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The *Glycine max* Xylem Sap and Apoplast Proteome

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Molecular signaling interactions in the plant apoplast are important for defense and developmental responses. We examined the soybean proteome of the apoplastic conduit of root-to-shoot communication, the xylem stream, using gel electrophoresis combined with two types of tandem mass spectrometry. We examined soybeans for the presence of a *Bradyrhizobium japonicum*-induced, long distance developmental signal that controls autoregulation of nodulation (AON) to determine if xylem proteins (XPs) were involved directly or indirectly in AON. The xylem and apoplast fluids collected in hypocotyl, epicotyl, and stem tissue contained a highly similar set of secreted proteins. The XPs were different from those secreted from imbibing seed implying they play important basic roles in xylem function. The XPs of wild-type and *nts1007* plants were indistinguishable irrespective of plant age, inoculation status, or time after inoculation suggesting that none was directly involved in AON. XPs were continuously loaded into the xylem stream, as they were present even 28 h after shoot decapitation. These results were consistent with semiquantitative RT-PCR studies that examined the expression of genes corresponding to the XPs under inoculated or uninoculated conditions. Monitoring the expression of XP genes by RT-PCR showed that four possessed root biased expression. This suggested that the corresponding protein products could be produced in roots and travel long distances to shoots. Of these, a species of lipid transfer protein is a candidate for a water-soluble, long-distance signal-carrier due to the presence of hydrophobic clefts that bind known plant signals in vitro. Two soybean XPs identified in this study, lipid transfer protein and Kunitz trypsin inhibitor (KTI), have known roles in plant signaling.

Keywords: model legume • root-to-shoot signalling • *Bradyrhizobium japonicum* • lipid transfer proteins • autoregulation-of-nodulation • MALDI-TOF/TOF

Introduction

Molecular interactions that occur in the extracellular spaces of plant cells (i.e., the apoplast) are attracting renewed and increasing attention. One reason is that root−shoot interactions are mediated by graft transmissible mobile signals of unknown origin,[1−6] and the connecting tissues in the xylem and phloem are the most likely conduits through which these novel signals are transmitted. Second, many important non-self- and self-recognition responses occur in the apoplast.[7] These include the recognition of (a) a range of bioactive molecules from symbionts, endophytes, and pathogens and (b) endogenous extracellular signal molecules that control plant development.[7] Endogenous extracellular apoplastic signals include bioactive (CLV3)/Endosperm Surrounding Region (ESR) or CLE peptides that have short and possibly long distance effects on Clavata like receptors.[8−12] These receptors are part of a large multigene family of membrane-bound, leucine-rich-repeat receptor like kinases (LRR-RLKs) proteins (e.g., *Clavata 1* and *2*) that mainly recognize protein and peptide ligands.[13] It is becoming increasingly clear that the unknown ligands and interacting partners for these receptors are targeted to the apoplast and could be identified directly by examining this plant compartment.

The xylem vessels in the roots and shoots are comprised of dead elongated cells that form an apoplastic space. The fluid in xylem vessels, xylem sap, is composed of inorganic and organic compounds that are known to be transported long distances. Recently, it has been shown that the xylem sap contains a discrete set of secreted proteins that are immunologically distinct to those contained within the phloem.[14] Since xylem vessels result from a process of programmed cell death, it is thought that some of the protein contents of the xylem vessels result from the dead xylem vessels.[15] However, a more deliberate targeting of protein to the xylem sap from, for example, adjacent xylem parenchyma is more likely to occur,

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since the vast majority of xylem proteins (XPs) pass through the plant secretion pathway and possess predicted N-terminal secretion signals.[16]

There are suggestions that proteins can move long distances in the xylem sap. RNA expression studies have shown that some genes encoding XPs appear to be expressed in roots and loaded into the xylem stream for long-distance transport to shoots.[17−19] Other evidence suggesting that proteins are involved in long-distance signal transduction comes from work with *Arabidopsis* where a mutant affected in the production of a well-known XP, a lipid transfer protein, was found to be crippled in systemic acquired resistance (SAR).[20] In addition, the protein product of the FT gene has recently been shown to move from leaves to the shoot apical meristem to regulate flowering;[5,6] however, this moves in the phloem.

Relatively little is known about the functions of XPs beyond roles in plant defense. XP composition tends to be conserved among plant species, suggesting an important role for these proteins in maintaining xylem function.[14] The types of XPs can be altered dramatically by pathogenic infection of plants.[18] The induction of pathogenesis related (PR) genes in the plant host is a well-recognized consequence of pathogenesis, and several of the corresponding proteins (including PR1 and PR5) become components of the xylem sap some time after infection and can be detected at distance from the infection site.[18]

During the infection of legumes by nitrogen fixing rhizobia, a close relationship exists between the symbiotic bacteria and the host. Legume root nodule numbers are controlled by signals exchanged between the root and shoot that lead to autoregulation of nodulation (AON).[1] Nodulation induces an unknown root-to-shoot signal, which, in turn, induces the production of a shoot-derived inhibitor (SDI) mediating AON.[1,21] Both signals are graft transmissible (i.e., mobile); however, the root-to-shoot signal is likely to be a mobile xylem sap component. Intergrafts do not alter the transmission of the signal, arguing that (a) the signal rather than its transport is affected in AON mutants and (b) the intergrafted stem segments do not produce SDI. In addition, although infection of roots by symbiotic rhizobia bears some similarities with that of pathogens,[22,23] the capacity of rhizobia to directly or indirectly alter the XPs or induce SAR-type proteins has not been examined. Rhizobia possess protein secretion systems,[24] but it is not known if these proteins are targeted to plant cells or can move away from infection sites or nodule tissues to become XPs as occurs with some plant pathogens.[25]

Here, we determine the identity of XPs of wild-type and *nts1007* mutants in the presence and absence of *Bradyrhizobium japonicum* at different times after infection and nodule initiation. We determined whether the XP constituents could be altered directly or indirectly by *B. japonicum* infection and nodulation. Both plants are competent for making the root-to-shoot initiator of AON signal, whereas *nts1007* is defective in SDI production. In *nts1007*, mutants there might be a higher concentration of the root-to-shoot signal due to lack of feed back inhibition by SDI.[21] In addition, we examined the possibility that XPs collected from shoots could result from expression of XP genes in the roots, implying a long-distance movement of XPs. To achieve these aims, we isolated and identified soybean XPs from the hypocotyl, epicotyl, and stem apoplast using one- and two-dimensional gel electrophoresis in combination with tandem mass spectrometry (MALDI-TOF/TOF and LC−MS/MS). We compared these XPs to proteins exuded from imbibing seed to show that XPs are secreted specifically to the xylem. By the comparison of XPs in inoculated and uninoculated wild-type and *nts1007* mutants, a possible protein candidate for the root-to-shoot initiator of AON could be directly identified, if present at detectable levels. A protein or peptide ligand for AON control is possible since the super-nodulating AON mutant *nts1007* is defective in a Clavata 1-like LRR-RLK[1] that recognizes a peptide ligand. Finally, we used semiquantitative reverse transcriptase-PCR to examine the gene expression patterns of the genes encoding for XPs. This approach provided (a) an independent means to determine if XPs were regulated differentially by *B. japonicum* inoculation and (b) a method to identify long-distance XP movement.

Materials and Methods

Plant Growth and Sap Collection. Seeds of *Glycine max* cv. Bragg wild-type and *nts1007* (AON mutant with a super-nodulation phenotype, mutated at Q106* of a CLAVATA1-like LLR-receptor kinase[1]) were used. Seeds were germinated and plants grown (4−6 per pot) in vermiculite under glasshouse conditions at (28 °C). After germination, the plants were watered with half strength B and D medium[26] solution three times a week and with water on the other 4 days. The B and D medium was supplemented with 2 mM KNO_3 on one watering day per week.

Between 10 and 21 days after planting, half of the plants in each experimental batch (numbers varied from 4 to 20 plants per treatment) were inoculated with a slurry of commercial soybean inoculum, Nodulaid 100 (*B. japonicum* strain CB 1809, Bio-Care Technology P/L Somersby NSW, 10^{10}/g) leaving the other half uninoculated. Equal numbers of inoculated or uninoculated plants were used for sap collection. Sap was collected at different time points after inoculation (e.g., 2, 4, 7, 9, or 12 days or as indicated in text) as the root-to-shoot signal is known to be produced within 2 days of inoculation.[21] After morning watering, plants were decapitated either below the cotyledons for hypocotyls and above the cotyledons for epicotyls, and the root stock was attached securely to 1 mL syringes by 3 cm pieces of plastic tubing (i.d. 3 mm) generating an airtight connection. The seal between the tubing and the hypocotyl was established by tying gift tape around the plastic tubing attached to the hypocotyl. After the syringe was attached to the tubing, a small vacuum was maintained by pulling back on the syringe and impeding its retraction.

For the time course experiment, sap from four plants was pooled and collected at 2 h intervals for the first 10 h. Plants also continued to bleed xylem sap via root pressure, and collections could be made up to 28 h after decapitation. The sap was regularly collected from syringes or the plastic tubing and immediately placed into precooled tubes (Eppendorf, Hamburg, Germany). Pooled sap from plants was immediately lyophilized, resuspended in 50 μL of water, and stored at −70 °C. The concentration of protein in undiluted sap was estimated using Bradford assays (Bio-Rad, Hercules, CA) with BSA as standard.

Hypocotyl and epicotyl sap proteins were compared to those in the soybean stem apoplast. Stem apoplastic fluid was isolated based on the method of Hon et al.[27] Soybean stem material (80−100 mm) was collected, weighed, washed with sterile water (18.2 MΩ·cm), blotted dry, cut into 30 mm segments, and washed with sterile water several times. The segments were submerged into precooled Apo solution (20 mM $CaCl_2$ and 20 mM ascorbic acid; 4 °C) and placed under vacuum

in a desiccator for 1 h, after which the vacuum was slowly released. The segments were then blotted dry on paper towels, placed into a 5 mL syringe within a 15 mL tube, and centrifuged in a swing out rotor (1500*g* at 4 °C for 30 min), and the apoplastic fluid was collected, freeze-dried, and stored at −80 °C.

Seed Exudate Isolation. Fifty wild-type cv. Bragg seed were surface-sterilized with 2.5% sodium hypochlorite for 5 min, rinsed four times with sterile water, and submerged in the dark in 50 mL of sodium acetate buffer (1 M, pH 4.8) for 24 h at 30 °C. The solution was centrifuged at 3000*g*, and the supernatant was freeze-dried before isolating the proteins using the Ready Prep 2-D clean up kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Proteins were reduced, alkylated (see below), and either separated on 1-D gels for examination by MALDI-TOF/TOF or introduced directly into the LC−MS after trypsin digestion (see below).

Protein Preparation and 1-D Gel Electrophoresis. Xylem or apoplast proteins (30 μL; containing approximately 10−15 μg of protein) were first reduced in 1 mM dithiothreitol (pH 8) for 30 min at 37 °C and alkylated with 20 mM iodoacetamide for 30 min at 37 °C. The preparation was boiled for 5 min in Invitrogen Tris tricine-SDS sample buffer, loaded, electrophoresed into precast 1-D gels (10−20% Tricine, Invitrogen), and stained with Coomassie Blue.

2-D Gel Separation. XPs (30−50 μg) collected from xylem sap pooled from six plants were resuspended in 150 μL of 2-D sample buffer, cup-loaded onto 18 cm pH 4−7 immobiline strips, and run as described previously.[28] After reduction and alkylation, proteins were electrophoresed into horizontal second-dimension gels as described previously before staining using colloidal Coomassie.[28]

Preparation of Tryptic Fragments. Proteins were excised from Coomassie stained 1- or 2-D gels, destained with 50% acetonitrile in 25 mM ammonium hydrogen carbonate (pH 7.8), dehydrated with acetonitrile, rehydrated with 10 μL of trypsin (Promega, sequencing grade, 15 ng/μL in 25 mM ammonium hydrogen carbonate), and digested overnight.[28] Peptides were extracted from gel pieces with 10 μL of 50% acetonitrile/1% trifluroacetic acid (TFA), and all digestions were purified with C18 Zip-Tips (Millipore). Purified peptides were eluted from the Zip-Tips with either 70% acetonitrile/0.1% TFA (MALDI-TOF/TOF) or 50% methanol/1% formic acid (ESI for LC−MS; see below). Alternatively, for samples where trypsin digestion resulted in poor data, samples were digested overnight with Lysyl endopeptidase (LysC, 1 pg in 25 mM ammonium hydrogen carbonate, pH 8; Wako, Japan) followed by trypsin digestion for 30 min.

Mass Spectrometry. LC−MS/MS mass spectrometry was done as follows. Aliquots of 100 μL containing 20−30 μg of total sap protein were cleaned and concentrated with a Bio-Rad (Hercules, CA) 2-D gel clean up kit before reduction and alkylization (as above) followed by overnight digestion with trypsin. The sample was subjected to multidimensional protein identification (Mudpit) analysis using a LCQ DECA XP Plus ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA). Peptides were eluted from the strong cation exchange column (Thermo BioBasic 5 μm, 100 × 0.32 mm) onto a C18 column (Thermo BioBasic C18 5 μm, 100 × 0.18 mm) with 5 salt elutions of 100, 200, 400, 600, and 800 mM ammonium chloride/0.1% formic acid. The C18 columns were eluted with an acetonitrile/0.1% formic acid gradient of 5−65% over 30 min. Data were collected in a sequence consisting of a full scan followed by three data-dependent MS−MS scans of the three most intense ions. Alternatively, individual 1-D gel protein bands and the intervening areas between gels bands were excised, destained, reduced, and alkylated followed by trypsin digestion, as above, before LC−MS analysis.

For MALDI-TOF/TOF mass spectrometry, individual proteins were excised from gels, destained, reduced, alkylated, and then subject to trypsin digestion and C18 reverse phase clean up using Zip Tips before being spotted onto plates. Alternatively, individual tracks from 1-D gels were systematically cut into 1 mm width × 3 mm gel pieces over the entire size range (from 4 to >70 kDa), including the clearly stained proteins, and treated as above after trypsin or LyC/trypsin sequential digestions. Mass spectra were generated using an ABI 4800 MALDI-TOF/TOF (Applied Biosystems, Foster City, CA) with recrystallized α-cyano-4-hydroxycinnamic acid matrix. Spectra were obtained in positive ion reflectron mode. For MS analyses, 1600 laser shots were accumulated with 50 shots per subspectrum for each spot. For MS/MS analysis, 4000 shots were accumulated with 50 shots per subspectrum for each parent ion selected using air as the collision gas at a collision energy of 1 kV and a collision gas pressure of 2.8×10^{-6} Torr. External calibration for MS was done using LaserBio Labs (Cedex, France) peptide calibration Mix 4, using angiotensin II $[M + H]^+$ 1046.54, neurotensin $[M + H]^+$ 1672.92, ACTH (18−39) $[M + H]^+$ 2465.20, and oxidized insulin B chain $[M + H]^+$ 3494.65. External 10 point calibration for MS/MS was done using ACTH (18−39) $[M + H]^+$ 2465.20.

Protein Identification. MS/MS data files generated from the LC−MS and the MALDI-TOF/TOF were used to search the soybean expressed sequence tag data base (Soy gene index 12.0, September 2004; http://compbio.dfci.harvard.edu/index.html) and the nonredundant database to detect bacterial (especially *B. japonicum*) proteins. Mascot (www.matrixscience.com) searches were done using an in-house Mascot server. For the LC−MS data, the Bioworks dta files were merged into a single file with a perl script, Merge, available from the Matrix Science Web site. For data base searches, a tolerance of 0.8 Da was allowed for peptide and MS−MS with variable modifications allowed for cysteine (carbamidomethyl) and methionine (oxidation) and one trypsin miscleavage. For the MALDI-TOF/TOF, tolerance was set to 0.3 Da for peptide and MS/MS determination with the same variable modifications allowed as above. Mascot protein identifications were accepted when the score was above the cutoff threshold for that data base (>39). At least two peptides were identified from each protein in at least two independent samples (except where indicated). N-terminal signal sequences were determined using the Signal P (http://www.cbs.dtu.dk/services/SignalP/) and Secretome P (http://www.cbs.dtu.dk/services/SecretomeP/) programs.

RNA Isolation and Semiquantitative RT-PCR. Soybean plants (cv Bragg) were grown in pots without bacterial inoculation and under the conditions stated above. After the first leaves appeared, material from leaves, stems, cotyledons, hypocotyls, roots, and root tips was harvested and frozen in liquid nitrogen. In a separate experiment to test for the effect of inoculation of gene expression, plants were prepared; half were inoculated for 10 days, and hypocotyl, stem, leaf, and root material was harvested and assayed. In the case of samples that were deliberately wounded, a second hypocotyl sample was taken 4 h after the first. RNA was prepared from 100 mg of each tissue using a Qiagen (Venlo, The Netherlands) RNeasy Plant mini kit (including the DNase treatment) according to the manu-

facturer's instructions. The concentration of each preparation was determined by measuring absorption at 260 nm. DNA was prepared from 200 mg of leaf tissue with a FastDNA kit (BIO 101 Systems, Seven Hills, NSW, Australia) according to the manufacturer's instructions.

Oligonucleotide primer pairs were designed to amplify products of about 300 base pairs in the corresponding mRNAs of sap proteins. The same primers were used to amplify a DNA product, and in many cases, the product was of a different size to the corresponding RNA product (indicating the presence of an intron in the DNA product). Primer pairs for the integrated amplicons of 16 of the secreted sap protein genes were synthesized by Gene Works (Adelaide, South Australia). The reverse transcriptase PCR reaction was performed using the Invitrogen Superscript III One-Step RT-PCR System. PCR reactions (50 μL) containing 500 ng of RNA template and 10 pmol of each primer were amplified in a FTS-960 Thermal Sequencer (Corbett Research, Sydney, Australia). Cycling conditions were as follows: 50 °C for 30 min; 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 48 °C for 30 s, and 68 °C for 30 s. After 25 and 30 cycles, 10 μL aliquots were removed, and 10 μL reaction products resulting from 25, 30, and 35 cycles were visualized on 1.5% agarose gels in TAE buffer (40 mM Tris, 1 mM Na_2EDTA, and 20 mM acetic acid, pH 8). Control reactions were performed with two separate pairs and RNA and DNA templates using Promega (Madison, WI) PCR Master Mix that contains no reverse transcriptase enzyme, to eliminate the possibility of DNA being the template.

Results

Analysis of XPs from Hypocotyl and Epicotyl Sap Compared to Stem Apoplast Proteins. XPs from hypocotyl and epicotyl were isolated and compared with those in the stem apoplast. The XP from hypocotyl and epicotyl sap were collected from wild-type Bragg and *nts1007* plants between 17 and 35 days postgermination with half the plants exposed to *B. japonicum* CB1809 for various times and the remainder untreated. Nodules could be seen from 8 days postinoculation (dpi) on wild-type and *nts1007* roots. Sap volumes ranged from 100 to 300 (μL/plant)/h, and the solution was clear and without observable chlorophyll contamination. Stem apoplastic fluid was isolated from the epicotyl at 4 and 7 dpi. The protein concentration of xylem sap and stem apoplast fluid was 10−12 μg/mL.

The XPs and apoplastic proteins from soybean stems were examined using 1- and 2-D gel electrophoresis. Irrespective of the method of protein isolation used, the genotype of the plants, the age of plants at collection time, the tissue location where the protein was isolated (stem, epicotyl, or hypocotyl), the treatment applied, or the day postinoculation when the fluids were collected, the 1-D gel electrophoresis of the fluids collected was qualitatively similar. Twelve major protein species ranging from ~10 to ~80 kDa were always observed in the gels. A representative gel is shown (Figure 1A). The XP and apoplast proteins from more than 20 independent experiments were examined by 1-D gel electrophoresis. A faint 6 kDa protein was just observable (Figure 1A arrow). The relative levels of individual protein bands varied between different experiments, but otherwise, the protein banding pattern was invariable.

Some samples were also examined by 2-D gel electrophoresis, which also gave near identical protein arrays irrespective of treatment or plant genotype. The only additional information gained from 2-D gel electrophoresis was that several of the

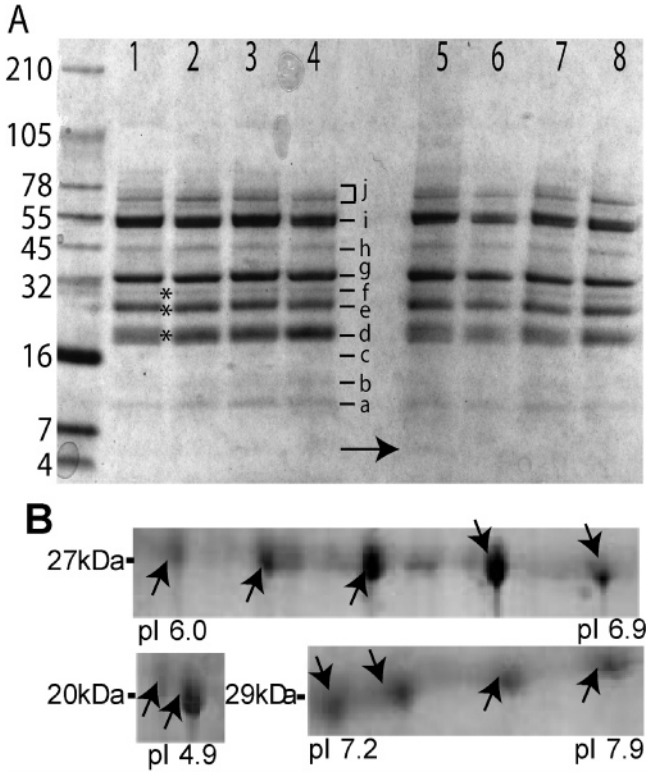

Figure 1. Analysis of apoplastic proteins in the hypocotyl of wild-type and mutant soybeans by gel electrophoresis. Panel A shows a reproducible and discrete array of XPs isolated from inoculated and untreated wild-type Bragg and *nts1007* plants at 2 and 7 days postinoculation with *B. japonicum* CB1809. Irrespective of whether the plants were treated or untreated and the time of collection, the XPs appeared similar. Twelve major protein bands (labeled alphabetically with J comprising 3 closely spaced bands) were always observed, and a faint 6 kDa protein was just observable in some lanes (best seen in lanes 3 and 5 at the level of the horizontal arrow). Lanes 1−4, no treatment; lanes 5−8, *B. japonicum* added. Lanes 1 and 5 and lanes 2 and 6 were wild-type plants collected 2 and 7 days after no treatment or *B. japonicum* treatment, respectively. Lanes 3 and 7 and lanes 4 and 8 were *nts1007* plants at 2 and 7 days after no treatment or *B. japonicum* treatment, respectively. Panel B shows select portions of 2-D gels where XPs were separated into distinct isoforms (arrows) that were not apparent on 1-D gels. The apparent molecular weights and p*I*s of these proteins are indicated. The corresponding bands on 1-D gels are asterisked in panel A. The species at 27, 20, and 29 kDa represent isoforms of vegetative storage protein, VspB, Kunitz trypsin inhibitor (KTI; TC215770), and Vsp25, respectively (see Table 1).

major protein bands, that occurred on the 1-D gels at 20, 27, and 29 kDa (bands d−f, indicted by an asterisk in Figure 1 A), occurred as multiple isoforms in 2-D gels (Figure 1B). This was confirmed by mass spectrometry of the individual protein spots in the 2-D gels at these molecular weights (see below). Some species that occurred on 1-D gels (e.g., bands a and b) were not apparent in 2-D gels, thus, validating the use of the two gel systems.

Examination of Xylem Sap over a 28 h Time Course. All previous experiments examined XPs collected within 2 h of decapitation (for example in Figure 1A). However, we observed that xylem sap continued to bleed from the hypocotyl or epicotyls for at least 24 h after decapitation even in the absence of syringe suction. The volume of sap generated by "root

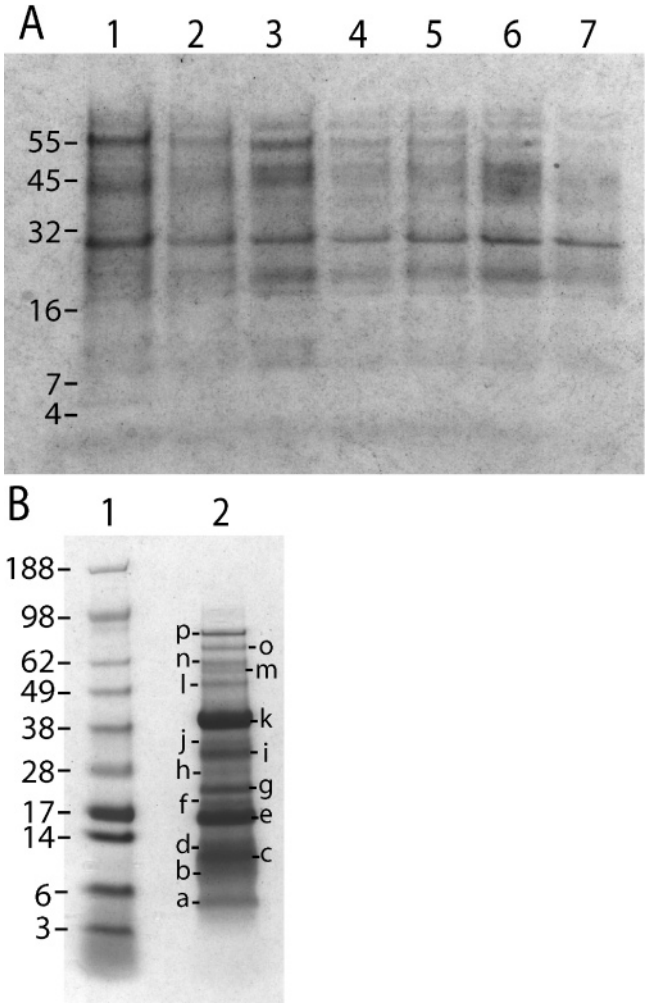

Figure 2. Gel electrophoresis of proteins found in xylem over a time course and released from imbibing seed. Panel A shows XPs collected from the same soybean plants at different times over a 28 h period separated on 1-D gels. The protein content of the xylem sap was consistent over the time course, although there appeared to be evidence of protein degradation at the final time point. Ten micrograms of protein per track is loaded. Panel B shows a 1-D gel separation of proteins released from imbibing soybean seeds. (A) Lanes 1−8 were collected from hypocotyls from inoculated wild-type plants at 2, 4, 6, 8, 10, 12, 24, and 28 h after shoot decapitation. (B) Lane 1, protein standards; lane 2, protein released from imbibing seed. The 16 protein bands are labeled alphabetically.

pressure" was less than that obtained when syringe suction was applied. Nevertheless, this allowed us to collect xylem sap at 2 h intervals at several time points over 28 h. The XPs that resulted after 1-D gel electrophoresis was again remarkably constant irrespective of the genotype or the treatment applied (Figure 2A), suggesting that these XPs are stable sap constituents and continuously produced. Mass spectrometry analysis (below) confirmed that the major protein species present over the 28 h period were the same as those collected within 2 h of decapitation.

Examination of Proteins Released from Imbibing Seed. To determine if the XPs and stem apoplast proteins were distinct from other secreted proteins, we isolated, separated, and identified proteins released from imbibing soybean seeds. Sixteen prominent protein bands were found in the washings of imbibed seed (Figure 2B), and the protein banding pattern was completely different from that found in Figure 1A. The protein concentration was 12 μg/mL for the seed exudate, and the protein pattern was consistent in five intrasample repeats.

Proteins Identified in Xylem and Apoplastic Fluid of Soybeans by Mass Spectrometry. We compared the XPs isolated from sap, the stem apoplast proteins, and those released from imbibing seeds using mass spectrometry. We expected the predicted open reading frames of putative secreted proteins in the xylem stream to possess N-terminal secretion signals, and this was examined using SignalP and Secretome P algorithms. Different strategies were employed to identify major and minor components of the xylem sap and apoplast materials. First, individual protein spots and bands from 1- and 2-D gels were directly examined using MALDI-TOF/TOF and LC−MS/MS. In addition, entire 1-D gel lanes were cut into discrete molecular weight cuts ranging from 4 to 80 kDa and examined using MALDI-TOF/TOF to determine if there were (a) low-abundance proteins present in the preparations and (b) evidence of protein degradation. Finally, the entire sample was subjected to multidimensional protein identification using 2-D LC−MS/MS. The material secreted from seed was examined by 2-D LC−MS/MS, and individual protein bands were examined using MALDI-TOF/TOF.

MS analysis showed that the major and minor components of xylem sap and apoplastic fluid were very similar (Table 1) as suggested by the near identical patterns observed in 1- and 2-D gels (Figure 1; data not shown). The protein identities obtained from using MALDI-TOF/TOF or 2-D LC−MS/MS instruments on the same sample were identical; however, different peptides of the same identified protein were sometimes more easily detected by either the MALDI-TOF/TOF or 2-D LC−MS/MS, thus, demonstrating the advantages of using two types of mass spectrometry. Nevertheless, the results obtained were consistent from sample to sample, irrespective of the genotype of the plant examined, the treatment or age of the plant, the tissue from which the proteins were derived, or the time of collection with respect to inoculation by *B. japonicum*.

Twenty-four soybean proteins were identified (Table 1). The most abundant proteins were identified in all samples, but several proteins, presumably of lower abundance, were found in some samples but not others (Table 1). The proteins identified had predicted molecular masses that corresponded to the M_r calculated by 1- or 2-D gel electrophoresis (Table 1 and see lettering indicated in Figure 1) with the exception of band(s) at j. Each protein identified generated a significant Mascot score. Nineteen of the proteins listed in Table 1, including those corresponding to all the prominent protein bands, possess N terminal signal sequences. Consistent with the removal of the N-terminal secretion signal, no peptide was identified in the region of the proteins that spanned the cleaved N-terminal regions. One protein, the Cu/Zn superoxide dismutase, is predicted to possess an atypical N-terminal secretion signal only with the Secretome P program (Table 1). The remaining five proteins (numbered 20−24 in Table 1) were identified predominantly using 2-D LC−MS/MS and were typically abundant cytoplasmic proteins that do not possess N-terminal secretion signals. These XPs are likely to be due to low level cellular contaminants from disrupted cells at the collection site. These were found predominantly in the stem apoplast preparation, which is subjected to more wounding, but as well in the XPs in hypocotyl and epicotyl tissue. None

Table 1. Summary of the Properties of the Hypocotyl and Epicotyl XPs Stem Apoplast Proteins

number	protein	gel band	TC	Mascot score	times identified[a]	size kDa	peptides found	signal sequence
1	Kunitz trypsin inhibitor	d	215770	801	27	20.6	5	Y
2	Kunitz trypsin inhibitor Dare and Forrest	d	204174	213	7	23.9	4	Y
3	β-1,3 endoglucanase	g	214925	290	18	38	15	Y
4	γ-glutamyl hydrolase	g	214792	245	13	37.6	9	Y
5	β-amylase	i	225462	188	17	53.6	13	Y
6	β-glucosidase	g	203307	71	5	38	5	Y
7	Lipase/hydrolase	g	226564	105	6	41.3	2	Y
8	Vegetative storage glycoprotein VspB	f	225143	435	26	29	8	Y
9	Vegetative storage glycoprotein Vsp25	e	225142	446	18	29	9	Y
10	Lipid transfer protein	a	215570	273	8	9.3	3	Y
11	Lipid transfer protein	a	225077	94	2	9.3	1	Y
12	Aspartyl protease/nucleoid binding protein	h	204568	153	3	41	3	Y
13	Blue Cu protein	a	204673	110	4	12.9	2	Y
14	Dehydration stress/elicitor-inducible protein Eig-J7	d	215094	137	4	20.7	3	Y
15	Peroxidase	g	204153	92	6	35.5	5	Y
16	Invertase inhibitor	c	229450	89	1	16.8	3	Y
17	Mln1−5 (Fasciclin like protein) arabinogalactan-protein	d	204373	188	2	24	3	Y
18	Nt Ppr27-like	d	229090	86	4	23	2	Y
19	Superoxide dismutase Cu/Zn	b	204404	60	3	15.1	1	Y weak
20	Nucleoside diphosphate kinase	_	215215	215	4	16.3	4	N
21	Glyceraldehyde phosphate dehydrogenase	_	224783	142	4	36.7	4	N
22	Actin depolymerising factor	_	214036	104	2	16.5	2	N
23	Rubisco small subunit	_	214143	101	4	20	4	N
24	Triose phosphate isomerase	_	214995	245	1	27	5	N

[a] A predominant protein was defined as being found in eight or more independent samples and being represented by a corresponding protein band after 1-D gel electrophoresis. The results are pooled from the analysis over 20 independent biological samples.

of these typically cytoplasmic proteins generated a visible protein band on 1- or 2-D gels, further supporting the conclusion that they are low level contaminants. One 1-D gel protein band of approximately 6 kDa (Figure 1A, arrow) was of low intensity and was unable to be identified.

Individual protein spots from the insets of the 2-D gels shown in Figure 1B were confirmed to be isoforms of the same protein. Finally, MS analysis of individual bands from the time course experiment shown in Figure 2A confirmed that these corresponded to the major proteins listed in Table 1. None of the protein samples matched to any *B. japonicum* protein when examined against the nonredundant database.

The results from dissecting the 1-D gel lanes into multiple size cuts (irrespective of whether a protein band was visible of not) and analyzing the peptide contents by MALDI-TOF/TOF suggested that slow proteolytic activity was present in the samples. For example, gel pieces corresponding to a size of 5−10 kDa that were extracted and digested with trypsin before MS analysis consistently contained tryptic fragments of abundant higher molecular weight species (e.g., KTI, 20−23 kDa; the vegetative storage proteins, 25−28 kDa γ-glutamyl hydrolase, 38 kDa; and β-1,3 endoglucanase, 38 kDa).

The only 1-D gel band(s) that did not generate a protein identification of the correct size was the band(s) at position j in Figure 1. Repeated examination of these bands identified them as proteins of smaller size than one would predict for band(s) j. The proteins at band(s) j were also found in band g of Figure 1, which was represented by four proteins of similar molecular weight: β 1,3 endoglucanase, γ-glutamyl hydrolase, β glucosidase, and peroxidase. Since the protein bands at position j were approximately double the molecular weight of each of the predicted molecular weight of the identified proteins, we concluded that these were protein dimers. Despite repeated attempts and employing several strategies to denature the proteins, these bands remained in the gel. Band g was not the only protein band where more than one protein species was represented; bands a, b, and d, also possessed more than one protein species (Table 1). Two KTIs were present in band d, and two lipid transfer proteins (LTPs) were in band a. We concluded that these were the products of different genes, since distinct peptides were found in each case that distinguished one gene product from another. However, TC215770 and TC215570 were the predominant species of KTI and LTP detected, respectively (Table 1). Examination of individual protein spots from 2-D gels indicated that the two vegetative storage proteins of xylem and apoplastic fluids (TCs 225143 and 225142) and the major KTI (TC215770) were present in distinct isoforms and varied slightly in molecular weight (Figure 1B; Table 1). The two vegetative storage proteins appeared to be representatives of two distinct subclasses of this protein each possessing distinctive peptides and each with a different molecular weight (see Figure 1B). All proteins, except TC229450 (Invertase inhibitor) and TC214995 (Triose phosphate isomerase), were found in multiple samples (Table 1).

Proteins Identified in Seed Exudate. Sixteen protein bands were evident in seed exudate, and the proteins present were of sizes and classes that were different from the XP and stem apoplast proteins (cf. Figure 2B and Figure 1A). Mass spectrometry and bioinformatics analysis identified 17 different protein species (Table 2), although proteins from two bands (a and o) could not be identified. As with the XPs and apoplastic proteins, there was good congruence between the predicted and experimental molecular weights; however, there was evidence of proteolytic breakdown occurring especially with the 7S globulin precursor and a species of the Kunitz trypsin inhibitor, which were found in independent protein bands (Table 2). Most of the proteins identified contained N-terminal processing sequences. Proteins that were predicted to be glycosylated occurred at higher molecular weights than predicted (e.g., the EDGP protein; Table 2). The only proteins common to seed secretion and the xylem and apoplast fluids were several isoforms of KTI and β1,3 endoglucanase. However, only one of the five KTIs identified in the XPs and seed exudate shared the same TC number (Dare and Forest KTI; TC 204174);

Table 2. Proteins Identified in the Seed Exudate of Imbibing Soybean Seed

number	identification	gel band	TC	Mascot score	times identified[a]	size kDa	signal sequence	peptides found
1	Seed lipoxygenase-1	p	215483	304	2	94	N	4
2	Lipoxygenase-3(Soybean) Non-Heme Fe(Ii) Metalloprotein	p	225495	191	1	92	N	3
3	LEXYL1 protein	n	228735	127	2	53	N	2
4	Proline-rich protein	n	215840	89	2	29	Y	1
5	Glycinin G2	n	204283	90	1	52	Y	2
6	(Q05929) EDGP	m, l	227346	139	4	44	Y	2
7	β-mannosidase enzyme	m	206723	54	1	36	Y	2
8	Basic 7S globulin	k, j, d	216733	139	5	46	Y	7
9	β-1,3 endoglucanase	j	228694	165	1	36	Y	1
10	Lectin (Agglutinin)	h,i	204072	103	3	31	Y	4
11	24 kDa Sc24 seed coat protein	g	216614	82	1	24	Y	2
12	Kunitz-type trypsin inhibitor KTI1	g	215049	86	4	20	Y	8
13	Kunitz-type trypsin inhibitor KTI1 Dare Forrest	g	204174	137	4	20	Y	4
14	Kunitz-type trypsin inhibitor KTI1	g, f, e, c	214846	120	4	20	Y	4
15	Cystatin	c	219907	50	1	12.3	Y	1
16	Thioredoxin H	c	204598	79	1	12.5	N	1
17	Napin 2S albumin	b	225756	107	2	18.5	Y	4

[a] Results are pooled from one biological sample analyzed five times.

Table 3. Sequence of Primers Used to Amplify XP Genes

protein	TC number	forward primer	reverse primer
Beta amylase	225462	CACTTCCGACAGTAACATGC	CCAACAGTTAGATATTCCTTGTTC
β-1,3 Glucanase	214925	GGCTAAGTATCATTCAAGTGGG	AGGCACTAGAAATTGTGCAAATG
β-glucosidase	203307	GTTGGGTTGGATTCATACAGAT	CAATGTAGGGCTCGGTGG
γ-Glutamyl hydrolase	214792	GCCAAACGACAGCGTTCTG	CACCAGTGAAGAGCACTCC
Lipase hydrolase	226564	GCTAATTGCATGCAACATTGTG	CAGATACTATGACTCTGTGATTTG
Dehydration/elicitor induced	215094	GCACCTGCAACACTGTCTC	CCCAGTGGAGGTGACCTG
Blue copper protein	204673	CTCTGGGAAGAGGCAGTG	CCCAGACTCGCAGTGACC
Invertase inhibitor	229450	CATTAGCTCTTATCTTCTACAGTAC	CTGCAGAGACGCAACGG
Kunitz trypsin inhibitor Dare and Forrest	204174	GAGGCACCATCTTCTTTGCTC	CCATCCATTGCATCTTTGTTCTCAC
Kunitz trypsin inhibitor	215770	GAGCCCTACCATTTTCATT	GAAGCACTAGATTATTCTG
Aspartyl protease	204568	GAAAACCACACTCTTCTCACTC	ACAGGCAGTGCAAGGGATC
Lipid transfer protein	215570	GGAGGGTTGTACTTATTGTGTG	GGCAGAGTGTAAGGTCCAC
Lipid transfer protein	215570	GGTTGTACTTATTGTGTGAAGTTGC	CAAGGCAGAGTGTAAGGTCCAC
Lipid transfer protein	225077	GAGGCCACTGATTCTCTTG	CCATCCATTGCATCTTTGTTCTCAC
Vegetative storage protein	225142	GTAGCATGGCAATGCCATGC	CCCATTCATCATACAAGGTCG
Vegetative storage protein	225143	GTAGCATGGCCATGCCATG	CCTCCACCCCATATCCATG
Peroxidase	204153	GGGTAGGATTAATCTCCTAC	CCAAGGCCAGAATATATGCAC
Lipid transfer protein (aerial tissue predominant expression)	225088	GCTAGCCTCAAGGTTGCAT	CCATCCATTGCATCTTTGTTCTCAC

the two β1,3 endoglucanase did not share the same TC number. The Dare and Forest KTI was a dominant protein of seed exudate but not in the xylem.

Examination of the Expression Pattern of Sap Protein Genes. To examine the possibility of long-distance movement of XPs (from roots-to-shoots) and to determine if *B. japonicum* inoculation affects the expression of XP genes, we generated unique primers (Table 3) to amplify mRNA and DNA corresponding to 16 of the genes encoding secreted proteins identified in Table 1. We amplified RNA isolated from several tissues (stem, hypocotyl, leaf, and root) using semiquantitative RT-PCR 10 days post *B. japonicum* inoculation or mock inoculation. *B. japonicum* inoculation did not reproducibly affect the expression of any of the genes assayed (e.g., Figure 3A−C for KTI, Lipase and LTP, respectively).

Of the 17 genes examined, four genes appeared to possess greater expression in root than in aerial tissues (TC215570, LTP; TC214925, β 1,3 endoglucanase; TC204568 aspartyl protease; and TC229450, invertase inhibitor). To confirm the root expression bias of this LTP, its expression pattern was compared to that of another LTP (TC225088), known to be predominantly expressed in the aerial tissues (Figure 3, panel D vs E). The results showed that the root-predominant major xylem sap LTP was indeed expressed preferentially in roots and the aerial tissue LTP (TC225088) was expressed predominantly in aerial tissues. By contrast, Figure 3F shows the expression pattern of blue copper protein, which is strong and consistent in all tissues examined. Finally, to ensure that the root-predominant LTP was not induced by the wound made during sap collection, we compared the expression of the root-predominant sap LTP and the predominant KTI (TC215770) in hypocotyl tissue at the time of shoot excision and 4 h after excision using semiquantitative RT PCR (data not presented). The results showed that wounding for 4 h did not change the expression of the two genes examined. This time point was selected because the vast majority of the sap examined resulted from collections within 2 h of shoot decapitation.

Discussion

Analysis of Soybean XPs Common to the Xylem Sap of Other Plants. Soybean XPs isolated from hypocotyl and epicotyl contain a discrete and stable set of secreted proteins that appear near identical to those in the stem apoplast but distinct from those secreted from imbibing seed. Only one of the five Kunitz trypsin inhibitors found in xylem sap and in seed exudate was common, and all other proteins were different. All major XPs and seed secreted proteins possess N-terminal signal sequences, and there was no evidence of *B. japonicum* proteins in these preparations. The predominant XPs listed in Table 1 were Kunitz trypsin inhibitor (TC 215770), β-1,3 endoglucanase (TC 214925), γ glutamyl hydrolase (TC 214792), β-amylase (TC 225462), stem 31 kDa glycoprotein VspB (TC

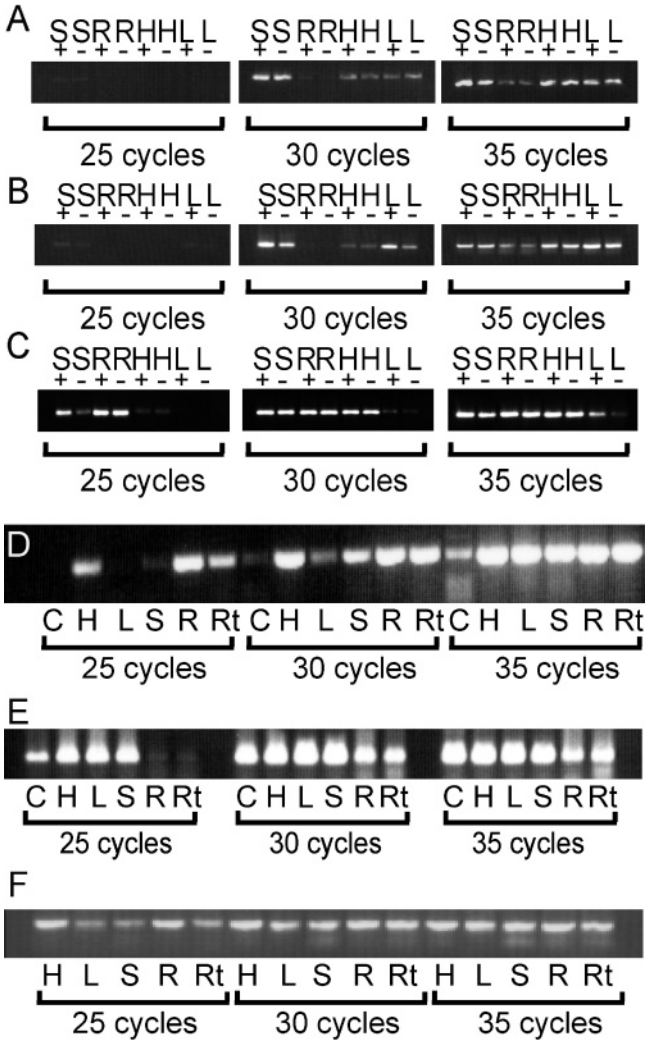

Figure 3. Semiquantitative RT-PCR of mRNA corresponding to XP and stem apoplast genes. (A−C) Amplification of products derived from inoculated (+) and uninoculated (-) plants corresponding to KTI, lipase, and LTP (TC215570). Amplification products of the expected sizes, 620, 380, and 360 bp, were generated that showed consistency in the presence or absence of *B. japonicum* inoculation. When DNA was used as a template, the amplification product for KTI and LTP was identical, whereas lipase generated a 600 bp product, suggesting the presence of an intron. The KTI and lipase genes were predominantly expressed in aerial tissues (shoots, hypocotyl, and leaves). The LTP was expressed predominantly in root tissue but was also present in the hypocotyl but not leaf. This was confirmed in panel D where other tissues (cotyledon and root tip) were examined also. Five to 10 more amplification cycles were required to amplify this LTP product from the other aerial tissues tested (C and D). (E) As a control, a LTP not found in xylem sap or stem apoplast fluid but which is known to be expressed in aerial tissue in soybean (TC225088) was amplified from the same RNA samples in panel D at the same time. Five to 10 more amplification cycles are required to generate similar levels of product from root tissues. This result supports the aerial tissue predominant expression pattern for this gene. The expression pattern of blue copper protein is shown in panel F. Its expression is strong (prominent bands resulted after 25 cycles) and found in all tissues examined. C, cotyledon; H, hypocotyl; L, leaf; S, stem; R, root; Rt root tip.

225143), stem 31 kDa glycoprotein Vsp25 (TC 225142), and lipid transfer protein (TC215570). The soybean XPs listed in Table 1 that are also found commonly in the xylem sap of a wide range of other plant species include lipid transfer proteins, peroxidases, aspartyl proteases, β 1,3 glucanases, β galactosidase, Kunitz trypsin inhibitors, arabinogalactan-proteins, lipases, invertases inhibitors, and blue copper proteins.[14−16,19] The major KTI was present in two isoforms on 2-D gels (Figure 1B) These results supports the finding that a conserved set of secreted proteins appear to serve an important basic role in maintaining xylem function in many plants.[14]

Analysis of "Soybean-Specific" XPs. γ-Glutamyl hydrolase, the two vegetative storage proteins, the dehydration stress/elicitor induced protein, and the Ppr-27-like (defense-related) protein (Table 1) appear to be more specific soybean XPs as we can find no report for these being XPs in other plants. Individual species of γ-glutamyl hydrolase (different to the ones identified here) have been found in the soybean leaf apoplast.[29,30] The vegetative storage glycoproteins are known to accumulate in soybean plants and are thought to provide temporary storage reserves.[31] The two vegetative storage glycoprotein subclasses were present in 2-D gels as multiple protein spots (possibly due to differential glycosylation patterns) and had distinctive isoelectric points (Figure 1B) and molecular masses. Alvarez et al.[15] found that Maize XPs are highly glycosylated and occur in multiple isoforms, and this could be a general attribute of secreted proteins and contribute to their resistance to proteolysis. The dehydration/elicitor-induced and Ppr-27 proteins appear to be defense-related, consistent with its EST expression pattern (see http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=soybean); however, our RT-PCR results and the EST expression patterns of the dehydration/elicitor-induced gene indicate it is expressed in a range of tissues without pathogen challenge.

Is There Long-Distance Movement of XPs from Roots to Shoots, and Is Any XP a Candidate for an AON Inducer? The major protein components of xylem sap were consistently found in the soybean lines examined irrespective of age, inoculation status, or time after inoculation. In addition, the protein contents remained stable for up to 28 h after decapitation as the proteins were found in the sap that resulted from root pressure alone. This result was supported by RT-PCR results that showed that the expression of the xylem sap genes was not affected by inoculation status, plant age, plant genotype, wounding, or time after inoculation. Most of the XP genes are expressed in several tissues, and this does not support the idea that a majority of XPs are transported long distances as reported in other systems,[16−18] but rather they are continuously produced and loaded into the xylem stream at multiple points. These results do not support any of the proteins being the root-derived, inoculation-dependent signal, which induces AON in shoots as the XPs of inoculated and uninoculated plants were indistinguishable. Therefore, none of the XPs identified in this study would be candidates for interacting with the LRR-RLK, NARK, which controls AON.

However, four of the XP genes examined were predominantly expressed in roots. The expression pattern of LTP gene (TC 215570) may be significant, since an LTP mutant in *Arabidopsis* has been shown to be defective in long-distance signaling (SAR[20]). In addition, the EST expression pattern of this gene also suggests a strong root and hypocotyl bias (see http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=soybean). The hydrophobic cleft(s) in LTP can bind potent lipophilic signals such as steroids, jasmonic acid (JA), and the JA intermediate, allele oxide.[32−38] Given that LTPs are known to

carry hydrophobic ligands, they could provide the basis of a long-distance shuttling system in plants; if so, we cannot exclude the possibility that this LTP is "loaded" with different ligands under different conditions, and we are currently exploring this possibility. To our knowledge, LTPs are not known to interact with LRR-RLKs.

Analysis of Seed Secreted Proteins. Soybean seeds secrete a distinctive set of proteins that include lipoxygenases, hydrolases, lectins, globulins, Kunitz trypsin inhibitors, an extracellular dermal glycoprotein (EDGP), and albumins. Lipoxygeneases, hydrolases, EDGP, and trypsin inhibitors are involved in plant defense responses,[29,39,40] and the LEXYL1 protein is involved in cell wall modification during fruit ripening.[41] Lipoxygenases and EDGP are also involved in the production of jasmonic acid and signal transduction, respectively.[40,41]

Protease Activity in the Secreted Fractions. There was evidence of proteolytic breakdown occurring in the proteins secreted from seed and in the xylem and apoplast fluids. For example, the basic 7S globulin precursor was found at several positions on 1-D gels in the seed exudate, and analysis of the parts of 1-D gels corresponding to a molecular weight of <10 kDa showed evidence of peptides derived from secreted proteins of higher molecular weights. Secreted proteases have been reported in the xylem fluid of several plants.[14,15] These proteases could be responsible for the slow degradation of the secreted proteins.

KTI and LTP: Proteins with Potential Signaling Ability. As discussed above, LTP could be a candidate for a water-soluble, long-distance signal carrier due to the affinity of its hydrophobic clefts to bind important signal molecules such as jasmonic acid and steroids.[34] Apart from these properties LTPs are also known to interact with cell surface receptors[33] and have direct antimicrobial activity.[37]

A Kunitz trypsin inhibitor has also been shown to have signaling ability in plant−microbe interactions in soybean. It acts as a "competency factor" for amplifying the extent of the activation of secondary metabolite defense pathways in response to fungal elicitors.[29] Protease inhibitors have roles in signaling processes in animals, and plant KTIs have 3-D conformational similarity to certain interleukins and fibroblast growth factor.[29] The xylem sap KTI and LTP species identified warrant further investigation.

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