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Proteome study of the phloem sap of pumpkin using multidimensional protein identification technology

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ABSTRACT

The phloem is the major transport route for both small substances and large molecules, such as proteins and RNAs, from their sources to sink tissues. To investigate the proteins present in pumpkin phloem sap, proteome analysis using multidimensional protein identification technology was carried out. Pumpkin phloem peptides obtained by liquid chromatography/mass spectrometry/mass spectrometry were searched against pumpkin protein data derived from the National Center for Biotechnology Information. A total of 47 pumpkin phloem proteins were identified. The identified proteins mainly corresponded to enzymes involved in gibberellin biosynthesis, antioxidation processes, or defense mechanisms. Interestingly, seven enzymes required for gibberellin biosynthesis were identified for the first time by this proteomics approach. In summary, the new phloem proteins identified in this study provide strong evidence for stress and defense signaling and new insights regarding the role of gibberellin in the developmental programming of higher plants through the phloem.

Introduction

Vascular plants have evolved two major tube systems, phloem and xylem, that originate from the vascular meristem and function in long-distance transport. In addition to these two primary conduit systems, some plant families have developed laticifers and secretory ducts, which play special roles in plant defense and other unknown functions (Le Hir et al., 2008; Pickard, 2008).

Increasing published evidence supports the theory that the phloem is the major transport route, not only for small substances such as water, inorganic ions, hormones, amino acids, and organic acids, but also for large molecules such as proteins, mRNAs, and small RNAs, from their sources to the sink tissues (Buhtz et al., 2008; Kehr and Buhtz, 2008; Lough and Lucas, 2006). The known or unknown phloem-sap components might be involved in communication throughout the plant or may have roles in the proper regulation of responses to developmental and environmental changes.

The long-distance translocation and distribution of molecules is assumed to be driven by mass flow primarily occurring in sieve elements (SEs). SEs lose their nuclei and ribosomes during development in angiosperms; thus, these cells lack transcriptional and translational abilities. Sieve-sap proteins are mainly synthesized in other phloem cell types, such as companion cells (CCs) and phloem parenchyma cells (PPCs). Subsequently, they are imported into SEs through specialized plasmodesmata (Le Hir et al., 2008; Oparka and Turgeon, 1999). Therefore, symplasmic connections through plasmodesmata between SEs and CCs/PPCs are thought to be the major functional units of the phloem systems.

Thus far, a large number of mRNAs and proteins present in the phloem of diverse plant species have been identified through transcriptomic and proteomic approaches. Transcriptome analyses based on expressed sequence tags (ESTs) have been successfully applied to many species (Le Hir et al., 2008). Small interfering RNA and micro-RNA analyses have been conducted on phloem sap collected from a wide range of plants, including cucumber and pumpkin (Yoo et al., 2004). Compared to transcriptome analysis, proteomic analyses of phloem have been conducted only in a limited number of plant species because sampling of sufficient quantities of phloem sap is difficult in most plant species. Consequently, most phloem proteins have been identified from cucurbits, rice, *Ricinus*, and *Brassica napus* (Aki et al., 2008; Balachandran et al., 1997; Barnes et al., 2004; Giavalisco et al., 2006; Haebel and Kehr, 2001; Walz et al., 2004). Furthermore, only a few phloem proteins from these species have been identified in proteomic analyses due to the lack of available genomic sequences and protein data, as opposed to the high-throughput results attainable from transcriptome analyses.

Abbreviations: MudPIT, multidimensional protein identification technology

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A recent study reported 1209 proteins from pumpkin phloem exudates using a large-scale proteomic approach providing a rich source of phloem proteome (Lin et al., 2009).

In this study, the phloem proteome present in pumpkin phloem sap was investigated using multidimensional protein identification technology (MudPIT), which is a higher-throughput technique compared to the method based on two-dimensional electrophoresis. The pumpkin phloem peptides obtained by liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS) were searched against pumpkin protein data acquired from the National Center for Biotechnology Information (NCBI). A total of 47 unique pumpkin phloem proteins were identified. The identified proteins mainly corresponded to enzymes that carry out pivotal roles in stress and defense pathways. Furthermore, proteins related to the pathway for gibberellin (GA) synthesis were detected for the first time in phloem sap, supporting the theory that the phloem is the route for transport and modification of GAs.

Materials and methods

Plant materials

A pumpkin (*Cucurbita maxima* Duchesne cv. Jungju) was used in this study. Plants were grown under natural light conditions during the spring season (from April to July). The main reason for choosing naturally grown pumpkin for sap collection is that natural conditions, as opposed to controlled ones, provide advantageous phenomena for the communication between plants and pathogens occurring in the phloem.

Phloem-sap sampling

Phloem sap was collected from two-month-old *Cucurbita maxima* plants. The stems of *Cucurbita* plants were excised and the initial fluid exuding from the upper stem was removed using water and filter paper to avoid contamination. Thereafter, the droplets of sap flowing out from the holes of the pumpkin stems were gathered with a hand-held pipette. The exuding phloem sap was collected into tubes containing PBS/EDTA buffer (10 mM Tris–HCl pH 8.3, 1 mM EDTA pH 8.0, 10% glycerol and 10 mM DTT), placed on ice, frozen in liquid nitrogen, and maintained at −80 °C for further analysis. The volume of PBS/EDTA buffer was five times more than that of the samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The isolated pumpkin phloem proteins were resuspended in 2 × sodium dodecyl sulfate (SDS) loading buffer by boiling for 5 min. After centrifugation, the supernatants of the sample were loaded onto 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) protein gels and run for 45 min at 100 V. Proteins on gels were visualized after staining for 30 min in Coomassie brilliant blue (CBB)-staining buffer (50% methanol, 10% glacial acetic acid, 40% H_2O, 0.5 g/L CBB). Gels were destained thoroughly in destaining buffer (30% methanol, 10% glacial acetic acid in H_2O). Three to five gels were prepared from three different protein extractions.

In-gel digestion and peptide sample preparation

All solvents used in this procedure were of high-performance liquid chromatography grade. Protein bands were excised from CBB-stained gels. Subsequently, the excised bands were washed three times with a 1:1 (v/v) solution of acetonitrile/deionized water for 10 min and dehydrated with 100% acetonitrile. The bands were finally washed with a 1:1 (v/v) solution of 100% acetonitrile:100 mM ammonium bicarbonate and dried using a Speed-Vac. Proteins contained in the gel pieces were reduced using 10 mM tris (2-carboxyethyl) phosphine in 0.1 M ammonium bicarbonate at 56 °C for 45 min and alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. Subsequently, the above-mentioned washing step was repeated with the alkylated sample. After the washing step, the gel pieces were dried and soaked in sequencing-grade trypsin solution (500 ng) and placed on ice for 45 min. Then, the gel pieces were immersed in 100 μL of 50 mM ammonium bicarbonate (pH 8.0) at 37 °C for 14–18 h. The resulting peptides were extracted sequentially for 20 min with 45% acetonitrile in 20 mM ammonium bicarbonate, 45% acetonitrile in 0.5% trifluoroacetic acid (TFA), and 75% acetonitrile in 0.25% TFA, with agitation. The extracts containing the tryptic peptides were pooled together and evaporated under vacuum.

Micro-LC/LC–MS/MS (MudPIT) analysis

A single-phase microcapillary column was constructed from 100-μm inner-diameter (i.d.) fused-silica capillary tubing pulled to a 5-μm-i.d. tip using a CO_2 laser puller (P-2000, Sutter Instruments, Novato, CA). The capillary column was packed sequentially with 7 cm of 5-μm i.d. Polaris C18-A (Metachem, West Warwick, RI), 3 cm of 5-μm i.d. Partisphere strong cation exchanger (Whatman, Florham Park, NJ), followed by another 3 cm of Polaris C18-A, using a homemade high-pressure column loader. The columns were equilibrated with a 5% acetonitrile/0.1% formic acid solution, and about 10–25 μg of protein digests were directly loaded onto the capillary column. The buffer solutions used to separate the protein digests were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Six steps of strong cation exchange liquid chromatography/reversed-phase high-performance liquid chromatography peptide separation were conducted. Step 1 consisted of a 100-min gradient from 0% to 100% buffer B. Steps 2–5 had the following profiles: 3 min of 100% buffer A, 2 min of buffer C, a 10-min gradient from 0% to 15% buffer B, and a 97-min gradient from 15% to 45% buffer B. The 2-min buffer C percentages were 10%, 20%, 40%, 60%, and 100%. Peptides eluted from the capillary column were electrosprayed onto an LTQ Linear ion-trap mass spectrometer (ThermoFinnigan, Waltham, MA) with the application of a distal 2.4-kV spray voltage. A cycle, consisting of one full scan (400–1400m/z) followed by nine data-dependent MS/MS scans at 35% normalized collision energy, was repeated throughout the LC separation. MS/MS spectra were compared against an in-house pumpkin protein sequence database (downloaded from the NCBI website), using the Sequest Cluster System (14 nodes). Bioworks Ver 3.1 was used to filter the search results. A delta C_n value of 0.1, SpR (ranking based on prescoring system) between 1 and 5, and the following X_{corr} values were applied to different peptide charge states: 1.8 for singly charged peptides, 2.5 for doubly charged peptides, and 3.5 for triply charged peptides.

Results and discussion

Identification of pumpkin phloem protein by MudPIT analysis

For a long time, pumpkin has been the best choice for the study of phloem proteomics because of the benefits of a large