Short report

Abscisic acid-inducible 25 kDa xylem sap protein abundant in winter poplar

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Abstract: To investigate the environmental regulation of root function, xylem sap was annually collected from *Populus nigra* and the proteins in the sap were analyzed. A 25 kDa xylem sap protein (XSP25) was found to be most abundant in the xylem sap in winter and mass spectrometry analyses showed its high similarity to abscisic acid (ABA)-inducible basic secretory protein reported in tobacco BY-2 cells. By utilizing the information of whole genome sequence of *Populus, PmXSP25* was cloned from *Populus maximowiczii,* naturally growing poplar in Japan. The expression of *PmXSP25* was abundant in root at December and February and strongly enhanced by ABA application to the autumn root. We suggest that dormancy-inducing short day length and low temperature promote the synthesis of XSP25 in root, possibly via ABA, as an adaptation to the winter environment.

Keywords: abscisic acid, basic secretory protein, *Populus maximowiczii*, *Populus nigra*, winter, xylem sap protein

Abbreviations: ABA, abscisic acid

Introduction

The long distance transport of water and minerals is the primary function of xylem vessels. However, xylem sap contains many organic components, such as plant hormones, sugars and proteins synthesized in root. Some xylem sap proteins are thought to be possibly involved in the protection of plant against biotic and abiotic stresses. For example, 39 xylem sap proteins were differentially regulated in maize in response to water stress (Alvarez et al., 2008). Xylem sap proteins are important to understand the mechanisms by which higher plants adapt to environmental conditions, but are difficult to study in trees because of the lack of model woody plants used for molecular research. However, the elucidation of the entire *Populus trichocarpa* genome (Tuskan et al. 2006) and identification of expressed sequence tags of *Populus* (Kohler et al. 2003, Sterky et al. 2004) have triggered molecular biological research in deciduous trees. Recently, proteomic analyses were performed on xylem sap from poplar (*Populus trichocarpa* × *Populus deltoides*) grown under long-day conditions (Dafoe and Constabel 2009).

Seasonal changes in the substances in the xylem sap of woody plants (Tromp and Ovaa 1990, Bertrand et al. 1997) suggests that root functions are regulated by environmental factors in the root and/or by shoot-synthesized signals. In this study, we identified the major xylem sap protein (XSP25) in winter condition, analyzed the seasonal expression of *XSP25*, and examined the effect of plant hormone ABA on *XSP25* expression in poplar. We suggest the involvement of XSP25 in winter adaptation under the control of ABA.

Materials and Methods

Plant materials

Xylem sap was collected from a *Populus nigra* plant (80 cm in diameter) growing at the University of

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Tsukuba in Ibaraki, Japan (latitude 36° 05' N, 140° 07' E; altitude 25 m). Analysis of gene expression was performed with *P. maximowiczii*, a native poplar in Japan. *P. maximowiczii* is involved in the same phylogenetic clade as *P. trichocarpa* whose whole genome has been sequenced (Tuskan et al. 2006). The original shoot cuttings had been obtained 2001 from the lateral branches of a mature *P. maximowiczii* planted in the National Science Museum, Tsukuba Botanical Garden; the ID number of the original plant was TBG10713, and used for the propagation with cutting culture. Two trees grown in pots with commercially available soil for 5 years after cutting culture were used for the analysis of seasonal expression of *XSP25*. For the analysis of ABA effect, cutting propagation for all potted plants started on March 2009 and roots were excised for the experiments on September 2009.

Collection of xylem sap from P. nigra

The collection of *P. nigra* xylem sap was performed according to Furukawa et al. 2011. Side branch was cut and connected via a tube to a sample container. The container was maintained at −0.08 MPa with a vacuum pump for 12 h. The xylem sap was then stored at −30°C until further analysis. Samples were collected in 2007 (December 3) and in 2008 (January 11, February 4 and 18, June 14, October 8).

Identification of xylem sap proteins

The total protein in 10 ml of xylem sap was precipitated with 80% (v/v) ethanol at 4°C overnight, collected by centrifugation at 18000 ×g for 30 min at 4°C, and re-dissolved in 200 µl of distilled water; 10 µl of this solution was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The gels were stained using a silver staining kit (Wako Pure Chemical Industries, Osaka, Japan), and the protein concentration determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Xylem sap (20 ml) obtained on February 18, 2008 was dialyzed (size 36 dialysis membrane, Wako) three times in distilled water at 4°C for 12 h, and proteins were precipitated with 80% (v/v) ethanol, re-dissolved in 20 µl of distilled water, and separated with SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250 (Wako), and the most intense bands at 25 kDa was excised from the gel.

MS and data analysis

The proteins in the excised gel were reduced, alkylated by acrylamide, and digested by trypsin (Trypsin Gold, MS Grade; Promega, Madison, WI, USA) in the presence of 0.01% (v/v) Protease MAX surfactant (Promega) at 50°C for 1 h. Resultant peptides were fractionated by Dina nanoHPLC system with reverse phase chromatography (KYA Technologies, Tokyo, Japan) and each fraction was directly spotted on matrix-assisted laser-desorption ionization (MALDI) plate with α-cyano-4-hydroxycinnamic acid containing diammonium hydrogen citrate by DiNa Map system (KYA Technologies). MALDI-TOF MS and MS/MS were performed on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). MS spectra were acquired in reflector positive ion mode and MS/MS spectra were acquired in positive ion mode with CID off. MS/MS data were analyzed by MASCOT searching software (Matrix Science, London, UK) in the NCBI non-redundant public protein database.

Expression analysis of XSP25 in the root of P. maximowiczii

Two potted *P. maximowiczii* plants (Plants A and B) were grown outdoors in 15 L of soil augmented with organic fertilizer (fermented oil cake) in March 2007 and 2008. The root sample was collected at December 11 in 2008 and February 14, April 13 and June 13 in 2009. The sampling time was fixed at 4 p.m. to avoid the diurnal fluctuation of gene expressions. The longest root was collected from the root tip to the basal part of root with liquid N_2 and was stored at -80°C until further analysis. At the sampling date of winter (December 11 in 2008 and February 14 in 2009), dormant bud was formed and no leaves were observed in the shoot of potted *P. maximowiczii*. After the dormancy break (April 13, 2008 and June 13, 2009), the new leaves were opened.

The nucleotide sequences of *XSP25* and *UBQ* (Brunner et al. 2004) in *P. trichocarpa* were obtained from JGI Genome Portal database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) and were used for primers design. The specificities of the primers were investigated by PCR using cDNA derived from the mRNA of *P. maximowiczii*. PCR fragments were recovered by MiniElute Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and the individual sequences were identified with Big Dye termination Cycle Sequence Kit (Applied Biosystems). The nucleotide sequences were compared with *P. trichocarpa* with GENETYX-MAC (Software Development). The primers used for RT-PCR were followings. PmXSP25U : 5'-CACTGGCATCTGCCAC-3', PmXSP25L : 5'-CCATTGCCATATGTGTG-3', UBQU : 5'-TGAGGCTTAGGGGAGGAACT-3', UBQL : 5'-TGTAGTCGCGAGCTGTCTTG-3'. The condition of RT-PCR was 98°C for 30 sec,

55°C for 30 sec and 72°C for 30 sec and the cycle was 35 both in *PmXSP25* and *UBQ* amplifications.

RNA extraction and cDNA synthesis

Total RNA was extracted from 200 mg root of *P. maximowiczii* with Total RNA Isolation Kit (Ambion, Austin, TX, USA). Complementary DNA was synthesized from 1 μg of total RNA with QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer's instructions.

Effect of ABA treatment on XSP25 expressions

In September 2009, the potted *P. maximowiczii*, which had formed dormant bud, were treated with 10^{-5} M (±)-ABA (Wako Pure Chemical Industries, Osaka, Japan). One week before experiment, two plants were transplanted to the one pot containing 5 L of soil. ABA solution dissolved in distilled water was added to the pot (1 L / pot) and then grown for 72 h under continuous light condition at 27 °C. As a control treatment, 1 L of distilled water was added to the other two plants potted in another pot by the same way. After these treatments, the longest root, from the root tip to the basal part of the root, was collected and stored at -80°C until further analysis. Gene expression was analyzed with RT-PCR.

Results and Discussion

Identification of 25-kDa xylem sap protein in Populus nigra

To elucidate the seasonal difference in protein pattern, total protein contained in 0.5 ml of xylem sap was loaded to each lane. The loaded amount of proteins were 4, 16, 9, 17, 4 and 1 μg at December 3, 2007, January 11, February 4 and 18, June 14 and October 8, 2008, respectively. The xylem sap was found to contain large amount of proteins in winter and the 25-kDa protein (XSP25) was the most abundant protein in the xylem sap (Fig. 1). MS analyses of XSP25 using the peptide mass fingerprinting method identified XSP25 as gi224060617 (NCBI Reference Sequence: XM_002300207.1) in the *Populus trichocarpa* database (Fig. 2), and has previously been identified by proteomic analysis of xylem sap collected from long day-grown poplar (Dafoe and Constabel 2009). The Pfam web site (http://pfam.sanger.ac.uk/; Finn et al. 2010) identified this protein as a basic secretory protein which is abundant in charged amino acids. A BLAST search identified homologous proteins in the Magnoliophyta, especially *Populus*, *Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor*, and *Selaginella moellendorffii* (club-mosses). Proteins with high sequence similarity to XSP25 include NtPRp27 (pathogenesis-related protein) and wheat ABA-induced secretory (WAS) protein-2, which are present in the culture medium of tobacco BY-2 cells (Okushima et al. 2000) and in wheat cultured cells (Kuwabara et al. 1999), respectively. These proteins were also induced by ABA application. The gene encoding NtPRp27 is mainly expressed in roots of intact tobacco plants, but the several stress treatments including ABA application induced *NtPRp27* in leaves (Okushima et al. 2000). Functions of NtPRp27 and WAS-2 have not been clear but hypothesized to be related to the plant response against ABA-mediated environmental stress and pathogen-infection. The sequence of XSP25 involves the signal sequence for secretion predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/; Emanuelsson et al. 2007). The presence of secretion-signal in XSP25 is consistent with the existence of this protein in the xylem sap. Based on the amino

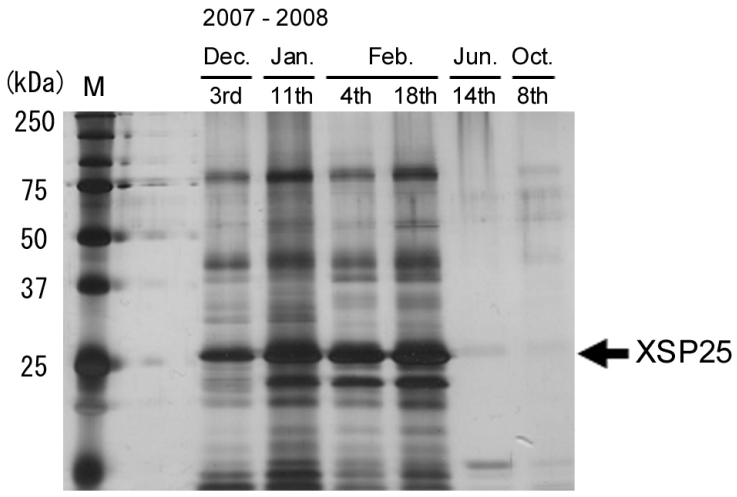

Fig. 1. SDS-PAGE profiles of *P. nigra* xylem sap proteins obtained at each sampling date from December 3, 2007 to October 8, 2008. The total protein in 0.5 ml of xylem sap was loaded to each lane. The loaded amount of proteins were 4, 16, 9, 17, 4 and 1 μg at December 3, 2007, January 11, February 4 and 18, June 14 and October 8, 2008, respectively. Markers (M) are indicated. Arrow indicates the most abundant protein, XSP25 (25 kDa).

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XSP25    ----------------MFHHVYVLSLLVLLAINT--VSAVDYTVTNRASATAGGARFTRDI
NtPRp27  MKKMTEAIFVFSQVKRAHHKIFFFYSLFVLAIFTQKIHAVDYSVTNTAANTAGGARFNRDI
WAS-2    ----------------MKLQVATVASFVLVALAAA-AQAVTFDASNKASGTSGGRRFNQAV
                          ::  .  :.::*: :    ** : .:* *: *:** **.: :

XSP25    GVDYSKQTLASATDFIWRTLQQSNAADRKNVQTVNLFIDVMGGVAYATNNEIHVSNDYIGN
NtPRp27  GAQYSQQTLAAATSFIWNTFQQNFPADRKNVQKVSMFVDDMDGVAYASNNEIHVSASYIQG
WAS-2    -VPYSKKVLSDASAFIWKTFNQRAVGDRKTVDAVTLVVEDISGVAFTSANGIHLSAQYVGG
          . **::.*: *: ***.*::*   .***.*: *.:.:: :.***::: * **:* .*: .

XSP25    YSGDVRREITGVLYHEMAHIWQWNGNGQTPGGLIEGIADFVRLKANYAPSHWVQAGQGDSW
NtPRp27  YSGDVRREITGVLYHESTHVWQWNGNGGAPGGLIEGIADYVRLKAGFAPSHWVKPGQGDRW
WAS-2    ISGDVKKEVTGVLYHEATHVWQWNGPGQANGGLIEGIADYVRLKAGFAPGHWVKPGQGDRW
          ****::*:******* :*:***** * : *********:*****.:**.***:.**** *

XSP25    DQGYDVTAKFLDYCNGLRNGFVAELNKKMKTGYSAQYFVDLLGKTVDQLWKDYKAKYGK
NtPRp27  DQGYDVTARFLDYCNSLRNGFVAQLNKKMRTGYSNQFFIDLLGKTVDQLWSDYKAKFRA
WAS-2    DQGYDVTARFLDYCDSLKPGFVAHVNAKMKSGYTDDFFAQILGKNVQQLWKDYKAKFRG
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Fig. 2. Amino acid sequence of *P. trichocarpa* gi224060617 (XSP25) aligned with tobacco NtPRp27 and wheat WAS-2. Bold characters show the amino acid sequences identified with MS analysis of *P. nigra* XSP25. Identical amino acid residues are marked with a star, strongly similar with two dots, and weakly similar with one dot. The underlined sequence shows the signal sequence for secretion predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/; Emanuelsson et al. 2007), and shaded areas indicate the Pfam-predicted basic secretory protein motifs.

acid sequence of XSP25, highly hydrophilic structure with abundant charged amino acids is expected. This property might suggest the possible participation of XSP25 in the anti-freezing mechanisms of protein and membrane as a molecular chaperon as reported in some LEA proteins (Close 1996, Goyal et al. 2005).

XSP25 expression in roots and its change by season and exogenous ABA

P. nigra has been used for chemical analysis of xylem sap (Furukawa et al. 2011) and for the identification of XSP25. However, two individual plants of *Populus maximowiczii* were used for the analysis of XSP25 gene expression by RT-PCR, for utilizing the genomic information. *P. maximowiczii* is a native poplar in Japan and belongs to the same phylogenetic clade as *P. trichocarpa* whose whole genome has been sequenced (Tuskan et al. 2006). In addition, the timings of the response to the short day length in autumn including leaf abscission and dormant bud formation and of the bud burst in spring in *P. maximowiczii* were almost the same as those in *P. nigra* in the experimental site (Satoh, unpublished data). Therefore, the molecular biological analyses were performed with *P. maximowiczii*. The XSP25 nucleotide sequence of *P. maximowiczii* was the same as that of *P. trichocarpa* and the gene encoding

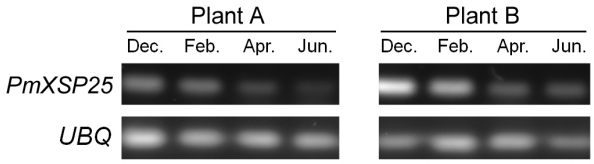

Fig. 3. Gene expression profile of *PmXSP25*. Total RNA was extracted at December 11th in 2008 and February 14th, April 13th and June 13th in 2009 from potted *P. maximowiczii* growing outdoor in the Tsukuba city. On December 11th, 2008 and on February 14th, 2009, dormant bud was formed and no leaves were observed in the shoot and, on April 13th, 2008 and on June 13th, 2009, the new leaves were opened. The cycle of PCR, 98°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, was 35 both in *PmXSP25* and *UBQ* amplifications.

XSP25 homolog in *P. maximowiczii* was designated as *PmXSP25*. The expression pattern of *PmXSP25* was shown in Fig. 3. The high transcription level of *PmXSP25* was observed at December and February, suggesting that the PmXSP25 was synthesized in the root at winter season and transported to the shoot via xylem sap.

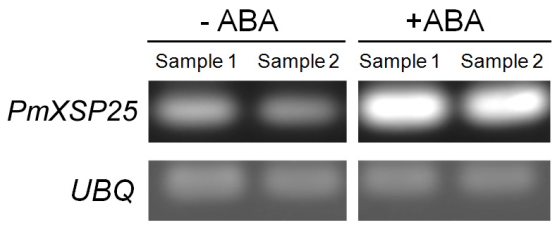

Fig. 4. Effects of ABA on *PmXSP25* expression in the root of potted *P. maximowiczii*. The plants that formed dormant buds in the autumn were treated with and without 10^{-5} M ABA for 72 h under continuous light condition at 27℃. After the treatments, the roots were collected and used for the analysis. The cycle of PCR, 98°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, was 35 both in *PmXSP25* and *UBQ* amplifications.

Because of sequence similarity between XSP25, NtPRp27 and WAS-2, ABA was focused as an internal factor for *PmXSP25* expression. In *A. thaliana*, ABA has been reported to be synthesized at the intrafascicular sieve tissue and translocated via sieve tube under drought stress condition (Ikegami et al. 2009). Since the effect of foliar application of ABA including its translocation in the sieve tissue was not clear in our experimental system, we employed the direct application of ABA to the root. The gene expressions in the root after ABA application were analyzed. In September 2009, the plant already formed dormant bud was treated with ABA (10^{-5} M) for 72 h. Although *PmXSP25* was expressed under ABA-free condition, the transcript amounts were drastically increased with exogenous ABA application both in two investigated plants (Fig. 4). It was known that ABA was synthesized in shoot cambium and apical bud triggered by the decrease of temperature (Druart et al. 2007, Ruttink et al. 2007). Therefore, we hypothesize that the ABA synthesized at the shoot moves to root and enhance the *PmXSP25* expression in winter.

To reveal the environmental factors controlling the gene expression more in detail, the experiments under artificially controlled condition will be needed. As to the function of XSP25, the analysis of translocation and localization of XSP25 in plant and the functional analysis by molecular biological techniques will be required.

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