Establishment of a Protein Reference Map for Soybean Root Hair Cells[1][W][OA]

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Root hairs are single tubular cells formed from the differentiation of epidermal cells on roots. They are involved in water and nutrient uptake and represent the infection site on leguminous roots by rhizobia, soil bacteria that establish a nitrogen-fixing symbiosis. Root hairs develop by polar cell expansion or tip growth, a unique mode of plant growth shared only with pollen tubes. A more complete characterization of root hair cell biology will lead to a better understanding of tip growth, the rhizobial infection process, and also lead to improvements in plant water and nutrient uptake. We analyzed the proteome of isolated soybean (*Glycine max*) root hair cells using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and shotgun proteomics (1D-PAGE-liquid chromatography and multidimensional protein identification technology) approaches. Soybean was selected for this study due to its agronomic importance and its root size. The resulting soybean root hair proteome reference map identified 1,492 different proteins. 2D-PAGE followed by mass spectrometry identified 527 proteins from total cell contents. A complementary shotgun analysis identified 1,134 total proteins, including 443 proteins that were specific to the microsomal fraction. Only 169 proteins were identified by the 2D-PAGE and shotgun methods, which highlights the advantage of using both methods. The proteins identified are involved not only in basic cell metabolism but also in functions more specific to the single root hair cell, including water and nutrient uptake, vesicle trafficking, and hormone and secondary metabolism. The data presented provide useful insight into the metabolic activities of a single, differentiated plant cell type.

Root hairs are single, specialized, tube-shaped epidermal cells present on primary and secondary roots that function to increase root surface area. Root hairs improve the capacity of the root to absorb water and nutrients (NO_3^-, Cl^-, Ca^{2+}, K^+, Zn^{2+}, and Mn^{2+}) from the soil (Gilroy and Jones, 2000). Root hairs also represent the preferred infection site for rhizobia on legume roots. This symbiotic interaction results in the formation of a nodule in which bacteria fix atmospheric nitrogen that can be utilized by the plant (Stacey et al., 2006).

The formation of a single root hair cell from an epidermal cell can be divided into several stages, including cell fate specification, root hair initiation (which is characterized by the development of a protuberance in the cell wall), and tip growth (in which plasma membrane and cell wall materials are added), leading ultimately to maturity and functionality (Gilroy and Jones, 2000). The specialization of epidermal cells (trichoblasts) into root hair cells depends on the position of the epidermal cell relative to the underlying cortical cell (Dolan et al., 1994) and constitutes an excellent model in which to study cell differentiation. Many plant mutants with root hair development abnormalities (ectopic formation, limited number, size, growth abnormality) were linked to a range of genes, including transcription factors (Lee and Schiefelbein, 1999; Ishida et al., 2007), a kinase (Kieber et al., 1993), a cellulose synthase (Favery et al., 2001; Chul et al., 2007), components of the exocytosis complex (Wen et al., 2005), and cytoskeleton proteins (Webb et al., 2002; Ringli et al., 2005). The hormones ethylene and auxin also have critical roles in root hair formation (Masucci and Schiefelbein, 1994; Tanimoto et al., 1995; Ringli et al., 2005; Fischer et al., 2007).

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Jasmonates are also known to stimulate root hair formation in conjunction with ethylene (Zhu et al., 2006).

It is expected that proteomic analysis of root hairs will contribute to a better biological understanding of this important cell type. A limiting factor for root hair proteomic studies is acquiring root hair cells in sufficient purity and quantity to allow analysis. Fortunately, the larger root size of soybean (*Glycine max*) enables isolation of gram quantities of root hairs required for proteomic approaches. Soybean is also an important crop for animal feed, oil, and, more recently, for biodiesel production (Wilson, 2008). Over 390,000 EST sequences are available in public databases (http://www.ncbi.nlm.nih.gov/dbEST/). Previously, Wan et al. (2005) published a procedure for the isolation of purified soybean root hairs and showed that these preparations were suitable for proteomic analysis. That study identified only a few soybean root hair proteins that appeared to respond to inoculation with *Bradyrhizobium japonicum*. However, in other studies, soybean was successfully used to identify proteins involved in the response to pathogen invasion (Mithofer et al., 2002), salt stress (Aghaei et al., 2009), flooding (Shi et al., 2008), seed germination (Xu et al., 2006b), seed development (Hajduch et al., 2005), and UV radiation (Xu et al., 2008). Soybean peroxisomal proteins, xylem sap, and glycinins have also been characterized by proteomics (Natarajan et al., 2006, 2007; Djordjevic et al., 2007; Krishnan et al., 2007; Arai et al., 2008). A protein reference map was also developed for soybean leaves, identifying 119 proteins (Xu et al., 2006a).

Proteome reference maps have been developed for different plant tissues and cell cultures, but in general, these have come from only four plant species Arabidopsis (*Arabidopsis thaliana*), *Medicago truncatula*, rice (*Oryza sativa*), and maize (*Zea mays*). Arabidopsis proteome reference maps were established for cell cultures (Sarry et al., 2006; Baerenfaller et al., 2008), mature pollen (Holmes-Davis et al., 2005; Noir et al., 2005), seedlings (Charmont et al., 2005; Giavalisco et al., 2005), leaves (Giavalisco et al., 2005; Baerenfaller et al., 2008), roots (Baerenfaller et al., 2008), flowers (Baerenfaller et al., 2008), and siliques (Giavalisco et al., 2005). *M. truncatula* proteome reference maps were established for cell cultures (Watson et al., 2003; Imin et al., 2004; Lei et al., 2005), cell culture exudates (Kusumawati et al., 2008), roots (Mathesius et al., 2001; Watson et al., 2003), root nodules (Djordjevic et al., 2003), leaves (Watson et al., 2003), stems (Watson et al., 2003), flowers (Watson et al., 2003), and pods (Watson et al., 2003). A small protein proteome of *M. truncatula* and proteins regulated during the infection of *Melilotus alba* by *Sinorhizobium meliloti* were also studied (Natera et al., 2000; Zhang et al., 2006). Rice proteome reference maps were established for seeds (Koller et al., 2002), leaves (Koller et al., 2002; Nozu et al., 2006), stems (Nozu et al., 2006), roots (Koller et al., 2002; Nozu et al., 2006), etiolated and green shoots (Komatsu et al., 1999), and anthers (Imin et al., 2001). A comprehensive rice proteome database representing a variety of tissues was also developed (Komatsu et al., 2004; Komatsu and Tanaka, 2005). Maize proteome reference maps were established for endosperm (Méchin et al., 2004, 2007), roots (Hochholdinger et al., 2005), xylem sap (Alvarez et al., 2006), egg cells (Okamoto et al., 2004), leaves (Porubleva et al., 2001), and pericycle cells (Dembinsky et al., 2007).

Here, we have exploited the preparative technique described above to attain a deep investigation of the soybean root hair proteome. Whole cell preparations and microsome fractions were analyzed separately. We identified 1,492 proteins using two-dimensional (2D)-PAGE and two shotgun protein separation methods, 1D-PAGE-liquid chromatography (LC) and multidimensional protein identification technology (MudPIT). A reference map and database were constructed. The resulting information provides insight into the proteome of the root hair cell, a single, differentiated plant cell type.

RESULTS AND DISCUSSION

The aim of this experiment was to identify as many proteins as possible from soybean root hair cells. A number of factors were considered and are summarized here and elaborated upon later. First, we carefully prepared gram amounts of root hairs to ensure that sample amounts would not be limiting. Proteins were extracted with ionic and nonionic detergents to increase their solubility and enhance their detection. In some cases, microsomes were specifically prepared from whole cells as a way to increase the detection of membrane-spanning proteins. Second, we used 2D-PAGE and two shotgun separation methods, 1D-PAGE-LC and biphasic HPLC (i.e. MudPIT), to resolve a wide variety of proteins; each identifies different proteins inherent to the method (Lee and Cooper, 2006). 2D-PAGE is reproducible, in that gels of replicate samples can have superimposable patterns, and this enhances identification precision. By contrast, shotgun separation demonstrates more run-to-run variability but can lead to the identification of more proteins in a sample. Also, 2D gels are pI-range dependent, whereas shotgun separation methods (i.e. HPLC) are less restrictive. Used together, the methods improve proteomic analysis. We coupled each separation method with a mass spectrometry (MS) method compatible with the detergents used in extraction (Lee et al., 2007). Individual protein spots separated by 2D-PAGE were excised from the gel and their derivatized peptides analyzed by matrix-assisted laser-desorption ionization time of flight/time of flight (MALDI-TOF/TOF) MS, whereas proteins and their derivatized peptides separated by 1D-PAGE and LC/LC were analyzed en mass using an ion trap mass spectrometer. This created a situation in which some identified proteins and their peptides were physically associated with 2D gel spots, whereas the shotgun identifications of other proteins resolved by 1D-PAGE and MudPIT were bioinformatically inferred from a set of identified peptides. Separate analyses were performed as a re-

sult. Third, because the complete genome sequence of soybean is not yet available, all spectra were matched to peptide sequences from a database composed of translated ESTs from soybean. To make up for soybean sequence deficiencies, translated ESTs from the model legumes *M. truncatula* and *Lotus japonicus* and other protein sequences from a wide variety of plant species were included to exploit the power of sequence relatedness to resolve soybean spectra. This created many situations in which a soybean spectrum was matched to a protein from another organism. Together, the identifications made from protein spots and from peptides made via shotgun analysis were assembled and the meanings of these findings with respect to root hair biology were assessed.

2D-PAGE Analysis

The quality of the isolated soybean root hairs was estimated by light microscopy, as described by Wan et al. (2005). In all cases, little or no root contamination (e.g. root epidermal cells) was seen. This was further confirmed by the analysis of gene expression, in which genes specifically expressed in root hairs or in stripped roots (roots lacking root hairs) could be easily distinguished (data not shown).

Total proteins were extracted from the root hair preparations and separated by 2D-PAGE to generate a root hair proteome reference map (Fig. 1). Four biological replicates were performed, and the four gels showed very high similarity in terms of number and location of spots over the pI range of 4 to 7, suggesting that the root hair isolation, like the protein separation, was very reproducible. The digital gel image analysis matched 1,165 spots present in at least three of the four biological replicates, which again attests to the reproducibility. The pI range 4 to 7 was selected since it was previously shown by 2D gel electrophoresis (pI 3–10) that most of the root hair proteins have their pI values between 4 and 7 (Wan et al., 2005). Furthermore, in our strategy, we expect to identify high-pI proteins by shotgun proteomic approaches and not by 2D gel. The best gel was chosen as the master to establish the root hair proteome reference map (Fig. 1). Out of the 1,165 spots, 988 spots were excised in duplicates from two different gels, digested with trypsin, and analyzed by MS and tandem mass spectrometry (MS/MS). The visualization of the other spots on the gels was difficult and could not be picked accurately. These spots represent those proteins of least abundance.

The soybean root hair protein reference map was constructed using the SpotLink software (Hajduch et al., 2005) and includes clickable spots linked to the corresponding best match protein identification (molecular mass [MM], pI, accession number, and plant origin), score data (protein score, percentage of protein coverage, average ppm, ion score, number of significant ions, and matched and unmatched peaks), and 2D gel spot parameters (number, normalized volume, MM, and pI). This reference map also contains clickable links to the peptide mass fingerprint data, which includes mass spectrum (matched peaks to the best protein match in red and unmatched peaks in blue), and the peak list used for database search detailing the observed mass, the cluster area, and the signal-to-noise ratio. A clickable link is also available to access MS/MS data and includes mass spectra for which significant scores were obtained (no color code for the peaks). A protein score higher than 70 and a total ion score higher than 40 indicate a confidence in the protein identification higher than 95%. All of these data are available at http://www.soyroothair.org/ (follow research link → research progress link → SpotLink link).

Information gained from 2D-PAGE led to the identification of 660 proteins out of 988 analyzed spots (Supplemental Table S1), which equates to a success rate of 67% for protein identification. Other proteins were not identified due to the fact that the database is not complete or to the weak abundance of the proteins within the spots. The MM and pI of all identified proteins fell within a range of 15.2 to 163 kD and 4.1 to 7, respectively. None of these proteins contained more than a single, predicted transmembrane domain. This result is expected, since it is difficult to identify membrane proteins by 2D-PAGE (Braun et al., 2007; Rabilloud et al., 2007). Fifty seven proteins were predicted to have a signal peptide using SignalP-NN and SignalP-HMM (Supplemental Table S1) and, therefore, represent putative secreted proteins. Theoretical MM and pI of the best matched proteins were compared with those of the corresponding 2D-PAGE spots to assess the quality of the protein identification. The

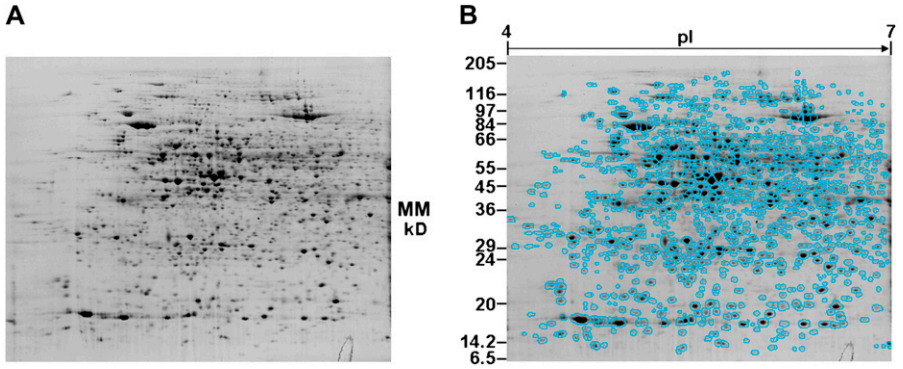

Figure 1. A, Colloidal Coomassie Brilliant Blue-stained 2D-PAGE gel of 600 μg of total protein extracted from soybean root hairs. B, The first dimension of protein separation was performed on an IPG strip (pH 4–7), and the second dimension was carried out on an 11% to 17% gradient SDS-PAGE gel. The images were analyzed using Phoretix software (Non linear dynamics), and the 988 spots matched in at least three biological replicates were excised for identification.

MM and pI of the majority of 2D-PAGE spots were consistent with the theoretical values of the identified proteins. About 75% of the gel spots had similar MM and pI (±20%) to the identified proteins. The differences observed for the other spots might be explained by the presence of a degraded form of the protein on the 2D gel, by different posttranslational modifications (PTMs), or by different amino acid sequences for matches occurring within different plant species. Proteins identified with translated ESTs were excluded from this analysis, since many of the sequences are truncated and the exact mass and pI of the sequence are unknown.

Among the 660 proteins, we identified 527 different proteins (different accession numbers), of which 437 were present in only one spot and 90 were present in several spots (minimum, two; maximum, eight). For example, Met synthase (gi|33325957|gb|AAQ08403.1|) and phosphoglucomutase (gi|12585330|sp|Q9ZSQ4|PGMU_POPTN) were identified in eight and four spots, respectively. Detection of isoforms encoded by distinct genes of a multigene family (different MM) and different PTMs of the same gene product (similar MM, different pI) can explain the presence of proteins with the same annotation but having different MM and pI. PTMs, such as phosphorylation, glycosylation, ubiquitylation, and methylation, are often involved in differential regulation; for example, protein activity and localization (Chen and Harmon, 2006; Rossignol, 2006). To our knowledge, no PTMs were already described for Met synthase. In contrast, PTMs of phosphoglucomutase, including ADP ribosylation, phosphorylation, and glycosylation, were already detected (Dey et al., 1994; Fu et al., 1995; Nomura et al., 1998).

Thirty-eight spots led to the identification of two different proteins. The resolution of 2D-PAGE was insufficient to separate proteins with similar MM and pI. The spot 2,295 mass spectrum (26,778 D, pI 5.5), for example, enabled the identification of a proteasome subunit (25,717 D, pI 5.53) and a glutathione *S*-transferase (25,893 D, pI 5.66).

Fifty-five percent of the gel spots had their best match with soybean proteins, 7% with Arabidopsis proteins, 8% with rice proteins, 3% with pea (*Pisum sativum*) proteins, 3% with *M. truncatula* proteins, and 24% with proteins from other plant species. In all, 660 protein identifications were made using sequences from 81 different plant species (Supplemental Table S1).

All of the proteins identified were classified into 16 different functional categories and subcategories, as described by Brechenmacher et al. (2008). Proteins involved in primary metabolism constituted the largest category (28.8%), followed by proteins implicated in protein synthesis and processing (18.8%), miscellaneous (9.8%), oxidation (6.4%), energy (5.8%), unknown (5.5%), secondary metabolism (5%), membrane (4.8%), signaling (4.5%), DNA/RNA (3.8%), cytoskeleton (2.6%), lipids (1.5%), stress (1.1%), defense (1.1%), and cell development (0.6%) categories (Fig. 2; Supplemental Table S1). The majority of categories were divided into

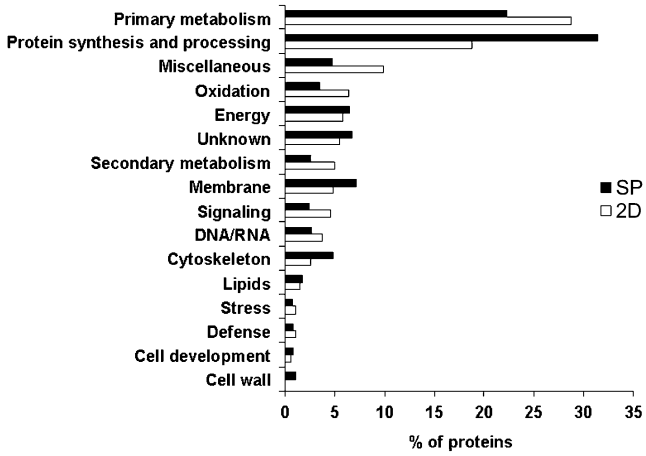

Figure 2. Distribution (by percentage) of proteins identified by shotgun proteomics (SP) and 2D-PAGE (2D) into 16 different functional categories. The protein distribution is expressed as a percentage of the total number of proteins identified by each approach.

subcategories to more accurately describe the function of the proteins (Supplemental Table S1). For example, the 124 proteins belonging to protein synthesis and processing categories were divided in chaperone (27.3%), degradation (31.5%), degradation inhibition (6.5%), folding (8.1%), ribosome (4%), and translation (22.6%) subcategories (Supplemental Table S1).

1D-PAGE-LC-MS/MS and MudPIT Shotgun Proteomics

Total root hair proteins were extracted with SDS to increase protein solubility and analyzed by a 1D-PAGE-LC-MS/MS method two times. Total root hair proteins were also extracted with a nonionic detergent and analyzed by MudPIT two times. To increase the detection of membrane-spanning proteins, root hair microsomes were extracted with a nonionic detergent and analyzed exclusively by MudPIT three times. For data analysis, total root hair protein spectra and microsome spectra were analyzed separately and also together in an effort to create a unified protein data set for root hair cells. The sum of all spectra obtained was 272,228, of which 154,395 were collected from microsomal proteins. For the three data sets, the spectra were searched with Mascot and the output analyzed with PANORAMICS, which assembled the matched peptide sequences into nonredundant protein complements using a probability model. Proteins exceeding a 95% confidence level were kept for further analysis. The unified protein data set contained 1,134 proteins or groups of proteins, and information on the peptide identification is summarized in Supplemental Table S2 (peptide sequence matches, Mascot scores, charge states of spectra, number of missed cleavages, peptide masses, peptide probabilities, and other relevant data). For each protein or group of proteins, sequence homology and annotation information, the protein group

probability, and the number of total and distinct peptides identified are indicated (Supplemental Tables S2 and S3). The analysis of the microsomal fraction led to the identification of 833 proteins, of which 443 were specific to this fraction. Total root hair protein analysis identified 613 proteins, of which 223 were specific to total proteins (Supplemental Table S3). The MM and pI of all the identified proteins fell within a range of 8.2 to 275.5 kD and 4.4 to 11.5, respectively. As described above, proteins identified with translated ESTs were excluded from this analysis. In all, 395 of 1,134 proteins were identified with a soybean record, and this was similar to the 2D-PAGE data set (Supplemental Table S3; Supplemental Fig. S1). In all, records from 186 different plant species were used in the identification (Supplemental Table S3).

All of the proteins identified were classified into the same 16 functional categories described above, with the following distribution: protein synthesis and processing (31.4%), primary metabolism (22.3%), membrane (7.1%), unknown (6.7%), energy (6.4%), cytoskeleton (4.9%), miscellaneous (4.7%), oxidation (3.4%), DNA/RNA (2.7%), secondary metabolism (2.6%), signaling (2.4%), lipids (1.8%), cell wall (1.1%), cell development (0.9%), defense (0.9%), and stress (0.8%; Fig. 2; Supplemental Table S3). The category distribution of proteins identified by the shotgun methods was similar to that identified by 2D-PAGE, except that no cell wall proteins were identified by 2D-PAGE (Fig. 2). One hundred twenty-seven proteins were predicted to have a signal peptide using SignalP-NN and SignalP-HMM (Supplemental Table S3).

The efficiency of the microsome isolation was first estimated by comparing functional categories of total and microsomal proteins. The proportion of proteins classified in the membrane category was 2.1 times higher for microsomal proteins than for total protein, suggesting an enrichment of membrane proteins (Supplemental Fig. S2). Cell wall, stress, and cytoskeleton protein distribution were also at least 2-fold different between microsomal and total proteins (Supplemental Fig. S2). The second approach was to predict transmembrane domains in microsomal and total proteins. More than two transmembrane domains were predicted in 45 proteins, of which 84% were specific to the microsomal fraction. These proteins are mainly involved in oxidative phosphorylation (H^+-ATPases), transport across the membrane (aquaporin, ATP-binding cassette [ABC] transporter), synthesis of cell wall cellulose (cellulose synthase), and proteins of unknown function (Supplemental Table S3). Furthermore, 70% of proteins annotated as transporters were specific to the microsomal fraction. All of these data suggest that the microsomal fraction was enriched in membrane proteins. Of the 148 identified peptides coming from proteins having three or more transmembrane domains, 97% were outside of transmembrane domains and 3% were partially within a transmembrane domain. None of the identified peptides was totally included within a transmembrane domain.

Comparison of 2D-PAGE and Shotgun Proteomic (1D-PAGE-LC-MS/MS + MudPIT) Results and Mapping of Identified Proteins to Metabolic Pathways

The same database was used to match proteins from 2D-PAGE, 1D-PAGE-LC-MS/MS, and MudPIT experiments. A total of 169 proteins were identified by both the 2D-PAGE and shotgun approaches. This represents an overlap of only 11%, which can be partially explained by the use of IPG strips (pH 4–7) for the 2D gel electrophoresis. This value is similar to the 18%, 10%, and 11% overlap obtained, respectively, for rice leaves, roots, and seeds (Koller et al., 2002) using similar approaches. The total number of root hair proteins (unique accession numbers) identified by combining all approaches is 1,492 (Supplemental Table S4). These proteins were also classified into functional categories, as described above (Supplemental Fig. S3). As expected, the largest number of identified proteins are predicted to be involved in the basal functions of the root hair cell. This is exemplified by the fact that the two major categories are protein synthesis and processing and primary metabolism (Supplemental Fig. S3).

Mapman was used to map 763 (i.e. roughly half of the identified proteins) root hair proteins onto metabolic pathways (no significant matches to Affymetrix probe identifiers were obtained for the other proteins; Supplemental Fig. S4). While the resulting picture is incomplete, it is helpful for gaining a global view of the functions of the root hair cell.

Proteins of Basal Metabolism

Many identified proteins were predicted to be involved in RNA transcription, RNA processing, protein synthesis (137 ribosomal proteins, 56 initiation and elongation translation factors), protein processing (101 chaperones), or protein degradation (Supplemental Fig. S4; Supplemental Table S4). The majority of proteins within the DNA/RNA category were predicted to play a role in nuclear organization (histone), RNA binding (which may affect its stability), processing RNA, or modifying secondary structure of RNA and DNA (helicases). We detected only a very small number of predicted transcription factors (Supplemental Table S4), probably due to the fact that such proteins are usually of low abundance and, therefore, not detectable by the methods used.

Root hair development and functioning may require posttranscriptional modifications and PTMs. One splicing factor and a single argonaute protein were identified, suggesting that posttranscriptional modifications occur in the root hair. Such RNA processing appears to be common during plant organ development (Wang and Li, 2007). Seven kinases and three phosphatases that presumably regulate protein phosphorylation were identified. Interestingly, one protein was annotated as a receptor kinase (Supplemental Table S4). This protein might have a critical role in sensing external stimuli. However, important receptor-

like kinases (RLKs) known to be present in root hair cells, such as the LysM-RLKs, essential for legume nodulation (Madsen et al., 2003; Holsters, 2008), were not identified in the root hair preparations. Again, this likely reflects a low relative abundance of these proteins. Protein quantity in a cell is regulated by both de novo synthesis and degradation. Eighty-two proteins predicted to be involved in protein degradation were identified, including proteases and different subunits of the proteasome or ubiquitin-conjugating complexes (Supplemental Table S4).

The root hair proteins identified spanned all aspects of energy production (mitochondrial electron transport, H^+-ATPases), lipid metabolism (fatty acid synthesis), and primary metabolism (glycolysis, tricarboxylic acid cycle, amino acid, nucleotides; Fig. 3; Supplemental Table S4).

Proteins of Secondary Metabolism

Plants have complex pathways for secondary metabolism, and these pathways are well represented in the root hair cell. For example, proteins involved in lignin, flavonoid, and phenylpropanoid synthesis were identified, including Phe ammonia lyase, chalcone reductase, chalcone synthase, chalcone isomerase, caffeoyl-CoA *O*-methyltransferase, isoflavone synthase, and isoflavone reductase (Fig. 3; Supplemental Fig. S4; Supplemental Table S4). Phe is the precursor of phenylpropanoids, flavonoids, isoflavonoids, and lignin biosynthetic pathways. These pathways lead to the formation of many secondary metabolites having various biological functions, including light absorption (anthocyanin pigment), UV protection (flavonoids), vascular tissue formation (lignin), defense against biotic stress (phytoalexin), and establishment of the symbiosis with rhizobia

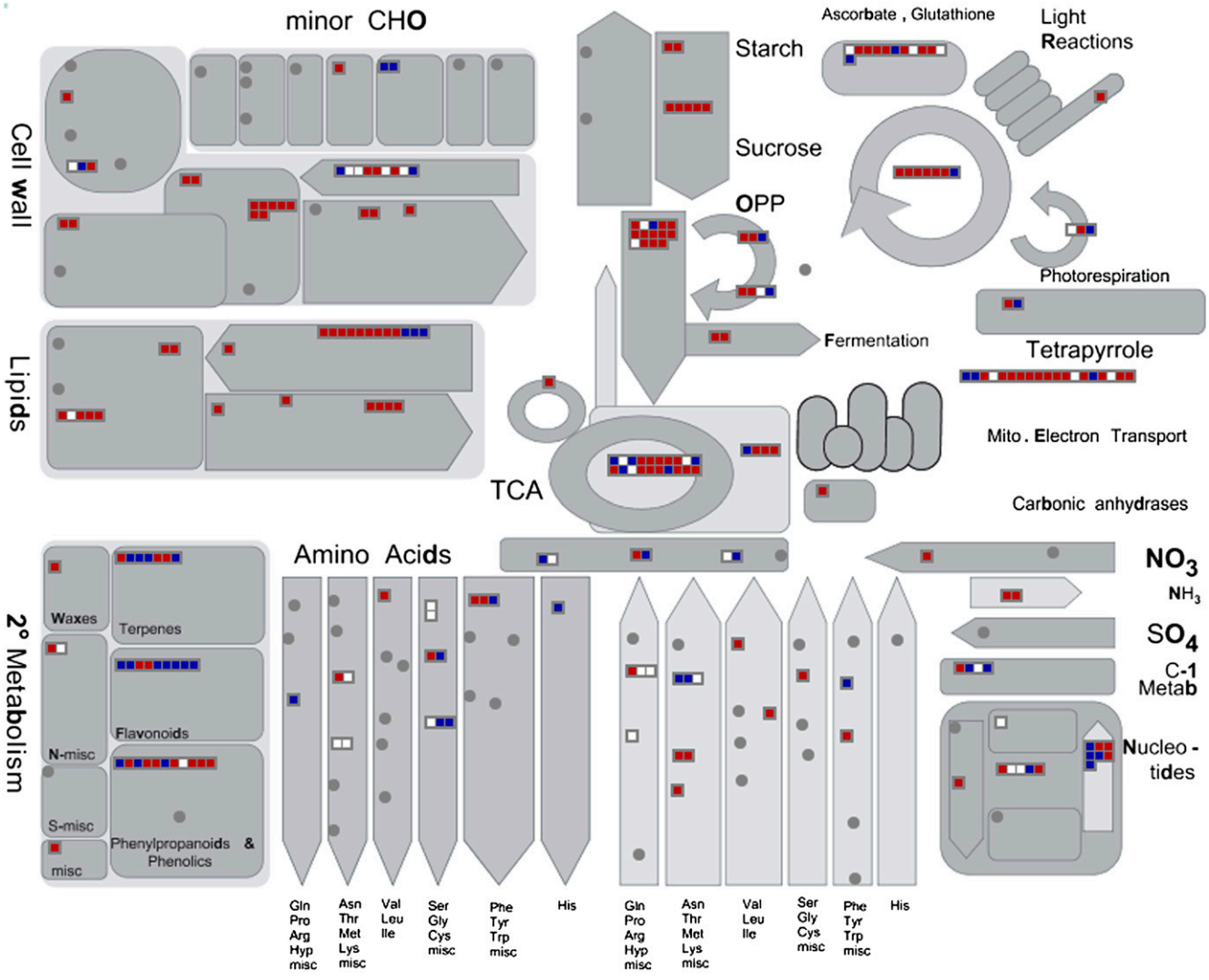

Figure 3. Visualization of metabolism overview of root hair proteins using Mapman (Thimm et al., 2004). Each small square represents a protein. Red, blue, and white squares correspond, respectively, to proteins identified only by shotgun proteomics, only by 2D-PAGE, and by both approaches. Gray circles indicate that no proteins of this study matched the pathway of Mapman. OPP, Oxidative pentose phosphate.

(flavonoids, isoflavonoids; Subramanian et al., 2007; Ververidis et al., 2007; Ferrer et al., 2008). With regard to the latter role, the products of the phenylpropanoid pathway play very important roles during establishment of the rhizobial-legume symbiosis either by serving as important signals to the invading symbiont or by modulating internal auxin transport (reviewed in Subramanian et al., 2007). Identification of the proteins involved in these complex secondary metabolic pathways will allow further investigation of their specific biological roles.

Proteins Involved in Signaling and Hormone Action

Many of the proteins identified in this study may be part of the adaptive response of the plant to its environment and, therefore, are proteins involved in signal transduction, defense/stress reaction, or hormone synthesis. Signal transduction proteins identified include 14-3-3 proteins, annexins, G proteins, and phospholipases. Betaine aldehyde dehydrogenase, dehydration-responsive proteins, pathogenesis-related proteins, chitinase, disease resistance proteins, and hypersensitivity-induced proteins are probably part of the basic spectrum of proteins that enable the plant to initially resist biotic and abiotic stresses (Supplemental Table S4).

Proteins known to be involved either in the response to hormone addition or in hormone synthesis were also detected (Table I; Supplemental Fig. S4; Supplemental Table S4). Ethylene and auxin both stimulate root hair formation and modulate positioning (Fischer et al., 2007; Dugardeyn and Van Der Straeten, 2008; Li et al., 2008). The enzymes needed for ethylene synthesis (i.e. *S*-adenosylmethionine synthetase, 1-aminocyclopropane-1-carboxylate synthase) were identified in the root hair preparations, as well as some proteins known to be responsive to ethylene treatment (i.e. ER6 protein; Table I; Supplemental Table S4). Similarly, one auxin-responsive protein was detected in the root hair preparations.

Jasmonate is involved in signaling in response to biotic and abiotic stress but also functions in flower and root growth or in senescence (Wasternack, 2007). Jasmonate also affects glandular trichome development in tomato (*Solanum lycopersicum*) and increases root hair density in Arabidopsis (Li et al., 2004; Zhu et al., 2006), clearly showing a role for this hormone in the differentiation of epidermal cell-derived organs. Our data suggest that jasmonates can be directly produced by the root hair cell, as evidenced by the identification of 12-oxophytodienoic acid 10,11-reductase and lipoxygenase implicated in jasmonate synthesis (Table I; Supplemental Table S4). Signaling between the root hair cell and the invading rhizobia is an essential step in the establishment of the symbiosis. For example, the products of the phenylpropanoid pathway are critical signals (Subramanian et al., 2007). Jasmonate is also known to be recognized by the invading symbiont (Mabood et al., 2006). The fact that soybean root hair cells may have the capacity to produce this hormone lends credence to its possible role in the rhizobial infection mechanism.

Brassinosteroid is a hormone that regulates plant growth by affecting cell elongation and proliferation. For example, this phytohormone is involved in vascular differentiation, seed germination, root development, senescence, and the response to biotic and abiotic stress (Asami et al., 2005; Bajguz, 2007). The brassinosteroid signal transduction pathway was partially characterized (Gendron and Wang, 2007), and its physiological cooperation with auxin was proposed (Hardtke et al., 2007). Squalene synthase, *S*-adenosyl-L-Met:Δ24-sterol-*C*-methyltransferase, and 2′-hydroxydihydrodaidzein reductase are examples of proteins identified in this study that are involved in the synthesis of brassinosteroid (Table I; Supplemental Table S4). Thus, the root hair proteome reveals that this single, differentiated cell has a broad capacity to synthesize various hormones and/or to respond to their presence.

Proteins Involved in Water and Nutrient Uptake

Root hairs increase the root surface area, permitting increased water and nutrient uptake. Consistent with these roles, the proteomic analysis revealed a variety of predicted transport proteins. Aquaporins, including two plasma membrane intrinsic proteins (PIP1,5 and PIP2,2), were identified (Table I; Supplemental Table S4). Root hairs are involved in the uptake of soil phosphorus (Bates and Lynch, 2000) and calcium (Very and Davies, 2000). The corresponding transporters of these elements were identified in the root hair preparations (Table I; Supplemental Table S4). Likewise, ABC transporters and a lipid transfer protein were detected in this study and were also shown previously to be present in Arabidopsis and *Vigna unguiculata* root hairs, respectively (Krause et al., 1994; Kim et al., 2007). While uptake of ammonium, chloride, nitrate, manganese, and zinc by root hairs was described previously (Gilroy and Jones, 2000), our proteomic analysis did not identify transporters for these compounds. These transporters might be present at too low a level to be detected, or the extraction procedures might not be sufficient to enable their detection. In contrast, expression of the adenine nucleotide translocator and a dicarboxylate/tricarboxylate carrier was not demonstrated previously in root hairs but was found in the soybean root hair preparations. The energy required to translocate nutrients from the soil into the root hair is generated by an electrochemical gradient established by H^+-ATPase activities, some of which were previously shown to be localized in root hair cells (Moriau et al., 1999). Indeed, many predicted H^+-ATPases were identified in soybean root hairs and probably have a role in generating the energy required for nutrient uptake (Supplemental Fig. S4; Supplemental Table S4).

Table I. *Soybean root hair proteins involved in membrane transport and hormone signaling*

The accession number, the organism of the best hit, the method by which they were identified (SP, shotgun proteomics on total protein; Mm, MudPIT on microsomal protein; 2D, 2D-PAGE), and the number of predicted transmembrane domains (TMHMM) are also indicated. The transmembrane domains were not defined (ND) on ESTs.

Protein Identifier	Accession No.	Organism	SP	Mm	2D	TMHMM
Membrane/transport						
AAA ATPase	gi|92877793|gb|ABE84767.1|	*Medicago truncatula*	No	Yes	No	13
ABC transporter	gnl|UG|Gma#S21566214	*Glycine max*	No	Yes	No	ND
ABC transporter	gi|92878433|gb|ABE85045.1|	*Medicago truncatula*	No	Yes	No	17
ABC transporter homolog	gi|7573600|dbj|BAA94511.1|	*Populus nigra*	Yes	Yes	No	0
Adenine nucleotide translocator	gi|2780194|emb|CAA05979.1|	*Lupinus albus*	No	Yes	No	3
Adenine nucleotide translocator	gnl|UG|Gma#S23072301	*Glycine max*	No	Yes	No	ND
ADP-ATP carrier-like protein (putative)	gi|84468306|dbj|BAE71236.1|	*Trifolium pretense*	No	Yes	No	3
Ca^{2+}-ATPase (plasma membrane)	gi|11066056|gb|AAG28436.1|AF195029_1	*Glycine max*	No	Yes	No	8
Calcium-transporting ATPase 4	gi|12643934|sp|Q9XES1|ECA4_ARATH	*Arabidopsis thaliana*	No	Yes	No	8
Dicarboxylate/tricarboxylate carrier	gi|19913109|emb|CAC84547.1|	*Nicotiana tabacum*	Yes	Yes	No	0
HvPIP1;5	gi|6692753|dbj|BAA23746.2|	*Hordeum vulgare*	Yes	Yes	No	6
Importin-α (putative)	gi|84453224|dbj|BAE71209.1|	*Trifolium pretense*	No	Yes	No	0
Importin-α 2	gi|13752562|gb|AAK38727.1|AF369707_1	*Capsicum annuum*	No	No	Yes	0
Lipid transfer protein	gnl|UG|Gma#S22952700	*Glycine max*	Yes	No	No	ND
Mitochondrial carrier protein	gnl|UG|Gma#S23072010	*Glycine max*	Yes	Yes	No	ND
Mitochondrial voltage-dependent anion-selective channel	gi|67848430|gb|AAY82249.1|	*Phaseolus coccineus*	No	Yes	No	0
Mitochondrial voltage-dependent anion-selective channel	gnl|UG|Gma#S4969810	*Glycine max*	No	Yes	No	ND
Mitochondrial voltage-dependent anion-selective channel	gnl|UG|Gma#S23062913	*Glycine max*	Yes	Yes	No	ND
MRP-like ABC transporter	gi|27263148|emb|CAD59448.1|	*Oryza sativa*	No	Yes	No	14
NLS receptor	gi|3273245|dbj|BAA31166.1|	*Oryza sativa*	No	No	Yes	0
PDR-type ABC transporter 1	gi|41052472|dbj|BAD07483.1|	*Nicotiana tabacum*	No	Yes	No	13
Phosphate transport protein G7	gi|7488693|pir||T05707	*Glycine max*	No	Yes	No	
Phosphate transporter	gi|18252510|gb|AAL66293.1|AF452453_1	*Glycine max*	No	Yes	No	0
PIP2,2	gi|62546339|gb|AAX86046.1|	*Glycine max*	Yes	No	No	6
VDAC2.1	gnl|UG|Gma#S22951075	*Glycine max*	No	Yes	No	ND
Mitochondrial import inner membrane translocase	gnl|UG|Gma#S5136170	*Glycine max*	No	No	Yes	ND
Protein transporter	gnl|UG|Gma#S21539204	*Glycine max*	No	No	Yes	ND
Protein transporter	gnl|UG|Gma#S23067564	*Glycine max*	No	No	Yes	ND
Signaling/hormone						
Progesterone-binding protein-like	gnl|UG|Mtr#S5299552	*Medicago truncatula*	No	Yes	No	ND
Progesterone-binding protein-like	gnl|UG|Lco#S19428065	*Lotus japonicus*	No	Yes	No	ND
Auxin-induced protein	gnl|UG|Gma#S23068301	*Glycine max*	No	No	Yes	ND
2′-Hydroxydihydrodaidzein reductase	gi|2687726|emb|CAA06028.1|	*Glycine max*	No	No	Yes	0
Brassinosteroid biosynthetic protein LKB	gnl|UG|Gma#S22952285	*Glycine max*	No	Yes	No	ND
Dehydrogenase-like protein (3-β-hydroxysteroid)	gnl|UG|Gma#S21565293	*Glycine max*	No	Yes	No	ND
Isopentenyl pyrophosphate isomerase	gi|35186998|gb|AAQ84167.1|	*Pueraria montana*	No	No	Yes	0
Mevalonate disphosphate decarboxylase	gnl|UG|Gma#S23070796	*Glycine max*	No	No	Yes	ND
S-Adenosyl-L-Met:Δ24-sterol-*C*-methyltransferase	gi|1399380|gb|AAB04057.1|	*Glycine max*	No	Yes	Yes	0
Squalene synthase	gi|2463569|dbj|BAA22559.1|	*Glycine max*	No	Yes	No	2
Sterol-*C*-methyltransferase	gnl|UG|Lco#S19430120	*Lotus japonicus*	No	Yes	No	ND
Sterol-*C*-methyltransferase	gnl|UG|Gma#S23068826	*Glycine max*	No	Yes	No	ND
Sterol-*C*-methyltransferase (putative)	gi|23397216|gb|AAN31890.1|	*Arabidopsis thaliana*	No	Yes	No	1
1-Aminocyclopropane-1-carboxylate synthase	gnl|UG|Gma#S23070964	*Glycine max*	No	No	Yes	ND
ER6 protein	gnl|UG|Gma#S23067712	*Glycine max*	No	No	Yes	ND
Ethylene-responsive protein	gnl|UG|Gma#S6667979	*Glycine max*	No	No	Yes	ND
12-Oxophytodienoic acid 10,11-reductase	gnl|UG|Gma#S23069937	*Glycine max*	No	No	Yes	ND

Proteins That May Be Involved in Root Hair Polar Growth

Sixty-four identified proteins were predicted to be associated with membranes. For example, these include proteins involved in vesicle trafficking (e.g. five ADP-ribosylation factors, four clathrins, 10 coatomer subunits, seven dynamins, and 22 GTP-binding proteins; Supplemental Table S4). Vesicle-trafficking proteins are essential for root hair polar growth due to their role in adding new plasma membrane and cell wall components to the growing tip. They are also involved in the recycling of these compounds by endocytosis (Samaj et al., 2006; Campanoni and Blatt, 2007). Tip growth was demonstrated to be highly regulated by small GTP-binding proteins, since mutants in RAB and ADP ribosylation factor exhibit abnormal root hair development (de Graaf et al., 2005; Xu and Scheres, 2005; Song et al., 2006). Such proteins were also identified in soybean root hairs.

The cytoskeleton is critical for root hair growth. Small GTP-binding proteins regulate the organization of actin filaments (Fu et al., 2002; Bloch et al., 2005; de Graaf et al., 2005). This study identified actins, which can play an important role in tip growth of the root hair cell (Voigt et al., 2005; Supplemental Table S4). For example, a mutant defective in actin synthesis also showed defective root hair development (Ringli et al., 2002). Furthermore, overexpression of an actin-interacting protein led to the disorganization of the actin cytoskeleton, resulting in the formation of thicker and shorter root hairs in comparison with the wild type (Ketelaar et al., 2007). Tubulins, detected in this study, are also involved in cytoskeleton function and likely also have critical roles in root hair development (Supplemental Table S4).

Proteins Involved in the Root Hair Cell Wall

Cell walls represent an extracellular matrix that structurally supports and shapes cells. They also constitute a physical barrier to pathogens (Humphrey et al., 2007; Huckelhoven, 2008) and are modified during the nitrogen-fixing symbiosis (Brewin, 2004). Cell walls are composed of polysaccharides (cellulose, hemicellulose, pectins) and structural and catalytic proteins (Galway, 2006; Jamet et al., 2006, 2008). We identified cellulose synthases that likely participate in the synthesis of the root hair cell wall (Supplemental Table S4). Cellulose synthases were shown to be critical in root hair development, since mutations in these genes result in plants that can initiate root hair development but the root hair cells cannot elongate (Favery et al., 2001; Wang et al., 2001; Chul et al., 2007). Root hair expansion requires cell wall loosening, which might be accomplished by proteins identified in this study, including endo-1,4-β-glucanase, xyloglucan endotransglycosylase, and pectinesterase, which target cellulose, xyloglucan, and pectin, respectively (Ohmiya et al., 2000; Marin-Rodriguez et al., 2002; Nishitani and Vissenberg, 2007; Pelloux et al., 2007).

CONCLUSION

Root hair cells represent a single, terminally differentiated plant cell type. The ability to isolate these cells in sufficient quantity and purity for functional genomic studies opens the possibility of a systems-level view of the interworkings of a single plant cell. While such studies are possible with plant tissue culture cells, these cells represent a highly artificial condition. The root hair proteome reference map provides a useful survey of the potential metabolism and regulatory pathways at work in this cell. As such, it presents a good foundation for further studies that will incorporate the proteome, transcriptome, and metabolome, with the eventual goal of a full understanding of the root hair cell. These studies will provide important insight into very important plant processes that may address important agronomic issues, such as water stress, nutrient uptake, and the establishment of the symbiotic association with rhizobia.

MATERIALS AND METHODS

Root Hair Isolation

Soybean seeds (*Glycine max* 'Williams 82') were surface sterilized twice for 10 min each in 20% bleach, rinsed five times in sterile water, soaked for 10 min in 0.1 N HCl, rinsed five more times in sterile water, and air-dried for 20 min. Sterilized seeds were sown in nitrogen-free B and D agar medium (Broughton and Dilworth, 1971) and incubated in a dark growth chamber (25°C, 80% humidity) for 3 d. About 1,500 soybean roots per experiment were frozen in liquid nitrogen and gently stirred for 20 min to break off root hairs from roots. Root hairs were separated from roots by filtering through a wire mesh, weighed (average of 1 g), and stored at −80°C until protein extraction. As shown previously (Wan et al., 2005), this procedure for root hair isolation results in preparations largely free of any other root cell contamination.

2D-PAGE

Protein Extraction

Proteins were extracted from 1 g of root hair by adding 2.5 mL of Tris-buffered phenol, pH 8.8, and 2.5 mL of extraction solution (0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, and 0.9 M Suc) to the root hairs. Root hairs were sonicated for 15 min in a water bath, agitated on a shaker for 30 min at 4°C, and centrifuged at 5,000*g* for 20 min at 4°C. The top (phenol) layer was transferred to a new tube. A second phenol extraction of the root hairs was performed as described above. The two phenol phases were then combined, back extracted with 1 volume of extraction solution, and precipitated overnight at −80°C with 0.1 M ammonium acetate in 100% methanol (10 volumes). Proteins were collected by centrifugation (4,000*g*, 30 min, 4°C), and the pellet was washed twice in 0.1 M ammonium acetate in methanol containing 10 mM dithiothreitol (DTT) and twice in 80% acetone containing 10 mM DTT. The pellet was resuspended in 100 μL of isoelectric focusing (IEF) buffer (8 M urea, 2 M thiourea, 4% CHAPS, 2% Triton X-100, 100 mM DTT, and 2% pH 4–7 ampholytes). Proteins were quantified using an EZQ protein quantification kit (Molecular Probes, Invitrogen Detection Technologies).

Protein Separation, Gel Staining, and Analysis

2D-PAGE was performed as described by Mooney et al. (2004). Briefly, for the first dimension, IPG strips (linear pH 4–7 gradient, 24 cm; GE Healthcare BioSciences) were rehydrated with 600 μg of total protein and then separated using a Protean IEF cell (Bio-Rad). Proteins were reduced and alkylated in the strip, which was loaded onto gradient acrylamide gels (11%−17%, w/v) for the second dimension separation.

Proteins were detected after 12 h of colloidal Coomassie Brilliant Blue staining (20% ethanol, 1.6% phosphoric acid, 8% ammonium sulfate, and 0.08% Coomassie Brilliant Blue G-250). Gels were destained in MilliQ water and scanned to obtain 16-bit TIF images that were used for analysis. The images were analyzed using Phoretix software version 2005 (Non linear dynamics). A gel was run for each biological replicate with a total of four replicates. The spots were detected on the four gels and manually edited to erase false positives. The background was then subtracted (mode of non spot), and the spots of all gels were matched. The matching efficiency was checked and manually edited when necessary. The total spot volume method was used for normalization. A gel reference map was created and harbored all spots that were detected in at least three of four biological replicates. For each spot, pI values were determined and MM was estimated by comparison with a wide range of MM markers (Sigma-Aldrich).

In-Gel Tryptic Digestion

Spots of interest were manually excised from the gels using a 1.5-mm spot picker (Gel Company). In-gel tryptic digestion was adapted from Mooney et al. (2004, 2006). Gel plugs were washed four times for 15 min each in acetonitrile:50 mM ammonium bicarbonate (50:50, v/v). Gel plugs were then briefly dried in 100% acetonitrile, incubated for 20 min in 100% acetonitrile, and completely air dried. They were rehydrated with a trypsin solution (20 units; Promega) and incubated for 2 h at 4°C. The trypsin solution was then removed and replaced by 15 μL of 40 mM ammonium bicarbonate and 10% acetonitrile. Trypsin digestion was performed overnight at 37°C. The samples were briefly centrifuged, and peptides were transferred to a new tube containing 4 μL of acetonitrile:MilliQ water:trifluoroacetic acid (60:39:1, v/v). Gel plugs were washed twice with the same solution for 10 min, and peptides were collected and transferred to the tubes, as described above. Samples were then lyophilized and reconstituted in 5 μL of acetonitrile:MilliQ water:88% formic acid (50:49:1, v/v).

MALDI-TOF MS, MS/MS, and Database Searching

Tryptic peptides (0.5 μL) were mixed with 0.5 μL of α-cyano-4-hydroxycinnamic acid (Fluka MS grade; Sigma-Aldrich) solution (5 mg mL^{-1} in acetonitrile:MilliQ water:10% trifluoroacetic acid:100 mM ammonium dihydrogen phosphate [50:38:2:10, v/v]). A minimum amount of phosphate was added to reduce matrix cluster, which improves peptide intensity as well as signal-to-noise ratio (Zhu and Papayannopoulos, 2003). The sample/matrix (0.3 μL) mix was deposited on a stainless-steel plate (ABI01-192-6-AB; Applied Biosystems). The analysis of the tryptic peptides was carried out on an Applied Biosystems 4700 MALDI TOF/TOF MS system with a 355-nm (200-Hz) laser in positive ion delayed extraction reflector mode. The calibration was performed using ABI peptide standards (4700 Mass Standards Kit; product no. 4333604). Both MS and MS/MS spectra were acquired for each spot. The peak list was generated using GPS Explorer software (version 3.0; Applied Biosystems). Spectra were analyzed using GPS Explorer software and Matrix Science's Mascot search engine (www.matrixscience.com) against the plant protein database from www.Arabidopsis.org (474,227 records; July 1, 2006 release) as well as translated soybean, *Lotus japonicus*, and *Medicago* species ESTs from The Institute for Genomic Research (42,799 combined records; June 19, 2006 release). The database from translated ESTs was created according to Cooper et al. (2007). Search parameters included a mass range from 700 to 4,000 D for the precursor ions, a maximum of 100 ions per MS spectrum with a signal-to-noise ratio higher than 20, a mass error of 100 ppm for the monoisotopic precursor ions, carbamidomethylation of Cys and Met oxidation, respectively, as fixed and variable modifications, a peak density filter of 50 peaks per 200 D, a maximum of one allowed miscleavage by trypsin, and an exclusion of peptide masses corresponding to the autolysis of the trypsin (100 ppm). For the MS/MS peak filtering, the search parameters included a mass range from 60 to 20 D below each precursor mass, a maximum of 100 ions per spectrum with a signal-to-noise ratio higher than 10, and the same peak density filter described above. Protein identification was considered significant based on peptide mass fingerprint data only if the Mascot score was confident (>95%), at least four peaks matched the protein, and a percentage of coverage higher than 10% was obtained. Based on MS/MS data, only protein identifications exceeding a 95% confidence level were accepted.

Shotgun Proteomics

Protein Extraction

Total Protein. Root hairs were resuspended in 5 mL of 10% TCA in acetone, sonicated for 20 min in a water bath, and precipitated overnight at −20°C. The precipitate was centrifuged at 20,000*g* for 1 h at 4°C. The pellet was washed three times in 100% acetone using the same parameters of centrifugation described above and dried. The precipitate was resuspended in 8 M urea, 2% dodecyl-β-maltoside, and 100 mM Tris-HCl, pH 8.5. Proteins were precipitated with 25% TCA, washed in acetone, and resolubilized with 8 M urea and 100 mM Tris-HCl, pH 8.5. The supernatants were then passed through 5.0- and 0.45-μm polyvinylidene difluoride membranes (Millipore).

Microsomal Fraction. Root hairs were sonicated in a water bath for 20 min in 0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, 0.4% β-mercaptoethanol, and 250 mM Suc. The cell wall debris was removed by centrifugation at 2,000*g* for 3 min at 4°C. The organelles were removed by centrifugation at 20,000*g* for 30 min at 4°C. The microsomal fraction was then obtained by centrifugation at 100,000*g* for 1 h at 4°C. This pellet was solubilized in 25 μL of 0.1 M Tris-HCl, pH 8.5, 8 M urea, and 2% dodecyl-β-maltoside. Proteins were precipitated using 25% TCA, washed in acetone, and resolubilized with 8 M urea and 100 mM Tris-HCl, pH 8.5. The supernatants were then passed through 5.0- and 0.45-μm polyvinylidene difluoride membranes (Millipore). Proteins were quantified using the bicinchoninic acid protein assay kit (Pierce Biotechnology).

Protein Separation and MS Analysis

1D-PAGE. Total proteins (50 μg) were separated by 1D-PAGE as described by Lee et al. (2007). Briefly, the gel was stained with Coomassie Brilliant Blue. The lane with the proteins was excised and divided into 32 pieces. No attempt was made to correlate gel slice positioning with the migration of proteins and their MM. Proteins on the gel slices were reduced, carbamidomethylated, and digested with trypsin. Peptides were extracted accordingly. Five microliters of peptide sample was loaded by an autosampler onto a ThermoFisher Scientific Biobasic-18, 100- × 0.18-mm column with 5 μm of reverse-phase packing material. A 30-min linear gradient from 5% to 60% acetonitrile (water acidified with 0.1% formic acid) was used to elute peptides at a flow rate of 3 mL min^{-1}. Peptides were microsprayed into the orifice of an LCQ Deca XP Plus quadrupole ion trap mass spectrometer (Thermo) operating in the MS/MS mode with Xcalibur 1.3 software (Thermo). A parent-ion scan was performed over the range 400 to 1,600 mass-to-charge ratio, and automated peak recognition, dynamic exclusion, and MS/MS ion scanning of the top three most intense parent ions were controlled by Xcalibur. This experiment was performed twice on root hair total protein.

MudPIT. For MudPIT, we used the method described by Lee et al. (2007). Briefly, total root hairs or microsomal proteins were reduced, carbamidomethylated, and digested with trypsin. Peptides were separated on homemade biphasic columns prepared from 365-μm (o.d.) × 75-μm (i.d.) fused silica with a 5-μm tip and packed with reverse-phase C18 resin followed by strong cation-exchange resin (Yates et al., 1996; Gatlin et al., 1998). Separation followed a 12-step elution procedure consisting of step-wise increasing concentrations of salt solution followed by increasing gradients of organic mobile phase (Washburn et al., 2001). Solvent flow of 200 nL min^{-1} was controlled with a Surveyor HPLC pump (Thermo Fisher Scientific) and a T-split junction where 1,800 V of electricity was also applied (Link et al., 1999). The eluent was electrosprayed directly into the orifice of an LCQ-Deca XP ion-trap mass spectrometer (Thermo Fisher Scientific) controlled by Xcalibur 1.3 software (Thermo Fisher Scientific). A parent-ion scan was performed over the range of 400 to 1,600 mass-to-charge ratio, and MS/MS was performed on the three most intense parent ions. Automated peak recognition and dynamic exclusion were enabled. The experiment was performed twice on total proteins and three times on microsomal proteins.

MS/MS Spectral Analysis. MS/MS spectra were extracted from the raw data with Bioworks 3.2 (Thermo) using the parameters 400 to 3,500 mass range, one group scan, one minimum group count, and 15 minimum ion counts. Three spectral data files were created. All microsomal spectra from the MudPIT replicates were combined in a file, and all total root hair spectra from the MudPIT and 1D-PAGE-LC-MS/MS replicates (all gel slices and all elution steps) were combined in a single file. Then, a unified file was created. Spectra were searched with Mascot 2.1 against the database described above. Search parameters were for tryptic digests, one possible missed cleavage, fixed amino acid modification (+57, C), averaged mass values, ±1.5-D parent ion mass

tolerance, and ±0.8 D fragment ion mass tolerance. Mascot output was processed by PANORAMICS, a probability-based program that determines the likelihood that peptides are correctly assigned to proteins (Feng et al., 2007). PANORAMICS first considers all peptide matches made by Mascot and calculates the probability that these matches are correct. Then, when computing the probability of a protein or group of proteins matched with the same set of identified peptide sequences, PANORAMICS takes into account the probabilities for both distinct peptides and shared peptides in a coherent manner and distributes the probabilities of shared peptides among all related proteins. The Mascot ion score, the database size, and the length and charge state of each peptide sequence are incorporated into the probability model. Only protein identifications exceeding a 95% confidence level with at least two peptides were accepted. The probability that a protein identification is not correct (false-positive rate) is 1 minus the calculated protein probability and agrees with false-positive rates that can be deduced by reverse database searching (Feng et al., 2007).

Reference Map, Transmembrane Domain, and Mapman Analysis

The reference map was built using SpotLink software, which is available at http://digbio.missouri.edu/SpotLink/. The reference map is available at http://www.soyroothair.org/ (follow research link → research progress link → SpotLink link). The transmembrane domains of proteins were predicted using TMHMM server version 2.0. (Krogh et al., 2001). The signal peptide domains were predicted using SignalP server version 3.0 (http://www.cbs.dtu.dk/services/SignalP/). A TBLASTN search was performed on protein sequences to identify corresponding Arabidopsis (*Arabidopsis thaliana*) Affymetrix probe identifiers (http://www.affymetrix.com/support/technical/byproduct.affx?product=arab). All Affymetrix identifiers having a match (e value $< e^{-12}$) with corresponding proteins were displayed on metabolic pathways (or other processes) using Mapman (Thimm et al., 2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Distribution of the best match protein by plant origin identified by 2D-PAGE and shotgun proteomics.

Supplemental Figure S2. Comparison of the distribution into 16 different functional categories of proteins identified in the microsomal and total root hair fractions.

Supplemental Figure S3. Distribution (by functional category) of all of the proteins identified by both 2D-PAGE and shotgun proteomics.

Supplemental Figure S4. Mapman display of root hair proteins involved in transcription modification, translation modification, PTM, secondary metabolism, transport, and hormone synthesis and response.

Supplemental Table S1. Proteins classified by functional categories identified by 2D-PAGE.

Supplemental Table S2. Peptides identified by shotgun proteomics.

Supplemental Table S3. Proteins classified by functional categories identified by shotgun proteomics.

Supplemental Table S4. Combined list of proteins identified by shotgun proteomics and 2D-PAGE.

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