

RESEARCH ARTICLE

Proteomic analysis of the sea-island cotton roots infected by wilt pathogen *Verticillium dahliae*

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Verticillium wilt of cotton is a vascular disease mainly caused by the soil-born filamentous fungus *Verticillium dahliae*. To study the mechanisms associated with defense responses in wilt-resistant sea-island cotton (*Gossypium barbadense*) upon *V. dahliae* infection, a comparative proteomic analysis between infected and mock-inoculated roots of *G. barbadense* var. Hai 7124 (a cultivar showing resistance against *V. dahliae*) was performed by 2-DE combined with local EST database-assisted PMF and MS/MS analysis. A total of 51 upregulated and 17 downregulated proteins were identified, and these proteins are mainly involved in defense and stress responses, primary and secondary metabolisms, lipid transport, and cytoskeleton organization. Three novel clues regarding wilt resistance of *G. barbadense* are gained from this study. First, ethylene signaling was significantly activated in the cotton roots attacked by *V. dahliae* as shown by the elevated expression of ethylene biosynthesis and signaling components. Second, the Bet v 1 family proteins may play an important role in the defense reaction against Verticillium wilt. Third, wilt resistance may implicate the redirection of carbohydrate flux from glycolysis to pentose phosphate pathway (PPP). To our knowledge, this study is the first root proteomic analysis on cotton wilt resistance and provides important insights for establishing strategies to control this disease.

Received: January 31, 2011

Revised: June 10, 2011

Accepted: August 8, 2011



Keywords:

Differential 2-D SDS-PAGE / *Gossypium barbadense* / Mass spectrometry / Plant proteomics / *Verticillium dahliae*

1 Introduction

Verticillium wilt is a highly destructive vascular disease that leads to serious loss of lint yield and threatens most cotton-producing areas [1, 2]. Like many vascular wilt diseases,

Verticillium wilt of cotton is particularly notorious because the fungus pathogen, *Verticillium dahliae*, is extremely persistent in soil. Moreover, no efficient chemical control was available against this pathogen [3]. Despite the great efforts that have been made in generation of wilt-resistant cotton cultivars by traditional breeding, very few varieties are resistant to the wilt in *Gossypium hirsutum*, the main species of cotton currently cultivated in the world [4]. Understanding the molecular mechanisms and identifying the molecular components involved in the defense responses of the wilt-resistant cotton cultivars and subsequently generating transgenic cotton plants with enhanced wilt resistance is a promising approach to restrain this disease.

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Abbreviations: **ACO**, 1-aminocyclopropane-1-carboxylate oxidase; **ADF1**, actin depolymerizing factor 1; **Bet v 1**, *Betula verrucosa* 1; **dpi**, days postinoculation; **ET**, ethylene; **hpi**, hours postinoculation; **NCBI**, NCBI non-redundant; **PPP**, pentose phosphate pathway; **PR10**, pathogen-related protein 10; **qRT-PCR**, quantitative real-time PCR

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Colour Online: See the article online to view Fig. 1 in colour.

Many works have been done to investigate the defense mechanisms against *V. dahliae* infection in cotton. Through several physiological and biochemical studies [5–11], the involvement of phytoalexin (sesquiterpenes, phenolics, and flavonoid), proteases (GST, superoxidase dismutase, glucose oxidase (GO), and lipid transfer protein), and some other substances (soluble sugar denning and alanine) in defense response were demonstrated. Meanwhile, progress has been made in the identification of defense responsive genes via transcriptional analysis of the upland and sea-island cotton cultivars infected by *V. dahliae*. About 50 uni-genes encoding pathogen-related protein 10 (PR10), phenylalanine ammonia-lyase (PAL), 14-3-3-like proteins and other oxidative stress-related proteins, etc., were identified by these studies [12, 13]. In addition, several individual genes were functionally characterized and their roles in the defense response against *Verticillium* infection were revealed. These genes include those coding for catalytic enzymes involved in phytoalexin biosynthesis [14, 15], pathogenesis-associated proteins [16], phenylalanine ammonia lyase [17], oxidized proteins participating in pathogenic factor detoxification [18], chitinase and glucanase [19] and nonsymbiotic hemoglobin [20, 21]. Recently, a major latex protein gene (*GhMLP*) from *MLP* subclass in the *Betula verrucosa* 1 (Bet v 1) family [22]; thaumatin-like protein (*GbTLP1*), encoding a thaumatin-like protein [23]; and *HpaIXoo*, encoding a harpin protein [24], were also found to be associated with *V. dahliae* toxin response or *Verticillium* wilt resistance. Together, these data indicate that interaction between cotton and *V. dahliae* is a complex process that may involve various defense pathways.

It is well known that proteome analysis provides critical information of final executors in different biological processes [25]. Accumulating evidence indicates that the application of proteomics for global investigation of microbe–plant interaction can provide novel insights for the molecular mechanisms involved in the defense processes [26–28]. Recently, El-Bebany et al. [29] have successfully identified many important protein factors involved in pathogenicity of *V. dahliae* by proteomic analysis. In this study, we performed a comparative proteomic analysis on mock- and *V. dahliae*-inoculated roots at different time points using the sea-island cotton Hai 7124, a cultivar that has been frequently used as a resistance source for transcription analysis and genetic mapping [12, 30]. To our knowledge, this is the first proteomic study on cotton roots upon *V. dahliae* infection. The present data provide important information on the molecular mechanisms of *Verticillium* wilt resistance in cotton at protein level.

2 Materials and methods

2.1 Cotton growth, *V. dahliae* inoculation, and sample collection

The cultivar, Hai 7124 (*Verticillium* wilt-resistant sea-island cotton) was used for this study. Seeds were treated with

H₂SO₄ (98%) to remove the fuzz, and surface-sterilized by immersion in 70% ethanol for a few seconds followed by treatment in 0.1% w/v mercuric chloride for 10 min. A hydroponic method was applied for the growth of plants according to the method described by Qu et al. [20, 21].

The defoliating *V. dahliae* isolate V592 [31], originated from an infected cotton plant in Xinjiang district of China, was used for cotton infection. For conidial production, the fungus was grown on Czapek's medium containing 2 g NaNO₃, 1 g K₂HPO₄, 1 g MgSO₄·7H₂O, 1 g KCl, 2 mg FeSO₄·7H₂O, and 30 g/L sucrose at 28°C for 3–5 days. For inoculation, a conidial suspension (1 × 10⁸ conidia/mL) in deionized water was prepared. Seedlings were removed and roots were dip-inoculated without injure in either the *V. dahliae* conidia suspension or sterile water for 15 min. The seedlings were grown continuously under hydroponic conditions until sample collection. For protein extraction, seedlings of Hai 7124 were harvested at 1, 3, 5, and 7 days after mock- and *V. dahliae*-inoculation. For quantitative real-time PCR (qRT-PCR) analysis, seedlings of Hai 7124 were harvested at 12, 24, 36, 48, 60, 72, 84, and 96 h after mock- and *V. dahliae*-inoculation. The tap and lateral roots were cut, patted dry between paper towels, and then stored at –80°C until use.

2.2 Protein extraction

Cotton root proteins were extracted according to a reported method [28] with minor modification. Each sample (inoculated or mock-inoculated cotton roots from 15 to 25 seedlings) was collected and ground with 10% w/w PVPP and 10% w/w quartz sand in a mortar in liquid nitrogen. The ground powder was mixed with plant total protein extraction buffer (P1258, Applygen, Beijing, China) and then centrifuged (10 000 × g, 10 min) at 4°C. Protein crude extract (3 mL) was re-extracted according to a phenol extraction procedure derived from that of Hurkman and Tanaka [32]. In summary, 3 mL of extract was mixed with the extraction buffer (30% sucrose, 0.1 M Tris-HCl, pH 8.0, 2 mM PMSF, 1% DTT, 100 mM KCl, and 5 mM EDTA) and an equal volume of phenol saturated with Tris-HCl (pH 8.0) was then added. The mixture was vortexed for 2 min and centrifuged at 10 000 × g for 5 min. Proteins were precipitated from the phenolic phase with five volumes of 0.1 M ammonium acetate in methanol at –20°C overnight and pelleted by centrifugation at 10 000 × g for 30 min. The pellet was rinsed twice with ice-cold 0.1 M ammonium acetate in methanol, three times with ice-cold methanol, and once with ice-cold acetone/water (80/20 v/v). After air drying, the pellet was dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 2% v/v IPG buffer (pH 4–7). Protein concentration was determined using 2-D quant kit (GE Healthcare Life Science, NJ, USA). The dissolved proteins were immediately subjected to 2-DE for protein separation. Equal aliquots of samples at 1, 3, 5, and 7 days

after mock inoculation were pooled and used as the control for 2-DE and Western blot analyses.

2.3 2-DE and image analysis

Total protein extracts (1 mg each sample) in lysis buffer were adjusted to a final volume of 450 μ L with the rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 0.5% v/v IPG buffer (pH 4–7), and then loaded onto 24 cm IPG (pH 4–7) strips (GE Healthcare Life Science). The IPG strips were rehydrated for 12 h at 30 V. IEF was carried out on the IPGphor III system (GE Healthcare Life Science) at total voltage-hours of 80 kVh using the following conditions: 1 h at 100 V, 1 h at 300 V, 1 h at 500 V, 1 h at 1000 V, gradient to 4000 V in 1 h, gradient to 8000 V in 1 h, and finally 10 h at 8000 V. Prior to the second dimension analysis, the strips were incubated first in equilibration buffer containing 1% DTT and then in equilibration buffer containing 2.5% iodoacetamide. Proteins in the equilibrated strips were then separated in 12.5% denatured SDS polyacrylamide gels (SDS-PAGE). After electrophoresis, proteins were visualized by Coomassie Brilliant Blue staining (50% methanol, 0.15% CBB G-250, and 0.75% acetic acid) for 48 h on an orbital shaker. The stained gels were scanned using Image Scanner III scanner (GE Healthcare Life Science) and comparative analysis of the protein spots was performed with Image Master 2D Platinum software (version 6.0, GE Healthcare Life Science). Detection and matching of the protein spots were carried out by the software, followed by visual re-evaluation. The detected protein spots were quantified using the percent volume criterion and the spots that showed a change of more than 1.75-fold between mock-inoculated and inoculated samples were selected. Statistical significance was defined as p -value of <0.05 according to Student's two-tailed t -test for unpaired means.

2.4 MALDI-TOF MS and MS/MS analyses

Relevant protein spots from preparative gels were manually excised and subjected to in-gel digestion using trypsin. The peptide mixtures in 0.7 μ L of CHCA matrix solution (5 mg/mL CHCA in 50% ACN/0.1% TFA) were spotted onto a target plate. The air-dried and crystallized spots were analyzed on a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA, USA). All mass spectra were recorded in a reflector mode within a mass range of 900–3700 Da, using a laser operated at a 200 Hz repetition rate with wavelength of 355 nm. MS calibration was automatically performed by a peptide standard Kit (Applied Biosystems) containing des-Arg1-bradykinin ($m/z = 904.4675$), angiotensin I ($m/z = 1296.6851$), Glu1-fibrinopeptide B ($m/z = 1570.6774$), ACTH (1–17, $m/z = 2903.0867$), ACTH (18–39, $m/z = 2465.1989$)

and ACTH (7–38, $m/z = 3657.9294$). MS/MS calibration was performed by the MS/MS fragment peaks of Glu1-fibrinopeptide B. The MS/MS mass spectra were acquired by data-dependent acquisition method with the ten strongest precursors selected from one MS scan. All the MS/MS spectra resulted from accumulation of at least 3000 laser spots. The combined MS and MS/MS peak lists were searched by the GPS Explorer software version 3.5 (Applied Biosystems) against a *Gossypium* peptide sequence database created from *Gossypium* EST database [33]. Search parameters were as follows: trypsin digestion with one missed cleavage, fixed modification for carbamidomethylation of cysteine, variable modification for methionine oxidation, and mass tolerance of precursor ion and fragment ion at 0.2 Da for singly charged ions. In all protein identifications, peptide mass fingerprint or MS/MS, the probability scores were greater than the score fixed as significant with a p -value of <0.05 . Cross-species identification was performed by searching the MS and MS/MS data against the Swiss-Prot database and NCBI nonredundant (NCBI nr) database newly downloaded from National Center for Biotechnology Information (NCBI) on 28 May 2011. In addition, for the differential proteins only appeared in the inoculated samples, the MS and MS/MS data were also searched against the *V. dahliae* VdLs.17 protein sequence database downloaded from the website: http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiDownloads.html, in order to find the *verticillium* proteins in the cotton roots infected with *V. dahliae*.

2.5 Western blotting analysis

Ten microgram of proteins from cotton roots was separated in 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The filter was blocked overnight with 5% w/v milk powder in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and then incubated with the polyclonal antibodies raised against human HSP70 (1:1000; Biosynthesis biotechnology, Beijing, bs-0126R) or cotton actin depolymerizing factor 1 (ADF1, 1:1000; Wang et al., 2009) for 1 h at 37°C. After three washes, the membranes were incubated for 1 h with anti-rabbit IgG AP-conjugated antibody (1:5000) (Promega, Madison, WI, USA), and the protein signals were detected using a chemiluminescent kit (Promega).

2.6 qRT-PCR

Total RNA of cotton roots was extracted by plant RNA extraction kit (Biomed, Beijing, China). First-strand cDNA was synthesized from 4 μ g of total RNA using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Aliquots of the reverse transcription (RT) product were used for qRT-PCR. Cotton *histone 3* gene was used as an internal control. Sequences of the primers used

for qRT-PCR analysis are summarized in Supporting Information Table 1. qRT-PCR assays were conducted using SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) and DNA Engine Opticon 2 Real-Time PCR Detection System (MJ research). All reactions were performed in triplicates. Equal aliquots of reverse transcription products from mock-inoculation samples at different time points were pooled and used as the control for the analysis.

2.7 cDNA cloning and sequence analysis

The corresponding EST sequences of identified proteins were used to search the NCBI database. The obtained ESTs were assembled using SeqMan program (DNASTar, version 7.1.0, Madison, WI, USA) and the silicon-cloned cDNA sequences were served for primer design. RT-PCR experiments were conducted to amplify the cDNA fragments. The verified cDNA sequences including ORFs and 3'-UTRs were submitted to GenBank (*GbVdI2*: JF261111; *pCPR10-16*: JF261106; *MLP*: JF261109; *MLP-like*: JF261110; *pCPR10-12*: JF261107). Amino acid sequences of the Bet v 1 family proteins were predicted and aligned by using the program DNAMAN (version 6.0.3.99, Lynnon Biosoft). The phylogenetic tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method, and 1000 trials of bootstrap analyses were used to provide confidence estimates for phylogenetic tree topologies.

3 Results and discussion

3.1 Identification and functional classification of cotton proteins involved in the defense responses against *V. dahliae*

3.1.1 Establishment of inoculation system

The wilt-resistant sea-island cotton cultivar Hai 7124 and the *V. dahliae* defoliating strain V592 were used to study the defense mechanisms of the host in response to the pathogen attack. As the root is the first barrier against *V. dahliae* infection and the appropriate organ for analyzing plants' responses to the soil-born pathogens [26, 27], we chose this part of the cotton plant for the proteomic analysis. To facilitate the procedure of inoculation and sample collection, Hai 7124 plants were grown under hydroponic conditions as described previously [20, 21]. For fungus inoculation, we adopted the unimpaired method reported by Gao et al. who inoculated a susceptible cotton cultivar using a 1×10^7 conidia suspension of the *V. dahliae* V592 [31], the same fungus isolate as we used in this study. To ensure effective inoculation, we tested different concentrations (1×10^6 , 1×10^7 , and 1×10^8) of *V. dahliae* conidia suspensions for inoculation in the trial experiments. It turned out that

inoculation with 1×10^8 of *V. dahliae* conidia suspension caused observable weak symptoms on the roots at 5 days postinoculation (dpi) under our experimental conditions. This concentration of conidia suspension was used for subsequent inoculation experiments.

3.1.2 Protein separation and identification

Total soluble proteins at 1, 3, 5, and 7 dpi were extracted from inoculated cotton roots together with mock-inoculated control and analyzed by 2-DE in pH 4–7 and molecular weight between 15 and 150 kDa as shown in Fig. 1A. Protein patterns of mock-inoculated samples at 1, 3, 5, and 7 dpi were pre-evaluated by 2-DE to determine whether there existed expression difference. Since similar patterns were observed among these samples, equal aliquots of them were pooled and used as control for subsequent 2-DE gel electrophoresis. Approximately, 1000 spots were resolved from both the inoculated and the control samples. Three biological replicates and three technical replicates per biological replicate were conducted. The reproducible 2-DE protein patterns were obtained for all the biological replicates and technical replicates and were independent of the experimental variations between gels. Moreover, the between gel variation was lower than the biological variation with an average CV of $26.33 \pm 18.08\%$. Protein amounts of the identified spots on normalized gels were quantified with ImageMaster 2D Platinum software and the average intensity of the spots was measured. About 90 differentially expressed protein spots ($p < 0.05$) between mock and inoculated samples at least in two time points were detected by image analysis. To reduce errors due to technical and biological variations, we selected 70 spots showing more than 1.75-fold changes from differentially expressed protein spots ($p < 0.05$) for MS analysis.

The differential protein spots were excised from the gels, in-gel digested by trypsin, and then analyzed by MALDI-TOF MS and MS/MS. The obtained MS and MS/MS spectra were searched utilizing the GPS Explorer software version 3.5 (Applied Biosystems) against a newly established cotton peptide sequence database [33]. A total of 68 proteins were identified in the cotton database of which 51 were upregulated, whereas 17 were downregulated (Fig. 1A) in response to *V. dahliae* infection. As proteins only appeared in the inoculated samples may include the fungal proteins, the MS and MS/MS spectra were also searched against the Verticillium database. It turned out that two proteins (an L-arabinitol 4-dehydrogenase, and a predicted protein) had a Verticillium origin. As we were mainly focused on the differentially expressed proteins from cotton roots after *V. dahliae* attack, these two proteins from Verticillium origin were excluded in the subsequent experiments. It should be mentioned that a recent article by El-Bebany et al. [34] reported the identification of transcripts putatively produced by *V. dahliae* in response to elicitation with potato root

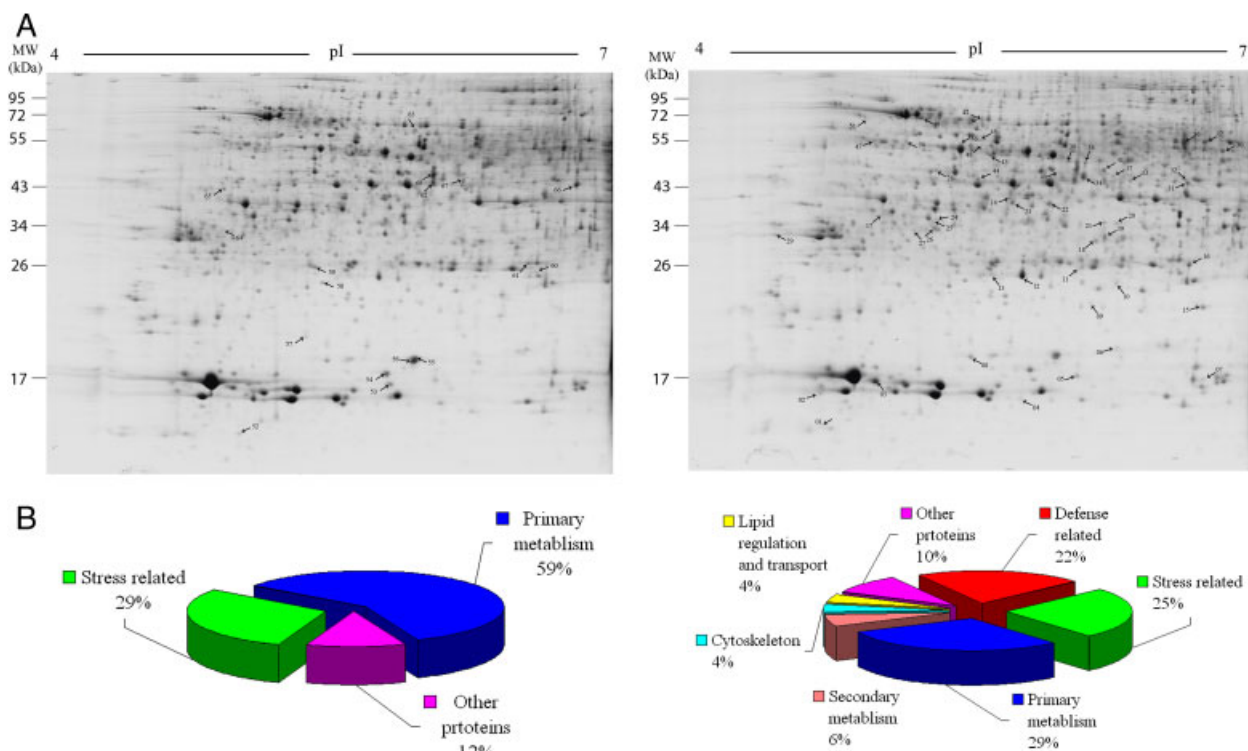


Figure 1. Representative 2-DE map of total proteins and functional categorization of the differently expressed proteins from mock-inoculated and inoculated cotton roots. (A) The downregulated proteins were labeled in the left image (mock inoculated control) and the upregulated proteins were labeled in the right image (inoculated at 5 dpi). These differentially expressed protein spots were submitted to MALDI-TOF PMF and MS/MS analyses. (B) Functional categorization of downregulated (left) or upregulated (right) proteins using the Gene Ontology Tool (<http://www.geneontology.org>) combined with manual analysis. The relative percentages of each category of proteins are shown.

extracts. The study deepens greatly our understanding of the pathogenesis of *V. dahliae* [34].

To further validate the results, the cross-species identification was also conducted by searching against the Swiss-Prot and NCBI database downloaded from NCBI. As shown in Supporting Information results, 24 and 35 proteins identified from the local *Gossypium* peptide sequence database were also successfully identified from Swiss-Prot and NCBI database, respectively (Supporting Information Tables 2 and 3). No extra protein was identified just by cross-species searching, demonstrating that a high quality of local *Gossypium* peptide sequence database was of great value in the study of *Gossypium* proteomics.

3.1.3 Functional classification of identified proteins

BLAST searches of individual identified cotton proteins were performed on NCBI based on their corresponding EST sequences and homologous proteins from cotton or other organisms were identified, these proteins were functionally categorized by the Gene Ontology Tool (<http://www.geneontology.org>) combined with manual analysis. Tables 1

and 2 summarize the accession numbers corresponding to protein spots shown in Fig. 1A, the protein names, the organism from which the protein has been identified, the values for experimental and theoretical pI, the MASCOT score, peptide matched, numbers of MS/MS fragments together with the sequence coverage, the molecular mass, and expression profile of individual protein spots as well as their putative functions. The differentially expressed proteins in the inoculated cotton roots were classified into seven categories, including defense response, stress response, primary metabolism, secondary metabolism, lipid synthesis and transport, cytoskeleton, and other proteins (Fig. 1B, Tables 1 and 2). For the upregulated proteins, defense-related (11 spots), stress-related (13 spots), and primary metabolism-related (15 spots) proteins are the major groups. The stress-related proteins (29%) and proteins involved in primary metabolism (59%) comprised the majority of the downregulated proteins.

Among these differentially expressed proteins, 12 have been identified previously by transcriptional studies. These included the 14-3-3a, two PR10s [12], and nine proteins in defense-related, stress-related, or primary metabolism categories [13]. In the later case, the authors also used the Hai 7124 as the experimental material. The consistence

Table 1. List of 51 upregulated proteins in response to *V. dahliae* infection in *G. barbadense*

Protein number	NCBI acc. no.	Protein name	Species	Theor.; Exp. Mol/Mass (kDa)/pI	MASCOT score	Peptides matched	No. of MS/MS fragments	Sequence coverage (%)	Molecular function	Expression pattern ^{a)}
Defense related										
02	CD486122	Novel Bet v 1 family protein	<i>G. hirsutum</i>	19.06/5.66; 15.86/4.68	134	10	2	66	Response to biotic stimuli	
03	CD486504	pCPR10-16	<i>G. hirsutum</i>	16.10/5.20; 16.87/4.98	426	18	5	91	Response to biotic stimuli	
04	CD486566	MLP	<i>G. hirsutum</i>	17.92/5.65; 15.76/5.77	108	9	1	68	Response to biotic stimuli	
05	ES809211	MLP-like protein	<i>G. hirsutum</i>	17.90/5.79; 17.50/6.07	298	18	3	67	Response to biotic stimuli	
26	DW519159	pCPR10-12	<i>G. hirsutum</i>	20.49/7.41; 33.59/5.27	277	14	6	49	Response to biotic stimuli	
29	DT465746	Family 19 chitinase	<i>G. hirsutum</i>	29.24/5.95; 31.46/4.41	259	15	5	45	Response to biotic stimuli	
01	CD485821	Meloidogyne-induced cotton 5	<i>G. hirsutum</i>	16.25/5.45; 13.55/4.70	167	12	5	50	Response to biotic stimuli	
36	DW519682	Mannose-specific lectin	<i>G. hirsutum</i>	32.72/6.49; 61.57/6.63	436	22	3	55	Response to biotic stimuli	
39	ES808410	ATP citrate lyase b-subunit	<i>G. hirsutum</i>	30.30/5.91; 53.36/5.96	73	8	1	28	Response to biotic stimuli	
41	ES808410	ATP citrate lyase b-subunit	<i>G. hirsutum</i>	30.30/5.91; 52.12/5.96	168	11	3	32	Response to biotic stimuli	
24	ES839366	Phenazine biosynthesis-like protein	<i>G. hirsutum</i>	34.35/5.13; 35.00/5.27	150	15	1	45	Response to biotic stimuli	
Stress related										
34	ES849354	Alcohol dehydrogenase 2a	<i>G. hirsutum</i>	33.64/5.67; 46.00/6.08	542	28	4	73	Response to oxidative stress	
38	ES849354	Alcohol dehydrogenase 2a	<i>G. hirsutum</i>	33.64/5.67; 47.02/6.09	663	31	5	74	Response to oxidative stress	
20	ES849354	Alcohol dehydrogenase 2a	<i>G. hirsutum</i>	33.64/5.67; 35.46/6.27	473	25	8	51	Response to oxidative stress	
27	ES849354	Alcohol dehydrogenase 2a	<i>G. hirsutum</i>	33.64/5.67; 50.71/6.08	542	31	8	64	Response to oxidative stress	
19	DT463387	Short-chain alcohol dehydrogenase	<i>G. hirsutum</i>	30.84/8.19; 32.85/6.20	397	21	7	43	Response to oxidative stress	
10	ES849598	BR2 benzoquinone reductase	<i>G. hirsutum</i>	23.10/5.97; 25.26/6.28	364	20	6	57	FMN binding; oxidoreductase	
16	DW500060	BR1 benzoquinone reductase	<i>G. hirsutum</i>	22.24/6.54; 27.86/6.67	649	22	8	56	FMN binding; oxidoreductase	
13	ES840989	GST	<i>G. hirsutum</i>	25.70/5.89; 61.70/6.57	145	12	4	45	Detoxification	
12	ES840989	GST	<i>G. hirsutum</i>	25.70/5.89; 25.74/5.76	610	23	7	57	Detoxification	
51	CO099920	Nucleoredoxin	<i>G. raimondii</i>	25.38/5.28; 72.00/4.88	178	17	3	53	Response to oxidative stress	
33	CO116573	Isovaleryl-CoA dehydrogenase	<i>G. raimondii</i>	21.69/5.43; 45.24/6.34	194	16	2	75	Response to oxidative stress	

Table 1. Continued

Protein number	NCBI acc. no.	Protein name	Species	Theor.; Exp. Mol.Mass (kDa)/pI	MASCOT score	Peptides matched	No. of MS/MS fragments	Sequence coverage (%)	Molecular function	Expression pattern ^{b)}
45	CO070834	Alanine aminotransferase	<i>G. raimondii</i>	22.55/7.55; 59.77/5.51	115	16	2	49	Detoxification	
25	ES816278	Annexin	<i>G. hirsutum</i>	37.00/7.79; 34.80/5.27	505	33	8	62	Vesicle transport	
Primary metabolism										
46	DT545135	ATP synthase β subunit 1	<i>G. hirsutum</i>	33.26/5.43; 57.66/5.40	838	29	6	73	ATP synthesis coupled protein transport	
48	CO130256	ATP synthase β subunit 1	<i>G. hirsutum</i>	30.35/5.62; 63.43/5.42	538	26	5	76	ATP synthesis coupled protein transport	
35	DW242450	ATP synthase α subunit	<i>G. hirsutum</i>	28.26/8.33; 62.19/6.74	274	11	3	35	ATP synthesis coupled protein transport	
15	DV850018	Vacuolar H ⁺ -ATPase subunit E	<i>G. hirsutum</i>	24.28/7.96; 23.27/6.75	205	20	2	65	Energy coupled proton transport	
37	CO090963	Transketolase	<i>G. raimondii</i>	29.62/5.67; 48.57/6.22	98	12	1	47	Enzyme of PPPs	
17	BE054627	Fructokinase	<i>G. arboreum</i>	25.25/5.28; 35.89/4.99	78	13	0	48	Enzyme of PPPs	
30	CO085233	6-Phosphogluconate dehydrogenase	<i>G. raimondii</i>	28.28/4.86; 60.00/6.84	396	20	4	66	Enzyme of PPPs	
31	DT567439	α Subunit of the pyruvate dehydrogenase	<i>G. hirsutum</i>	29.87/8.57; 44.87/6.65	174	16	5	44	Response to oxidative stress	
43	DT555398	Copper ion binding/phosphopyruvate hydratase	<i>G. hirsutum</i>	30.90/5.63; 52.56/5.57	283	21	4	72	Response to oxidative stress	
06	DW228443	Ribosomal protein	<i>G. hirsutum</i>	13.77/6.71; 19.63/6.29	197	13	3	60	Protein synthesis	
22	DT567326	Elongation factor 2	<i>G. hirsutum</i>	32.25/5.40; 39.15/5.90	220	24	4	74	Protein synthesis	
23	CO124104	Elongation factor 2	<i>G. raimondii</i>	31.11/5.31; 39.20/5.70	329	27	4	68	Protein synthesis	
14	ES850809	Elongation factor 2	<i>G. hirsutum</i>	35.15/6.38; 40.12/5.68	252	22	7	49	Protein synthesis	
44	DW508093	Putative elongation factor τ	<i>G. hirsutum</i>	15.80/9.43; 51.63/5.53	157	4	1	18	Protein synthesis	
07	EV483951	Eukaryotic translation initiation factor 5A-1	<i>G. hirsutum</i>	18.13/5.78; 17.59/6.76	230	13	3	71	Protein synthesis	
Secondary metabolism										
11	CO074418	Isopentenyl diphosphate isomerase	<i>G. raimondii</i>	23.19/6.62; 26.68/6.07	147	19	2	74	Isoprenoid biosynthesis process	
21	DT558026	1-Aminocyclopropane-1-carboxylate	<i>G. hirsutum</i>	29.74/5.53; 32.78/6.10	237	16	5	58	ET biosynthesis	

Table 1. Continued

Protein number	NCBI acc. no.	Protein name	Species	Theor.; Exp. Mol. Mass (kDa)/pI	MASCOT score	Peptides matched	No. of MS/MS fragments	Sequence coverage (%)	Molecular function	Expression pattern ^{a)}
42	CO098975	cdn1-C4	<i>G. raimondii</i>	32.30/5.77; 69.06/5.52	204	17	5	46	Terpenoid biosynthesis	
Cytoskeleton										
8	DW506006	ADF1	<i>G. hirsutum</i>	17.65/6.81; 18.75/5.49	259	18	5	71	Cytoskeleton change	
28	CO125022	Actin 11	<i>G. raimondii</i>	28.92/4.93; 46.76/5.28	292	22	5	77	Cytoskeleton change	
Lipid regulation and transport										
47	CO091608	Phosphoethanolamine N-methyltransferase 2	<i>G. raimondii</i>	32.82/5.44; 56.32/4.86	192	12	4	34	PtdCho synthesis	
09	ES836864	Glycolipid transfer protein 1	<i>G. hirsutum</i>	21.56/6.25; 23.41/6.13	122	15	2	71	glycolipids transfer	
Other proteins										
40	DT544347	HSP70	<i>G. hirsutum</i>	35.83/6.08; 45.68/5.97	95	14	1	44	Stabilize denaturing proteins	
32	DN779299	DUF26 containing serine/threonine kinases	<i>G. hirsutum</i>	24.30/6.54; 45.93/6.64	234	18	5	73	Serine/threonine kinases	
50	ES797289	TPR, tetratricopeptide repeat domain	<i>G. hirsutum</i>	7.12/7.67; 72.66/5.18	276	19	2	54	GA signaling	
18	ES819290	Protein transport protein sec13	<i>G. hirsutum</i>	26.42/5.41; 31.85/6.16	252	20	5	76	Protein transport	
49	BO408539	Predicted Zn-dependent peptidases	<i>G. arboreum</i>	12.81/8.15; 54.25/5.09	69	8	1	70	Protein degradation	

BR, benzoquinone reductase.

a) Y axis: normalized expression volumes of the spots. X axis: column1, mock; columns 2–5, 1, 3, 5, and 7 dpi, respectively. Fold changes of expression volumes for upregulated spots were averaged from three biological replicates and three technical replicates per biological replicate. * Results significantly different to at least a significance level of 0.05 and ** indicates results significantly different to at least a significance level of 0.01.

Table 2. List of downregulated proteins in response to *V. dahliae* infection in *G. barbadense*

Protein number	NCBI acc. no.	Protein name	Species	Theor.; Exp. Mol.Mass (kDa/pI)	MASCOT score	Peptides matched	No. of MS/MS fragments	Sequence coverage (%)	Molecular function	Expression pattern ^{a)}
Stress related										
52	ES829521	Glycine-rich RNA-binding protein	<i>G. hirsutum</i>	13.05/5.52; 13.67/5.02	169	15	5	71	Response to biotic or abiotic stress	
53	BE054076	Copper/zinc superoxide dismutase	<i>G. arboreum</i>	11.14/5.48; 17.23/5.84	428	12	5	77	Antioxidant enzyme	
54	BQ416013	Peroxiredoxin	<i>G. arboreum</i>	15.60/6.33; 17.88/5.81	364	18	6	77	Antioxidant protein	
65	DT468069	Spermidine Synthase	<i>G. hirsutum</i>	30.13/4.85; 69.07/5.52	167	15	3	52	Response to biotic or abiotic stress	
60	CO088138	Aldo-keto reductases	<i>G. raimondii</i>	32.36/9.25; 27.18/6.61	433	23	6	57	Oxidoreductase activity	
Primary metabolism										
67	DT559559	Phosphoglycerate kinase	<i>G. hirsutum</i>	31.00/7.18; 45.56/6.19	421	26	8	64	Transferase of glycolysis	
62	CO072970	Phosphoglycerate kinase	<i>G. raimondii</i>	19.25/6.49; 45.18/6.03	194	11	2	62	Transferase of glycolysis	
68	DT571881	Phosphoglycerate kinase	<i>G. raimondii</i>	31.34/8.85; 47.15/6.05	343	18	4	59	Transferase of glycolysis	
59	CO106577	Triosephosphate isomerase	<i>G. hirsutum</i>	29.70/7.10; 26.80/5.4	472	22	6	54	Glycolysis and Energy production	
61	CO079858	Triose-phosphate isomerase	<i>G. raimondii</i>	27.48/6.33; 28.58/6.56	450	18	5	66	Glycolysis and Energy production	
63	DN780852	Malate dehydrogenase	<i>G. hirsutum</i>	26.35/7.70; 34.84/6.83	265	19	6	63	Enzyme in the citric acid cycle	
66	DT563677	Pyruvate dehydrogenase	<i>G. hirsutum</i>	34.85/8.33; 44.56/6.82	225	23	6	47	Pyruvate decarboxylation process	
55	DW224254	Translation initiation factor 5A	<i>G. hirsutum</i>	18.00/5.44; 18.95/5.95	332	14	3	58	Protein synthesis	
56	EV492786	Translation initiation factor 5A	<i>G. hirsutum</i>	19.27/5.98; 18.77/5.93	383	17	4	63	Protein synthesis	
64	DT543807	Translation initiation factor 2	<i>G. raimondii</i>	26.13/4.88; 33.50/4.89	385	22	9	66	Protein synthesis	
Other proteins										
58	DW485344	Cp10-like protein (CLP)	<i>G. hirsutum</i>	27.48/8.93; 25.19/5.45	279	15	4	55	Protein folding	
57	ES815190	14-3-3a protein mRNA	<i>G. hirsutum</i>	29.46/4.85; 20.44/5.37	258	18	3	47	Protein folding	

a) Y axis: normalized expression volumes of the spots. X axis: column1, mock; columns 2–5, 1, 3, 5, and 7 days post-*V. dahliae* inoculation, respectively. Fold changes of expression volumes for downregulated spots were averaged from three biological replicates and three technical replicates per biological replicate. “*”, “**”, “***” indicates results significantly different to at least a significance level of 0.05 and “***” indicates results significantly different to at least a significance level of 0.01.

between these studies highlighted the roles of defense-related, stress-related, and primary metabolism proteins in response to *V. dahliae* invasion. On the other hand, we also identified a number of proteins whose corresponding genes were not reported by those studies at transcriptional levels. This difference may reflect a contribution of post-transcriptional mechanisms, indicating that proteomic analysis served as an indispensable approach to unravel the pathogen-responsive proteins in the cotton host.

3.1.4 Data verification

Western blot analysis was conducted to verify the differential expression of identified proteins. HSP70 and ADF1 proteins were chosen as representatives for the test based on their expression levels (moderately or highly induced by *V. dahliae* infection). As shown in Fig. 2A, both HSP70 and ADF1 expression was enhanced compared with mock-inoculated plant. This was in agreement with their expression patterns from 2-DE analysis. In addition, transcription levels of six selected proteins were quantified by qRT-PCR to examine if the expression changes were consistent at the mRNA and protein levels (Fig. 2B–G). Concordant differential expression at the mRNA level was observed with four upregulated proteins (chitinase, ATP citrate lyase (ACLY), EP1, and HSP70) and one downregulated protein (Cu/Zn superoxide dismutase, Cu/Zn SOD1). The mRNA level of another upregulated protein (ADF1) was almost not changed at early stage and downregulated at later stage of infection. These results indicated that most of the tested proteins had consistent expression changes with their transcripts, thus confirming the data from 2-DE analysis. On the other hand, the different expressions between

mRNA and protein levels of ADF1 may be due to a post-transcriptional event triggered by the invading fungus.

3.2 Functional categories of cotton proteins involved in the defense responses against *V. dahliae*

3.2.1 Categories of defense-related, secondary metabolism, and cytoskeleton organization

Most of the proteins identified in this study belonging to the categories of defense-related, secondary metabolism, and cytoskeleton organization were previously shown to participate in fighting fungus invasion. Lectin and chitinase were found to interact directly with the fungi in cell wall and disrupt the growth of the invading pathogen [19, 35]. Phenazines could disrupt the oxidation/reduction status of various pathogens and cause the accumulation of toxic superoxide radicals in the cells [36]. The actin cytoskeleton was observed to undergo polarized rearrangements upon fungi attack to prevent pathogen invasion [37]. In our study, the expression of a number of proteins in these functional classes such as lectin, chitinase, actin, ADF1, and phenazines synthesis-related proteins was upregulated upon *V. dahliae* invasion, indicating that the first barrier for plant defense was activated in cotton roots. Apart from these proteins, several others may be involved in the indirect combat with fungal infection. These include the ATP citrate lyase (two spots), a pathogen inducible enzyme required for the biosynthesis of phytochemicals in combating with various abiotic or biotic stresses [38, 39]; the isopentyl diphosphate isomerases, an enzyme crucial for biosynthesis of isoprenoids that are important for gossypol and related

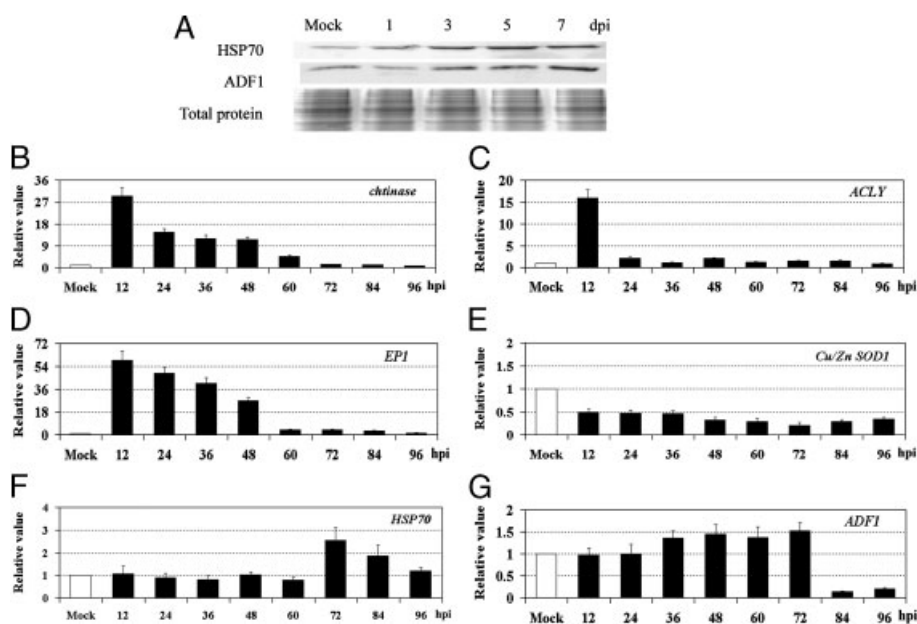


Figure 2. Verification of differential expression via Western blotting analysis and qRT-PCR. (A) Western blot analysis of two upregulated proteins, HSP70 and ADF1, in mock inoculated and inoculated roots at 1, 3, 5, and 7 dpi. (B–G) qRT-PCR analysis of transcription levels of selected proteins in response to *V. dahliae* infection in mock inoculated and inoculated roots at 12, 24, 36, 48, 60, 72, 84, and 96 hpi (hpi, hours postinoculation). The data are mean values and standard errors (bar) of three independent qRT-PCR experiments. The transcription level is represented as the ratio of the Ct value of the studied gene calibrated to the mock-inoculated control and normalized to the Ct value of the *histone 3* gene.

phytoalexins synthesis [40]; the (+)-delta-cadinene synthase (CDNS), an enzyme responsible for biosynthesis of cadinane-type sesquiterpenes such as gossypol to provide constitutive and inducible protection against pests and diseases in cotton [14]. Together, these results point to critical roles of proteins in categories of defense-related, secondary metabolism, and cytoskeleton organization against *V. dahliae* in cotton plants.

3.2.2 Category of stress-related proteins

A number of stress-related proteins were found to be upregulated or downregulated in response to *V. dahliae* attack in our study. Based on the literature information, their functions in defending responses against *V. dahliae* infection can be speculated. Upregulation of alcohol dehydrogenase (five spots), benzoquinone reductase (two spots), nucleoredoxin, and annexin in *V. dahliae*-infected cotton roots may alter cellular redox state by keeping NAD^+ homeostasis, thus preventing the production of free radical intermediates or alleviating H_2O_2 stress [41–46]. Downregulation of some protective proteins including glycine-rich RNA-binding protein, copper/zinc superoxide dismutase, and peroxiredoxin indicated that components of oxidant protection were tightly regulated upon *V. dahliae* infection [47–49]. Upregulation of GST (two spots) suggested that GST induction and GSH pool homeostasis had a role in the defense responses [50].

3.2.3 Other proteins

Several other proteins may also be important for cotton defense response against *V. dahliae*. For example, upregulation of SEC13 suggested that control of the protein secretory system may be important to adapt to pathogen stress as reported by previous studies [51]. Upregulation of HSP70 and downregulation of CP10 and 14-3-3a indicated that different protein folding pathways were activated or repressed upon *V. dahliae* invasion in cotton roots.

3.3 Novel clues regarding wilt resistance of *G. barbadense*

3.3.1 Activation of ethylene synthesis and signaling

Plant signaling and response systems under pathogen infection are operated through the action of plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) [52]. The role of ET is even more complicated in disease-resistance mechanisms and it appears to be involved in only particular classes of pathogens [53]. In cotton, transcriptional upregulation of a gene-encoding ET signal transduction kinase in the ET signaling pathway upon

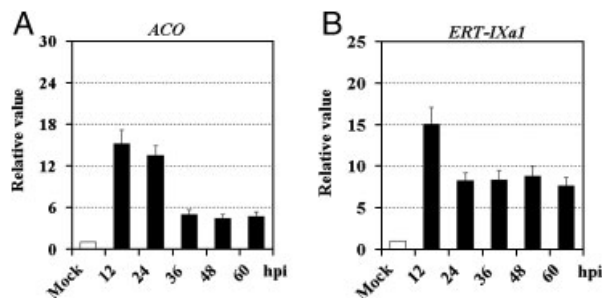


Figure 3. Expression analyses of ET biosynthesis and signaling genes in response to *V. dahliae* infection within 60 hpi in Hai 7124 by qRT-PCR. The data are mean values and standard errors (bar) of three independent qRT-PCR experiments. The transcript level is represented as the ratio of the Ct value of the studied gene calibrated to the mock-inoculated control and normalized to the Ct value of the *histone 3* gene.

V. dahliae infection has been reported [13], but the relationship between ET synthesis and signaling and the host resistance was not established. In this study, the key biosynthetic enzyme of ET, 1-aminocyclopropane-1-carboxylate oxidase (ACO), was identified as upregulated protein, suggesting that this plant hormone was implicated in the regulation of defense responses of Hai 7124 against *V. dahliae* infection. To investigate and verify the participation of ET synthesis and signaling in response to *V. dahliae* attack, transcriptional changes of two marker genes in ET biosynthesis (*ACO*) and signaling (ethylene response factor-IXa, *ERT-IXa*) [54] were detected upon *V. dahliae* infection in Hai 7124. As shown in Fig. 3, the highly activated transcription of *ACO* and *ERT-IXa* (above tenfolds) in wilt-resistant Hai 7124 suggested that the strength of ET signaling is related to the defense against *V. dahliae* infection in cotton roots. Consistent with activation of ET synthesis, protein level of a spermidine synthase that uses the same precursor (*S*-adenosylmethionine) as ET biosynthesis was found to be downregulated in our study. Additional evidence supporting the involvement of ET signaling is the transcriptional activation or inhibition of some identified proteins after ET treatment. Of seven identified proteins tested for their mRNA levels in response to ET treatment, five showed induced expression, whereas two showed inhibited expression (data not shown).

3.3.2 Induced expression of Bet v 1 family proteins

Bet v 1 family proteins have various functions including ribonuclease activity and binding ability for amphiphilic molecules such as cytokinins, sterols, and fatty acids [55]. In previous root proteomic studies, PR10s in the Bet v 1 family have been identified under various biotic and abiotic stresses [27, 56]. Additionally, recent studies indicated that Bet v 1 family proteins other than PR10s including *Gossypium hirsutum* major latex protein (Gh-MLP) and norcochloraurine

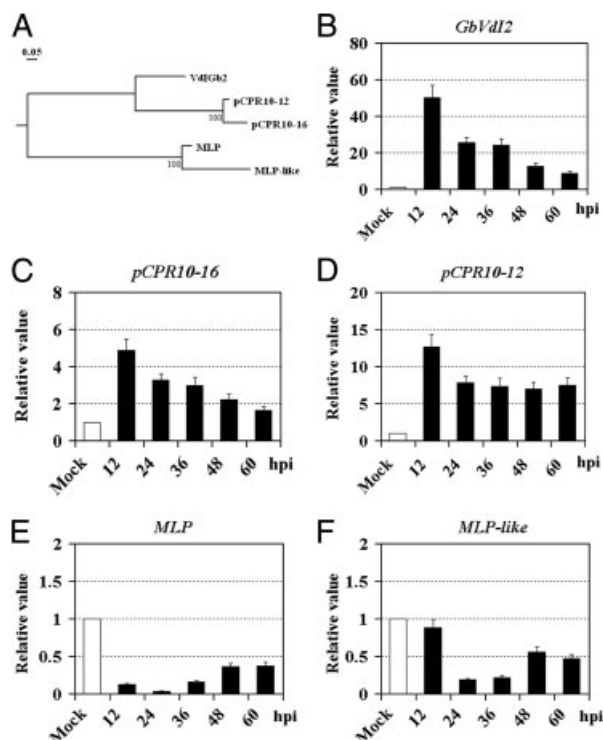


Figure 4. Phylogenetic analysis of identified Bet v 1 proteins and expression analysis of corresponding genes in response to *V. dahliae* infection within 60 hpi in Hai 7124. (A) Phylogenetic tree of five identified Bet v 1 proteins based on amino acid sequences. (B–F) qRT-PCR analysis of the expression patterns of the five Bet v 1 family genes in response to *V. dahliae* infection within 60 hpi in Hai 7124. The data are mean values and standard errors (bar) of three independent qRT-PCR experiments. The transcript level is represented as the ratio of the Ct value of the studied gene calibrated to the mock-inoculated control and normalized to the Ct value of the *histone 3* gene.

synthase were involved in secondary metabolism changes upon abiotic or biotic stresses [22, 57]. In our study, about 10% of the upregulated proteins belong to the Bet v 1 family. tBlastx analysis indicated that these proteins consist of two homologues of GhPR10s (GhpC-PR10-12 and GhpC-PR10-16), two MLPs (MLP and MLP-like), and one novel Bet v 1 family protein GbVdI2. Phylogenetic relationships among these proteins based on the amino acid sequences are shown in Fig. 4A. To further investigate the function of this family of proteins in the defense responses, we analyzed the transcriptional changes of these genes in Hai 7124 within 60 h after *V. dahliae* inoculation. As shown in Fig. 4B–F, transcription of the *GbVdI2* and two *PR10*s genes enhanced remarkably, whereas the expression of *MLP* gene and *MLP-like* gene was downregulated. *MLP* and *MLP-like* genes (64% identity) belong to the same subfamily. Expression changes of both genes at mRNA and protein level were inconsistent, pointing to a more complex mechanism for their responses to *V. dahliae* infection. These results indicate that the expression of some Bet v 1 family proteins is tightly

associated with Verticillium wilt defense response in cotton roots and may contribute significantly to the disease resistance in cotton plants.

3.3.3 Redirecting of carbohydrate flux

In this study, many proteins involved in primary metabolism underwent significant changes in expression levels upon *V. dahliae* infection. Among these proteins, three enzymes in the pentose phosphate pathway (PPP), including transketolase, fructokinase fructose-bisphosphate aldolase, and phosphogluconate dehydrogenase were upregulated and meanwhile two enzymes in the glycolysis pathway including phosphoglycerate kinase and triosephosphate isomerase (TPI) were downregulated. These changes suggested a redirection of metabolic flux from glycolysis to PPP, which was previously found to be a strategy for plants to survive under oxidative stress [58, 59]. Upregulation of the PPP pathway components found in our study is consistent with the previous reports which indicate that the PPP can be activated under pathogen infection, resulting in the production of more intermediates such as 1-deoxy-D-xylulose 5-phosphate for the generation of isoprenoid [60, 61]. Thus, the switch from glycolysis to PPP may be an active response of the cotton plant against *V. dahliae* infection to enhance the wilt tolerance or resistance.

4 Concluding remarks

We report for the first time the protein profiling upon *V. dahliae* attack in Verticillium wilt-resistant cotton plants, using roots as the experimental material. A number of differently expressed proteins with various biological functions from *V. dahliae*-challenged cotton roots were identified, which revealed the participation of ET signaling components and Bet v 1 family proteins as well as the redirection of carbohydrate flux from glycolysis to PPP in response to *V. dahliae* infection in *G. barbadense*. This study deepens our understanding of cotton defense mechanisms against *V. dahliae* and may provide candidate genes/proteins for antidisease transgenic breeding and for the development of antifungal agent against cotton vascular disease.

This work was supported by grants from the National Natural Science Foundation (Grant No.30671116) and the Ministry of Agriculture of China (Grant No. 2009ZX08005-001B and 2009ZX08010-001B).

The authors have declared no conflict of interest.

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