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FUNGAL DISEASES

Proteomics-based analysis reveals that *Verticillium dahliae* toxin induces cell death by modifying the synthesis of host proteins

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Abstract *Verticillium dahliae* is one of the most destructive soil-borne fungal pathogens that cause vascular wilt diseases in a wide range of important crop plants, including cotton. However, the mechanisms used by this pathogen to infect cotton have not been fully elucidated. In the present study, we first investigated changes in protein abundance during the initial interaction between cotton roots and *V. dahliae*. Among the proteins that were upregulated upon infection, some were related to reactive oxygen species (ROS); among those downregulated upon infection were proteins involved in normal metabolism or cell structure. Further experiments confirmed that a sudden release of ROS and cell death accompany *V. dahliae* infection in the cotton vasculature. Further analysis indicated that a culture supernatant of *V. dahliae* induced lesion formation in tobacco leaves; de novo protein synthesis not active gene expression was required for this induction. Lesion formation was dependent on the age of leaves, but neither the known ROS burst nor the ubiquitin/26S proteasome system are prerequisites.

Keywords *Verticillium dahliae* · Cotton · Root proteome · Cell death · ROS

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Introduction

Verticillium wilt, a vascular disease of cotton that substantially reduces cotton production every year, is caused by the soil-borne fungus *Verticillium dahliae* Kleb. The microsclerotial form of *V. dahliae* can remain viable in the soil for more than 20 years (Wilhelm 1955). In 1967, the pathogen was identified as the causal agent of this important disease of cotton under field conditions, and it has been a major threat to cotton production since then (Fradin and Thomma 2006).

Verticillium dahliae generally enters the cotton plant either by penetrating roots or via wounds in root tissues (Vallad and Subbarao 2008). After penetrating the cortex of the root, the fungus passes through the endodermis, invades the xylem vessels, eventually reaching the aerial parts of the plant (Tzima et al. 2011). Typical disease symptoms may comprise wilting, stunting, leaf necrosis, vascular plugging and brown vascular discoloration (Fradin and Thomma 2006). There are currently no fungicides available to control Verticillium wilt once plants have been infected (Klosterman et al. 2009).

Despite the economic importance of Verticillium wilt, not much is known about the molecular mechanisms and crucial components underlying this disease (Klosterman et al. 2009). Historically, there are two seemingly contradictory hypotheses concerning the mechanism of Verticillium wilt. One hypothesis states that Verticillium wilt results from toxin activity, whereas the other states that it is predominantly the result of vessel occlusion in the plant (Fradin and Thomma 2006). Some studies suggest that crude *V. dahliae* extracts containing some phytotoxic factors, such as protein–lipopolysaccharide complex (PLPC), glycoproteins, small peptides and/or cell-wall-degrading enzymes, can damage leaf and root cell membranes,

resulting in ion leakage (Gour and Dube 1985). Meyer's group purified PLPC from culture filtrates of a virulent *V. dahliae* strain, and this PLPC showed high binding activity on cotton plasma membranes and protoplasts, especially from root tissues (Meyer et al. 1994). However, evidence for a dominant role for these toxins in causing Verticillium wilt is often inconsistent and contradictory and therefore not universally accepted (Cooper 2000).

Studies on tomato Verticillium wilt have shown that in this host, vessel occlusion is the primary cause of water stress (Street and Cooper 1984). The occlusion can be due to a physical blockage of the xylem by the pathogen or due to host defense responses that result in vessel plugging (Fradin and Thomma 2006). In recent years, some genes related to *V. dahliae* pathogenicity have been identified, such as a mitogen-activated protein kinase (VMK1) (Rauyaree et al. 2005), a cAMP-dependent protein kinase A (VdPKAC1) (Tzima et al. 2010), a glutamic acid-rich protein (VdGARP1) (Gao et al. 2010) and a sucrose non-fermenting 1 gene (VdSNF1) (Tzima et al. 2011). Proteins that can induce plant cell death have also been isolated from a culture supernatant of *V. dahliae* such as VdNEP (Wang et al. 2004); however, there is still doubt whether any of these play key role in *V. dahliae* pathogenicity (Zhou et al. 2012). So the critical elements and the molecular mechanisms involved in pathogenicity of Verticillium wilt have not yet been discovered.

Because the early biochemical responses of cotton roots after *V. dahliae* infection can reflect the plant reaction to *V. dahliae* invasion, in the present study, we use proteomic methods to identify proteins involved in roots responses to *V. dahliae* and investigate the response of cotton to *V. dahliae* infection.

Materials and methods

Conidial culture, plant growth, and inoculation system

Verticillium dahliae isolate V9 (a highly virulent strain isolated from upland cotton that causes defoliation) was obtained from the Department of Plant Protection, Southwest University, China. The strain was used throughout the study and stored in our laboratory. The isolate was grown on potato dextrose broth (PDB) (200 g potato, 20 g glucose, and distilled water; final volume, 1 L) at 25 °C for 20 days. Acid-delinted upland cotton seeds (*Gossypium hirsutum* cv. Xinluzao-13) were kindly provided by Dr. Wangfeng Zhang (Shihezi University, China). The seeds were germinated in sterile distilled water (containing 0.9 % v/v hydrogen peroxide) for 2 days at 25 °C. Germinated seeds were grown in sterile vermiculite under artificial light (12-h photoperiod at 500 μmol m^{-2} s^{-1}) at 25 °C and watered using autoclaved MS basal liquid medium (Murashige et al. 1962) every third day for 15 days. At the fourth true-leaf stage of cotton seedlings, the roots of the seedlings were dipped into a conidial suspension of *V. dahliae* (10^6 conidia/mL) or into sterile distilled water without conidia as the control. The seedlings were incubated at 25 °C with a 12-h light/12-h dark photoperiod and 90 % relative humidity, then harvested at various times and rinsed using deionized water. The roots were cut, patted dry between paper towels, frozen in liquid nitrogen and then stored at −80 °C until protein extraction.

Protein extraction and quantification

Proteins were extracted from cotton roots using our previously described method with minor modifications (Xie et al. 2009). The sample was ground in liquid nitrogen in a pre-chilled mortar containing 10 % w/v silicon dioxide and 10 % w/v polyvinylpolypyrrolidone (PVPP). The finely ground powder (~1 g per tube) was immediately transferred into a 50 mL centrifuge tube and weighed. The sample was resuspended in 10 mL of extraction buffer [0.1 M Tris–HCl, pH 8.0, containing 30 % w/v sucrose, 2 % w/v SDS, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 % v/v β-mercaptoethanol], and 7 mL of 1 M Tris-saturated phenol (pH 8.0) was added. The mixture was homogenized and incubated on ice for 5 min, the upper phenol phase was collected, and this extraction step was repeated once again. The phenol phases were combined in a new tube and washed once with an equal volume of extraction buffer, and the proteins were precipitated with 5 volumes of 0.1 M ammonium acetate in methanol overnight at −20 °C. After centrifugation at 12,000×*g* for 15 min (4 °C), the protein pellets were washed once with 3 mL of methanol and then washed twice with 3 mL of cold 80 % v/v acetone in water. The pellets were dried in a cold vacuum dryer for 10 min and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS, 65 mM dithioithreitol (DTT), and 0.5 % v/v carrier ampholytes, pH 3–10 (nonlinear gradient) and shaken for 1 h (IKA Vortex Genius 3, Staufen, Germany). After centrifugation at 15,000×*g* for 20 min to remove debris, the supernatant was collected. Protein concentration was determined using the Bradford (1976) method with bovine serum albumin as a standard.

Two-dimensional gel electrophoresis

The first electrophoresis was performed using immobilized pH gradient (IPG) strips (13 cm, pH 3–10 nonlinear gradient; GE Healthcare, Piscataway, NJ, USA) on an IPGphor isoelectric focusing (IEF) system (Amersham Pharmacia, San Francisco, CA, USA). The IPG strips were rehydrated with 250 μL of rehydration buffer (containing

370 μg proteins), and the IEF was then done at 20 °C as follows: active rehydration at 30 V for 12 h, 200 V for 2 h, 500 V for 3 h, 1,000 V for 4 h, 8,000 V for 5 h, with a gradient increase in voltage between 8,000 and 40,000 V. After IEF, the proteins in the strips were reduced with 1 % w/v DTT in 10 mL of equilibration buffer (6 M urea, 20 % w/v glycerol, 2 % w/v SDS, and 50 mM Tris–HCl, pH 8.8) for 15 min and alkylated with 2.5 % w/v iodoacetamide in 10 mL of equilibration buffer for 15 min. The strips were transferred onto vertical 10.5 % w/v SDS-PAGE selfcast gels. The second electrophoresis (SDS-PAGE) was performed on an Amersham Hoefer SE600 system (Amersham Pharmacia) at 10 mA for 1 h and 20 mA for 6 h at 15 °C.

Protein visualization and image analysis

The 2D gel was stained with blue silver (Candiano et al. 2004). The gel was fixed in a solution of 40 % v/v methanol and 10 % v/v acetic acid for 30 min, washed in distilled water four times for 15 min, and finally incubated in a staining solution (0.12 % w/v Coomassie brilliant blue G-250, 10 % w/v ammonium sulfate, 10 % w/v phosphoric acid, 20 % v/v methanol) overnight with gentle shaking. The gel was decolorized in distilled water. After the 2D gel was stained with blue-silver, it was scanned using an image scanner (Amersham Pharmacia). The images from the 0-, 24-, and 72-h samples were analyzed using Image Master 2D Platinum Version 5.0 Analysis Software (Amersham Pharmacia) according to the user's manual. The density of each spot was determined by the software and normalized against the total gel density. To ensure reproducibility, we did the experiment three times independently for each time point (root samples harvested from at least three seedlings at each time), and Student's t test was used to determine any significant differences among samples harvested at the same time ($p < 0.05$).

Protein identification and database search

Spots of interest were excised using a modified plastic pipette tip. The bottom of a 200 μL pipette tip was cut off such that its opening was approximately 1.5 mm in diameter. These excised spots were transferred to a microcentrifuge tube. The gel particles were washed twice with deionized water, and 50 μL decolorizing solution [50 mM NH_4HCO_3/CH_3CN (1:1)] was added. The samples were vortexed for 20 min at 37 °C, and the decolorization step was repeated until the blue color disappeared. To shrink the gel particles, 50 μL acetonitrile was added until the particles became white. The gel particles were dried in a vacuum centrifuge for 10 min and mixed with a 20 μL solution of 10 mM DTT in 25 mM NH_4HCO_3 for 1 h at 56 °C to reduce the proteins. At ambient temperature, the gels were dried in a vacuum centrifuge for 30 min before incubation in 20 μL alkylation solution (50 mM iodoacetamide in 25 mM NH_4HCO_3) for 45 min in the dark. Several wash steps were performed in succession: 25 mM NH_4HCO_3 (2 × 10 min), 25 mM NH_4HCO_3 in 50 % v/v CH_3CN (2 × 10 min), CH_3CN (10 min). The gel particles were completely dried in a vacuum centrifuge for 10 min and enzymatically digested at 37 °C overnight with 5 ng/mL trypsin. Digested peptides were extracted in 0.1 % v/v trifluoroacetic acid (TFA) in 50 % CH_3CN and analyzed in the Ultraflex TOF/TOF (Bruker, Germany). Maldi Matrix: a-cyano-4-hydroxy-cinnamic acid (4 mg/mL, 70 % v/v acetonitrile: 30 ddH_2O, containing 0.1 % v/v TFA). Parent ion masses were measured in the delayed extraction mode with an accelerating voltage of 20 kV. The autolytic fragments of trypsin acted as internal calibrants. The spectra were acquired in a positive reflectron mode (20 kV) and collected within the mass range of 700–4200 Da. Mass fingerprint spectra were processed with the FlexAnalysis 2.0 software (Bruker). Database searches were performed using the online Mascot search engine (http://www.matrixscience.com). Because a complete cotton protein database is not available, the searches were run against all available higher plant proteins. The following search parameters were applied using the NCBI protein database: a mass tolerance of 0.2 Da and one missed cleavage was allowed; carboxyamidomethylation of cysteine was specified because fixed modification and oxidation of methionine were allowed as variable modifications.

In vivo detection of H_2O_2 in cotton roots

H_2O_2 was detected in the roots of cotton using 3,3′-diaminobenzidine (DAB) as a substrate (Orozco-Cardenas and Ryan 1999). Briefly, the primary roots were excised from seedlings with a knife and immersed in a solution containing 1 mg/mL DAB dissolved in HCl-acidified (pH 3.8) distilled water for 5 h at 25 °C. The surface dye was washed with distilled water, and root pieces were fixed and decolorized in a boiling acetic acid–glycerol–ethanol solution (1:1:3) for 10 min. The samples were then stored in 60 % v/v glycerol for microscopic examination. Very thin, free-hand sections of root pieces were mounted on a glass slide in microscopy solution and examined with a light microscope Nikon 80i (Nikon, Tokyo, Japan).

Detection of dead plant cells in cotton tissues

Staining with trypan blue to detect dead cells in cotton tissues was done as described by van Wees (2008) with some modification. The trypan blue working solution was prepared by diluting the stock solution (10 g phenol,

10 mL glycerol, 10 mL lactic acid, 10 mL water and 0.02 g trypan blue) with ethanol (96 %; 1:2 v/v). Whole seedlings were harvested and transferred to plastic test tubes with a lid containing diluted trypan blue solution. The tubes were boiled in the staining solution for 1 min and left overnight in the solution. Samples were stored in 60 % glycerol. Transverse and longitudinal hand sections of cotton stems and roots were cut using a razor blade and mounted on glass slides in 60 % glycerol and examined with the light microscope.

Preparation of crude culture supernatant of *V. dahliae* and infiltration experiments

To prepare a crude culture supernatant for *V. dahliae*, we cultured *V. dahliae* in PDB for 20 days. The culture was centrifuged at 12,000×*g* for 15 min (4 °C). The supernatant was filtered twice through Whatman filter paper (Maidstone, Kent, UK) and observed with a light microscope to ensure the removal of all propagules of *V. dahliae*. In the tests on supernatant pathogenicity, 10 mL of *V. dahliae* supernatant was poured onto sterile vermiculite for root-dip inoculation. For the cotyledon infiltration, cotton cotyledons were vacuum-infiltrated with either 50 µL of *V. dahliae* supernatant or a mixture of supernatant and inhibitors.

DPI, MG132, ActD and CHX treatments

Diphenyleneiodonium (DPI) (NADPH oxidase inhibitor), actinomycin D (ActD) (inhibitor of RNA synthesis) and *N*-benzyloxycarbonyl(Z)-Leu–Leu-leucinal (MG132) (26S proteasome-specific inhibitors) were dissolved in DMSO, and cycloheximide (CHX) (protein synthesis inhibitor) was dissolved in methanol. Tobacco (*Nicotiana tabacum* cv. Xanthi) leaves were vacuum-infiltrated with 50 µL of *V. dahliae* supernatant containing either these inhibitors or DMSO (methanol) only. The same volume of ddH_2O containing the inhibitors or DMSO (methanol) was used as a negative control. To prepare crude *V. dahliae* upernatant, we cultured *V. dahliae* in PDB for 20 days. The culture was centrifuged at 12,000×*g* for 15 min (4 °C). The supernatant was filtered twice through Whatman filter paper and observed with a light microscope to ensure the absence of *V. dahliae.*

Results and discussion

Proteomics analysis of cotton roots during early infection by *V. dahliae*

In our study, acid-delinted cotton seeds were grown in sterile vermiculite under artificial light and MS liquid medium (Murashige and Skoog 1962) was autoclaved to ensure that growth of other microorganisms was as much as possible restricted. The infected seedlings showed significant and consistent dehydration and wilt symptoms in comparison with the control plants 72 h after inoculation with *V. dahliae* (Fig. 1). Later, the seedlings developed typical leaf lesions and became defoliated (from base to apex) (Fig. S1a–c), and *V. dahliae* was isolated from the stems 25 days after inoculation (Fig. S1d).

Detection of differentially expressed proteins

To examine the initial interaction between cotton roots and *V. dahliae*, we harvested seedling root tissue at 0, 24, and 72 h after *V. dahliae* inoculation because the infected seedlings were significantly dehydrated and wilted by 72 h after *V. dahliae* inoculation (Fig. 1). Every sample for protein extraction included at least three cotton seedlings to reduce potential differences among plants. The proteins were analyzed on two-dimensional (2D) gels using pH 3–10 nonlinear gradient strips and 10.5 % SDS-PAGE. On average, 830 ± 11 protein spots were detected on the gels after blue-silver staining (Fig. S2). Fifteen differentially expressed protein spots were identified (Table 1). One protein (Fig. 2c, spot No. 15) was significantly upregulated, whereas 10 (Fig. 2a, spot Nos. 1–10) were suppressed. Accumulation of four proteins peaked 24 h after inoculation with *V. dahliae* (Fig. 2b, spot Nos. 11–14). The proteins that were differentially regulated during early infection by *V. dahliae* are highlighted in Fig. 2. The proteins identified belong to various classes of defense- or compatibility-related plant proteins, including those related to pathogenesis (PR proteins), reactive oxygen species (ROS), protein folding and modification, glycolysis and energy generation and others. In general, however, many of these proteins are associated with ROS regulation and cell death. These observations will be detailed in the following section.

Proteins related to ROS regulation

To protect against oxidative damage, plants have developed a wide range of antioxidant systems that scavenge excessive ROS (Ahsan et al. 2008). In this respect, protein spots b11–14 (Fig. 2) showed a temporary upregulation; their expression increased by 24 h and subsequently decreased by 72 h after inoculation. Temporarily upregulated proteins that have a potential role in modulating plant responses to ROS were also reported for cotton infected by *Thielaviopsis basicola* (Coumans et al. 2009). Spot b11 (Fig. 2), *S*-adenosylmethionine (SAM) synthetase, can be determined through its action as a precursor for intracellular glutathione (GSH, a major cellular antioxidant) or direct radical scavenging (Evans et al. 1997;

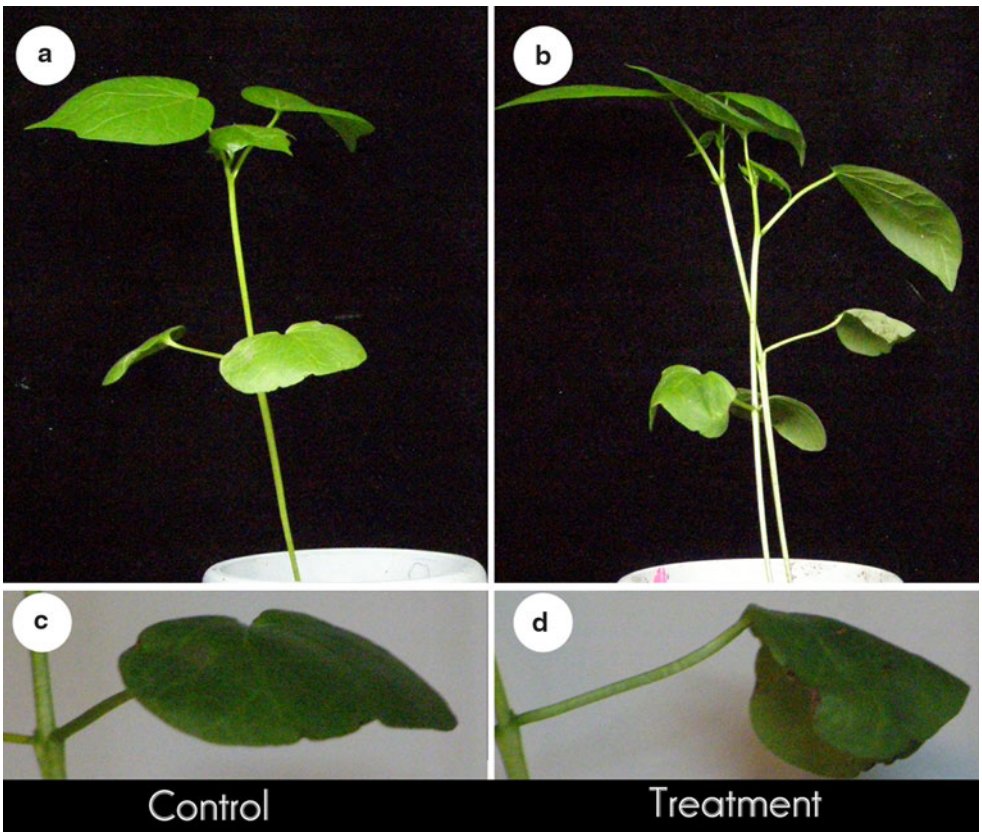

Fig. 1 Cotton seedlings without wilting symptoms after inoculation with sterile water (**a**) or with typical wilt symptoms after inoculation with *Verticillium dahliae* (**b**). Cotyledons of water control (**c**) and infected plant (**d**). All images were taken at 72 h after inoculation

Tchantchou et al. 2008). Ascorbate peroxidase (APX) (Fig. 2, b12) and its isozyme APX1 (Fig. 2, b13) play a key role in scavenging ROS to protect cells of algae and higher plants (Davletova et al. 2005). Their expression was responsive to biotic and abiotic stress (Shigeoka et al. 2002). The family of pathogenesis-related-10 proteins (PR-10) (Fig. 2, b14) is usually induced upon attack by various pathogens (El-kereamy et al. 2009). Moreover, some research suggests that PR-10 expression can be induced directly by ROS and is a marker for oxidative stress in plants (Mur et al. 2000). Phosphoglycerate kinase (Fig. 2, spot c15) is an enzyme that catalyzes the formation of ATP (Varga et al. 2009); it is a biomarker for oxidative stress and can be induced by environmental stress (Jang et al. 2008). In summary, all these proteins (Fig. 2, spot b11–14 and c15) are related to ROS. The significant upregulation suggests that infection by *V. dahliae* leads to an ROS burst in the cotton tissues; however, further experimental is needed to detect ROS in cotton tissue.

Proteins related to cell death

The proteins that had decreased in level by 72 h after inoculation were involved in glycolysis, energy metabolism, phosphoglycerate metabolism, and potentially in cell structure modifications. Among these proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 2, a1 and a2) and enolase (Fig. 2, a3), enzymes involved in glycolysis (Pancholi 2001; Tarze et al. 2007), are abundantly expressed in most cells (McAlister and Holland 1982). Malate dehydrogenase (MDH) (Fig. 2, a4) is involved in the citric acid cycle and glyoxylate cycle. Qin et al. (2009), while investigating fruit senescence, discovered that protein oxidation is accompanied by a loss of catalytic activity of MDH. Ubiquitin modification (Fig. 2, a5) and heat-shock proteins (HSPs) (Fig. 2, a6) are involved in the regulation of protein function and location in plants (Acconcia et al. 2009). The ubiquitin/26S proteasome system is involved in plant–microbe interactions and plant defense (Dielen et al. 2010), and most HSPs have strong cytoprotective effects and ensure plant homeostasis (Baniwal et al. 2004). Transitional endoplasmic reticulum ATPase (TER ATPase) (Fig. 2, a7 and a8) binds ubiquitinated proteins and is necessary for the export of unfolded proteins from the ER to the cytoplasm, where they are degraded by the proteasome (Zhang et al. 1994). Short et al.'s (2007) study suggests that TER ATPase and HSPs are involved in apoptosis. Actin (Fig. 2, a9) and alpha-tubulin (Fig. 2, a10), both components of the cytoskeleton, are commonly used to normalize molecular expression

Table 1 List of proteins identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) analysis

Spot No.[a]	Protein name	Accession No.[b]	Organism	Mr (kDa/)/pI theoretical; experimental	Mascot score[c]	Sequence coverage (%)[d]	%Vol ± SD[e]		
							0 h	24 h	72 h
Modulation of ROS-related proteins									
b11	*S*-adenosylmethionine (SAM) synthetase	ADN96174	*Gossypium hirsutum*	43539/5.51; 46564/5.52	116	26	0.211 ± 0.028	0.379 ± 0.09	0.111 ± 0.016
b12	Ascorbate peroxidase	ABR18607	*Gossypium hirsutum*	27738/5.93; 25290/6.31	112	48	0.286 ± 0.039	0.352 ± 0.076	0.131 ± 0.046
b13	Cytosolic ascorbate peroxidase 1	ABR18607	*Gossypium hirsutum*	27738/5.93; 24525/5.99	84	38	0.416 ± 0.058	0.478 ± 0.04	0.19 ± 0.032
Pathogenesis-related protein (PR)									
b14	PR-10	AAG18451	*Gossypium hirsutum*	17258/5.21; 15673/5.11	71	42	0.488 ± 0.15	0.695 ± 0.132	0.494 ± 0.039
Glycolysis and energy-related proteins									
a1	Glyceraldehyde 3-phosphate dehydrogenase	AAA32794	*Populus trichocarpa*	37241/7.67; 36905/7.82	77	32	0.553 ± 0.059	0.53 ± 0.095	0.251 ± 0.017
a2	Glyceraldehydes 3-phosphate dehydrogenase	NP_187062	*Arabidopsis thaliana*	37005/6.62; 37000/6.47	75	29	0.553 ± 0.109	0.508 ± 0.118	0.318 ± 0.035
a3	Enolase	ABW21688	*Gossypium hirsutum*	47982/5.49; 54515/5.63	74	23	0.242 ± 0.035	0.241 ± 0.034	0.122 ± 0.019
a4	Malate dehydrogenase	P57106	*Gossypium hirsutum*	35873/6.10; 36445/6.33	104	37	0.396 ± 0.052	0.38 ± 0.009	0.156 ± 0.032
c15	Phosphoglycerate kinase	NP_001141324	*Zea mays*	38249/5.40; 48371/5.99	100	25	0.186 ± 0.03	0.202 ± 0.025	0.285 ± 0.025
Protein folding and modification-related protein									
a5	Ubiquitin	XP_002305847	*Populus trichocarpa*	41678/6.56; 38970/6.48	73	25	0.336 ± 0.023	0.358 ± 0.045	0.169 ± 0.045
a6	Heat shock protein	XP_002305263	*Populus trichocarpa*	80954/5.01; 82992/4.91	134	22	0.209 ± 0.11	0.124 ± 0.073	–[f]
a7	Transitional endoplasmic reticulum ATPase	EEF42916	*Ricinus communis*	90181/5.15; 105556/5.13	114	16	0.138 ± 0.039	0.109 ± 0.02	–
a8	Transitional endoplasmic reticulum ATPase	EEF42916	*Ricinus communis*	90181/5.16; 105159/5.11	185	25	0.213 ± 0.102	0.093 ± 0.008	–
Proteins of cell cytoskeleton									
a9	Actin	AAP73454	*Gossypium hirsutum*	41902/5.23; 42794/5.21	109	49	0.425 ± 0.09	0.399 ± 0.073	0.204 ± 0.058
a10	Alpha-tubulin	NP_001060075	*Gossypium hirsutum*	50224/4.93; 52075/4.92	114	35 %	0.185 ± 0.008	0.123 ± 0.031	0.032 ± 0.031

[a] Spot number as given on the 2D gel image

[b] Accession number from NCBI database of matched protein

[c] Mascot score for each identified protein

[d] Percentage sequence coverage (%)

[e] Average expression value of identified proteins at various times (0, 24 and 72 h); results are mean ± SD of three independent experiments. Expression value is relative volume (%Vol) for each spot, which was calculated using Image Master 2D Platinum Version 5.0 Analysis Software (Amersham Pharmacia). The bar charts of different expression pattern are shown on the left of Fig. 3

[f] No protein was detected

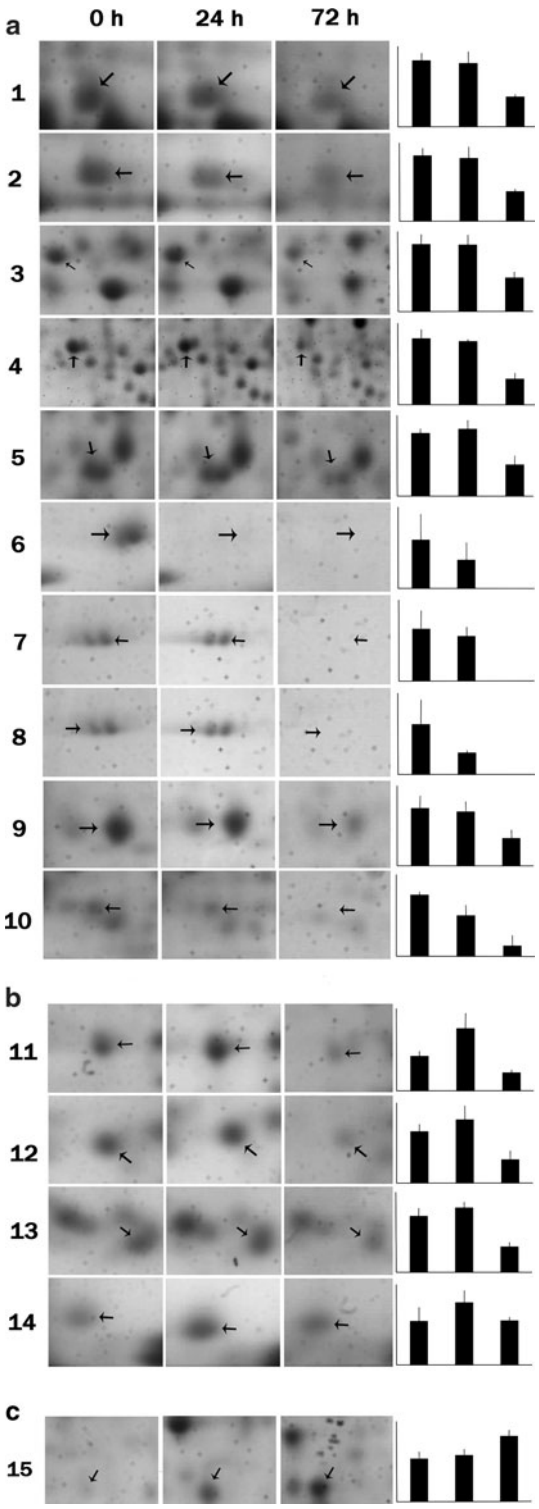

Fig. 2 Magnification of spots of differentially expression proteins on the two-dimensional gel electrophoresis gel of protein samples in cotton roots at 0-, 24-, and 72-h after inoculation with *Verticillium dahliae*. **a** Downregulated proteins, spot No. 1–10; **b** temporary upregulated proteins at 24 h, spot No. 11–14; **c** upregulated proteins, spot No. 15. *Bar graphs* on the right show mean change in protein levels at each time (left to right, 0-, 24-, and 72-h); *error bars* represent the standard deviation of three repetitions

studies because of their functions as endogenous housekeeping genes (Ruan and Lai 2007). In summary, these downregulated proteins participate in cell metabolism, structure, and stability and are expressed at a high level when the physiological status of cotton is normal. At 72 h after *V. dahliae* inoculation, the downregulation of these two proteins could indicate degradation of cell structures and necrosis in cotton roots, but further experimental evidence is needed.

ROS accumulation and cell death in cotton roots after infection with *V. dahliae*

The generation of ROS in cotton roots after infection with *V. dahliae* was monitored using the DAB staining assay, an efficient method for detecting H_2O_2 accumulation or localization, an indicator of ROS generation (Orozco-Cardenas and Ryan 1999). As shown in Fig. 3d, a deep-brown polymerization product was clearly seen in cotton roots 72 h after *V. dahliae* inoculation, but was absent in control plants (Fig. 3c), indication that cotton generated ROS, as the so-called oxidative burst, after infection with *V. dahliae*, consistent with the results of Robb et al. (2012). To examine cell death after infection with *V. dahliae* in more detail, we stained infected stem and root tissue of cotton seedlings with trypan blue to visualize dead cells (van Wees 2008). At 72 h after *V. dahliae* inoculation, numerous blue-stained cells were observed around the vasculature (Fig. 3f, h, j, and l). In contrast, the control plants treated with water had no blue-stained cells in these areas; only a slight background color was present (Fig. 3e, g, i, and k).

Culture supernatant of *V. dahliae* induces cotton cell death

Phytotoxins or effectors secreted by *V. dahliae* have been considered to be important in pathogenesis by *Verticillium* (Tzima et al. 2011). When cotton cotyledons were treated with the culture supernatant of *V. dahliae*, whole *V. dahliae* culture (including supernatant and conidia, as positive control) and PDB (as negative control), respectively. The culture supernatant (Fig. 4c) caused typical symptoms such as dehydration, consistent with that caused by the whole culture (Fig. 4b), and brown discoloration of the vascular tissues in cotton stem sections (Fig. 4e). Because leaf wilt also is a key symptom of cotton plants infected with *V. dahliae* (Wang et al. 2004), we infiltrated a cotton leaf with 50 μL of the culture supernatant, and the characteristic wilting was induced in cotton leaves within 24 h (Fig. 4g), These results indicated that a compound(s) in the supernatant was involved in cell death and wilt.

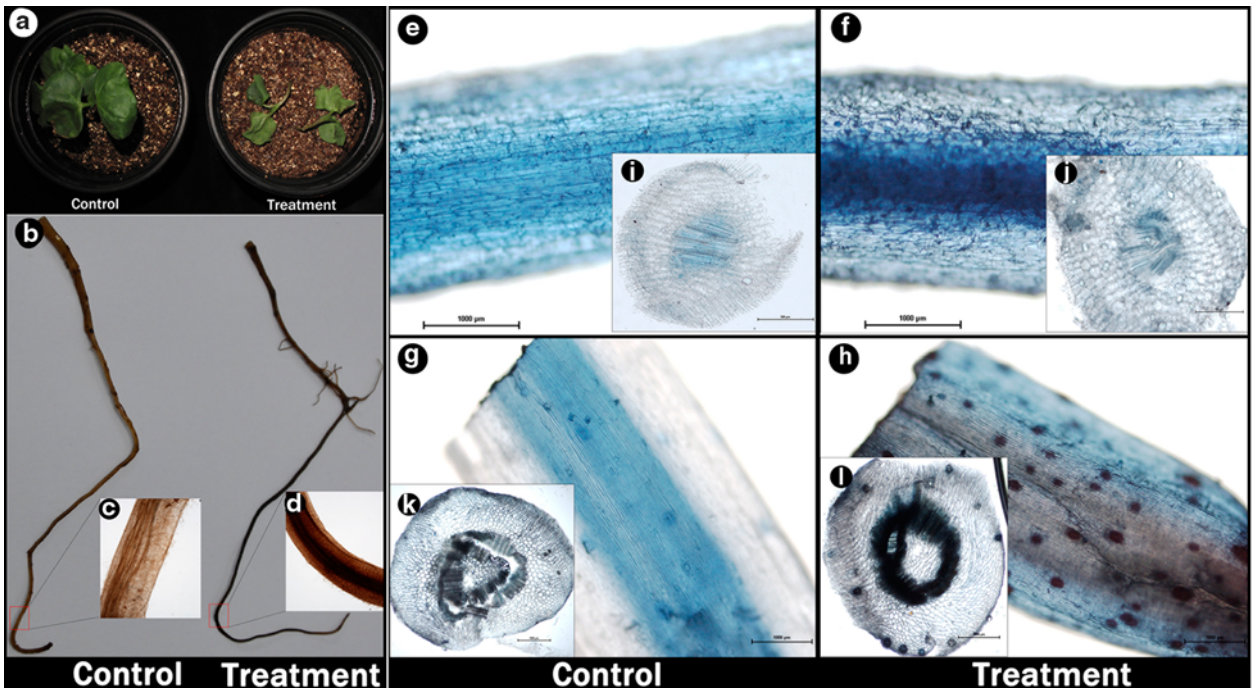

Fig. 3 Disease symptoms caused by *Verticillium dahliae* in cotton seedlings. **a** At 72 h after inoculation with *V. dahliae*. The control was treated with sterile distilled water instead of *V. dahliae*. **b** DAB-stained cotton roots showing H_2O_2 accumulation. **c**, **d** Light micrograph of H_2O_2 accumulation in the root sections. Longitudinal sections of infected cotton stained with *trypan blue*. **e**, **f** roots, **g**, **h** stems. **i–l** Transverse sections of samples in **e–h**. Compared to control plants, significant, dense blue staining was observed around the vasculature of cotton roots infected by *V. dahliae*. Tests with DAB and *trypan blue* staining were repeated twice again to verify the result

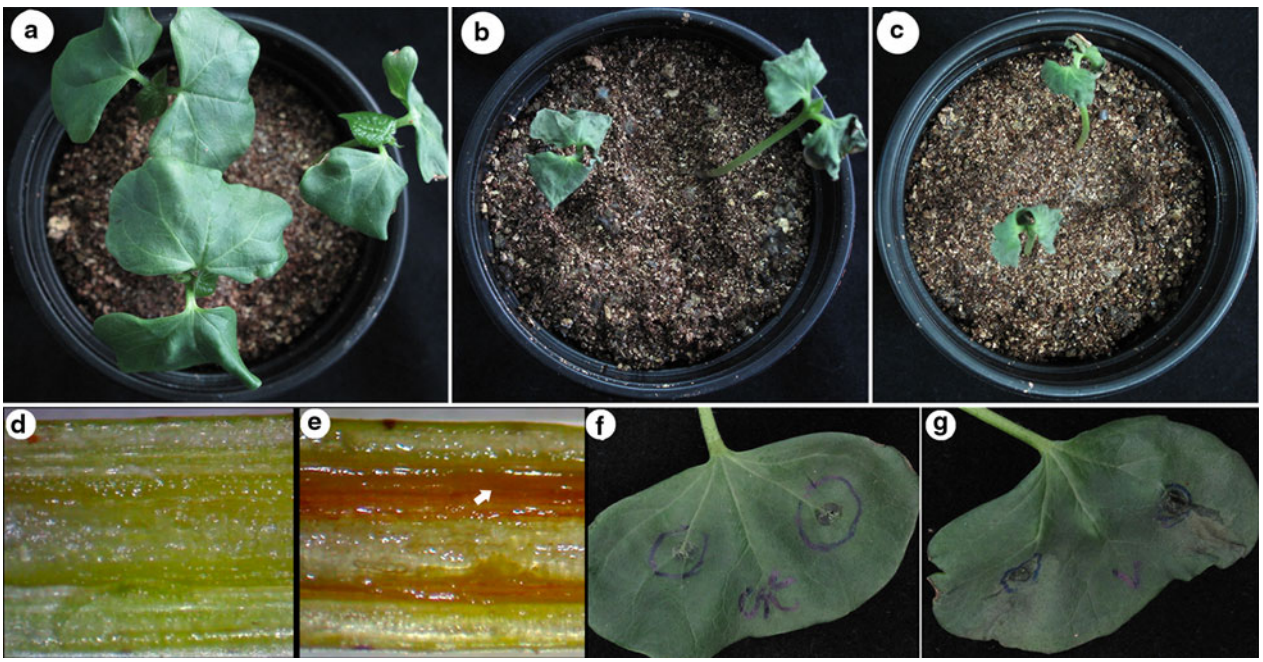

Fig. 4 Typical *Verticillium* symptoms were caused by the supernatant of a *Verticillium dahliae* culture. **a** No symptoms on the negative control inoculated with PDB only. **b** Wilt symptoms were induced by treatment with whole culture of *V. dahliae* as positive control. **c** Cotton cotyledons developed wilt symptoms after treatment with culture supernatant of *V. dahliae*. Three repetitions of the experiment gave consistent results. **d** No visible vessel discoloration was observed in cotton stem shown in Fig. 4a. **e** *Brown* discoloration of vessel (*white arrow*) in longitudinal section of cotton stem in Fig. 4c. The above photographs were obtained 72 h after *V. dahlia* or supernatant inoculation. When cotyledons were infiltrated with supernatant, necrotic lesions developed on cotyledons (**f**), but no symptoms on the negative control (PDB infiltation) (**g**). Photographs (**f** and **g**) were obtained 24 h after infiltration. The leaf infiltration were done three times, respectively

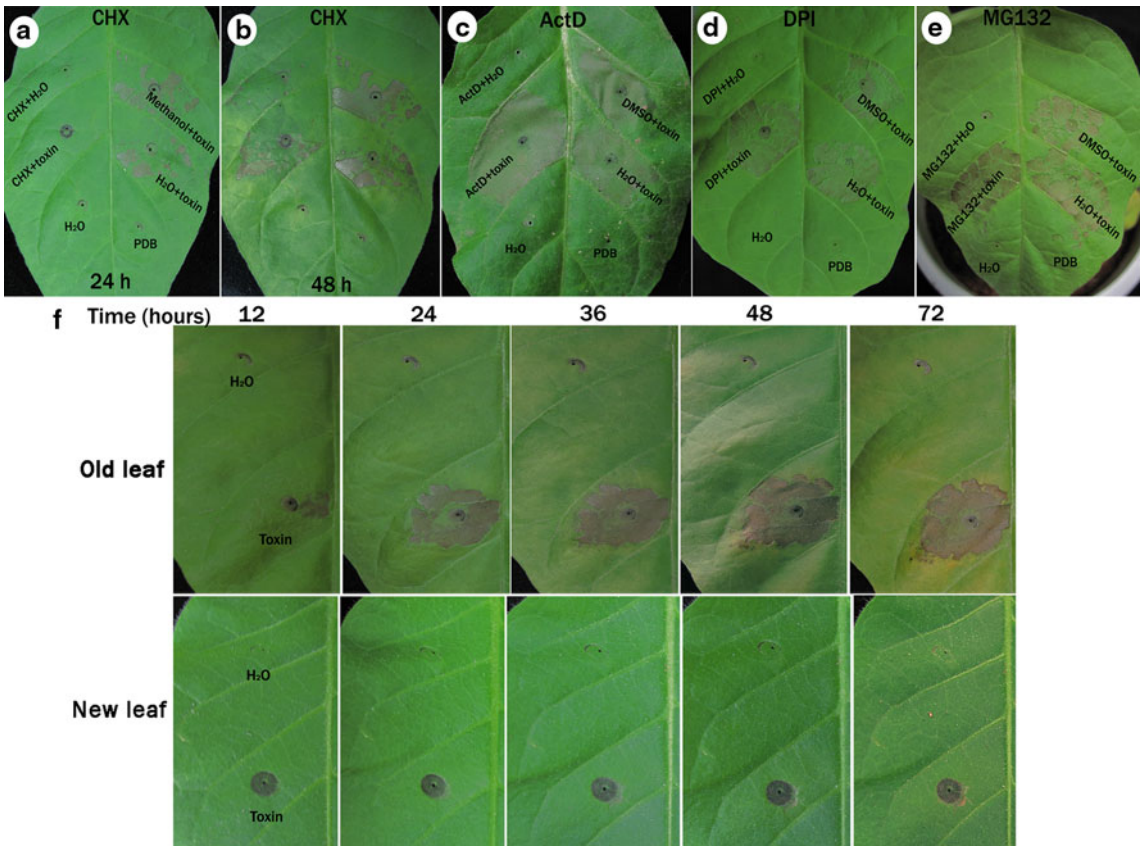

Fig. 5 Infiltration of old and new tobacco (*Nicotiana tabacum* cv. Xanthi) leaves with different inhibitors and analysis of inhibitory effect on lesion formation induced by *Verticillium dahliae* supernatant. **a** and **b** CHX (50 μg/mL) significantly inhibited lesion formation, but (**c**) ActD (100 μg/mL), **d** DPI (5 μM) and **e** MG132 (50 μM) did not. Images **a** and **c**–**e** were obtained 24 h after infiltration, image **b** was taken at 48 h after infiltration. **f** *V. dahliae* supernatant induced leaf lesions in old and young leaves. Images were taken 0, 24, 48, and 72 h after infiltration. The experiments were repeated at least three times with the same results

V. dahliae supernatant triggers cell death by an age-dependent active process requiring de novo protein synthesis

Necrotrophic pathogenic fungi are generally thought to use simpler mechanisms to destroy host tissue via a series of degrading enzymes or toxins (Oliver and Solomon 2010). However, recent studies have suggested that necrotrophic fungal pathogens can also manipulate the host during infection in subtle ways (Govrin and Levine 2000). To determine whether *V. dahliae* uses supernatant-induced cell death to manipulate its host, tobacco leaves were infiltrated with cycloheximide (CHX) (an inhibitor of protein synthesis) and actinomycin D (ActD) (an inhibitor of RNA synthesis), together with *V. dahliae* supernatant (Duval et al. 2005). CHX efficiently delayed the development of leaf lesions (Fig. 5a and b, CHX + supernatant) but ActD had no inhibitory effect (Fig. 5c, ActD + supernatant), suggesting that *V. dahliae* supernatant-triggered cell death involves an active modification of the host (requiring de novo protein synthesis rather than new gene expression) and is not only the result of toxicity.

Some necrotrophic pathogens induce the ROS burst and HR cell death to facilitate their growth in host plants (Hammond-Kosack and Rudd 2008). To test whether cotton-generated ROS facilitate *V. dahliae* infection, we infiltrated tobacco leaves with diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase and of other flavoprotein enzymes, together with *V. dahliae* supernatant. DPI did not suppress *V. dahliae* supernatant-triggered lesions to any great extent (Fig. 5d, DPI + supernatant). This finding suggests that the ROS burst was likely a plant defense response from *V. dahliae*, not the cause of cell death (Dorey et al. 1999).

Previous studies have shown that the ubiquitin/26S proteasome system plays a central role in the activation of programmed cell death (PCD) by pathogens (Bos et al. 2010). However, the proteasome inhibitor MG132 did not prevent the induction of leaf lesions by the culture supernatant (Fig. 5e, MG132 + supernatant). These results suggest that the culture supernatant actively induces plant cell death by modifying protein synthesis in the host. This modification, however, does not involve the ROS burst or the ubiquitin/26S proteasome system.

Interestingly, we observed that *V. dahliae* supernatant did not cause lesions on young leaves (Fig. 5f, young leaves). Moreover, lesions formed quicker on older leaves (Fig. 5f, old leaves), indicating that the supernatant-triggered cell death is correlated with leaf age. This finding is consistent with the observation that lesions and defoliation start at the base (old leaves) and move to the apex (young leaves) of the infected plant. However, the molecular mechanism is still unclear and needs further study.

In the present study, we investigated differential expression of proteins in cotton roots infected by *V. dahliae*. Some ROS-related proteins were upregulated after *V. dahliae* infection, and other proteins involved in normal metabolism or cell structure were downregulated. We further detected the release of ROS and death of cells in the cotton vasculature. Leaf wilt and brown discoloration of the vascular tissues were also caused by the supernatant of a *V. dahliae* culture. We used several inhibitors to analyze the mechanism by which the culture supernatant induced cell death in tobacco and found that lesion formation in tobacco leaves required de novo protein synthesis but not active gene expression. The fungus thus seems to regulate host protein synthesis to successfully infect cotton. Robb et al. (2012) considered that Verticillium wilt actually may be the result of an exaggerated plant response induced by *V. dahliae*. Based on the present results, however, toxins play a crucial role of Verticillium wilt, but the effective molecule or effectors in the toxins remain a scientific puzzle. Overall, our results suggest a new view of Verticillium wilt, and we will focus on identifying effectors of *V. dahliae* in further research.

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