Identification of a xylem sap germin-like protein and its expression under short-day and non-freezing low-temperature conditions in poplar root

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Abstract In the shoots of photoperiod-sensitive deciduous trees, including poplar, short-day and non-freezing low-temperature conditions induce bud dormancy and its break, respectively, and these conditions also induce shoot cold acclimation. In a previous study, levels of organic and inorganic components, including proteins, increased in the xylem sap of *Populus nigra* in winter, suggesting seasonal changes in root functions. Here, analysis of a major xylem sap protein (XSP24) of *P. nigra* in winter by mass spectrometry together with the whole genome sequence of *P. trichocarpa* and transcript abundance in roots under short-day conditions identified *PtXSP24* to be a germin-like protein of the cupin superfamily, which was reported to be associated with various stresses and to have oxalate oxidase and/or superoxide dismutase activities in the cell wall. Expression of XSP24, which corresponds to *PtXSP24* in *P. maximowiczii*, a potentially useful Japanese native poplar in the same phylogenetic clade as *P. trichocarpa*, was enhanced under short-day and non-freezing low-temperature conditions, as well as by application of abscisic acid. These results suggest that XSP24 is involved in tolerance to environmental stresses in autumn and early winter.

Key words: Abscisic acid, acclimation, apoplast, autumn, *Populus*.

Poplar, or aspen, is a deciduous tree used for ornamentation, timber, pulp, biomass and biofuel production. It includes species of the genus *Populus*, which inhabits the temperate zone of the Northern Hemisphere, and various hybrids are cultivated in this region. Because the *Populus trichocarpa* genome has been sequenced (Tuskan et al. 2006) and a hybrid aspen transformation system has been established (Nilsson et al. 1992), poplar is used as a model woody plant for molecular biological studies, particularly of wood formation (Ohtani et al. 2011).

Because perennial plants, including deciduous trees, live in an environment that changes annually, they have a seasonal cycle of growth and dormancy that is regulated by environmental cues, such as photoperiod and temperature (Welling et al. 2002). In photoperiod-sensitive plant species, such as poplar, short days in late summer result in a decrease in gibberellin (GA) level in shoots (Olsen et al. 1995) and an increase in abscisic acid (ABA) level in buds (Li et al. 2005). In early winter, non-freezing low temperatures lead to increased GA levels by upregulating the expression of GA biosynthesis enzymes, and induce *FLOWERING LOCUS T* to break endo-dormancy (Rinne et al. 2011). These short days and non-freezing low temperatures are also associated with acclimation to cold mid-winter conditions (Welling and Palva 2006).

In deciduous trees, root growth and functions are activated before bud break from late winter to early spring, and the transport of water, mineral nutrients and free sugars to shoots from roots is initiated prior to shoot growth (Canam et al. 2008; Teskey and Hinckley 1981). Although the annual rhythms of growth and functions differ between shoots and roots in woody plants, the mechanisms that regulate root functions are poorly understood compared to those of shoots.

In a previous study, we analyzed xylem sap seasonally collected from the branches of *Populus nigra* planted on the campus of the University of Tsukuba, and found that the levels of calcium, potassium, glucose and proteins in xylem sap peaked from winter to spring (Furukawa et al. 2011a). Among the proteins, 25 and 24kDa proteins,
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named XSP25 and XSP24, were highly abundant in xylem sap; the former was most abundant and the latter was second-most abundant. Mass spectrometry analysis of XSP25 revealed its high similarity to ABA-inducible basic secretory protein (Furukawa et al. 2011b). Using the whole genome sequence of Populus trichocarpa, PmXSP25 was cloned from Populus maximowiczii, a poplar native to Japan, which is included in the same phylogenetic clade as P. trichocarpa and a potential candidate Populus species for production of pulp and/or biomass in Japan (Ministry of Agriculture, Forestry and Fisheries 2011). Analysis of gene expression in P. maximowiczii plants propagated by cutting culture and grown in pots with soil revealed that PmXSP25 expression was abundant in roots in December and February and enhanced by ABA application to the roots in autumn (Furukawa et al. 2011b).

In this study, we analyzed XSP24 in Populus nigra xylem sap using MALDI-TOF MS and MS/MS according to Furukawa et al. (2011b). Proteins from xylem sap (20 ml) obtained on February 18, 2008 were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250. The second-most intense band at 24 kDa (XSP24), below XSP25, was excised and subjected to MALDI-TOF mass spectrometry in the positive-ion reflector mode, followed by data analysis using the Mascot software. (B) Amino acid sequence of PmXSP25, registered as gi66229978 in the Populus trichocarpa database, aligned with GLPs of other species. The underlined sequence is the amino acid sequence of Populus nigra XSP24 identified by mass spectrometry. The broken underlining is the signal sequence for secretion predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/; Emanuelsson et al. 2007). The bold characters indicate the cupin conserved domain according to the Pfam website. Using the Clustal W software, the sequence was aligned with the closest homologs in cotton, grapevine and Arabidopsis (GrGLP14: gi823124638, VvGER3: gi526118118, AtGLP9: gi15233510), belonging to the GER 7 clade, and with wheat oxalate oxidase (TaGermin GF-2.8: gi2112129), belonging to the GER 1 clade, in the germin-like protein family (Barman and Banerjee 2015). Identical amino acid residues are indicated by asterisks, strongly similar sequences by two dots, and weakly similar sequences by one dot.

Figure 1. Identification of XSP24 and the amino acid sequence of P. trichocarpa gi566229978 (PmXSP24) with homologous germin-like proteins from other species. (A) Proteins prepared from 20 ml xylem sap collected from a branch of Populus nigra on February 28, 2008 were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250. The second-most intense band at 24 kDa (XSP24), below XSP25, was excised and subjected to MALDI-TOF mass spectrometry in the positive-ion reflector mode, followed by data analysis using the Mascot software. (B) Amino acid sequence of PmXSP25, registered as gi66229978 in the Populus trichocarpa database, aligned with GLPs of other species. The underlined sequence is the amino acid sequence of Populus nigra XSP24 identified by mass spectrometry. The broken underlining is the signal sequence for secretion predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/; Emanuelsson et al. 2007). The bold characters indicate the cupin conserved domain according to the Pfam website. Using the Clustal W software, the sequence was aligned with the closest homologs in cotton, grapevine and Arabidopsis (GrGLP14: gi823124638, VvGER3: gi526118118, AtGLP9: gi15233510), belonging to the GER 7 clade, and with wheat oxalate oxidase (TaGermin GF-2.8: gi2112129), belonging to the GER 1 clade, in the germin-like protein family (Barman and Banerjee 2015). Identical amino acid residues are indicated by asterisks, strongly similar sequences by two dots, and weakly similar sequences by one dot.
ATG AGA AGT GTT CAT TTC CTA CTA G-3’ and reverse primer 5’-GACAAA TCCAA CATA GAGTGT G-3’) was designed in regions conserved among these six genes and used for PCR under the following conditions: 10 s denaturing at 98°C, 30 s annealing at 60°C and 30 s amplification at 68°C, for a total of 35 cycles. The amplified cDNA fragments were separated by agarose gel electrophoresis, and fragments of ca. 0.4 kb were recovered from the gel, cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* (DH5α) cells. Then the nucleotide sequences of the inserts in the plasmids obtained from 15 independent *E. coli* colonies were determined using a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Fourteen clones harbored the sequence of gi566229978 and another clone that of gi566230121, indicating that gi566229978 is the gene (*PtXSP24*) that was most abundantly expressed in the roots of *P. trichocarpa* under SD conditions (Figure 1B).

The Pfam website (Finn et al. 2010) revealed that *PtXSP24* belongs to the cupin superfamily. Cupin is one of the largest groups of proteins, and all members share two conserved motifs, [G(X)5HXH-(X)3,4E(X)6G] and [G(X)5PXG(X)2H(X)3N]. Among the cupin superfamily, *PtXSP24* belongs to the germin-like protein (GLP) family. GLPs are functionally and taxonomically diverse (Dunwell et al. 2008). The GLP with high sequence similarity to *PtXSP24* belongs to the GER 7 clade of the GLP family (Barman and Banerjee 2015). The closest homologs in cotton, grapevine and *Arabidopsis* (*GrGLP14*: gi823124638, *VvGER3*: gi526118118, *AtGLP9*: gi15233510), which belong to the GER 7 clade, and wheat oxalate oxidase (*TaGermin GF-2.8*: gi121129), which belongs to the GER 1 clade, were aligned using the ClustalW software (Figure 1B). All genes harbored the predicted secretion signal sequence (Figure 1B, broken underline).

To elucidate the effects of environmental factors on shoot growth and XSP24 expression, *P. maximowiczii*, which is phylogenetically close to *P. trichocarpa*, and had been propagated by cutting culture, was hydroponically cultured under an artificial annual environmental cycle, as described previously (Dunwell et al. 2008). Root and shoot samples were collected at each time point. Gray, black and white triangles indicate the timings of cessation of shoot growth, dormant bud formation (left photograph) and bud break (right photograph), respectively. Bars indicate 1 cm. *XSP24, plasma membrane aquaporin (PIP)* (gi27362897) and *ubiquitin (UBQ)* (gi7862065) expressions were measured using RT-PCR. The following primers were used for RT-PCR: *PtXSP24* forward 5′-AGATCAAATACCAGGGCTTAAC-3′, *PtXSP24* reverse 5′-GGCCAATTTGAGTTGAGGATT-3′, *PtXSP24* reverse 5′-GGCCACAAATGCAAAACAGACC-3′, *PIP* forward 5′-CCGCTGATCCTGATCCTTCTCC-3′, *UBQ* forward 5′-TGAGGCTTAGGGGAGGAACT-3′, *UBQ* reverse 5′-TGTAGTCGCAAGGATCCG-3′.

![Figure 2](image_url)  
**Figure 2.** Expression of *XSP24* in *P. maximowiczii* plants cultured hydroponically under an artificial annual environmental cycle. Plants hydroponically cultured in 0.1% (w/v) Hypoxen solution at 26°C under 16 h light/8 h dark (LD) conditions with 60 µmol m⁻² s⁻¹ light intensity were sequentially transferred to culture at 26°C under 8 h light/16 h dark (SD) for 8 weeks, at 4°C in the dark (LT) for 4 weeks, and at 26°C under LD conditions for 3 weeks with 60 µmol m⁻² s⁻¹ light intensity. Root and shoot samples were collected at each time point. Gray, black and white triangles indicate the timings of cessation of shoot growth, dormant bud formation (left photograph) and bud break (right photograph), respectively. Bars indicate 1 cm. *XSP24, plasma membrane aquaporin (PIP)* (gi27362897) and *ubiquitin (UBQ)* (gi7862065) expressions were measured using RT-PCR. The following primers were used for RT-PCR: *PtXSP24* forward 5′-AGATCAAATACCAGGGCTTAAC-3′, *PtXSP24* reverse 5′-GGCCAATTTGAGTTGAGGATT-3′, *PtXSP24* reverse 5′-GGCCACAAATGCAAAACAGACC-3′, *PIP* forward 5′-CCGCTGATCCTGATCCTTCTCC-3′, *UBQ* forward 5′-TGAGGCTTAGGGGAGGAACT-3′, *UBQ* reverse 5′-TGTAGTCGCAAGGATCCG-3′.

at SD8 and then an increase at LT2 and LT4, followed by a drastic decrease under LD conditions. In contrast, plasma membrane aquaporin (*PIP*: gi27362897) expression in roots, which may be involved in water mobility in root tissues, decreased at low temperatures, similar to the observation that most *PIP* genes are downregulated by cold stress in *Arabidopsis* (Jang et al. 2004). This tendency of the *XSP24* expression response in roots was confirmed by real-time quantitative RT-PCR (qRT-PCR) using *P. maximowiczii* plants grown in pots with commercial soil with a similar artificial annual environmental cycle as the hydroponic culture, except that LT was under SD conditions (Figure 3A). The pattern of *XSP24* gene expression was similar to that in hydroponic culture (Figure 2).

In several deciduous tree species, such as poplar, SD conditions induce ABA accumulation in shoots to induce bud dormancy (Welling et al. 1997). To examine whether ABA produced in shoots and/or roots under SD conditions is involved in the induction of *XSP24* expression in roots, ABA (10 µM) was applied to shoots and roots of plants at SD8, when *XSP24* expression fully decreased after transient expression upon transfer into
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The following primers were used for qRT-PCR:

Forward 5′-GTTTAAGCAGCCAAAATCCTGGTAC-3′, reverse 5′-GTTTAAGCAGCCAAAATCCTGGTAC-3′.

SD condition to clearly detect the effects of exogenously applied ABA. After 1 week of culture under SD conditions, XSP24 expression was markedly increased compared with the control (Figure 3B).

PtXSP24, which belongs to the GER 7 subfamily of GLP, has homologs in cotton, grapevine and Arabidopsis (GrGLP14, VvGER3 and AtGLP9, respectively) (Barman and Banerjee 2015). Although wheat germin was originally found to have oxalate oxidase activity (Lane et al. 1993), it was revealed to be a bifunctional enzyme, which also exhibits superoxide dismutase activity (Dunwell et al. 2008; Woo et al. 2000). Physiological analysis of germin and germin-like proteins has revealed their association with mitigation of various biotic and abiotic stresses in some plant species (Dunwell et al. 2008; Wan et al. 2009). VvGER3 is induced by powdery mildew infection and has superoxide dismutase activity in grapevine (Ficke et al. 2004; Godfrey et al. 2007). AtGLP9 is induced by salt stress in Arabidopsis roots (Jiang et al. 2007) and binds to calmodulin (Banerjee et al. 2013). Oxalate, the substrate of oxalate oxidase, is produced from ascorbate and/or glyoxylate (Yu et al. 2010) and accumulated in the form of insoluble calcium oxalate crystals in the vacuole and cell wall (Franceschi and Nakata 2005; Nakata 2012). XSP24 catalyzes the production of CO₂ and H₂O₂ from calcium oxalate in the cell wall, and H₂O₂ might be used for oxidative cross-linking of extensin, a structural cell wall protein which has been reported to be induced during cold acclimation (Weiser et al. 1990) therein, which may confer tolerance to freezing on the cell wall by increasing its rigidity. Free Ca released from the crystals may be the cause of the increased Ca level in winter xylem sap reported by Furukawa et al. (2011a). Because Ca²⁺ is associated with stress responses and organ growth in trees (Lautner and Fromm 2010), the release of apoplastic Ca²⁺ from calcium oxalate crystals may be another physiological function of XSP24 in winter. The superoxide dismutase functions of XSP24 might mitigate cold and/or dry stresses in winter by scavenging active oxygen species (Bowler et al. 1992; Mittler 2002).

This study revealed that XSP24 expression in root was induced under SD conditions probably perceived by leaf, possibly via ABA, as well as under non-freezing low temperature perceived by root itself and/or shoot. Once root cells produce and secrete proteins into the cell wall in stele, they can be efficiently delivered to the shoot cell wall via xylem by transpiration stream (Satoh 2006).

At higher latitudes, short days with fewer than 12 h of daylight and a temperature lower than 10°C occur almost simultaneously in autumn, and in early winter, the transpiration stream slows due to leaf fall and a decrease in water translocation in roots due to suppression of PIP expression by non-freezing low temperature (Figure 2). Therefore, the xylem sap proteins produced in root from autumn to winter should be retained in xylem until early spring before bud break. Functional analysis of the role of XSP24 in tolerance to cold and/or dry stresses in winter within apoplasts, such as the cell wall and xylem vessels, is warranted.
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References


