Proteomics of Plasma Membranes from Poplar Trees Reveals Tissue Distribution of Transporters, Receptors, and Proteins in Cell Wall Formation*

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By exploiting the abundant tissues available from Populus trees, 3–4 m high, we have been able to isolate plasma membranes of high purity from leaves, xylem, and cambium/phloem at a time (4 weeks after bud break) when photosynthesis in the leaves and wood formation in the xylem should have reached a steady state. More than 40% of the 956 proteins identified were found in the plasma membranes of all three tissues and may be classified as “housekeeping” proteins, a typical example being P-type H⁺-ATPases. Among the 213 proteins predicted to be integral membrane proteins, transporters constitute the largest class (41%) followed by receptors (14%) and proteins involved in cell wall and carbohydrate metabolism (8%) and membrane trafficking (8%). ATP-binding cassette transporters (all members of subfamilies B, C, and G) and receptor-like kinases (four subfamilies) were two of the largest protein families found, and the members of these two families showed pronounced tissue distribution. Leaf plasma membranes were characterized by a very high proportion of transporters, constituting almost half of the integral proteins. Proteins involved in cell wall synthesis (such as cellulose and sucrose synthases) and membrane trafficking were most abundant in xylem plasma membranes in agreement with the role of the xylem in wood formation. Twenty-five integral proteins and 83 soluble proteins were exclusively found in xylem plasma membranes, which identifies new candidates associated with cell wall synthesis and wood formation. Among the proteins uniquely found in xylem plasma membranes were most of the enzymes involved in lignin biosynthesis, which suggests that they may exist as a complex linked to the plasma membrane. Molecular & Cellular Proteomics 9:368–387, 2010.

As a model for trees, the Populus genome was recently sequenced (1), chosen because of its relatively small size. Thus, it is now possible to perform proteomics on poplar material to obtain information on e.g. tissue and intracellular distribution of proteins in a tree. We have used this possibility to determine the protein composition of plasma membranes obtained from different tissues of young poplar trees. The plasma membrane constitutes the interface between the cell and the surrounding environment, a position that imposes a number of important functions on the plasma membrane. These include transport of compounds into and out of the cell, communication with the cell exterior, defense against invading pathogens, and cell wall synthesis, functions that are fulfilled by transport proteins, receptors, glucan synthases, proteins involved in membrane trafficking, etc. (2–8). The explicit demand put on plant plasma membranes to support development of a surrounding cell wall is of particular interest, especially the formation of secondary cell wall, which has important economical value as a renewable source in paper and biofuel production. During leafing of poplar trees in spring, there is a rapid development not only of leaves but also of the wood-forming tissue next to the bark in the stem. Using MS, we have determined the protein composition of plasma membranes isolated from leaves, xylem, and cambium/phloem. The protein composition of plasma membranes obtained from different tissues should give important information on the biological activities in these tissues and reveal which proteins are highly expressed in a particular tissue and which are more evenly expressed in all tissues and therefore may be regarded as “housekeeping” proteins. The plasma membranes were obtained from young poplar trees, 3–4 m high, harvested in early summer about 4 weeks after bud break. At this stage, leaves were essentially fully expanded, and both leaf photosynthesis and wood formation in the xylem were expected to have reached a steady state (9, 10). Our understanding of cell wall synthesis has mainly been gathered using Arabidopsis as a model system (11) where the transition from primary to secondary cell wall synthesis has been analyzed at the transcript level (12, 13). To study secondary cell wall formation without interference of multiple cell types, tra-
cheary element differentiation in cell culture is a useful tool (14). Utilizing the system for proteomics, however, is problematic as described by Millar et al. (15). In *Populus*, a considerable amount of secondary cell wall is produced, which has made high resolution transcript profiling across the wood-forming zone possible. Thus, the different developmental stages from cambium meristem to phloem or xylem have been resolved at the transcript level (16, 17), but characteristics of different cell types, including vessels, fibers, and ray cells, still need further investigation. These studies relied on frozen tissue and cryosectioning not applicable for membrane isolation. The sample treatment and volumes required for the present proteomics study limited the developmental resolution in stem tissue to xylem and cambium/phloem. Our study has so far resulted in the identification of more than 900 proteins of which more than 20% are predicted to be integral membrane proteins. More than 40% of total proteins were found in the plasma membrane fractions of all three tissues, and about one-third if only integral membrane proteins are considered. Twenty-one percent of the integral proteins were only found in leaf plasma membranes, 12% were unique to xylem plasma membranes, and 11% were only found in plasma membranes isolated from cambium/phloem.

**EXPERIMENTAL PROCEDURES**

**Plant Material—**Young poplar (*Populus tremula* × *Populus tremuloides*, clone S21K884036) trees, 3–4 m high and with a diameter of about 5 cm at the base, were obtained from a field trial at Ekebo, south Sweden (latitude, 55.95°; longitude, 13.12°), belonging to the Forestry Research Institute of Sweden. The ~3-year-old trees were harvested on June 1, 2006, about 4 weeks after bud break, when leaves were essentially fully expanded. The lowest 1–1.5 m of the stem from five trees was divided (by saw) into pieces of about 2 dm, and the bark was ripped off. Using a knife, the cambium/phloem layer on the inside of the bark was scraped off (yield, 31 g) into a beaker containing preparation medium: 0.33 M sucrose, 50 mM MOPS-KOH, pH 7.5, 5 mM EDTA, 0.2% (w/v) casein hydrolysate, 10% (w/v) polyethylene glycol, 0.6% (w/v) polyvinylpolypropyridine, 5 mM ascorbate, 5 mM DTT (polyvinylpolypropyridine, ascorbate, and DTT were added immediately before use). Similarly, the xylem on the outside of the remaining stem pieces was scraped off (yield, 44 g). Finally, 50 g of leaves was taken from branches.

**Plasma Membrane Isolation**—The three plant materials were homogenized, using a knife blender, in 150 ml of preparation medium. Immediately after homogenization, PMSF was added to a final concentration of 0.5 mM together with 1.5 ml of a “protease inhibitor mixture for plant cell and tissue extracts” (Sigma P 9599) containing 4-(2-aminoethyl)benzenesulfon fluoride, bestatin, pepstatin A, E-64, leupeptin, and 1,10-phenantroline in DMSO. The homogenates were filtered through a 200-μm nylon mesh and centrifuged at 10,000 × g for 15 min; the supernatants were saved and centrifuged at 30,000 × g for 55 min. The resulting microsomal pellets were resuspended in 5 ml of xylem and cambium/phloem and 10 (leaves) ml, respectively, of resuspension medium: 0.33 M sucrose, 5 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 1 mM DTT (DTT was added immediately before use). Resuspended membranes (4.50 ml for xylem and cambium/phloem and 9.00 ml for leaves) were added to 13.50 ml of suspension (xylem and cambium/phloem) and 27.00 g (leaves) of phase mixtures to produce two 18.00-g and one 36.00-g aqueous polymer two-phase systems with a final composition of 6.1% (w/w) dextran 500, 6.1% (w/w) polyethylene glycol 3350, 5 mM potassium phosphate, pH 7.8, and 3 mM KCl. Plasma membranes were then purified by aqueous polymer two-phase partitioning as described previously (18). The final upper phases were diluted at least 2-fold with 0.33 M sucrose, 5 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, and plasma membranes were pelleted by centrifugation at 100,000 × g for 1 h. The whole preparation procedure was performed at 4 °C. The plasma membrane pellets were resuspended in 0.3 ml of resuspension medium and stored in liquid nitrogen until used. Similarly, the final lower phases from the phase systems, containing intracellular membranes, as well as the remaining parts of the microsomal fractions were diluted —10-fold, pelleted, and stored as above. Protein concentration was determined according to Bearden (19).

The plasma membrane vesicles, which on isolation were largely cytoplasmic side-in, were turned inside-out by treatment with the detergent Brij 58 (20) and pelleted again. This was done by mixing, at room temperature, stock solutions of 2 M KCl and of 20 mg/ml Brij 58 in 0.33 M sucrose, 5 mM potassium phosphate, pH 7.8 with plasma membranes to give a detergent to protein ratio of 5:1 (w/w) and a KCl concentration of 0.2 M. Plasma membranes were then pelleted at 100,000 × g for 2 h at 4 °C and resuspended in half the original volume of resuspension medium. The membranes recovered from the lower phases and the microsomal fractions were washed similarly but with Brij 58 excluded from the wash medium. All membrane fractions were then subjected to SDS-PAGE.

**SDS-PAGE and Immunoblotting**—Samples were solubilized at room temperature in standard sample buffer, and polypeptides were separated by SDS-PAGE (12% acrylamide, 0.3% bisacrylamide) according to Laemmli (21). Gels were either stained with Coomassie Brilliant Blue R-250, or polypeptides were electrophoretically transferred to an Immobilon PVDF transfer membrane (Millipore) for immunostaining. After blocking in 2% (w/v) BSA in PBS (0.15 M NaCl, 0.01 M potassium phosphate, pH 7.5) overnight, the blots were incubated with one of the following rabbit polyclonal antisera diluted in PBS: 1) anti-Lhc1b (Agrisera, Vännäs, Sweden), an antiserum raised against a peptide corresponding to a sequence of the Lhc1b1 protein (one of three Photosystem II light-harvesting complex (LHClI) isoforms) of *Arabidopsis*; 2) anti-COXII (Agrisera), an antiserum raised against a peptide corresponding to a widely conserved sequence of the mitochondrial cytochrome oxidase subunit II; 3) anti-ArF1 (Agrisera), an antiserum raised against a peptide corresponding to a full-length Arabidopsis protein and therefore probably recognizing all ADP-ribosylation factors; 4) anti-H+-ATPase, an antiserum raised against a polypeptide corresponding to amino acids 851–949 of the C terminus of the *Arabidopsis* H+-ATPase isoform 2 (AHA2) (a kind gift from Professor R. Serrano (Universidad Politecnica, Valencia, Spain)); 5) anti-sucrose synthase, an antiserum raised against sucrose synthase isoform 2 purified from maize (Zea mays) kernels (22) (a kind gift from Professor C. B. Chourey (University of Florida, Gainesville, FL)); 6) anti-PiP2 aquaporin, an antiserum raised against a peptide corresponding to

The abbreviations used are: LHCCI, Photosystem II light-harvesting complex; DRM, detergent-resistant plasma membrane microdomains; FRAI, fasciclin-like arabinoxylan; GPN, glycosylphosphatidylinositol; GT, glycosyltransferase; LOPIT, localization of organelle proteins by isotope tagging; UPLC, ultra-performance LC; PIP, plasma membrane intrinsic protein; TAR, The Arabidopsis Information Resource; AGI, Arabidopsis Genome Initiative; ABC, ATP-binding cassette; ID, identification number; PPi-ase, inorganic pyrophosphatase; TIP, tonoplast intrinsic protein; RLK, receptor-like kinase; LRR, leucine-rich repeat; PERK, proline extensin-like receptor kinase; SD, S-locus glycoprotein-like domain; SUS, sucrose synthase; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; CES, Celulose synthase.
amino acids 271–281 of the C terminus of the spinach (*Spinacia oleracea*) aquaporin isoform So PIP2-1; this amino acid sequence is conserved in plant aquaporins belonging to the PIP2 subfamily (23), and the antisera thus recognizes all PIP2 isoforms (a kind gift from Professor P. Kjellbom [Lund University, Lund, Sweden]); and 7) anti-calreticulin, a serum raised against maize calreticulin and also recognizing the closely related calnexin (24). The horseradish peroxidase-conjugated secondary antibody was visualized by ECL (GE Healthcare).

**Digestion of Proteins and Recovery of Peptides**—After SDS-PAGE, the three lanes containing plasma membranes were each cut into 34 sections, and samples were also taken from some of the major bands in the intracellular membrane fractions (compare Fig. 1). Bands were further cut into 0.5–1 mm cubes and washed with 100 μl of water in microcentrifuge tubes. Gel pieces were destained by repeated incubation in 100 μl of 35% ACN, 50 mM NH₄HCO₃ for 10 min each. Dehydration was performed twice using 100 μl of ACN for 5 min; in between, 100 μl of 50 mM NH₄HCO₃ was used in a 30-min rehydration. The second dehydration was completed by evaporation for 10 min at 40 °C (Labconco CentriVap concentrator). Finally, 19 μl of ice-cold trypsin (Gold MS grade; Promega) was added per tube (5 ng/μl in 50 mM NH₄HCO₃), and gel pieces were rehydrated on ice for 1 h. Excess trypsin was removed, and 50 mM NH₄HCO₃ was added to cover the gel pieces. Tubes were transferred to a 37 °C incubator for overnight digestion. Extraction of peptides was started by adding 50 μl of 50 mM NH₄HCO₃ to each tube for a 10-min incubation with occasional gentle vortexing. Tubes were centrifuged for 30 s at 21,000 × g, and supernatants were collected in fresh tubes. The extraction step was repeated twice, and the supernatants were pooled. Extracted peptides were evaporated until dry, dissolved in 10 μl of 0.1% (v/v) formic acid, and stored at −20 °C until used.

**Identification of Proteins by Peptide Fragmentation**—Extracted peptides were separated and analyzed by reversed-phase liquid chromatography-MS/MS using a nanoACQUITY UPLC™ system (Waters) and a Q-Tof Ultima™ (Waters). Solvent gradients and columns used with the UPLC system were as described previously (25). To optimize peptide ionization parameters and supervise splitting of concentrated samples, 0.5 μl of each sample was first analyzed in MS mode. The complexity of the samples was assessed by inspecting the resulting data set (e.g. file size), and some samples were then chosen to be analyzed in the mass range m/z 400–1500, and others were split in two (m/z 400–650 and 650–1500) or three (m/z 400–500, 500–650, and 650–1500) ranges using the include list option. Peptide fragmentation data were generated by automated data-dependent acquisition. The three most abundant signals of a survey scan (400–1500 m/z range, 0.8-s scan time, 0.2-s interdelay) were selected by include list; charge state and collision energy were applied accordingly for subsequent MS/MS fragmentation scanning (50–2000 m/z range, 1-s scan time, 0.1-s interdelay). Conversion of raw data to peak lists for the database search was performed with the ProteinLynx Global Server (v2.0.5) software. The following criteria were applied: MS channel: background subtraction (third order, 30% below curve), smoothing (two iterations, Savitzky-Golay, four-channel window), and centering (minimum, six channels; 80% peak height); MS/MS channels: same settings as the MS channel except for background subtraction (55%), centering (minimum four channels), and deisotoping. Two different database searches were performed using a *Populus* protein database (45,555 entries, assembly release v1.1) created from predicted and translated gene models from the *Populus trichocarpa* genomic sequence. Searches were performed individually for each sample with analyzed mass ranges merged using a local version of the Mascot search program (v2.2.04; Matrix Science Ltd.) and the Mascot Daemon application (v2.1.6). The settings used for the database search were: trypsin-specific digestion with one missed cleavage allowed, propionamide cysteine (26) set as fixed modification, acetylated N-terminal and oxidized methionine set as variable modifications, peptide tolerance set to 80 ppm, and fragment tolerance set to 0.06 Da. In a primary database search, only proteins that contained one or more top ranking peptide (“bold red” filtering) was exported from Mascot to csv files using a threshold for statistical significance of <0.05 (referred to as “top rank” proteins). A second search on the top 100 proteins was exported including “same set” and “subset” proteins. Multidimensional protein identification technology scoring was applied in both searches. Files were merged by in-house software and arranged in Excel before removal of overlapping peptides within each sample (tissue) as well as peptides with a score below 25. To limit the number of subset proteins with a single peptide hit in supplemental Table 2, a peptide score above 40 was set as the threshold. A false positive rate of 4.7% (47/[847 + 47]) was estimated by decoy database (randomized *Populus* protein database, 45,555 entries) search. Contamination by keratin was determined by searching a human database downloaded from the International Protein Index (September 25, 2009, 84,032 entries) and linked to *Populus* data. Assessing the result, we found one protein (ID 493) possible to be background contamination.

**Phylogenetic Analysis**—Alignments and the phylogenetic tree based on amino acid sequences were computed by MEGA version 4 (27) and presented by TreeView 1.6.6. The following settings were used: Gonnet matrix series, gap opening penalty of 10, gap extension penalty of 0.2, neighbor-joining method, and 2000 bootstrap replicates.

**RESULTS**

**Plasma Membrane Preparations**—In the preparation procedure used, the membrane vesicles in the microsomal fraction are partitioned between the upper and lower phases of an aqueous polymer two-phase system (28). The composition of the phase system is adjusted such that plasma membranes partition to the upper phase, whereas intracellular membranes partition to the interface and to the lower phase (18). The yield of plasma membrane protein was 1.7, 1.2, and 1.4 mg from leaves, xylem, and cambium/phloem, respectively, which corresponds to 2, 3, and 6% of total microsomal membrane protein, respectively. SDS-PAGE of the membrane fractions (Fig. 1A) showed very similar polypeptide patterns for the microsomal fraction and the intracellular membrane fraction of each tissue, which was expected because very small proportions of the membranes in the microsomal fractions ended up in the upper phases, i.e. in the plasma membrane fractions. Also, the plasma membrane fractions showed similar polypeptide patterns, although they were derived from different tissues. In Fig. 1B, the positions of some of the major proteins in the plasma membrane and intracellular membrane fractions are indicated, assuming that the number of peptides identified by MS reflects abundance (29) (Table I and supplemental Table 3; data not shown for the intracellular membrane fractions). H⁺-ATPase isoforms and aquaporins are well-known major integral proteins of the plasma membrane (30, 31), but several of the major proteins were peripheral proteins involved in membrane trafficking and as components of the cytoskeleton, such as clathrin heavy chain, annexin, tubulin, and actin. Sucrose synthase was found in a major band in the
xylem plasma membranes. The intracellular membrane fractions derived from xylem and cambium/phloem were dominated by mitochondrial membranes, and the ADP/ATP antiporter as well as the α- and β-subunits of the mitochondrial ATP synthase were major polypeptide bands in these fractions. The leaf intracellular membrane fraction was instead dominated by chloroplast membranes, and major bands were due to LHCII protein and the α- and β-subunits of the chloroplast ATP synthase.

Plasma membranes prepared by aqueous two-phase partitioning are usually of high purity (about 95%) and consist mainly of right side-out (cytoplasmic side-in) vesicles (Ref. 4 and references therein). A very low degree of contamination by other membranes was supported also by the present investigation. Immunoblotting (Fig. 1C) showed a strong enrichment of the P-type H⁺-ATPase, a canonical plasma membrane protein, and also of PIP2 aquaporin in the plasma membrane fractions. However, substantial amounts of PIP2 aquaporin were also found in the intracellular membrane fractions, particularly those from xylem and cambium/phloem, in agreement with previous reports on the presence of plasma membrane intrinsic proteins (PIPs) also in intracellular membranes (32). By contrast, LHCII, a major component of chloroplast thylakoid membranes, and cytochrome oxidase (subunit II), a major component of mitochondrial inner membranes, were almost exclusively found in the intracellular membrane fractions. Calreticulin and calnexin, which are usually regarded to be located in the endoplasmic reticulum (33), were both mainly found in the intracellular membrane fractions. Notably, calreticulin has also been located to both Golgi and plasma membranes in tobacco using immunogold labeling (34). Calnexin is probably a more reliable marker for the endoplasmic reticulum because calnexin, unlike calreticulin, is an integral membrane protein. Sucrose synthase, a soluble enzyme that associates with cellulose synthase (35), was used as a marker for cellulose synthesis and was clearly most abundant in the xylem plasma membrane fraction where wood formation occurs. The antiserum raised against the full-length Arabidopsis Arf1 protein will most probably detect all ADP-ribosylation factors because they form a closely related family with 21 members in Arabidopsis (36). The ADP-ribosylation factors are small GTPases that are regulators of complex, and ADP-ribosylation factor. Expected locations for these proteins are indicated to the right. Sucrose synthase is a marker for cellulose/cellulose synthesis in the plasma membrane, and ADP-ribosylation factor is a marker for vesicle transport, i.e. membrane trafficking. The molecular masses given are the calculated masses and do not necessarily reflect the positions in the gel in A and B. For instance, the P-type H⁺-ATPase bands is next to the 97-kDa marker (compare A and B), although its isoforms have molecular masses of about 105 kDa. ant, antiporter; AQP, PIP2, PIP2 subfamily aquaporin; chl, chloroplast; COXII, cytochrome oxidase subunit II; er, endoplasmic reticulum; hc, heavy chain; im, intracellular membranes; mit, mitochondria; pm, plasma membrane; synth, synthase.

**Fig. 1.** Polypeptide patterns of poplar membrane fractions and distribution of marker proteins. A, poplar microsomal fractions obtained from xylem, cambium/phloem (C/P), and leaves, respectively, were subjected to aqueous two-phase partitioning to produce plasma membrane and intracellular membrane fractions. Polypeptides were separated by SDS-PAGE (20 μg of protein/lane) and stained with Coomassie Blue. B, positions in the gel of some of the major proteins identified by mass spectrometry. C, immunoblot using sera directed against the plasma membrane P-type H⁺-ATPase, sucrose synthase, calnexin and calreticulin, PIP2 subfamily aquaporin, cytochrome oxidase subunit II, Photosystem II light-harvesting
### Table 1

Integral membrane proteins detected by mass spectrometry in plasma membranes from leaves, xylem, and cambium/phloem

Integral membrane proteins were identified by Phobius (38), and the number of predicted transmembrane domains (TM) are given in column 10. Proteins are grouped according to function and identified by an ID in column 1, also used in figures and text. Only top rank proteins are included, and the number of peptides identifying each protein is given in columns 7–9 for plasma membranes from leaves (L), xylem (X), and cambium/phloem (C/P), respectively; a number followed by “u” indicates that at least one peptide unique to the protein was found, and a star indicates that the protein is classified as top rank in that tissue. The color code is yellow for proteins found in the plasma membranes from all three tissues and green, red, and blue for proteins found only in leaf, xylem, and cambium/phloem plasma membranes, respectively. All annotation is via the *Arabidopsis* database at TAIR. Thus, amino acid sequences corresponding to identified gene models in the poplar database (column 2) were blasted against the *Arabidopsis* database to identify the closest *Arabidopsis* homolog of each protein, which is identified by its AGI accession number (column 4) followed by its short name in column 5 and description in column 6. Blast p values (column 3) are included to indicate how well the poplar and *Arabidopsis* amino acid sequences agree. The calculated molecular mass in column 11 is from Mascot. *Arabidopsis* genes previously suggested to be involved in cell wall formation are indicated in column 4. Predicted lipid anchors are indicated in column 10. For ABC transporters, the new nomenclature according to Verrier et al. (2) was used, and for RLK receptors, we used the nomenclature in Shiu and Bleecker (5). For a complete list of integral proteins including the subset, see supplemental Table 2. PM, plasma membrane; PEP, phosphoenolpyruvate; MATE, multidrug and toxin extrusion.

#### Transporters

| ID     | *Populus* JGI Gene Model | BLAST P-value | *Arabidopsis* name | TAIR description | peptides | L | X | C/P | TM kD |
|--------|--------------------------|---------------|--------------------|------------------|----------|---|---|-----|-------|
| 001    | eugene3.00180800         | 0             | 2g18960            | AHA1             | *11u 4 4 | 10 | 105 |     |       |
| 002    | estExt* Genesew1_v1.C_LG_XVIII2227 | 0 4g30190 | AHA2              | P-type H^-ATPase | *13u 3 *9u | 10 | 105 |     |       |
| 004    | estExt_fgenes4_pg.C_1470038 | 0 2g24520 | AHA5              | P-type H^-ATPase | *15u 2 *6 | 10 | 105 |     |       |
| 005    | eugene3.00400133         | 0 3g60330 | AHA7              | P-type H^-ATPase | 8 4u *4u | 10 | 106 |     |       |
| 007    | fgenes4_pm.C_LG_V1000025 | 0 3g24640 | AHA8              | P-type H^-ATPase | *8u 3 3u | 10 | 104 |     |       |
| 008    | fgenes4_pg.C_LG_V1001183 | 0 3g24640 | AHA8              | P-type H^-ATPase | *7 2 3 | 10 | 105 |     |       |
| 009    | gw1.l.17475.1            | 0 1g80660 | AHA9              | P-type H^-ATPase | *9 4 4 | 10 | 105 |     |       |
| 010    | eugene3.00031490         | 0 1g80660 | AHA9              | P-type H^-ATPase | *9 4 4 | 10 | 106 |     |       |
| 012    | estExt_fgenes4_pg.C_290018 | 0 1g17260 | AHA10             | P-type H^-ATPase | *13u 0 *6u | 10 | 105 |     |       |
| 014    | gw1.l.XIII.988.1         | 0 5g62670 | AHA11             | P-type H^-ATPase | 20u *6u *11u | 10 | 107 |     |       |
| 015    | gw1.l.XV.1202.1          | 0 5g62670 | AHA11             | P-type H^-ATPase | 20u *5 *9u | 10 | 107 |     |       |
| 018    | estExt_fgenes4_pm.C_LG_XVI10121 | 0 2g41560 | ACA4              | Ca^2+^-ATPase    | *1 0 0 | 8 | 114 |     |       |
| 020    | fgenes4_pg.C_LG_VIII000059 | 0 3g21180 | ACA9              | Ca^2+^-ATPase    | *1 0 0 | 10 | 119 |     |       |
| 022    | gw1.l.XVIII.392.1        | 0 4g29900 | ACA10             | Ca^2+^-ATPase    | 0 *1 *2 | 8 | 116 |     |       |
| 024    | estExt* Genesew1_v1.C_LG_VIII2173 | 0 1g68710 | ALA9              | P-type ATPase, P-lipid transporter | 0 *1u 0 | 10 | 155 |     |       |
| 026    | gw1.l.XVIII.929.1        | 1.2E-243 5g39040 | ABCB27          | ABC transporter | *4u 0 0 | 5 | 63 |     |       |
| 027    | gw1.l.129.177.1         | 8.4E-241 5g39040 | ABCB27          | ABC transporter | *4 0 0 | 5 | 63 |     |       |
| 030    | fgenes4_pg.C_LG_VIII000415 | 0 3g55320 | ABCB20          | ABC transporter | *2 0 *3 | 12 | 154 |     |       |
| 032    | fgenes4_pg.C_LG_VIII000406 | 0 3g28345 | ABCB15          | ABC transporter | 0 *1 0 | 8 | 136 |     |       |
| 036    | estExt* Genesew1_v1.C_LG_IJ3719 | 0 2g47000 | ABCB4            | ABC transporter | 0 *4u *2u | 9 | 132 |     |       |
| 042    | fgenes4_pm.C_LG_IJ000094 | 0 1g27940 | ABCB13          | ABC transporter | 0 0 *1u | 11 | 133 |     |       |
| 044    | eugene3.00290217         | 0 4g25960 | ABCB2            | ABC transporter | *1 0 1 | 11 | 135 |     |       |
| 045    | estExt_fgenes4_pg.C_LG_XVII0355 | 0 3g28860 | ABCB19          | ABC transporter | 0 *4u *3u | 9 | 136 |     |       |
| 046    | fgenes4_pg.C_LG_XVI000833 | 0 2g36910 | ABCB1            | ABC transporter | *1 *4u *11u | 12 | 144 |     |       |
| 047    | gw1.l.28.733.1          | 0 2g36910 | ABCB1            | ABC transporter | 1 *4u *10 | 12 | 139 |     |       |
| 048    | estExt_fgenes4_pg.C_LG_V11067 | 0 5g06530 | ABCG22          | ABC transporter | *1 0 0 | 6 | 82 |     |       |
| 051    | fgenes4_pm.C_LG_V000052 | 2.4E-181 1g17840 | ABCG11          | ABC transporter | 0 0 *1 | 6 | 67 |     |       |
| 053    | gw1.l.918.1             | 0 2g29940 | ABCG31          | ABC transporter | 1 *1 1 | 14 | 161 |     |       |
| 054    | estExt* Genesew1_v1.C_LG_I0381 | 0 1g59870 | ABCG36          | ABC transporter | *1 0 0 | 14 | 162 |     |       |
| 069    | fgenes4_pg.C_LG_III001690 | 0 3g13100 | ABCC7            | ABC transporter | *1 0 0 | 15 | 164 |     |       |
| 070    | gw1.l.XIII.304.1        | 0 3g59140 | ABCC10          | ABC transporter | *1 0 0 | 7 | 113 |     |       |
| 074    | estExt_fgenes4_pg.C_LG_XI0622 | 0 2g34660 | ABCC2            | ABC transporter | *16u 0 *3u | 17 | 182 |     |       |
| 078    | estExt_fgenes4_pg.C_LG_XIII00040 | 0 1g15690 | AVP1            | H^-pumping PP^-ase | *7u 5 *5 | 13 | 79 |     |       |
| 079    | eugene3.00181092        | 0 1g15690 | AVP1            | H^-pumping PP^-ase | *10u *9u *7u | 13 | 80 |     |       |
| 080    | estExt_fgenes4_pg.C_1520062 | 0 1g15690 | AVP1            | H^-pumping PP^-ase | *9u *7u *7u | 13 | 80 |     |       |
| Protein Name | Molecular Weight | pI | Function | Expression | Source |
|-------------|------------------|----|----------|------------|--------|
| 108 gwL.VIII.3134.1 | 2.1E-173 | 1.5g54730 | Sugar transporter | 0 *1u | 11 47 |
| 101 genesh4.pg.C.LG.II02066 | 1.1E-212 | 1.1g75220 | Sugar transporter | 0 *1u | 12 52 |
| 102 genesh4.pg.C.LG.VI001084 | 9.6E-201 | 1.5g75220 | Sugar transporter | 0 *1u | 12 52 |
| 103 genesh4.pg.C.LG.II0020052 | 9.8E-292 | 1.5g35300 | Sugar transporter | 0 *1u | 11 79 |
| 104 gwL.IX.162.1 | 0 | 1.5g35300 | Sugar transporter | 0 *1u | 11 79 |
| 105 gwL.IX.162.1 | 0 | 1.5g35300 | Sugar transporter | 0 *1u | 11 79 |
| 106 gwL.IX.155.1 | 4.2E-202 | 5g17010 | Sugar transporter | 0 *1u | 13 51 |
| 107 genesh4.pg.C.LG.II00447 | 1.2E-232 | 1.5g46850 | INT4 | Inositol transporter | 0 *1u | 12 52 |
| 108 genesh4.pg.C.LG.II02194 | 2.9E-199 | 1.0g9960 | SUT4 | Sucrose transporter | 0 *1u | 12 52 |
| 109 genesh4.pg.C.LG.II01940 | 2.5E-257 | 1.5g58030 | CAT2 | Cationic amino acid transporter | 0 *1u | 14 68 |
| 110 genesh4.pg.C.LG.II000146 | 1.9E-218 | 1.5g9540 | CAT9 | Cationic amino acid transporter | 1 *1u | 15 *1u |
| 112 genesh4.pg.C.LG.II0002004 | 2.4E-266 | 1.5g47670 | Amino acid transporter | 0 *1u | 11 58 |
| 113 genesh4.pg.C.LG.IX000568 | 7.2E-196 | 1.5g25530 | Lys/His transporter | 0 *1u | 9 47 |
| 114 genesh4.pg.C.LG.II02555 | 4.7E-198 | 1.5g40780 | THT1 | Lys/His transporter | 0 *1u | 9 47 |
| 120 Genesys1.v1.C.LG.I3156 | 0 | 1.5g2070 | YSL6 | Oligopeptide transporter | 0 *1u | 14 73 |
| 121 genesh4.pg.C.LG.II000336 | 1.8E-240 | 1.5g6730 | Oligopeptide transporter | 0 *1u | 14 73 |
| 127 genesh4.pg.C.LG.IX000568 | 1.7E-219 | 1.5g3140 | Oligopeptide transporter | 0 *1u | 10 63 |
| 128 genesh4.pg.C.LG.II01298 | 0 | 1.5g0940 | KUP7 | K+ transporter | 0 *2 | 12 93 |
| 129 genesh4.pg.C.LG.III1502 | 0 | 1.5g0940 | KUP7 | K+ transporter | 0 *3u | 12 94 |
| 130 genesh4.pg.C.C scaffold_273000008 | 4.2E-230 | 2g39840 | PT2 | Phosphate transporter | 0 *1u | 10 57 |
| 132 genesh4.pg.C.LG.II000463 | 2.1E-269 | 3g51895 | SULTR3:1 | Sulfate transporter | 0 *1u | 12 69 |
| 133 genesh4.pg.C.LG.IX000517 | 1.9E-275 | 3g51895 | SULTR3:1 | Sulfate transporter | 0 *1u | 12 79 |
| 134 gwL.IX.2878.1 | 2E-161 | 2g25680 | MOT1 | Molbdate transporter | 0 *3u | 8 43 |
| 135 genesh4.pg.C.LG.II04555 | 1.1E-204 | 1.5g3270 | Na+/Ca2+ transporter | 0 *1u | 10 65 |
| 136 genesh4.pg.C.LG.II000713 | 7.3E-258 | 5g62890 | NAT6 | Nucleobase-ascorbate transporter | 0 *1u | 11 57 |
| 137 genesh4.pg.C.LG.II000713 | 2.3E-257 | 5g62890 | NAT6 | Nucleobase-ascorbate transporter | 0 *4u | 10 58 |
| 140 genesh4.pg.C.LG.II0511 | 7.7E-199 | 3g5030 | TT12 | Sulfit/H+ antiporter | 0 *1u | 12 54 |
| 141 gwL.IX.2310.1 | 8.7E-136 | 3g5030 | MATE efflux protein | 0 *1u | 12 50 |
| 142 gwL.IX.2326.1 | 6.8E-136 | 3g5030 | MATE efflux protein | 0 *1u | 12 50 |
| 143 gwL.IX.42.1 | 1.9E-172 | 5g25450 | MATE efflux protein | 0 *1u | 12 49 |
| 144 genesh4.pg.C.LG.II12333 | 4.2E-175 | 3g21690 | MATE efflux protein | 0 *1u | 11 46 |
| 145 genesh4.pg.C.LG.II006153 | 7.7E-199 | 3g5030 | TT12 | Sulfit/H+ antiporter | 0 *1u | 12 54 |
| 146 gwL.IX.2197.1 | 8.8E-175 | 2g01970 | Transporter? | 0 *1u | 9 73 |
| 147 gwL.IV.5578.1 | 1.2E-152 | 2g46800 | ZAT | Zn2+ transporter | 0 *1u | 6 43 |
| 150 genesh4.pg.C.LG.II00271 | 2.5E-138 | 1g01620 | PIP1;3 | Aquaporin | 0 *1u | 6 31 |
| 151 genesh4.pg.C.LG.IV0408 | 6.6E-138 | 4g00430 | PIP1;4 | Aquaporin | 0 *1u | 5 31 |
| 154 genesh4.pg.C.LG.IV0408 | 6.6E-138 | 4g00430 | PIP1;4 | Aquaporin | 0 *1u | 5 31 |
| 155 genesh4.pg.C.LG.IX0002165 | 1.1E-128 | 3g54820 | PIP2;5 | Aquaporin | 0 *1u | 5 30 |
| 156 genesh4.pg.C.LG.IX0002165 | 3.1E-131 | 4g35100 | PIP2;7 | Aquaporin | 0 *1u | 5 30 |
| 157 genesh4.pg.C.LG.IX0002165 | 3.1E-131 | 4g35100 | PIP2;7 | Aquaporin | 0 *1u | 5 30 |
| 158 genesh4.pg.C.LG.IX0002165 | 3.1E-131 | 4g35100 | PIP2;7 | Aquaporin | 0 *1u | 5 30 |
### Receptors

| Accession | Description | Cytosolic Domain | Voltage-Activated | Response | 
|-----------|-------------|------------------|-------------------|----------| 
| 163 grail3.001068301 | 0 2g01950 | BRL2 | LRR RLK, BR11-like | 0 | 0 | 1 | 123 |
| 164 estExt_fgenesh4_pg_C_LG_J0469 | 0 2g33170 |  | LRR RLK | 0 | 0 | *1 | 1 | 121 |
| 166 eugene3.00060471 | 0 2g41820 |  | LRR RLK | 0 | 0 | *1 | 1 | 97 |
| 168 estExt_Genewise1_v1C_LG_VII0023 | 0 5g65700 | BAM1 | LRR RLK | 1u | 0 | 1 | 94 |
| 169 fgenesh4_pm_C_LG_IV000169 | 0 1g28440 | HSL1 | LRR RLK | 0 | 0 | *2 | 1 | 109 |
| 171 estExt_fgenesh4_pg_C_LG_VI1628 | 5.8E-281 2g25790 |  | LRR RLK | 0 | 0 | *1 | 1 | 106 |
| 173 estExt_fgenesh4_pg_C_LG_XVIII0976 | 4.2E-191 5g51350 |  | LRR RLK | 0 | *1 | 2u | 1 | 104 |
| 174 eugene3.00131289 | 1.9E-236 5g58300 |  | LRR RLK | 0 | 0 | *1u | 2 | 69 |
| 176 fgenesh4_pg_C_LG_IV000713 | 4.3E-182 5g16590 | LRR RLK | 0 | 0 | *1u | 1 | 68 |
| 177 gw1.XII.830.1 | 3.3E-214 1g48480 | RKL1 | LRR RLK | 0 | 0 | *1 | 1 | 66 |
| 178 estExt_fgenesh4_pg_C_LG_XV0398 | 3.1E-218 1g48480 | RKL1 | LRR RLK | 0 | 0 | *1 | 3 | 71 |
| 179 eugene3.00002566 | 1.5E-280 3g24660 | TMKL1 | LRR RLK | 0 | *1u | 3u | 1 | 74 |
| 180 eugene3.00060911 | 1.1E-286 3g51740 | IMK2 | LRR RLK | 0 | 0 | 1 | 3 | 85 |
| 184 gw1.18.291.1 | 5E-227 5g49760 | LRR RLK | 0 | *2 | 1 | 1 | 98 |
| 186 gw1.28.1090.1 | 0 1g79629 | LRR RLK | 0 | 0 | 1 | 3u | 3u | 1 | 101 |
| 188 gw1.120.65.1 | 2.2E-265 4g03960 | SRF3 | LRR RLK | 0 | *1 | 0 | 1 | 81 |
| 190 fgenesh4_pm_C_LG_VII000290 | 0 1g56130 | LRR RLK | 0 | 0 | *1 | 0 | 1 | 114 |
| 193 estExt_fgenesh4_pg_C_LG_XVI0807 | 2.1E-132 1g53430 | LRR RLK | 0 | 0 | *2u | 1 | 94 |
| 194 fgenesh4_pg_C_LG_XVI00066 | 1.1E-120 1g53430 | LRR RLK | 0 | 0 | *2 | 0 | 1 | 87 |
| 195 gw1.XVI.709.1 | 2.6E-95 1g53430 | LRR RLK | 0 | 0 | *2 | 0 | 1 | 52 |
| 207 eugene3.00110972 | 0 5g54380 | THE1 | CrRLK1L | 0 | 0 | 1 | 93 |
| 208 gw1.134.227.1 | 0 5g54380 | THE1 | CrRLK1L | 0 | 0 | 1 | 82 |
| 210 eugene3.00060962 | 0 3g51550 | FER | CrRLK1L | 0 | 0 | 1 | 97 |
| 211 fgenesh4_pm_C_LG_VIII00441 | 4.2E-255 5g28680 | CrRLK1L | 0 | *2 | 0 | 1 | 84 |
| 216 gw1.29.53.1 | 6.2E-183 4g21390 | B120 | SD RLK | 0 | 0 | 0 | 1 | 91 |
| 218 estExt_fgenesh4_pg_C_9540001 | 1.3E-182 5g69090 | RLK1 | SD RLK | 0 | 0 | 2 | 0 | 1 | 91 |
| 219 gw1.18.454.1 | 2.8E-185 4g34440 | PERK (RLK) | 0 | 0 | *1 | 0 | 1 | 68 |
| 222 eugene3.00020079 | 0 1g42470 | Hedgehog receptor | 0 | 0 | 1 | 1 | 135 |
| 224 estExt_Genewise1_v1C_LG_IX0199 | 0 1g42470 | Hedgehog receptor | 0 | 0 | 1 | 14 | 143 |

### Cell wall and carbohydrate metabolism

| Accession | Description | Cytosolic Domain | Voltage-Activated | Response | 
|-----------|-------------|------------------|-------------------|----------| 
| 232 eugene3.00002636 | 0 5g44030PC | CES4A | Cellulose synthase | 0 | 2u | 0 | 8 | 119 |
| 233 gw1.XL.3218.1 | 0 5g44030PC | CES4A | Cellulose synthase | 0 | 1 | 0 | 9 | 114 |
| 236 grail3.012400291 | 0 4g04970 | GSL1 | Glucan synthase | 0 | 2 | *2 | 1 | *1 | 17 | 199 |
| 238 estExt_fgenesh4_pg_C_1480042 | 0 2g31960 | GSL03 | Glucan synthase | 0 | 1 | 4 | *4 | 17 | 225 |
| 241 fgenesh4_pg_C_LG_I000551 | 0 1g06490 | GSL07 | Glucan synthase | 0 | 1 | 2 | 17 | 225 |
| 242 gw1.I.6689.1 | 0 3g07160 | GSL10 | Glucan synthase | 0 | 4u | 5u | 4u | 15 | 222 |
| 243 gw1.XV.1929.1 | 0 3g07160 | GSL10 | Glucan synthase | 0 | 2u | 2u | 5u | 15 | 217 |
| 244 estExt_fgenesh4_pg_C_LG_I0109 | 0 1g05570 | CALS1 | Callose synthase | 0 | 2 | 8u | 10u | 17 | 223 |
| 245 estExt_fgenesh4_pg_C_LG_VII013 | 5.5E-189 5g66680 | DGL1 | Glycosyl transferase | 0 | 0 | 1 | 1 | 49 | 49 |
| 247 estExt_fgenesh4_pm_C_LG_I0174 | 1.5E-234 4g21150 | GGT | Galactosyl transferase | 0 | 1 | 0 | 3 | 0 | 75 |
| 248 eugene3.00160929 | 8.5E-106 1g07250 | UDP-glucosyl transferase | 0 | 0 | 1 | 3 | 52 |
| 250 gw1.IV.3970.1 | 4E-140 5g17310 | UDP-glucose pyrophosphorylase | 0 | 0 | 3 | 3 | 2 | 46 |
| 253 gw1.L.683.1 | 1.9E-234 3g06510 | SFR2 | β-glucosidase | 0 | 0 | 2 | 70 |
| 254 estExt_fgenesh4_pm_C_LG_V0631 | 5.9E-201 1g75680 | GH9B7 | Glycosyl hydrolase, family 9 | 0 | 0 | 1 | 0 | 19 | 57 |
### Table I—continued

| Accession | GO Term | GO Description | Cond. | Score | P-value |
|-----------|---------|----------------|-------|-------|---------|

**Membrane trafficking**

| Accession | GO Term | GO Description | Cond. | Score | P-value |
|-----------|---------|----------------|-------|-------|---------|

**Others**

| Accession | GO Term | GO Description | Cond. | Score | P-value |
|-----------|---------|----------------|-------|-------|---------|

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284 grail3.0018000201 7.1E-118 5g46700 TRN2 TORNADO-2 0 *1 *1 4 30
287 grail3.0155003301 0 3g02260 BIG Involved in auxin polar transport 0 *1 0 1 168
288 estExt_Genewise1_v1.C_LG_III1005 2E-51 1g32210 DAD1 Defender against apoptosis *1 *1 *1 3 13
289 estExt_Genewise1_v1.C_LG_VI2154 2.6E-104 2g23810 TET8 Senescence-associated *1 *2 *2 4 31
290 gw1.II.2836.1 4.9E-126 3g25290 TOM2A, virus multiplication 1 *1 *1 0 1 30
291 estExt_Genewise1_v1.C_LG_VI1395 2E-271 4g14360 Dehydration-responsive protein 0 *2 0 1 69
292 estExt_Genewise1_v1.C_LG_VI1503 0 5g64030 Dehydration-responsive protein 0 *2 0 1 92
294 gw1.I.4474.1 3.2E-264 1g30360 ERD4 Early responsive to dehydration *3 *3 *3 11 81
298 estExt_Genewise1_v1.C_LG_III0903 7.1E-260 4g12420 SKU5 Cu⁺ binding, root tip growth 0 *2 *1 1 66
299 grail3.000080784 0 3g19870 Binding 0 0 0 0 1 127
301 grail3.00008044 4E-195 1g16860 Merozoite surface protein-related 0 *1 0 2 52
304 estExt_Genewise1_v1.C_LG_IV1455 7.6E-224 2g20990 SYTA C2 domain-containing protein *2 *1 *1 1 60
307 grail3.011800051 2.2E-100 1g32400 TOM2A, virus multiplication *2 0 0 4 30
308 gw1.II.2836.1 7.4E-107 3g32400 TOM2A, virus multiplication 1 *1 *1 0 1 30
309 grail3.00140182 1.2E-147 2g45510 CYPT04A2 Cytochrome P450 *1 0 0 1 58
310 estExt_Genewise1_v1.C_LG_VI1133 1E-86 4g25570 ACYB-2 Cytochrome b561 *1 *1 *1 0 6 25
311 estExt_Genewise1_v1.C_LG_XII0286 9.7E-50 5g53560 BS-A CYTOCHROME b5 *2 0 *1 1 15
314 estExt_Genewise1_v1.C_LG_VI1705 0 2g25730 Heme binding 0 *5 *6 1 276
315 grail3.00080606 1.3E-267 3g19820 DF1 DWF1, photosynthesis *5 9 10 1 66
316 estExt_Genewise1_v1.C_LG_XI518 1.2E-266 3g19820 DF1 DWF1, photosynthesis *3 6 8 1 66
318 grail3.0018000201 9.2E-148 1g52760 Esterase/lipase/thioesterase 0 *2 0 3 37
319 grail3.007000243 0 1g55020 LOX1 Lipoxygenase 0 0 *5 1 92
320 gw1.IV.984.1 6E-121 1g18180 Oxidoreductase, lipid metabolism *3 0 *1 7 32
321 estExt_Genewise1_v1.C_LG_XIV0228 1.3E-139 2g46890 Oxidoreductase, lipid metabolism 0 *1 *1 6 39
vesicular trafficking and thus present in all membranes involved in that process (37). Judging from the staining by the Arf1 antiserum, membrane trafficking was most intense in the xylem engaged in wood formation and least intense in the essentially fully expanded leaves (Fig. 1C). A low degree of contamination of the plasma membrane fractions was also

### Table I—continued

| Gene          | P1  | P2  | G | m | g | f   | Description                                      | Coefficient | Coefficient | Coefficient |
|---------------|-----|-----|---|---|---|-----|-------------------------------------------------|-------------|-------------|-------------|
| 323 fgn8h4  | 5.3E-232 | 4g26690p1 | MRH5 | Glycero-P-diester P-diesterase | 0    | 0   | *1  | 10 | 20 |
| 325 estFxG   | 3.2E-90  | 1g52600 | Signal peptidase, putative | 2u  | 0   | 0   | 3   | 20 |
| 326 estFxG   | 1.5E-90  | 152600  | Signal peptidase, putative | 1   | 0   | *1  | 3   | 20 |
| 327 fgn8h4  | 1.3E-79  | 2g39960 | Signal peptidase, putative | *1  | 0   | 1   | 2   | 21 |
| 329 estFxG   | 4.8E-199 | 4g01320 | STE24 | CAAX protease | *1  | 0   | 5   | 49 |
| 331 estFxG   | 2.8E-162 | 1g7550  | Nodulin MtN21 family protein | *3  | *3  | *3  | 10  | 42 |
| 334 gw1.88227 | 6.7E-248 | 3g01930 | Nodulin family protein | 0   | 1u  | *4u | 13  | 64 |
| 335 estFxG   | 2.2E-185 | 3g01930 | Nodulin family protein | 0   | 0   | *3  | 8   | 46 |
| 336 estFxG   | 1.2E-37  | 1g22480 | Plastocyanin-like domain | *1  | 0   | 0   | 10  | 19 |
| 337 eugene3.00130865 | 2.1E-45  | 3g20570 | Plastocyanin-like domain | 0   | 0   | *1  | 10  | 24 |
| 338 eugene3.00081064 | 6.5E-40  | 1g8750  | PPC4 | PEP carboxylase? | 0   | 0   | *1  | 2   | 22 |

### Unknown

| Gene          | Coefficient |
|---------------|-------------|
| 342 eugene3.28120001 | 1.9E-62  2g07707 |
| 343 gw1.X.41471 | 4.3E-189 4g14240 |
| 345 fgn8h4 pm.C_LG_XV000060 | 5.8E-59 5g24170 |
| 339 fgn8h4 pg.C_LG_VIII000635 | 9.6E-86 2g40316 |
| 347 estFxG fgn8h4 pg.C_LG_X0743 | 2.7E-93 3g22845 |
| 348 fgn8h4 pg.C_LG_I002256 | 7.5E-27 3g03341 |
| 349 eugene3.00061972 | 1.4E-82 5g10780 |
| 350 estFxG fgn8h4 pg.C_LG_I1877 | 8E-126 5g12470 |
| 355 eugene3.00700232 | 6.7E-26 1g54860 |
| 356 fgn8h4 pm.C_LG_XVI000041 | 4.9E-158 2g32240 |
| 357 eugene3.00151111 | 5E-37 5g62200 |
| 358 estFxG Genesigv 1.1.C_LG_XV3031 | 2.4E-53 5g62200 |
| 359 estFxG fgn8h4 pg.C_LG_I0430 | 1.1E-87 3g24160 |
| 361 estFxG fgn8h4 pm.C_LG_I1168 | 8.4E-83 4g28770 |
| 362 eugene3.00070307 | 8.7E-65 4g33625 |
| 363 estFxG fgn8h4 kg.C_LG_XIII0023 | 2.6E-49 5g20090 |
| 364 gw1.X.834.1 | 6.9E-143 2g03510 |
| 365 estFxG fgn8h4 pg.C_LG_X1459 | 1.9E-234 1g69450 |
| 366 gw1.XVIII.829.1 | 7.6E-137 2g06005 |
| 367 eugene3.00060644 | 3.8E-32 2g31490 |

### Probable contaminants

| Gene          | Coefficient |
|---------------|-------------|
| 370 eugene3.00111115 | 2.5E-154 5g54800 |
| 372 estFxG fgn8h4 pg.C_LG_I0752 | 4.5E-100 1g44575 |
| 373 eugene3.00013110 | 2.8E-52 1g55670 |
| 374 estFxG fgn8h4 pg.C_LG_I1918 | 1.3E-173 3g08580 |
| 377 g2a1.0161000402 | 4.3E-166 5g13490 |

p1 Highly coregulated gene for At CESA1, -3, and -6, i.e. primary cell wall formation (12).
p2 Highly coregulated gene for At CESA4, -7, and -8, i.e. secondary cell wall formation (12).
GPI
Myristoyl
Geranylgeranyl
Farnesyl (predictions described in Ref. 49).
suggested by the MS data (Table I); only peptides identifying the ADP/ATP antiporter (compare Fig. 1B), one Photosystem I and one Photosystem II subunit, and the glucose 6-phosphate/Pi antiporter, markers for the mitochondrial inner membrane, the chloroplast thylakoid membrane, and the plastid envelope inner membrane, respectively, were found in the plasma membrane fractions. As a final step in the purification of the plasma membranes, they were treated with the detergent Brij 58 and 0.2 M KCl to turn the cytoplasmic side-in vesicles inside-out and thus remove soluble proteins enclosed in the vesicles as well as loosely bound proteins. Plasma membranes are not solubilized by this detergent, but the vesicles are simply turned cytoplasmic side-out (20). However, we do not know to what extent other membranes are solubilized by Brij 58, and the detergent was therefore excluded from the wash medium for the microsomal and intracellular membrane fractions. If some of the intracellular membranes were solubilized, the Brij treatment of the plasma membrane fractions would constitute an additional purification step also with respect to contaminating integral membrane proteins. Proteins remaining after this procedure were separated by SDS-PAGE, sections of the lanes were excised, and the excised proteins were further processed for analysis by nano-LC-MS/MS.

Protein Identification—A total of 956 proteins was identified by matching peptide fragment ion mass data to the Populus sequence database (Fig. 2). These proteins were either identified by one or more peptides unique to a particular protein, or they were ranked as first choice (top rank) by the identification software used (Mascot v2.2.04, Matrix Science Ltd.) based on one or more peptides shared by e.g. several members of a protein family. Thus, in addition to the 956 top rank proteins, about 800 proteins ranked as subset (proteins also matching peptide fragment ion mass data but with lower ranking) were also identified. In Figs. 2, 3, 4, and 6; Table I, and supplemental Table 3, only top rank proteins are included. However, in Fig. 5, displaying a large protein family, and in supplemental Table 2, subset proteins are also included. This should be justified because already the Arabidopsis genome contains a large number of closely related genes coding for members of protein families, a feature that should be even more emphasized in the 40–60% larger protein-coding genome of Populus (1). In supplemental Tables 2 and 3, each protein is identified with a number running from 1 to 1122. These IDs are kept in Table I and used in Figs. 5 and 6 and in the text to refer to the respective proteins. All annotations in Table I and supplemental Tables 2 and 3 were via the Arabidopsis database at TAIR. Thus, amino acid sequences corresponding to identified gene models in the Populus v1.1 (45,555 entries) database were blasted against the Arabidopsis TAIR8 (32,825 entries) data set to identify the closest Arabidopsis homolog of each protein, which in Table I and supplemental Tables 2 and 3 is identified by its AGI accession number. p values are included to give an indication of how well the poplar and Arabidopsis amino acid sequences agree and thus of the reliability of the annotation for the poplar protein.

A relatively large part (42%) of the 956 top rank proteins was found in the plasma membranes of all three tissues, and only 10–11% were unique to a particular tissue (Fig. 2). Using the software Phobius (38) to predict transmembrane domains, 213 integral proteins were identified, and the remaining 743 were classified as soluble proteins (Fig. 2). Among these 743 soluble proteins, seven are predicted to have transmembrane domains but judged by us to be soluble proteins based on previous knowledge of these proteins (protein IDs 622, 623, 628, 629, 963, 964, and 1104 in supplemental Table 3); most of them are subunits of the proteasome. Many of the soluble proteins...
proteins are likely to be true peripheral proteins of the plasma membranes. However, because of the difficulties involved in differentiating between true peripheral proteins and contaminating soluble proteins, the present study is focused on the integral membrane proteins.

**Tissue Distribution of Integral Membrane Proteins**—The 213 top rank proteins predicted to be integral membrane proteins are listed in Table I where they are divided according to function into “transporters,” “receptors,” “cell wall and carbohydrate metabolism,” “membrane trafficking,” “others,” “unknown,” and “probable contaminants.” The integral protein composition of plasma membranes from leaves, xylem, and cambium/phloem is shown in Fig. 3, and the overlap between tissues for each class of integral protein is shown in Fig. 4. Notably, almost half of the identified leaf plasma membrane integral proteins are transporters (Fig. 3), partly reflecting the photosynthetic activity, which not only involves CO2 fixation and hence carbohydrate transporters but also reduction of nitrate to produce amino acids and reduction of sulfate to produce thiol. Thus, 75% of the transporters were found in the leaf plasma membranes, 32% were found in the plasma membranes of all three tissues, and only 25% were exclusively found in the plasma membranes of xylem and/or cambium/phloem (Fig. 4). The plasma membranes isolated from cambium/phloem had the highest proportion of receptors (Figs. 3 and 4), possibly reflecting the position of this tissue in the periphery of the stem. More than 70% of the proteins involved in cell wall and carbohydrate metabolism and all of the proteins engaged in membrane trafficking were identified in the xylem plasma membranes (Figs. 3 and 4) in agreement with the role of the xylem in wood formation. Furthermore, about proteins are likely to be true peripheral proteins of the plasma membranes. However, because of the difficulties involved in differentiating between true peripheral proteins and contaminating soluble proteins, the present study is focused on the integral membrane proteins.

![Fig. 4. Distribution of major classes of integral proteins between plasma membranes from leaves, xylem, and cambium/phloem.](image)

*The distribution (number of proteins) of integral membrane proteins among plasma membranes from leaves (green), xylem (red), and cambium/phloem (blue) is shown. Only the 213 integral proteins (Fig. 2) classified as top rank are included. Carboh, carbohydrate metabolism.*

![Fig. 5. Phylogenetics analysis of plasma membrane ABC transporters and their tissue localization.](image)

*ABC transporters detected by mass spectrometry, both top rank and subset, are included (one truncated sequence, ID 75 in Table I, was excluded). The numbers outside each symbol refer to the gene model ID in Table I (top rank integral proteins; numbers in bold) and supplemental Table 2 (both top rank and subset integral proteins). The phylogenetic analysis (27) was supervised by using all Arabidopsis proteins annotated to subfamilies B, C, and G (2). The color code is green for leaf, red for xylem, and blue for cambium/phloem plasma membranes. A star in a colored field indicates that the protein is classified as top rank in that tissue.*
60% of the proteins involved in membrane trafficking were identified in the plasma membranes of all three tissues, and not a single protein was uniquely identified in the leaf plasma membranes (Fig. 4). This suggests that membrane trafficking is a process that relies mainly on housekeeping proteins in agreement with the data of Uemura et al. (8). Very few obvious contaminants were found (Fig. 3).

**Transporters**—Transporters were by far the largest class of integral proteins identified in the plasma membranes of all three tissues (Table I and Fig. 3). In Table I and supplemental Table 2, they are listed in the following order: pumps (IDs 1–92), carriers (IDs 93–149), and channels (IDs 150–161).

Assuming that the number of identified peptides reflects protein abundance, the H\(^+\)-ATPase (IDs 1–15; Table I) was the most abundant transporter. This is expected from its role in the plasma membrane where it creates the electrochemical gradient driving secondary transport and therefore should be an abundant housekeeping protein. It is also in agreement with earlier findings that the H\(^+\)-ATPase constitutes several percent of total integral protein in spinach leaf plasma membranes (calculated from recovery of the protein upon isolation (30)). At least 13 H\(^+\)-ATPase isoforms were detected. Based upon data from Arabidopsis, tobacco, and rice, which all contain 10 or more H\(^+\)-ATPase isoforms, the isoforms have been divided into five subfamilies, and subfamily I and II members have been suggested to be the most highly expressed (3). AHA1, -2, and -5 belong to subfamily II, and AHA11 belongs to subfamily I, and indeed, homologs to these.

### Proteins involved in wood formation

A schematic model of the cellulose-synthesizing complex and other proteins associated with wood formation, such as enzymes involved in lignin biosynthesis, is shown. The numbers outside each symbol refer to the gene model ID in Table I for integral proteins and supplemental Table 3 for soluble proteins. Only proteins detected in the xylem plasma membranes are included, and the color code is green for leaf, red for xylem, and blue for cambium/phloem plasma membranes. A star in a colored field indicates that the protein is classified as top rank in that tissue. When available, Populus names are stated below the symbol. Abbreviations for the NAD-dependent epimerase dehydratase family (11) are: UGE, UDP-\(\alpha\)-glucose 4-epimerase; GME, GDP-\(\alpha\)-mannose 3,5-epimerase; RHM, UDP-\(\alpha\)-rhamnose synthase; UXS, UDP-\(\alpha\)-apiose/xylene synthase. Abbreviations for the lignin enzymes are: CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl-CoA 3-O-methyltransferase; HCT, hydroxycinnamoyltransferase; PAL, phenylalanine ammonia-lyase.
isoforms are among the most highly expressed in poplar. However, homologs to AHA7, -8, -9 and -10 also show relatively high levels of expression in poplar in contrast to what is known for Arabidopsis where these isoforms are weakly expressed and/or show a very tissue-specific expression: AHA8 and -9 are highly expressed only in the flower, and AHA10 is only expressed in the seed coat (Genevestigator (39)). The Arabidopsis AHA10 is not even localized to the plasma membrane but seems to have a role in acidifying transport vesicles in the seed coat (40). Notably, the poplar homolog of AHA10 (ID 12) was the only isoform that was not identified in all three tissues.

ATP-binding cassette (ABC) transporters constitute the largest family of pumps found in the present investigation (IDs 25–77; Table I and supplemental Table 2). ABC transporters are able to transport a broad array of compounds. Substrates ranging from lipids to ions have been reported, and ABC transporters are involved in many different processes, such as resistance to pathogens, detoxification, and cutin accumulation at the plant surface (2). According to a recent inventory and new nomenclature for plant ABC proteins (2), they can be divided into nine subfamilies, ABCA–I, with all subfamilies except H found in plants. A phylogenetic analysis of the ABC transporters identified by us (both top rank and subset; Table I and supplemental Table 2) showed that they all fall within three subfamilies, B, C, and G (Fig. 5), with a rather specific “tissue” distribution. Thus, only a few of the ABC transporters were identified in the plasma membranes of all three tissues, two in subfamily B (IDs 46 and 47), five in G (IDs 53, 63, 64, 66, and 67), and none in C. The two subfamily B members (IDs 46 and 47) are homologs to At ABCB1 (earlier PGP1) reported to catalyze auxin efflux (41). Most of the ABC transporters were identified in leaves and many of these in leaves only; notably all were in subfamily C (IDs 68–77) except one (ID 74), which was also present in cambium/phloem. Just a few were identified in cambium/phloem only, one in subfamily B (ID 42) and three in G (IDs 50–52). Only one, a subfamily B member (ID 32), was specific to xylem, and its Arabidopsis homolog, ABCB15 (MDR13), has been suggested to excrete lignin monomers into the apoplast (42). Subfamily B is a large and diverse subfamily not only involved in export of secondary metabolites. Thus, At ABCB4 (PGP4; homolog to IDs 34, 36–39, and 41) appears to catalyze import of auxin (43), and At ABCB27 (TAP2/ALS1; homolog to IDs 25–29) has been suggested to have a role in aluminum resistance, albeit in root tissue (44). Subfamily G is markedly expanded in plants with more than 40 members in both Arabidopsis and rice (2). At ABCG36 (PEN3/PDR8; homolog to IDs 54 and 61) is suggested to export antifungal materials at attempted invasion sites and in that way confer resistance to powdery mildews (45), and the wheat homolog, LR34, is associated with resistance to multiple fungal pathogens (46). Both poplar homologs (IDs 54 and 61) were only identified in leaves. At ABCG11 (WBC11; homolog to IDs 50–52) is involved in the export of wax components and is required for normal cutin accumulation at the cell surface (47). Notably, the poplar ABCG11 homologs (IDs 50–52) were only detected in the plasma membranes from cambium/phloem, i.e. in a tissue that should be involved in secretion of cuticular lipids.

Two pumps usually associated with the vacuolar membrane were also detected: H+-pumping PP-ase (IDs 78–81; Table I) and V-type ATPase (IDs 84–87). Both pumps have been reported earlier in proteomics studies of plasma membranes from e.g. Arabidopsis leaves and cell cultures (48, 49). Both pumps have also been localized to plant plasma membranes by immunostaining in situ (50, 51), and it was suggested that the plasma membrane constitutes “a temporary repository for tonoplast proteins en route to the vacuole” (50).

Most of the carriers (IDs 93–147; Table I) are predicted to have 9–15 transmembrane domains and are therefore likely to belong to the “major facilitator superfamily” (4), the members of which typically have around 12 transmembrane domains. Only a few of the carriers were found in all three tissues (IDs 93, 110, 120, and 135), and as few were specific to xylem (IDs 109, 145, and 146) or cambium/phloem (IDs 100, 112, and 113). Most of the carriers were found in the leaf plasma membranes and are likely associated with the photosynthetic reduction of CO2 to carbohydrate, nitrate to amino nitrogen, and sulfate to thiol. Thus, a number of carriers of carbohydrates (IDs 93–108), amino acids and oligopeptides (IDs 110–114, 116–117), and sulfate (IDs 132 and 133) were localized to the leaf plasma membranes as well as a putative molybdate transporter (ID 134). The Arabidopsis homolog to this protein was recently identified as a high affinity molybdate transporter and named MOT1 (52) and shown to be present in the plasma membrane of both roots and shoots in agreement with the localization of its poplar homolog (ID 134). The largest sink for molybdate in plants is probably nitrate reductase, which catalyzes the first step in the mainly photosynthetic conversion of nitrate to amino nitrogen.

Only a few channels (IDs 150–161; Table I) were detected. Eight aquaporins, six PIPs (IDs 150–157) expected to be localized in the plasma membrane as indicated by the name, and one tonoplast intrinsic protein (TIP) (ID 158) rather expected to be found in the vacuolar membrane. TIPs have been recorded earlier in proteomics studies of plant plasma membranes (e.g. Ref. 48) and also localized to plasma membranes by immunostaining in situ (50). Thus, the PIP and TIP nomenclature as an indication of location has been questioned (32). Aquaporins should be housekeeping proteins; still some “tissue specificity” was observed, and two aquaporins were only detected in leaf tissue (IDs 151 and 154). Aquaporins are also really major integral proteins of plant plasma membranes, and PIPs may constitute about 20% of total integral protein (31) in contrast to ion channels, which because of their high transport capacity are only needed in small amounts and therefore easily escape detection by MS. Thus, only two ion channels, a “xylem-specific” Ca2+ channel (ID 160) and a “leaf-specific”
Cl⁻ channel (ID 161), were found. Chloride channels are involved in turgor regulation and may therefore be relatively abundant.

Receptors—Two types of receptors (Table I), receptor-like kinases (RLKs; IDs 163–219) and Hedgehog receptors (IDs 222 and 224) were identified. The RLKs constitute by far the largest family of integral proteins found in the present survey and indeed the largest family of receptors in plants with over 600 members in Arabidopsis and over 1100 in rice (5, 6). They typically have an N-terminal extracellular domain for signal perception followed by a transmembrane domain and a C-terminal intracellular protein kinase domain for propagation of the signal. All RLKs detected by us fall within four subfamilies according to the classification of their Arabidopsis homologs (IDs 163–219; Table I). The largest subfamily, the leucine-rich repeat (LRR) RLKs (IDs 163–195), have an extracellular domain with 1–32 LRRs, which e.g. may interact with pathogen-specific molecules (so-called pathogen-associated molecular pattern) to trigger innate immunity (53) or with brassinosteroids and thus serve as hormone receptors (e.g. At BRI1 (54)). A clear tissue specificity was observed, and only four of the 20 LRR RLKs were detected in the plasma membranes of all three tissues.

The CrRLK1L (Catharanthus roseus RLK1-like) receptors are the second largest group (IDs 207–211), and all four members were found in the plasma membranes of all three tissues. The N-terminal extracellular domains of this group do not show any similarity to domains of known function. However, the functions of two members of the group, At THE1 (THESEUS1; homolog to IDs 207 and 208) and At FER (FERONIA; homolog to ID 210), have recently been elucidated. Both are involved in growth regulation; THESEUS inhibits cell expansion by somehow affecting cellulose synthesis, and FERONIA inhibits the growth of pollen tubes at the target, and it is suggested that also other members of the CrRLK1L subfamily control cell growth during development and perhaps upon pathogen attack (55).

A member of the proline extensin-like receptor kinase (PERK) subfamily was found in cambium/phloem plasma membranes only (ID 219). The extracellular domain is proline-rich, but there is no information on ligands or the function of these receptors. The two SD RLKs (IDs 216 and 218) were both found in leaf plasma membranes only. SD is for S-locus glycoprotein-like domain, and this extracellular domain has lectin properties and specifically binds α-D-mannose. The Brassica S-locus receptor kinase mediates the self-incompatibility response (56), and a recent report suggests that SD RLKs may function as negative regulators in plant defense responses (57). An additional seven putative RLKs can be found among the soluble proteins in supplemental Table 3 (IDs 458–460, 470, 471, 515, and 608). The reason that these RLKs have not been classified as integral proteins could either be due to the program used to predict transmembrane domains (38) or be due to mistakes in the gene model. These additional RLKs all fall within the subfamilies already identified (Table I).

Receptors belonging to the patched family, and hence with putative Hedgehog receptor activity, were also detected (IDs 222 and 224; Table I). The Hedgehog signaling pathway was first discovered in Drosophila and is a fundamental signal transduction pathway in animal development, controlling axial patterning and stem cell fate (58).

Cell Wall and Wood Formation—To identify proteins involved in cell wall formation, 660 Arabidopsis genes were extracted from the Cell Wall Navigator database (11) and matched to our data set. In addition, a number of genes identified to be involved in wood formation by analyzing coexpression of genes during secondary cell wall formation (12) were added. Several proteins, both integral and peripheral, corresponding to such genes were identified in this study, particularly in the xylem plasma membranes (Table I and supplemental Table 3). Fig. 6 shows a schematic model of the cellulose-synthesizing complex and other proteins associated with wood formation (modified from Refs. 59–61) where proteins identified by us are indicated.

The cellulose synthase complex is highly integrated with the cytoskeleton (59, 61), and an elevated expression of α- and β-tubulin genes in xylem tissue compared with leaf tissue was shown by Oakley et al. (62). Our results confirm these findings at the protein level, and essentially all tubulins were identified in xylem (IDs 845–859; supplemental Table 3). Notably, no α-tubulin (IDs 845–849) was identified in the leaf plasma membranes. Furthermore, the large (≈220-kDa) homolog (ID 813) of the At MOR1 protein was found, at the top of the gel, in the xylem plasma membranes. This protein has been suggested to have a role in microtubule stabilization and in linking microtubules to the plasma membrane via plasma membrane-associated proteins (63). We also detected an enrichment of actin (IDs 860–864) in the stem tissues, all homologs of At ACT7 and -11, as well as actin-binding villin (IDs 777 and 778), profilin (IDs 779 and 780), and actin-depolymerizing factor (IDs 781 and 782). Cellulose synthases of the CESA (GT2 family (7)) subfamily should have a central role in the complex, and homologs of At CES1A (IDs 229–231), CES4A (IDs 232 and 233), and CES7A (IDs 234 and 235) were detected, although only the two At CES4A homologs, Pt CES4A (ID 232) and Pt CES8A (ID 233), were classified as top rank proteins (Table I and supplemental Table 2). At CES4A, -7, and -8 are associated with secondary cell wall formation in Arabidopsis (12).

Sucrose synthase (SUS) is a soluble enzyme that associates with the plasma membrane and has been suggested to provide the substrate, UDP-glucose, for cellulose synthase and callose synthase (35). We identified a large number of peptides from Pt SUS1 (ID 983) and Pt SUS2 (ID 984) in the plasma membranes from the wood-forming tissue and less of Pt SUS3 (ID 985) and another member of the GT4 family (7) (ID 986) in agreement with the gene expression data of Geisler-
Lee et al. (7). This is also consistent with the immunolocalization of sucrose synthase predominantly to the xylem plasma membranes (Fig. 1C). Within the GT48 family (7) (IDs 236–244), most peptides were recovered from a likely callose synthase (ID 244) with highest sequence similarity to At CALS1, slightly enriched in cambium/phloem as ID 243. Annexin (IDs 599–601), a regulator of callose synthase activity (64) and a putative membrane-cytoskeleton linker (65), was enriched in the stem tissue. KORRIGAN (ID 255), an O-glycosyl hydrolase required for cellulose microfibril synthesis (66), was only found in the xylem.

Several peptides were recorded for the homologs of At UXS6, UDP-xylulose synthase 6 (IDs 691 and 692), in stem tissues. This gene is coregulated with the secondary cell wall-specific CESAs4, -7, and -8 in Arabidopsis (12). A homolog of the membrane-bound Arabidopsis UDP-xylulose synthase 2, At UXS2 (ID 259), was also enriched in stem tissues in agreement with the data of Pattathil et al. (67). Peptides were also recovered from fasciclin-like arabinogalactan (FLA) proteins in the xylem plasma membranes, particularly from the homolog of At FLA10 (ID 1040) with a recognized abundance of peripheral proteins in the xylem plasma membranes, and these are predicted to be integral membrane proteins (Fig. 2). Thus, the majority of the proteins are soluble proteins and putative peripheral proteins of the plasma membranes. However, because of the problems involved in differentiating between true peripheral proteins and contaminating soluble proteins, we have chosen to focus on the proteins predicted to have one or more transmembrane domains. Among these 213 integral membrane proteins, transporters constitute the largest class (41%) followed by receptors (14%) and proteins involved in cell wall biosynthesis (14%)”
teins in Arabidopsis (81–83) and rice (79) plasma membranes. Thus, many transporters are shown to be phosphorylated, probably as a means to regulate their activities, as well as many receptors in the RLK family, not surprisingly, because they undergo autophosphorylation. Using localization of organelle proteins by isotope tagging (LOPIT), the main location for 527 proteins obtained from an Arabidopsis callus culture were determined (84). Of these 527 proteins, 417 are classified as integral membrane proteins by Phobius (38), and 50 of these integral proteins are found among our poplar homologs (Table I). These include 10 of their 130 endoplasmic reticulum proteins, four of the 69 Golgi proteins, two of the 71 mitochondrial/plastid proteins, six of the 68 not classified, eight of the 17 vacuolar proteins, and 20 of the 62 plasma membrane proteins. The LOPIT data should reflect the steady state distribution of proteins, which will vary depending on e.g. tissue and developmental stage. Thus, endomembrane proteins are in a constant flux, and for example, plasma membrane proteins travel through the endoplasmic reticulum and Golgi before they reach the cell surface and may then again be internalized and recycled (73). When 22 of the proteins in the LOPIT study were expressed as green fluorescent protein fusion proteins in tobacco leaves, 16 were targeted as predicted, two were not targeted as predicted, and the localization of four was inconclusive, and it was suggested that location may differ between tissues (84).

In the present study, the aim was to identify plasma membrane proteins common to leaves, xylem, and cambium/phloem of young poplar trees as well as proteins specific to these tissues. The leaves were essentially fully expanded at harvest, and the overlap with published transcript profile clusters (9, 16, 17) suggests sampling of xylem from the secondary cell wall-forming zone and cambium/phloem at earlier stages of cell wall development. One-third of the integral proteins and 44% of the soluble proteins were found in all three tissues (Fig. 2). These proteins, which are common to all tissues, may be regarded as housekeeping proteins providing “everyday service.” A typical example is the P-type H^+-ATPase, which creates the electrochemical gradient across the plasma membrane, driving secondary transport (IDs 1–15; Table I), and therefore should be present in virtually all living plant cells. However, many of the proteins only found in one (31%) or two (27%) tissues (Fig. 2) are not necessarily “tissue-specific” proteins. The identification of a protein by MS is partly dependent on protein abundance. Generally, highly expressed proteins will have a larger probability to be detected than lowly expressed proteins. For instance, there are very few ion channels identified because of their very high transport capacities and therefore low copy numbers. Still, tissue-specific is a useful expression if it is understood that it may often mean highly abundant rather than unique and that “absence” of a protein in a tissue often just reflects low abundance. Most of the proteins identified in the present study show tissue specificity, and the tissue distribution largely agrees with what is presently known about the activities in these tissues.

The leaf plasma membranes were characterized by a very high proportion of transporters, constituting almost half of the integral proteins (Fig. 3). This agrees well with the role of the leaves as the source of carbohydrates, amino acids, and other products of photosynthesis to the rest of the plant. Moreover, based on the number of peptides found, leaf plasma membranes harbor more P-type H^+-ATPase than the plasma membranes from the other two tissues, which is supported by the immunostaining in Fig. 1C. This reflects well the larger number of carriers (and hence the workload for the P-type H^+-ATPase in supporting secondary transport) recorded for the leaf plasma membranes compared with xylem and cambium/phloem plasma membranes (Table I).

Very few proteins were uniquely shared between the plasma membranes from leaves and the other two tissues, respectively (2–4% of total; Fig. 2), whereas xylem and cambium/phloem plasma membranes shared many proteins not found in leaf plasma membranes (21% of total; Fig. 2). This may partly be due to the fact that xylem and cambium/phloem are neighboring tissues with a diffuse border, which makes it difficult to obtain pure preparations of each tissue with the preparation procedure used by which these two tissues are simply torn apart. Few of the integral proteins specific to cambium/phloem plasma membranes have a known function that can be related to known functions of that tissue; the exception is the ABC transporters that are homologs of Arabidopsis ABCG11 (IDs 50–52, Fig. 5, Table I, and supplemental Table 2). In Arabidopsis, ABCG11 has a role in the secretion of cuticular lipids (47), which should be a function for a tissue close to the surface of the stem. This position may also be the reason for the relatively high proportion of receptors in the cambium/phloem plasma membranes (Figs. 3 and 4). Many of these receptors likely have a role in sensing changes in the environment, such as emerging pathogen attacks.

Proteins involved in cell wall and carbohydrate metabolism were most abundant in the xylem plasma membranes (Figs. 3 and 4) in agreement with the role of the xylem in wood formation. Coexpression of genes during secondary cell wall formation has identified a number of proteins likely to be involved in this process (12), and several of these proteins were also identified in this study, particularly in xylem plasma membranes (summarized in Fig. 6). These include a number of integral proteins, such as cellulose synthase, but also peripheral proteins, such as sucrose synthase suggested to provide the substrate, UDP-glucose, for cellulose synthase and callose synthase (35). The model in Fig. 6 also includes both actin filaments and microtubules. Thus, we detected an enrichment of both tubulin and actin in the stem tissues (supplemental Table 3), and notably, α-tubulin was only found in xylem and cambium/phloem plasma membranes and was “absent” in leaf plasma membranes. Using live cell imaging on intact roots of Arabidopsis, Wightman and Turner (85) recently
 further clarified the important relationship among microtu-
bules, actin, and the cellulose synthase complex during sec-
ondary cell wall formation. They found that bundles of micro-
tubules localized the cellulose synthase complex to the edges
of developing cell wall thickenings and that actin cables were
essential for the rapid trafficking of cellulose synthase com-
plex-containing "organelles" around the cell. Using a tobacco
cell culture and transgenic xylogenic cells, the secondary cell
wall and secretory proteome were recently analyzed (15). Of
the 109 proteins identified in multiple green plants, 26 soluble
proteins were similar to proteins identified in our study, in-
cluding vacuole-associated annexin, vacuolar H^+-ATPase,
14-3-3 protein, phospholipase D, phosphoglycerate kinase,
fructose-bisphosphate aldolase, glutathione peroxidase, me-
thionine synthase, malate dehydrogenase, and phosphoenol-
pyruvate carboxylase. Interestingly, one integral protein, a
callose synthase (Oryza sativa), was reported with a sequence
similarity closest to subset protein ID 237 (supplemental Table
2), although several proteins in the GT48 family (7) are closely
related. By BLAST-matching proteins identified in isolated
detergent-resistant plasma membrane microdomains (DRMs),
also from a tobacco cell culture (86), an overlap was found
with several GT48 proteins identified in the present study (IDs
242–244). Other cell wall-related proteins found in these
DRMs were similar to KORRIGAN (ID 255) and FLA10 (ID
1040). Novel data on hybrid aspen (P. tremula \times P. tremu-
loides) DRMs (87) showed that more than 70% of total glucan
synthase activities present in the original plasma membrane
preparation are associated with DRMs. Post-translational
processing of the enzymes seemed necessary for catalytic
activity, highlighting some of the difficulties in determining the
activities of callose (GT48) and cellulose synthases (GT2) (87).

In this study, we have largely focused on cell wall forma-
tion in xylem and less on phloem development. A recent
proteomics study of the pumpkin phloem sap identified
1121 proteins (88). Our study shows a 28% overlap with these
proteins by BLAST matching; all but three (IDs 177, 178, and
287) are non-transmembrane proteins. Among
their 45 most abundant and common phloem proteins, we
found 19 in leaf, 30 in xylem, and 32 in cambium/phloem
plasma membranes. We therefore conclude that few of
these proteins are highly enriched in cambium/phloem
plasma membranes compared with the xylem plasma mem-
branes. We found only a small number of proteins to be
enriched in cambium/phloem plasma membranes, such as
homologs to Arabidopsis FLA1 (IDs 1038 and 1039). Tran-
script data to support a specific cambium/phloem expres-
sion in Populus (89) for an FLA1 homolog, however, are not
conclusive. One protein only found in cambium/phloem
plasma membranes was a homolog to Arabidopsis lipoxy-
genase LOX1 (ID 319), a component of jasmonate signal
transduction, regulating a variety of processes (90). Jas-
monic acid has for instance been shown to induce rapid
changes in carbon transport and partitioning in Populus
(91), probably regulated by changes in phloem cell wall
structure and composition (92–94).

A large number of proteins involved in membrane trafficking
were recorded in the xylem plasma membranes (Figs. 3 and
4). This is in agreement with the important role of exocytosis
in cell wall formation where, e.g., matrix polysaccharides made
in the Golgi are delivered to the cell wall via secretory vesicles
(74). Indeed, judging from the number of peptides recovered
(Table I) and the presence of ADP-ribosylation factors (Fig.
1C), membrane trafficking was most intense in xylem and
least intense in leaves, which were essentially fully expanded
at harvest.

Of particular interest is the finding of almost all enzymes
involved in lignin biosynthesis in the xylem plasma mem-
branes. These are soluble proteins and have earlier been
located to the xylem but intracellularly to the cytosol, poly-
somes, endoplasmic reticulum, and the Golgi apparatus (95–
98). The organization of these enzymes into complexes was
suggested already in 1974 (99), and biochemical and genetic
data supporting the presence of complexes have been re-
viewed (100). Our data suggest that the lignin-forming en-
zymes may exist as a complex linked to the plasma
membrane, possibly in close proximity to a transporter trans-
locating lignin monomers across the plasma membrane. The
Arabidopsis ABC transporter ABCB15 has been suggested to
have such a role based on its expression profile, which closely
resembles those of known monolignol biosynthetic genes
(42), and its poplar homolog (ID 32) was exclusively found in
the xylem plasma membranes.

Analyses of genes coexpressed during wood formation
have identified many proteins likely to be involved in second-
ary cell wall formation (12). Several of these candidate pro-
teins were found in the present proteomics study (Fig. 6),
which identifies 25 integral proteins (Table I) and 83 soluble
proteins (supplemental Table 3) exclusively found in xylem
plasma membranes and thereby also provides additional can-
didates to the list of proteins putatively involved in wood
formation.

Acknowledgments—We thank Adine Karlsson for skillful technical
assistance and senior researcher Lars-Göran Stener at the Forestry
Research Institute of Sweden (Ekebo, Svalöv, Sweden) for the supply of
poplar trees. We are in debt to Professor R. Serrano (Universidad
Politecnica, Valencia, Spain), Professor P. S. Choorey (University of
Florida, Gainesville, Florida), and Professor P. Kjellbom (Lund Univer-
sitet, Lund, Sweden) for gifts of antisera.

* This work was supported by grants from the Swedish Foundation
for Strategic Research (to C. L) and the Formas excellence center
FUNCFIBER, Kempe foundation, Troedssons foundation (to G. W.).

§ The on-line version of this article (available at http://www. mcponline.org) contains supplemental Tables 2–5.

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