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Note

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 24 kDa proteins,

Abbreviations: ABA, abscisic acid; GA, gibberellin; GLP, germin-like protein; LD, long day; LT, non-freezing low temperature; qRT-PCR, real time quantitative RT-PCR; SD, short day; SDS, sodium dodecyl sulfate; XSP, xylem sap protein.

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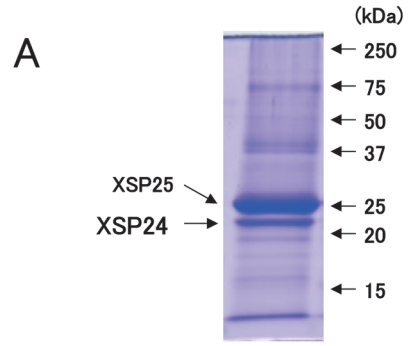

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                    10        20        30        40        50        60        70        80
PtXSP24          --MRSVHFLL AFVLLTLASS LASASDPSPL QDFCVAINDP KAAVFVNGKF CKDPKLATAN DFSFSG-LNI PRDTGNRVGS
GrGLP14          --MKSAHFLL AFCLLALASP LAYASDPSPL QDFCVAINDP KDGVFVNGKF CKDPKLAKAE DFYYSG-LNI PRNTSNPVGS
VvGER3           -MMKGVCFLV TVALLALASS LASASDPSPL QDICVAISDP KDGVFVNGKF CKDLKLASAD DFFYYG-LHI PGNITNPVGS
AtGLP9           MTIKSISFIA ALSLFALTLP LVIASDPSPL QDFCVGVNTP ADGVFVNGKF CKDPRIVFAD DFFFSS-LNR PGNTNNAVGS
TaGermin GF-2.8  -MGYSKTLVA GLFAMLLLAP AVLATDPDPL QDFCVADLDG K-AVSVNGHT CK-PMSEAGD DFLFSSKLAK AGNTSTPNGS
Clustal Consensus     .  ::   .  : *  .  . *:**.** **:**.       .* ***:  **      .: ** : . *   . :  .  **

                    90       100       110       120       130       140       150       160
PtXSP24          NVTLLNVDQI PGLNTLGISL ARIDYAPNGG LNPPHTHPRA TEILVVVEGT LYVGFVTSNP D-NRFISKVL YPGDVFVFPF
GrGLP14          TVTPVNVAQI PGLNTLGISL VRIDYAPNGG LNPPHTHPRA TEILVVVEGT LYVGFVTSNT D-NRLIAKVL YPGDVFVFPI
VvGER3           MVTPVNVEQI PGLNTLGISM VRIDYAPYG- QNPPHTHPRA TEILVVLEGT LLVGFVTSNN E-NRLISKVL YKGDVFVFPI
AtGLP9           NVTTVNVNNL GGLNTLGISL VRIDYAPNG- QNPPHTHPRA TEILVVQQGT LLVGFISSNQ DGNRLFAKTL NVGDVFVFPE
TaGermin GF-2.8  AVTELDVAEW PGTNTLGVSM NRVDFAPGG- TNPPHIHPRA TEIGIVMKGE LLVGILGSLD SGNKLYSRVV RAGETFLIPR
Clustal Consensus  ** ::* :   * ****:*:  *:*:** *   **** **** *** :* :*  * **:: *   . *:: ::.:   *:.*::*

                   170       180       190       200       210       220       230
PtXSP24          GLIHFQLNIA KTPAVVFAGL SSQNPGTITI ANAVFGSDPL INPDVLAKAF HLDIKIVNYL Q-KLFGGNSE
GrGLP14          GLIHFQFNVG KTKAVAFAGL SSQNPSVITI ANAVFGPNPS INPDILVKAL QLDKNVVKKL QSKLWWANN-
VvGER3           GLIHFQFNVG KAKAVAFAGL SSQNPGVITI ANAVFGSDPP IDPDVLTRAF QLDEDVVKDL QSRFWWDNN-
AtGLP9           GLIHFQFNLG GTPAVAIAAL SSQNAGVITI ANTIFGSKPD VDPNVLARAF QMDVNAVRNL QARF------
TaGermin GF-2.8  GLMHFQFNVG KTEASMVVSF NSQNPGIVFV PLTLFGSNPP IPTPVLTKAL RVEARVVELL KSKFAAGF--
Clustal Consensus **:***:*:.  : *  ...: .***.. : : . ::**..*  : . :*.:*: :::   *. * : ::
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Figure 1. Identification of XSP24 and the amino acid sequence of *P. trichocarpa* gi566229978 (PtXSP24) 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 PtXSP24, registered as gi566229978 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: gi121129), 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.

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. The gel was stained with Coomassie brilliant blue R-250, and the second-most intense band at 24 kDa (XSP24), below the band with the highest intensity corresponding to XSP25 (Figure 1A), was excised from the gel and subjected to MS and data analyses. The peptide sequence of XSP24 (IDYAPNGGLNPPHTHPR) was identified. Six *P. trichocarpa* genes were found to encode the peptide by means of a BLAST search against the poplar genome database (https://phytozome.jgi.doe.gov/). To identify the gene responsible for the production of XSP24 in roots, cDNA was synthesized using total RNA extracted from the roots of *P. trichocarpa* aseptically cultured on agar medium under SD conditions (8 h light/16 h dark) for 6 weeks in plastic pots. Then, a set of primers, (forward primer 5′-

ATG AGA AGT GTT CAT TTC CTA CTA G-3′ and reverse primer 5′-GAC AAA TCC AAC ATA GAG TGT 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* (DH5*α*) 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 *Pt*XSP24 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, *Pt*XSP24 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 *Pt*XSP24 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 (at 26°C under 16 h light/8 h dark (LD)>at 26°C under 8 h light/16 h dark (SD)>at 4°C in the dark (LT)>at 26°C under LD). Cessation of shoot growth, dormant bud formation and bud break were observed after 2 weeks under SD, 4 weeks under SD and 1 week under LD after 4 weeks of LT, respectively (Figure 2). At each time point, RNA was extracted from the roots and shoots and subjected to RT-PCR using the primer set for *PtXSP24* because the *XSP24* gene is thought to be similar in *P. maximowiczii* and *P. trichocarpa*, which belong to the same group. Although *XSP24* expression in shoots was low, with an exception at SD8, expression in roots increased at SD6, followed by a slight decrease

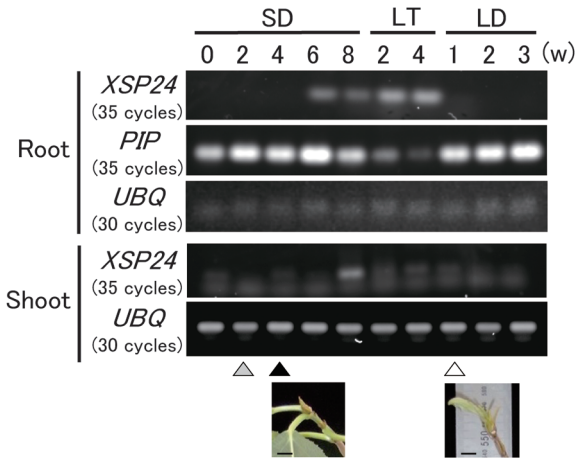

Figure 2. Expression of *XSP24* in *P. maximowiczii* plants cultured hydroponically under an artificial annual environmental cycle. Plants hydroponically cultured in 0.1% (w/v) Hyponex solution at 26°C under 16 h light/8 h dark (LD) conditions with 60 μmol m^{-2} s^{-1} light intensity were sequentially 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′-GGCAATATTGAGTTGGAAGTG-3′, *PIP* forward 5′-GGCCACAAGTCAAACAAAGACC-3′, *PIP* reverse 5′-CAGCCCTGATCAGTGATACTTTCC-3′, *UBQ* forward 5′-TGAGGCTTAGGGGAGGAACT-3′, *UBQ* reverse 5′-TGTAGTCGC GAGCTGTCTTG-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

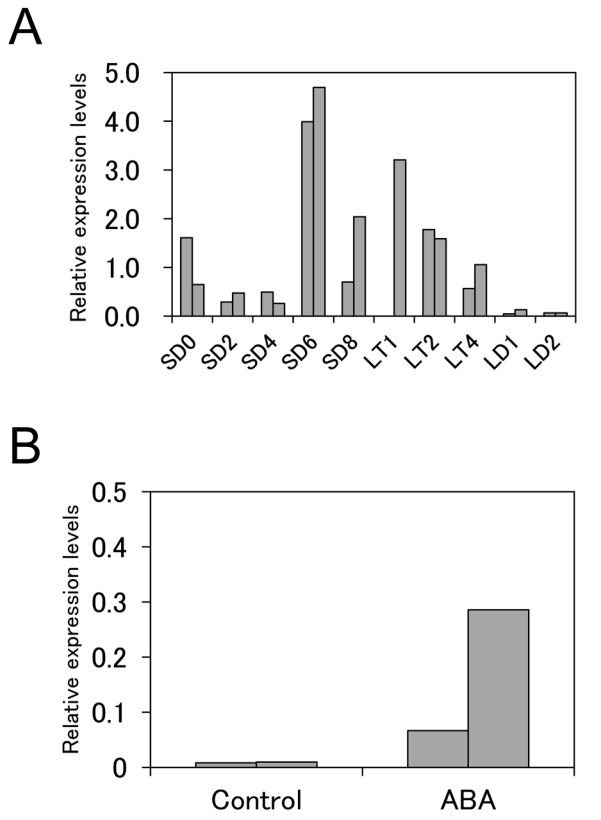

Figure 3. Expression of *XSP24* in *P. maximowiczii* plants cultured with soil under an artificial annual environmental cycle and the effect of ABA on *XSP24* expression. (A) Plants grown in pots with commercial soil at 26°C under 16 h light/8 h dark (LD) conditions with 60 μmol m^{-2} s^{-1} light intensity were sequentially transferred to culture at 26°C under 8 h light/16 h dark (SD) conditions for 8 weeks, at 4°C under SD conditions (LT) for 4 weeks, and at 26°C under LD for 2 weeks with 60 μmol m^{-2} s^{-1} light intensity. Root samples (two samples per time point, with the exception of one sample at LT1) were collected at each time point and used for analysis of *XSP24* expression by qRT-PCR with *ubiquitin* (gi566172648) as an internal standard (=1). (B) At SD8, ABA (10 μM) was sprayed onto shoots of two plants and showered on the soil. Excess solution was allowed to drain from the holes in the pots, and plants underwent a further 1 week of culture under SD conditions; roots were then sampled for qRT-PCR analysis. PCR was performed using a SYBR Premix Ex Taq II kit (Takara Bio Inc., Shiga, Japan) under the following conditions: 5 s denaturation at 95°C, 10 s annealing at 63°C and 31 s extension at 72°C, for a total of 50 cycles. PCR products were detected using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR: *PtXSP24* forward 5′-GTTTAAGCAGCCAAAATCCTGGTAC-3′, *PtXSP24* reverse 5′-AATGGAAGGCCTTGGCAAG-3′, *UBQ* forward 5′-TGA ACCAAATGATACCATTGATAG-3′, and *UBQ* reverse 5′-GTAGTC GCGAGCTGTCTTG-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 CO_2 and H_2O_2 from calcium oxalate in the cell wall, and H_2O_2 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 Ca^{2+} is associated with stress responses and organ growth in trees (Lautner and Fromm 2010), the release of apoplastic Ca^{2+} 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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