Peptide Separation Methodologies for In-Depth Proteomics in Arabidopsis

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In the post-genome era, several tools that have increased our global understanding of the molecular basis of several cell-based phenomena have been developed. However, proteomics has not been efficiently integrated with the other ‘omics’ (e.g. transcriptomics and metabolomics), because of the relatively low number of proteins identified by mass spectrometry (MS). Peptides from low-abundance proteins are often not detected by MS due to ionization suppression. To improve the number of peptide identifications in MS analyses, we propose three separation methodologies; namely, OFFGEL electrophoresis, 2D-liquid chromatography (LC) and the long monolithic silica-C18 capillary column method, with the common aim to decrease peptide complexity prior to MS analyses. Proteomics using the above three separation methods were separately applied to protoplasts collected from the epidermal cell layer of Arabidopsis roots using fluorescence-activated cell sorting. In each method alone, 1,132, 836 and 795 proteins were specifically identified, respectively. This has allowed the identification of 1,493 proteins with no redundancy and with <1.0% false discovery rate. Moreover, approximately two-thirds of these proteins are identified here for the first time in the epidermal cell layer. These results show that use of different proteomic approaches can increase the total number of proteins identified. We propose that the integration of data from these methodologies represents a powerful tool for generation of proteome maps by enabling identification of low-abundance proteins in the various Arabidopsis root cell layers.

Keywords: Arabidopsis root • Epidermal cells • FACS • In-depth proteomics • Peptide separation • Protoplast.

Abbreviations: FACS, fluorescence-activated cell sorting; FDR, false discovery rate; GFP, green fluorescent protein; LC, liquid chromatography; MS, mass spectrometry; TM, transmembrane.

Introduction

Since the term proteome was introduced by Marc Wilkins in 1994, numerous proteomic analyses have contributed to the development of several biological fields (Wilkins et al. 1995, Wilkins and Appel 2007). However, the low numbers of identified proteins have hampered their integration into a comprehensive ‘omics’ approach, including transcriptomics and metabolomics (Alba et al. 2005, Carrari et al. 2006, Deluc et al. 2007, Wang et al. 2009, Enfissi et al. 2010, Fortes et al. 2011, Osorio et al. 2012). Recently, the integration of 12,285 transcripts, 758 proteins and 408 metabolites provided a comprehensive understanding of development and post-harvest drying in grapevines; indeed, putative biomarkers were identified (Zamboni et al. 2010). Furthermore, 158 proteins were integrated with transcriptomic data using a microarray of approximately 8,500 tomato genes and specific primary metabolites (Osorio et al. 2011). However, the number of identified proteins is still insufficient for a full comparison with other ‘omics’ data.

On the other hand, many efforts have gradually but significantly allowed an increase in the total numbers of proteins identified in proteomic analyses linked with mass spectrometry (MS) (Wilkins et al. 1995, Fukao, 2012). Several thousands of proteins are now identifiable using advanced methodologies (Ahrens et al. 2010). It is widely recognized that to increase the number of proteins identified, three factors must be taken into consideration: (i) increasing the efficiency of protein extraction; (ii) a suitable peptide purification method; and (iii) decreasing peptide complexity prior to MS analysis. With regard to the first factor, the use of detergents can affect the extraction efficiency of insoluble proteins, such as membrane proteins, significantly (Bodzon-Kulakowska et al. 2007, Masuda et al. 2008). Whether an extraction procedure that includes detergents is suitable for in-solution or in-gel digestion following SDS–PAGE is critical. Even if detergents increase the
recovery rate of protein samples, including membrane proteins, they are not always suitable for the following procedures, because some inhibit trypsin activity during in-solution digestion. In addition, while C-18 resin is widely used for peptide purification, the purification efficiencies of C-18 resins differ among manufacturers (Rappsilber et al. 2003, Ishihama 2008). Moreover, chloroplastic, storage, ribosomal and cytoskeletal proteins are the most abundant proteins in plant samples (Chen and Harmon 2006). As a consequence, the ionization of peptides from low-abundance proteins is suppressed by highly abundant peptides from major proteins during MS analysis, leading to lower detection of low-abundance peptides. Therefore, development of an enhanced proteomics methodology by addressing the above three points is necessary.

In this study, to establish methodologies that enable the identification of the maximum number of proteins, three peptide separation methods were evaluated. To examine whether these methodologies are applicable to low-abundance protein samples, we established an Arabidopsis line in which green fluorescent protein (GFP) was specifically expressed in the epidermis under the control of the GL2 promoter (Masucci et al. 1996). Epidermal cell protoplasts were sorted by fluorescence-activated cell sorting (FACS), and GFP-expressing protoplasts were used as the test protein sample. FACS is an efficient method for the establishment of a proteome profile for a particular cell type within an organ or tissue of interest, although the low number of cells of interest can result in a reduced protein yield, which limits the success of subsequent MS analysis. Therefore, substantially improved methodologies for identification of large numbers of proteins are needed, in particular when a relatively small quantity of protein, such as protoplast extracts, is subjected to MS. To increase the peptide density, trypsin-digested peptides were fractionated by OFFGEL electrophoresis (Hörth et al. 2006, Hubner et al. 2008, Malmström et al. 2009) or two-dimensional-liquid chromatography (2D-LC) approaches. Finally, these fractionated peptides were analyzed by LTQ-Orbitrap XL. In addition to these two methods, purified peptides were separated on a 200 cm long monolithic silica-C18 capillary column and then directly analyzed using the LTQ-Orbitrap XL as a one-shot approach (Iwasaki et al. 2010). Our results highlight the usefulness of integration of the three proteomic methodologies for efficient analysis of low-abundance protein samples. We also emphasize the necessity of using various proteomic methodologies to establish a complete proteome map.

Results

Collection of GFP-positive protoplasts using FACS

To investigate the efficacy of OFFGEL electrophoresis, 2D-LC and a long monolithic silica-C18 capillary column, protoplasts prepared from the epidermal cells of GL2pro-GFP roots were used as a model case of proteomics for samples containing low-abundance proteins. The protoplasting procedures were performed using GL2pro-GFP roots grown for 5 d (Supplementary Fig. S1A). The purity of GFP-positive protoplasts after FACS-mediated sorting was confirmed by fluorescence microscopy. A protoplasting solution was prepared by mixing equal amounts of cellulase R-10 and cellulase RS. The cellulase R-10 yielded a soft pellet after centrifugation, with some protoplasts lost during supernatant removal. However, the cellulase RS yielded a harder pellet, and some protoplasts burst during resuspension. As a result, we obtained a mixture of GFP-positive and -negative protoplasts (Supplementary Fig. S1B), and finally a fraction with only GFP-positive protoplasts, each of which had a diameter <40 μm and was successfully collected by FACS, as indicated by fluorescence microscopy (Supplementary Fig. S1C). The average protein concentration in the collected GFP-positive protoplast fraction was 70.9 μg per ~5.0 × 10⁵ protoplasts.

OFFGEL electrophoresis

Protein samples were extracted from pelleted GFP-positive protoplasts using an extraction buffer containing 6 M urea by vortexing and ultrasonication, for in-solution digestion. Then, 10 μg of trypsin-digested peptides were separated by OFFGEL electrophoresis into 24 fractions (Fig. 1; Supplementary Table S1). OFFGEL electrophoresis is an established method for proteomic analysis of complex protein samples, and was recently

![Fig. 1 Schematic diagram of proteomic methodologies. GFP-positive protoplasts were sorted by FACS using protoplasts prepared from the epidermal cells of GL2pro-GFP roots. Digested peptides by in-solution digestion were fractionated by OFFGEL electrophoresis, 2D-LC and the long-column method. Then the fractionated peptides were analyzed by the MASCOT software following LC-MS (LTQ-Orbitrap XL) analysis.](https://academic.oup.com/pcp/article-abstract/54/5/808/1854638)
applied to the identification of low-abundance proteins and quantitative proteomics by label-free or iTRAQ analysis (Malmsröm et al. 2009; Ernoult et al. 2010; Warren et al. 2010; Zhang et al. 2010; Abdallah et al. 2012; Sudhir et al. 2012). Samples after OFFGEL electrophoresis are not suitable for LC–MS analysis without peptide purification, because the solution used during electrophoresis includes glycerol and other impurities from plant materials. These non-peptidic molecules would be likely to block the flow of the HPLC system. To purify the large number of samples required efficiently and reproducibly, an automated method with an autosampler was used. Each of the 24 purified fractions was separated on a 15 cm reverse-phase column and then directly analyzed by MS. In three independent biological replicates, 952, 663 and 517 proteins were identified, with a <1.0% false discovery rate (FDR). In total, 1,132 proteins were identified in three biological replicates with no redundancy (Fig. 2; Supplementary Table S2). The identified proteins were not always identical in MS analyses, even for identical samples, because identification depended on column conditions, temperature and other operational variables. Therefore, analysis of additional biological replicates should be necessary for the completion of an accurate proteome map. Along with the above analysis, we have performed a classical shotgun analysis to evaluate whether the numbers of proteins identified by OFFGEL electrophoresis were effectively increased. For the classical shotgun analysis, the numbers of proteins identified by OFFGEL electrophoresis per performed a classical shotgun analysis to evaluate whether proteome map. Along with the above analysis, we have replicates should be necessary for the completion of an accur- operational variables. Therefore, analysis of additional biological replicates should be necessary for the completion of an accurate proteome map. Along with the above analysis, we have performed a classical shotgun analysis to evaluate whether the numbers of proteins identified by OFFGEL electrophoresis were effectively increased. For the classical shotgun analysis, the numbers of proteins identified by OFFGEL electrophoresis per fraction were separated under acidic conditions in a 15 cm reverse-phase column by the gradient as the first dimension, prior to LC–MS analysis (Gilar et al. 2005). Then, each peptide fraction was separated under acidic conditions in a 15 cm reverse-phase column by the gradient as the second dimension and directly analyzed by MS. In three independent biological replicates, 620, 604 and 570 proteins were identified, and a total of 836 proteins were identified with no redundancy and with an FDR <1.0% (Fig. 2; Supplementary Table S3). Of these, 581 were common to both OFFGEL electrophoresis and 2D-LC, although 255 and 551 proteins were identified by each methodology alone (Fig. 2). Abundant proteins, such as ribosomal or cytoskeletal proteins, were commonly identified using both methodologies. Thus, varying the elution conditions appears to increase the number of proteins identified, because detection of minor peptides differed depending on the method applied.

Long monolithic silica-C18 capillary column

In the two methods described above, complex peptides were fractionated prior to LC–MS analysis. We next examined the peptide separation efficiency of the LC column prior to MS analysis (Fig. 1; Supplementary Table S1). A 200 cm long monolithic silica-C18 capillary column was recently developed and is now commercially available for proteomics applications (Miyamoto et al. 2008; Iwasaki et al. 2010). Peptides were separated on a 15 cm reverse-phase column using a 26 min gradient by the two methods described above. However, here, a 320 min gradient was applied for more efficient peptide separation. Trypsin-digested peptides (5 μg) were manually purified by Sep-Pak, and then directly analyzed by MS (Fig. 1; Supplementary Table S1). In three independent biological replicates, 607, 581 and 540 proteins were identified, and a total of 795 proteins were identified with no redundancy and with an FDR <1.0% (Fig. 2; Supplementary Table S4). The long-column method increased the number of proteins identified to the same level as that achieved by 2D-LC, although 328 and 287 proteins were identified exclusively by the long-column and 2D-LC methods alone, respectively. In comparison with the OFFGEL electrophoresis and 2D-LC methods, the time required for sample analyses was significantly reduced in the LC–MS. The total analysis time, including column washing, of the long-column, OFFGEL electrophoresis and 2D-LC methods was 360, 1,200 and 500 min, respectively. Hence, the long-column method
has the advantage not only of increasing the total number of proteins identified, but also of reducing the total analysis time. Consistently, these results indicate that the long-column method has a greater advantage vs. the classical shotgun. Indeed, the number of proteins identified was >5-fold higher in the long-column method. This suggested that the fine separation of peptides by the 200 cm long monolithic silica-C18 capillary column allowed the detection of minor peptides, which were previously undetectable by the classical shotgun method.

**Discussion**

To increase the number of proteins identified, including those of low abundance, it is essential to decrease the peptide density in LC–MS. In this study, we investigated three methodologies based on in-solution digestion, and successfully identified a total of 1,493 proteins with no redundancy using protoplasts collected from a single epidermal cell layer (FDR <1.0%: **Supplementary Table S5**). The numbers of proteins uniquely identified using the OFFGEL electrophoresis, 2D-LC and long-column methods were 370, 208 and 106, respectively (**Fig. 2**). Furthermore, the numbers of proteins identified with two or more transmembrane (TM) regions were evaluated using the SOSUI system (Hirokawa et al. 1998; http://bp.nuap.nagoya-u.ac.jp/sosui/) (**Fig. 3A**). These results indicated that 120, 64 and 64 proteins were predicted to be membrane bound with two or more TM regions, and 63, 26 and 14 proteins were identified by only OFFGEL electrophoresis, 2D-LC and the long-column method, respectively (**Supplementary Fig. S2**). This result demonstrates that the identification of membrane proteins also differs among the three methodologies. In addition, our preliminary results further confirmed that these methodologies effectively increased the number of proteins identified using microsomal fractions from whole root or leaves (unpublished results). Therefore, these methodologies are potentially applicable to various plant tissues and probably to various plant species.

The identities and functions of several key genes expressed in each cell layer of Arabidopsis primary roots have been determined using developmental genetic and other molecular biology approaches. To establish a transcriptome map of each root cell layer, FACS has been applied (Birnbaum et al. 2003, Brady et al. 2007). This approach has enabled identification of genes expressed in response to high salinity and iron deficiency (Dinneny et al. 2008), and was later expanded to studies of plant hormones (Petersson et al. 2009, Bargmann and Birnbaum 2010). Importantly, the first report in which proteomics were combined with FACS was published recently. Petricka et al. (2012) performed proteomic analysis following protoplast sorting by FACS and compared the proteins expressed by the various root cell layers. In-gel digestion following SDS–PAGE resulted in identification of several proteins, including some which had been identified previously (Petricka et al. 2012). Then, our data on epidermal cells were compared with the previous proteomics data, that have identified 714 proteins in non-hair epidermis cells of the WER marker line (Petricka et al. 2012). We identified 1,032 and 253 proteins, respectively; moreover, 461 proteins were identified in both epidermal cell samples (**Supplementary Fig. S3A**). Furthermore, 163 and 94 of the respective proteins were predicted to have two or more TM domains. The proteins containing 8–12 TM domains were predominantly identified in this study (**Fig. 3B**). However, a single method alone would not be sufficient for uncovering proteomes that include minor proteins, because the results of in-solution and in-gel digestion matched less well. We suspect that further technical advances will allow the identification of proteins that move between cell layers, such as the...
SHORT-ROOT transcription factor, by comparison with the transcriptome maps available (Nakajima et al. 2001).

The proteins identified in this study were also compared with transcriptomic data reported in Birnbaum et al. (2003). In the NCBI Gene Expression Omnibus (GEO) (Barrett and Edgar 2006; http://www.ncbi.nlm.nih.gov/geo/), 10,119 genes are registered above the threshold of detection in each of three independent microarray analyses. In a comparison between our proteomic data and these 10,119 genes, 1,317 proteins were consistent with the transcriptomics data and the remaining 176 proteins were identified only in this study (Supplementary Fig. S3B). These results are fundamentally important, because transcriptomic data do not reflect protein degradation or movement between adjacent root cell layers. The transcriptomics of the sorted protoplasts suggest that the proteins corresponding to the identified mRNAs do not necessarily function within the same cell layer, indicating the necessity of establishing root proteome and transcriptome maps. Comparisons of proteomic and transcriptomic data are hindered by the inability to determine whether non-detected proteins are not expressed or just not identified by MS due to their low abundance.

Proteomics combined with FACS technology has been developed for V-ATPase-enriched, dendritic, colon stem cells in mice, prior to their application in Arabidopsis (Da Silva et al. 2010, Luber et al. 2010, Di Palma et al. 2011). Protein identification was performed by in-gel or in-solution digestion with hydrophilic-interaction liquid chromatography (HILC). Using the in-gel digestion approach, 6,664 proteins were identified in dendritic cells (Luber et al. 2010). It is technically possible to identify almost all expressed proteins in unicellular organisms, because the genome size and the number of protein-coding genes are relatively small (Ahrens et al. 2010). In addition, several efficient methods of protein extraction from these organisms have been established. On the other hand, only about 1,000 proteins were identified in proteomic analysis following protoplast sorting by FACS from Arabidopsis roots in both this and a previous study (Petricka et al. 2012). In plants, contamination of non-protein molecules has become problematic, interfering with protein extraction and affecting peptide preparation in proteomic analyses (Bodzon-Kulakowska et al. 2008). Therefore, to better understand cellular function at the protein level in differentiated cells, such as in plants, more effective protein extraction, peptide purification and peptide separation methods are needed. We believe that this study represents a significant technical advance, which with further fine tuning should facilitate elucidation of the complete proteome map in Arabidopsis roots, and also advances the application of an integrative ‘omics’ approach in plants.

**Materials and Methods**

**Construction of GL2pro-GFP lines**

The endoplasmic reticulum (ER)-targeted GFP-coding sequence was amplified from pBIN-3SS-GFPer (provided by Jim Haseloff) (Haseloff 1999), with the primers Hind-GFPer-Nt and Xho-GFPer-3’R, and cloned into the HindIII-Xhol site of pBluescript II SK(−) (Stratagene) to give pBS-GFPer. A polyadenylation sequence derived from Agrobacterium Gene 7 (Velten and Schell 1985) was amplified from the pBl vector (Becker 1990) with the primers Xho-Genec7-ter-F and Kpn-Genec7-ter-R, and inserted downstream of the GFPer-coding sequence of pBS-GFPer to give pBS-GFPer-Genec7ter. A 2.0 kb GL2 promoter sequence was amplified from genomic DNA prepared from Arabidopsis thaliana accession Columbia (Col-0) with the primers Bam-GL2-(−)2047 and Hind-GL2pro-End-R, and inserted upstream of the GFPer-coding sequence of pBS-GFPer-Genec7ter to give pBS-GL2pro-GFPer-Genec7ter. The GL2pro-GFPer-Genec7ter fragment was excised with BamHI and KpnI, and inserted into the pBl-Kan binary vector (Waki et al. 2011) to give pBl-Kan-GL2pro-GFP. The primers used this study were summarized in Supplementary Table S6.

**Preparation of protoplasts**

Col-0 or GL2pro-GFP seeds were sterilized and germinated on MGRL medium (Fujiwara et al. 1992) containing 1.0% (w/v) sucrose and 1.2% purified agar (Nacalai Tesque). Approximately 1,500 Col-0 or 15,000 GL2pro-GFP seedlings were grown vertically for 5 d at 22°C under 16 h light/8 h dark conditions. Protoplasts were prepared from roots as described previously with minor changes (Bargmann and Birnbaum 2010). Roots were harvested and incubated with protoplasting solution using 10 ml per 1,500 seedlings for 1 h with gentle agitation. For preparation of protoplasting solution, 0.625% cellulase RS, 0.625% cellulase RS and 0.3% Macerozyme R-10 (all Yakult) with mannitol solution (0.6 M mannitol, 20 mM MES and 20 mM KCl, adjusted to pH 5.7 with 1 M Tris–HCl pH 7.5) was incubated at 55°C for 10 min, and then 0.1% (w/v) bovine serum albumin, 10 mM CaCl2 and 5 mM β-mercaptoethanol were added after cooling to room temperature. Roots treated with the cellulase solution were filtered through a 40 μm Cell Strainer (BD Falcon). Filtrates were centrifuged at 500 × g at 4°C for 10 min to collect protoplasts. After decanting the supernatant, the pellets were washed once in the mannitol solution to remove cellulase completely. Protoplast pellets were then dissolved in the mannitol solution for FACS. Fluorescence images of GL2pro-GFP roots and protoplasts were obtained using a confocal laser scanning microscope (FV1000, Olympus).

**Sorting of protoplasts by FACS analysis**

Protoplasts were isolated as described previously (Birnbaum et al. 2003, Birnbaum et al. 2005). In brief, the FACSAria III cell-sorting solution was substituted for mannitol solution 1 d before use to avoid bubble formation. The protoplasts were filtered through a 100 μm Cell Strainer (BD Falcon) immediately before FACS. When setting gates to capture GFP-positive protoplasts, Col-0 protoplasts were used to determine...
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In-solution digestion
The protoplast pellets were dissolved in 100 μl of 6 M urea/100 mM ammonium bicarbonate by vortexing for 1 min and ultrasonication for 1 min. Then ~50 μg of protein was reduced with DTT solution (100 mM dithiothreitol/100 mM ammonium bicarbonate), alkylated with 200 mM iodoacetamide/100 mM ammonium bicarbonate, and then treated again with DTT solution to block the reaction of extra iodoacetamide at 37°C for 1 h in each step. Samples were digested with ~2.0 μl of trypsin (1 mg ml−1) at 37°C for 16 h.

OFFGEL fractionation
The trypsin-digested peptides (20 μg) were mixed with the supplied OFFGEL buffer without any peptide purification, and then subjected to isoelectric focusing using immobilized pH gradient strips in the liquid phase (Hörth et al. 2006). The peptides were separated into 24 fractions with a 3100 OFFGEL fractionator (Agilent Technologies) using a 24 cm IPG gel, pH 3–10 (GE Healthcare, UK) at 4,500 V for 50,000 Vh at 50 μA, according to the manufacturer’s instructions. The fractionated peptides were loaded on a C-TIP (AMR) after conditioning of C18 resin with 0.1% formic acid and 5% (v/v) acetonitrile. The loaded peptides were washed with 0.1% (v/v) acetic acid and 90% (v/v) acetonitrile and then 0.1% (v/v) acetic acid and 5% (v/v) acetonitrile. The loaded peptides were washed with 0.1% (v/v) acetic acid and 5% (v/v) acetonitrile, and then eluted from the column into vials with 0.1% (v/v) acetic acid and 90% (v/v) acetonitrile. This desalting procedure was performed automatically using a HTC-PAL autosampler (CTC analytics, Switzerland) according to the set-up program. The samples were dried in air and dissolved in 20 μl of 0.1% (v/v) acetic acid and 5% (v/v) acetonitrile in water.

2D-LC
Digested peptides (10 μg) were adjusted to 0.1% formic acid solution. The solution was loaded on Sep-Pak (Waters) after conditioning of C18 resin with 0.1% (v/v) formic acid and 60% (v/v) acetonitrile and then 0.1% (v/v) formic acid. The loaded peptides were washed with 0.1% (v/v) formic acid, and then eluted from the column into vials with 0.1% (v/v) formic acid and 60% (v/v) acetonitrile. Purified peptides (5 μg) were then loaded on a column (200 μm internal diameter, 10 cm, Zaplus column×P; AMR) using a HTS-PAL autosampler (CTC analytics). Buffers were 20 mM ammonium formate adjusted to pH 9.2 (A) and 20 mM ammonium formate and 45% (v/v) acetonitrile in water adjusted to pH 9.2 (B). A linear gradient from 2 to 100% B for 20 min, remaining at 100% B for 4 min, decreasing to 2% B in 1 min and remaining at 2% B for 5 min was applied. Peptides eluted from the column were collected into 10 vials over equal time periods, and were dried in air. The same procedure was performed again and fractionated peptides were collected in the same vials, because the maximum capacity of the column was 5 μl. In total, 10 μg of fractionated peptides were dried in air and dissolved in 20 μl of 2% acetonitrile/0.1% acetic acid without vortexing.

The long-column method
Peptides (5 μg) were desalted on Sep-Pak (Waters) as described for 2D-LC. The peptides were then concentrated in a vacuum concentrator to ~5 μl and adjusted to 20 μl with 0.1% (v/v) acetic acid and 5% (v/v) acetonitrile in water.

LC-MS analysis
The prepared peptides were analyzed by an LTQ-Orbitrap XL-HTC-PAL-Paradigm MS4 system, as described previously (Fukao et al. 2011). The peptides were loaded onto the column (100 μm internal diameter, 15 cm; L-Column, CERI) for samples separated by the OFFGEL fractionator or 2D-LC (100 μm internal diameter, 200 cm; monolithic silica-C18 capillary column, GL Sciences) for the long-column method using a Paradigm MS4 HPLC pump (Michrom BioResources) and an HTC-PAL autosampler (CTC analytics). Buffers were 0.1% (v/v) acetic acid and 2% (v/v) acetonitrile in water (A) and 0.1% (v/v) acetic acid and 90% (v/v) acetonitrile in water (B). Linear gradients from 5 to 45% B for 26 min for samples separated by the OFFGEL fractionator and 2D-LC, or from 5 to 40% B for 320 min for the long-column method, were applied. Peptides eluted from the column were introduced directly into an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) with a flow rate of 500 nl min−1 and a spray voltage of 2.0 kV. The range of the MS scan was m/z 450–1,500 and the top three peaks were subjected to MS/MS analysis. The obtained spectra were compared against data in The Arabidopsis Information Resource (TAIR10; http://www.arabidopsis.org/) using the MASCOT software (version 2.4, Matrix Science) with the following search parameters: threshold set-off at 0.05 in the ion-score cut-off; protein identification cut-off set to two assigned spectra per predicted protein; peptide tolerance at ±0.5 Da; peptide charge of 2+ or 3+; trypsin as the enzyme and allowing up to one missed cleavage; carboxymethylation on cysteines as a fixed modification, and oxidation on methionine as a variable modification. In this study, only proteins identified with an FDR <1% (as calculated by MASCOT software) were considered.

Supplementary data
Supplementary data are available at PCP online.

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