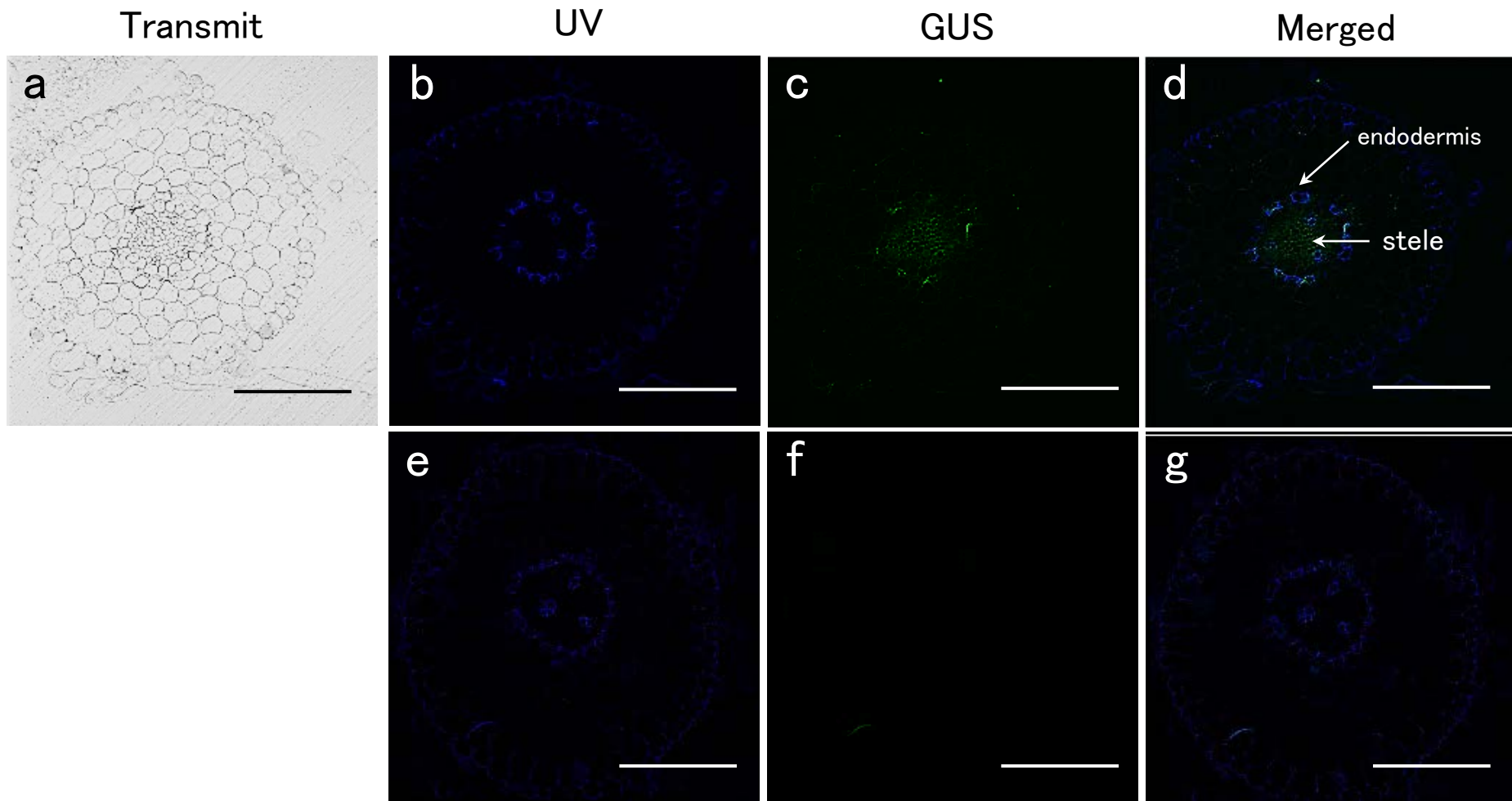


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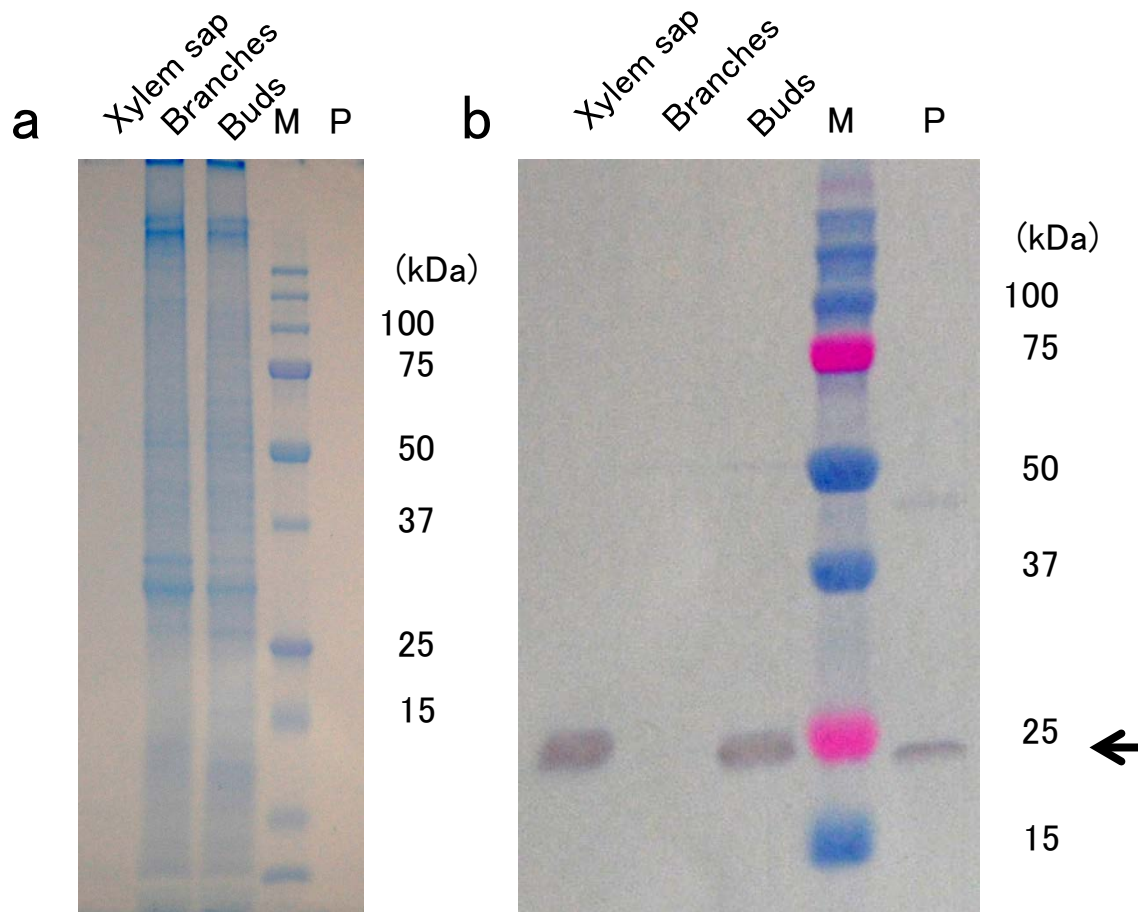


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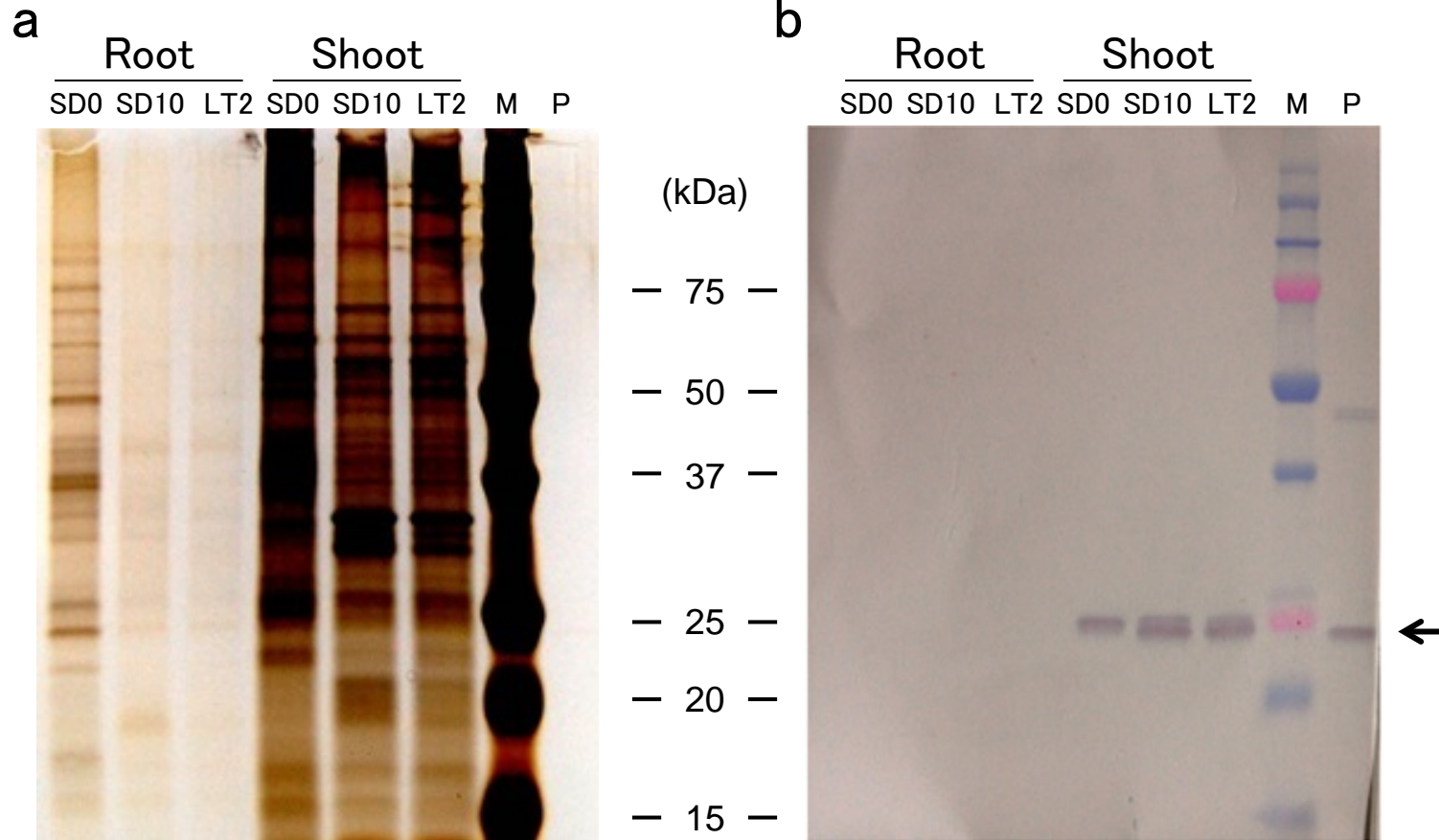


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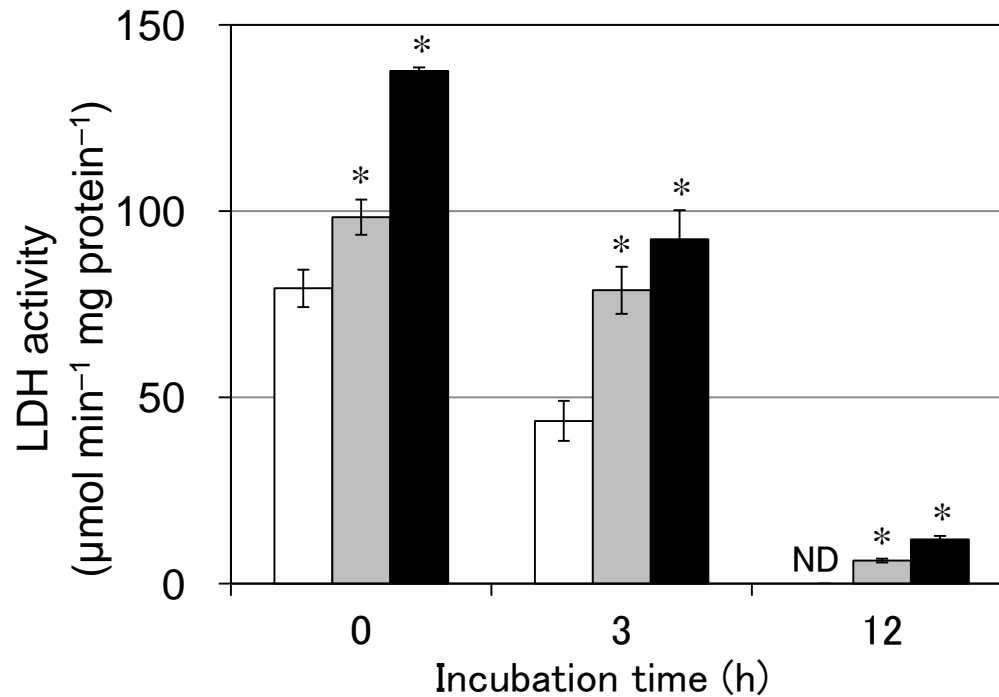


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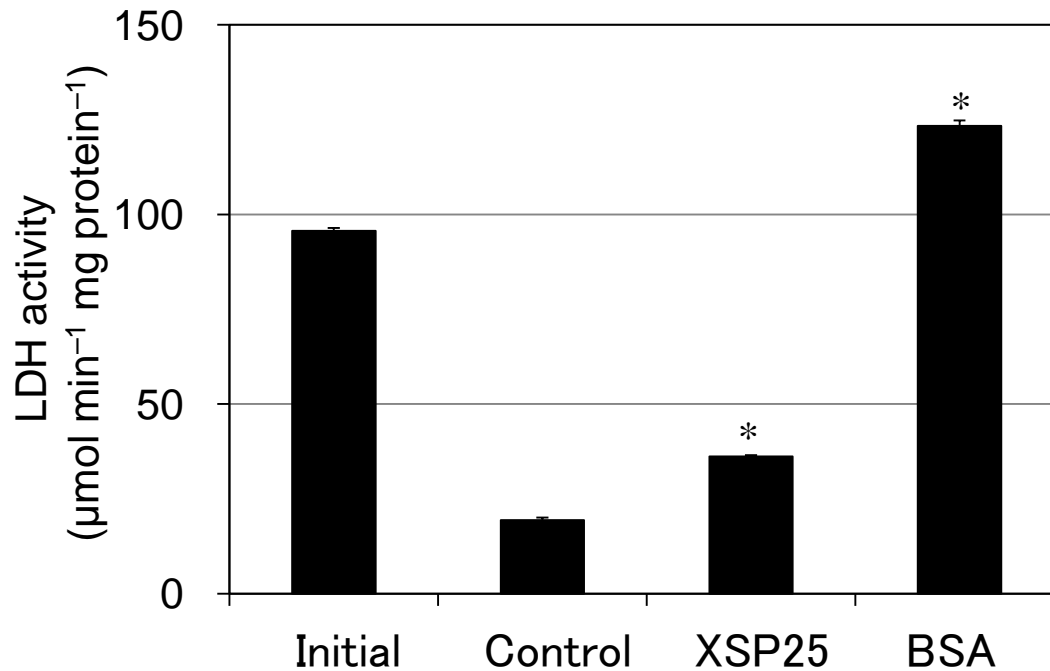


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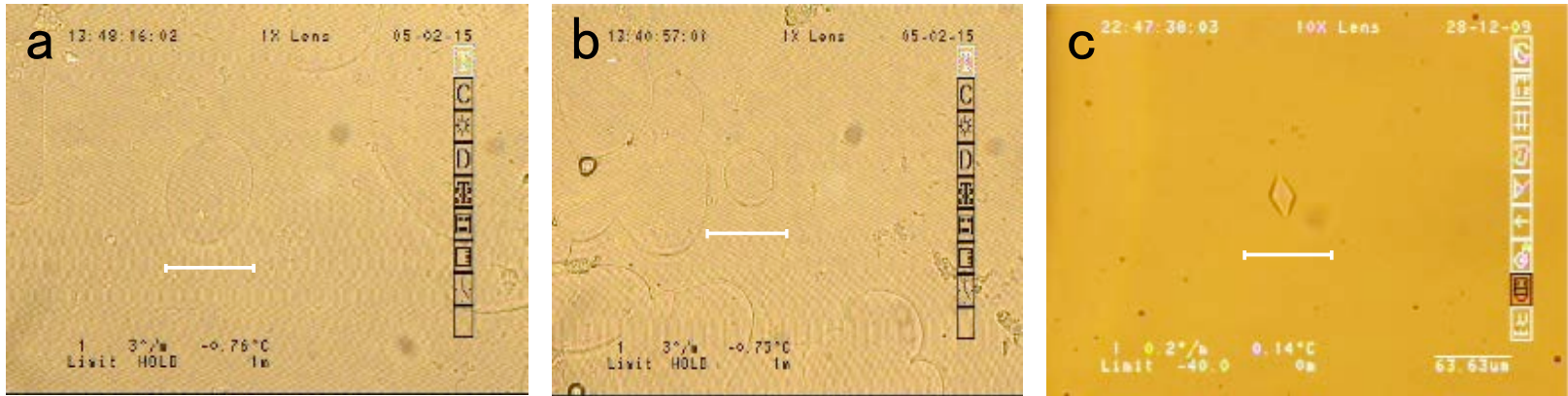


Fig. 8. Photomicrographs of ice crystals in PtXSP25 protein solution. Droplets of recombinant PtXSP25 protein (5 mg/mL) (a, b) and type III AFP (from *Zoarces elongatus Kner*, Notched-fin eelpout) (70 μ g/mL) (c) solutions in water were frozen at a cooling rate of 0.05° C/min, and the morphology of the 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 μ m (a, b), 50 μ m (c).

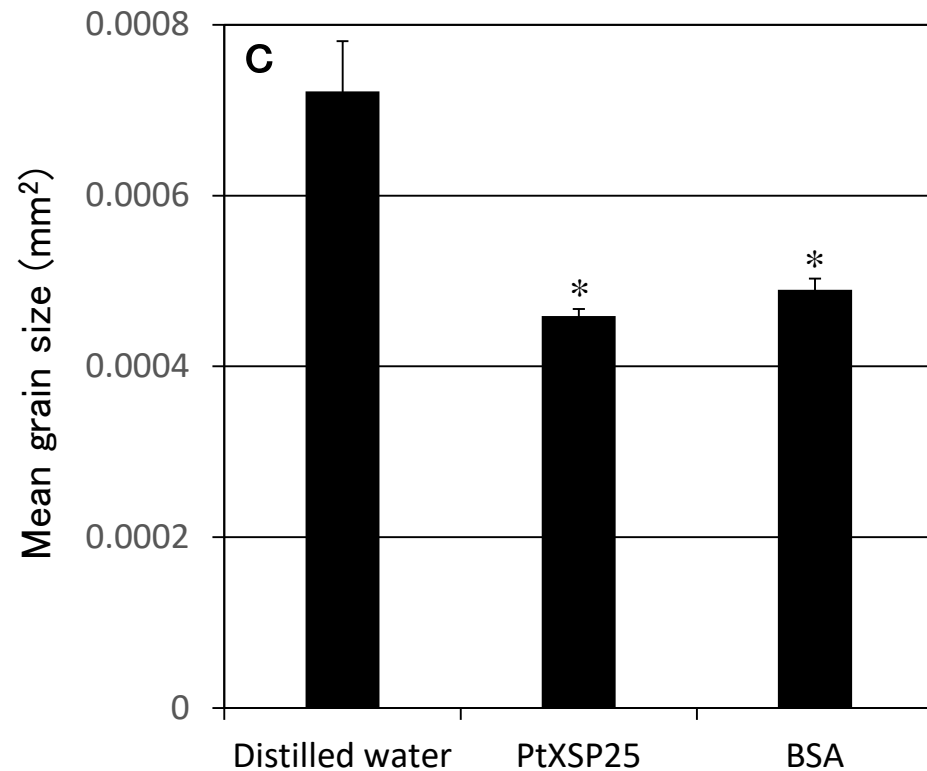
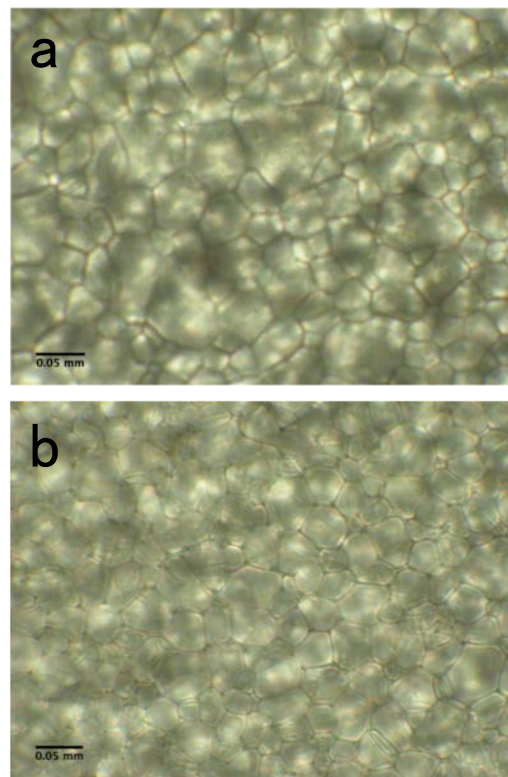


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Electronic supplementary materials

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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Journal:

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Content:

Figs. S1–S2

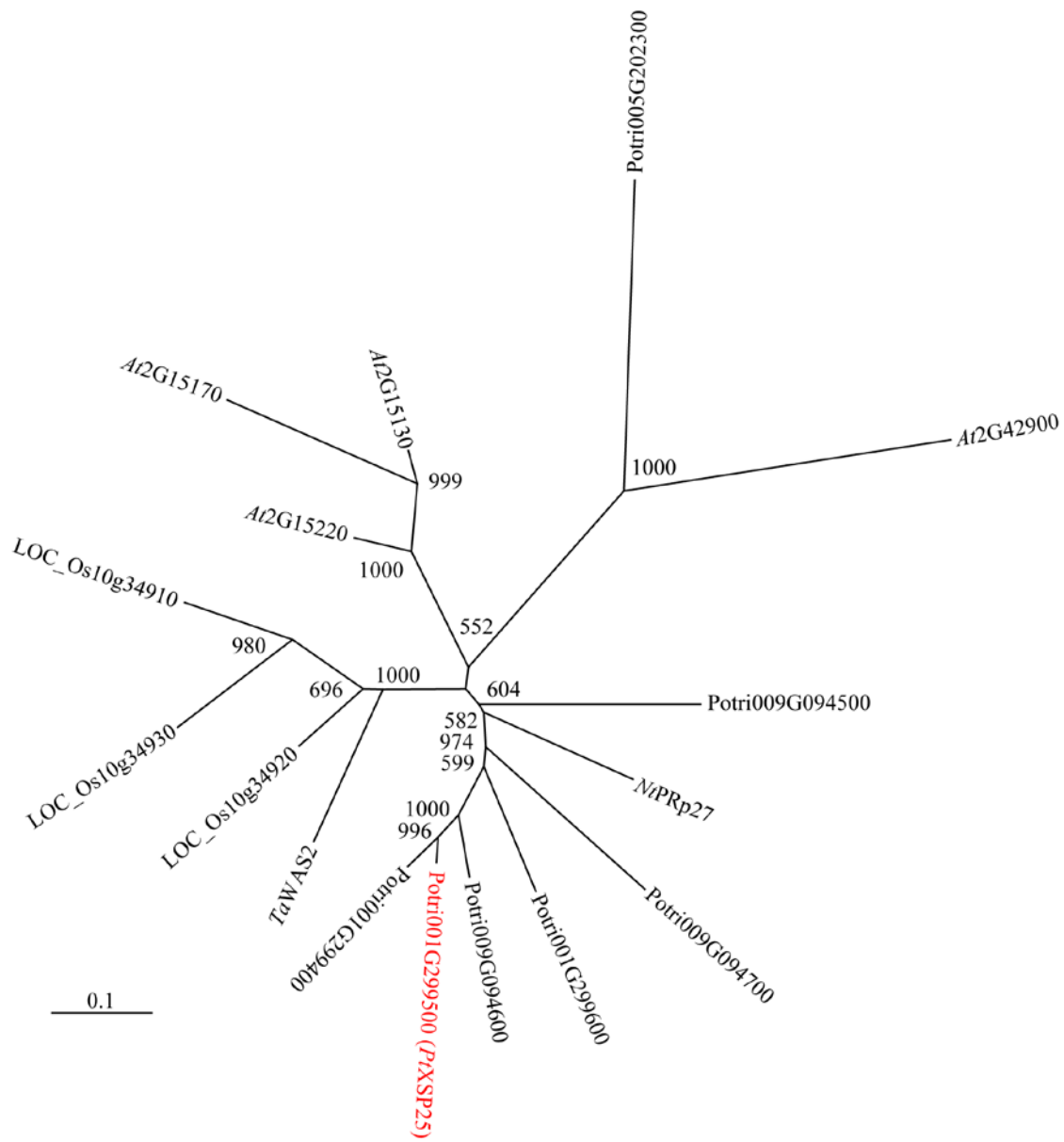


Fig. S1. Phylogenetic tree of PtXSP25 and its homologs in poplar (Potri), tobacco (NtPRp27), wheat (TaWAS2), Arabidopsis (At), and rice (LOC_Os), generated by the ClustalW program.

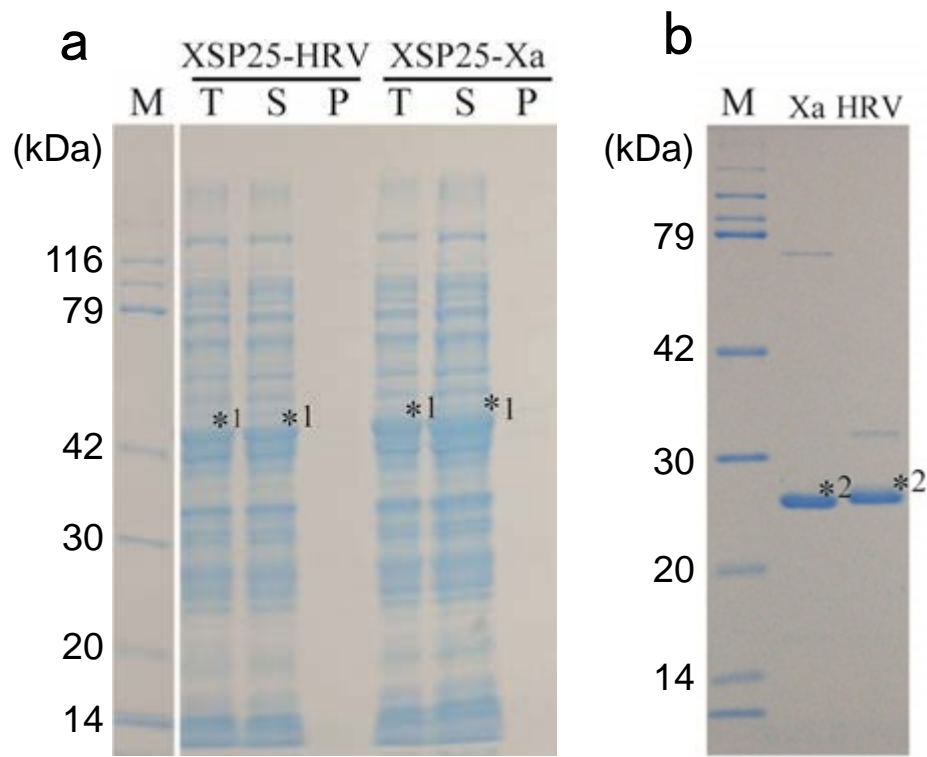


Fig. S2. Production of recombinant PtXSP25 protein and its purification by Ni-chelate affinity resin. (a) *PtXSP25* coding region without signal peptide cloned into the pCold ProS2 vector was expressed in *E. coli*, and total cell lysate (T) was separated into soluble (S) and insoluble (P) fractions. (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.