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Characterization of the plasma membrane proteins and receptor-like kinases associated with secondary vascular differentiation in poplar

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Abstract The constituents of plasma membrane proteins, particularly the integral membrane proteins, are closely associated with the differentiation of plant cells. Secondary vascular differentiation, which gives rise to the increase in plant stem diameter, is the key process by which the volume of the plant body grows. However, little is known about the plasma membrane proteins that specifically function in the vascular differentiation process. Proteomic analysis of the membrane proteins in poplar differentiating secondary vascular tissues led to the identification 226 integral proteins in differentiating xylem and phloem tissues. A majority of the integral proteins identified were receptors (55 proteins), transporters (34 proteins), cell wall formation related (27 proteins) or intracellular trafficking (17 proteins) proteins. Gene expression analysis in developing vascular cells further demonstrated that cambium differentiation involves the expression of a group of receptor kinases which mediate an array of signaling pathways during secondary vascular differentiation. This paper provides an outline of the protein composition of the plasma membrane in differentiating secondary vascular tissues and sheds light on the role of receptor kinases during secondary vascular development.

Keywords Plasma membrane protein · Integral protein · Receptor-like kinase · Proteomics · Vascular differentiation · Poplar

Introduction

Secondary vascular differentiation occurs mainly in angiosperm dicot and gymnosperm trees. Tree trunks grow in diameter through the activity of its vascular cambium, which is a secondary meristem that divides inwards to produce secondary xylem cells and outwards to develop secondary phloem cells. To date, many studies have profiled the global gene expression during secondary vascular differentiation in order to understand the molecular mechanisms underlying this secondary growth process. Gene transcripts profiled in poplar and other tree species during cell differentiation after vascular cambium division indicated that the differentiation is under stage-specific transcriptional regulation and that a number of genes are found to be expressed in association with the differentiation (Hertzberg et al. 2001; Allona et al. 1998; Pavy et al. 2008; Schrader et al. 2004). Meanwhile, proteomic profiling has also provided an outline of which genes are expressed during the various stages of secondary meristem cell differentiation in poplar. Regulatory proteins for cell cycle progression and cell fate were found to be expressed in the early stages while proteins for secondary wall formation were found predominantly in the later stages of differentiation (Du et al. 2006).

Secondary vascular tissue of tree species features several types of specialized cells including fiber cells, sieve and vessel elements, which are formed during the differentiation process after cambium cell division. Membrane proteins are believed to play important roles over the course of cell differentiation via various functions such as

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cell signaling, catalysis and cross-membrane transport (Tan et al. 2008). For the characterization of the plasma proteins related to plant cell wall formation, detergent-resistant plasma membrane microdomains was analyzed in aspen cell suspensions and found to contain a group of key carbohydrate synthases (Bessueille et al. 2009). Subcellular proteomic analysis was conducted for protein inventory of cell organelles such as mitochondria, chloroplast, and peroxisomes (Lilley and Dupree 2007; Baginsky 2009; Yu et al. 2008; Reiland et al. 2009). However, the particular protein constituents in the plasma membrane of secondary vascular tissues has been little studied. While this manuscript was in the process of being prepared, a study reported the detection of 956 proteins from the membrane preparation of *Populus* (Nilsson et al. 2010). Among them, transporter and receptor proteins were found to be major constituents of membrane proteins which displayed a pronounced distribution among leaf, xylem and phloem tissues. Leaf plasma membrane contained a high proportion of transporters, constituting almost half of the integral proteins while xylem plasma membranes contained an abundance of membrane trafficking proteins. Overall, those results demonstrated that membrane proteins are differentially distributed in the various tissues of poplar (Nilsson et al. 2010).

In plants, receptor-like kinases (RLKs) have been shown to be a crucial class of transmembrane proteins for the perception of various signals on the cell surface. It has been reported that signaling mediated by ligand-RLK pathways play an essential role in regulating cell-to-cell communication and cell differentiation during postembryonic development in plants (Fletcher et al. 1999; Lenhard and Laux 1999; Fukuda 2004; De Smet et al. 2009). Recently, a RLK has been studied for its implication in vascular cell differentiation. PXY/TDR (PHLOEM INTERCALATED WITH XYLEM/TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR RECEPTOR) has been reported as an important receptor-like kinase that controls the orientation of cell division during vascular development (Hirakawa et al. 2008; Fisher and Turner 2007). A peptide, TDIF (TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR), which is encoded by CLE41 and CLE44 in *A. thaliana* has been demonstrated as a ligand which binds specifically to TDR/PXY (Hirakawa et al. 2008). TDIF–PXY/TDR forms a ligand-receptor system involved in regulating vascular cell differentiation in Arabidopsis.

In the present paper, we present the proteomic profile of the plasma membrane isolated after a two-phase separation from the differentiating xylem and phloem tissues in poplar. More than 1,500 proteins were found to be associated with the plasma membrane isolation. Of those, a total of 226 proteins were identified as integral plasma membrane proteins. Overall, the results of the present study offer an independent categorization of the plasma membrane proteins isolated after a rigorous separation procedure. In particular, a group of RLKs were identified in the plasma membrane. Analysis of the cell-specific gene expression revealed that a group of the RLK genes were differentially expressed in a pattern which suggests that different RLKs may mediate different signaling pathways during secondary vascular development. Profiling of the expression of RLK genes provides a line of new information for dissecting how secondary vascular tissues are developed through serial signaling regulation on the plasma membrane.

Materials and methods

Plant materials and micro-dissection

A group of *Populus* female cloning trees (*Populus* × *euramericana cv. 'Nanlin895'*), which were grown in an experimental field with 3 years old, were used for collection of a large amount of tissue samples. The sample collection was carried out in the morning of May 11, 2008, when the leaves of the trees were fully developed. The upper part of tree stems was sectioned for tissue collection. After the stem bark was peeled, differentiating phloem and xylem were examined and harvested directly into liquid nitrogen and stored for later use.

The same clone of the tree, which was grown in a greenhouse was used for micro-dissection. Cell samples of the differentiating secondary vascular tissue were acquired from cross-sections of poplar stem by micro-dissection as described (Song et al. 2010). Vascular cambium, differentiating xylem and phloem, and cortex cells were collected from dissecting a total of 40 stem sections, which amounted to approximately 12,000 cells in each sample. Total transcript preparation from the sampled cells was as described (Song et al. 2010).

Real-time quantitative PCR quantification of cell-specific gene expression

For real-time quantitative PCR measurement, primers were designed to amplify a specific fragment (100–300 bp in length) of the detected genes. The primer specificity was confirmed by amplification of a single specific band. Measurements were performed on a MyiQ Real-Time PCR Detection System (Bio-Rad, Winston-Salem, NC, USA). The PCR reaction was carried out in a volume of 20 ul containing 50 ng of cDNA template using SYBR Green Master Mix (TOYOBO, Osaka, Japan). PCR program was: one cycle of 95°C for 2 min, followed by 45 cycles of 95°C

for 15 s, 58°C for 15 s and 72°C for 20 s. After amplification, the PCR product was examined by measuring their melting curves to ensure the accuracy of the reaction. The abundance of the gene transcripts was normalized against *Actin2* expression. The expression scale of ST651, a characterized cytokinin receptor gene, was set to 1, equivalent to 1/1000 of the *Actin2* transcript abundance, for the relative comparison of gene expression.

Microsomal fraction preparation

Differentiating xylem and phloem samples (100 g) were ground in liquid nitrogen together with 1% PVPP to fine powder, and then homogenized at 4°C in 500 ml extraction buffer containing 0.5 M Tris–HCl, pH 8.5, 0.7 M sucrose, 0.1 M KCl, 50 mM EDTA, 1 mM PMSF, 2% (v/v) β-mercaptoethanol, 1 mM leupeptin and 1 mM pepstatin (Saravanan and Rose 2004; Suzuki et al. 2006). Afterwards the homogenate was centrifuged at 10,000*g* for 10 min at 4°C and filtered with Miracloth. The filtrate was diluted by an equal volume of ice cold water and centrifuged at 150,000*g* for 30 min to collect the microsomal fraction. The pellet was then washed three times with ice cold water at 4°C to remove residual supernatant proteins.

Plasma membrane separation and quality assay

The plasma membrane fraction was separated from the above microsomal preparation using a dextran-PEG aqueous two phase system with minor modification(Tanaka et al. 2004). The microsomal fraction was suspended in 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.8) and then added to a partition system consisting of 6.3% PEG3350, 6.3% dextran T-500, 0.3 M sucrose and 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.8). After the first partition, the upper phase was recovered and partitioned with fresh lower phase twice. The final upper phase was diluted 1:5 with ice cold water. The plasma membrane pellet was collected by centrifugation at 150,000*g* for 30 min at 4°C.

The quality of the isolated plasma membrane was estimated by monitoring different types of H^+-ATPase as indicated by P type H^+-ATPase for plasma membrane, F type H^+-ATPase for mitochondrion or chloroplast and V type H^+-ATPase for vacuoles, respectively, (Sze 1985). ATPase activity was measured according to the methods described (Sandstrom et al. 1987; Tanaka et al. 2004) with modification. Suspended plasma membrane protein (about 5 μg) was added to assay buffer (50 mM Tris-Mes (pH 6.5), 5 mM $MgSO_4$, 50 mM KCl, 5 mM NaATP, 0.1 mM Na_2MoO_4, 125 mM sucrose, 0.0125%(w/v) Triton ×100) with or without inhibitor (inhibitors: 100 μM Na_3VO_4 for P- H^+-ATPase, 2 mM NaN_3 for F- H^+-ATPase and 50 mM KNO_3 for V- H^+-ATPase). Assay mixture was incubated at 37°C for 10 min and then terminated by adding stop solution containing 2% H_2SO_4, 5% SDS, 0.5% Na_2MoO_4. After being stopped, 10% ascorbic acid was added into the reaction mixture and incubated for 10 min at room temperature, ATPase activity was determined spectrophotometrically at A_{660}.

For membrane protein identification, the plasma membrane pellet was dissolved in SDS buffer (0.5 M Tris–HCl pH 8.5, 2% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 4%SDS, 1 mM PMSF, 1 mM leupeptin and 1 mM pepstatin) and heated for 5 min at 80°C, the dissolvent was subsequently centrifuged at 12,000*g* for 30 min at room temperature to remove insoluble debris. Then the supernatant was extracted with an equal volume of water-saturated phenol. After the phenol phase was recovered, it was re-extracted three times with the microsomal extraction buffer. Then the membrane proteins were precipitated from the phenol phase by adding 5 volumes of cold methanol containing 0.1 M ammonium acetate. After overnight precipitation at −20°C, the proteins were pelleted by centrifugation at 12,000*g* for 10 min at 4°C. The pellet was washed three times with 90% cold methanol, followed by another wash with 90% acetone. After drying under vacuum at 4°C, the pellet was resuspended in 500 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM DTT, 1% v/v IPG buffer, pH 4–7). After centrifugation at 20,000*g* for 30 min at 4°C, the plasma proteins were divided into two portions: one was soluble in the rehydration buffer and the other insoluble.

Protein separation and digestion

The above soluble portion of protein (about 1.2 mg) was applied to 2-DE analysis according to (Fiorani Celedon et al. 2007). After 2-DE separation, proteins were detected by silver or CBB G-250 staining method. Three protein extract replicates were performed and gel images were analyzed with the Image Master Platinum software (v. 6.0) (GE Healthcare, Amersham Bioscience). Meanwhile the insoluble portion of protein was analyzed by SDS–PAGE following our previous used protocol (Song et al. 2010). After the gels were stained, 46 protein bands were detected in each sample.

Protein samples excised from 2-DE spots or 1-DE bands were cut into 1 mm cubes. Samples were destained with 100 μl of 50% v/v ACN/25 mM ammonium bicarbonate solution. The digestion was incubated with a 10 ng/μl of trypsin solution in 25 mM ammonium bicarbonate at 37°C for 12 h. The peptide mixtures were extracted twice with 8 μl of 50% v/v ACN, 0.5% v/v formic acid (FA). The extracts were dried under protection of N_2 and resuspended in 5% ACN, 0.1% FA. The protein samples from 2-DE spots were analyzed by MALDI-TOF–MS/MS and the proteins from 1-DE bands were identified by nano-LC–MS/MS analysis.

Protein identification by MALDI-TOF–MS/MS

Digested proteins from the spot samples were redissolved in 50% ACN, 0.1% TFA and 5 mg/ml CHCA. Then the samples were spotted on a target plate. The MALDI-TOF–MS was performed on an ABI 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) instrument, followed by MS/MS analysis. Mass spectra were obtained on a mass range of 700–3,200 Da using a laser beam (335 nm, 200 Hz). The instrument was performed in a positive ion mode using an acceleration voltage of 20 kV. The mass spectra were acquired by the data-dependent acquisition method with 5–6 of the strongest precursor ions selected for MS/MS analysis. Myoglobin digested by trypsin was used to calibrate the mass instrument. MALDI-TOF–MS/MS data were analyzed using MASCOT (Matrix Science, London) search software against *Populus trichocarpa* protein database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.download.ftp.html) assuming the digestion enzyme trypsin and the search parameters for MASCOT as described (Zhang et al. 2010).

Protein analysis by Nano-flow LC–MS/MS

Analyses of the digested proteins from band samples were performed on a LC-20AD system (Shimadzu, Tokoya, Japan) connected to an LTQ Orbitrap mass spectrometer (ThermoFisher, San jose, CA, USA) as described (Song et al. 2010). Tandem mass spectra were extracted by BioWorks version 3.3.1 sp1 (ThermoFisher). All MS/MS samples were analyzed using Sequest (ThermoFisher, version 28). The parameters for the Sequest were: peptide tolerance, 50 ppm; MS/MS tolerance, 1.0 Da. Peptide identifications were accepted only if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003).

Results

Preparation of the plasma membrane proteins from differentiating xylem and phloem

According to microscopic analysis, cambium cells, which are usually restricted to only 2–4 layers of cells, were found to be attached to both differentiating phloem and xylem tissue when the bark was peeled from the poplar stem (Fig. 1). This is different from previous report in eucalyptus, in which cambium cells are found to be stuck only to the side of the phloem (Fiorani Celedon et al. 2007). Thus, both differentiating phloem and xylem tissues collected for our study actually contained vascular cambium cells and were used for protein isolation.

To study the plasma membrane protein constituents of differentiating vascular tissues, the collected tissue was first isolated for the crude microsomal fraction, from which the plasma membrane fraction was then purified using an aqueous two phase partition system (Schindler and Nothwang 2006). To ensure the high quality of the preparation, the purified plasma membrane was examined for the membrane-specific marker activities. H^+-ATPase is widely used as a specific marker for distinguishing between subcellular membranes. Vanadate-sensitive P type H^+-ATPase is found in plasma membrane while azide-sensitive F type H^+-ATPase is specific to mitochondrion and chloroplast, and nitrate-sensitive V type H^+-ATPase is specific to vacuoles (Sze 1985; Nohzadeh Malakshah et al. 2007; Tanaka et al. 2004; Komatsu et al. 2007). As shown in Fig. 2, the H^+-ATPase activity in the prepared plasma membrane samples from both differentiating xylem and phloem was sensitive to Na_3VO_4, but insensitive to NaN_3 and KNO_3. Quantitatively, 79.1% and 80.5% of the H^+-ATPase activity in the preparations of differentiating xylem and phloem cells were inhibited by Na_3VO_4, respectively. Meanwhile, only 9.3% and 10.6% of the H^+-ATPase activity was inhibited by NaN_3 and 11.7% and 12.9% by KNO_3, in differentiating xylem and phloem cells, respectively. The sensitivity of the H^+-ATPase activity to Na_3VO_4 but not to NaN_3 or KNO_3 suggests the predominance of P-type H^+-ATPase, which is specific to the plasma membrane. Overall the results demonstrated that the plasma membrane was isolated with a high degree of purity.

Protein isolation from the plasma membrane preparations

In previous studies, the plasma membrane proteins are generally directly analyzed using the SDS/acetone method after the aqueous two-phase separation (Santoni et al. 1998; Hurkman and Tanaka 1986; Nohzadeh Malakshah et al. 2007). However, when we first used this method to isolate the plasma membrane proteins from the poplar samples, the resolution quality of the subsequent electrophoresis analysis was poor (data not shown). Phenol extraction has been shown to enhance the qualitative and quantitative comparisons of plasma membrane proteins on 2-DE (Hurkman and Tanaka 1986; Saravanan and Rose 2004; Isaacson et al. 2006). To improve the resolution of the protein separation, the poplar plasma membrane preparation was first solubilized by SDS buffer and then extracted with water-saturated phenol. After extraction, the plasma membrane proteins were divided into a soluble and insoluble portion. The soluble portion was analyzed on 2-DE, yielding a high

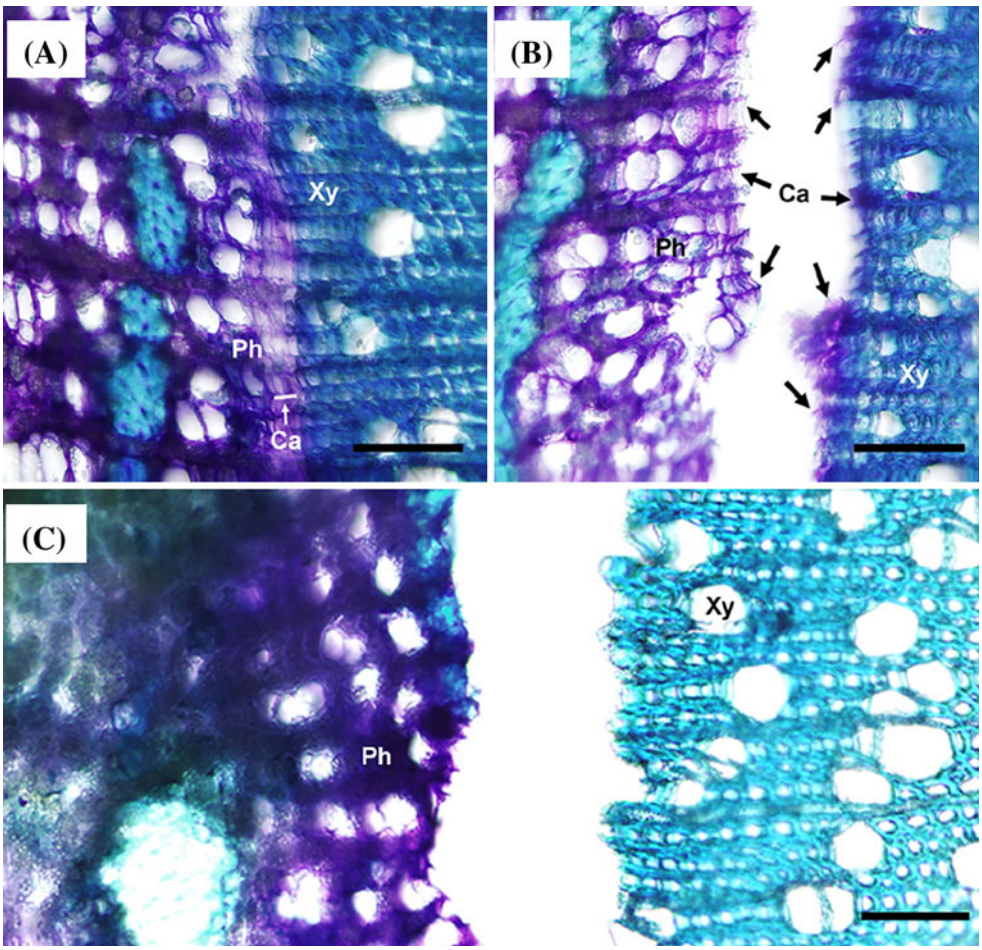

Fig. 1 Tissue sampling of Popolus secondary developing xylem and phloem. **a** Transverse section of *Popolus* stem before sampling. Four layers of cambium cells were underlined and indicated by *arrows*. **b** Transverse section of *Popolus* stem after bark separation. Cambium cells were found to be adhered to both xylem and phloem sides as indicated by *arrows*. **c** Transverse section of *Popolus* stem after developing xylem and phloem tissues were harvested. The sections were stained with toluidine blue. *Bar* 50 μm. *Xy* xylem, *Ca* Cambium, *Ph* phloem

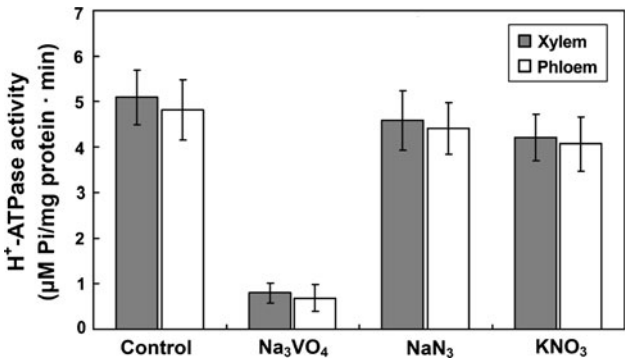

Fig. 2 H^+-ATPase activities in the isolated plasma membranes. Plasma membranes isolated from developing xylem (*grey column*) and phloem (*white column*) were measured for their H^+-ATPase activities. H^+-ATPase inhibitors, Na_3VO_4, NaN_3 and KNO_3 were used to examine the H^+-ATPase type. H^+-ATPase activity in the isolated plasma membranes was strongly inhibited by Na_3VO_4, but barely affected by NaN_3 and KNO_3

resolution separation, while the insoluble portion was partitioned on SDS–PAGE. Through this enhanced isolation procedure, plasma membrane proteins were effectively isolated and used for subsequent proteomic analysis.

Identification of the plasma membrane proteins by MS/MS analysis

The soluble portion of the plasma membrane proteins was profiled through 2D protein separation, which resulted in the identification of approximately 1,350 protein spots from differentiating xylem and about 1,351 protein spots from differentiating phloem (Fig. 3). The difference between xylem and phloem was compared using the Image Master Platinum software (v. 6.0). The results showed that while most of the protein spots matched each other in the two tissue samples, 55 protein spots were preferentially identified in

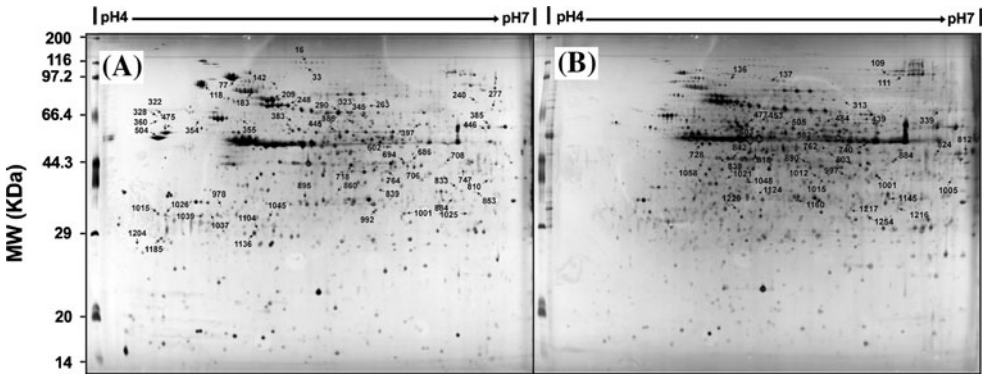

Fig. 3 2-DE profile of plasma membrane proteins from developing xylem and phloem tissue in poplar. The soluble portion of the plasma membrane proteins was profiled though 2-DE. *Arrow* indicates proteins that were preferentially expressed in (**a**) xylem and (**b**) phloem tissue. The 1-D pI ranges are indicated at the top. *MW* molecular weight

xylem and 40 in phloem. After MS/MS analysis and search against a *Populus* protein database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.download.ftp.html; http://www.phytozome.net/poplar), the corresponding annotation and gene model of these proteins were characterized (Tables S1, S2).

Among the identified 95 proteins, their protein sequences were analyzed for the presence of a transmembrane domain using the TMHMM Server, v. 2.0 program (http://www.cbs.dtu.dk/services/TMHMM/). Domain prediction indicated that only 8 proteins contained a single transmembrane domain and no protein contained multiple transmembrane domains. This suggests the majority of proteins in the soluble portion were not transmembrane and could therfore be peripheral or other proteins.

Meanwhile, the insoluble portion was partitioned by SDS–PAGE. After the gels were visualized, forty-six protein bands were detected and the proteins in each band were analyzed by LC–MS/MS analysis. The yielded peptide information was used to search a *Populus* protein database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.download.ftp.html; http://www.phytozome.net/poplar). Table 1 lists the proteins that were identified to contain at least one peptide unique to a particular protein. A total of 397 proteins were identified in xylem (Table 1 and Table S3) and 519 proteins identified in phloem (Table 1; Table S4).

A total of 678 different proteins were found in the phloem and xylem combined (Table 1; Tables S3, S4). Among them, xylem and phloem tissues shared 238 (~35%) proteins in common while 159 (~23%) proteins were only detected in xylem and 281 (~42%) proteins only in phloem (Fig. 4a). Among the detected proteins, 226 proteins (~33%) contained at least one transmembrane domain and the number of transmembrane domains varied in a range from 1 to 13 (Table 1; Fig. 4b). On the other hand, 452 (~67%) proteins were detected which did not contain transmembrane domains (Fig. 4b; Table S5). Within the transmembrane proteins, 133 of them (~59%) were identified in both xylem and phloem samples while 40 proteins were identified to be xylem-specific and 53 proteins were found to be phloem-specific (Table 1; Fig. 4c). A comparison of the non-transmembrane proteins indicated that the two tissues had 105 proteins in common (~23%), while 119 (~26%) proteins were only identified in xylem and 228 (~51%) proteins were found only in phloem (Fig. 4d; Table S5).

In a previously reported study, analysis of the plasma membrane preparations from leaf, xylem and phloem/cambium led to the identification of 213 out of 956 proteins as integral proteins(Nilsson et al. 2010). Here we identified 226 proteins with transmembrane domains from differentiating xylem and phloem which could be integral proteins. Although the total number of integral proteins detected in the two studies is similar, fewer non-transmembrane proteins (452) were detected in our study due to an additional separation procedure which resulted in the separation of more non-transmembrane proteins into a soluble portion.

The identification of 226 integral proteins in the specific tissues provides useful information to further investigate how plasma membrane proteins can regulate the differentiation of xylem and phloem tissues. For the non-transmembrane proteins it was difficult to determine whether they actually belonged to peripheral membrane proteins or another source.

Function classification of the plasma integral proteins from xylem and phloem

Being major functional players on the plasma membrane, integral proteins play important roles in signaling, cell inward/outward transportation and specific cell wall formation during cell differentiation. In the present study, the identified integral proteins were analyzed for their possible functions by sequence homology comparisons against the Arabidopsis Information Resource (TAIR) database. According to the annotation of their homolog genes in Arabidopsis and the results of structure domain analysis, a majority (51%) of the integral proteins identified in the study had functions related to signaling (55 proteins), transportation (34 proteins), cell wall formation (27 proteins), or intracellular trafficking (17 proteins). The rest of

Table 1 The transmembrane proteins identified from the plasma membrane of poplar differentiating xylem and phloem

Protein ID	*Populus* gene model	TMN	NPDX	NPDP	Mw (Da)	Arabidopsis Homolog	TAIR description
Signal transduction (ST)							
ST157	gw1.XI.248.1	1	3	0	92433	At4g21380	Lectin-receptor-like protein kinase
ST245	eugene3.00191036	1	3	4	118701	At4g03230	Lectin-receptor-like protein kinase
ST384	gw1.XI.174.1	1	4	2	92581	At4g21380	Lectin-receptor-like protein kinase
ST151	eugene3.00131031	1	1	0	103786	At3g56370	LRR receptor-like protein kinase
ST191	gw1.29.518.1	1	3	5	72467	At4g22130	LRR receptor-like protein kinase
ST247	eugene3.00071196	1	2	3	121959	At4g36180	LRR receptor-like protein kinase
ST330	gw1.I.6094.1	1	5	4	112782	At4g20940	LRR receptor-like protein kinase
ST358	eugene3.00002215	1	12	13	103458	At1g66150	LRR receptor-like protein kinase
ST359	eugene3.00040713	1	10	13	103230	At1g66150	LRR receptor-like protein kinase
ST360	fgenesh4_pg.C_LG_VI000556	1	23	33	100181	At3g23750	LRR receptor-like protein kinase
ST363	estExt_fgenesh4_pg.C_LG_XVIII1177	1	25	32	97108	At3g23750	LRR receptor-like protein kinase
ST371	eugene3.00160451	2	10	15	96327	At5g06940	LRR receptor-like protein kinase
ST372	eugene3.01520008	2	12	20	95757	At5g06940	LRR receptor-like protein kinase
ST374	eugene3.00100968	1	12	13	100989	At1g66150	LRR receptor-like protein kinase
ST375	eugene3.00130345	1	4	4	100274	At3g23750	LRR receptor-like protein kinase
ST386	fgenesh4_pm.C_LG_XVIII000357	1	4	12	94301	At2g24230	LRR receptor-like protein kinase
ST391	fgenesh4_pg.C_LG_X001647	2	3	8	88905	At3g51740	LRR receptor-like protein kinase
ST656	eugene3.00190594	1	0	3	103453	At3g56370	LRR receptor-like protein kinase
ST163*	eugene3.00060911	1	4	7	84818	At3g51740	LRR receptor-like protein kinase
ST343*	fgenesh4_pm.C_LG_IV000169	1	12	13	108851	At1g28440	LRR receptor-like protein kinase
ST373*	eugene3.00060471	1	4	10	96585	At2g41820	LRR receptor-like protein kinase
ST385*	estExt_Genewise1_v1.C_LG_VII0023	1	6	6	94198	At5g65700	LRR receptor-like protein kinase
ST390*	eugene3.00161196	2	7	7	89070	At3g51740	LRR receptor-like protein kinase
ST263*	grail3.0010068301	1	3	2	123520	At2g01950	LRR receptor-like protein kinase, BRL2
ST8	estExt_Genewise1_v1.C_290164	1	6	0	81436	At1g14390	Receptor-like protein kinase
ST38	fgenesh4_pm.C_LG_XIII000012	2	9	0	58921	At1g56720	Receptor-like protein kinase
ST39	fgenesh4_pg.C_scaffold_3857000001	1	6	0	20706	At1g07650	Receptor-like protein kinase
ST173	gw1.IV.3076.1	2	3	3	80902	At4g04960	Receptor-like protein kinase
ST223	eugene3.00100444	1	2	3	58001	At1g67510	Receptor-like protein kinase
ST367	eugene3.00160778	1	25	23	101857	At2g37050	Receptor-like protein kinase
ST394	grail3.0022032801	1	7	16	91591	At3g46290	Receptor-like protein kinase
ST395	grail3.0076008101	1	15	15	90959	At3g51550	Receptor-like protein kinase
ST416	fgenesh4_pg.C_LG_XVI000918	1	0	1	75825	At3g55550	Receptor-like protein kinase
ST426	gw1.XI.3269.1	1	0	6	71870	At4g18640	Receptor-like protein kinase
ST165*	gw1.134.227.1	2	7	11	84850	At5g54380	Receptor-like protein kinase
ST174*	gw1.XIII.3434.1	1	2	2	83247	At4g03390	Receptor-like protein kinase
ST198*	eugene3.00131289	2	4	4	69403	At5g58300	Receptor-like protein kinase
ST206*	estExt_fgenesh4_pg.C_LG_XV0398	2	2	4	67406	At1g48480	Receptor-like protein kinase
ST207*	eugene3.00002256	1	3	5	67607	At1g48480	Receptor-like protein kinase
ST208*	fgenesh4_pg.C_LG_IV000713	1	15	14	67735	At1g48480	Receptor-like protein kinase
ST346*	gw1.XVI.567.1	1	11	24	107565	At1g79620	Receptor-like protein kinase
ST351*	gw1.28.1090.1	1	7	10	104012	At1g79620	Receptor-like protein kinase
ST366*	eugene3.00060962	2	19	20	97278	At3g51550	Receptor-like protein kinase
ST387*	gw1.I.6134.1	2	5	9	93233	At1g30570	Receptor-like protein kinase
ST388*	eugene3.00110972	2	7	10	92737	At5g54380	Receptor-like protein kinase
ST392*	grail3.0001120501	2	10	13	90916	At3g46290	Receptor-like protein kinase

Table 1 continued

Protein ID	*Populus* gene model	TMN	NPDX	NPDP	Mw (Da)	Arabidopsis Homolog	TAIR description
ST393*	gw1.I.1449.1	1	8	12	91099	At3g46290	Receptor-like protein kinase
ST665*	gw1.86.291.1	1	0	5	101997	At5g49760	Receptor-like protein kinase
ST651	gw1.VIII.2924.1	2	0	3	111360	At2g01830	Receptor histidine kinase, CRE1
ST221	fgenesh4_pg.C_scaffold_21924000001	1	8	6	19070	No hit	Hybrid histidine kinase
ST7	estExt_Genewise1_v1.C_LG_XVIII0587	1	2	0	84654	At3g43220	Phosphoinositide phosphatase
ST75	eugene3.00150591	2	9	0	39135	At5g63050	Emb2759, embryo defective 2759
ST179	gw1.41.218.1	1	9	8	79168	At1g34550	Emb2756, embryo defective 2756
ST284*	gw1.II.2836.1	5	6	5	43018	At3g25290	Auxin-responsive protein
ST356	fgenesh4_pm.C_LG_IX000015	3	2	2	99384	At4g35290	Glutamate receptor
Transporter (TR)							
TR531	gw1.I.4875.1	6	0	4	11815	At3g13220	ABC transporter
TR578*	estExt_Genewise1_v1.C_LG_II3719	10	0	19	132305	At2g47000	ABC transporter
TR5	gw1.44.184.1	10	11	0	84023	At3g21250	ABC transporter
TR119	eugene3.00061718	7	1	0	26131	At2g25810	Aquaporin
TR271	estExt_Genewise1_v1.C_LG_XVI2799	6	2	2	30400	At2g37170	Aquaporin
TR283	estExt_fgenesh4_pg.C_LG_IX1411	6	2	3	30433	At2g37170	Aquaporin
TR565	eugene3.00011331	7	0	1	24955	At3g16240	Aquaporin
TR256*	eugene3.00280238	6	3	3	26043	At2g36830	Aquaporin
TR281*	grail3.0045020302	6	2	2	29585	At4g35100	Aquaporin
TR282*	estExt_Genewise1_v1.C_LG_III0271	6	4	5	30753	At4g00430	Aquaporin
TR296*	grail3.0049030302	6	3	3	30949	At4g00430	Aquaporin
TR303*	eugene3.00102165	6	8	9	30303	At3g54820	Aquaporin
TR543*	estExt_fgenesh4_pm.C_LG_XVI0408	6	0	1	30621	At4g00430	Aquaporin
TR389	gw1.VI.1514.1	6	1	1	89766	At4g30110	Cadmium-transporting ATPase
TR211*	estExt_Genewise1_v1.C_LG_I4955	11	2	2	64816	At1g53210	Calcium-binding EF hand protein
TR143	gw1.148.178.1	9	11	0	106054	At1g07670	Calcium-transporting ATPase
TR314	fgenesh4_pg.C_LG_IX001309	8	16	8	116598	At1g07670	Calcium-transporting ATPase
TR600	gw1.135.25.1	8	0	12	124965	At5g23630	Cation-transporting ATPase
TR145	fgenesh4_pg.C_LG_III000552	8	3	0	107764	At5g44790	Copper-transporting ATPase
TR357	eugene3.00010321	8	6	7	105221	At1g63440	Copper-transporting ATPase, HMA5
TR299*	estExt_fgenesh4_pg.C_LG_II0267	10	2	2	41736	At1g75500	MtN21-like protein
TR300*	estExt_fgenesh4_pg.C_LG_V1470	10	4	5	41898	At1g75500	MtN21-like protein
TR33	gw1.158.66.1	8	2	0	61016	At1g72480	Multiple transmembrane protein
TR336	eugene3.00011076	5	8	10	106164	At1g52780	Multiple transmembrane protein
TR225	gw1.XVI.376.1	8	1	1	57747	At1g61670	Multiple transmembrane protein
TR265	gw1.XV.1560.1	7	1	1	45776	At5g33320	Phosphate translocator
TR495	fgenesh4_pg.C_LG_I000711	8	0	15	142941	At1g17500	Phospholipid-transporting ATPase
TR349*	gw1.XII.988.1	8	24	19	104609	At5g62670	Plasma membrane H^+-ATPase
TR361*	gw1.XV.1202.1	8	18	15	104511	At5g62670	Plasma membrane H^+-ATPase
TR182*	gw1.66.623.1	11	2	2	79166	At4g35300	Sugar transporter
TR60*	fgenesh4_pg.C_LG_II002606	12	1	0	52470	At1g75220	Sugar transporter
TR379*	estExt_fgenesh4_pg.C_LG_IX0438	6	14	12	93123	At2g21410	VHA-A2
TR158	fgenesh4_pg.C_LG_II000263	6	2	0	91988	At2g21410	VHA-A2
TR380*	estExt_fgenesh4_pm.C_LG_IV0476	6	12	10	92736	At4g39080	VHA-A2
Cell wall formation and carbohydrate metabolism (CW)							
CW662	estExt_fgenesh4_pg.C_LG_X0451	1	0	2	96660	At1g67490	Alpha-glucosidase I
CW258	eugene3.00011928	1	3	5	120061	At5g14950	Alpha-mannosidase

Table 1 continued

Protein ID	*Populus* gene model	TMN	NPDX	NPDP	Mw (Da)	Arabidopsis Homolog	TAIR description
CW304	estExt_fgenesh4_pm.C_LG_III0447	1	5	7	38374	At5g53340	Beta-1,3-galactosyltransferase
CW232*	estExt_fgenesh4_pg.C_13980001	8	5	6	121143	At4g32410	CesA1-A
CW270	eugene3.00060479	8	3	4	118944	At5g05170	CesA3-A
CW234	estExt_fgenesh4_pg.C_LG_IX0979	8	4	2	120652	At5g05170	CesA3-C
CW302	eugene3.00160483	8	7	13	119929	At5g05170	CesA3-D
CW308*	eugene3.00002636	8	10	12	118579	At5g44030	CesA4
CW278	fgenesh4_pm.C_LG_XIII000084	8	14	8	122381	At5g64740	CesA6-E
CW310*	gw1.XVIII.3152.1	8	10	22	116943	At5g17420	CesA7-A
CW289*	estExt_Genewise1_v1.C_LG_VI2188	8	12	25	116336	At5g17420	CesA7-B
CW341*	gw1.XI.3218.1	8	14	16	110349	At4g18780	CesA8-A
CW321	eugene3.00040363	8	19	18	114553	At4g18780	CesA8-B
CW18	estExt_fgenesh4_pg.C_LG_X0013	1	1	0	70938	At4g16120	Cobl (Cobra-like protein)
CW229*	estExt_fgenesh4_pm.C_LG_V0631	1	2	5	57112	At1g19940	Endo-beta-1,4-glucanase family protein
CW196*	grail3.0263001401	1	5	9	68495	At5g49720	Endo-beta-1,4-glucanase, KOR homolog
CW220	eugene3.00071182	1	13	17	58428	At4g36220	Ferulate-5-hydroxylase
CW216	estExt_fgenesh4_pg.C_LG_III0527	1	13	11	59876	At1g17270	GDP-fucose protein-o-fucosyltransferase
CW644	gw1.II.3117.1	1	0	2	113954	At4g01210	Glycosyltransferase family protein
CW253	gw1.VII.2855.1	2	5	7	48551	At4g36890	GT family 43 protein, IRX14
CW231	estExt_Genewise1_v1.C_LG_V4069	1	5	7	56558	At5g67230	GT family 43 protein, IRX14 homolog
CW461	eugene3.00130489	5	0	2	59094	At5g18480	GT family 8 protein
CW114	eugene3.00190332	8	2	0	128266	At3g03050	PtCslD6
CW65	eugene3.00880019	1	7	0	50504	At3g23820	UDP-glucuronate 4-epimerase
CW250*	estExt_Genewise1_v1.C_LG_XVI2527	1	12	9	48929	At3g62830	UDP-glucuronic acid decarboxylase
CW246*	eugene3.00140737	1	9	9	49744	At3g62830	UDP-glucuronic acid decarboxylase
CW520*	fgenesh4_pm.C_LG_II000873	1	0	1	48435	At2g47650	UDP-glucuronic acid decarboxylase
Intracellular trafficking (IT)							
IT144*	gw1.I.9637.1	1	1	0	107845	At1g52360	Coatomer beta subunit
IT261	grail3.0020010802	1	20	18	33638	At2g19950	Golgin-84
IT181	estExt_Genewise1_v1.C_440243	1	2	4	79746	At5g45160	GTP-binding protein
IT396	gw1.XV.2621.1	1	2	2	90530	At5g45160	GTP-binding protein
IT410	gw1.246.2.1	1	0	11	79912	At5g45160	GTP-binding protein
IT592*	Eugene3.00060912	3	0	3	23984	At2g38360	Prenylated rab acceptor
IT601	Fgenesh4_pm.C_LG_XIX000312	4	0	1	22640	At2g38360	Prenylated rab acceptor
IT17	estExt_fgenesh4_pm.C_700083	1	5	0	71860	At5g27540	Rac-GTP binding protein
IT288	Eugene3.00140364	3	2	2	28100	At2g46170	Reticulon family protein
IT574	gw1.122.33.1	3	0	8	29014	At4g23630	Reticulon family protein
IT617*	estExt_fgenesh4_pm.C_LG_X0012	1	0	7	28624	At3g17440	SNARE, protein transporter
IT291	gw1.X.834.1	1	12	18	41243	At2g03510	Synaptobrevin-related protein
IT106*	estExt_fgenesh4_pg.C_LG_XV0909	1	2	0	24596	At1g04760	Synaptobrevin-related protein
IT315	estExt_fgenesh4_pm.C_LG_XIX0109	1	7	9	34275	At3g03800	Syntaxin 131
IT628	estExt_fgenesh4_pg.C_290237	1	0	5	30179	At4g17730	Syntaxin 23
IT629*	Eugene3.00280153	1	0	15	30759	At3g09740	Syntaxin 71
IT631*	estExt_fgenesh4_pg.C_LG_XVI0751	1	0	15	29693	At3g09740	Syntaxin 71
Function unknown (FU)							
FU161	estExt_Genewise1_v1.C_LG_XVI1567	3	2	2	85234	At3g57880	C_2 domain-containing protein

Table 1 continued

Protein ID	*Populus* gene model	TMN	NPDX	NPDP	Mw (Da)	Arabidopsis Homolog	TAIR description
FU498	gw1.VIII.297.1	3	0	2	51259	At4g14240	CBS domain-containing protein
FU50*	gw1.X.4147.1	3	3	0	54367	At4g14240	CBS domain-containing protein
FU215	fgenesh4_pg.C_scaffold_70000199	1	6	7	18462	No hit	Duf1068
FU559	gw1.X.1601.1	1	0	2	43883	At2g40320	Duf213
FU239	estExt_fgenesh4_pg.C_LG_X1673	1	6	4	53094	At3g55990	Duf231
FU448	estExt_fgenesh4_pg.C_LG_XVI0458	1	0	2	64313	At5g06700	Duf231
FU517	estExt_Genewise1_v1.C_LG_XI3238	1	0	11	47601	At1g29200	Duf246
FU170	estExt_fgenesh4_pg.C_LG_II2588	1	1	2	83319	At1g19430	Duf248
FU184	eugene3.00080530	1	5	7	75654	At2g39750	Duf248
FU193	estExt_fgenesh4_pg.C_290162	1	13	15	68792	At4g18030	Duf248
FU194	estExt_fgenesh4_pg.C_290313	1	1	3	68364	At1g31850	Duf248
FU429	gw1.I.2672.1	1	0	2	69704	At5g14430	Duf248
FU434	estExt_fgenesh4_pg.C_LG_VIII1310	1	0	5	69331	At1g26850	Duf248
FU435	estExt_fgenesh4_pg.C_LG_X0857	1	0	4	69638	At1g26850	Duf248
FU442	fgenesh4_pg.C_LG_XIV000203	1	0	2	67005	At4g00740	Duf248
FU160*	fgenesh4_pg.C_LG_V000057	1	5	7	87575	At5g64030	Duf248
FU195*	estExt_fgenesh4_pg.C_LG_V1395	1	4	11	69308	At1g04430	Duf248
FU381*	estExt_Genewise1_v1.C_LG_VII1503	1	7	8	92340	At5g64030	Duf248
FU162	estExt_fgenesh4_pg.C_LG_VI0421	1	2	6	87693	At2g41770	Duf288
FU47	eugene3.00080638	1	5	0	55079	At3g55990	Duf321
FU79	eugene3.00050506	1	1	0	35496	At5g67210	Duf579
FU85	gw1.XIX.1870.1	1	1	0	33668	At1g33800	Duf579
FU103	gw1.41.566.1	1	2	0	30634	At1g33800	Duf579
FU120	gw1.86.114.1	1	2	0	32443	At1g09610	Duf579
FU297	gw1.VII.2881.1	1	5	6	36120	At5g67210	Duf579
FU449	eugene3.01240052	1	0	1	66720	At1g28240	Duf616
FU273	estExt_fgenesh4_pm.C_LG_IX0699	1	1	1	43885	At2g28310	Duf707
FU185	eugene3.00570132	1	2	3	77002	At3g51050	Fg-gap repeat-containing protein
FU605	eugene3.01200101	1	0	16	131690	No hit	Tir-nbs-tir type disease resistance protein
FU430	fgenesh4_pg.C_LG_IX000173	1	0	12	64478	At5g21990	Trp-containing protein
FU340	fgenesh4_pm.C_LG_VI000737	2	4	5	109593	At5g11560	Unknown protein
FU9	eugene3.00400141	2	1	0	83569	At3g60380	Unknown protein
FU13	eugene3.00081921	1	3	0	75211	At3g06150	Unknown protein
FU30	estExt_Genewise1_v1.C_LG_IV3066	1	2	0	66269	At1g28240	Unknown protein
FU66	fgenesh4_pm.C_LG_I000553	1	1	0	50584	At3g16200	Unknown protein
FU76	eugene3.00130055	3	1	0	20866	At1g09330	Unknown protein
FU90	eugene3.00180117	1	1	0	44673	At5g11730	Unknown protein
FU166	eugene3.00180314	1	5	6	85916	At5g11560	Unknown protein
FU167	eugene3.01340010	1	1	1	87295	At4g27290	Unknown protein
FU187	gw1.XIX.2110.1	1	6	5	79107	At1g34550	Unknown protein
FU214	eugene3.00141171	1	2	3	65644	At2g04280	Unknown protein
FU252	estExt_fgenesh4_pm.C_LG_X0570	1	2	3	48530	At4g16170	Unknown protein
FU267	eugene3.00160295	1	3	3	36620	At3g56750	Unknown protein
FU269	estExt_Genewise1_v1.C_1400180	4	2	2	23818	At5g56020	Unknown protein
FU293	estExt_fgenesh4_pg.C_LG_IX0791	1	1	1	28677	At3g49720	Unknown protein
FU335	eugene3.01240046	3	2	6	107178	At4g21700	Unknown protein

Table 1 continued

Protein ID	*Populus* gene model	TMN	NPDX	NPDP	Mw (Da)	Arabidopsis Homolog	TAIR description
FU397	gw1.I.2846.1	1	4	3	90914	At3g01720	Unknown protein
FU419	eugene3.00081920	1	0	3	75132	At3g06150	Unknown protein
FU476	fgenesh4_pg.C_scaffold_192000004	1	0	8	54884	No hit	Unknown protein
FU481	gw1.28.115.1	1	0	11	53253	At5g20680	Unknown protein
FU513	estExt_Genewise1_v1.C_LG_X3098	2	0	1	50210	At1g16860	Unknown protein
FU537	estExt_Genewise1_v1.C_LG_XVIII0915	1	0	5	30200	At5g11890	Unknown protein
FU597	eugene3.00060357	1	0	2	28946	At3g56750	Unknown protein
FU669	fgenesh4_pg.C_LG_XVIII001074	1	0	5	15019	No hit	Unknown protein
FU292*	estExt_fgenesh4_pm.C_LG_II1168	4	2	3	31667	At2g20230	Unknown protein
FU62*	eugene3.00080044	2	2	0	51817	At1g16860	Unknown protein
FU307	grail3.0010063802	5	1	1	39805	At1g68070	Zinc finger family protein
FU569	eugene3.00141007	2	0	7	42604	At5g41060	Zinc finger family protein
Unclassified (UC)							
UC333*	eugene3.00170157	1	4	7	109369	At2g32730	26 s proteasome regulatory subunit
UC459	estExt_fgenesh4_pg.C_LG_II2102	10	0	2	59079	At1g05820	Aspartic-type endopeptidase
UC12	fgenesh4_pg.C_scaffold_28000065	7	15	0	197526	At2g03140	CAAX amino terminal protease
UC92	gw1.XVI.2464.1	1	22	0	116765	At2g36200	Kinesin motor protein-related
UC240*	eugene3.00102346	2	2	2	51843	At1g16860	Merozoite surface protein-related
UC464	grail3.0003069401	1	0	14	56153	At1g77510	Protein disulfide isomerase
UC199	estExt_fgenesh4_pm.C_LG_II0175	1	6	9	69918	At1g04430	SAM-dependent methyltransferase
UC243	fgenesh4_pg.C_LG_I000592	1	10	11	55513	At1g11680	Sterol 14-demethylase
UC557	estExt_fgenesh4_pg.C_LG_II0150	1	0	8	40593	At1g20330	Sterol C-24 methyltransferase
UC213	fgenesh4_pg.C_LG_VIII000736	1	16	22	65583	At3g19820	Sterol C-24 reductase
UC280*	estExt_Genewise1_v1.C_290374	4	1	1	31173	At1g32400	TOM2A
Possible contaminants (PC)							
PC210*	estExt_fgenesh4_pg.C_LG_X1518	1	16	16	65824	At3g19820	24-dehydrocholesterol reductase
PC620	gw1.2627.7.1	1	0	7	21843	No hit	3-octaprenyl-4-hydroxybenzoate carboxy-lyase
PC108*	gw1.64.623.1	3	17	0	40899	At4g28390	ADP/ATP antiporter
PC279*	estExt_fgenesh4_pg.C_LG_I1918	3	23	24	42074	At5g13490	ADP/ATP antiporter
PC295*	gw1.IX.3274.1	3	4	5	40779	At5g13490	ADP/ATP antiporter
PC242	fgenesh4_pm.C_LG_XIX000083	1	1	1	51119	At5g18280	Apyrase
PC248*	estExt_fgenesh4_pg.C_LG_VII1013	1	5	5	48905	At5g66680	Dolichyl-diphosphooligosaccharide-protein glycotransferase
PC51	gw1.III.1232.1	9	3	0	54282	At1g10950	Endomembrane protein 70, putative
PC202	grail3.0038018602	10	15	4	67928	At2g01970	Endomembrane protein 70, putative
PC443	eugene3.00440214	10	0	3	68212	At5g37310	Endomembrane protein 70, putative
PC583	estExt_Genewise1_v1.C_27420001	10	0	3	41321	At5g37310	Endomembrane protein 70, putative
PC188*	eugene3.00061953	10	3	2	73326	At5g10840	Endomembrane protein 70, putative
PC424*	estExt_fgenesh4_pm.C_LG_I0291	1	0	3	73510	At5g42020	ER luminal-binding protein
PC501	fgenesh4_pg.C_scaffold_896000002	1	0	10	50806	No hit	Integrase protein
PC275	estExt_fgenesh4_pm.C_LG_XV0031	1	8	5	43274	At4g27680	Msp1 protein, putative
PC46	eugene3.00031308	1	11	0	55427	At1g11680	Probable obtusifoliol 1,4-alpha-demethylase
PC40	fgenesh4_pg.C_LG_II001639	1	4	0	58769	At1g01120	Putative beta-ketoacyl-CoA synthase
PC180	gw1.XVIII.267.1	1	9	5	80153	At1g15690	Vacuolar H^+-pyrophosphatase
PC183*	estExt_fgenesh4_pg.C_1520062	13	7	4	80345	At1g15690	Vacuolar H^+-pyrophosphatase

Table 1 continued

Protein ID	*Populus* gene model	TMN	NPDX	NPDP	Mw (Da)	Arabidopsis Homolog	TAIR description
PC432	fgenesh4_pm.C_LG_I000245	2	0	2	71461	At1g30900	Vacuolar sorting receptor
PC433	fgenesh4_pg.C_LG_VI000894	2	0	2	69479	At3g52850	Vacuolar sorting receptor
PC549	estExt_fgenesh4_pm.C_LG_III0520	1	0	6	12202	At1g30900	Vacuolar sorting receptor
PC23	estExt_Genewise1_v1.C_LG_I4100	2	5	0	69567	At2g14740	Vacuolar-sorting receptor

TMN: transmembrane domain number, NPDX: number of peptides detected in xylem, NPDP: number of peptides detected in phloem. Note: * indicates that the protein was also detected in Nilsson's study

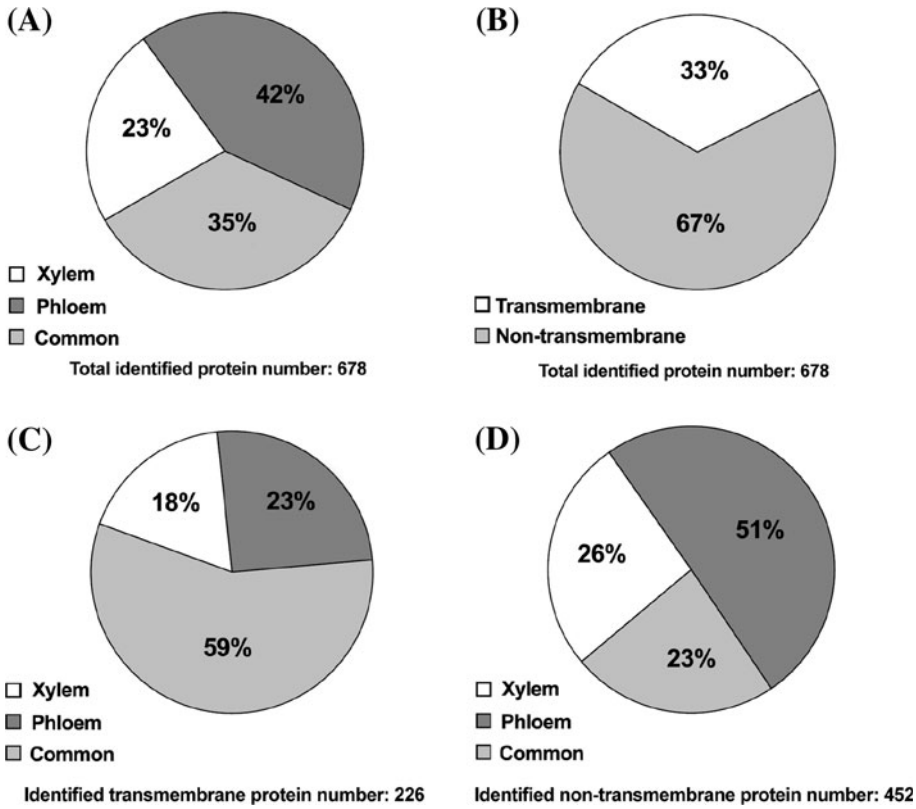

Fig. 4 Distribution of the identified plasma membrane proteins from developing xylem and phloem tissue. (**a**) Total number of the identified plasma membrane proteins in the insoluble portion, (**b**) proportion of the transmembrane and non-transmembrane proteins, (**c**) distribution of transmembrane proteins and (**d**) distribution of non-transmembrane proteins

the proteins could not be classified (11 proteins) due to either unknown function (59 proteins) or possible containments (23 proteins) (Table 1; Fig. 5a, b). Many overlapping proteins were identified in xylem and phloem tissues (Fig. 5b) which may reflect a set of similar biological processes such as intensive cell wall biosynthesis that occur over the course of the differentiation of both sets of tissues. At the same time, a number of proteins were also identified as being specifically related to either xylem or phloem formation, suggesting that these proteins may be involved in the biological processes underlying tissue-specific differentiation.

Proteins with potential functions related to signal transduction formed the largest group (55 proteins) of integral proteins identified in the plasma membrane of differentiating xylem and phloem (Table 1). Among the detected receptors, the function of most of them is yet to be investigated and only a few have been characterized for their roles in mediating signal pathways. ST263, a homolog of BRL2, was a receptor-like kinase protein detected in xylem. In Arabidopsis, BRL2 affects provascular cells differentiation and serves as an integrator of brassinosteroids (BRs) and Auxin signals with the help of its interacting proteins VIT [VH1-interacting tetratricopeptide repeat (TPR)-containing protein] and VIK (VH1-interacting kinase) (Ceserani et al. 2009; Cano-Delgado et al. 2004; Clay and Nelson 2002). ST651, a homolog of CRE1 that is a receptor histidine kinase mediating cytokinin signaling (Nieminen et al. 2008; Mahonen et al. 2000),

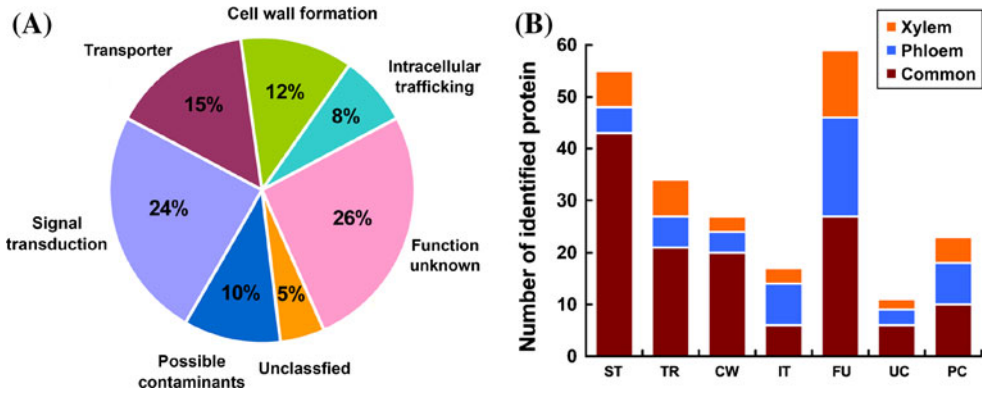

Fig. 5 Function classification of the identified plasma integral proteins from developing xylem and phloem tissue. (**a**) Functional classification of the identified plasma integral proteins, (**b**) distribution of the identified plasma integral proteins from xylem and phloem tissue. *ST* signal transduction, *TR* transporter, *CW* cell wall formation, *IT* intracellular trafficking, *FU* function unknown, *UC* unclassified, *PC* possible contaminants

was detected in cambium. In addition to receptor kinases, auxin-responsive family proteins, glutamate receptor, GTP-binding family proteins and other signaling proteins were also detected. In the other reported study, a total of 24 signaling proteins are identified in the xylem and cambium/phloem (Nilsson et al. 2010), of which, 21 were also identified in our study, indicating a consistency in the detection of the signaling-related proteins between our results.

Transporters are a major class of proteins in the plasma membrane and include a variety of pumps, carriers and channels. In our study, 34 transporter proteins were identified, including aquaporins, ABC transporters, sugar transporters, cadmium-transporting ATPase, copper-transporting ATPase, H^+-ATPase, and other likely transporter proteins. Aquaporins formed the largest group of transporter proteins identified in the plasma membrane. A total of 10 aquaporins proteins (TR119, TR271, TR283, TR565, TR256, TR281, TR282, TR296, TR303, TR543) were identified, of which 7 (TR271, TR283, TR256, TR281, TR282, TR296, TR303) were present in both xylem and phloem, 1 (TR119) in xylem and 2 (TR565, TR543) in phloem. This finding consistently reflects the fact that the vascular system is heavily engaged in water distribution which enables the developing xylem or phloem cells to effectively transport nutrients and photosynthetic products.

Twenty-seven integral proteins related to cell wall formation and carbohydrate metabolisms were identified, including cellulose synthases (CesAs) and other proteins known to be localized on the plasma membrane. In Populus, 18 gene loci encode 17 different CesA proteins. In the present study, a total of 10 CesAs (CW232, CW270, CW234, CW302, CW308, CW278, CW310, CW289, CW341, CW321), corresponding to CesA1-A, CesA3-A, CesA3-C, CesA3-D, CesA4, CesA6-E, CesA7-A, CesA7-B, CesA8-A and CesA8-B were detected in xylem and phloem tissues. Two endo-1,4-β-D-glucanases were detected in both developing xylem and phloem tissues. One of them (CW196) is a homolog of the Arabidopsis KORRIGAN protein which is suggested to have a role in regulating cellulose crystallinity (Nicol et al. 1998; Takahashi et al. 2009). The other endo-1,4-β-D-glucanase (CW229) is a poplar homolog of Arabidopsis AtGH9B5, belonging to Arabidopsis GH9 family(Urbanowicz et al. 2007), indicating this gene may play a role in secondary xylem differentiation in poplar. A COBRA-like protein (CW18) was detected in xylem. The Cobra gene encodes an extracellular glycosyl-phosphatidyl inositol-anchored protein and its mutation results in a disordered deposition of cellulose microfibrils and cellulose synthesis reduction (Roudier et al. 2005; Schindelman et al. 2001).

A number of other proteins involved in intracellular trafficking were also detected, such as SNAREs (IT617), syntaxins (IT315, IT628, IT629, IT631) and prenylated rab acceptors(IT592, IT601). These proteins are involved in membrane trafficking for the recycling of plasma membrane proteins (Chen and Scheller 2001; Sanderfoot et al. 2001; Martincic et al. 1997; Gougeon et al. 2002). During secondary vascular development, cells undergo a rapid process of differentiation from cambium divided cells to specialized wall-thickened cells. Thus intracellular trafficking could become active as proteins on the plasma membrane turnover.

A fairly large group of the detected proteins (55 proteins) have functions which were unknown. Some of these proteins could play a role in various biological events over the course of vascular cell differentiation and cell wall formation. For example, the homolog of the DUF231 proteins (FU239 and FU448) in Arabidopsis was reported recently to be required for cellulose synthesis (Bischoff et al. 2010). The identification of these proteins, which may participate in vascular cell differentiation, presents new targets for further investigations.

A total of 452 soluble proteins (Table S5) were detected in association with plasma membrane. A few of them are known to be associated with the plasma membrane or involved in cell wall formation. Sucrose synthase (SUSY, ID 377, 378), which affects cellulose synthesis, has been investigated for its association with the cellulose synthase complex on the plasma membrane (Haigler et al. 2001; Amor et al. 1995; Fujii et al. 2010). Kinesin proteins (ID 675) and katanin-like proteins (ID 36) may play a role in oriented deposition of cellulose microfibrils and cell wall biosynthesis (Burk and Ye 2002; Zhong et al. 2002). However, the function as well as the association between the soluble proteins and the plasma membrane remains to be determined.

Expression of the receptor kinase genes in secondary vascular cells

The plasma proteins found in poplar secondary vascular tissues include a large group of yet to be characterized receptor-like kinases (Table 1). We are particularly interested in understanding how this group of protein are involved in intercellular communication during xylem differentiation. We analyzed their domain structures and found that the 50 proteins could be classified into 3 lectin-receptor-like kinases (Lectin-RLK), 21 leucine-rich-repeat receptor kinases (LRR-RLK), 22 receptor-like kinases (RLK), and 2 receptor histidine kinases (RHK) (Fig. 6). In order to measure the quantitative expression of these kinases in cells from the xylem, cambium, phloem and cortex, gene-specific primers (Table S6) were designed for real-time RT–PCR analysis of the 50 RLK transcripts. The expression of 46 of the 50 RLKs detected initially was confirmed in cambium meristem and differentiating cells (Fig. 7). These RLK genes displayed distinct cell-specific expression patterns. As showed in Figs. 7, 5 RLK genes were found to be specifically expressed in xylem. 12 RLK genes were expressed in cambium and xylem cells. 14 RLK genes were specifically expressed in cambium cells. 4 RLK genes were predominantly expressed in phloem cells. 11 RLK genes were expressed in cortex cells as well in xylem, phloem, or cambium cells. During differentiation from cambium meristem cells to xylem and phloem, the results showed that the RLKs were differentially expressed at various stages. However, what roles these RLKs play in intercellular communications during vascular differentiation remain yet to be investigated. As our results indicated that the process of vascular differentiation involves expression of a large group of RLK genes, the expressions of some of these RLKs are also reported in transcriptomic profiling of the secondary growth in poplar and the xylem differentiation in Arabidopsis (Dharmawardhana et al. 2010; Schrader et al. 2004; Zhao et al. 2005; Ko et al. 2006). For example, ST385 was found to be expressed in cambium and was also detected in the region adjacent to cambium cells in a high-resolution transcript profile study on poplar (Schrader et al. 2004). ST198 was found to be expressed in cambium and xylem, while the expression of

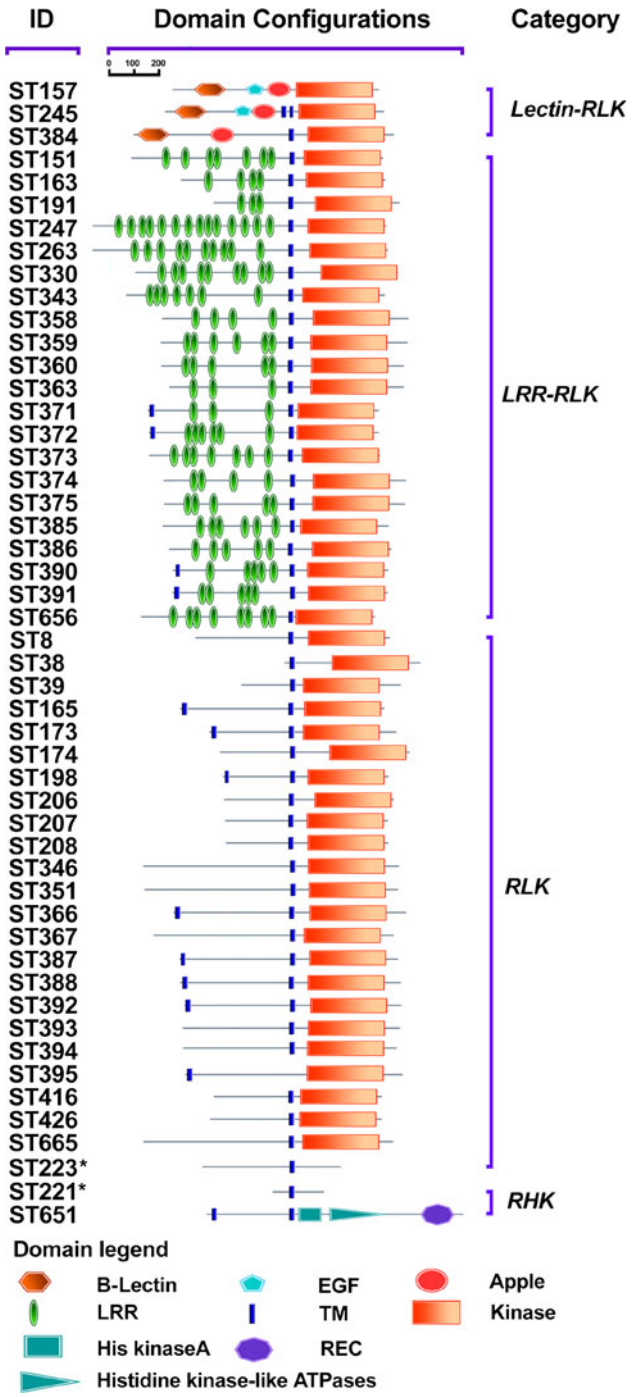

Fig. 6 Schematic structure of the RLKs identified in poplar differentiating vascular tissues. The RLKs were classified according to their domain structures. Protein domain configurations were predicted by the SMART program (http://smart.embl-heidelberg.de). *Star* indicates proteins with only partial sequences available

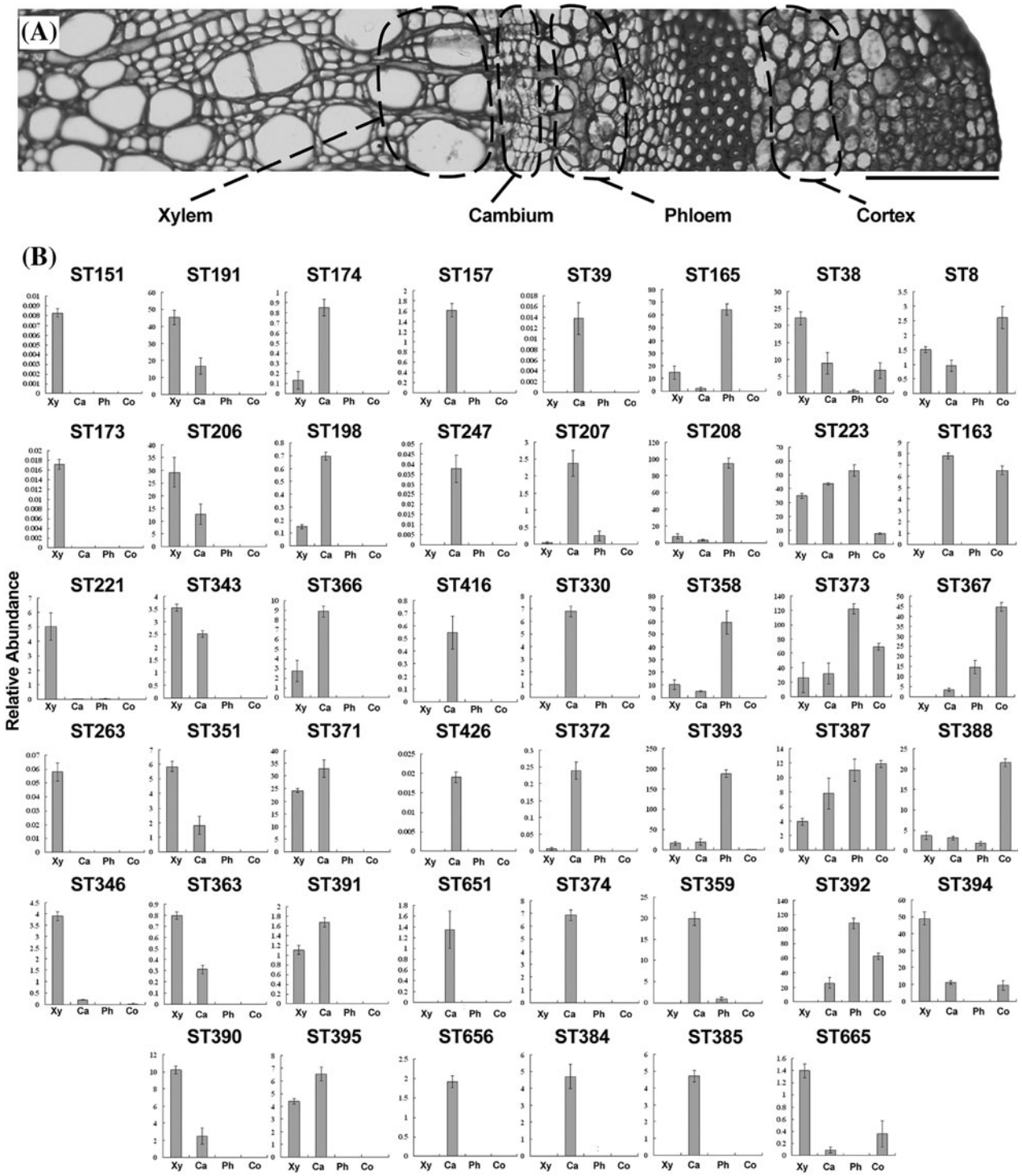

Fig. 7 Expression of receptor-like kinase genes in differentiating vascular cells. Four types of tissue cells: xylem, cambium, phloem and cortex, were collected by laser microdissection. Transcript abundance of the detected RLK genes was measured via quantitative real-time PCR analysis. (**a**) Transverse sections of *Populus* stem at the sixth internode. Samples of the collected cells were *circled* by *broken line*. *Bar* 100 μm. (**b**) Relative transcript abundance of the RLK genes in xylem, cambium, phloem and cortex cells. 5 RLK genes: ST151, ST173, ST221, ST263 and ST346, were specifically expressed in xylem; 12 RLK genes: ST191, ST174, ST206, ST198, ST343, ST366, ST351, ST371, ST363, ST391, ST390 and ST395, were expressed in cambium and xylem cells; 14 RLK genes: ST157, ST39, ST246, ST207, ST416, ST330, ST426, ST372, ST651, ST374, ST359, ST656, ST384 and ST385, were specifically expressed in cambium cells; 4 RLK genes: ST165, ST208, ST358 and ST393, were predominantly expressed in phloem cells; 11 RLK genes: ST38, ST8, ST223, ST163, ST373, ST367, ST387, ST388, ST392, ST394 and ST665, were expressed in cortex cells and other cells. *Xy* xylem, *Ca* Cambium, *Ph* phloem, *Co* cortex

its homolog in Arabidopsis was detected in root cambium and upregulated during stem xylem differention (Zhao et al. 2005; Ko et al. 2006). ST346 and ST351 were found to be expressed mainly in xylem, while the expression of their homolog in Arabidopsis was detected in root secondary xylem and upregulated during stem xylogensis (Zhao et al. 2005; Ko et al. 2006). The expression of ST223 and ST208 which were detected in secondary phloem was found to be upregulated during the transition from primary to secondary stem development in popar (Dharmawardhana et al. 2010). The expression of ST247, ST263, ST360, ST363, ST366, ST375, ST426 was also consistent with that of their homologs in the process of Arabidopsis secondary tissue development (Zhao et al. 2005; Ko et al. 2006).

Discussion

The plasma membrane hosts a large number of proteins that are involved in a variety of cellular processes including cell-to-cell communication, cross membrane transportation, catalysis, intercellular attachment, et cetera. Membrane proteins in plants, particularly those that participate in tissue differentiation, are rarely studied. In this study, we carried out a proteomic profiling of plasma membrane from the differentiating xylem and phloem tissues of poplar. As a result, more than 1,500 proteins were detected in association with the isolated plasma membrane. Among them, a total of 226 proteins were characterized via bioinformatics as integral membrane proteins with functions mainly related to signaling, cross membrane transport, cell wall formation and carbohydrate metabolism, and intracellular trafficking. A group of proteins with unknown functions were also identified which presents potentially new targets for future studies of the plasma membrane. Recently, another study also reported the detection of a total of 956 proteins including 213 integral membrane proteins from the plasma membrane of *Populus* leaf, xylem and phloem (Nilsson et al. 2010). The results of the two studies showed a considerable degree of consistency in their classification of protein function and offered two independent categorizations of the integral proteins found in differentiating vascular tissues. Assessment of the two sets of independently obtained data will provide valuable information towards a better understanding of the mechanisms underlying the secondary growth process in plants, an important biological process which remains little understood.

Increases in the diameter of plant stems, a main consequence of secondary growth, depend on the activities of the secondary vascular cambium and involves a sequence of biological events including vascular cambium cell division, orientated cell differentiation, specialized cell wall thickening and programmed cell death.

A major process in cell wall thickening is cellulose synthesis, which is believed to be mediated by CesA function. CesAs in Arabidopsis are known to be divided into two types according to their involvement in primary or secondary wall formation (Desprez et al. 2007; Persson et al. 2007; Taylor et al. 2003). In poplar, recent studies have suggested that both types of CesAs are simultaneously involved in secondary wall formation (Suzuki et al. 2006; Song et al. 2010). Here, a total of 10 CesA proteins are detected in developing xylem tissue, providing additional evidence to suggest that both types of CesAs participate in secondary wall formation during poplar vascular differentiation. Detected proteins also included KORRIGAN and COBRA, which are believe to play a role in cell wall formation. Mutation in korrigan leads to a significant reduction in cellulose content and crystallinity (Nicol et al. 1998; Szyjanowicz et al. 2004; Maloney and Mansfield 2010). Overexpression of *kor1* and its poplar homolog leads to decreased cellulose crystallinity in Arabidopsis stem (Takahashi et al. 2009). COBRA may play a role in regulating microfibril orientation and deposition (Roudier et al. 2005; Schindelman et al. 2001). Though the mechanisms of how these proteins regulate the cell wall formation are still not fully understood, our results here again confirmed that KOR1 and COBRA are localized in the plasma membrane.

Cell-to-cell communication plays a crucial role in determining cell fate and differentiation, especially for immobile plant cells. In the present study, a group of RLKs were identified in the differentiating xylem and phloem, suggesting their involvement in secondary vascular cambium differentiation. Generally, ligand-RLK signaling is believed as a crucial pathway regulating cell differentiation in plants (De Smet et al. 2009). Thus, the identification of a group of RLKs which may play a role in secondary vascular differentiation is of particular interest. It is know that the Arabidopsis genome contains more than 600 RLK genes (Shiu and Bleecker 2001). In our study, 50 RLK proteins were detected specifically in the plasma membrane of secondary differentiating tissues of poplar. Among them, 46 genes are further confirmed to be expressed in the cambium which differentiates into xylem and phloem cells. However, only 2 of these RLK genes, ST263 and ST651, have been studied for their function in Arabidopsis. ST263 is a homolog of VH1/AtBRL2, which is reported to mediate brassinosteroids (BRs) and Auxin signaling and play a role in vascular differentiation in Arabidopsis (Ceserani et al. 2009; Cano-Delgado et al. 2004; Clay and Nelson 2002). During secondary growth, the ST263 gene is found to be expressed in xylem cells, suggesting that brassinosteroids (BRs) and auxin signaling also play a role in secondary vascular differentiation. ST651 is a homolog of CRE1 which is a receptor histidine kinase mediating

cytokinin signaling and is involved in many cellular processes, including cambial development in Arabidopsis, poplar and birch (Nieminen et al. 2008; Mahonen et al. 2000). The ST651 gene was expressed specifically in cambium cells, strongly suggesting that cytokinin signaling plays an important role in cambium cell division during secondary growth.

In addition, homologs of several RLK genes were found to be specifically regulated by xylem differentiation. For example, the ST346 and ST351 genes were found to be expressed in the xylem and cambium of poplar plants used in our study. Their homolog in Arabidopsis *At1g79620* is specifically expressed in xylem and induced by a vessel regulator VND6 (Zhao et al. 2005; Ko et al. 2006; Ohashi-Ito et al. 2010). Also the ST198 gene, which was found to be expressed in xylem and cambium cells in poplar and its Arabidopsis homolog, *At5g58300*, is directly regulated by transcription factor VND6. (Zhao et al. 2005; Ko et al. 2006; Ohashi-Ito et al. 2010). The results here would help in the construction of yet to be characterized signaling networks which play important roles in regulating xylem (vessel cell) differentiation in poplar.

On the other hand, an Arabidopsis LRR receptor kinase, PXY/TDR and its ligand CLE41/TDIF peptide, have been recently reported play a key role in xylem-phloem patterning through controlling procambial cell division in a non-cell-autonomous manner (Hirakawa et al. 2008; Hirakawa et al. 2010; Etchells and Turner 2010). In the *Populus* genome, the gw1.29.276.1 gene model is the closest homolog of PXY (At5g61480) with a sequence homology of 77%. However, neither our study nor the other study (Nilsson et al. 2010) detected a unique peptide which matches the *Populus* PXY homolog. The reason may be the protein identification in the two studies was unable to fully include all possible membrane proteins due to limitation of the proteomic analysis (Garbis et al. 2005). Meanwhile, whether the mechanism of the xylem-phloem patterning regulated by PXY is the same in poplar secondary growth remain to be confirmed.

Different from primary growth that is derived from apical meristems, the process of secondary growth occurs through the activities of the secondary vascular meristem. After the first tree genome was sequenced (Tuskan et al. 2006), experimental attempts to uncover the basic biological networks underlying secondary growth has yielded interesting insights into this biological process (Du and Groover 2010). The tree genome also provided a valuable database to enable an understanding of the secondary growth of trees at a proteomic level. Tissue-specific proteins profiles and even more specifically, the identification of subcellular proteins are among the key information required for the characterization of secondary growth. In this study, the identification of tissue-specific plasma membrane proteins as well as cell types which specifically expressed RLKs may serve as a first step to further dissect how secondary growth could have developed through various biological activities which originated from the plasma membrane.

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