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

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SUMMARY

Guard cells are highly specialized cells, forming tiny pores called stomata on leaf surface. The opening and closing of stomata control leaf gas exchange and water transpiration, as well as allow plants to quickly respond and adjust to new environmental conditions. Mesophyll cells are specialized for photosynthesis. Despite of 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 proteomic analysis of purified guard cells and mesophyll cells. With the use of iTRAQ tagging and two-dimensional liquid chromatography mass spectrometry, we have identified 1458 non-redundant proteins in both guard cells and mesophyll cells of *Brassica napus* leaves. Based on stringent statistical criteria, a total 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 have observed several well-characterized guard cell proteins. By contrast, proteins involved in photosynthesis, starch synthesis, disease/defense/stress, and other metabolism 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 percent of these proteins follow the same trend of expression at 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.

INTRODUCTION

Guard cells (GC) are highly specialized cells, forming tiny pores called stomata on 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 to MC, GC contain few chloroplasts with very limited structures, and thus possess very low photosynthetic capability. Calvin cycle in GC only assimilates 2-4% of CO_2 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. (2004) 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 (5). This only available large scale genomic study identified 1309 guard cell expressed genes, of which 64 transcripts mainly involved in transcription, signaling and cytoskeleton were preferentially expressed in GC compared to 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 protein level.

Up 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 proteomic analysis 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. Dr. Assmann laboratory at Pennsylvania State University started guard cell proteomics work several years ago and have identified more than 1800 unique guard cell proteins in Arabidopsis (personal communications). *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 towards 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 (2-DE) 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 *Brassica napus* var Global were obtained from the USDA National Plant Germplasm System (http://www.ars-grin.gov/npgs/searchgrin.html). Seeds were

germinated in Metro-Mix 500 potting mixture (The Scotts Co., USA) and plants were grown in a growth chamber under a photosynthetic flux of 160 µmol photons $m^{-2}s^{-1}$ with a photoperiod of 10 hours at 24°C light and 20°C dark. Fully expanded leaves from two 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 gram fully expanded leaves with main veins removed were blended three times, 30 seconds each in cold tap water, using a 14-speed Osterizer blender (Oster Inc., Mexico). The first enzyme digestion of epidermal peels was one hour 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 digestion was 100 µm and 30 µm, respectively. After Histopaque purification, the cells were resuspended in 1 ml basic solution. Ten microliters of the suspension was then taken and the number of protoplasts 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 previously described (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 protein as one replicate. Three guard cell replicates and three different mesophyll preparations, each with 100 µg 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 four-plex kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA). The guard cell replicates were labeled with iTRAQ tag 114, 115 and 114, and the mesophyll cell replicates were labeled with tag 116, 117 and 116, respectively. From our experience, these isotope 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 x 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% acetic acid v/v) plus 0.01% trifluoric acetic 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 id, 3 µmm, 100 A) (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 previously described (21). Peptides were eluted from the HPLC column by a linear gradient from 3% solvent B (96.9% acetonitrile v/v, 0.1% acetic acid v/v) to 40% solvent B for 2 hours, followed by ramping up to 90% solvent B in 10 min. Peptides were sprayed into the orifice of the mass spectrometer, which is operated in an information-dependent data acquisition mode where a MS scan followed by three MS/MS scans of 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 NCBI nonredundant fasta database (5,222,402 entries, downloaded on July 2, 2007) using the Paragon™ algorithm (22) of ProteinPilot v2.0.1 software suite (Applied Biosystems, USA). Plant species, fixed modification of methylmethanethiosulfatelabeled 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 peptides 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 confidence which 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 MSMS spectra unique to a particular protein and where the sum of the signal-to-noise ratio for all of the peak pairs greater than nine were used for quantification (software default settings, Applied Biosystems, USA). The mean, standard deviation, 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 6 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 in the method section. From eight gram fully expanded leaves, the yield of guard cell protoplasts is on average $5x10^5$/ml, which corresponds to approximately 30 µg protein. The purity of final guard cell preparation is 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 proteomic analysis.

Protein Identification by Offline 2D HPLC-MS/MS

After iTRAQ labeling and combination of guard cell and mesophyll cell samples, the

peptides were fractionated by strong cation exchange (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 online 2D LC-MS, the offline 2D LC-MS workflow 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 additional 342 guard cell proteins. So altogether 1458 GC proteins were identified (Supplemental Table I; Fig. 2). 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 the 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 *Brassica napus* genome is not yet available. Thus we have included other plant species in our database searching to enhance the success rate of protein identification. For cross-species identification, we have 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 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. (1998) (26). A Venn diagram for the functional classification is shown in Figure 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 II). 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, while 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 the fold-change of at least 2 are included as guard cell or mesophyll cell preferentially expressed proteins (Tables I, II, Fig. 2). While most published results are based on a fold change threshold of 1.2 to 1.5 (20, 27-29), our criterion of two 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 nucleosome and three in cell structure were highly expressed in GC (Table I, Fig. 2). On the contrary, in MC the majority of proteins (approximately 50%) are involved in photosynthesis, followed by 23 proteins involved in metabolism and 17 disease/defense/stress proteins (Table II, Fig. 2). Representative MS/MS spectra for peptides identified from a photosystem II protein and a plasma membrane H^+-ATPase AHA1 are shown in Fig. 3. The peaks of iTRAQ signature ions

(114.1 for guard cells and 116.1 for mesophyll cells) are shown as inserts, representing the relative abundance of the proteins in MC and GC, respectively. It is important to note that we have 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 D1 (34), protein kinase C (35, 36), OST protein kinase (6), Atrboh NADPH oxidase (37), potassium channel (38), chloride channel (38), lipid transfer protein (39), calreticulin (40) and profilin (41).

Comparative Analysis of Transcriptome Data and Proteome Data

The only available transcriptomic 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 has revealed specific functions associated with the two types of cells (see previous paragraph). When comparing proteome data with transcriptome data, 60 genes out of the 1309 genes could be matched to 110 proteins by identity (proteins identified in Arabidopsis database) or by high homology (proteins identified in Brassica or other species database) (Supplemental Table II). When the relative protein expression levels were compared with mRNA levels, 80 displayed similar expression trend and 30 showed opposite trend of expression. For those that follow similar expression trend at mRNA and protein levels, the fold changes at the two levels were mostly different. The correlation coefficient is only 0.37 (Fig. 4). Among the 74 GC-enriched proteins described in the previous section, 15 are represented on the microarray and nine transcripts were identified as GC-enriched (Supplemental Table II). These results confirm the general observation that mRNA levels are not always consistent with protein levels because of posttranscriptional, translational or posttranslational regulations

(32-44). They also highlight the importance of proteomic analysis.

DISCUSSION

While most proteomic 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 several folds. First, *B. napus* guard cells are larger and have higher protein contents than Arabidopsis guard cells. Usually 30 µg protein can be obtained from each preparation of eight gram fully expanded leaves. While with Arabidopsis, the yield of GC is about 10 µg per preparation. Second, the knowledge gained in Arabidopsis and *B. napus* may be inter-extendable 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 recently developed 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). While 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 labs (e.g., 46). We have 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 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 of MS/MS spectra for identification of low abundance proteins. In this study, we have 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, since 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 has been obtained from at least one of the three different experiments (Supplemental Table I). However, the relative expression ratios of over a half of the proteins either have high p-values (> 0.05) or no p-values. This seems to be generally a case when iTRAQ experiments were 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 the peak pairs with the sum of the signal-to-noise ratio over nine 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 as those described in the result section 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 (GAPDH), mitochondrial ATPase beta 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, 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, 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 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, while 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 are novel proteins identified in *B. napus* GC. While 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 previously identified in GC. Recently, a 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 abscisic acid (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 MAP kinase, MPK3, downstream of H_2O_2 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) A 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 homolog 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 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 disease and defense category, two plant peroxiredoxins and a dehydroascorbate reductase showed high levels of expression in GC. Peroxiredoxins 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, 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 I). 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 similar trend at the transcriptional level, the exact fold changes were mostly of low degree of consistency (Supplemental Table II, Fig. 4). This is not surprising since post-transcriptional, translational and post-translational mechanisms regulate protein isoforms and their quantities. The iTRAQ proteomics is important to identify quantitative changes of different protein species, for which little can be reflected at the mRNA levels. iTRAQ proteomics approach allows the analysis of relative abundance of all proteins in a sample including both membrane and soluble proteins, while the traditional 2D gel electrophoresis based proteomics tends to focus on identifying soluble proteins and to quantify gel spots, each often contains more than one proteins (18). With the development eight-plex 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, which indicate 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.

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TABLES

Table I. Proteins expressed at least two fold higher in guard cells (GC) ($p < 0.05$). The proteins, whose expression changes determined only in one of the replicates, were highlighted with a star.

Table II. Proteins expressed at least two fold higher in mesophyll cells (MC) ($p < 0.05$). The proteins, whose expression changes determined only in one of the replicates, were highlighted with a star.

FIGURE LEGENDS

FIG 1. Isolation of guard cell protoplasts from *Brassica napus* leaves. *A.* guard cells and epidermal cells on a leaf epidermal peel (arrows); *B.* after the second enzyme digestion, guard cells round up and are released from stomata; *C.* guard cell protoplasts collected after separating from epidermal peels. Note contamination by a mesophyll cell; *D.* guard cell protoplasts purified by Histopaque centrifugation (400 x).

FIG 2. Classification of the 1458 identified proteins into molecular functions. The pie chart shows the distribution of the non-redundant proteins into their functional classes in percentage. The classification was performed with reference to Bevan et al.

(26). *A*. all the 1458 proteins; *B*. proteins enriched in GC; *C*. proteins enriched in MC.

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 DSNIASIPVEELIEK (confidence 99%) derived from plasma membrane P-type ATPase AHA1 and its relative abundance in the two types of cells.

FIG 4. Scatter plot of GC/MC ratio at mRNA level and protein level. The correlation coefficient is estimated to be 0.37.

SUPPLEMENTAL DATA

Supplemental Table I. List and functional classification of all 1458 proteins identified using ProteinPilot software. Peptides from mesophyll cell proteins were labeled with iTRAQ tag 116 or 117 and from guard cells were labeled with iTRAQ tag 114 or 115. Ratios and p-values were calculated with the Paragon algorithm built in the ProteinPilot software. Functional classification was performed with reference to Bevan et al. (26)

Supplemental Table II. Comparison of relative protein expression levels with mRNA levels of homologous Arabidopsis genes. The microarray data were obtained using Affymetrix GeneChips representing approximately 8100 genes (5). The Arabidopsis

genes displaying the highest possible homology (determined by BLAST e-values) with the sequences identified in *Brassica napus* were chosen for comparative analysis of expression levels.

Supplemental Fig. 1. MS/MS spectra of major peptides (A) and protein sequence coverage (B) showing all the peptides supporting a confident protein identification using Paragon algorithm.

Supplemental Fig. 2. Example of cation exchange chromatography of iTRAQ labeled peptides derived from guard cell (iTRAQ tag 114 or 115) and mesophyll cell proteins (iTRAQ tag 116 or 117). Nineteen fractions were collected for downstream reverse phase separation.

Supplemental Fig. 3. Volcano plot representation of iTRAQ results. (A) Ratios of replicate samples of guard cells and mesophyll cells combined against p-value of ratio; (B) ratios of guard cells versus mesophyll cells against p-value of ratio. Ratios and p-values were calculated with ProteinPilot using the Paragon algorithm. Only proteins with multiple peptide measurements (and thus calculated p-values) are included. Dotted vertical lines indicate the 2-fold-change threshold selected for inclusion in Tables I & II, and the dotted horizontal line indicates the minimum p-value for inclusion in Tables I & II of 0.05.

Table 1 Proteins predominantly expressed in guard cells (GC) ($p < 0.05$)

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				GC/MC	P-value	GC/MC	P-value	GC/MC	P-value	GC/MC
		Energy (16)								
1.	gi|15226479	triose-phosphate isomerase (TIM)	*Arabidopsis thaliana*	1.781	0.000	2.044	0.000	2.226	0.001	2.000
2.	gi|15229231	glyceraldehyde-3-phosphate dehydrogenase C subunit	*A. thaliana*	5.087	0.070	2.777	0.004	5.453	0.020	3.680
3.	gi|15233272	cytosolic triose phosphate isomerase	*A. thaliana*	1.920	0.000	2.018	0.000	4.702	0.007	2.441
4.	gi|15236591	aldose 1-epimerase family protein	*A.thaliana*	2.370	0.001	2.603	0.001	4.070	0.063	2.481
5.	gi|15238151	6-phosphogluconate dehydrogenase	*A.thaliana*	2.051	0.012	2.582	0.009			2.286
6.	gi|15240075	succinate dehydrogenase 1-1(SDH1-1)	*A.thaliana*	2.248	0.023	2.041	0.026	1.001	0.998	2.139
7.	gi|21536853	putative phosphoglycerate kinase	*A.thaliana*	2.166	0.026	2.467	0.020	2.779	0.043	2.445
8.	gi|21592878	inorganic pyrophosphatase-like protein	*A.thaliana*	2.132	0.003	2.027	0.001			2.078
9.	gi|30689081	phosphoenolpyruvate carboxylase 2(ATPPC2)	*A.thaliana*	7.017	0.000	4.701	0.001	3.180	0.232	5.630
10.	gi|30696904	xylose isomerase family protein	*A.thaliana*	2.252	0.043	2.169	0.019	1.939	0.381	2.210
11.	gi|3676296	mitochondrial ATPase beta subunit	*Nicotiana sylvestris*	3.088	0.003	2.567	0.003	2.121	0.009	2.532
12.	gi|79314806	mithochondrial ATP synthase D chain(ATPQ)	*A.thaliana*	2.171	0.017	1.919	0.011			2.037
13.	gi|51102306	putative glyceraldehyde 3-phosphate dehydrogenase	*Brassica juncea*	2.142	0.014	2.089	0.021			2.115
14.	gi|34597332	enolase	*B.napus*	1.807	0.003	1.516	0.206	2.525	0.003	2.106
15.	gi|4874272*	strong similarity to gb|Y09533 involved in starch metabolism with a PF|01326 Pyruvate phosphate dikinase, PEP/pyruvate binding domain.	*A. thaliana*	2.223	0.045	1.870	0.219			2.223
16.	gi|68426	triose-phosphate isomerase	*Zea mays*	2.021	0.000	2.043	0.000	2.276	0.135	2.032
		Metabolism (13)								
17.	gi|15222072	UDP-D-glucose/UDP-D-galactose 4-epimerase 1 (UGE1)	*A. thaliana*	1.923	0.004	2.187	0.001			2.046
18.	gi|147742770	hypothetical protein,containing PRK10675 UDP-galactose-4-epimerase domain	*B. rapa*	1.902	0.018	2.190	0.008			2.036
19.	gi|15242351*	reversibly glycosylated polypeptide-3	*A.thaliana*	0.181		0.635		3.872	0.013	3.872
20.	gi|15241721*	putative protein,containing pfam02719 Polysaccharide biosynthesis protein domain	*A. thaliana*	3.630	0.383	3.064		2.055	0.025	2.055

Table 1 continued

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				GC/MC	P-value	GC/MC	P-value	GC/MC	P-value	GC/MC
21.	gi|15239735*	thiazole biosynthetic enzyme precursor (ARA6)	*A. thaliana*	2.438	0.005					2.438
22.	gi|15239772	aspartate aminotransferase 2 (ASP2)	*A. thaliana*	2.293	0.005	2.127	0.045			2.207
23.	gi|3334244	S-D-lactoylglutathione methylglyoxal lyase	*B. juncea*	2.464	0.043	1.994	0.005	1.338	0.338	2.205
24.	gi|599625*	aconitase	*A. thaliana*	1.132	0.749	1.135	0.589	3.311	0.020	3.311
25.	gi|7385217*	beta-ketoacyl-ACP synthetase 1	*B. napus*	1.406	0.161	2.113	0.036	1.375	0.526	2.113
26.	gi|15226618*	putative fumarase	*A. thaliana*	2.467	0.006	1.860	0.052			2.467
27.	gi|116077986	pterocarpan reductase	*Lotus japonicus*	2.479	0.021	3.614	0.021			2.941
28.	gi|157890952*	putative lactoylglutathione lyase	*B. rapa*	3.311	0.254	2.559	0.320	3.114	0.011	3.114
29.	gi|15241492	formate dehydrogenase (FDH)	*A. thaliana*	2.419	0.007	2.347	0.007	1.491	0.566	2.383
		Protein synthesis (3)								
30.	gi|15228111*	40S ribosomal protein S5	*A. thaliana*	1.019	0.909	1.080	0.744	2.085	0.049	2.085
31.	gi|7489746*	golgi associated protein se-wap41	*Z. mays*	0.229		0.462		3.535	0.015	3.535
32.	gi|79322680	40S ribosomal protein S25 (RPS25E)	*A. thaliana*	2.109	0.006	2.621	0.015			2.338
		Protein folding, transporting and Degradation (7)								
33.	gi|15234781*	peptidylprolyl isomerase ROC1, containing cd01926 cyclophilin domain	*A. thaliana*	1.191	0.097	1.021	0.886	13.072	0.004	13.072
34.	gi|15229559*	mitochondrial chaperonin hsp60	*A. thaliana*	1.019	0.909	1.080	0.744	2.085	0.049	2.085
35.	gi|15232760	polyubiquitin (ubq8)	*A. thaliana*	3.297	0.001	3.373	0.001	1.449	0.010	2.326
36.	gi|40060485	heat shock protein HSP101	*Z.mays*	2.315	0.007	1.867	0.008			2.067
37.	gi|15229559	mitochondrial chaperonin hsp60	*A. thaliana*	2.364	0.024	2.132	0.028	1.600	0.083	2.242
38.	gi|15224993*	20S proteasome subunit (PAA2)	*A.thaliana*	1.149	0.790	1.189	0.810	3.018	0.004	3.018
39.	gi|5921735*	10 kDa chaperonin (Protein CPN10) (Protein groES)	*B.napus*	2.335	0.026	2.249	0.285			2.335
		Membrane and transport (9)								
40.	gi|124360090	plasma-membrane proton-efflux P-type ATPase	*Medicago truncatula*	4.576	0.007	4.317	0.016	5.797	0.065	4.443

Table 1 continued

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				GC/MC	P-value	GC/MC	P-value	GC/MC	P-value	GC/MC
41.	gi|1352830	vacuolar ATP synthase catalytic subunit A	*Z. mays*	3.352	0.004	2.785	0.036	2.184	0.003	2.690
42.	gi|15224264	plasma membrane proton ATPase (PMA), AHA1	*A. thaliana*	1.977	0.005	2.333	0.003	3.674	0.008	2.486
43.	gi|15232300*	plasma membrane H^+-ATPase, AHA7	*A.thaliana*	7.210	0.083	6.631	0.035	2.181		6.631
44.	gi|15234666	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.	gi|18844793	putative H^+-exporting ATPase	*Oryza sativa*	2.316	0.010	2.546	0.003	2.580	0.067	2.426
46.	gi|2493131	vacuolar ATP synthase subunit B 1	*A. thaliana*	1.932		3.050	0.004	1.905	0.005	2.345
47.	gi|758355*	H^+ -transporting ATPase	*Z. mays*	1.590	0.099	1.565	0.627	2.694	0.015	2.694
48.	gi|15231937	adenylate translocator	*A. thaliana*	2.163	0.000	2.150	0.002	1.766	0.246	2.157
		Stress and Defence (9)								
49.	gi|15222163*	putative GSH-dependent dehydroascorbate reductase 1	*A.thaliana*	3.586	0.017	1.750	0.070	1.903		3.586
50.	gi|15225245*	Bet v I allergen family protein	*A. thaliana*	3.134	0.064	2.572	0.031			2.572
51.	gi|15231718	putative peroxiredoxin type 2	*A. thaliana*	5.085	0.010	4.522	0.006	9.075	0.148	4.787
52.	gi|15239697	resistant to agrobacterium transformation 5	*A. thaliana*	2.372	0.015	4.025	0.032	4.783	0.003	3.412
53.	gi|157849770	early-responsive to dehydration 12 (ERD12 protein)	*B.rapa*	2.039	0.039	2.058	0.040	1.454	0.272	2.048
54.	gi|18397457	peroxiredoxin IIF (ATPRXIIF/PRXIIF)	*A. thaliana*	2.038	0.000	1.999	0.002	1.935		2.018
55.	gi|18404709	unknown protein,containing pfam00407 Pathogenesis-related protein Bet v I family domain	*A. thaliana*	2.532	0.019	3.279	0.041	11.657		2.857
56.	gi|21555213*	vegetative storage protein-like	*A. thaliana*	2.030	0.023	1.019	0.911	4.052		2.030
57.	gi|9795585*	Putative GSH-dependent dehydroascorbate reductase	*A. thaliana*	2.255	0.011	2.046	0.068	2.275	0.113	2.255
		Signal transduction (8)								
58.	gi|15219510	14-3-3 protein, putative	*A. thaliana*	2.428	0.006	1.995	0.014	2.099	0.051	2.190
59.	gi|21553354	glycine-rich RNA binding protein 7	*A. thaliana*	2.161	0.002	2.280	0.004	1.766	0.158	2.219
60.	gi|3702349	putative mitogen-activated protein kinase	*A. thaliana*	3.134	0.000	3.250	0.002			3.191
61.	gi|78102508	cytokinin-binding protein CBP57	*Nicotiana sylvestris*	2.791	0.034	3.798	0.031	1.770		3.217
62.	gi|79317272	calmodulin binding/translation elongation factor	*A. thaliana*	3.253	0.013	3.799	0.001	1.459	0.044	2.389
63.	gi|81248479*	mitogen-activated protein kinase 4	*B.napus*	2.140	0.138	2.609	0.077	5.013	0.034	5.013

Table 1 continued

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				GC/MC	P-value	GC/MC	P-value	GC/MC	P-value	GC/MC
64.	gi|899058	calmodulin	*B. juncea*	2.340	0.002	3.475	0.002	2.025	0.000	2.482
65.	gi|9958062*	putative protein phosphotase 2a 65kd regulatory subunit	*A. thaliana*	2.063	0.045	2.075	0.155			2.063
		Transcription related (4)								
66.	gi|145323776	histone H4	*A. thaliana*	1.988	0.000	2.621	0.000	4.369	0.014	2.695
67.	gi|15224536	histone H1	*A. thaliana*	6.491	0.000	8.080	0.002	6.445	0.090	7.199
68.	gi|15241858	histone H2B, putative	*A.thaliana*	1.877	0.001	2.299	0.000	5.443	0.000	2.605
69.	gi|5777792*	histone H2A	*B. napus*	2.282	0.223	2.173	0.182	4.860	0.001	4.860
		Cell structure (3)								
70.	gi|34733239*	putative tubulin alpha-2/alpha-4 chain	*B. napus*	1.650	0.184	1.639	0.397	2.697	0.034	2.697
71.	gi|4139264*	actin	*B. napus*	1.181	0.073	1.210	0.059	2.238	0.010	2.238
72.	gi|77549556*	tubulin alpha-3 chain	*O. sativa*					3.413	0.016	3.413
		Unknown (2)								
73.	gi|30681554	unknown protein	*A. thaliana*	1.696	0.000	1.855	0.000	2.994	0.048	2.051
74.	gi|30690673*	CP12 domain-containing protein 1 (CP12-1)	*A. thaliana*	1.095	0.800	2.112	0.014			2.112

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

Table 2. Proteins predominantly expressed in mesophyll cells (MC) (p < 0.05)

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				MC/GC	P-value	MC/GC	P-value	MC/GC	P-value	MC/GC
		Photosynthesis (71)								
1.	gi|108796808	photosystem I subunit VII	*Staurastrum punctulatum*	2.759	0.000	3.149	0.000	2.397	0.004	2.768
2.	gi|6006283	photosystem I subunit PSI-L	*Arabidopsis thaliana*	2.856	0.001	3.849	0.001			3.353
3.	gi|15222757	putative photosystem I subunit V precursor	*A. thaliana*	5.941	0.011					5.941
4.	gi|15221681	putative photosystem I subunit III precursor	*A.thaliana*	2.388	0.000	2.777	0.000	6.347	0.000	3.837
5.	gi|121582119	photosystem I P700 apoprotein A1	*Brassica oleracea*	2.568	0.000	3.643	0.000	7.325	0.000	4.512
6.	gi|3914442	photosystem I reaction center subunit VI, PSI-H	*B. rapa*	2.514	0.032	2.847	0.011	2.170		2.681
7.	gi|75107089	photosystem I reaction center subunit N (PSI-N)	*Pisum sativum*	1.741	0.012	2.528	0.009			
8.	gi|15237593	photosystem I reaction center subunit PSI-N	*A. thaliana*	2.102	0.000	1.987	0.000	4.536	0.000	
9.	gi|15235503	putative photosystem I reaction center subunit II precursor	*A. thaliana*	3.541	0.000	3.855	0.000	3.259	0.000	3.552
10.	gi|15235490	probable photosystem I chain XI precursor	*A. thaliana*	2.316	0.000	3.125	0.000	6.426	0.006	3.956
11.	gi|34393511	putative photosystem I antenna protein	*Oryza sativa*	3.388	0.000	4.382	0.000	9.827	0.000	5.865
12.	gi|56784285	chloroplast photosystem I P700 apoprotein A2	*O.sativa*	1.977	0.010	3.737	0.198	7.440	0.034	4.709
13.	gi|125656346	chloroplast PSI type III chlorophyll a/b-binding protein	*B. juncea*	3.966	0.000	3.969	0.000	6.431	0.006	4.789
14.	gi|3885892	photosystem-1 F subunit precursor	*O.sativa*	2.056	0.001	2.550	0.002	3.757	0.009	2.788
15.	gi|21554335	PSI type III chlorophyll a/b-binding protein, putative	*A. thaliana*	3.194	0.000	3.684	0.000	5.179	0.002	4.019
16.	gi|407769	PSI-D1 precursor	*Nicotiana sylvestris*	3.536	0.001	3.996	0.012	9.398	0.043	5.644
17.	gi|15219418	photosystem II 22kDa protein, putative	*A. thaliana*	2.464	0.000	3.370	0.000	5.037	0.000	
18.	gi|15230324	photosystem II subunit O-2 (PSBO-2/PSBO2)	*A. thaliana*	3.155	0.000	3.520	0.000	4.605	0.005	
19.	gi|15234637	photosystem II subunit Q-2; calcium ion binding	*A. thaliana*	2.198	0.000	2.053	0.000	5.744	0.016	
20.	gi|15237225	photosystem II stability/assembly factor HCF136	*A.thaliana*	1.688	0.000	1.532	0.001	3.280	0.032	
21.	gi|29470191	photosystem II protein D1	*Bassia scoparia*	2.009	0.000	2.897	0.000	6.049	0.000	
22.	gi|49359169	photosystem II protein	*B. oleracea*	3.717	0.000	4.621	0.001	6.645	0.003	4.994
23.	gi|81176267	photosystem II protein V	*Lactuca sativa*	1.889	0.001	2.299	0.001	4.801	0.118	2.094
24.	gi|7525059	photosystem II 47 kDa protein	*A.thaliana*	1.777	0.000	2.711	0.000	6.051	0.005	3.513
25.	gi|83641952	PSII cytochrome b559 8kDa subunit	*N. glutinosa*	2.381	0.056	3.952	0.001	3.971	0.003	3.962
26.	gi|902201	PSII 32 KDa protein	*Zea mays*	3.543	0.001	6.049	0.000			4.796

Table 2 continued

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				MC/GC	P-value	MC/GC	P-value	MC/GC	P-value	MC/GC
27.	gi|967968	photosystem II 10kDa polypeptide	*B. rapa*	2.206	0.018	1.942	0.145	6.386	0.009	4.296
28.	gi|902207	PSII 43 KDa protein	*Z. mays*	6.378	0.000	6.886	0.000	7.120	0.000	6.795
29.	gi|91983987	photosystem II protein D2	*Vitis vinifera*	2.271	0.000	3.056	0.000	5.407	0.001	3.578
30.	gi|91983988	photosystem II 44 kDa protein	*V. vinifera*	1.900	0.000	2.476	0.000	8.091	0.000	4.155
31.	gi|1620920	23kD protein of oxygen evolving system of photosystem II	*B. juncea*	1.717	0.000	1.850	0.000	3.043	0.005	2.203
32.	gi|109389998	chloroplast chlorophyll a/b binding protein	*B.napus*	3.072	0.000	4.745	0.000	8.345	0.000	5.387
33.	gi|110377793	chloroplast pigment-binding protein CP26	*N. tabacum*	2.427	0.000	3.157	0.000	8.325	0.000	4.636
34.	gi|12805303	putative Chlorophyll A-B binding protein..	*Beta vulgaris*	3.877	0.000	5.377	0.000			4.627
35.	gi|15232815	chlorophyll A-B biding protein 4 percursor homolog	*A. thaliana*	2.989	0.000	3.937	0.000	7.748	0.000	4.891
36.	gi|15241005	chlorophyll A-B binding protein CP29 (LHCB4)	*A. thaliana*	2.668	0.000	3.712	0.000	7.691	0.000	4.690
37.	gi|1644289	chlorophyll a/b-binding protein CP26 in PS II	*B. juncea*	3.098	0.000	4.077	0.000	3.554	0.000	3.576
38.	gi|145688411	LHCA2 protein	*B. juncea*	3.576	0.000	4.421	0.000	8.994	0.000	5.664
39.	gi|15225630	LHCB4.3 (light harvesting complex PSII)	*A. thaliana*	2.896	0.000	3.081	0.000	3.356	0.025	3.111
40.	gi|50313237	LHCB6 protein	*B. rapa*	2.760	0.000	3.545	0.000	7.888	0.043	4.731
41.	gi|515616	LHC II Type III chlorophyll a /b binding protein	*B. napus*	2.591	0.000	3.611	0.000	3.215	0.058	3.101
42.	gi|15231990	Light harvesting complex PSII (LHCB4.2)	*A. thaliana*	4.652	0.000	6.258	0.000	8.524	0.001	6.478
43.	gi|15233115	LHCA1; chlorophyll binding	*A. thaliana*	3.008	0.000	3.606	0.000	4.962	0.000	3.859
44.	gi|147864470	hypothetical protein	*V. vinifera*	2.848	0.001	3.886	0.002			3.367
45.	gi|15222551	phosphoribulokinase precursor	*A. thaliana*	2.382	0.000	2.562	0.000	2.958	0.000	2.634
46.	gi|15222956	plastocyanin	*A.thaliana*	2.261	0.000	1.918	0.000	2.504	0.025	2.228
47.	gi|15223331	starch synthase, putative	*A. thaliana*	2.894	0.000	5.181	0.000	7.577	0.000	5.217
48.	gi|15236722	H^+-transporting ATP synthase chain 9 - like protein	*A. thaliana*	2.222	0.000	1.949	0.000	5.552	0.005	3.241
49.	gi|15240013	33 kDa polypeptide of oxygen-evolving complex	*A. thaliana*	4.216	0.000	4.515	0.000	8.552	0.001	5.761
50.	gi|18405061	thylakoid lumen 18.3 kDa protein	*A. thaliana*	1.961	0.000	2.821	0.000	4.095	0.000	2.959
51.	gi|28141361	granule bound starch synthase	*B. rapa*	1.469	0.007	3.509	0.001	8.831	0.020	4.603
52.	gi|401249	cytochrome b6-f complex iron-sulfur subunit 2, chloroplast	*N. tabacum*	4.364	0.000	2.852	0.000	4.349	0.002	3.855
53.	gi|42571761	nonphotochemical quenching (NPQ4)	*A. thaliana*	1.916	0.000	2.760	0.000	4.849	0.002	3.175
54.	gi|58700507	chloroplast oxygen-evolving protein 16 kDa subunit	*N. benthamiana*	2.135	0.000	2.103	0.000	4.720	0.008	2.986

Table 2 continued

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				MC/GC	P-value	MC/GC	P-value	MC/GC	P-value	MC/GC
55.	gi|9843639	rieske FeS protein	*A. thaliana*	2.690	0.000	3.375	0.000			3.033
56.	gi|62318781	protochlorophyllide reductase precursor like protein	*A. thaliana*	2.633	0.026	2.716	0.019			2.675
57.	gi|110377772	chloroplast pigment-binding protein CP24	*N. tabacum*	3.143	0.000	4.320	0.000	7.529	0.004	4.997
58.	gi|17852	ribulose bisphosphate carboxylase /oxygenase small subunit	*B. napus*	2.018	0.001	2.742	0.000	3.667	0.000	2.809
59.	gi|79013990	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit precursor	*B. napus*	1.738	0.000	2.227	0.000	4.068	0.000	2.678
60.	gi|8745521	ribulose-1,5-bisphosphate carboxylase/oxygenase	*B.napus*	2.008	0.000	2.079	0.000	3.706	0.000	2.598
61.	gi|15229349	putative ribose 5-phosphate isomerase	*A. thaliana*	2.198	0.000	2.381	0.002	4.961	0.016	3.180
62.	gi|92884121	4Fe-4S ferredoxin, iron-sulfur binding	*Medicago truncatula*	2.147	0.098	2.158	0.035	6.393	0.000	4.275
63.	gi|116309995	Chlorophyll A-B binding protein, containing pfam00504	*O.sativa*	3.495	0.024	4.319	0.007	3.689	0.077	3.907
64.	gi|126633416	unnamed protein product, containing pfam00504	*B. napus*	2.981	0.000	4.176	0.000	5.965	0.001	4.374
65.	gi|24210533	chlorophyllase 1	*B. oleracea*	2.121	0.022	3.635	0.002			2.878
66.	gi|29839389	Ferritin-1, chloroplast precursor	*B.napus*	1.557	0.018	3.280	0.001	3.540	0.185	2.418
67.	gi|458797*	cytochrome b	*B. napus*	1.264		2.175		3.970	0.012	3.970
68.	gi|54043095	glycolate oxidase	*B. napus*	1.990	0.002	1.784	0.018	6.224	0.000	3.333
69.	gi|544122	apocytochrome f precursor	*B. rapa*	2.174	0.000	2.641	0.000	6.393	0.000	3.736
70.	gi|67463833*	plastocyanin with cytochrome f	*B. rapa*					7.934	0.000	7.934
71.	gi|81301580*	cytochrome f	*N.tomentosiformis*	1.265	0.845	1.372	0.773	4.468	0.005	4.468
		Energy (7)								
72.	gi|1480014	putative delta subunit of ATP synthase	*B. rapa*	3.540	0.000	2.721	0.000	4.664	0.000	3.641
73.	gi|15234900	putative fructose-bisphosphate aldolase	*A. thaliana*	1.811	0.000	1.619	0.000	4.383	0.000	2.604
74.	gi|20339362	ribulose-5-phosphate-3-epimerase	*Pisum sativum*	1.743	0.009	2.295	0.011	2.899	0.018	2.312
75.	gi|27530932	cytosolic NADP-malic enzyme	*Lithospermum erythrorhizon*	2.388	0.024	2.327	0.005			2.357
76.	gi|15233597	containing PRK05621 F0F1-ATP synthase γ domain	*A. thaliana*	1.957	0.000	2.091	0.000	5.336	0.000	3.128
77.	gi|4995091	malate dehydrogenase 2	*B.napus*	2.392	0.001	2.322	0.008	4.982	0.051	2.357
78.	gi|75336517	ATP synthase subunit beta	*B. napus*	1.772	0.000	1.775	0.000	3.254	0.000	2.267

Table 2 continued

	Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
				MC/GC	P-value	MC/GC	P-value	MC/GC	P-value	MC/GC
		Metabolism (23)								
79.	gi|114324489	geranylgeranyl reductase	*B.rapa*	2.582	0.000	1.613	0.005	1.835		2.097
80.	gi|15217485	containing PRK10675 UDP-galactose-4-epimerase domain	*A. thaliana*	2.619	0.000	2.426	0.000			2.523
81.	gi|15221892	unknown protein containing the COG1512 Beta-propeller domains of methanol dehydrogenase	*A. thaliana*	2.103	0.000	2.989	0.000	4.391	0.000	3.161
82.	gi|15225026	alanine-glyoxylate aminotransferase	*A. thaliana*	2.071	0.000	2.099	0.000	3.575	0.021	2.582
83.	gi|15232133	carbonic anhydrase, chloroplast precursor	*A. thaliana*	2.907	0.000	3.599	0.001	7.765	0.001	4.757
84.	gi|1711296	myrosinase binding protein	*B. napus*	2.916	0.000	4.833	0.000	6.517	0.000	4.755
85.	gi|1769968	myrosinase-associated protein	*B. napus*	2.110	0.003	3.561	0.000	4.843	0.027	3.505
86.	gi|18410661	unknown protein	*A. thaliana*	3.384	0.035	2.543	0.008			2.963
87.	gi|28192642	cystine lyase BOCL-3	*B. oleracea*	1.737	0.013	2.714	0.005	3.658	0.027	2.703
88.	gi|42408130	putative aminotransferase	*O. sativa*	2.063	0.007	2.003	0.001	2.793	0.025	2.286
89.	gi|50508805	putative (S)-2-hydroxy-acid oxidase	*O. sativa*	2.087	0.000	2.299	0.000	4.892	0.020	3.093
90.	gi|79313265	Jacalin lectin family protein (JR1)	*A. thaliana*	4.524	0.014	4.677	0.007			4.600
91.	gi|15220620	hydroxypyruvate reductase	*A. thaliana*	1.813	0.027	1.482	0.161	3.574	0.033	2.693
92.	gi|15221119	aminomethyltransferase-like precursor protein	*A. thaliana*	1.707	0.002	1.819	0.003	4.426	0.021	2.650
93.	gi|15225449	putative transketolase precursor	*A.thaliana*	2.424	0.013	2.195	0.327	2.369	0.044	2.396
94.	gi|15239032*	allene oxide synthase	*A. thaliana*	3.500	0.054	4.163	0.049			4.163
95.	gi|15239406	p-nitrophenylphosphatase-like protein	*A. thaliana*	2.126	0.001	2.017	0.004	2.634	0.006	2.259
96.	gi|157849706	catalytic/coenzyme binding protein	*B. rapa*	1.746	0.067	2.301	0.043			2.301
97.	gi|1617272*	AMP-binding protein	*B. napus*	1.502	0.091	2.404	0.017			2.404
98.	gi|296223	glutamate--ammonia ligase precursor	*B. napus*	2.353	0.000	2.249	0.000	5.250	0.002	3.284
99.	gi|30692947	putative phosphoglycolate phosphatase	*A. thaliana*	1.956	0.000	1.826	0.005	2.569	0.006	2.117
100.	gi|5281016*	hydroperoxide lyase (HPOL) like protein, containing pfam00067 Cytochrome P450 domain	*A. thaliana*	2.933	0.215	7.273	0.011			7.273
101.	gi|6966930*	glutamine synthetase	*B. napus*	1.173	0.395	1.301	0.317	5.327	0.000	5.327
		Protein synthesis (2)								
102.	gi|15232276*	50S ribosomal protein L12-C	*A. thaliana*	2.399	0.002	0.958	0.764	0.982	0.962	2.399
103.	gi|30692346*	RPS1 (ribosomal protein S1); RNA binding	*A. thaliana*	2.148	0.040	1.323	0.326	0.975		2.148

Table 2 continued

Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
			MC/GC	P-value	MC/GC	P-value	MC/GC	P-value	MC/GC
	Protein folding, tansporting and degradation (11)								
104. gi|15241314	ATP-dependent Clp protease, ATP-binding subunit	*A. thaliana*	2.283	0.014	2.597	0.002	2.167	0.148	2.440
105. gi|30684767*	ATP-dependent peptidase/ATPase/ metallopeptidase	*A. thaliana*	1.597	0.170	1.592	0.186	5.722	0.031	5.722
106. gi|167117	60-kDa beta-polypeptide of plastid chaperonin-60 precursor	*B. napus*	2.155	0.013	2.945	0.013	1.693	0.059	2.550
107. gi|18399551	complex 1 family protein / LVR family protein	*A. thaliana*	1.412	0.038	2.675	0.014			2.044
108. gi|42565672	plastid transcriptionally active 18 (PTAC16)	*A. thaliana*	1.924	0.000	2.218	0.001			2.071
109. gi|75321947	DEAD-box ATP-dependent RNA helicase 35B	*O. sativa*	6.686	0.001	12.884	0.000	12.754	0.007	10.775
110. gi|15225545	hypothetical protein,containing COG1222 ATP-dependent 26S proteasome regulatory subunit domain	*A. thaliana*	2.303	0.000	1.600	0.000	3.433	0.000	2.446
111. gi|15238369*	ribosomal protein L29 family protein	*A. thaliana*	2.135	0.019	1.127	0.216			2.135
112. gi|2565436	DegP protease precursor	*A. thaliana*	1.481	0.007	2.053	0.003	4.659	0.040	2.731
113. gi|77554415	stromal 70 kDa heat shock-related protein, chloroplast	*O. sativa*	2.745	0.008	3.427	0.001			3.086
114. gi|841208	trypsin inhibitor propeptide	*B. oleracea*	1.907	0.005	2.707	0.016	4.022	0.024	2.879
	Membrane and transport (5)								
115. gi|124360831	H^+-transporting two-sector ATPase, alpha/beta subunit	*M. truncatula*	1.693	0.000	1.587	0.001	5.066	0.004	2.782
116. gi|15224625	translocon at the inner envelope membrane of chloroplast 55	*A. thaliana*	2.460	0.004	1.934	0.005			2.197
117. gi|15228268*	apolipoprotein D-related	*A. thaliana*	2.077	0.082	2.246	0.013	1.529		2.246
118. gi|2199574*	aquaporin PIP1b2	*B. oleracea*	2.809	0.086	3.693	0.000			3.693
119. gi|5081423	plasma membrane intrinsic protein 2	*B. napus*	3.546	0.030	5.095	0.013	7.000	0.013	5.214
	Stress and defence (17)								
120. gi|15227359	unknown protein cotaining cd01958 and pfam00234 domain, putative seed storage proteins and lipid transfer proteins	*A. thaliana*	7.321	0.000	6.978	0.002	11.961	0.008	8.754
121. gi|1755154	germin-like protein	*A. thaliana*	3.626	0.000	4.533	0.000	8.589	0.000	5.583
122. gi|17813	BnD22 drought induced protein	*B. napus*	2.490	0.000	2.913	0.000	9.806	0.000	5.070
123. gi|27372775	lipoxygenase 2	*B. napus*	2.180	0.001	3.319	0.000	9.880	0.000	5.126
124. gi|336422	triazine-resistance	*B. napus*	2.444	0.000	3.408	0.000	5.522	0.000	3.791
125. gi|5487875	catalase	*B. napus*	2.816	0.000	3.485	0.000	5.072	0.000	3.791
126. gi|15229440	unknown protein,containingg pfam04755 PAP-fibrillin domain	*A. thaliana*	2.011	0.000	3.419	0.000	4.592	0.005	3.341

Table 2 continued

Accession	Protein	Species	Experiment 1		Experiment 2		Experiment 3		Average
			MC/GC	P-value	MC/GC	P-value	MC/GC	P-value	MC/GC
127. gi|158523427	myrosinase	*B.napus*	3.835	0.002	4.232	0.001	4.544	0.022	4.204
128. gi|18405273*	kelch repeat-containing protein	*A. thaliana*	4.795		9.490		13.694	0.032	13.694
129. gi|18423233	early responsive to dehydration 1; ATP binding / ATPase	*A. thaliana*	2.670	0.003	1.837	0.008			2.253
130. gi|1883008*	jasmonate inducible protein containing pfam01419 Jacalin-like lectin domain	*B. napus*	1.390		1.902		7.666	0.000	7.666
131. gi|21554102	putative chloroplast drought-induced stress protein	*A. thaliana*	2.094	0.001	2.036	0.006	2.494		2.065
132. gi|30688146	plastid-lipid associated protein PAP / fibrillin family protein	*A. thaliana*	2.093	0.000	2.967	0.000	3.739		2.530
133. gi|33285912*	putative myrosinase-binding protein 3	*B. rapa*	2.068	0.079	4.370	0.040	3.839		4.370
134. gi|62900701	plastid lipid-associated protein 1, chloroplast precursor	*B. rapa*	1.638	0.000	2.574	0.000	2.811		2.106
135. gi|6522943	myrosinase-associated protein	*B. napus*	1.956	0.033	4.132	0.016			3.044
136. gi|66734182	epithiospecifier protein	*B. oleracea*	2.099	0.001	2.909	0.001	8.182	0.002	4.397
	Signal transduction (2)								
137. gi|15220216	Ca^{2+}-dependent membrane-binding protein annexin	*A. thaliana*	1.447	0.029	2.911	0.018	5.720	0.085	2.179
138. gi|89513072*	annexin 1	*B. juncea*	3.404	0.003					3.404
	Transcription (1)								
139. gi|15229384	putative mRNA-binding protein	*A. thaliana*	1.861	0.006	1.447	0.228	3.902	0.023	2.882
	unknown (2)								
140. gi|15232724	unknown protein	*A. thaliana*	2.832	0.001	2.670	0.015			2.751
141. gi|18394322*	unknown protein	*A. thaliana*	2.073	0.030	2.258	0.365			2.073
	Miscellaneous (2)								
142. gi|15237201	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|18418200*	rubredoxin family protein	*A. thaliana*	2.141	0.047	2.783	0.089	1.976	0.474	2.141

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

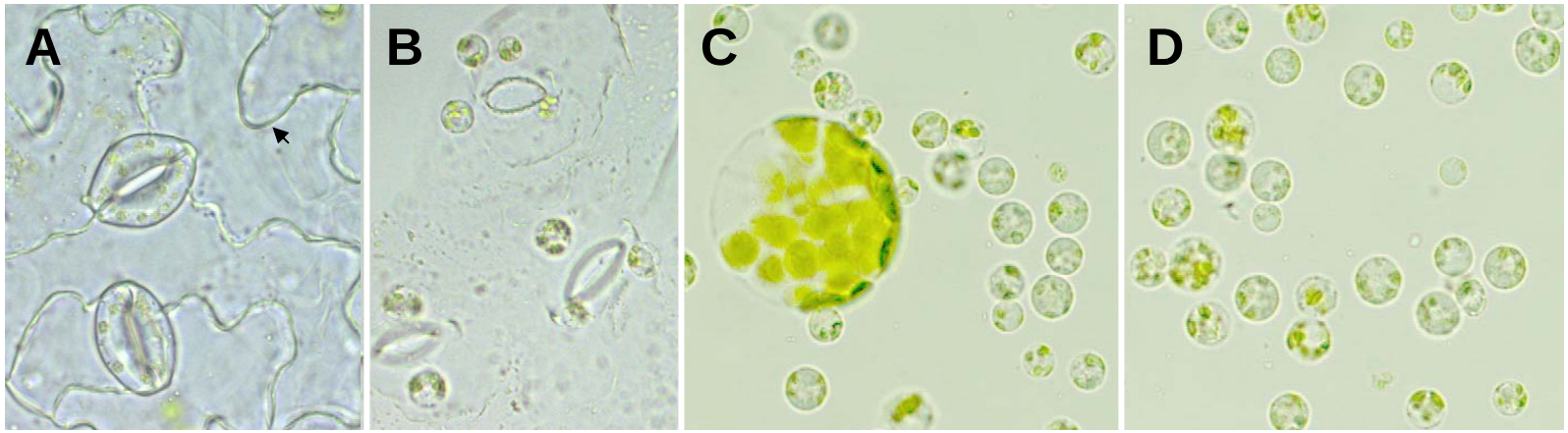

FIG 1. Isolation of guard cell protoplasts from *Brassica napus* leaves. *A.* guard cells and epidermal cells on a leaf epidermal peel (arrows); *B.* after the second enzyme digestion, guard cells round up and are released from stomata; *C.* guard cell protoplasts collected after separating from epidermal peels. Note contamination by a mesophyll cell; *D.* guard cell protoplasts purified by Histopaque centrifugation (400 x).

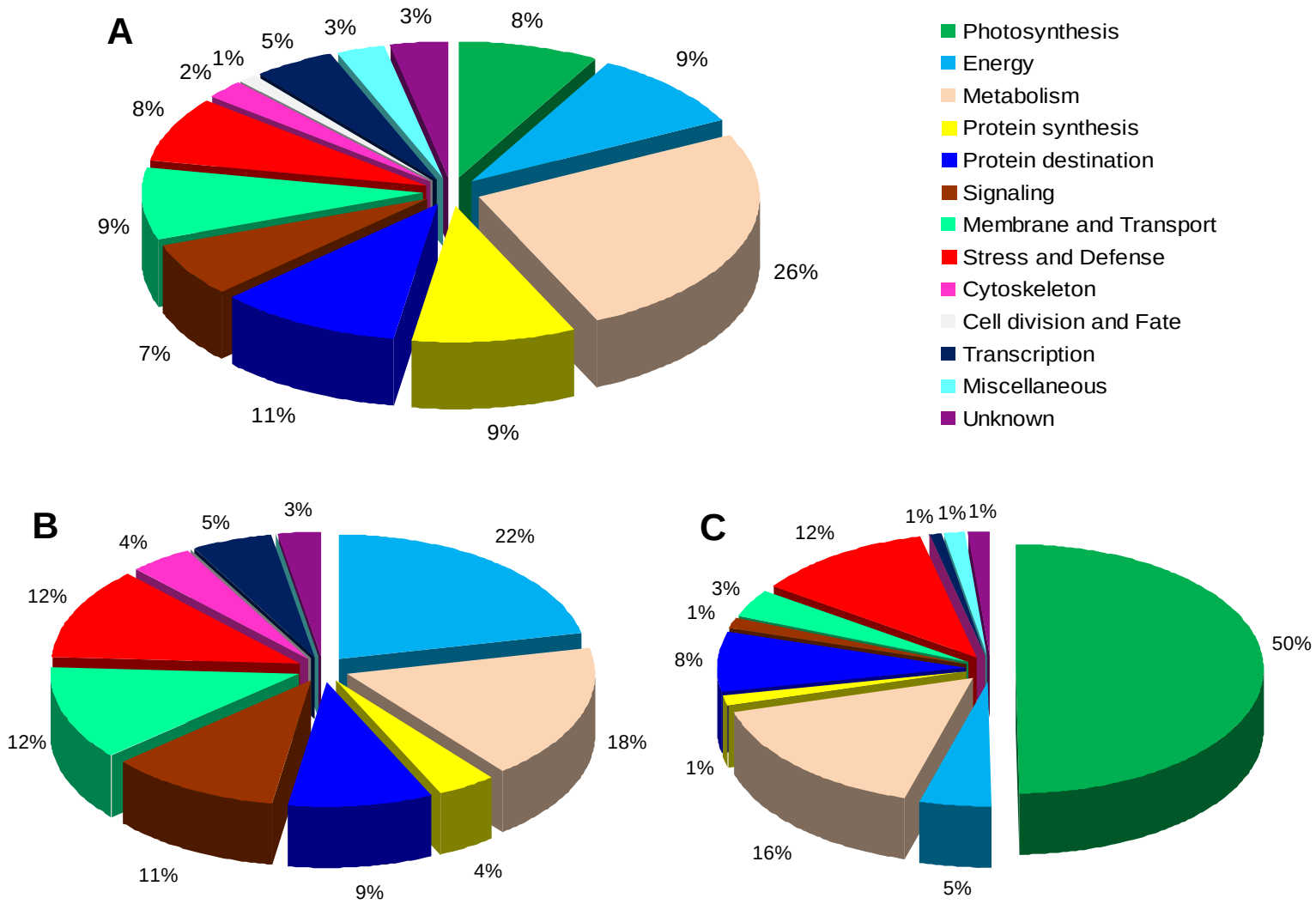

FIG 2. Classification of the 1458 identified proteins into molecular functions. The pie chart shows the distribution of the non-redundant proteins into their functional classes in percentage. *A*. all the 1458 proteins; *B*. proteins enriched in GC; *C*. proteins enriched in MC.

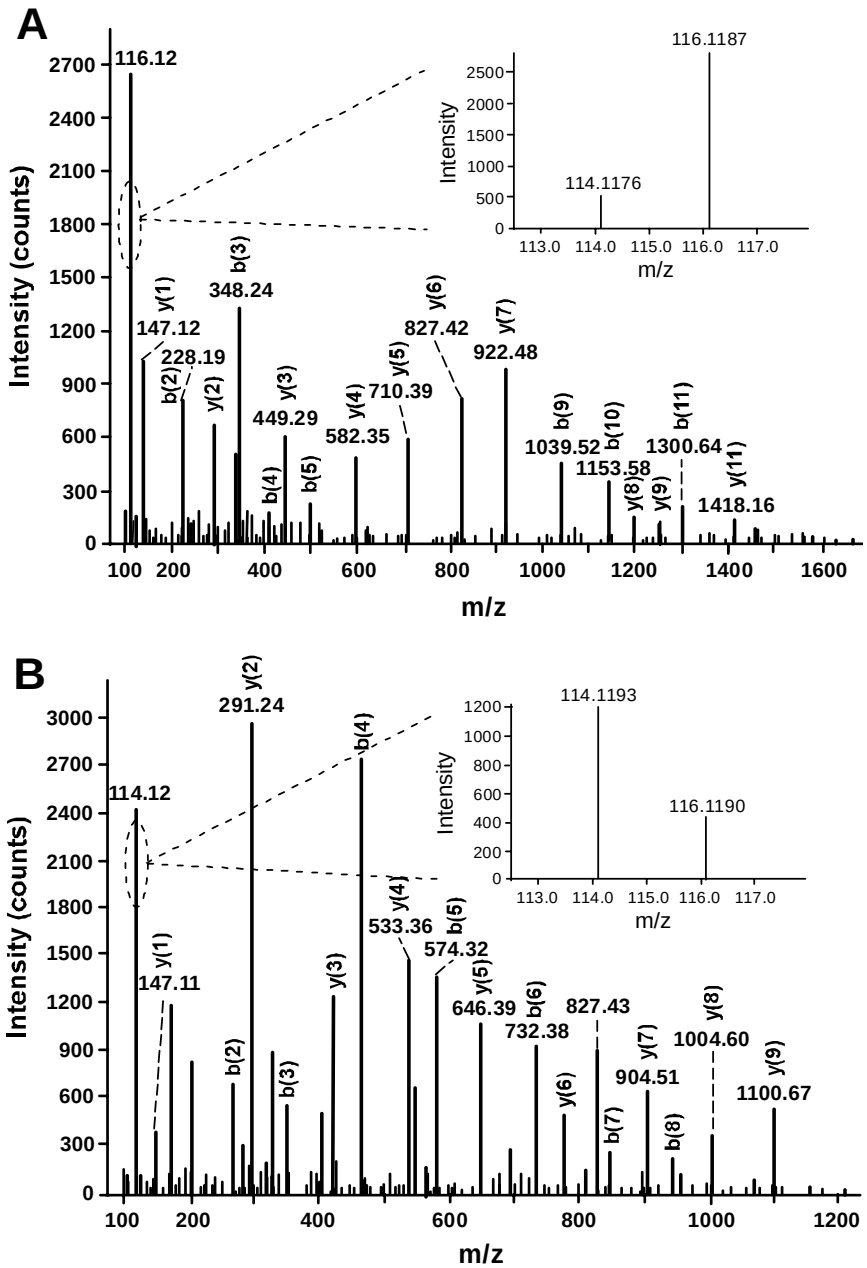

FIG 3. Representative MS/MS spectra showing protein identification and relative quantification in guard cells (iTRAQ tag 114) and mesophyll cells (iTRAQ tag 116). *A*. an MS/MS spectrum identified the peptide QLDASGKPDNFTGK derived from photosystem II protein and its relative abundance in GC and MC; *B*. an MS/MS spectrum identified the peptide DSNIASIPVEELIEK derived from plasma membrane P-type H^+-ATPase AHA1 and its relative abundance in GC and MC.

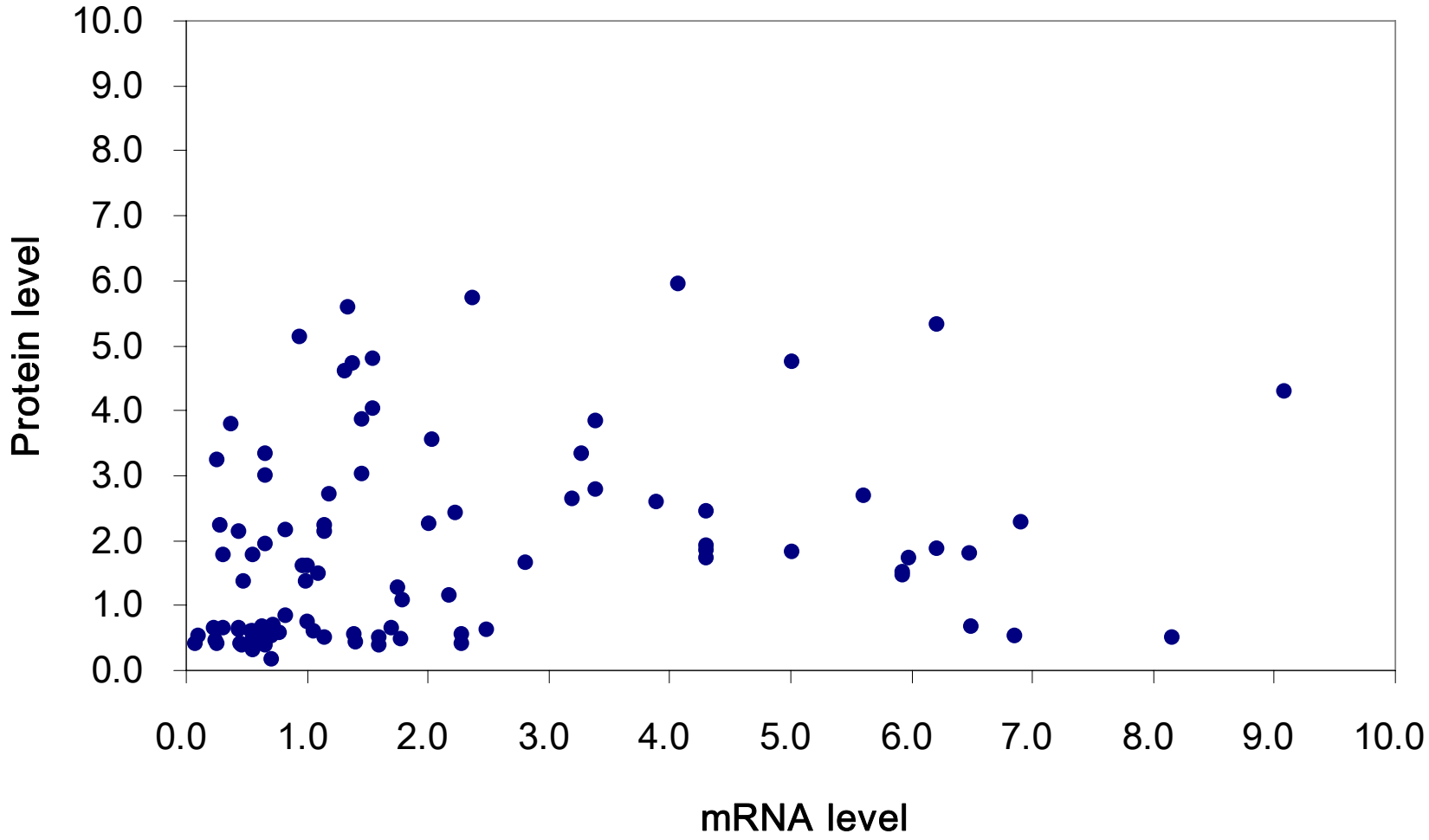

FIG 4. Scatter plot of GC/MC ratio at mRNA level and protein level. The correlation coefficient is estimated to be 0.37.