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ORIGINAL ARTICLE

Protein and metabolite composition of xylem sap from field-grown soybeans (*Glycine max*)

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Abstract The xylem, in addition to transporting water, nutrients and metabolites, is also involved in long-distance signaling in response to pathogens, symbionts and environmental stresses. Xylem sap has been shown to contain a number of proteins including metabolic enzymes, stress-related proteins, signal transduction proteins and putative transcription factors. Previous studies on xylem sap have mostly utilized plants grown in controlled environmental chambers. However, plants in the field are subjected to high light and to environmental stress that is not normally found in growth chambers. In this study, we have examined the protein and metabolite composition of xylem sap from field-grown cultivated soybean plants. One-dimensional gel electrophoresis of xylem sap from determinate, indeterminate, nodulating and non-nodulating soybean cultivars revealed similar protein profiles consisting of about 8–10 prominent polypeptides. Two-dimensional gel electrophoresis of soybean xylem sap resulted in the visualization of about 60 distinct protein spots. A total of 38 protein spots were identified using MALDI-TOF MS and LC–MS/MS. The most abundant proteins present in the xylem sap were identified as 31 and 28 kDa vegetative storage proteins. In addition, several proteins that are conserved among different plant species were also identified. Diurnal changes in the metabolite profile of xylem sap collected during a 24-h cycle revealed that asparagine and aspartate were the two predominant amino acids irrespective of the time collected. Pinitol (D-3-*O*-methyl-chiro-inositol) was the most abundant carbohydrate present. The possible roles of xylem sap proteins and metabolites as nutrient reserves for sink tissue and as an indicator of biotic stress are also discussed.

Keywords Xylem sap · Proteomics · Soybean · Vegetative storage protein

Abbreviations

MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
LC–MS/MS	Liquid chromatography–mass spectrometry
IPG	Immobilized pH gradient
2D PAGE	Two-dimensional polyacrylamide gel electrophoresis

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Introduction

Transport of nutrients and signal molecules is facilitated by vascular bundles which are made up of xylem and phloem. Phloem mediates the translocation of organic compounds such as amino acids and sugars from the source organ (e.g., mature leaves) to sink organs such as seeds (Oparka and Cruz 2000). Xylem is involved in the transport of water and inorganic nutrients from the soil to the aerial parts of the plant. Both xylem and phloem sap have been shown to contain a number of proteins involved in defense response and environmental stresses (Biles and Abeles 1991; Masuda et al. 2001; Aki et al. 2008). Additionally, signal transduction proteins, putative transcription factors, stress response factors, and small interfering RNAs were also detected in the vascular sap (Mlotshwa et al. 2002; Aki et al. 2008).

Previous studies have detected the presence of proteins in the phloem and xylem sap of several plants including canola (Kehr et al. 2005; Giavalisco et al. 2006), cucumber (Walz et al. 2004), castor bean (Barnes et al. 2004), maize (Alvarez et al. 2006, 2008) and pumpkin (Haebel and Kehr 2001). Partial amino acid sequencing and database searches have shown that xylem sap protein composition is conserved among different plant species. Pathogenesis-related (PR) proteins, peroxidases, chitinases and serine proteases were abundantly present in the xylem sap of several plant species (Buhtz et al. 2004). The composition of xylem sap proteins can be altered significantly by pathogen infection. In addition, these proteins can be detected in the xylem sap at some distance from the infection site (Rep et al. 2002).

Xylem sap protein composition of soybean seedlings has been previously investigated (Li et al. 2000; Djordjevic et al. 2007; Subramanian et al. 2009). A stress-induced protein (FISP 17) showing homology to PR-proteins was identified in the stem sap of soybean seedlings infected with *Fusarium solani* f. sp. *glycines*, the causal agent of sudden death syndrome of soybean (Li et al. 2000). FISP 17 was detected only in infected but not in non-infected seedlings stem exudates suggesting that this protein may play a role in defense response. However, FISP 17 was found in the stem exudates of both sudden death syndrome susceptible and partially resistant soybean seedlings (Li et al. 2000). The involvement of xylem sap proteins in the control of autoregulation of nodulation was invested by 2D PAGE followed by MALDI-TOF MS and LC–MS (Djordjevic et al. 2007). This study demonstrated not only that the xylem protein composition can be altered by *Bradyrhizobium japonicum* infection, but also that proteins synthesized in the roots can travel long distances to shoots. Additionally, lipid transfer protein and Kunitz trypsin inhibitor (KTI), proteins with potential signaling ability, were identified in the xylem sap (Djordjevic et al. 2007). A similar proteomic study was conducted to identify protein changes in soybean seedling xylem sap in response to symbiotic (*Bradyrhizobium japonicum*) and pathogenic (*Phytophthora sojae*) interactions (Subramanian et al. 2009).

All these studies were conducted with soybean seedlings grown in a controlled environment. Plants in the field are subjected to high light and to environmental stress that is not normally found in a controlled environment chamber. Little information is available on the xylem sap composition of field-grown soybean. The purpose of the present study was to identify the proteins and metabolites present in the xylem sap collected from the stem exudates of healthy field-grown soybeans. To achieve this goal we collected xylem sap at different developmental stages and examined its protein composition using 1D PAGE and 2D PAGE. A total of 38 xylem sap proteins were identified by MALDI-TOF MS and LC–MS/MS. Additionally, we also report on the metabolite composition of xylem sap and diurnal changes in these metabolites.

Materials and methods

Plant growth and xylem sap collection

Soybean cultivars Williams *Dt1* (indeterminate growth habit), Williams *dt1* (determinate growth habit), Williams *NN5* (non-nodulating mutant) and Williams 82 were grown in Mexico silt loam soil at Bradford Research and Extension Center, Columbia, Missouri. Xylem sap was obtained directly from intact field-grown soybean plants by cutting the stems with a sharp razor blade and collecting the sap (forced by the root pressure) from the cut stem ends. Since this procedure was time consuming and resulted in low quantities of xylem sap, an alternative procedure was also carried out. In this procedure whole plants were pulled from the soil and placed in 20-L buckets containing water. To facilitate the of harvesting plants the plots were surface irrigated the previous day. Immediately after excision from soil the plants were taken to the laboratory for processing. The stem was severed from the root system above the cotyledonary node. A 5-mL syringe filled with sterile distilled water was connected to the stem using latex tubing and the juncture sealed with parafilm. To facilitate collection of xylem sap, the main stem was again severed above the 12th node. Hydraulic pressure was applied using the water-filled syringe and sap was collected in 1.5 mL tubes, to which phenylmethanesulfonyl fluoride (PMSF) was added to a final concentration of 1 μM. A maximum of 0.5 mL sap was collected to prevent dilution by the ensuing water column. Immediately following collection the samples were frozen in liquid nitrogen. The entire process

starting from uprooting soybean plants to sap collection was completed in less than 30 min.

1D PAGE analysis

Xylem sap proteins pooled from 10 plants (ranging from 0.3 to 0.5 mg protein mL^{-1} sap) were first concentrated by acetone precipitation. Precipitated proteins were pelleted by centrifugation at 12,000*g* for 15 min. The resulting protein pellet was resuspended in 1D PAGE sample buffer containing SDS, and aliquots were resolved on 13.5% resolving gels using the Hoefer SE260 minigel electrophoresis apparatus (GE Healthcare, Piscataway, NJ, USA). Typically, 30–50 μg of protein from each sample was loaded per well. Electrophoretic separation was achieved with 20 mA per gel (constant current) and a 1.5 h run time. Visualization of proteins was achieved by either staining the gel overnight with Coomassie Blue G-250 or by silver staining (Marek et al. 1995).

2D PAGE analysis

Isoelectric focusing was performed, essentially as described previously (Krishnan et al. 2009), using 13 cm, pH 3–10 linear IPG strips in the Protean II apparatus (BioRad Laboratories, Hercules, CA, USA). IPG strips were rehydrated with 300 μg of protein. For the second dimension, the IPG strips were incubated with 50 mM Tris–HCl pH 6.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue; reduced with 2% DTT for 20 min and alkylated with 2.5% iodoacetamide for 20 min, and subsequently separated in the second dimension using 13.5% polyacrylamide gels as described by Laemmli (1970). The 2D PAGE separations were visualized by staining with Colloidal Coomassie Blue G-250 and imaged using an Epson Perfection V700 scanner (Epson, Long Beach, CA, USA). Triplicate samples were used for 2D PAGE analysis.

Protein identification

Soybean xylem sap proteins resolved by 1D and 2D PAGE were excised with a 1.5 mm Spot Picker (The Gel Company, San Francisco, CA, USA) from Colloidal Coomassie G-250 stained gels. Both 1D and 2D gel pieces were washed briefly in distilled water and destained in 50% acetonitrile (v/v) containing 25 mM ammonium bicarbonate. A final 100% acetonitrile wash was performed and the protein contained in the acrylamide gel was digested using 20 μL (10 μg/mL) of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate. 2D PAGE peptides resulting from the tryptic digest were analyzed using a Voyager DE-STR (Applied Biosystems, Framingham, MA, USA) MALDI-TOF mass spectrometer (Natarajan et al. 2009). The peptides were co-crystallized with alpha-cyanohydroxycinnamic acid (CHCA) matrix on a MALDI-TOF MS plate, briefly dried, and ionized using a 337 nm nitrogen laser operating at 20 Hz. 2D PAGE peptide mass searches were performed via Mascot v2.3 (Matrix Sciences, http://www.matrixscience.com) using NCBI non-redundant plant database (2010.09.24) and/or Protein Prospector (University of California-San Francisco, http://prospector.ucsf.edu) using NCBI non-redundant (2010.09.24), SwissProt or UniProtKB (2010.08.10) *Glycine max* databases. All searches were performed with a fragment mass tolerance of 50 ppm or less, allowance of only one missed cleavage, and carbamidomethyl fixed modification. Peptides from unmatched proteins were searched using identical search criteria, but within "all-plant" databases. 1D PAGE peptides resulting from the tryptic digest were analyzed using an LTQ Orbitrap XL (ThermoFisher Scientific, San Jose, CA, USA) hybrid linear ion trap mass spectrometer (Krishnan et al. 2006). Peptides were separated by reverse phase on a 100 × 0.18 mm BioBasic-18 column; 30 min linear gradient (5–60% ACN in 0.1% formic acid) and a flow rate of 3 μL min^{-1}. When two MS/MS spectra of an ion were acquired, dynamic exclusion circumvented continuous analysis. Electrospray voltage was set at 3.5 kV and desolvation was assisted with 10 units of sheath gas. The capillary transfer tube temperature was set to 200°C, and the minimum ion count required to trigger spectrum acquisition was set to 5,000. The normalized collision energy was set at 30%. Initial processing of the data was done using Sequest (University of Washington, Seattle, WA, USA). Multiple Sequest DTA files were then concatenated into a single Mascot file using merge pl. script (Matrix Science, Boston, MA, USA). 1D PAGE peptide mass searches were performed via Mascot v2.3 (Matrix Sciences, http://www.matrixscience.com) using NCBI non-redundant "all-plant" database (2010.09.24). All searches were performed with a fragment mass tolerance of 50 ppm or less, allowance of only one missed cleavage, and carbamidomethyl fixed modification. Mascot Distiller ver. 2.3 was used to prepare searchable peak lists for both MALDI-TOF MS and LC–MS/MS data.

Metabolite quantization in soybean xylem sap

Soluble amino acids were determined by a UPLC procedure using a pre-column derivatization method (Waters Corp., Milford, MA, USA). Known volumes of each sample and amino acid standards were derivatized in a total volume of 100 μL at 50°C using the AccQFluor Ultra kit from Waters. Separations were performed on Acquity UPLC equipped with a 2.1 × 100 mm AccQTag Ultra

Amino Acid column at 55°C. The mobile phase consisted of AccQTag Ultra Eluent A1 and AccqTag Ultra Eluent B as provided by the instrument manufacturer. The column was preconditioned with 99.9% Eluent A1 and 0.1% Eluent B for 15 min at a flow rate of 0.7 ml min^{-1}. The injection volume for both standards and samples was 1 µL. This was obtained using a calibrated 2-µL sample loop. The sample loop was washed with mixtures of acetonitrile and H_2O between injections. Amino acids were eluted from the column by linearly increasing concentrations of Eluent B in the mobile phase. Eluent B was 0.1% between 0 and 0.54 min, 9.1% at 5.74 min, 21.2% at 7.74 min and 59.6% at 8.04 min. The column was then washed with 90% Eluent B and 10% Eluent A1 for 1.5 min before regenerating the column. Absorbance was detected with an Acquity TUV detector using a wavelength of 260 nm and a sampling rate of 10 datapoints s^{-1}. The system was controlled and the detector output monitored using Empower2 Plus software from Waters. Standard curves were prepared with a known mixture of 17 amino acids obtained from Waters (WAT088122). In addition, three individual amino acids—Gln, Asn and γ-aminobutyric acid—were added to this mixture prior to derivatization. All of the amino acid standards, plus ammonia, were resolved over 90% using the method described above. Quantitation was based on standard curves using 0, 25, 50 and 100 pmol of each standard amino acid, except for Cys which was present in one-half the usual amount. Each individual sample set was quantified using a separate standard curve. The values reported are from three replicates, assayed independently.

Organic acids and soluble carbohydrate quantization in soybean xylem sap

Organic acids and soluble carbohydrates were determined using gas chromatography coupled to mass spectrometry essentially as described previously (Sicher 2008). Either 10 or 20 µL of each sap sample was transferred to a 1 mL reactivial and dried overnight in a desiccator under vacuum. Dried samples and appropriate standards were dissolved in 100 µL of pyridine containing 2 mg of methoxyamine and were then incubated in a H_20 bath at 30°C for 90 min with continuous shaking. Subsequently, 100 µL of MSTFA [*N*-methyl-*N*-(trimethylsilyl) fluoracetamide] was added to each vial which and was then incubated as above for 30 min at 37°C. Total ion chromatograms were obtained with an Agilent 6890A gas chromatograph and an Agilent 7125C mass selective detector (Agilent Technologies, Wilmington, DE, USA). Sample injections were 1 µL using an inlet temperature of 250°C operated in splitless mode. Separations were performed on a 30 m × 0.25 mm Supelco SPB-50 column (Sigma-Aldrich, St. Louis, MO, USA) using high-purity helium as a carrier gas at 1.2 mL min^{-1}. The oven temperature was increased at 5°C min^{-1} from 70–310°C with a solvent delay of 8.5 min. The detector was operated in full scan mode at 50 scans min^{-1} with a range of 0–550 *m/z*. Total ion chromatograms were quantified using peak identification and calibration parameters within the Agilent MSD Chemstation software. Peaks were identified by retention times and by mass spectra that were obtained using known compounds. Standard curves were prepared with mixtures of specific standards prepared at four concentrations between 0 and 10 ng. The values reported are from three replicates, assayed independently.

Results

SDS-PAGE analysis of soybean xylem sap proteins

We first examined the protein composition of xylem sap from field-grown soybeans by 1D gel electrophoresis. The protein composition of the xylem sap collected irrespective of the method of sap collection (directly from the field or collected in the lab with the help of syringe suction) was very similar (Fig. 1). Several polypeptides ranging from 8 to 100 kDa (numbered sequentially in descending

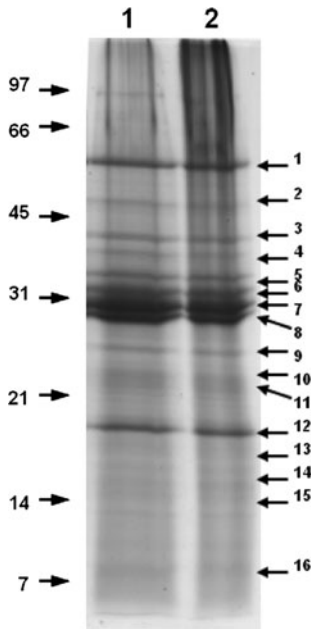

Fig. 1 Xylem sap protein composition from field-grown soybeans. Xylem sap collected directly from the field (lane *1*) or collected in the lab with the help of syringe suction (lane *2*) were concentrated by acetone precipitation, fractionated by 15% SDS-PAGE and stained with Colloidal Coomassie G-250. Each lane contains approximately 50 µg of protein. The position of the protein molecular weight markers in kDa are shown on the *left* of the figure. The *numbers on the right* side of the figure correspond to the proteins bands that were excised from the gel for identification by LC–MS/MS (Table 1)

molecular weight) were observed in the xylem sap. The most abundant proteins were in the range of 50–18 kDa. Some low molecular weight proteins (less than 18 kDa) were also faintly visible. Since very little change in xylem sap protein profile was observed irrespective of the isolation method, for all subsequent experiments xylem sap collected with the help of syringe suction was used. An examination of the protein profile of soybean xylem sap from six different independent experiments revealed only minor variation in the abundance of individual protein bands. Next, we examined if the genotype of the soybean cultivars had any influence on the soybean xylem sap protein composition. For this purpose we collected xylem sap from field-grown soybean cultivars that showed differences in their growth habit and ability to enter into symbiotic association with *Bradyrhizobium japonicum*. Soybean cultivar Williams *dt1* stops vegetative growth once reproductive development starts (determinate growth habit), while the indeterminate type Williams *Dt1* continues vegetative growth even after reproductive growth begins. The protein profile of the xylem sap from determinate, indeterminate, nodulating (Williams 82) and non-nodulating (Williams *NN5*) soybean cultivars in spite of their differences in genotype were very similar, though minor changes were detected (Fig. 2). An 18, 22, 25, 28, 30, 32, 34, 36 and 50 kDa protein is present abundantly in all the examined soybean genotypes. These proteins were consistently observed from the xylem sap from four independent experiments even though the relative abundance of some of the other proteins was variable (data not shown).

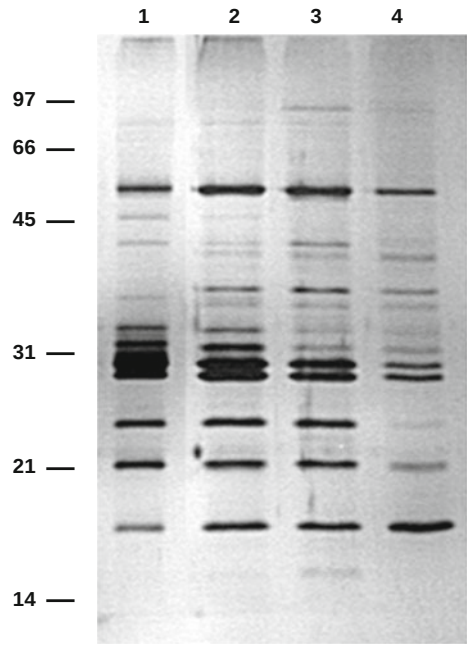

Fig. 2 Xylem sap protein composition of different field-grown soybean genotypes. Xylem sap collected from indeterminate (Williams *Dt1*, lane *1*), determinate (Williams *dt1*, lane *2*) nodulating (Williams 82, lane *3*) and non-nodulating (Williams *NN5*, lane *4*) soybean cultivars were concentrated by acetone precipitation, fractionated by 13.5% SDS-PAGE and stained with Coomassie Blue. Each lane contains approximately 30 μg of protein The position of the protein molecular weight markers in kDa are shown on the *left* of the figure

Diurnal changes in xylem sap proteins

We collected xylem sap from Williams 82 soybean cultivar at 3 h intervals during a 24-h cycle to monitor diurnal changes in protein profile. 1D PAGE analysis revealed very similar protein profile of the xylem sap collected at different time points, indicating that these proteins were constantly produced. Interestingly, the accumulation of some of the proteins showed differences. For example, the 35 and 37 kDa proteins were seen to gradually increase in abundance from 12 p.m. to 12 a.m. and thereafter declined (Fig. 3). Several smaller molecular weight proteins were also abundant in samples collected during the dark period than during the day. This observation indicates that the abundance of the xylem sap proteins is subject to changes throughout the day.

Identification of xylem sap proteins by 1D and 2D PAGE and mass spectrometry

A total of 16 soybean xylem sap proteins excised from Coomassie stained 1D gels (Fig. 1) were identified by LC–MS/mass spectrometry (Table 1). The predicted and

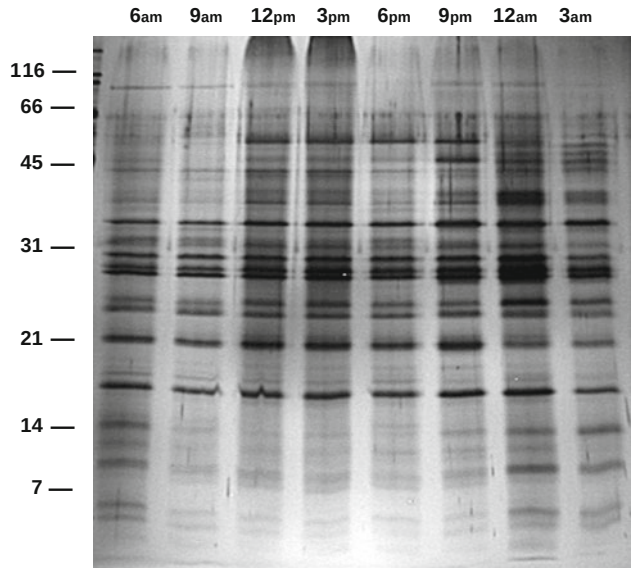

Fig. 3 Diurnal changes in xylem sap protein composition. Xylem sap from soybean cultivar Williams 82 collected every 3 h during a 24 h cycle was concentrated by acetone precipitation, fractionated by 15% SDS-PAGE and silver stained. *Numbers above the lanes* refer to the time of collection of the xylem sap. The position of the protein molecular weight markers in kDa are shown on the *left* of the figure

Table 1 Proteins identified from soybean xylem sap after 1D-PAGE separation and peptide LC–MS/MS

Band #	Protein ID	MOWSE	Peptides matched	% Cov.	M_r E/T	MS-Fit DB: Acc. #	NCBInr BLAST DB: Acc. #
1	Beta amylase (*G. max*)	827	22	32	59,000/56,384	NCBInr: gil62122631	
2	Unknown protein (*G. max*); [94% homology to putative lipase/hydrolase (*A. thaliana*)]	328	7	13	50,000/43,055	NCBInr: gil255636449	[NCBInr: gil21592417]
3	Unknown protein (*G. max*); [99% homology to globulin (*Vitis* spp.)	600	18	32	42,000/38,733	NCBInr: gil255645488	[NCBInr: gil290578589]
4	Gamma-glutamyl hydrolase (*G. max*)	386	13	24	40,000/37,824	NCBInr: gil6016129	
5	Lectin (*G. max*)	539	12	36	36,000/30,168	NCBInr: gil289465309	
6	Lectin (*G. max*)	640	14	45	32,000/30,168	NCBInr: gil289465309	
7	Stem 31 kDa glycoprotein (*G. max*)	475	28	38	30,000/29,433	NCBInr: gil134146	
8	Stem 28 kDa glycoprotein (*G. max*)	631	27	40	29,000/29,218	NCBInr: gil134145	‘
9	Unknown protein (*G. max*); [Kunitz trypsin inhibitor (*G. max*)	285	5	30	26,000/23,666	NCBInr: gil255629211	[NCBInr: gil39725577]
10	Unknown protein (*G. max*); [93% homology to Kunitz trypsin inhibitor p20-1-like protein (*G. max*)]	122	4	17	23,000/21,990	NCBInr: gil255625877	[NCBInr: gil168259026]
11	Gamma-glutamyl hydrolase (*G. max*)	102	3	8	22,000/37,824	NCBInr: gil6016129	
12	Trypsin inhibitor subtype A (*G. max*)	85	3	14	19,000/24,346	NCBInr: gil18770	
13	Nucleoside diphosphate kinase (*G. max*)	211	5	22	17,000/16,489	NCBInr: gil2498078	
14	Unknown (*G. max*); [98% homology to protease inhibitor/seed storage/lipid transfer protein family protein (*A. thaliana*)]	113	2	20	15,000/13,023	NCBInr: gil255626149	[NCBInr: gil15232384]
15	Rubisco small subunit (*Pisum sativum*)	55	2	9	14,000/18,211	NCBInr: gil169156	
16	Unknown (*G. max*); [94% homology to protease inhibitor/seed storage/lipid transfer protein family protein (*A. lyrata*)]	59	1	13	9,000/11,281	NCBInr: gil255626313	[NCBInr: gil297793017]

Band identification #’s correspond to those proteins labeled in Fig. 1. Molecular weight (M_r) values are given as experimental/theoretical values. MOWSE scores represent those searches performed via Mascot using a peptide mass tolerance of 50 ppm. Peptides matched, % coverage and accession numbers within each respective database are given. Unknown proteins from the soybean database were blasted against the NCBI all-plant database and the resulting homologous proteins and accession numbers are given in brackets. Database used was National Center for Biotechnological Information (NCBI non-redundant)

estimated molecular weight of most of the identified proteins was very similar. Some of the proteins identified in the xylem sap of field-grown soybean include β-amylase, γ-glutamyl hydrolase, 31 and 28 kDa vegetative storage glycoprotein, trypsin inhibitor, nucleoside diphosphate kinase, Rubisco small subunit and lipid transfer protein. These proteins were also previously identified in the xylem sap obtained from soybean hypocotyls and epicotyls (Djordjevic et al. 2007). Attempts to identify several faintly visible bands in the range of 6–15 kDa by LC–MS/MS were unsuccessful. Our failed attempts to identify these proteins may be related to low protein concentration or possibly due to the presence of some interfering compounds such as lipopolysaccharides. We also examined the complexity of xylem sap proteins collected from field-grown soybeans by 2D PAGE. Colloidal Coomassie Blue staining of the gel resulted in the visualization of approximately 60 protein spots ranging in molecular weight from 15 to 90 kDa (Fig. 4). Abundant protein spots, which covered a range of pIs, were excised from the gel and analyzed by MALDI-TOF mass spectrometry. Analysis of the 45 xylem sap protein spots led to the identification of 38 spots while the other 7 spots did not generate a significant MOWSE score (Supplementary Table 1). Five prominent proteins (spot 20, 21, 22, 43, and 44) all with similar molecular weights were identified as a stem 31 kDa vegetative storage protein. Similarly, another group of five proteins (spot 23, 24, 25, 26, and 45) having the same molecular weights were identified as a stem 28 kDa vegetative storage protein. Both the 31 and 28 kDa stem proteins are glycoproteins and thus the charge heterogeneity exhibited by these two proteins could be related to

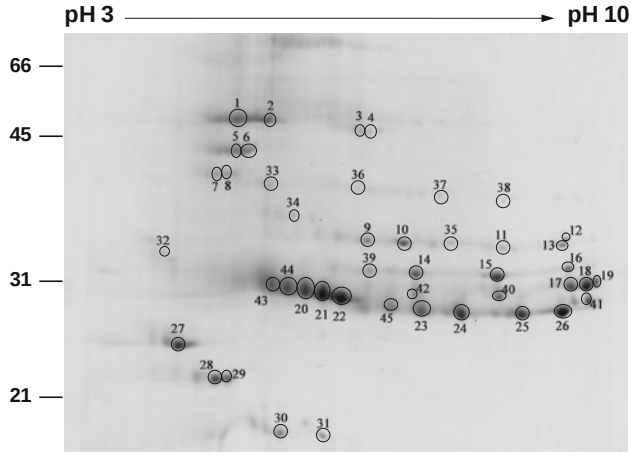

Fig. 4 2D PAGE of xylem sap proteins from field-grown soybean cultivar Williams 82. Proteins were first separated by isoelectric focusing on IPG strips (13 cm, pH 3–10) then by 2D SDS-PAGE on a 13.5% gel. The gels were stained with Colloidal Coomassie Blue G-250. The *numbers at the top of circles* point to different proteins that were identified by MALDI-TOF MS

post-translational modification. Protein spots 9, 10, 11, 13, and 35, which have similar molecular weights but a different isoelectric point, were all identified as gamma-glutamyl hydrolase (Supplementary Table 1). Other identified proteins that had isoforms of the same molecular weight were β-amylase (spots 1 and 2), 3-phosphoshikimate 1-carboxyvinyltransferase (spots 5 and 6), thiamine pyrophosphokinase (spots 17, 18 and 19) and Kunitz trypsin inhibitor (spots 28 and 29). Spot 30 was also identified as thiamine pyrophosphokinase but the experimental molecular weight of this protein is much smaller than the theoretical molecular weight. Proteolytic degradation of the mature protein may have resulted in the appearance of this protein in different spot on the gel. Other proteins identified in the xylem sap include LRR-receptor-like protein kinase (spot 32), SGNH plant lipase-like protein (spot 7 and 8), peroxiredoxin (spot 14), caspase (spot 15), nucleosome assembly-related protein (spot 33), F-box family protein (spot 40) and DUF1997 superfamily related protein (spot 41).

Amino acid content of xylem sap

We also examined changes in the amino acid profile of xylem sap collected at 9 a.m., 3 p.m. and 12 a.m. during a 24 h cycle (Table 2). The concentration of total soluble amino acids were 701, 983, and 701 μmol μg^{-1} protein in the xylem sap collected at 9 a.m., 3 p.m. and 12 a.m., respectively. Asparagine and aspartate were the predominant amino acids found in the xylem sap irrespective of the time point at which xylem sap was collected. These two amino acids represented 50–57% of the total soluble amino acids. An examination of the concentration of these two amino acids revealed an inverse relationship. The percent contribution of asparagine to the total amino acid pool was about 49% in the 9 a.m. samples, dropped to 40% by 3 p.m. and further declined to 27% by 12 a.m. In contrast, the percent contribution of the aspartate increased from 8 to 13

Table 2 Amino acid profile of Williams 82 soybean xylem sap collected at 9 a.m., 3 p.m. and 12 a.m

Amino acid	Xylem sap 9 a.m. (mean ± SE)	Xylem sap 3 p.m. (mean ± SE)	Xylem sap 12 a.m. (mean ± SE)
Histidine	20,623 ± 1,238	25,232.2 ± 435	25,662 ± 58
Asparagine	345,711 ± 20,578	39,0425 ± 13,381	209,035 ± 1,565
Serine	30,248 ± 1,867	48,301 ± 1,459	18,950 ± 170
Glutamine	31,828 ± 1,421	30,230 ± 682	19,234 ± 490
Arginine	19,534 ± 1,146	39,608 ± 953	45,902 ± 409
Glycine	905 ± 98	2,819 ± 174	696 ± 8
Aspartate	57,420 ± 3,362	132,887 ± 6,130	179,069 ± 2,741
Glutamate	7,458 ± 441	40,747 ± 1,745	7,664 ± 101
Threonine	24,395 ± 1,466	42,199 ± 1,290	33,028 ± 285
Alanine	14,101 ± 858	18,079 ± 714	2,515 ± 35
Proline	23,696 ± 1,424	32,554 ± 878	14,406 ± 158
Cysteine	325 ± 9	1,208 ± 13	361 ± 36
Lysine	28,804 ± 1,857	58,959 ± 2,903	59,746 ± 1,467
Tyrosine	5,491 ± 247	8,714 ± 108	3,670 ± 14
Methionine	5,332 ± 293	4,509 ± 100	4,597 ± 29
Valine	29,908 ± 1,783	41,905 ± 1,292	35,684 ± 419
Isoleucine	24,592 ± 1,435	27,505 ± 812	19,718 ± 232
Leucine	12,905 ± 751	16,934 ± 790	8,222 ± 100
Phenylalanine	18,518 ± 771	20,813 ± 284	13,142 ± 43

Values are in picograms per microgram protein. Values (mean) and standard errors (SE) are reported as that from three replicates, assayed independently

to 23% during the same sampling period. The percent contribution of glutamine and glutamate to the total amino acid content of the xylem sap varied from 3.5 to 7.1%. The concentration of the sulfur containing amino acids methionine and cysteine was very low (less than 0.8%) in the xylem sap irrespective of the sampling time. Similarly, glycine was also found at very low concentration in the xylem sap samples.

Changes in organic acids and soluble carbohydrates in xylem exudates

Diurnal changes in the concentration of select organic acids and soluble carbohydrates quantified at three time points during a 24 h cycle was also noted (Table 3). The concentration of these metabolites was 710 ng μg^{-1} protein in samples harvested at 9 a.m., increased to 2,927 ng μg^{-1} protein at 3 p.m. and declined to 430 ng μg^{-1} protein at 12 a.m. Between 9 a.m. and 3 p.m., there was a 4.1-fold increase in the concentration of these metabolites in the xylem sap. Pinitol was the most abundant soluble carbohydrate representing about 32–45% of the total metabolites identified in this study. Even though the concentration of fructose and glucose was the highest in the xylem sap collected at 3 p.m., the relative percentage contribution of these sugars to the total metabolite pool remained mostly unchanged during other sampling periods. In contrast the concentration of sucrose, which was about 1.5–2% in samples collected at 9 a.m. and 12 a.m., increased substantially in the samples collected at 3 p.m. and represented about 17% of the total metabolite pool (Table 3). The xylem sap also contains significant amounts of maltose and raffinose whose concentration are highest in saps collected at 3 p.m. Greater than 300-fold change in raffinose content was observed between 12 a.m. and 3 p.m. Malate and citrate were the two most abundant organic acids detected in the xylem sap examined here. The concentration of malate was highest in the 3 p.m. samples; however, the relative percentage concentration of this organic acid to the total metabolite pool was unchanged during the diurnal cycle. In contrast, the percent concentration of citrate increased from 1.5 to 2.8% in samples collected during the day time to 13.8% in the 12 a.m. samples.

Discussion

In this study we have examined the composition and identity of xylem sap proteins from field-grown Williams 82 soybean. The most abundant proteins present in the xylem sap were identified as 31 kDa (spots 20, 21, 22, 43, and 44) and 28 kDa (23, 24, 25, 26, and 45) glycoproteins. Previous studies have shown that these two abundant glycoproteins accumulate in leaves, stems, pods, flower petals, and germinated cotyledons (Staswick 1989a, b). It is estimated that these proteins can account for about 6–15% of soluble leaf protein (Wittenbach 1983a; Staswick 1989a). Because of their abundance they have been designated as

Table 3 Metabolite profile of Williams 82 soybean xylem sap collected at 9 a.m., 3 p.m. and 12 a.m

Metabolite	Xylem sap 9 a.m. (mean ± SE)	Xylem sap 3 p.m. (mean ± SE)	Xylem sap 12 a.m. (mean ± SE)
Ribose	1.0 ± 0	2.3 ± 0.3	0.4 ± 0.1
Fructose	89.8 ± 1.4	427.4 ± 1.7	52.9 ± 1.3
Sucrose	2.6 ± 0.9	7.9 ± 2.8	6.7 ± 0.2
Maltose	103.0 ± 1.8	511.2 ± 2.6	0.8 ± 0.1
Trehalose	7.9 ± 0.7	28.8 ± 0.5	0.3 ± 0.0
Raffinose	15.4 ± 0.2	507.4 ± 4.0	1.5 ± 0.9
Glucose	1.0 ± 0.1	5.2 ± 0.2	68.4 ± 1.6
Galactionol	0.2 ± 0	1.1 ± 0	2.4 ± 0.0
Myo-inositol	1.5 ± 0.7	9.8 ± 5.7	4.5 ± 0.2
Pinitol	326.4 ± 1.1	960.0 ± 3.0	142.8 ± 1.7
Pyruvate	0.2 ± 0	0.4 ± 0	0.1 ± 0.0
Fumarate	15.3 ± 0	27.6 ± 0.1	2.7 ± 0.0
Succinate	3.4 ± 0	15.4 ± 0.1	1.4 ± 0.0
Glycerate	27.4 ± 1.5	15.2 ± 0.3	28.8 ± 1.7
Malate	88.8 ± 1.0	353.3 ± 4.1	53.8 ± 0.4
2-Oxoglutarate	2.9 ± 0.4	0.2 ± 0.1	0.1 ± 0.0
Shikimate	1.8 ± 0	5.8 ± 0	1.1 ± 0.0
Citrate	20.4 ± 0.3	45.8 ± 1.1	59.7 ± 0.4
Ascorbate	1.1 ± 0.2	2.8 ± 0.7	1.8 ± 0.3

Values are in nanograms per microgram protein. Values (mean) and standard errors (SE) are reported as that from three replicates, assayed independently

vegetative storage proteins. Removal of reproductive sink tissue has shown to drastically increase the accumulation of these vegetative storage proteins (Staswick 1990; Wittenbach 1983a, b). Interestingly, the concentration of these vegetative proteins in soybean pods, which was highest at 3 weeks after flowering, declined 37% within 3 weeks after seed development. This drastic decline indicated that these vegetative proteins are mobilized to meet the nutrient needs of the developing seeds (Staswick 1989a, b). Since these proteins are also abundant in the xylem sap we examined their abundance from the xylem sap collected from soybean plants at vegetative and reproductive phase. However, we were unable to detect any appreciable changes in the concentration of vegetative storage proteins at either developmental stage (data not shown). The role of these vegetative proteins in the xylem sap needs further investigation. These vegetative storage proteins appear to be unique to soybeans since they have not been reported in xylem sap of other plant species. Since soybean accumulates a large amount of seed storage proteins (about 40%), there is a tremendous need for nutrients and amino acids during seed filling. It is likely the vegetative storage proteins in the xylem sap may be transported to the developing seeds. However, the 27 and 31 kDa glycoproteins are not found in soybean seeds indicating that these proteins may not be transported intact to the developing seeds. Instead, they may serve as a temporary storage pool for amino acids and utilized by the developing soybean seeds to support the massive synthesis of seed storage proteins.

Recently, the xylem sap proteome of soybean seedlings was reported (Djordjevic et al. 2007; Subramanian et al. 2009). Kunitz trypsin inhibitor, vegetative storage proteins (VspB and Vsp25), gamma-glutamyl hydrolase, and β-amylase were the most abundant proteins found in the soybean xylem sap. In this study, in addition to the abundant xylem sap proteins reported earlier, we were able to identify thiamin pyrophosphokinase, LRR-receptor-like protein kinase and 3-phosphoshikimate 1-carboxyvinl-transferase. Interestingly, the xylem sap of field-grown soybeans contained a few proteins involved in plant defense such as peroxidase, chitinase and serine proteases. Plant defense-related proteins have been reported to be abundant in the xylem sap of several plants (Buhtz et al. 2004; Kehr et al. 2005; Alvarez et al. 2006; Aki et al. 2008). Even though peroxidase and subtilisin have been detected in the soybean seedling xylem sap (Subramanian et al. 2009), it is not abundant in the xylem sap of field-grown soybeans. In our study we collected xylem sap from healthy field-grown soybeans and the apparent absence of defense-related proteins may indicate that these proteins may be induced by pathogen attack. This possibility is strengthened by the observation that *Phytophthora sojae* elicitor treatment of soybean seedlings resulted in an increase in serine protease and peroxidase in xylem sap (Subramanian et al. 2009).

Previous studies have shown that most of the xylem sap proteins contain N-terminal secretion signals and pass through the plant secretion pathway (Satoh 2006). We have also examined the sequences of the identified xylem sap proteins and found putative N-terminal secretion signals in several of these proteins suggesting that these proteins pass through secretory pathway. Our observation is consistent with several other reports which claim that the xylem sap proteins belong to a class of secreted proteins (Rep et al. 2002; Buhtz et al. 2004; Alvarez et al. 2006; Djordjevic et al. 2007; Subramanian et al. 2009).

Soybean require substantial amount of nitrogen to support protein synthesis in the developing seeds. Biological nitrogen fixation is a major source of nitrogen for soybean. The relative ureide (allantoin and allantoic acid) content in the xylem sap can be used as a reliable indicator of nitrogen fixation in soybeans (McClure et al. 1980). In this study, we observed that the concentration of free amino acids was higher during daytime than at night and asparagine was the dominant amino acid found in the xylem sap. Our finding is consistent with an earlier report that showed an increase in the proportion of asparagine in xylem sap from 40 to 70% during seed development (Ohtake et al. 1995). The relative abundance of asparagine in the xylem sap of soybean highlights the importance of this amino acid as a major nitrogen transport amino acid. We also found diurnal changes in the asparagines/aspartate concentration in the xylem sap. It has been earlier reported that changes in the nitrogen metabolism can affect the relative composition and concentration of amino acids in the xylem sap. Such changes can also be exerted by several stress conditions. For example, flooded roots which results in impaired nitrogen fixation led to a significant reduction in xylem glutamine levels and alters the asparagines/aspartate ratios in both ureide and amide exporting legumes (Lima and Sodek 2003). Since the soybean plants used in our study were healthy and not subjected to any obvious biotic stresses, the observed diurnal changes in the concentration of asparagine may reflect the difference in the rate of biological nitrogen fixation during the day and night.

The occurrence of maltose in the xylem sap is intriguing. Maltose is derived from starch breakdown mediated by the action of β-amylase and is usually concentrated in plastids. Phloem-specific β-amylase has been characterized in Arabidopsis (Wang et al. 1995). However, starch breakdown mediated by xylem-specific β-amylase has not been reported. Since proteomic analyses have revealed the presence of β-amylase in the xylem sap (Djordjevic et al. 2007; Subramanian et al. 2009), the maltose detected in the xylem sap could be derived from xylem-specific β-amylase activity. However, the possibility that the β-amylase

identified in the xylem sap is a contaminant from the phloem sap may not be completely excluded.

Pinitol (D-3-*O*-methyl-chiro-inositol) has been shown to be a major low molecular weight carbohydrate in soybean plants (Phillips and Smith 1974; Streeter 1985). A significant amount of pinitol accumulates in the leaves and its concentration exceeds the combined concentration of all mono- and disaccharides (Streeter 2001). It has been suggested that the accumulation of pinitol is an adjustment mechanism of the soybean to reduce high-temperature damage (Guo and Oosterhuis 1995). Two- to three-fold gradients in pinitol concentration from the bottom to the top portion of soybean plants have been reported (Streeter et al. 2001). The spatial differences in the concentration of pinitol were attributed to translocation of the pinitol from lower to upper nodes. Streeter (1981) examined seasonal distribution of carbohydrates in nodules and stem exudates from soybean plants and found a tenfold decline in pinitol transport during seed set to seed maturation. In this study, we found pinitol as an abundant compound in the xylem sap of field grown soybean and observed marked temporal differences in their concentration. The pinitol concentration, which was higher in the daytime declined at night. Their abundance during daytime may suggest a protective role for pinitol against high temperatures that are frequently encountered by field grown soybeans. It should be pointed that previous studies have provided correlative evidence for the role of pinitol in drought tolerance in soybean (Streeter et al. 2001). Many years ago people thought of the xylem as a conduit for water, inorganic ions and little else. However, it is clear from this and related studies that the xylem has a number of important functions, such as signal transduction, stress avoidance and plant defense. Therefore, the xylem is an active and complex vascular system in plants that performs an array of functions that deserve further study.

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