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Original Paper

Continuous expression of genes for xylem cysteine peptidases in long-lived ray parenchyma cells in *Populus*

Satoshi Nakaba[1,*], Naoki Takata[2], Makoto Yoshida[1], Ryo Funada[1,3]

[1]Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan; [2]Forest Bio-Research Center, Forestry and Forest Products Research Institute, Hitachi, Ibaraki 319-1301, Japan; [3]College of Life Science, Kyung Hee University, Yongin 446-701, Korea
*E-mail: nakaba@cc.tuat.ac.jp Tel: +81-42-367-5814 Fax: +81-42-334-5700

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Abstract XYLEM CYSTEINE PEPTIDASE 1 (XCP1) and XCP2 are key autolytic enzymes in programmed cell death of short-lived tracheary elements in plants. However, the patterns of expression of *XCP* genes remain to be clarified in long-lived ray parenchyma cells, which survive for several years after the completion of secondary wall formation. We isolated full-length cDNAs that encoded three XCPs (*PsgXCP1*, *PsgXCP2A* and *PsgXCP2B*) of *Populus*. The deduced amino acid sequences of the XCPs revealed three conserved catalytic residues, namely, cysteine (Cys), histidine (His) and asparagine (Asn), indicating that PsgXCP1, PsgXCP2A and PsgXCP2B might function as papain-like cysteine proteases. Ray parenchyma cells in hybrid poplar remained alive for several years, with continuous expression of *XCP* genes even after obvious secondary wall thickenings. Levels of expression of *XCP* genes were lower in the innermost annual ring of sapwood than in one and/or two outer annual rings from beyond the innermost sapwood. This result implies that autolysis of ray parenchyma cells might not be fully explained by dramatically enhanced expression of *XCP* genes just before their programmed death. In addition, the expression of *PtaSND1-A1*, *PtaVND6-A1* and *PtaMYB003* genes was detected in all annual ring in sapwood. Our observations suggest that the expression of genes associated with xylem differentiation such as secondary wall formation and cell death continues for several years in long-lived ray parenchyma cells before their death.

Key words: Cell death, cysteine protease, heartwood formation, Populus, ray parenchyma.

The programmed death of ray parenchyma cells is involved in the formation of heartwood, which is a phenomenon unique to long-lived woody plants. Heartwood is defined as "the inner layers of wood, which, in the growing tree, have ceased to contain living cells, and in which the reserve materials (e.g., starch) have been removed or converted into heartwood substances" (IAWA 1964). Ray parenchyma cells, prior to their death, synthesize heartwood substances, such as polyphenols, which contribute to enhancement of the resistance of the tree trunk to decay and which to the coloration, in some species, of the heartwood (Bamber and Fukazawa 1985; Hillis 1987; Magel 2000; Spicer 2005; Taylor et al. 2002). The quality and quantity of heartwood are important parameters in the production of lumber and furniture. A better understanding of the mechanism of the programmed death of ray parenchyma cells might provide useful information for manipulating the properties of wood.

Time-dependent programmed cell death in short-lived tracheary elements has been widely studied (Escamez and Tuominen 2014; Fukuda 1997, 2004). During the differentiation of tracheary elements from *Arabidopsis*, master switches of genes involved in vessel differentiation, namely, VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 induce secondary wall formation and the subsequent autolysis of vessel elements (Kubo et al. 2005). However, little analogous information is available about the programmed death of long-lived cells. Therefore, we have been investigating the programmed death of ray parenchyma cells as a model of the programmed death of long-lived xylem cells in situ (Nakaba et al. 2006, 2008, 2012). We showed previously that death does not occur successively in ray parenchyma cells, even within the same radial cell lines of a ray. The observation suggested that the programmed death of ray parenchyma cells might not be controlled in a time-dependent manner. Our findings indicated that the programmed death of long-lived ray parenchyma cells might involve reactions distinct from those that occur

Abbreviations: Asn, asparagine; Cys, cysteine; His, histidine; JTT, Jones–Taylor–Thornton; NJ, neighbor-joining; PBS, phosphate-buffered saline; Psg, *Populus sieboldii*×*P. grandidentata*; Pta, *P. tremula*×*P. alba*; RACE, rapid amplification of cDNA ends; SKP1, SUPPRESSOR OF KINETOCHORE PROTEIN 1; SND/NST, SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN/NAC SECONDARY WALL THICKENING PROMOTING FACTOR; SP, signal peptide; UBQ, ubiquitin; VND, VASCULAR-RELATED NAC-DOMAIN; XCP, xylem cysteine peptidase.

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during the programmed death of short-lived tracheary elements.

The molecular biology of the death of ray parenchyma cells during heartwood formation has already received some attention. Yang et al. (2004) found that the expression of *SUPPRESSOR OF KINETOCHORE PROTEIN 1* (*SKP1*) genes, which are involved in proteolysis during cell differentiation, cell death and the defense response, was enhanced in the transition zone between the sapwood and heartwood of *Robinia pseudoacacia*. Expression of a *KNAT3*-like homeobox gene for a transcription factor that regulates the specification and patterning of cells was enhanced in the transition zone and inner sapwood of *Juglans nigra* (Huang et al. 2009). In *Cryptomeria japonica*, expression of the gene for a putative papain-type cysteine protease, which has been shown to be involved in some case of programmed cell death, was also induced in the transition zone (Yoshida et al. 2012). In order to clarify the differences between the molecular mechanisms of programmed death in ray parenchyma cells and short-lived xylem cells, we focused on autolytic enzymes that are involved in the programmed cell death of tracheary elements.

The *XYLEM CYSTEINE PEPTIDASE 1* (*XCP1*) and *XCP2* genes are homologous to genes for papain-like proteases, which are key enzymes in the programmed cell death of tracheary elements (Bollhöner et al. 2012; Petzold et al. 2012). The *Arabidopsis* genes *XCP1* and *XCP2* are directly activated by the products of the *VND6* and *VND7* genes that participate in xylem vessel differentiation (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011). In *Populus*, the expression of *XCP* genes is enhanced by the products of orthologs of the *VND* and *SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN*/*NAC SECONDARY WALL THICKENING PROMOTING FACTOR* (*SND*/*NST*) genes (Mitsuda et al. 2007; Zhong et al. 2006, 2007), master regulator for differentiation of xylem fibers (Ohtani et al. 2011). In *Populus*, the products of the *VND* and *SND*/*NST* genes also induce expression of genes associated with secondary wall formation, such as genes for MYB transcription factors, cellulose synthases and laccases. Secondary wall formation and programmed cell death occurs successively during differentiation of tracheary elements and wood fibers (Courtois-Moreau et al. 2009; Fukuda 2004). However, ray parenchyma cells survive for several years after the completion of secondary wall formation (Nakaba et al. 2012). In such long-lived ray parenchyma cells, the patterns and regulation of expression of *XCP* genes remain to be clarified.

In the present study, we studied the expression of *XCP* genes in ray parenchyma cells of *Populus*. Full-length cDNAs (*PsgXCP1*, *PsgXCP2A* and *PsgXCP2B*) were isolated from hybrid poplar (*P. sieboldii*×*P. grandidentata*). Levels and patterns of expression of the *XCP* genes were examined in ray parenchyma cells of *Populus* by real-time PCR. We also investigated the expression of upstream regulators that are associated with cell wall formation and programmed cell death in short-lived xylem cells.

Materials and methods

Plant materials

Samples were collected from a cloned hybrid poplar (*P. sieboldii*×*P. grandidentata*; *Psg*) of approximately 14 years of age (height, 14.0 m; diameter at breast height, 20.0 cm; width of sapwood, 2.6 cm) that was growing in the field nursery of the Tokyo University of Agriculture and Technology in Fuchu-Tokyo, Japan. Small blocks containing phloem, cambium and xylem and incremental cores containing sapwood and heartwood were taken from the main stem at breast height in October 2007 and June 2012. Three leaves were collected from the same tree in August 2014. Six- to seven-year-old *P. tremula*×*P. alba* trees (*Pta*; sample No. 1: height, 12.8 m; diameter at breast height, 10.5 cm; width of sapwood, 5.5 cm; sample No. 2: height, 12.7 m; diameter at breast height, 12.0 cm; width of sapwood, 5.2 cm), which were growing in the Sapporo Experimental Nursery of Hokkaido University in Sapporo, Hokkaido, were used to evaluate survival rates of ray parenchyma cells and levels of expression of *XCP*s. Tree disks containing sapwood and heartwood were collected from main stems in September 2013. Samples for light microscopy were soaked in a 4% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Samples for extraction of RNA were frozen immediately in liquid nitrogen and stored at −80°C until use.

Cloning full-length cDNA of PsgXCP1*,* PsgXCP2A *and* PsgXCP2B

The previous year's xylem was collected from small blocks of tissue in June 2012. Total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and then treated with DNase I (Life Technologies, Carlsbad, CA, USA) to remove contaminant genomic DNA. The 3′- and 5′-rapid amplification of cDNA ends (RACE) was performed with total RNA and a GeneRacer™ kit (Life Technologies) according to the manufacturer's instructions. The primer sequences for RACE are shown in Supplemental Table S1. The products of PCR were ligated into the pGEM®-T Easy vector (Promega Co., Madison, WI, USA) and then subjected to dideoxy-nucleotide sequencing.

Isolation of genomic DNA that encoded PsgXCP1b

Genomic DNA was extracted with a DNeasy Plant Mini Kit (Qiagen) from the leaves of *P. sieboldii*×*P. grandidentata*. The genomic region of PsgXCP1 was isolated by PCR with KOD plus (Toyobo, Osaka, Japan) using the primer set shown in Supplemental Table S1. The fragment amplified by PCR was subcloned into the pCR®-XL-TOPO® vector (Life

Technologies) and subjected to dideoxy-nucleotide sequencing by the primer-walking method.

Sequence alignment and phylogenetic analysis

Amino acid sequences were deduced from the cDNA sequences of the *papain* of *Carica papaya* (*Cp*), the *XCP*s of *A. thaliana* (*At*) and *PsgXCP*s and aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The Boxshade program (http://www.ch.embnet.org/software/BOX_form.html) was used to highlight the conserved amino acid residues in the aligned amino acid sequences. The signal peptides of PsgXCPs were predicted with SignalP 4.1 (Petersen et al. 2011).

We retrieved *XCP* genes from genomic databases for *A. thaliana* (The Arabidopsis Information Resource, TAIR), *C. papaya*, *Medicago truncatula* (*Mt*), *P. trichocarpa* (*Ptr*) and *Vitis vinifera* (*Vv*) (Phytozome v9.1). The locus identification numbers of *AtXCP1*, *AtXCP2*, *CpXCP*, *MtXCP*, *PtrXCP1*, *PtrXCP2A*, *PtrXCP2B*, *VvXCP1* and *VvXCP2* are, respectively, At4g35350, At1g20850, evm. TU.supercontig_232.4, Medtr3g116080, Potri.004G207600, Potri.005G256000, Potri.002G005700, GSVIVG01023863001 and GSVIVG01013420001. Amino acid sequences were aligned with the ClustalW program. The numbers of amino acid substitutions in comparisons of pairs of XCP proteins were estimated by the Jones–Taylor–Thornton (JTT) model with the complete-deletion option. A phylogenetic tree was reconstructed by the neighbor-joining (NJ) method (Saitou and Nei 1987). Bootstrap values were calculated with 1,000 replications (Felsenstein 1985). Phylogenetic analysis was performed using MEGA5 software (Tamura et al. 2011).

Light microscopy

Samples were fixed overnight at room temperature in a 4% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Radial sections of approximately 40-μm thickness were cut on the freezing stage of a sliding microtome (Yamatokohki, Saitama, Japan) and washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM $NaHPO_4$, adjusted to pH 7.2). Sections were stained with a 1% aqueous solution of acetocarmine for observations of nuclei (Nakaba et al. 2006) and examined under a light microscope (Axioscop; Carl Zeiss, Oberkochen, Germany).

Survival rates of ray parenchyma cells

Survival rate of ray parenchyma cells in xylem tissues of *P. tremula*×*P. alba* was calculated for the outer region (cambial side) and inner region (pith side) of each annual ring in three independent sections, as described previously (Nakaba et al. 2012). The rate of survival is presented as the number of ray parenchyma cells that contained a nucleus among 100 ray parenchyma cells and given as a percentage. Values are means±SD (n=3).

Extraction of RNA and real-time PCR

For analysis of gene expression in *P. sieboldii*×*P. grandidentata*, we collected samples of xylem tissues (diameter, 5 mm; radial length, 1 cm) in October 2007 from two different parts of each incremental core that contained outer sapwood without the current year's xylem and inner sapwood with a small part of the outer heartwood. In *P. tremula*×*P. alba*, we identified the presence of heartwood by light microscopy and collected xylem tissue from each annual ring in the sapwood. We isolated total RNA using an RNeasy Plant Mini Kit (Qiagen) and then treated it with DNase I (Qiagen) to remove contaminant genomic DNA. Single-stranded cDNAs were synthesized by SuperScript™ III Reverse Transcriptase (Life Technologies) or with a High Capacity RNA-to-cDNA™ kit (Life Technologies) according to the manufacturer's instructions.

Real-time PCR was performed with StepOne Plus™ and Power SYBR® Green PCR Master Mix (Life Technologies). The gene-specific primers for real-time PCR are shown in Supplemental Table S1. The *ubiquitin 11* (*UBQ*) gene was used as a control for normalization. To determine the relative abundance of *PsgXCP* mRNAs, we subcloned the amplicons into the pCR®-XL-TOPO® vector (Life Technologies). Vectors containing *PsgXCP1*, *PsgXCP2A* and *PsgXCP2B*, respectively, were digested with *Xba*I and *Spe*I to excise DNA fragments that contained *PsgXCP1*, *PsgXCP2A* and *PsgXCP2B*. The individual fragments were introduced into the *Xba*I site of the pTAC1 vector that harbored the *UBQ* gene by conventional cloning methods (Takata et al. 2009). Each vector containing a fragment of *PsgXCP1*, *PsgXCP2A*, *PsgXCP2B* plus *UBQ* was used to generate a standard curve of real-time PCR amplification. We analyzed levels of expression of genes for three transcriptional factors, namely, *SND1-A1* (POPTR_0011s15640; Li et al. 2012), *VND6-A1* (POPTR_0015s14770; Li et al. 2012) and *MYB003* (POPTR_0001s27430; McCarthy et al. 2009; Zhong et al. 2013) in *P. tremula*×*P. alba* by real-time PCR. For the analysis by real-time PCR of the expression of *PtaSND1-A1*, *PtaMYB003* and *PtaVND6-A1*, a mixture of cDNAs was used to generate a standard curve of real-time PCR amplification. Levels of transcripts of these genes were normalized by reference to the level of the transcript of *UBQ*. Each sample of RNA was assayed in triplicate.

Accession numbers

The accession numbers of the sequences of cDNAs of *PsgXCP1*, *PsgXCP2A* and *PsgXCP2B* are AB845718, AB845719 and AB845720, respectively; that of the genomic DNA of *PsgXCP1b* is LC005520.

Results

Isolation and characterization of XCP *genes from* Populus

There are two putative *XCP1* genes and two putative *XCP2* genes in the genomic database of *P. trichocarpa* (Tuskan et al. 2006). We isolated full-length cDNAs of *XCP1*, *XCP2A* and *XCP2B* from *P. sieboldii*×*P. grandidentata*. We failed to detect the expression of

PsgXCP1b in xylem tissue by RT-PCR (data not shown). We isolated genomic DNA that encoded *XCP1b* from *P. sieboldii*×*P. grandidentata* and found that five short nucleotide deletion sites and a single nucleotide insertion has occurred in the genomic sequence of *PsgXCP1b* that corresponded to the coding region of *XCP1* of *Populus* (Supplemental Figure S1). If the first atg site is the start codon, the predicted amino acid length of the PsgXCP1b is only 20 caused by the first nucleotide deletion. These results suggested that the *XCP1b* gene might be a pseudogene in *Populus* and therefore, we only investigated three *XCP* genes (*XCP1*, *XCP2A* and *XCP2B*) in this study. The amino acid sequences deduced from *PsgXCP1*, *PsgXCP2A* and *PsgXCP2B* retain the cathepsin propeptide inhibitor domain and the papain family cysteine protease domain that are conserved in papain (Figure 1). The catalytic triad of cysteine (Cys), histidine (His) and asparagine (Asn) residues, which forms the active site, is conserved in each of the deduced PsgXCPs (asterisks in Figure 1A). PsgXCP1, PsgXCP2A and PsgXCP2B are 75%, 69% and 72% homologous to AtXCP1, respectively, at the amino acid level and all three are 77% homologous to AtXCP2. The XCPs of *Arabidopsis* are members of the C1 family of cysteine proteases, which are synthesized as preproenzymes with a signal peptide (Barrett et al. 1998). A search for signal peptides in PsgXCPs, using the SignalP program (Petersen et al. 2011), revealed that all three deduced PsgXCPs include a putative signal peptide sequence with a cleavage site in the N-terminal region (arrowhead in Figure 1A).

We constructed phylogenetic trees using 12 genes from *angiosperms*, such as *A. thaliana*, *C. papaya*, *M. truncatula*, *P. sieboldii*×*P. grandidentata*, *P. trichocarpa* and *V. vinifera* (Figure 1B). Phylogenetic analysis indicated that the *XCP* genes could be clearly separated into two clades. One of the clades was composed of *AtXCP1*, *CpXCP*, *PsgXCP1*, *PtrXCP1* and *VvXCP1* and the other of *AtXCP2*, *MtXCP*, *PsgXCP2A*, *PsgXCP2B*, *PtrXCP2A*, *PtrXCP2B* and *VvXCP2*. In the latter clade, *Populus XCP2A* and *XCP2B* were more closely related than the *XCP* genes of the other plants examined.

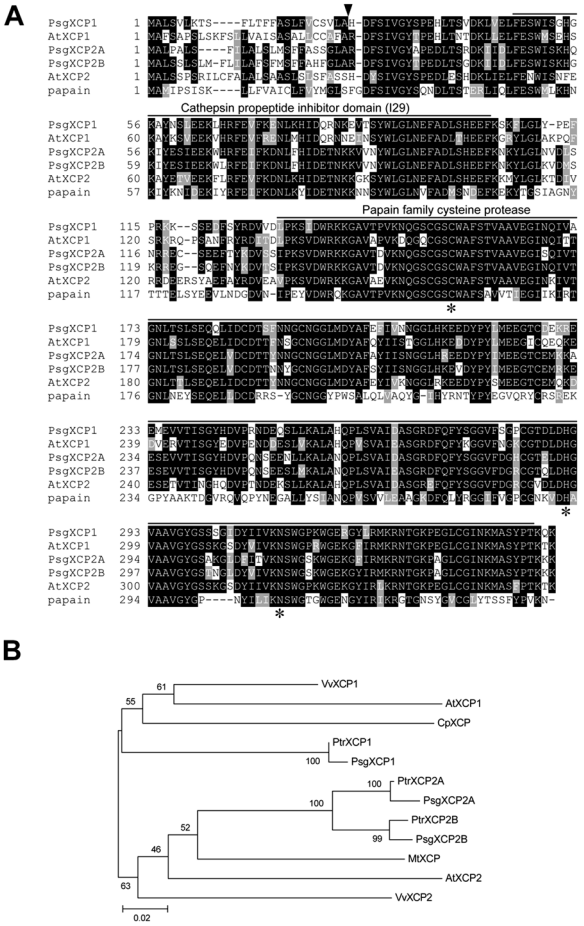

Figure 1. Deduced amino acid sequences of XCPs from *Populus* and a phylogenetic tree of XCPs. (A) Alignments of the amino acid sequences encoded by *PsgXCP1*, *PsgXCP2A*, *PsgXCP2B*, *AtXCP1* and *AtXCP2* and *papain* from *Carica papaya*. Amino acid sequences were aligned using the ClustalW program. Identical and similar amino acid residues are shown in black and gray boxes, respectively. The conserved catalytic residues Cys, His and Asn are indicated by asterisks. The predicted cleavage site is shown with an arrowhead. (B) Phylogenetic tree of XCPs from angiosperms. Full-length amino acid sequences were aligned using the ClustalW program. The phylogenetic tree was constructed by the NJ method from numbers of amino acid substitutions, as estimated by application of the JTT model. The numerals at the branch points indicate bootstrap values, calculated by the NJ method with 1,000 replications.

Levels of expression of XCP*s in ray parenchyma cells of* P. sieboldii×P. grandidentata

To evaluate the progression of cell death, we monitored morphological changes in nuclei after staining nuclei with acetocarmine in ray parenchyma cells from the current year's xylem to the fifth annual ring from the cambium in 14-year-old *P. sieboldii*×*P. grandidentata* (Figure 2A–C). Ray parenchyma cells contained fusiform nuclei in samples of outer sapwood (Figure 2A, D). No vessel elements and no wood fibers were alive in the samples taken from the previous year's xylem, where vessel elements and fibers had completed cell differentiation. In samples of inner sapwood, some ray parenchyma cells had fusiform, spherical or deformed nuclei (Figure 2B, E, F), while other cells no longer contained nuclei (Figure 2C). In our previous research, we demonstrated that these morphological changes in nuclei progressed towards the inner region of the tree trunk of *P. sieboldii*×*P. grandidentata* (Nakaba et al. 2012). Such morphological changes in nuclei indicate the progression of the process of cell death. Therefore, we concluded that the ray parenchyma cells in the inner sapwood were undergoing programmed cell death.

To investigate changes in the expression of *PsgXCP* genes during programmed death of ray parenchyma cells, we evaluated gene expression in the outer and

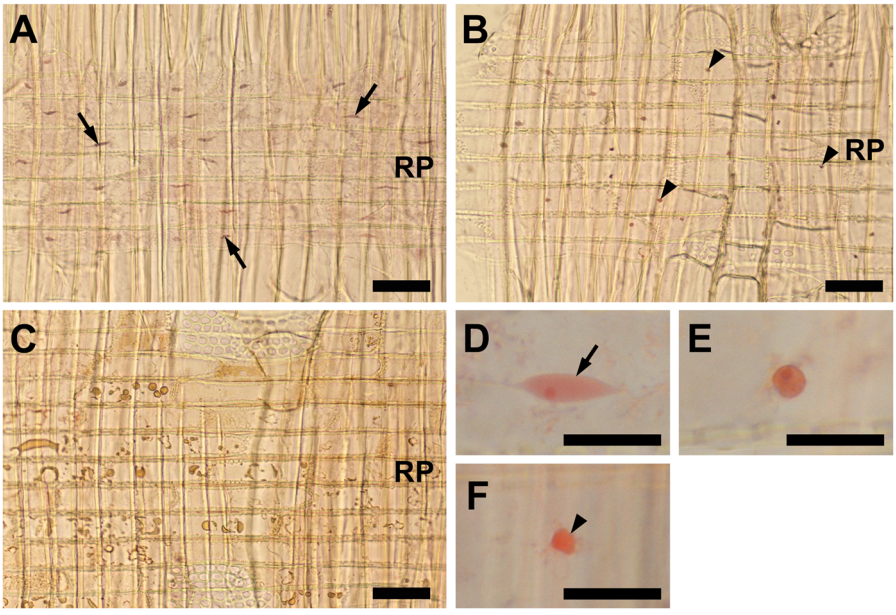

Figure 2. Light micrographs of radial sections, stained with acetocarmine, showing nuclei (A) in the ray parenchyma cells in the previous year's xylem, (B) in the outer region of the fifth annual ring from the cambium and (C) in the inner region of the fifth annual ring from the cambium in *P. sieboldii*×*P. grandidentata*. Transmission images, after staining with acetocarmine, of (D) a fusiform nucleus, (E) a spherical nucleus and (F) a deformed nucleus. Arrows indicate fusiform nuclei. Arrowheads indicate deformed nuclei. The left side of each micrograph corresponds to the outer side of the tree. RP, Ray parenchyma cell. Bars=50 μm in (A)–(C); 10 μm in (D)–(F).

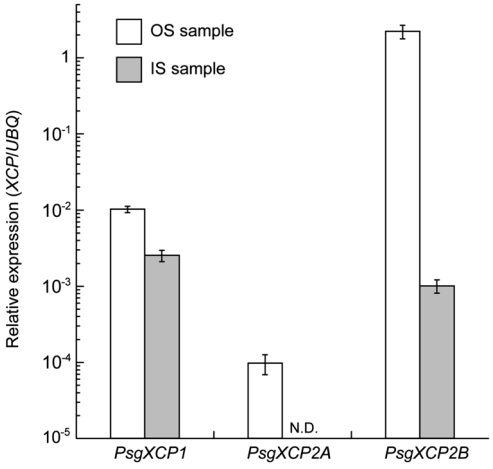

Figure 3. Relative levels of expression of *XCP*s in outer and inner sapwood of *P. sieboldii*×*P. grandidentata*. Values shown represent results normalized by reference to results for *UBQ*. Values are means±SD (n=3). OS, Outer sapwood; IS, inner sapwood; N.D., not detected. See text for details.

inner sapwood by real-time PCR (Figure 3). No vessel elements and wood fibers were alive in these sapwood tissues. Therefore, RNA extracted from these tissues should have originated in living or dying ray parenchyma cells. Real-time PCR analysis showed that *PsgXCP* genes were detected in outer and inner sapwood and were expressed at lower levels in the inner sapwood than in the outer sapwood. Levels of expression of *PsgXCP1* and *PsgXCP2B* in the outer sapwood were approximately 5- and 2,000-fold higher, respectively, than those in the inner sapwood. Levels of expression in the inner sapwood decreased in the following order: *PsgXCP1*, *PsgXCP2B* and *PsgXCP2A*. In the outer sapwood, the level of expression was highest for *PsgXCP2B* and lowest for *PsgXCP2A*.

Survival rates of ray parenchyma cells and levels of expression of XCPs *in* P. tremula×P. alba

For more detailed characterization of patterns of expression of *XCP*s during programmed cell death, we examined younger poplar (*P. tremula*×*P. alba*) trees that had wider and multiply divisible sapwood in the radial direction. We monitored morphological changes and the disappearance of nuclei in the inner sapwood (Figure 4A–F). Ray parenchyma cells were alive from the first to the third annual rings from the cambium (Figure 4G). Dying ray parenchyma cells were observed inside the third and the fourth annual rings from the cambium. There were no living ray parenchyma cells in the inner region of the fourth and the fifth annual rings from the cambium.

We performed real-time PCR analyses using total RNA extracted from each annual ring to evaluate changes in the expression of the *PtaXCP* genes (Figure 5). The expression of *PtaXCP* genes was observed in all annual ring in sapwood. The patterns of expression of *PtaXCP* genes varied in the radial direction among the trees examined. However, we noted common characteristics of the expression of these genes in the inner sapwood. The expression of *PtaXCP* genes was lower in the innermost

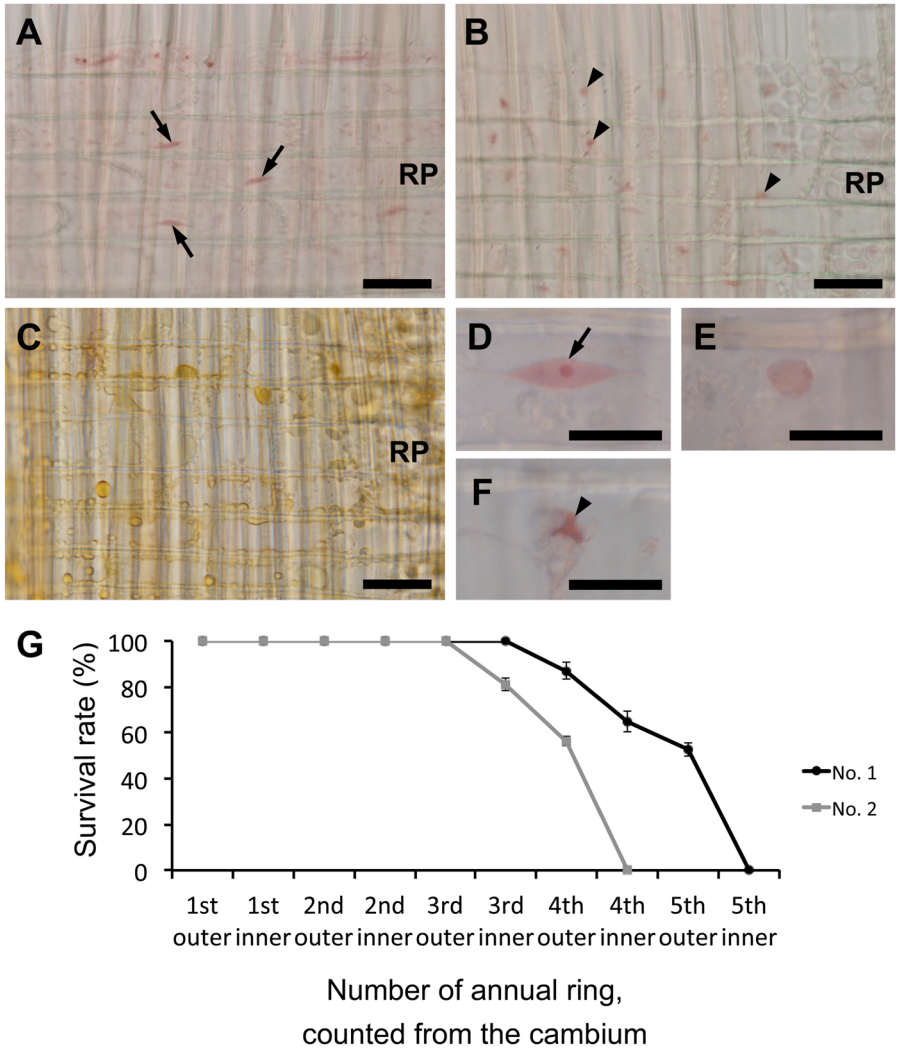

Figure 4. Light micrographs of radial sections, stained with acetocarmine, showing (A) nuclei in the ray parenchyma cells in the current year's xylem, (B) in the outer region of the fifth annual ring from the cambium and (C) in the inner region of the fifth annual ring from the cambium in sample No. 1 of *P. tremula*×*P. alba*. Transmission images, stained with acetocarmine, of (D) a fusiform nucleus, (E) a spherical nucleus and (F) a deformed nucleus. Arrows indicate fusiform nuclei. Arrowheads indicate deformed nuclei. The left side of each micrograph corresponds to the outer side of the tree. RP, Ray parenchyma cell. Bars=30 μm in (A)–(C); 10 μm in (D)–(F). (G) The rate of survival of ray parenchyma cells, as determined from the current year's xylem to the annual ring in which all ray parenchyma cells had lost their organelles, in *P. tremula*×*P. alba*. Values are means±SD (n=3).

annual ring of sapwood than in one and/or two outer annual rings from the innermost sapwood in both trees examined. The patterns of expression in *P. tremula*×*P. alba* were similar to those in *P. sieboldii*×*P. grandidentata*.

Finally, we next examined patterns of expression of genes for upstream regulators, namely, *SND1-A1*, *VND6-A1* and *MYB003* that participate in cell wall formation and programmed cell death in xylem fibers and vessels. The expression of *PtaSND1-A1*, *PtaVND6-A1* and *PtaMYB003* genes was detected in all annual ring in sapwood (Figure 6). The respective levels of expression of the *PtaSND1-A1* and *PtaMYB003* genes were higher in the current year's annual ring than those in the other annual rings. The expression of *PtaVND6-A1* was stronger in one and/or two outer annual rings beyond the innermost sapwood than in the innermost annual ring of the sapwood.

Discussion

In the present study, we isolated full-length cDNA of *XCP*s from *P. sieboldii*×*P. grandidentata*. The deduced amino acid sequences of PsgXCPs revealed strong homology to the cysteine proteases XCP1 and XCP2 of *A. thaliana* (Figure 1). Each PsgXCP included the cathepsin propeptide inhibitor domain and the papain family cysteine protease domain in addition to the catalytic residues Cys, His and Asn (asterisks in Figure 1A). These observations suggest that PsgXCP1, PsgXCP2A and PsgXCP2B might function as papain-like cysteine proteases.

In short-lived xylem cells in plants, the expression of

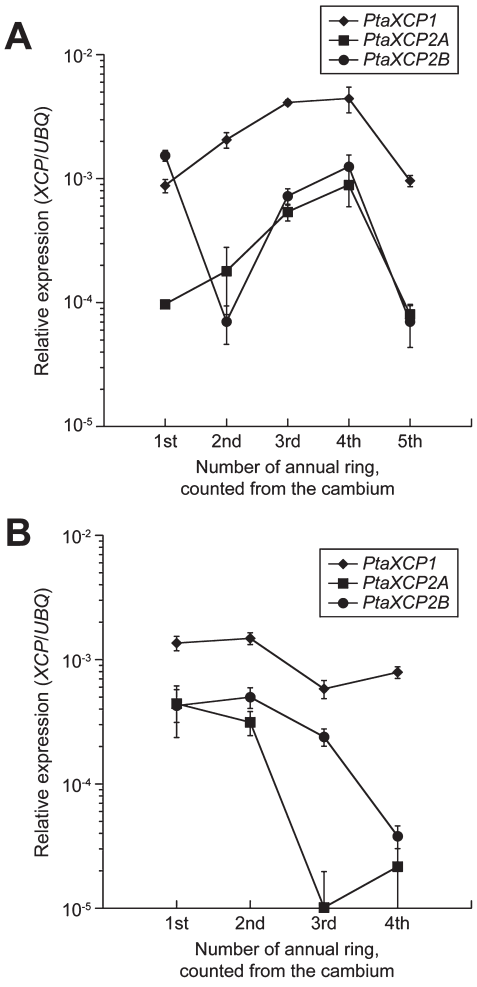

Figure 5. Relative levels of expression of genes for XCPs in the first through fifth annual rings in the sapwood of *P. tremula*×*P. alba*. The relative levels of expression of genes for XCPs in sample No. 1 (A) and No. 2 (B) are shown separately. Values shown represent results normalized by reference to results for *UBQ*. Values are means±SD (n=3).

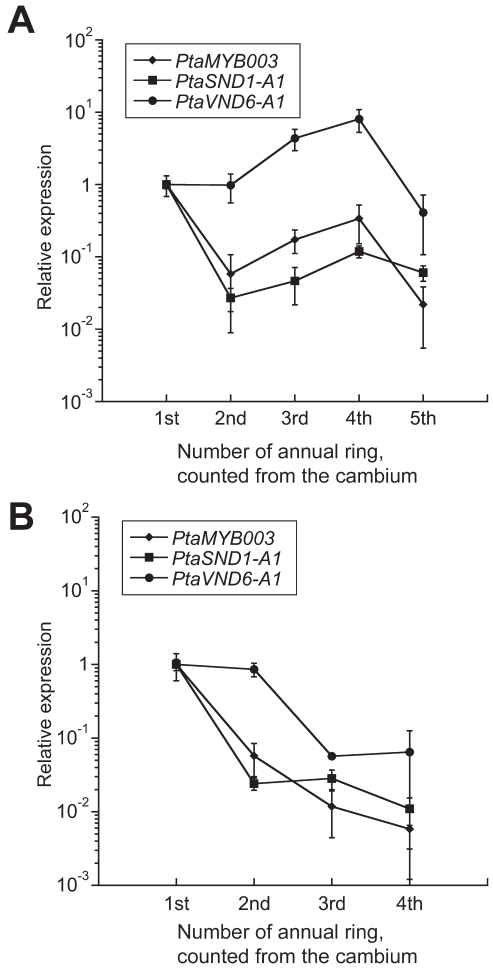

Figure 6. Relative levels of expression of genes for SND1-A1, MYB003 and VND6-A1 in the first through fifth annual rings in the sapwood of *P. tremula*×*P. alba*. The relative levels of expression of genes in sample No. 1 (A) and No. 2 (B) are shown separately. Values shown represent results normalized by reference to results for *UBQ*. Values are means±SD (n=3).

XCP genes increases dramatically before the cell death of vessel elements and wood fibers (Courtois-Moreau et al. 2009; Ohashi-Ito et al. 2010). In the present study, we detected the expression of *XCP* genes in ray parenchyma cells of the outer and the inner sapwood (Figures 3, 5). Ray parenchyma cells remained bioactive until the fourth or the fifth annual ring from the cambium (Figures 2, 4). Our observations indicate that the *XCP* genes are expressed for several years in ray parenchyma cells before they die. It is noteworthy that the levels of expression of *PtaXCP* genes were lower in the innermost annual ring of the sapwood than in annual rings that were one and/or two rings further out from the innermost annual ring of the sapwood (Figure 5). This observation implies that autolysis of ray parenchyma cells might not be fully explained by dramatically enhanced expression of *XCP* genes just before their programmed death.

In *A. thaliana*, the expression of *XCP* genes is directly regulated by the products of *VND6* and *VND7*, which have been identified as master switches of xylem vessel elements (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011). In *Populus*, we detected the expression of *VND6-A1* for several years in ray parenchyma cells and expression was enhanced in one and/or two outer annual rings from the innermost sapwood (Figure 6). The pattern of expression of *PtaVND6-A1* appeared to be correlated with that of the *PtaXCP* genes, implying that the transcriptional activation of *Populus XCP*s by the products of the *VND* gene is conserved in ray parenchyma cells.

In *Populus*, the products of the *SND1* and *MYB003* genes regulate the expression of genes associated with secondary wall formation (McCarthy et al. 2009; Ohtani et al. 2011; Zhong et al. 2013). The products of the *SND1* also induce the enhanced expression of *XCP* genes in *Populus* (Ohtani et al. 2011). The *PtaSND1-A1* and *PtaMYB003* genes are expressed for several years in ray parenchyma cells before they die (Figure 6). We found that the expression of *PtaSND1-A1* and *PtaMYB003*

was the strongest in the current year's xylem. This result indicates that the *PtaSND1-A1* and *PtaMYB003* genes regulate secondary wall formation in the cells of the current year's xylem. In addition, the expression of *PtaSND1-A1* and *PtaMYB003* was weakly detected in ray parenchyma cells in inner annual rings of sapwood from the current year's xylem, implying that the expression of genes associated with secondary wall formation might continue for several years in long-lived ray parenchyma cells as well as genes associated with cell death.

During the differentiation of short-lived xylem cells, such as tracheary elements and wood fibers, secondary wall formation and programmed cell death occurs successively (Courtois-Moreau et al. 2009; Fukuda 2004). By contrast, ray parenchyma cells in hybrid poplar remain alive for several years, with continuous expression of *XCP* genes, even after obvious secondary wall thickenings (Figures 3, 5). The difference in the timing between the expression of *XCP* genes and autolysis of ray parenchyma cells raises the question of whether the products of *XCP* genes accumulate in the cells for several years until the cells die. Further studies on the subcellular and intercellular behavior of XCP proteins should help to clarify the functions of XCPs in the programmed death of long-lived ray parenchyma cells in *Populus*.

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References

Bamber RK, Fukazawa K (1985) Sapwood and heartwood: A review. *For Abstract* 46: 567–580

Barrett AJ, Rawlings ND, Woessner JF (eds) (1998) *Handbook of Proteolytic Enzymes*. Academic Press, San Diego

Bollhöner B, Prestele J, Tuominen H (2012) Xylem cell death: emerging understanding of regulation and function. *J Exp Bot* 63: 1081–1094

Courtois-Moreau CL, Pesquet E, Sjödin A, Muñiz L, Bollhöner B, Kaneda M, Samuels L, Jansson S, Tuominen H (2009) A unique program for cell death in xylem fibers of *Populus* stem. *Plant J* 58: 260–274

Escamez S, Tuominen H (2014) Programmes of cell death and autolysis in tracheary elements: when a suicidal cell arranges its own corpse removal. *J Exp Bot* 65: 1313–1321

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evol* 39: 783–791

Fukuda H (1997) Tracheary element differentiation. *Plant Cell* 9: 1147–1156

Fukuda H (2004) Signals that control plant vascular cell differentiation. *Nat Rev Mol Cell Biol* 5: 379–391

Hillis WE (ed) (1987) *Heartwood and Tree Exudates*. Springer-Verlag, New York

Huang Z, Meilan R, Woeste K (2009) A *KNAT3*-like homeobox gene from *Juglans nigra* L., *JnKNAT3*-like, highly expressed during heartwood formation. *Plant Cell Rep* 28: 1717–1724

IAWA (Committee on Nomenclature, International Association of Wood Anatomists) (1964) Multilingual glossary of terms used in wood anatomy. Verlagsanstalt Buchdruckrei Konkordia Winterthur, Switzerland

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Miura T, Fukuda H, Demura T (2005) Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev* 19: 1855–1860

Li Q, Lin YC, Sun YH, Song J, Chen H, Zhang XH, Sederoff RR, Chiang VL (2012) Splice variant of the SND1 transcription factor is a dominant negative of SND1 members and their regulation in *Populus trichocarpa*. *Proc Natl Acad Sci USA* 109: 14699–14704

McCarthy RL, Zhong R, Ye ZH (2009) MYB83 is a direct target of SND1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in Arabidopsis. *Plant Cell Physiol* 50: 1950–1964

Magel E (2000) Biochemistry and physiology of heartwood formation. In: Savidge R, Barnett J, Napier R (eds) *Molecular and Cell Biology of Wood Formation*. BIOS Scientific Publishers, Oxford, pp 363–376

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. *Plant Cell* 19: 270–280

Nakaba S, Sano Y, Kubo T, Funada R (2006) The positional distribution of cell death of ray parenchyma in a conifer, *Abies sachalinensis*. *Plant Cell Rep* 25: 1143–1148

Nakaba S, Kubo T, Funada R (2008) Differences in patterns of cell death between ray parenchyma cells and ray tracheids in the conifers *Pinus densiflora* and *Pinus rigida*. *Trees (Berl)* 22: 623–630

Nakaba S, Begum S, Yamagishi Y, Jin HO, Kubo T, Funada R (2012) Differences in the timing of cell death, differentiation and function among three different types of ray parenchyma cells in the hardwood *Populus sieboldii*×*P. grandidentata*. *Trees (Berl)* 26: 743–750

Ohashi-Ito K, Oda Y, Fukuda H (2010) *Arabidopsis* VASCULAR-RELATED NAC-DOMAIN6 directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. *Plant Cell* 22: 3461–3473

Ohtani M, Nishikubo N, Xu B, Yamaguchi M, Mitsuda N, Goué N, Shi F, Ohme-Takagi M, Demura T (2011) A NAC domain protein family contributing to the regulation of wood formation in poplar. *Plant J* 67: 499–512

Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* 8: 785–786

Petzold HE, Zhao M, Beers EP (2012) Expression and functions of proteases in vascular tissues. *Physiol Plant* 145: 121–129

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425

Spicer R (2005) Senescence in secondary xylem: heartwood formation as an active developmental program. In: Holbrook

NM and Zwieniecki MA (eds) *Vascular Transport in Plants*. Elsevier Academic Press, Amsterdam, pp 457–475

Takata N, Saito S, Saito CT, Nanjo T, Shinohara K, Uemura M (2009) Molecular phylogeny and expression of poplar circadian clock genes, *LHY1* and *LHY2*. *New Phytol* 181: 808–819

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739

Taylor A, Gartner BL, Morrell JJ (2002) Heartwood formation and natural durability—a review. *Wood Fiber Sci* 34: 587–611

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, et al. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313: 1596–1604

Yamaguchi M, Mitsuda N, Ohtani M, Ohme-Takagi M, Kato K, Demura T (2011) Vascular-related NAC-DOMAIN7 directly regulates expression of a broad range of genes for xylem vessel formation. *Plant J* 66: 579–590

Yang J, Kamdem DP, Keathley DE, Han KH (2004) Seasonal changes in gene expression at the sapwood-heartwood transition zone of black locust (*Robinia pseudoacacia*) revealed by cDNA microarray analysis. *Tree Physiol* 24: 461–474

Yoshida K, Futamura N, Nishiguchi M (2012) Collection of expressed genes from the transition zone of *Cryptomeria japonica* in the dormant season. *J Wood Sci* 58: 89–103

Zhong R, Demura T, Ye ZH (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. *Plant Cell* 18: 3158–3170

Zhong R, Richardson EA, Ye ZH (2007) Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of Arabidopsis. *Planta* 225: 1603–1611

Zhong R, McCarthy RL, Haghighat M, Ye ZH (2013) The poplar MYB master swithces bind to the SMRE site and activate the secondary wall biosynthetic program during wood formation. *PLoS ONE* 8: e69219

Supplemental Figure S1 Alignments of the nucleotide sequences of *PtrXCP1*_gDNA and *PtrXCP1*_cDNA from *Populus trichocarpa* and *PsgXCP1*_cDNA and *PsgXCP1b*_gDNA from *P. sieboldii* × *P. grandidentata*. Nucleotide sequences were aligned using the ClustalW program. Asterisks indicate identical nucleic acid residues.

Supplemental Table S1. Oligonucleotides used in the present study

Primer name	Sequence (5'-3')	Target gene	Experiment
PsgXCP1.5'RACE.rev	CACTGAGAGGCTGGTGAGCTAGTG	*PsgXCP1*	5' RACE
PsgXCP1.3'RACE.for	CCAGAGCACTTGACTTCTGTTGACAAAC	*PsgXCP1*	3' RACE
PsgXCP2A.5'RACE.rev	GGGAAAAAACACAAATTTAGGACATTTAGCATG	*PsgXCP2A*	5' RACE
PsgXCP2A.3'RACE.for	GCACTTGTGAGATGAGAAAGGCAGAATC	*PsgXCP2A*	3' RACE
PsgXCP2B.5'RACE.rev	CTCTGATTCTTCCTTTCTCATCTCACAG	*PsgXCP2B*	5' RACE
PsgXCP2B.3'RACE.for	GTCATTCTTCGCCAATTCTGGTTTAGC	*PsgXCP2B*	3' RACE
PsgXCP1b.for	CAGTTCTCGTCTCTCATTTTACTATCAT	*PsgXCP1b*	Cloning of genomic region of *PsgXCP1b*
PsgXCP1b.rev	TCTGTTGCTCAGGCTTGGTAGTCT	*PsgXCP1b*	Cloning of genomic region of *PsgXCP1b*
PsgXCP1.for	GTAGAAAATCCATCTCTTCTACTGCTCTG	*PsgXCP1*	Cloning of *PsgXCP1*
PsgXCP1.rev	CAACTTTCATCTCGTTTTATTGATGGACATGAG	*PsgXCP1*	Cloning of *PsgXCP1*
PsgXCP2A.for	CCTTCCACTCAACAATTTTTCCCATTCTC	*PsgXCP2A*	Cloning of *PsgXCP2A*
PsgXCP2A.rev	GGGGATGCTAATAGATTTTTTCGGGG	*PsgXCP2A*	Cloning of *PsgXCP2A*
PsgXCP2B.for	GAAAGACTGGAGCACGAGGAC	*PsgXCP2B*	Cloning of *PsgXCP2B*
PsgXCP2B.rev	GTGATGAAAGGGAGTTTCTTTATTGCAAACTTC	*PsgXCP2B*	Cloning of *PsgXCP2B*
PsgXCP2B.nested.for	GCACGAGGACACTGACATGG	*PsgXCP2B*	Cloning of *PsgXCP2B*
PsgXCP2B.nested.rev	GCAAACTTCTTCGAACTTTAATTGATAGCCAG	*PsgXCP2B*	Cloning of *PsgXCP2B*
PtUBQ.for	GGTTGATTTTTGCTGGGAAGC	*UBQ*	Real-time PCR
PtUBQ.rev	GATCTTGGCCTTCACGTTGT	*UBQ*	Real-time PCR
PsgXCP1.RT.for	ACGTTCCAAGAAATGACGAACA	*PsgXCP1*	Real-time PCR
PsgXCP1.RT.rev	CTCTTCCAGAAGCGTCAATAGCA	*PsgXCP1*	Real-time PCR
PsgXCP2A.RT.for	TGGCTTGGATTGAATGAGTTC	*PsgXCP2A*	Real-time PCR
PsgXCP2A.RT.rev	TCCTTGTATGTGAATTCCTCAGAGC	*PsgXCP2A*	Real-time PCR
PsgXCP2B.RT.for	ACCAATGGCTTGGATTACGTTATC	*PsgXCP2B*	Real-time PCR
PsgXCP2B.RT.rev	GCAGGCTTGCCAGTGTTTCTCTT	*PsgXCP2B*	Real-time PCR
MYB003.RT.for	TCATCAGAACCTAGAGATCATGTCG	*MYB003*	Real-time PCR
MYB003.RT.rev	CCTTGCACAGAGTCATAACGTC	*MYB003*	Real-time PCR
SND1-A1.RT.for	CGACATGACCTCATCCTCAATGAC	*SND1-A1*	Real-time PCR
SND1-A1.RT.rev	GTCTCTACTTCATGCCCATTAAGC	*SND1-A1*	Real-time PCR
VND6-A1.RT.for	TTGAGCCACGAAGATGTGATCAAG	*VND6-A1*	Real-time PCR
VND6-A1.RT.rev	AGGTCTTCCTCATTCCGATCAAGTCT	*VND6-A1*	Real-time PCR