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MOLECULAR BIOLOGY AND PHYSIOLOGY

The Characterization of Major Proteins Expressed in Roots of Four *Gossypium* Species

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ABSTRACT

Root proteins have not been examined extensively for cultivated and wild *Gossypium* species. This study identified unique cotton root proteins from *G. hirsutum, G. barbadense, G. arboreum,* and *G. longicalyx* in 2-D gels, which were then characterized by Q-TOF tandem mass-spectral sequencing. The subsequent *in silico* bioinformatics annotation of Q-TOF sequenced fragments from selected major spots revealed proteins that were associated with primary and secondary metabolism, defense and stress conditions, and growth and development functions. Most annotated proteins were common and shared among the species, but a few were unique to a species. The major constituents of root proteome included pathogenesis-related and latex proteins along with ubiquitously expressed proteins, such as 3-phosphoshikimate 1-carboxyvinyltransferase, NDP-kinase, and protease. Latex proteins, auxin-responsive proteins, actin, and annexin, which were annotated in this study, participate in cotton root elongation, growth, and regeneration processes. The differences in sequenced proteins of various cotton species were observed by spot intensity, concentration, and isoelectric points. This study was undertaken to document expressed proteins observed in the root that should be useful as a preliminary inventory of root proteome in different cotton species.

Roots function as the interface with the edaphic environment. Understanding roots at functional and cellular levels is critical to both rhizosphere ecology and plant production. Roots are highly plastic and are able to adapt developmentally and physiologically to changing environmental conditions, including disease infection and feeding by nematodes. Cotton roots are typical of herbaceous dicotyledonous species (Oosterhuis and Jernstedt, 1999). The proteomic studies of plant roots have elucidated root tissue differentiation and development in response to internal growth regulators as well as environmental signals (Song et al., 2007). *Gossypium* root proteins have been examined mainly from a pathogenesis perspective in response to infestations of *Meloidogyne incognita* (Kofoid and White) (Callahan et al., 1997; Zhang et al., 2002), black root rot *Thielaviopsis basicola* (Berk. & Broome) Ferraris (Coumans et al., 2009), and seedling blight *Rhizoctonia solani* Kühn (Chernin et al., 1997). An initial survey of benchmark proteins in cotton roots is a necessary first step toward understanding the mechanism of molecular responses against one or more pathogens or abiotic stresses.

There are 50 species recognized in the *Gossypium* genus, and cultivated species include two diploids *Gossypium arboreum* L. and *G. herbaceum* L. as well as two allotetraploids *G. hirsutum* L. and *G. barbadense* L. Of these, all are wild species except for two diploids (Kohel and Lewis, 1984). Wild diploid species are subdivided into three geographical groups: Australian, American, and Afro-Arabian. Eight diploid genome groups exist, designated with genomes A, B, C, D, E, F, G, and K (Percival et al., 1999). Although all diploid species share the same chromosome number (2n = 26), they exhibit more than a threefold variation in DNA content per genome. Our laboratory is embarking on a long-term effort to identify cotton genes, proteins, or regulatory mechanisms especially in wild cotton species that impart resistance to the reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira). This preliminary inventory was undertaken to observe any notable differences in the profile of the most abundant root proteins of resistant and susceptible *Gossypium* species.

The root proteome has been a topic of intense scrutiny. Initial studies focused on the *Arabidopsis* expression profiling (Birnbaum et al., 2003) based on

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mRNA of GFP-expressing root cells analyzed by microarrays to report localization of thousands of genes. Similarly in maize seedling roots, the Serial Analysis of Gene Expression (SAGE) defined the relative abundance of thousands of transcripts. Less than 5% of the most abundant transcripts were shared between maize and *Arabidopsis* (Poroyko et al., 2005). These studies are excellent for the global view of mRNA expression, but post-transcriptional regulatory mechanisms might reduce the correlation between mRNA and protein abundance. Therefore, in this study we attempted to determine the protein profile that differs between diploid and allotetraploid *Gossypium* species with varying response to reniform nematodes from being highly susceptible *G. hirsutum* to highly resistant *G. longicalyx* J.B.Hutch. & B.J.S.Lee species.

Our goal was to study cotton root proteins in cultivated allotetraploid and wild diploid cotton species and compare proteome profiles in each species. The roots of *G. arboreum* and *G. longicalyx* were included because of their known resistance to the reniform nematode *Rotylenchulus reniformis* (Avila et al., 2003), and compared them with the root proteins for the two major cultivated tetraploid cotton species *G. hirsutum* and *G. barbadense*. Our preliminary inventory of root proteome profiles of these species included important and distinctive metabolic proteins that are necessary for root growth and development, and those proteins responsive to naturally occurring pathogens.

MATERIALS AND METHODS

Plant Materials. Two cultivated cotton species—*G. hirsutum* (TM-1,Texas Marker-1, $[AD]_1$-genome) and *G. barbadense* (Pima 3-79, $[AD]_2$-genome)—as well as two wild cotton species—*G. arboreum* (accession #2-160, A_2-genome) and *G. longicalyx* (F-genome)—were used in this experiment. Seeds were obtained from the Cotton Germplasm Unit of USDA/ARS at College Station, TX, USA. Before germination, seeds of all cotton genotypes were surface sterilized in 0.6% sodium hypochlorite/0.1% SDS mixture for 15 min, then washed with 70% ethanol, rinsed 3 times with sterile water, and imbibed in sterile water overnight. Sterilized seeds were germinated in rolled sterile filter paper (Whatman, Inc. Florham Park, NJ 1003320), wetted by 1:10 diluted MS medium, and covered with aluminum foil for 24 h in dark at 30 ºC. Filter papers were sterilized by soaking in bleach (10%, 15 min), then in 70% ethanol, and dried under UV lamp. Seed coats of *G. longicalyx* were scarified by nicking the seed coat with a scalpel before sterilization. Seedlings at the expanded cotyledon stage were transferred to 12.7-cm pots containing potting medium (Pro-mix, Quaker Town, PA.) and placed in a growth chamber (16 h light at 28 ºC and 8 h dark at 25 ºC). Plants were irrigated each week with 100 ppm N using 20-20-20 Peter's fertilizer (J.R. Peters, Allentown, PA). Plant tissues were harvested when the third leaf attained its full expansion in each species.

Protein Extraction. A phenol extraction method (Ferguson et al., 1996; Hurkman and Tanaka, 1986) was adapted to extract total protein from cotton root tissues. Fresh plant roots were frozen in liquid N_2, blended in a household blender, and then homogenized in mortar and pestle with 1:1 mixture of extraction buffer and water-saturated phenol. Root tissue (1 g) was extracted with 3 ml each of buffer-saturated phenol and extraction buffer (500 mM Tris-HCl pH 8.6, 0.4M KCl, 2 mM PMSF, 20 mM EDTA, 1% CHAPS or Triton X-100, and 150 mM DTT). The homogenate was centrifuged for 10 min at 6000 rpm/min at 20 ºC to separate aqueous and phenol phases. The aqueous phase and the pelleted material were discarded and the phenol phase was washed twice with an equal volume of extraction buffer and centrifuged to separate the phenol and water phases. Proteins from the phenol phase were precipitated by adding cool (-20 ºC) methanol, containing 0.1M ammonium acetate in the proportion of 1:5 (per ml protein extract: methanol). The protein pellet was collected by centrifugation at 4000 rpm (Type 28 rotor, 4 ºC; Beckman, Fullerton, CA) for 15 min. Precipitated protein pellet was washed with cool acetone, containing 0.07% of 2-mercaptoethanol and dried in a SpeedVac rotary evaporator (Thermo Scientific, Waltham, MA) or in a vacuum desiccator and kept at -80 ºC.

Protein Determination. A solid-phase modification of the Bradford method (Said-Fernandez et al., 1990) was utilized for protein quantification. Aliquots of root sample fraction and standard protein (bovine serum albumin) were spotted on filter paper segments fixed by 20% TCA in acetone for 2 to 3 min and then stained by 0.1% Coomassie G or R at standard polyacrylamide gel (PAG) staining conditions for 15 min. The filter was de-stained by washing in 10% ethanol and 7.5% acetic acid. Coomassie-stained protein spots were cut and extracted in a solution of 1 ml of 1% SDS, 40% ethanol, and 50 mM Tris-HCl with pH 8.8. For fast extraction, tubes

were heated at 37–40 ºC. Protein concentration was determined by Bio-Rad SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA) at 600 nm.

Electrophoresis Procedures. Dry protein extracts were dissolved in a buffer containing 8M urea, 4% CHAPS or Triton X-100, 0.1% ampholytes with appropriate pH (pH range 3–10, linear), and adding a trace of bromophenol blue. Sample solution was centrifuged at 4000 rpm (Type 28 rotor, 25 °C) to separate the pellet. Protein concentration was determined and the sample diluted with rehydration buffer (6M urea, 1% CHAPS), if required. Isoelectric focusing (IEF) of protein extracts was performed using a Bio-Rad PROTEAN IEF Cell with 7-cm ReadyStrip IPG gels with a linear pH range of 3–10. Rehydration was passively carried out overnight at room temperature. IEF run was initiated at 250 V, 20 min; pre-IEF with linear ramp from 250 to 4000 V, 2 h; and actual IEF at 4000 V, for filling 10,000 V-h; total time was approximately 5 h. Gels were kept at 500 V after IEF and before the next treatment to prevent band diffusion. After completing the IEF run, IPG strips were re-equilibrated for the second dimension and stored at -20 ºC to minimize diffusion of protein bands. Two-dimensional electrophoresis (2-D) of samples was run utilizing Bio-Rad Dodeca Cell apparatus with gel sizes of 1.5 mm x 20 cm x 20 cm. Procedures for IPG strip re-equilibration, reduction of protein S-S bonds, alkylation reactions, electrophoresis, and gel staining were performed according to protocols as described by Simpson (2003). Polyacrylamide gradient concentration gels (7–15%) were made using Bio-Rad Model 495 Gradient Former using Bio-Rad-ready monomer mixture (T = 40%, C = 2.6 for light monomer mixture, and T = 40%, C = 3.1% for heavy monomer mixture). Separations on the second dimension were carried out using the Laemmli SDS disc-buffer system (Laemmli, 1970). The concentration and pH of Tris-HCl, SDS, TEMED, and ammonium persulfate used in PAG gel preparations were according to manufacturer's protocol. Electrophoresis was carried out at 10 V/cm constant voltages. Gels were fixed in a mixture of 20% TCA (w/v), and 40% (v/v) ethanol for 20 to 30 min. Protein bands in PAG were stained in 0.1% Coomassie R-250 for 2 to 4 h (Simpson, 2003). De-staining was carried out by few changes of mixture: 7.5% (v/v) and 12.5% (v/v) ethanol. Stained gels were scanned using Alfa Imager (Bio-Rad), and a relative molecular mass (Mr) of bands was determined using version 4.0.1 Alfa Ease-FC™ software. Isoelectric points (pI) of protein spots were estimated according to the linear immobilized pH gradient on the IPG strips. Every IPG strip series was checked on a pH gradient by isoelectrofocusing of Bio-Rad IEF standards (cat. No 161-0310) before using for 2-D electrophoresis experiments. Deviation of pH value ± 0.15 was obtained (data not shown).

Spot Cutting and Protein Identification. Protein bands of interest from the Coomassie-stained 2-D gel were cut using a razor blade cleaned with 1% SDS. The excised gel bands were placed in a 0.5-ml microcentrifuge tube (pre-washed with 50% acetonitrile, containing 0.1% trifluoroacetic acid) and kept at room temperature. Tandem mass-spectral analysis was performed at the Mass Spectrometry Facility, University of Alabama at Birmingham, AL, with Waters/Micromass Q-TOF2 mass spectrometer (Micromass, Manchester, UK) using electro-spray ionization. Peptides from a 16 h (37 ºC) trypsin-digested sample were purified using ZipTips C_{18} (Millipore, Billerica, MA) to concentrate and desalt the samples. The samples were then analyzed by Liquid Chromatograph Mass Spectrometer detectors (LC/MS/MS). The tandem mass spectra were processed with the MassLynx MaxEnt3 software software (Micromass, Manchester, UK). Proteins were identified on the basis of a sequenced-fragments similarity search on the proteomics site ExPASy database (www.expasy.org).

RESULTS AND DISCUSSION

The similarity in physiological age of cotton plant species is critical in comparative proteome analysis. The days-after-transplanting criterion was not utilized and instead fully expanded third leaf as the physiological age criterion was employed. *G. hirsutum* and *G. barbadense* formed all elements of a root system within 4 to 5 d but *G. arboreum* and especially, *G. longicalyx*, formed a mature root system 8 to 10 d after germination in sterile conditions. Therefore, 2-D electrophoresis was undertaken on plant roots at the three-leaf stage.

The 2-D root protein profile was obtained using a pH range of 3–10 for isoelectric focusing and 7–15% polyacrylamide concentration gradient gel in the second dimension, this facilitated optimal separation of polypeptides differing in molecular mass (5–120 kDa) from the root extract. From *G. hirsutum* root proteins (Fig. 1) 14 relevant spots were successfully sequenced by Q-TOF-MS/MS (Table 1). The identified proteins were from several functional classes: four defence-related latex proteins (GH-6, GH-10, GH-12, GH-13) and one PR protein (GH-11); three stress-related pro-

teins, apoplastic gaiacol peroxidase (GH-1), dehydrin (GH-4), and glutathione-S-transferase (GH-8); two primary metabolism-associated proteins, carboxyvinyltransferase (GH-2) and NDP-kinase (GH-14); one secondary metabolism-associated dirigent-like protein (GH-5); and several proteins with diverse functions such as actin (GH-3), annexin (GH-7), and auxin-responsive protein (GH-9).

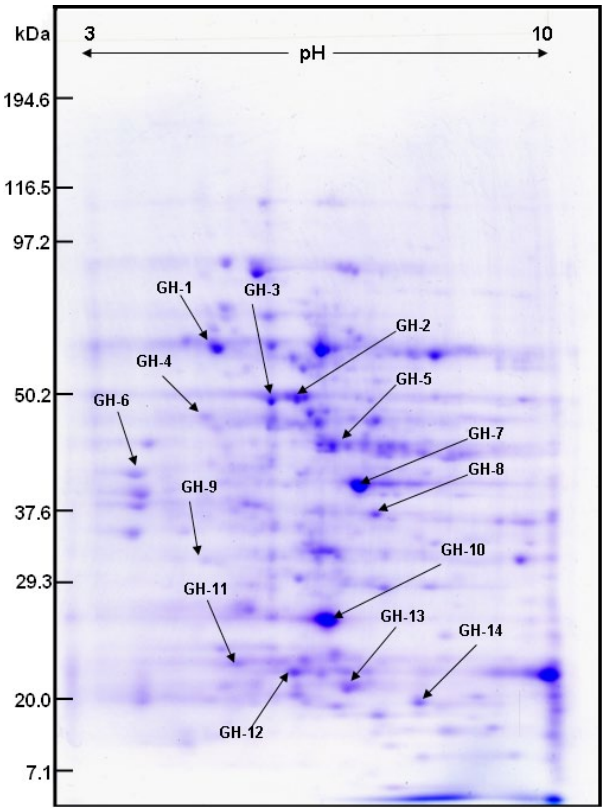

Figure 1. Two-dimensional electrophoresis of *G. hirsutum* (TM-1) plant root extract. Spots marked only if they are successfully Q-TOF MS/MS sequenced. Numbers in left vertical board of figure frames indicate molecular mass of pre-stained protein standards (Bio-Rad catalog no. 161-0318).

From *G. barbadense* root proteins (Fig. 2), 10 protein spots (Table 2) were sequenced that included two isozymes of *G. hirsutum* apoplastic anionic gaiacol peroxidase (GB-1, GB-2), *G. barbadense* dirigent-like protein (GB-3), plant cytochrome c oxidase (GB-4), a protein similar to the latex allergen-family protein from *Arabidopsis thaliana* (L.) Heynh (GB-5), which was also reported in our earlier work in developing cotton fibers (Wu et al., 2005). The remaining proteins were protease subunit alpha type 5 from soybean (GB-6), plant glyoxalase (GB-7), Cu-binding redox protein from wild tomato, *Solanum habrochaites* S.Knapp & D.M.Spooner (GB-8), and two other proteins that were identified as *G. hirsutum* PR protein class 10 (GB-9, GB-10). Of these, four proteins of *G. barbadense* were dissimilar to the *G. hirsutum* root proteome profile. Cytochrome c oxidase and Cu-binding redox protein (both stress-related proteins) might be present in all species but accumulated to levels adequate for isolation and sequencing only in *G. barbadense*. The other two proteins sequenced only in *G. barbadense* were protease subunit alpha type 5 and glyoxalase, which represents a class of primary metabolism-associated proteins. The corresponding spots might be in insufficient concentration in *G. hirsutum* 2-D gel to be analyzed by mass spectrometry. One additional feature of *G. barbadense* root proteome was the presence of a high concentration of apoplastic gaiacol peroxidase, represented by two isozymes with a close pI of 4.9 and 5.1. Gaiacol peroxidase functions as a member of class III peroxidases in detoxifying, in the production of reactive oxygen species, or as antimicrobial agents at the interface of the cell wall or plasma membrane (Mika and Lüthje, 2003). Abundant occurrence of dirigent-like protein that participates in lignification processes and synthesis of secondary metabolites such as gossypol was observed in *G. barbadense* (Liu et al., 2008). The dirigent protein also helps in guiding or aligning the stereochemistry of a compound synthesized by other enzymes. Originally they were discovered in lignan biosynthesis. Lignans are one of the major classes of phytoestrogens, which also include isoflavones and coumestans (Davin and Lewis, 2000).

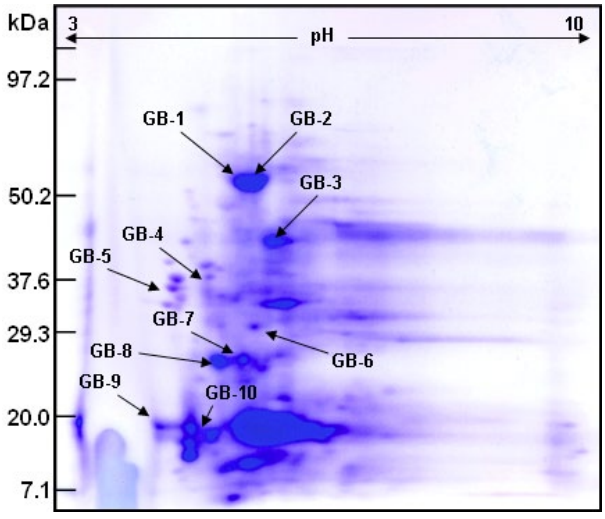

Figure 2. Two-dimensional electrophoresis of *G. barbadense* plant root extract. Informative part of criterion gel is presented.

Table 1. MS/MS sequencing of *G. hirsutum* root proteins and their identification

Spot no	Sequenced fragments	EMBL accession and protein name	Mr, kDa		pI	E-value
			Theor.	Exp.		
GH-1	sdqnlfstegadtieivnr; mgnispltgtegeir; yevidamk; aqcltftsr; igaslir	Q8RVP3 G. hirsutum apoplastic anionic gaiacol peroxidase	37.4	53.5	5.1	1e-05
GH-2	itgllegedvintgk; sfmfgglasgetr; laggedvadlr; vpmasaqvk; tptpityr	Q71LY8 Soybean 3-phosphoshikimate 1-carboxyvinyltransferase	47.6	49.8	6.1	5e-04
GH-3	vapeehpvlltevplnpk; fpsivgrpr	Q7XZK0 G. hirsutum actin	41.7	49.0	5.8	0.002
GH-4	papaaephhevssk; glfdfmgk	P31168 Arabidopsis thaliana and Q9SBI8 Hordeum vulgare dehydrin	29.9	44.8	4.9	1.4
GH-5	qyysdtlpyhpqppk; pyhpqppk	Q4U1X1 G. barbadense dirigent-like	18.6	42.5	6.7	0.01
GH-6	levasvqtalheek	Q8L5H5 Manihot esculenta allergenic related or Q6XQ13 M. esculenta glu-rich	18.9	39.4	3.9	1.4
GH-7	sleedvahhttgdfhk; liadeyqr; pttvpsvsedceqlrk; yegeevnmnlak; tyaetygedllk; adpkdeflallr; aldkelsndfer; lllvlaghven; aysdddvir; sanqllhar	O82090 G. hirsutum fiber annexin	36.0	38.2	7.0	0.003
GH-8	plnmatgehk; vldvyear	Q96266 A. thaliana glutathione-S-transferase	29.2	37.0	7.3	0.32
GH-9	gsdaiglapr; dlstalek; pqapaak	P93830 A. thaliana auxin responsive	25.3	30.4	4.9	0.022
GH-10	vqgcdlhegefgtpgvvicwr; gsivhwtldyek; mlegdlmeeyk; sfvitiqtspk; vevmdhekk	Q7X9S3 G. barbadense Putative major latex-like	14.6	24.7	6.5	0.005
GH-11	aftveapkvwptaapnavk; isyenkfeaaagggsick; gvvtydyentspvapar; fytvgdnvitedeik; infveglpfqymk	Q9FUI6 G. hirsutum PR protein class 10 or Q6Q4B4 G. barbadense PR-10-12	17.2	21.2	5.3	3e-20
GH-12	dpdknlvtfr; pdknlvtfr	B3RFK7 G. hirsutum PR bet vI	17.6	20.7	6.1	0.031
GH-13	akevveavdpdknlvtfr; evveavdpdknlvtfr; dpdknlvtfr; pdknlvtfr	B3RFK7 G. hirsutum PR bet vI	17.6	20.1	6.9	5e-05
GH-14	meqtfimikpdgvqr; iigatnpaesapgtir; eqtfimikpdgvqr	Q6L8H5 Codonopsis lanceolata nucleotide diphosphate kinase	16.1	19.8	7.8	3e-09

Table 2. MS/MS sequencing of *G. barbadense* root proteins and their identification

Spot no	Sequenced fragments	EMBL accession and protein name	Mr, kDa		pI	E-value
			Theor.	Exp.		
GB-1	sdqnlfstegadtieivnr; mgnispltgtegeir; gyevidamk; igaslir	Q8RVP3 *G. hirsutum* apoplastic anionic gaiacol peroxidase	37.4	53.5	4.9	3e-13
GB-2	sdqnlfstegadtieivnr; mgnispltgtegeir; gyevidamk; aqcltftsr; igaslir	Q8RVP3 *G. hirsutum* apoplastic anionic gaiacol peroxidase	37.4	53.5	5.1	3e-13
GB-3	gdpglavvggr; aglavvggr	Q4U1X1 & Q6Q4B7 *G. barbadense* dirigent-like	18.6	42.5	5.3	0.01
GB-4	letapadfrfpttnqtr	Q9SXV0 *Oryza sativa* Cytochrome c oxidase	18.9	38.2	4.6	6e-08
GB-5	vveavdpdknlvtfr; viegdllmeyk	B3RFK7 *G. hirsutum* PR Bet vI subunits	17.6	35.5	4.0	9e-15
GB-6	lfqveyaleaik; gvntfspegr; lgstaiglk; evvlavek; itspllepssvek; vtpnnvdiak	A9PE34 *Populus trichocarpa* proteasome subunit alpha type	26.0	30.2	5.2	6e-14
GB-7	gyimqqtmfr; fqnlgvefvk; imqqtmfr	Q8H0V3 *Arabidopsis thaliana* and other plants glyoxalase	20.8	24.7	5.0	0.002
GB-8	vvevndelsgspak; sdydncntgnalk	Q4KQZ4 *Solanum habrochaites* Cu-binding redox protein	29.4	24.5	4.7	0.04
GB-9	isyenkfeaaagggsick; gvvtydyentspvapar; fytvgdnvitedeik; sieveanpssgsivk; vwptaapnavk; infveglpfqymk	Q6Q4B4 *G. barbadense* PR-10-12	17.2	19.8	3.9	8e-15
GB-10	gvvtydyestspvapsr; fytvgdnvitedeik; sieveanpssgsivk; vwptaapnavk; infveglpfqymk; faaagggsick	Q5Y366 *G. klotzschianum* or Q6Q4B4 *G. barbadense* PR protein	17.2	19.8	4.5	7e-22

From *G. arboreum* root proteome (Fig. 3), nine proteins were identified (Table 3): two isozymes of apoplastic anionic gaiacol peroxidase (GA-1, GA-2), dirigent-like protein (GA-3), annexin (GA-4), glutathione transferase (GA-5), three proteins identical to PR protein class 10 (GA-6, GA-7, GA-8), and a plant ubiquitin. There were additional corresponding gel spots in 2-D gels of the other species, but they occurred in low concentrations that were not adequate for mass spectrometric analysis. Ubiquitin was the only unique protein identified in *G. arboreum.* The remaining proteins were similar to *G. hirsutum* and *G. barbadense*.

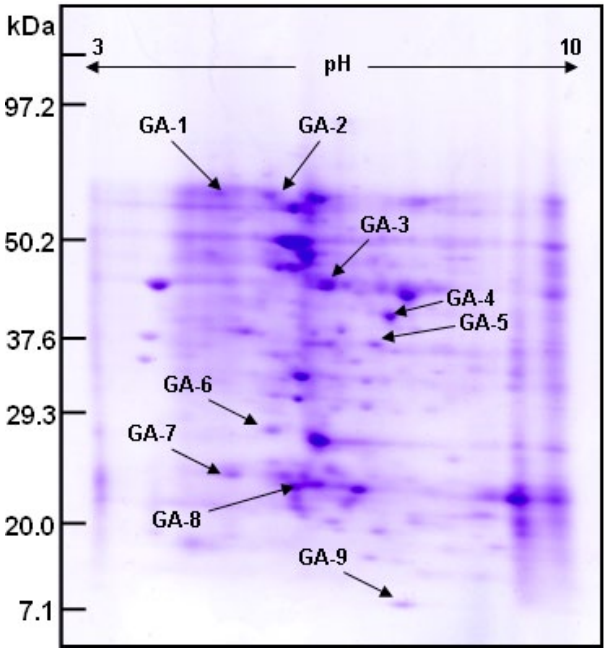

Figure 3. Two-dimensional electrophoresis of *G. arboreum* plant root extract. Informative part of criterion gel is presented.

In *G. longicalyx,* five spots were successfully sequenced (Fig. 4 and Table 4). They were apoplastic anionic gaiacol peroxidase (GL-1), soybean phosphoshikimate carboxyvinyltransferase (GL-2), actin (GL-3), putative major latex-like protein (GL-4), and PR protein class 10 (GL-5). These proteins were also common in *G. hirsutum*, *G. barbadense,* and *G. arboreum* root proteome. Phosphoshikimate carboxyvinyltransferase is the sixth enzyme of the shikimate pathway that leads to the biosynthesis of aromatic amino acids and secondary metabolites. It catalyzes the transfer of the intact 1-carboxyvinyl moiety of phosphoenolpyruvate to the 3'-hydroxyl group of the glucosamine moiety of UDP–(2')-N-acetylglucosamine with the concomitant release of inorganic phosphate (Wanke and Amrhein, 2005). Actin proteins that were identified here are ubiquitous in plants and are critical to cell division and expansion. Multitudes of accessory proteins are responsible for specifying whether cellular actin exists in the filamentous (F-actin) monomeric or globular form (Schenkel et al., 2008). The two diploid cotton species in our samples have known resistance to reniform nematodes; however, no proteins unique to these species were identified in this study.

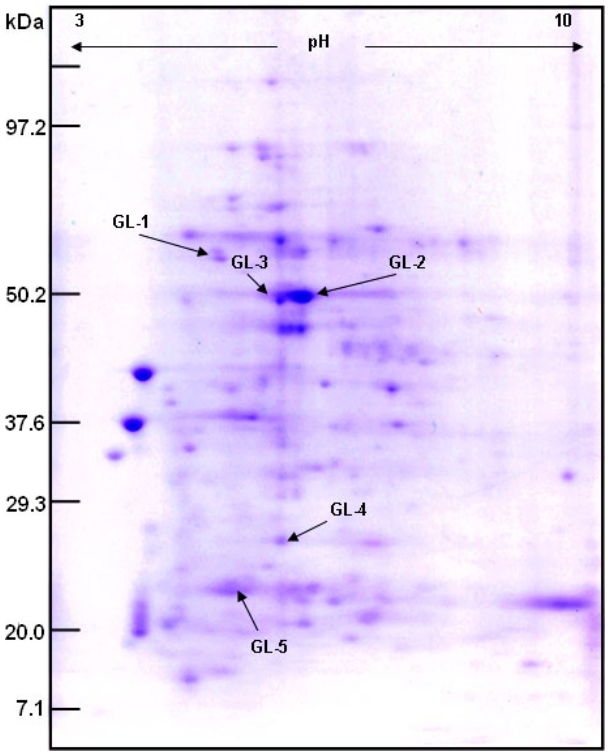

Figure 4. Two-dimensional electrophoresis of *G. longicalyx* plant root extract. Informative part of criterion gel is presented.

Cotton root protein profile has been characterized recently following root-rot fungus *Thielaviopsis basicola* infestation (Coumans et al., 2009). The expression of 32% of all sequenced root proteins was repressed, 10% sequenced root proteins were induced, and the remainder was constitutively expressed. The majority of these belonged to known PR-protein family. PR-protein presence is also observed during *Fusarium oxysporum* Schltdl. fungus-infested cotton roots (Dowd et al., 2004). The focus of their work was primarily on *G. hirsutum* response to the root-rot fungus, whereas our study used direct extraction of uninfected roots from four divergent *Gossypium* species. In our experiments, we found a number of spots in 2-D gels of cotton root proteins with a molecular mass less than 25 kDa. Such se-

quenced proteins in four *Gossypium* species were identified as PR or latex proteins. Some of these plant-defense proteins have steroid-binding properties (Liu and Ekromoddoullah, 2006). These protein groups are organ specific because of their putative biological functions in the root. The proteome study of developing cotton fiber (Yang et al., 2008) demonstrated the absence of both PR and latex proteins. Our results revealed minor difference for PR-protein content among cotton species. At least five rice PR-10 proteins have been characterized, and they are induced in roots by both biotic and abiotic stresses (Kim et al., 2008).

The group of redox enzymes and electron-carrier proteins (such as peroxidise-family isozymes, cytochrome c oxidase, Cu-binding redox) are integral to root metabolism and showed differences among them. Apoplastic gaiacol peroxidase was first sequenced from cotton cotyledons (Delannoy et al., 2003); however, it is absent in developing fibers where catalase isozyme was found as a peroxide homeostasis enzyme (Yang et al., 2008). We also observed high concentrations of apoplastic gaiacol peroxidase and their two isoforms in *G. arboreum* and *G. barbadense*. Apoplastic gaiacol peroxidase seems to be critical for cotton root peroxidase homeostasis and a prerequisite for lignification (Agudelo et al., 2005) and synthesis of terpenoids (Liu et al., 2008) during normal root development. Cytochrome c oxidase and Cu-binding redox protein were only found in root extract of *G. barbadense* and their concentration in the other species was insufficient for sequencing.

The dirigent-like protein in the roots of *G. hirsutum, G. barbadense,* and *G. arboreum* are responsible for stereospecific synthesis of lignin, gossypol, and its derivatives. Annexin, a calcium-dependent phospholipid binding protein family member was found in the root of *G. hirsutum* and *G. arboreum*, which is responsible for membrane functionality and cell growth regulation (Shin and Brown, 1999). In the corresponding spot of 2-D gels of *G. barbadense*, its quantity was insufficient for sequencing.

Actin, essential for motility and cell elongation was found in the roots of *G. hirsutum* and *G. longicalyx* (Song and Allen, 1997). Annexin and actin are abundant in cotton fiber proteome (Yang et al., 2008). In addition to these proteins, dehydrins, induced by dehydration, cold/heat stress, and abscisic acid (Kiyosue et al., 1994) were observed in root proteome of only *G. hirsutum*. It is a stress-response protein and acts as an intracellular stabilizer (Campbell and Close, 2008). The auxin-responsive protein (Kim et al., 1997) detected in *G. hirsutum* is essential for meristematic development and root hair formation (Ridge and Karsumi, 2002). Corresponding spots in other cotton species were in insufficient quantities for LC/MS/MS characterization.

Table 3. MS/MS sequencing of *G. arboreum* root proteins and their identification

Spot no	Sequenced fragments	EMBL accession and protein name	Mr, kDa		pI	E-value
			Theor.	Exp.		
GA-1	ptpdgfdnnyftnlqvnr; mgnispltgtegeir; gyevidamk; igaslir	Q8RVP3 *G. hirsutum* apoplastic gaiacol peroxidase	37.4	53.5	5.0	2e-11
GA-2	mnispltgtegeir; gyevidamk; aqcltftsr; igaslir	Q8RVP3 *G. hirsutum* apoplastic gaiacol peroxidase	37.4	53.5	5.5	8e-05
GA-3	qysdtlpyhpqppk; geqglavvggr; pyhpqppk	Q4U1X1 & Q6Q4B7 *G. barbadense* dirigent-like	18.6	42.5	6.2	9e-04
GA-4	seedvahhttgdfhk; pttvpsvsedceqlrk; yegeevnmnlak; tyaetygedllk; adpkdeflallr; aldkelsndfer; aysdddvir; sanqllhar; liadeyqr	O82090 *G. hirsutum* fiber annexin	36.0	38.5	7.4	6e-13
GA-5	pnmatgehk; vldvyear	Q96266 *A. thaliana* glutathione S transferase	29.0	37.0	7.1	0.32
GA-6	ftvgdnvitedeik; infveglpfqymk; vwptaapnavk	Q9FUI6 *G. hirsutum* PR protein class 10	17.2	27.0	5.6	0.05
GA-7	atveapkvwptaapnavk; isyenkfeaaagggsick; gvvtydyentspvapar; fytvgdnvitedeik; infveglpfqymk	Q5Y371 *G. herbaceum* PR protein	12.1	22.5	5.0	8e-04
GA-8	gvtydyentspvapar; fytvgdnvitedeik; infveglpfqymk; feaaagggsick	Q9FUI6 *G. hirsutum* PR protein class 10	17.2	21.0	5.8	3e-14
GA-9	ttlevessdtidnvk; iqdkegippdqqr; tladyniqk; estlhlvlr; lifagk	P69325 Soybean and other plants ubiquitin	8.5	8.5	7.6	2e-20

Table 4. MS/MS sequencing of *G. longicalyx* root proteins and their identification

Spot no	Sequenced fragments	EMBL accession and protein name	Mr, kDa		pI	E-value
			Teor.	Exp.		
GL-1	pttpdgfdnnyftnlqvnr; mgnispltgtegeir; gyevidamk; aqcltftsr; igaslir	Q8RVP3 *G. hirsutum* apoplastic anionic gaiacol peroxidase	37.4	53.5	5.1	2e-11
GL-2	itgllegedvintgk; sfmfgglasgetr; laggedvadlr; tptpityr	Q71LY8 soybean phosphoshikimate carboxyvinyltransferase	47.6	49.8	6.1	6e-06
GL-3	vapeehpvlltevplnpk; fpsivgrpr	Q7XZK8 O81221 *G. hirsutum* actin	41.7	49.0	5.8	2e-08
GL-4	vqgcdlhegefgtpgvvicwr; gsivhwtldyek; mlegdlmeeyk; sfvitiqtspk; vevmdhekk	Q7X9S3 *G. barbadense* Putative major latex-like	14.6	24.7	5.8	0.005
GL-5	gvvtydyentspvapar; vwptaapnavk; feaaagggsick	Q9FUI6 *G. hirsutum* PR protein class 10	17.2	21.2	5.3	4e-14

CONCLUSIONS

This study elucidated the constituents of root proteome in four cotton species—*G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. longicalyx*. Root-tissue extract demonstrated the presence of PR and latex proteins as more abundant in cotton root tissue. Along with ubiquitously expressed proteins, such as primarily metabolism enzymes (3-phosphoshikimate 1-carboxyvinyltransferase, NDP-kinase, and protease), additional proteins that are important for root homeostasis, defense, and synthesis of specific secondary metabolites were identified. Latex proteins, auxin-responsive proteins, actin, and annexin, annotated in this study, regulate cotton root elongation, growth, and regeneration processes. The observed differences in sequenced proteins of various cotton species were represented by spot intensity, concentration, and isoelectric points. Identification of novel proteins unique to *Gossypium* species provides an insight in root proteome signatures. Among the proteins analyzed by Q-TOF, few differences were observed between the cultivated tetraploid species and the two diploid species that are known to carry resistance to reniform nematodes, a menacing root-feeder of *Gossypium* species.

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