Functional Differentiation of Brassica napus Guard Cells and Mesophyll Cells Revealed by Comparative Proteomics*§

Mengmeng Zhu‡, Shaojun Dai‡§, Scott McClung¶, Xiufeng Yan§, and Sixue Chen‡¶

Guard cells are highly specialized cells that form tiny pores called stomata on the leaf surface. The opening and closing of stomata control leaf gas exchange and water transpiration as well as allow plants to quickly respond to new environmental conditions. Mesophyll cells are specialized for photosynthesis. Despite the phenotypic and obvious functional differences between the two types of cells, the full protein components and their functions have not been explored but are addressed here through a global comparative proteomics analysis of purified guard cells and mesophyll cells. With the use of isobaric tags for relative and absolute quantification (iTRAQ) tagging and two-dimensional liquid chromatography mass spectrometry, we identified 1458 non-redundant proteins in both guard cells and mesophyll cells of Brassica napus leaves. Based on stringent statistical criteria, a total of 427 proteins were quantified, and 74 proteins were found to be enriched in guard cells. Proteins involved in energy (respiration), transport, transcription (nucleosome), cell structure, and signaling are preferentially expressed in guard cells. We observed several well characterized guard cell proteins. By contrast, proteins involved in photosynthesis, starch synthesis, disease/defense/stress, and other metabolisms are preferentially represented in mesophyll cells. Of the identified proteins, 110 have corresponding microarray data obtained from Arabidopsis guard cells and mesophyll cells. About 72% of these proteins follow the same trend of expression at the transcript and protein levels. For the rest of proteins, the correlation between proteomics data and the microarray data is poor. This highlights the importance of quantitative profiling at the protein level. Collectively this work represents the most extensive proteomic description of B. napus guard cells and has improved our knowledge of the functional specification of guard cells and mesophyll cells.

Molecular & Cellular Proteomics 8:752–766, 2009.

Guard cells (GC)1 are highly specialized cells that form tiny pores called stomata on the leaf surface. When environmental conditions change, guard cells can rapidly change shape so that the pores open or close to control leaf gas exchange and water transpiration. Mesophyll cells (MC) are mainly parenchyma cells between the upper and lower epidermis specialized for photosynthesis. Previous studies that focused on guard cell metabolism and response to environmental signals have revealed important features of functional differentiation of GC (1, 2). Compared with MC, GC contain few chloroplasts with very limited structures and thus possess very low photosynthetic capability. The Calvin cycle in GC only assimilates 2–4% of CO2 fixed in MC (3). In contrast, GC contain abundant mitochondria and display a high respiratory rate, suggesting that oxidative phosphorylation is an important source of ATP to fuel the guard cell machinery (4). Using microarrays covering just one-third of the Arabidopsis genome, Leonhardt et al. (5) observed a differential abscisic acid (ABA) modulation of many guard cell ABA signaling components as well as key enzymes involved in carbon metabolism in GC and MC. This only available large scale genomics study identified 1309 guard cell expressed genes of which 64 transcripts mainly involved in transcription, signaling, and cytoskeleton were preferentially expressed in GC compared with MC. However, functional grouping of the genes revealed only a 1.9% higher representation of photosynthesis genes in MC than in GC. The percentages of genes in all other categories such as protein turnover, defense, signaling, channels and transporters, and metabolism are similar between the two distinct cell types (5). These proteins are known to play specific roles in guard cell functions (6). This highlights the necessity of studying guard cell functions at the protein level.

To date, there have been very few analyses of single cell-type proteomes in plants. Proteome analyses of trichomes from Arabidopsis (7) and tobacco (8) and root hairs from soybean (9) exist but have identified fewer than 100 proteins per proteome. The proteomes of pollen from different species have been relatively well studied, but the pollen grains are not single cells because they contain two/three-cell gametophytes (10). A critical factor for large scale proteomics anal-

1 The abbreviations used are: GC, guard cells; MC, mesophyll cells; ABA, abscisic acid; 2D, two-dimensional; iTRAQ, isobaric tags for relative and absolute quantification; SCX, strong cation exchange.
ysis of single cells is to obtain adequate amounts of sufficiently pure cells. MC are large cells present in high abundance in leaves and can be easily isolated from leaves in large quantities and purified by sucrose gradient centrifugation (11). However, GC are much smaller and comprise a minor fraction of the total cells in a leaf. Although guard cell protoplasts have been isolated mostly at a small scale from several plant species (12–16), it is often technically challenging to obtain GC with good quantity and good quality. With a full genome sequenced, Arabidopsis has become a model species for plant biology research. The large scale guard cell preparation method (5, 15) has great potential to enhance functional genomics of guard cell functions. The Assmann laboratory at the Pennsylvania State University started guard cell proteomics work several years ago and have identified more than 1800 unique guard cell proteins in Arabidopsis. Brassica napus, an important crop species, is genetically closely related to Arabidopsis. The ancestral lineages diverged about 15 million years ago, and the two species share extensive co-linearity and 87% sequence identity in their protein coding regions (17). The rich source of genomic sequences available for both organisms dramatically improves our ability to apply functional genomics tools in guard cell research. However, to the best of our knowledge isolation and purification of GC from B. napus has not been reported.

Recent years have seen proteomics moving beyond simple cataloging toward quantitative characterization of protein dynamics and modifications (18). Investigation of protein levels in single cell systems is important because it offers the most accurate determination of the protein components and dynamics that are directly related to the cell function. However, sensitive protein expression analysis is still challenging. The popular 2D gel electrophoresis-based approach tends to identify mostly abundant proteins and soluble proteins. In addition, quantitative analysis by image analysis is tedious and can be complicated by the presence of multiple proteins in one gel spot (18). An alternative approach, which has been proven powerful, is isotope labeling coupled to multidimensional liquid chromatography and mass spectrometry for protein identification and quantification (18–20). Here we report the isolation and purification of B. napus GC and MC and comparative proteomics of GC and MC using isobaric tags for relative and absolute quantification (iTRAQ) to identify qualitative and quantitative differences in proteomes of the two types of cells. Our results revealed that proteins involved in energy, transport, transcription, cell structure, and signaling are preferentially expressed in GC, whereas proteins important to photosynthesis, starch synthesis, defense/disease/stress, and other metabolisms are highly represented in MC.

### EXPERIMENTAL PROCEDURES

#### Plant Growth—Seeds of the B. napus var. Global were obtained from the United States Department of Agriculture National Plant Germplasm System. Seeds were germinated in Metro-Mix 500 potting mixture (The Scotts Co.), and plants were grown in a growth chamber under a photosynthetic flux of 160 μmol of photons m⁻² s⁻¹ with a photoperiod of 10 h at 24 °C in light and 20 °C in dark. Fully expanded leaves from 2-month-old plants were used for preparation of guard cell protoplasts and mesophyll cell protoplasts.

#### Isolation of Guard Cell Protoplasts and Mesophyll Cell Protoplasts—Guard cell protoplasts from B. napus leaves were isolated and purified mainly as described in the protocol developed for Arabidopsis (15) with the following modifications. Eight grams of fully expanded leaves with main veins removed were blended three times for 30 s each in cold tap water using a 14-speed Osterizer blender (Oster Inc.). The first enzyme digestion of epidermal peels was 1 h at a shaking speed of 140 rpm. The second enzyme digestion was 40 min at a speed of 50 rpm. The pore size of the nylon mesh used after the first and the second digestions was 100 and 30 μm, respectively. After Histopaque purification, the cells were resuspended in 1 ml of basic solution. Ten microliters of the suspension was then taken, and the number of protoplasts were estimated with a hemocytometer. The cells were pelleted at 1000 rpm at 4 °C, frozen in liquid nitrogen immediately, and stored in a −80 °C freezer. Mesophyll cell protoplasts were isolated as described previously (11) except the sucrose concentration for protoplast purification was 0.7 m instead of 0.5 m.

#### Protein Digestion, iTRAQ Labeling, and Strong Cation Exchange Fractionation—Three guard cell preparations were pooled to yield 100 μg of protein as one replicate. Three guard cell replicates and three different mesophyll preparations, each with 100 μg of protein, were used for acetone precipitation overnight. After protein precipitation, the pellet of each replicate was dissolved in 1% SDS, 100 mM triethylammonium bicarbonate, pH 8.5. The samples were reduced, alkylated, trypsin-digested, and labeled using the iTRAQ Reagents fourplex kit according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The guard cell replicates were labeled with iTRAQ tags 114, 115, and 114, and the mesophyll cell replicates were labeled with tags 116, 117, and 116, respectively. From our experience, these isoype tags do not exhibit significant differences in labeling efficiency. After labeling, the two types of cell samples were mixed sequentially to make three independent experiments. The combined peptide mixtures were dried down and dissolved in strong cation exchange (SCX) solvent A (25% (v/v) acetonitrile, 10 mM ammonium formate, pH 2.8). The peptides were fractionated on an Agilent HPLC system 1100 using a polysulfoethyl A column (2.1 × 100 mm, 5 μm, 300 Å; PolyLC, Columbia, MD). Peptides were eluted at a flow rate of 200 μl/min with a linear gradient of 0–20% solvent B (25% (v/v) acetonitrile, 500 mM ammonium formate) over 50 min followed by ramping up to 100% solvent B in 5 min and holding for 10 min. The absorbance at 214 nm was monitored, and a total of 19 fractions were collected.

**Reverse Phase Nanoflow HPLC and Tandem Mass Spectrometry—** Each SCX fraction was lyophilized and dissolved in Solvent A (3% acetonitrile (v/v), 0.1% (v/v) acetic acid, 0.01% (v/v) trifluoroacetic acid). The peptides were loaded onto a C₁₈ capillary trap cartridge (LC Packings) and then separated on a 15-cm nanoflow C₁₈ column (PepMap, 75-μm inner diameter, 3 μm, 100 Å (LC Packings) at a flow rate of 200 nL/min. The HPLC instrument and the quadrupole time-of-flight (QSTAR XL) MS system were the same as those described previously (21). Peptides were eluted from the HPLC column by a linear gradient from 3% Solvent B (96.9% acetonitrile (v/v), 0.1% (v/v) acetic acid) to 40% Solvent B for 2 h followed by ramping up to 90% Solvent B in 10 min. Peptides were sprayed into the orifice of the
mass spectrometer, which was operated in an information-dependent data acquisition mode where an MS scan followed by three MS/MS scans of the three highest abundance peptide ions were acquired in each cycle (21).

Data Analysis—The MS/MS data were processed by a thorough search considering biological modification and amino acid substitution against National Center for Biotechnology Information (NCBI) non-redundant fasta database (5,222,402 entries, downloaded on July 2, 2007) using the Paragon algorithm (22) of ProteinPilot version 2.0.1 software suite (Applied Biosystems). Plant species, fixed modification of methylmethane thiosulfate-labeled cysteine, fixed iTRAQ modification of free amine in the N terminus and lysine, and variable iTRAQ modifications of tyrosine were considered. Parameters such as trypsin digestion, precursor mass accuracy, and fragment ion mass accuracy are built-in settings of the software. The raw peptide identification results from the Paragon algorithm were further processed by the ProGroup algorithm. The ProGroup algorithm uses the peptide identification results to determine the minimal set of confident proteins. For each protein identification, two types of scores are reported, i.e. unused ProtScore and total ProtScore. The total ProtScore is a measurement of all the peptide evidence for a protein and is analogous to protein scores reported by other protein identification software. The unused ProtScore is a measurement of all the peptide evidence for a protein that is not better explained by a higher ranking protein. The unused ProtScore prevents reuse of the same peptide evidence to support the detection of more than one protein. Thus, it is the real indicator of protein confidence. The software calculates a percentage of confidence that reflects the probability that the hit is a false positive so that at the 99% confidence level there is a false positive identification rate of 1%. Low confidence peptides do not identify a protein by themselves but support the identification of the protein (22). For proteins with only one significant contributing peptide, the MS/MS spectrum of the peptide was manually inspected and confirmed (supplemental Fig. 1). The false discovery level was estimated by performing the search against a concatenated database containing both forward and reversed sequences.

For protein relative quantification using iTRAQ, only MS/MS spectra unique to a particular protein and for which the sum of the signal-to-noise ratio for all of the peak pairs was greater than 9 were used for quantification (software default settings, Applied Biosystems). The mean, S.D., and p values to estimate statistical significance of the protein changes were calculated by ProGroup. For the identification of expression differences, each experimental run was initially considered separately. To be identified as being differentially expressed, a protein had to be quantified with at least three spectra (allowing generation of a p value), a p value < 0.05, and a ratio -fold change of at least 2 in more than two independent experiments (i.e. at least six peptides).

**RESULTS**

**Isolation of Guard Cells and Mesophyll Cells from B. napus Leaves**—The objective of this study was to compare the proteome of GC and MC in B. napus. The initial step is to isolate and purify guard cell protoplasts and mesophyll cell protoplasts. The isolation of mesophyll cell protoplasts from B. napus has been reported, and the procedure is straightforward for obtaining large numbers of pure protoplasts (11). For guard cell isolation, we modified the procedures established for Arabidopsis leaves (15) as described under “Experimental Procedures.” From 8 g of fully expanded leaves, the yield of guard cell protoplasts was on average 5 × 10⁶/ml, which corresponds to ~30 μg of protein. The purity of final guard cell preparation was above 99.6% on a cell basis with little contamination originating from mesophyll cells and epidermal cells (Fig. 1). Three preparations were pooled to make one “biological” replicate, and three independent experiments were conducted for proteomics analysis.

**Protein Identification by Off-line 2D HPLC-MS/MS**—After iTRAQ labeling and combination of guard cell and mesophyll cell samples, the peptides were fractionated by SCX chromatography (supplemental Fig. 2). A total of 19 SCX fractions were collected. Each fraction was further separated by nanoflow reverse phase HPLC-MS/MS. Compared with on-line 2D LC-MS, the off-line 2D LC-MS work flow has been shown to display superior overall outcome in protein identification and sequence coverage (23, 24). After merging the data obtained from different experiments, a total of 1116 unique proteins were identified to be present in both guard cells and mesophyll cells. A second set of iTRAQ LC-MS experiments using purified GC only identified an additional 342 guard cell proteins. So altogether 1458 proteins from GC were identified (Fig. 2 and supplemental Table 1). The 1458 proteins were all confidently identified in guard cells because the signals for iTRAQ 114 and 115 tags that had been used to label specifically guard cell proteins were clearly present in the MS/MS spectra of all 1458 proteins. Searching against a reversed database allowed calculation of false discovery rates for these experiments as 4% at the protein level. A complete annotated sequence of the B. napus genome is not yet available. Thus we included other plant species in our database searching to enhance the
success rate of protein identification. For cross-species identification, we carefully inspected the mass spectra and identification quality. The identified proteins were functionally assigned according to 1) their homology with other proteins based on protein-protein basic local alignment search tool (BLAST) searches with an enabled conserved domain option (25), 2) protein family database information, and/or 3) available literature information. The proteins were classified with reference to the functional categories established by Bevan et al. (26). A Venn diagram for the functional classification is shown in Fig. 2. The identified proteins cover a wide range of molecular functions, including photosynthesis (8%), energy (respiration) (9%), metabolism (26%), transcription (5%), protein synthesis (9%), protein destination (11%), signaling (7%), membrane and transport (9%), stress and defense (8%), cell structure (2%), cell division and fate (1%), miscellaneous (3%), and unknown (3%). It should be noted that the percentages of proteins identified in different functional categories do not imply their representation in GC because GC and MC were combined for identification in the iTRAQ experiments. Those proteins that are of low abundance in GC would probably not be identified if only GC were used.

**Functional Specialization of Proteins in Guard Cells and Mesophyll Cells**—Of the proteins identified, 427 proteins could be quantified with at least three different peptide MS/MS spectra and a p value smaller than 0.05 in at least one of the experiments, and 311 proteins could be quantified in at least two of the three independent experiments (supplemental Table 2). To determine the significance threshold, ratios of replicate samples were plotted against p values of the ratios. Repetition of the same sample type, i.e. identical iTRAQ experiment, showed very similar overall quantification results, whereas comparison between GC and MC revealed differentially expressed proteins (supplemental Fig. 3). Based on this analysis, only proteins with calculated p values (based on multiple peptide measurements) smaller than 0.05 and a -fold change of at least 2 are included as guard cell or mesophyll cell preferentially expressed proteins (Tables I and II and Fig. 2). Although most published results are based on a -fold change threshold of 1.2–1.5 (20, 27–29), our criterion of 2-fold is stringent. There are 74 proteins and 143 proteins differentially expressed in GC and MC, respectively. Proteins involved in energy (respiration), signaling, transport, and transcription account for the majority of proteins that show preferential expression in GC. In addition, four proteins involved in nu-
### Proteins predominantly expressed in GC (p < 0.05)

| Accession no. | Protein | Species | Energy (16) | Metabolism (13) | Protein synthesis (5) | Protein folding, transporting, and degradation (7) |
|---------------|---------|---------|-------------|-----------------|-----------------------|---------------------------------|
| 1             | Triose-phosphate isomerase | Arabidopsis thaliana | 1.781 0.000 | 2.044 0.000 | 2.226 0.001 | 2.000 |
| 2             | Glyceraldehyde-3-phosphate dehydrogenase C subunit | A. thaliana | 5.087 0.070 | 2.777 0.004 | 5.453 0.020 | 3.680 |
| 3             | Cytosolic triose phosphate isomerase | A. thaliana | 1.920 0.000 | 2.018 0.000 | 4.702 0.007 | 2.441 |
| 4             | Aldose 1-epimerase family protein | A. thaliana | 2.370 0.001 | 2.603 0.001 | 4.070 0.063 | 2.481 |
| 5             | 6-Phosphogluconate dehydrogenase | A. thaliana | 2.051 0.012 | 2.582 0.009 | 2.296 |
| 6             | Succinate dehydrogenase 1-1 (SDH1-1) | A. thaliana | 2.248 0.023 | 2.041 0.026 | 1.001 0.998 | 2.139 |
| 7             | Putative phosphoglycerate kinase | A. thaliana | 2.166 0.026 | 2.467 0.020 | 2.779 0.043 | 2.445 |
| 8             | Inorganic pyrophosphatase-like protein | A. thaliana | 2.132 0.003 | 2.027 0.001 | 2.078 |
| 9             | Phosphoenolpyruvate carboxylase 2 precursor (ARA8) | A. thaliana | 7.017 0.000 | 4.701 0.001 | 3.180 0.232 | 5.630 |
| 10            | Xylose isomerase family protein | A. thaliana | 2.252 0.043 | 2.169 0.019 | 1.939 0.381 | 2.210 |
| 11            | Mitochondrial ATPase β subunit | Nicotiana sylvestris | 3.088 0.003 | 2.567 0.003 | 2.121 0.009 | 2.532 |
| 12            | Mitochondrial ATP synthase D chain (ATPQ) | A. thaliana | 2.171 0.017 | 1.919 0.011 | 2.037 |
| 13            | Putative glyceraldehyde-3-phosphate dehydrogenase | Brassica juncea | 2.142 0.014 | 2.089 0.021 | 2.115 |
| 14            | Enolase | B. napus | 1.807 0.003 | 1.516 0.206 | 2.525 0.003 | 2.106 |
| 15            | Strong similarity to gb/Y09533 involved in starch metabolism with a PF01325 pyruvate, phosphate dikinase, PEP/pyruvate binding domain | A. thaliana | 2.223 0.045 | 1.870 0.219 | 2.223 |
| 16            | Triose-phosphate isomerase | Zea mays | 2.021 0.000 | 2.043 0.000 | 2.276 0.135 | 2.032 |
| 17            | UDP-α-glucose/UDP-α-galactose 4-epimerase 1 (UGE1) | A. thaliana | 1.923 0.004 | 2.187 0.001 | 2.046 |
| 18            | Hypothetical protein, containing PRK10675 UDP-galactose 4-epimerase domain | Brassica rapa | 1.902 0.018 | 2.190 0.008 | 2.036 |
| 19            | Putative glycosylated polypeptide-3 | A. thaliana | 0.181 0.000 | 0.635 | 3.872 0.013 | 3.872 |
| 20            | Putative protein, containing pfam02719 polysaccharide biosynthesis protein domain | A. thaliana | 3.630 0.383 | 3.064 | 2.055 0.025 | 2.055 |
| 21            | Thiazole biosynthetic enzyme | A. thaliana | 2.438 0.005 |  | 2.438 |
| 22            | Aspartate aminotransferase 2 (ASP2) | A. thaliana | 2.293 0.005 | 2.127 0.045 | 2.207 |
| 23            | S-α-Lactoylgulactohione methylglyoxal lyase | B. juncea | 2.464 0.043 | 1.994 0.005 | 1.338 0.338 | 2.205 |
| 24            | Acotase | A. thaliana | 1.132 0.749 | 1.135 0.589 | 3.311 0.020 | 3.311 |
| 25            | β-Ketoacyl-ACP synthetase 1 | B. napus | 1.406 0.161 | 2.113 0.036 | 1.375 0.526 | 2.113 |
| 26            | Putative fumarase | A. thaliana | 2.467 0.006 | 1.860 0.052 | 2.467 |
| 27            | Peroxocarbox reductase | Lotus japonicus | 2.479 0.021 | 3.614 0.021 | 2.941 |
| 28            | Putative lactoylgulactohione lyase | B. rapa | 3.311 0.254 | 2.559 0.320 | 3.114 0.011 | 3.114 |
| 29            | Formate dehydrogenase (FDH) | A. thaliana | 2.419 0.007 | 2.347 0.007 | 1.491 0.566 | 2.383 |
| 30            | 40 S ribosomal protein S5 | A. thaliana | 1.019 0.909 | 1.080 0.744 | 2.085 0.049 | 2.085 |
| 31            | Golgi-associated protein se-wap41 | Z. mays | 0.229 0.462 |  | 3.535 0.015 | 3.535 |
| 32            | 40 S ribosomal protein S25 (RPS25ε) | A. thaliana | 2.109 0.006 | 2.621 0.015 | 2.338 |
| 33            | Peptidylprolyl isomerase ROC1, containing c919826 cyclolin domain | A. thaliana | 1.191 0.097 | 1.021 0.868 | 13.072 0.004 | 13.072 |
| 34            | Mitochondrial chaperin hsp60 | A. thaliana | 1.019 0.909 | 1.080 0.744 | 2.085 0.049 | 2.085 |
| 35            | Polyubiquitin (ubQ) | A. thaliana | 3.297 0.001 | 3.373 0.001 | 1.449 0.010 | 2.326 |
| 36            | Heat shock protein HSP101 | Z. mays | 2.315 0.007 | 1.867 0.008 | 2.067 |
| 37            | Mitochondrial chaperin hsp60 | A. thaliana | 2.364 0.024 | 2.132 0.028 | 1.600 0.083 | 2.242 |
| 38            | 20 S proteasome subunit (PAA2) | A. thaliana | 1.149 0.790 | 1.189 0.810 | 3.018 0.004 | 3.018 |
| 39            | 10-kDa chaperin (protein CPN10) (protein groES) | B. napus | 2.335 0.026 | 2.249 0.285 | 2.335 |
Proteomics of Guard Cells and Mesophyll Cells

### TABLE I—continued

| Accession no. | Protein                                                                 | Species                  | GC/MC | p value | GC/MC | p value | GC/MC | p value | Average GC/MC |
|---------------|-------------------------------------------------------------------------|--------------------------|-------|---------|-------|---------|-------|---------|----------------|
| 40            | Plasma membrane proton efflux P-type ATPase                            | *Medicago truncatula*    | 4.576 | 0.007   | 4.317 | 0.016   | 5.797 | 0.065   | 4.443          |
| 41            | Vacular ATP synthase catalytic subunit A                               | *Z. mays*                | 3.352 | 0.004   | 2.785 | 0.036   | 2.184 | 0.003   | 2.690          |
| 42            | Plasma membrane proton ATPase (PMA), AHA1                              | *A. thaliana*            | 1.977 | 0.005   | 2.333 | 0.003   | 3.674 | 0.008   | 2.486          |
| 43            | Plasma membrane H\(^+\)-ATPase, AHA7                                   | *A. thaliana*            | 7.210 | 0.083   | 6.631 | 0.035   | 2.181 |          | 6.631          |
| 44            | Plasma membrane H\(^+\)-transporting ATPase type 2, AHA2               | *A. thaliana*            | 3.209 | 0.038   | 2.171 | 0.763   | 5.012 | 0.070   | 3.209          |
| 45            | Putative H\(^+\)-exporting ATPase                                      | *Oryza sativa*          | 2.316 | 0.010   | 2.546 | 0.003   | 2.580 | 0.067   | 2.426          |
| 46            | Vacuolar ATP synthase subunit B 1                                      | *A. thaliana*            | 1.932 | 0.050   | 3.050 | 0.004   | 1.905 | 0.005   | 2.345          |
| 47            | H\(^+\)-transporting ATPase                                            | *Z. mays*                | 1.590 | 0.099   | 1.565 | 0.067   | 2.694 | 0.015   | 2.694          |
| 48            | Adenylyl translocator                                                   | *A. thaliana*            | 2.163 | 0.000   | 2.150 | 0.002   | 1.766 | 0.246   | 2.157          |
| 49            | Putative GSH-dependent dehydroascorbate reductase 1                    | *A. thaliana*            | 3.586 | 0.017   | 1.750 | 0.070   | 1.903 |          | 3.586          |
| 50            | Bet v 1 allergen family protein                                        | *A. thaliana*            | 3.134 | 0.064   | 2.572 | 0.031   |        |          |                |
| 51            | Putative peroxiredoxin type 2                                          | *A. thaliana*            | 5.085 | 0.010   | 4.522 | 0.006   | 9.075 | 0.148   | 4.787          |
| 52            | Resistant to agrobacterium transformation 5                            | *A. thaliana*            | 2.372 | 0.015   | 4.025 | 0.032   | 4.783 | 0.003   | 3.412          |
| 53            | Early responsive to dehydration 12 (ERD12 protein)                      | *B. napus*               | 2.039 | 0.039   | 2.058 | 0.040   | 1.454 | 0.272   | 2.048          |
| 54            | Peroxiredoxin IIF (ATPRIIIF/PRIIIFII)                                   | *A. thaliana*            | 2.038 | 0.000   | 1.999 | 0.002   | 1.935 |          | 2.018          |
| 55            | Unknown protein, containing pflam00407 pathogenesis-related protein     | *A. thaliana*            | 2.532 | 0.019   | 3.279 | 0.041   | 11.657| 0.033   | 2.857          |
| 56            | Vegetative storage protein-like                                        | *A. thaliana*            | 2.030 | 0.023   | 1.019 | 0.911   | 4.052 |          | 2.030          |
| 57            | Putative GSH-dependent dehydroascorbate reductase                      | *A. thaliana*            | 2.255 | 0.011   | 2.046 | 0.068   | 2.275 | 0.113   | 2.255          |
| 58            | 14-3-3 protein, putative                                               | *A. thaliana*            | 2.428 | 0.006   | 1.995 | 0.014   | 2.099 | 0.051   | 2.190          |
| 59            | Glycin-rich RNA-binding protein                                          | *A. thaliana*            | 2.161 | 0.002   | 2.280 | 0.004   | 1.766 | 0.158   | 2.219          |
| 60            | Putative mitogen-activated protein kinase                               | *A. thaliana*            | 3.134 | 0.000   | 3.250 | 0.002   |        |          | 3.191          |
| 61            | Cytokinin-binding protein CBP5                                           | *N. sylvestris*          | 2.791 | 0.034   | 3.798 | 0.031   | 1.770 |          | 3.217          |
| 62            | Calmodulin binding/translational elongation factor                      | *A. thaliana*            | 3.253 | 0.013   | 3.799 | 0.001   | 1.459 | 0.044   | 2.389          |
| 63            | Mitogen-activated protein kinase 4                                      | *B. napus*               | 2.140 | 0.138   | 2.609 | 0.077   | 5.013 | 0.034   | 5.013          |
| 64            | Cymodulin                                                               | *B. juncea*              | 2.340 | 0.002   | 3.475 | 0.002   | 2.025 | 0.000   | 2.482          |
| 65            | Putative protein phosphatase 2a 65-kDa regulatory subunit               | *A. thaliana*            | 2.083 | 0.045   | 2.075 | 0.155   |        |          | 2.063          |

**Proteins with p value smaller than 0.05 in only one of the independent replicates.**

cleosome and three involved in cell structure were highly expressed in GC (Table I and Fig. 2). On the contrary, in MC the majority of proteins (~50%) are involved in photosynthesis followed by 23 proteins involved in metabolism and 17 disease/defense/stress proteins (Table II and Fig. 2). Representative MS/MS spectra for peptides identified from a photosystem II protein and plasma membrane H\(^+\)-ATPase AHA1 are shown in Fig. 3. The peaks of ITFAQ signature ions (114.1 for guard cells and 116.1 for mesophyll cells) are shown as insets, representing the relative abundance of the proteins in MC and GC, respectively. It is important to note that we identified proteins known to specifically function in GC (most of low abundance) but were not able to obtain reproducible quantitative information. These proteins include G protein (30, 31), Rac GTPase (32, 33), phospholipase D\(_1\) (34), protein kinase C (35, 36), open stomata protein kinase (6), Atrboh
# Proteomics of Guard Cells and Mesophyll Cells

**Table II**

Proteins predominantly expressed in MC (p < 0.05)

| Accession no. | Protein | Species | Experiment 1 | Experiment 2 | Experiment 3 | Average MC/GC |
|---------------|---------|---------|--------------|--------------|--------------|---------------|
| 1             | Photosystem I subunit VII | Staurastrum punctulatum |              |              |              |               |
| 2             | Photosystem I subunit PSI-L | A. thaliana |              |              |              |               |
| 3             | Putative photosystem I subunit V precursor | A. thaliana |              |              |              |               |
| 4             | Putative photosystem I subunit III precursor | A. thaliana |              |              |              |               |
| 5             | Photosystem I P700 apoprotein A1 | Brassica oleracea |              |              |              |               |
| 6             | Photosystem I reaction center subunit VI | B. rapa |              |              |              |               |
| 7             | Photosystem I reaction center subunit N (PSI-N) | Pisum sativum |              |              |              |               |
| 8             | Putative photosystem I reaction center subunit PSI-N | A. thaliana |              |              |              |               |
| 9             | Putative photosystem I reaction center subunit II | A. thaliana |              |              |              |               |
| 10            | Putative photosystem I chain X precursor | A. thaliana |              |              |              |               |
| 11            | Putative photosystem I antenna protein | O. sativa |              |              |              |               |
| 12            | Chloroplast photosystem I P700 | A. thaliana |              |              |              |               |
| 13            | Chloroplast PSI type III chlorophyll a/b-binding protein | B. juncea |              |              |              |               |
| 14            | Photosystem-I F subunit precursor | O. sativa |              |              |              |               |
| 15            | PSII type III chlorophyll a/b-binding protein | A. thaliana |              |              |              |               |
| 16            | Probable photosystem I chain XI precursor | N. sylvestris |              |              |              |               |
| 17            | Chloroplast PSII 22-kDa protein | A. thaliana |              |              |              |               |
| 18            | Chloroplast PSII 23-kDa protein | A. thaliana |              |              |              |               |
| 19            | Chloroplast PSII 24-kDa protein | A. thaliana |              |              |              |               |
| 20            | Chloroplast PSII 25-kDa protein | A. thaliana |              |              |              |               |
| 21            | Chloroplast PSII 26-kDa protein | A. thaliana |              |              |              |               |
| 22            | Chloroplast PSII 27-kDa protein | A. thaliana |              |              |              |               |
| 23            | Chloroplast PSII 28-kDa protein | A. thaliana |              |              |              |               |
| 24            | Chloroplast PSII 29-kDa protein | A. thaliana |              |              |              |               |
| 25            | Chloroplast PSII 30-kDa protein | A. thaliana |              |              |              |               |
| 26            | Chloroplast PSII 31-kDa protein | A. thaliana |              |              |              |               |
| 27            | Chloroplast PSII 32-kDa protein | A. thaliana |              |              |              |               |
| 28            | Chloroplast PSII 33-kDa protein | A. thaliana |              |              |              |               |
| 29            | Chloroplast PSII 34-kDa protein | A. thaliana |              |              |              |               |
| 30            | Chloroplast PSII 35-kDa protein | A. thaliana |              |              |              |               |
| 31            | Chloroplast PSII 36-kDa protein | A. thaliana |              |              |              |               |
| 32            | Chloroplast PSII 37-kDa protein | A. thaliana |              |              |              |               |
| 33            | Chloroplast PSII 38-kDa protein | A. thaliana |              |              |              |               |
| 34            | Chloroplast PSII 39-kDa protein | A. thaliana |              |              |              |               |
| 35            | Chloroplast PSII 40-kDa protein | A. thaliana |              |              |              |               |
| 36            | Chloroplast PSII 41-kDa protein | A. thaliana |              |              |              |               |
| 37            | Chloroplast PSII 42-kDa protein | A. thaliana |              |              |              |               |
| 38            | Chloroplast PSII 43-kDa protein | A. thaliana |              |              |              |               |
| 39            | Chloroplast PSII 44-kDa protein | A. thaliana |              |              |              |               |
| 40            | Chloroplast PSII 45-kDa protein | A. thaliana |              |              |              |               |
| 41            | Chloroplast PSII 46-kDa protein | A. thaliana |              |              |              |               |
| 42            | Chloroplast PSII 47-kDa protein | A. thaliana |              |              |              |               |
| 43            | Chloroplast PSII 48-kDa protein | A. thaliana |              |              |              |               |
| 44            | Chloroplast PSII 49-kDa protein | A. thaliana |              |              |              |               |
| 45            | Chloroplast PSII 50-kDa protein | A. thaliana |              |              |              |               |
| 46            | Chloroplast PSII 51-kDa protein | A. thaliana |              |              |              |               |
| 47            | Chloroplast PSII 52-kDa protein | A. thaliana |              |              |              |               |

758 Molecular & Cellular Proteomics 8.4
TABLE II—continued

| Accession no. | Protein | Species |
|---------------|---------|---------|
| 48            | gi|15236722| H+ -transporting ATP synthase chain 9-like protein |
| 49            | gi|15240013| 33-kDa polyepitope of oxygen-evolving complex |
| 50            | gi|18405061| Thylakoid lumen 18.3-kDa protein |
| 51            | gi|28141361| Granule-bound starch synthase |
| 52            | gi|401249| Cytochrome b6-f complex iron-sulfur subunit 2, chloroplast |
| 53            | gi|42571761| Nonphotochemical quenching (NPQ) |
| 54            | gi|58700507| Chloroplast oxygen-evolving protein 16-kDa subunit |
| 55            | gi|9843639| Rieske FeS protein |
| 56            | gi|82318781| Proteochlorophyllide reductase precursor-like protein |
| 57            | gi|11037772| Chloroplast pigment-binding protein CP24 |
| 58            | gi|17852| Ribulose-bisphosphate carboxylase/oxygenase small subunit |
| 59            | gi|7903990| Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit precursor |
| 60            | gi|8745521| Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| 61            | gi|15229349| Putative ribose-5-phosphate isomerase |
| 62            | gi|92884121| 4Fe-4S ferredoxin, iron-sulfur binding protein |
| 63            | gi|11630999| Chlorophyll a/b-binding protein, containing pfam05004 |
| 64            | gi|12663416| Unnamed protein product, containing pfam05004 |
| 65            | gi|2410533| Phospholipid 1 |
| 66            | gi|29893389| Ferritin-1, chloroplast precursor |
| 67            | gi|458797| Cytochrome b |
| 68            | gi|5404095| Glycolate oxidase |
| 69            | gi|544122| Apocarboxyphilome f precursor |
| 70            | gi|87468333| Phleomycin with cytochrome f |
| 71            | gi|81031850| Cytochrome f |

Energy (7)

| Accession no. | Protein | Species |
|---------------|---------|---------|
| 72            | gi|1480014| Putative α subunit of ATP synthase |
| 73            | gi|15234900| Putative fructose-bisphosphate aldolase |
| 74            | gi|20339362| Ribulose-5-phosphate 3-epimerase |
| 75            | gi|27530932| Cytochrome oxidase |
| 76            | gi|15233597| Containing PRK005621 F1,F1-ATP synthase y domain |
| 77            | gi|4995091| Malate dehydrogenase 2 |
| 78            | gi|75363517| ATP synthase subunit β |

Metabolism (23)

| Accession no. | Protein | Species |
|---------------|---------|---------|
| 79            | gi|11432449| Geranylgeranyl reductase |
| 80            | gi|15217485| Containing PRK010675 UDP-galactose 4-epimerase domain |
| 81            | gi|15221892| Unknown protein containing the COG1152 glycerolipid operon domains of methanol dehydrogenase |

| Accession no. | Protein | Species |
|---------------|---------|---------|
| 82            | gi|15252026| Alaniine-glyoxylate aminotransferase |
| 83            | gi|15232133| Carbonylreductase, chloroplast precursor |
| 84            | gi|1711296| Myrosinase-binding protein |
| 85            | gi|1769968| Myrosinase-associated protein |
| 86            | gi|18410661| Unknown protein |
| 87            | gi|28126624| Cysteine lyase DOCL-3 |
| 88            | gi|42408130| Putative aminotransferase |
| 89            | gi|50508805| Putative (S)-2-hydroxy-acid oxidase |
| 90            | gi|79313265| Jacalin lectin family protein (JR1) |
| 91            | gi|15202620| Hydroxyprotein reductase |
| 92            | gi|15221119| Aminomethyltransferase-like precursor protein |
| 93            | gi|15225449| Putative transketolase precursor |
| 94            | gi|15239032| Allene oxide synthase |
| 95            | gi|15239406| p-Nitrophenol-phosphatase-like protein |
| 96            | gi|51784970| Catalytic,coenzyme-binding protein |
| 97            | gi|16172720| AMP-binding protein |
### Proteomics of Guard Cells and Mesophyll Cells

#### TABLE II—continued

| Accession no. | Protein Description | Species | Experiment 1 | Experiment 2 | Experiment 3 | Average |
|---------------|---------------------|---------|--------------|--------------|--------------|---------|
| 98            | Glutamate-ammonia ligase precursor B. napus | B. napus | 2.353 0.000 | 2.249 0.000 | 5.250 0.002 | 3.284 |
| 99            | Putative phosphoglycerate phosphatase | A. thaliana | 1.956 0.000 | 1.826 0.005 | 2.569 0.006 | 2.117 |
| 100           | Hydroperoxide lyase (HPOL)-like protein, containing pfam00067 cytochrome P450 domain | A. thaliana | 2.933 0.215 | 7.273 0.011 | 7.273 |
| 101           | Glutamine synthetase B. napus | B. napus | 1.173 0.395 | 1.301 0.317 | 5.327 0.000 | 5.327 |
| Protein synthesis (2) |          |         |              |              |              |         |
| 102           | 50 S ribosomal protein L1-C | A. thaliana | 2.399 0.002 | 0.958 0.764 | 0.982 0.962 | 2.399 |
| 103           | RPS1 (ribosomal protein S1); RNA binding | A. thaliana | 2.148 0.040 | 1.323 0.326 | 0.975 0.004 | 2.148 |
| Protein folding, transporting, and degradation (11) | | | | | | |
| 104           | ATP-dependent Cip protease, ATP-binding subunit A. thaliana | A. thaliana | 2.283 0.014 | 2.597 0.002 | 2.167 0.148 | 2.440 |
| 105           | ATP-dependent peptide ATPase/metalloproteinase A. thaliana | A. thaliana | 1.597 0.170 | 1.592 0.186 | 5.722 0.031 | 5.722 |
| 106           | 60-kDa 30S ribosome | B. napus | 2.155 0.013 | 2.945 0.013 | 1.693 0.059 | 2.550 |
| 107           | Complex 1 family protein/LVR family protein A. thaliana | A. thaliana | 1.412 0.038 | 2.675 0.014 | 2.044 |
| 108           | Plastid transcriptionally active 18 (PTAC16) A. thaliana | A. thaliana | 1.924 0.000 | 2.218 0.001 | 2.071 |
| 109           | DEAD box ATP-dependent RNA helicase 3B O. sativa | O. sativa | 6.686 0.001 | 12.884 0.000 | 12.754 0.007 | 10.775 |
| 110           | Hypothetical protein, containing COG1222 ATP-dependent 26 S proteasome regulatory subunit domain A. thaliana | A. thaliana | 2.303 0.000 | 1.600 0.000 | 3.433 0.000 | 2.446 |
| 111           | Ribosomal protein L29 family protein A. thaliana | A. thaliana | 2.135 0.019 | 1.127 0.216 | 2.135 |
| 112           | DegP protease precursor A. thaliana | A. thaliana | 1.481 0.007 | 2.053 0.003 | 4.659 0.040 | 2.731 |
| 113           | Stromal 70-kDa heat shock-related protein, chloroplast O. sativa | O. sativa | 2.745 0.008 | 3.427 0.001 | 3.086 |
| 114           | Trypsin inhibitor propeptide B. oleracea | B. oleracea | 1.907 0.005 | 2.707 0.016 | 4.022 0.024 | 2.879 |
| Membrane and transport (5) | | | | | | |
| 115           | H+ -transporting two-sector ATPase, αβ-subunit M. truncatula | M. truncatula | 1.693 0.000 | 1.587 0.001 | 5.066 0.004 | 2.782 |
| 116           | Translocon at the inner envelope membrane of chloroplast 55 A. thaliana | A. thaliana | 2.460 0.004 | 1.934 0.005 | 2.197 |
| 117           | Apolliprotein D-related A. thaliana | A. thaliana | 2.077 0.082 | 2.246 0.013 | 1.529 |
| 118           | Aquaporin PIP1b2 B. oleracea | B. oleracea | 2.809 0.086 | 3.693 0.000 | 3.693 |
| 119           | Plasma membrane intrinsic protein 2 A. thaliana | A. thaliana | 3.546 0.030 | 5.095 0.013 | 7.000 0.013 | 5.214 |
| Stress and defense (17) | | | | | | |
| 120           | Unknown protein containing cd01958 and pfam0234 domain, putative seed storage proteins and lipid transfer proteins A. thaliana | A. thaliana | 7.321 0.000 | 6.978 0.002 | 11.961 0.008 | 8.754 |
| 121           | Germin-like protein A. thaliana | A. thaliana | 3.626 0.000 | 4.533 0.000 | 8.589 0.000 | 5.583 |
| 122           | Bn22 drought-induced protein B. napus | B. napus | 2.490 0.000 | 2.913 0.000 | 9.806 0.000 | 5.070 |
| 123           | Lipoxigenase 2 B. napus | B. napus | 2.180 0.001 | 3.319 0.000 | 9.880 0.000 | 5.126 |
| 124           | Triazine resistance B. napus | B. napus | 2.444 0.000 | 3.408 0.000 | 5.522 0.000 | 3.791 |
| 125           | Catalase B. napus | B. napus | 2.816 0.000 | 3.485 0.000 | 5.072 0.000 | 3.791 |
| 126           | Unknown protein, containing pfam04753 PAP-fibrillin domain A. thaliana | A. thaliana | 2.011 0.000 | 3.419 0.000 | 4.592 0.005 | 3.341 |
| 127           | Myrosinase B. napus | B. napus | 3.835 0.002 | 4.232 0.001 | 4.544 0.022 | 4.204 |
| 128           | Kelch repeat-containing protein A. thaliana | A. thaliana | 4.795 0.000 | 9.490 0.000 | 13.694 0.032 | 13.694 |
| 129           | Early response to dehydration 1; ATP binding/ATPase B. napus | B. napus | 2.670 0.003 | 1.837 0.008 | 2.253 |
| 130           | Jasmonate-inducible protein containing pfam01419 jacalin-like lectin domain B. napus | B. napus | 1.390 0.000 | 1.902 0.000 | 7.666 0.000 | 7.666 |
| 131           | Putative chloroplast drought-induced stress protein A. thaliana | A. thaliana | 2.094 0.001 | 2.036 0.006 | 2.494 0.004 | 2.065 |
| 132           | Plastid lipid-associated protein PAP/fibrillin family protein A. thaliana | A. thaliana | 2.093 0.000 | 2.967 0.000 | 3.739 |
| 133           | Plastid lipid-associated protein 3 B. rapa | B. rapa | 2.068 0.079 | 4.370 0.040 | 3.839 |
| 134           | Plastid lipid-associated protein 1, chloroplast precursor B. rapa | B. rapa | 1.638 0.000 | 2.574 0.000 | 2.811 |
| 135           | Myrosinase-associated protein B. napus | B. napus | 1.956 0.033 | 4.132 0.016 | 3.044 |
| 136           | Epithiospecifier protein B. oleracea | B. oleracea | 2.099 0.001 | 2.909 0.001 | 8.182 0.000 | 4.397 |
NADPH oxidase (37), potassium channel (38), chloride channel (38), lipid transfer protein (39), calreticulin (40), and prolinol (41).

**Comparative Analysis of Transcriptome Data and Proteome Data**—The only available transcriptomics analysis of guard cell and mesophyll cell genes was carried out in *Arabidopsis* using a microarray covering one-third of the *Arabidopsis* genome (5). Although 1309 genes were identified to be guard cell-expressed, the study did not identify functional specialization of guard cells. Our comparative proteomics of GC and MC revealed specific functions associated with the two types of guard cells. Among the 74 proteins enriched in GC described in the previous section, 15 are represented on the microarray, and 9 transcripts were identified as enriched in GC (supplemental Table 2). These results confirm the general observation that mRNA levels are not always consistent with protein levels because of post-transcriptional, translational, or post-translational regulations (32–44). They also highlight the importance of proteomics analysis.

**DISCUSSION**

Although most proteomics studies of multicellular organisms tend to use intact organs and tissues that contain many different cells, proteomics of individual cell types or organelles has become increasingly important because it allows fine dissection of cellular or organelle functions (45–47). Although the guard cell isolation procedure is tedious and the yield is relatively low, obtaining proteomics quality and quantity material is not limiting. Actually the advantages of working with a crop plant closely related to *Arabidopsis* are severalfold. First, *B. napus* guard cells are larger and have higher protein contents than *Arabidopsis* guard cells. Usually 30 μg of protein can be obtained from each preparation of 8 g of fully expanded leaves. Although with *Arabidopsis*, the yield of GC is about 10 μg per preparation. Second, the knowledge gained in *Arabidopsis* and *B. napus* may be interextendable based on the conservation of the two plant species (17). Third, the genomics resources and functional genomics tools developed in *Arabidopsis* can be harnessed to address fundamental questions of guard cell functions. Last and not the least, results obtained in *B. napus* can be applied to the enhancement of stress tolerance and production of oilseeds and biofuels.

iTRAQ reagent technology is a recently developed method for relative and absolute quantification of proteins. It has immense potential to improve the sensitivity and quality of mass spectrometric analysis of the proteome (18, 48, 49). Although such a powerful technology has been widely implemented in mammalian research (20, 27–29, 49–51), its application in plant proteomics has been limited to only a few laboratories (e.g. 46). We demonstrated the usefulness of the technology to label peptide mixtures derived from proteins extracted from GC and MC and, using LC-MS/MS, to identify and quantify the relative levels of the peptides emanating from the two types of samples. In the iTRAQ approach, the peptides from different samples are combined and appear as one peak in MS, thus increasing the total ion current for that peptide. This is advantageous for obtaining good quality MS/MS spectra for identification of low abundance proteins. In this study, we identified 1458 unique proteins, many of which are of low abundance and would otherwise have escaped identification if the samples had not been combined. For quantification, because it is based on individual iTRAQ tags associated with different samples, the combination of different samples has little enhancing effect on quantitative results. For almost every identified protein, relative quantitative information was ob-

---

**TABLE II—continued**

| Accession no. | Protein | Species | Experiment 1 | Experiment 2 | Experiment 3 | Average |
|---------------|---------|---------|--------------|--------------|--------------|---------|
| 137 gi| Ca²⁺-dependent membrane-protein annexin | A. thaliana | 1.447 0.029 | 2.911 0.018 | 5.720 0.085 | 2.179 |
| 138 gi| Annexin 1 | B. juncea | 3.404 0.003 | 3.404 |
| 139 gi| Putative mRNA-binding protein | A. thaliana | 1.861 0.006 | 1.447 0.228 | 3.902 0.023 | 2.882 |
| 140 gi| Unknown protein | A. thaliana | 2.832 0.001 | 2.670 0.015 | 2.751 |
| 141 gi| Unknown protein | A. thaliana | 2.073 0.030 | 2.258 0.365 | 2.073 |
| 142 gi| Unknown protein, containing cd00158 rhodanese homology domain (RHOD) domain | A. thaliana | 2.037 0.007 | 2.271 0.015 | 3.465 2.154 |
| 143 gi| Rubredoxin family protein | A. thaliana | 2.141 0.047 | 2.783 0.089 | 1.976 0.474 | 2.141 |

* Proteins with *p* value smaller than 0.05 in only one of the independent replicates.
Proteomics of Guard Cells and Mesophyll Cells

Fig. 3. Representative MS/MS spectra showing protein identification and relative quantification in guard cells (iTRAQ tag 114) and mesophyll cells (iTRAQ tag 116). A, an MS/MS spectrum identified the peptide QLDASGKPDNFTGK (confidence, 99%) derived from photosystem II protein and its relative abundance in the two types of cells. B, an MS/MS spectrum identified the peptide DSNIAVIPVEELIEK (confidence, 99%) derived from plasma membrane P-type ATPase AHA1 and its relative abundance in the two types of cells.

tained from at least one of the three different experiments (supplemental Table 1). However, the relative expression ratios of over half of the proteins either have high \( p \) values (\( p < 0.05 \)) or no \( p \) values. This generally seems to be the case when iTRAQ experiments are done and analyzed using the current version of ProteinPilot software (20, 28, 29, 51). ProteinPilot software only uses MS/MS spectra unique to a particular protein and peak pairs with a sum of the signal-to-noise ratio over 9 for quantification (default software settings). In addition, at least three spectra are needed to determine the statistical significance of a change in protein levels (22). This software algorithm aiming at high quality and high accuracy quantitation may compromise the end results of the total number of proteins with confident changes. For instance, some guard cell-specific proteins such as those described under “Results” were missed. Other factors such as mass spectrometer interference, relative protein abundance, variation in sample preparation, and especially biological variations may all affect quantification outcomes that are based on the signal intensity and the variation of different iTRAQ tags (48).

Comparative proteomics using iTRAQ technology and LC-MS/MS has revealed the functional differences between MC
and GC. Proteins involved in respiration were much more abundant in GC than in MC. For instance, phosphoenolpyruvate carboxylase 2 (ATPPC2), glyceraldehyde-3-phosphate dehydrogenase, mitochondrial ATPase β subunit, phosphoglycerate kinase, triose-phosphate isomerase, and aldose 1-epimerase were highly expressed in GC (Table I). Proteins associated with transport and signal transduction, including channels, ATP synthase, protein kinases, 14-3-3 protein, calmodulin, and phosphatases, were also more abundant in GC. In contrast, proteins associated with photosystems, the Calvin cycle, and starch synthesis were more abundant in MC than in GC. A higher proportion of proteins for respiration, signal transduction, and transport and a lower proportion of the photosynthetic proteins indicate that GC devote more cellular activities to processing environmental or endogenous stimuli than to metabolic activities. This is consistent with the expected roles of guard cells in processing diverse signals to regulate stomatal movement. The lower proportion of photosynthetic activity, in particular, is consistent with the previously known physiological data (3, 4). Interestingly the myrosinase system including myrosinases, myrosinase-binding proteins, and myrosinase-associate proteins generally did not show high expression in B. napus GC. This is in strong contrast to what was found in Arabidopsis (52, 53). The functional significance of the difference is not known. In the transport functional category, three plasma membrane ATPases (the homologs of Arabidopsis plasma membrane proton ATPases AHA1, AHA2, and AHA7) were found to be highly expressed in B. napus GC. In Arabidopsis, AHA1 and AHA2 genes were shown to be preferentially expressed in GC, whereas AHA7 gene was uniquely expressed in GC (54). The iTRAQ data here correlate very well with the transcriptional results. The other three plasma membrane ATPases did not match to any other Arabidopsis AHA sequences; thus they are novel proteins identified in B. napus GC. Although plasma membrane ATPases are relatively well studied in GC, literature on the function of vacuolar ATPases has been few. Here we identified two sequences related to vacuolar ATPases (vacuolar ATP synthase catalytic subunit A and subunit B 1) that were abundantly expressed in B. napus GC. Vacuolar ATPase activities were found to be much higher in GC than in MC of Commelina (14). In Arabidopsis, guard cell vacuolar ATPases play an important role in cytosolic calcium oscillations that are essential for stomatal closure (55). Like ATPases, a dicarboxylate/tricarboxylate carrier is preferentially expressed in B. napus GC. This transporter has not been identified previously in GC. Recently slow anion channel-associated 1 (SLAC1; At1g12480) was identified in Arabidopsis GC to be a homolog of fungal and bacterial dicarboxylate/malic acid transporter (56). However, the B. napus dicarboxylate/tricarboxylate transporter has little homology to SLAC1.

Several signaling proteins were found to be highly expressed in B. napus GC (Table I). 1) Calmodulin is known to play an important role in guard cell signaling (13, 33). GC and epidermal cells were found to contain higher levels of calmodulin and calmodulin-binding proteins than MC (57). 2) 14-3-3 proteins play important roles in guard cell ABA and blue light signaling (16, 58). 3) Two mitogen-activated protein kinases (MPKs) displayed high expression levels in B. napus GC. Recently an ABA-activated mitogen-activated protein kinase, MPK3, downstream of H2O2 has been characterized in Arabidopsis (59). 4) A protein phosphatase 2A protein was identified in GC. This protein regulates K+ channels (60) and may be involved in auxin transport and/or ABA-induced stomatal closing (61). 5) Glycine-rich RNA-binding protein 7 was preferentially expressed in B. napus GC. In Arabidopsis, this protein (AtGRP7) is involved in the regulation of ABA and stress responses. It is part of a negative feedback loop through which it regulates the circadian oscillations of its own transcript, and gene transcription is induced by cold (62, 63). One of its homologs in Arabidopsis, AtGRP2, is predominantly expressed in GC (5). 6) A cytokinin-binding protein was highly expressed in B. napus GC. Functional studies of this protein are not available.

In the protein turnover category, several proteins preferentially expressed in GC are involved in ubiquitination and proteasome degradation (Table I). Protein degradation activity has rarely been studied in GC. This finding highlights the potential significance of protein turnover in guard cell function. Several histone proteins were found to be preferentially translated in B. napus GC (Table I). This result correlates well with the Arabidopsis microarray data (5). Consistent with the possibly high protein turnover rate, GC may have high gene transcriptional activities or regulations, facilitating efficient responses to environmental factors. In the disease and defense category, two plant peroxiredoxins and a dehydroascorbate
Proteomics of Guard Cells and Mesophyll Cells

reductase showed high levels of expression in GC. Peroxiredoxin isoforms are small proteins linked to reduced thioredoxin or glutaredoxin and function as peroxidases to remove hydrogen peroxide (64, 65), which is an important signaling molecule produced in GC in response to a variety of environmental conditions (33, 66). Peroxiredoxins seem to play important roles in regulating hydrogen peroxide levels and thus guard cell signaling processes. Dehydroascorbate reductase is responsible for regenerating ascorbate from an oxidized state. The ascorbate redox state controls guard cell signaling and stomatal movement and affects leaf growth, development, and function (67, 68). Overexpression of dehydroascorbate reductase led to plant resistance to salt stress (69). The preferential expression of these proteins in GC indicates that cellular redox state or redox control plays an essential role in guard cell function. In the cell structure category, several tubulin and actin proteins were identified in GC. They are cytoskeleton proteins (41, 70). Profilin was also identified in GC, but its relative expression levels did not pass the statistical criteria (supplemental Table 1). Profilin is an actin-binding protein that affects actin polymerization (70). These proteins together play an important role in regulating guard cell movements.

Despite advances in transcriptomics, global analysis of protein components is important. Comparison of the iTRAQ proteomics data set with the Arabidopsis cDNA microarray data set allows estimation of the correlation between transcripts and proteins. Although many proteins shown to be highly abundant in guard cells displayed a similar trend at the transcriptional level, the exact -fold changes were mostly of a low degree of consistency (Fig. 4 and supplemental Table 2). This is not surprising because post-transcriptional, translational, and post-translational mechanisms regulate protein isoforms and their quantities. iTRAQ proteomics is important to identify quantitative changes of different protein species for which little can be reflected at the mRNA levels. The iTRAQ proteomics approach allows the analysis of relative abundance of all proteins in a sample including both membrane and soluble proteins, whereas the traditional 2D gel electrophoresis-based proteomics tends to focus on identifying soluble proteins and to quantify gel spots, each often containing more than one protein (18). With the development of eightplex iTRAQ reagents, protein identification and quantification technology will be greatly advanced, especially the possibility of including more replicates within the same sample preparation, and mass spectrometry analysis will greatly improve reliability and accuracy of protein quantification (48, 49). In conclusion, we have shown the utility of iTRAQ proteomics technology in identifying and quantifying proteins in GC and MC. This study has unveiled many differentially expressed proteins, indicating functional specialization of the two types of cells in B. napus. Although the homologs of some of the proteins have been studied in other species, rarely any has been functionally characterized in B. napus GC. Future experiments using biochemical, molecular, and genetics tools are needed to unravel the roles that these proteins play. The functional information may be directly applied to the enhancement of stress tolerance and production of oilseeds and biofuels.

Acknowledgments—Dr. Sally Assmann and her student Zhixin Zhao are acknowledged for providing excellent training for guard cell isolation. Dr. David Oppenheimer is thanked for providing training and access to microscopes in his laboratory.

* This work was supported by faculty startup funding from the University of Florida and recently by the National Science Foundation. [S] The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

To whom correspondence should be addressed: Dept. of Botany, Genetics Inst., University of Florida, Gainesville, FL 32610. Tel.: 352-273-8330; Fax: 352-273-8284; E-mail: schen@ufl.edu.

REFERENCES
1. Assmann, S. M. (1993) Signal transduction in guard cells. Annu. Rev. Cell Biol. 9, 345–375
2. Vavasseur, A., and Raghavendra, A. S. (2005) Guard cell metabolism and CO2 sensing. New Phytol. 165, 665–682
3. Outlaw, W. H., and De Vlieghere-He, X. (2001) Transpiration rate, an important factor controlling the sucrose content of the guard cell apoplast of broad bean. Plant Physiol. 126, 1716–1724
4. Parvath, K., and Raghavendra, A. S. (1997) Both rubisco and phosphoenolpyruvate carboxylase are beneficial for stomatal function in epidermal strips of Commelina benghalensis. Plant Sci. 124, 153–157
5. Leonard, N., Kwak, J. M., Robert, N., Waner, D., Leonard, G., and Schroeder, J. I. (2004) Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell 16, 596–615
6. Li, S., Assmann, S. M., and Albert, R. (2006) Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol. 4, 1732–1748
7. Wienkoop, S., Zeiler, D., Ebert, B., Simon-Rosin, U., Fisahn, J., Glinski, M., and Weckwerth, W. (2004) Cell-specific protein profiling in Arabidopsis thaliana trichomes: identification of trichome located proteins involved in sulfur metabolism and detoxification. Phytochemistry 65, 1641–1649
8. Amme, S., Rutten, T., Melzer, M., Sorsmann, G., Vissers, J., Schlesier, B., and Mock, H. (2005) A proteome approach defines protective functions of tobacco leaf trichomes. Proteomics 5, 2508–2518
9. Wan, J., Torres, M., Ganapathy, A., Thelen, J., DaGue, B., Mooney, B., Xu, D., and Stacey, G. (2005) Proteomic analysis of soybean root hairs after infection by Bradyrhizobium japonicum. Mol. Plant-Microbe Interact. 18, 458–467
10. Dai, S., Wang, T., Yan, X., and Chen, S. (2007) Proteomics of pollen development and germination. J. Proteome Res. 6, 4556–4563
11. Chen, S., and Halkier, B. A. (2000) Characterization of glucosinolate uptake by leaf protoplasts of Brassica napus. J. Biol. Chem. 275, 22955–22960
12. Fairley-Grenot, K. A., and Assmann, S. M. (1992) Whole cell K+ current across the plasma membrane of guard cells from a grass: Zea mays. Planta 166, 282–293
13. Shimazaki, K. I., Kinoshita, T., and Nishimura, M. (1992) Involvement of calmodulin and calmodulin-dependent myosin light chain kinase in blue light-dependent H pumping by guard cell protoplasts from Vicia faba L. Plant Physiol. 99, 1416–1421
14. Willmer, C. M., Grammatikopoulos, G., LascÈve, G., and Vavasseur, A. (1999) Characterization of the vacuolar-type H+-ATPase from guard cell protoplasts of Commelina. J. Exp. Bot. 46, 383–389
15. Pandey, S., Wang, X., Coursol, S. A., and Assmann, S. M. (2002) Preparation and applications of Arabidopsis thaliana guard cell protoplasts. New Phytol. 153, 517–526
16. Takahashi, Y., Kinoshita, T., and Shimazaki, K. (2007) Protein phosphorylation and binding of a 14-3-3 protein in Vicia guard cells in response to ABA. Plant Cell Physiol. 48, 1182–1191
17. Love, C. G., Robinson, A. J., Lim, G. A. C., Hopkins, C. J., Batley, J., Barker, G., Spangenberg, G. C., and Edwards, D. (2005) Brassica ASTR: an
integrated database for Brassica genomic research. Nucleic Acids Res. 33, D656–D659.
18. Chen, S., and Harmon, A. (2006) Advances in plant proteomics. Proteomics 6, 5504–5516.
19. Zieke, L. R. (2006) A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. J. Exp. Bot. 57, 1501–1508.
20. Girard, Y., Singleton, P. A., Rowshan, A., Gucek, M., Cole, R. N., Graham, D. R. M., Van Eyk, J. E., and Garcia, J. G. N. (2007) Quantitative proteome analysis of Human endothelial cell membrane rafts. Mol. Cell. Proteomics 6, 689–696.
21. Chen, S. (2006) High throughput protein identification using direct infusion nanoelectrospray ionization mass spectrometry. Proteomics 6, 16–25.
22. Shilov, I. V., Seymour, S. L., Patel, A. A., Loboda, A., Tang, W. H., Keating, S. R., Singleton, P. A., Rowshan, A., Gucek, M., Cole, R. N., Graham, D. R. M., Van Eyk, J. E., and Garcia, J. G. N. (2007) Quantitative proteome analysis of Human endothelial cell membrane rafts. Mol. Cell. Proteomics 6, 689–696.
23. Nagele, E., Vollmer, M., Horth, P., and Vad, C. (2004) 2D-LC/MS techniques for the identification of proteins in highly complex mixtures. Exp. Rev. Proteomics 1, 37–46.
24. Qian, W., Jacobs, J. M., Camp, D. G., II, Monroe, M. E., Moore, R. J., Finn, R. D., Mistry, J., Schuster-Boocksler, B., Griffiths-Jones, S., Hollich, V., and Sonnhammer, E. L. L. (2006) Pfam: clans, web tools and services. Nucleic Acids Res. 34, D247–D251.
25. Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirks, W., Van Staveren, M., Stiekenka, W., Drost, L., Ridley, P., Hudson, S. A., Patel, K., Murphy, G., Pilfanelli, P., Weider, H., Weider, E., Wambutt, R., Wietzenegger, T., Pohl, T. M., Teyn, N., Gieler, J., Villarroyo, R., De Clerck, R., Van Montagu, M., Lechynar, A., Abou, S. R., Luy, L., Kran, A., Lao, N., and Schafer, C. A. (2005) The Arabidopsis proteome database TAP: a paradigm algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol. Cell. Proteomics 6, 1638–1655.
26. Chen, S., Hayes, P. M., Mulrooney, D. M., and Pan, A. (1994) Identification and characterization of cDNA clones encoding plant calreticulin in barley. Planta 189, 635–843.
27. Smart, L. B., Cameron, K. D., and Bennett, A. B. (2000) Isolation of genes predominantly expressed in guard cells and epidermal cells of Nicotiana glauca. Plant Mol. Biol. 42, 857–869.
28. Chen, F., Hayes, P. M., Mulrooney, D. M., and Pan, A. (1994) Identification and characterization of cDNA clones encoding plant calreticulin in barley. Planta 189, 635–843.
29. Kim, M., Hepler, P. K., Eun, S.-O., Ha, K. S., and Lee, Y. (1995) Actin filaments in mature guard cells are rapidly distributed and involved in stomatal movement. Plant Physiol. 109, 1077–1084.
30. Sui, J., Zhang, J., Tan, T.-L., Ching, C. B., and Chen, W. N. (2008) Comparative proteome analysis of Human endothelial cell membrane rafts. Proteomics 8, 2623–2633.
31. Chen, S., Hayes, P. M., Mulrooney, D. M., and Pan, A. (1994) Identification and characterization of cDNA clones encoding plant calreticulin in barley. Planta 189, 635–843.
32. Entian, K. D., Benes, V., Rechman, S., Ansorge, W., Cooke, R., Berger, C., Delseny, A., Voukelatou, E., Milioni, D., Hatzopoulos, P., Piravandi, E., Obermaier, P., Entian, K. D., Rieger, M., Schaeffer, M., Funk, B., Mueller-Auer, S., Auborg, S., Gy, I., Kreis, M., Lao, N., and Schafer, C. A. (2005) The Arabidopsis proteome database TAP: a paradigm algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol. Cell. Proteomics 6, 1638–1655.
33. Nygren, J. M., China, Z., Sun, Q., and van Wijk, K. J. (2005) Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. Plant Cell 17, 3111–3140.
34. Washburn, M. P., Koller, A., Oshiro, G., Ulaszek, R. R., Plouffe, D., Deciu, C., Winzeler, E., and Yates, J. R., III (2003) Protein pathway and complex clustering of correlated mRNA and protein expression analyses in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 100, 3107–3112.
35. Sarry, J., Chen, S., Collum, R., Liang, S., Peng, M., Lang, A., Yuan, C., Si, M., and Schaffer, C. A. (2007) The Arabidopsis proteome database TAP: a paradigm algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol. Cell. Proteomics 6, 1638–1655.
36. Mustilli, A. C., Merlot, S., Vavasseur, A., Freni, F., and Giraudeau, J. (2002) Arabidopsis mutants, A protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell 14, 3089–3099.
37. Kassie, F., Anderson, L. B., Higgins, L., Pan, Y., Matise, I., Negia, M., Pierce, A., Unwin, R. D., Evans, C. A., Griffiths, S., Carney, L., Zhang, L., and Sonnhammer, E. L. L. (2006) Pfam: clans, web tools and services. Nucleic Acids Res. 34, D247–D251.
38. Sokolovski, S., Hills, A., Gay, R., Lamattina, L., and Blatt, M. R. (2005) Protein phosphorylation is a prerequisite for intracellular Ca2+ release and ion channel control by nitric oxide and asbsic acid in guard cells. Plant J. 43, 520–529.
guard cell protoplasts of *Arabidopsis thaliana* in response to blue light. *Plant Cell Physiol.* **46**, 955–963

55. Allen, G. J., Chu, S. P., Schumacher, K., Shimazaki, C. T., Vafeados, D., Kemper, A., Hawke, S. D., Tallman, G., Tsien, R. Y., Harper, J. F., Chory, J., and Schroeder, J. I. (2000) Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis *det3* mutant. *Science* **289**, 2338–2342

56. Vahisalu, T., Kollist, H., Wang, Y. F., Nishimura, N., Chan, W. Y., Valerio, G., Lamminmäki, A., Broschê, M., Moldau, H., Desikan, R., Schroeder, J. I., and Kangasjärvi, J. (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature* **452**, 487–491

57. Ling, V., and Assmann, S. M. (1992) Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba*. *Plant Physiol.* **100**, 970–978

58. Kinoshita, T., and Shimazaki, K. (2002) Biochemical evidence for the requirement of 14–3-3 protein binding in activation of the guard-cell plasma membrane H⁺-ATPase by blue light. *Plant Cell Physiol.* **43**, 1359–1365

59. Gudesblat, G. E., Iusem, N. D., and Morris, P. (2007) Guard cell-specific inhibition of Arabidopsis MPK3 expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide. *New Phytol.* **173**, 713–721

60. Li, W., Luan, S., Schreiber, S. L., and Assmann, S. M. (1994) Evidence for protein phosphatase 1 and 2A regulation of K⁺ channels in two types of leaf cells. *Plant Physiol.* **106**, 963–970

61. Kwak, J. M., Moon, J. H., Murata, Y., Kuchitsu, K., Leonhardt, N., DeLong, A., and Schroeder, J. I. (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. *Plant Cell* **14**, 2849–2861

62. Cao, S., Jiang, L., Song, S., Jing, R., and Xu, G. (2006) AtGRP7 is involved in the regulation of abscisic acid and stress responses in Arabidopsis. *Cell. Mol. Biol. Lett.* **11**, 526–535

63. Schöning, J. C., Streitner, C., Page, D. R., Hennig, S., Uchida, K., Wolf, E., Furuya, M., and Staiger, D. (2007) Auto-regulation of the circadian slave oscillator component AtGRP7 and regulation of its targets is impaired by a single RNA recognition motif point mutation. *Plant J.* **52**, 1119–1130

64. Rouhier, N., and Jacquot, J. P. (2002) Plant peroxiredoxin: alternative hydroperoxide scavenging enzymes. *Photosynth. Res.* **74**, 259–268

65. Buchanan, B. B., and Balmer, Y. (2005) Redox regulation: a broadening horizon. *Annu. Rev. Plant Biol.* **56**, 187–220

66. Kwak, J. M., Nguyen, V., and Schroeder, J. I. (2006) The role of reactive oxygen species in hormonal response. *Plant Physiol.* **141**, 323–329

67. Chen, Z., and Gallie, D. R. (2004) The ascorbic acid redox state controls guard cell signalling and stomatal movement. *Plant Cell* **16**, 1143–1162

68. Chen, Z., and Gallie, D. R. (2006) Dehydroascorbate reductase affects leaf growth, development, and function. *Plant Physiol.* **142**, 775–787

69. Ushimaru, T., Nakagawara, T., Fujioka, Y., Daicho, K., Naito, M., Yam-auchi, Y., Nonakad, H., Amakoc, K., Yamawakib, K., and Murata, N. (2006) Transgenic Arabidopsis plants expressing the rice dehydroascor-bate reductase gene are resistant to salt stress. *J. Plant Physiol.* **163**, 1179–1184

70. Babich, M., Foti, L. R. P., Sykaluk, L. L., and Clark, C. R. (1996) Profilin forms tetramers that bind to G-actin. *Biochem. Biophys. Res. Commun.* **216**, 125–131