Plant Science 185–186 (2012) 176–184

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Proteomic identification of differentially expressed proteins in *Gossypium thurberi* inoculated with cotton *Verticillium dahliae*

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ARTICLE INFO

Article history:
Received 19 August 2011
Received in revised form 11 October 2011
Accepted 11 October 2011
Available online 14 October 2011

Keywords:
Gossypium thurberi
Cotton *Verticillium dahliae*
Proteome
Two-dimensional electrophoresis
Mass spectrometry
Genetically modified crops

ABSTRACT

Thurber's cotton (*Gossypium thurberi*) is the wild relative of cultivated cotton. It is highly resistant to cotton Verticillium wilt, a disease that significantly affects cotton yield and quality. To reveal the mechanism of disease resistance in *G. thurberi* and to clone resistance-related genes, we used two-dimensional electrophoresis (2-DE) and tandem time-of-flight mass spectrometry (MALDI-TOF-MS) to identify differentially expressed proteins in Thurber's cotton after inoculation with *Verticillium dahliae*. A total of 57 different protein spots were upregulated, including 52 known proteins representing 11% of the total protein spots. These proteins are involved in resistance to stress and disease, transcriptional regulation, signal transduction, protein processing and degradation, photosynthesis, production capacity, basic metabolism, and other processes. In addition, five disease resistance proteins showed intense upregulation, indicating that resistance genes (R genes) may play a critical role in resistance to Verticillium wilt in Thurber's cotton. Our results suggest that disease and stress resistance are the combined effects of multiple co-expressed genes. This provides a basis for further, detailed investigation into the mechanisms underlying Verticillium wilt resistance of *G. thurberi* and for cloning essential genes into cotton cultivars to produce Verticillium wilt resistant plants.

1. Introduction

As a crop, cotton is hugely important. It is not only the main raw material for the textile industry but is also essential to national security. In China, Verticillium wilt is the primary disease attacking cotton crops. It has a strong adverse effect on cotton yield, quality, and production. It leads to discoloration of the cotton leaves and stems' vascular bundles, inhibits photosynthesis and increases respiration [1]. Verticillium wilt is caused by the Verticillium fungus pathogen, *Verticillium dahliae*. The coexistence of defoliating and non-defoliating strains of *V. dahliae* makes it difficult to explicate the pathogenic function. It is known that the defoliating strains can cause symptoms typical of Verticillium wilt, that they lead to the complete defoliation of infected plants, and that they are more virulent than other strains, but the genetics and molecular biology of Verticillium-host interactions still remain unknown.

Breeding Verticillium-wilt-resistant cotton is the most economical, environmentally friendly, and effective means of controlling Verticillium wilt. The fact that the genetic mechanisms of resistance to this cotton disease remain unknown may be why attempts to breed Verticillium-wilt-resistant cottons from existing cultivars have been unsuccessful. Thurber's cotton (*Gossypium thurberi*), the wild relative of domestic cotton, is highly resistant to Verticillium wilt. It could be used as a source of resistance in breeding. However, hybridization between distantly related species (such as between wild and cultivated cotton species) is extremely difficult.

Genetic engineering offers one alternative to conventional breeding methods. Introducing plant genes that confer resistance can lead to improved resistance to pathogens, insects, and herbicides. Several attempts have been made to generate transgenic cottons with a higher tolerance to Verticillium wilt. For example, a bean chitinase gene was transferred into cotton plants, and it was found that crude leaf extracts from this transgenic cotton line inhibited the growth of *V. dahliae in vitro* [2]. Furthermore, a transgenic cotton line overexpressing foreign *Gastrodia* anti-fungal protein was more resistant to Verticillium wilt than untransformed cotton [3]. Transferal, through agrobacterium-mediated transformation, of *hpa1Xoo* into cotton line Z35, generated a transgenic cotton line (T-34) with improved resistance to *V. dahliae*. Cells of the transgenic T-34 cotton line, when mixed with the conidia suspension of *V. dahliae*, showed higher tolerance to *V. dahliae* than cells of untransformed Z35 [4].

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doi:10.1016/j.plantsci.2011.10.007

A thorough understanding of the molecular biology of Verticillium-cotton will reveal more candidate genes for genetic engineering. In recent years, proteomics has had a profound effect on the study of plant biology. It has seen use in many studies. For example, it has been used to explore plant developmental mechanisms, such as the mechanisms involved in seed filling and in cell differentiation and de-differentiation [5,6]. Proteomics has also provided a means of studying signal transduction mechanisms, such as hormone signal transduction in the brassinosteroid, ABA, and jasmonate signaling pathways [7–9]. In addition, proteomics has been applied to the study of light signal transduction and the examination of plant responses to various kinds of environmental stressors, including cold, heavy metals, and pathogens [10–12]. However up until now, there has been no published study that used proteomics to investigate the response of plants to the pathogen *V. dahlia*.

In this study, we used two-dimensional electrophoresis (2-DE) and tandem TOF mass spectrometry (MALDI-TOF-MS) technology to identify differentially expressed proteins in Thurber's cotton inoculated with *V. dahliae*. Our findings may be helpful in revealing the mechanisms of disease resistance in cotton, in cloning key genes, and in developing methods of breeding *Verticillium*-resistant cotton lines.

2. Materials and methods

2.1. Thurber's cotton (Gossypium thurberi)

Thurber's cotton seeds were provided by the Cotton Research Institute of the Chinese Academy of Agricultural Sciences. The seed surfaces were immersed in 0.1% mercuric chloride solution for 8 min and rinsed with sufficient sterile water. They were then planted in culture soil in a bottomless bowl (pre-autoclaving, building sand: vermiculite = 6:4), which was placed in a greenhouse (day temperature 28 ± 2 °C, night temperature 25 ± 2 °C, relative humidity 60%) for seed germination. Inoculation was performed after the expansion of the second true leaf. To ensure uniformity of the test materials, 3–5 seedlings per bowl were collected and stored for future use.

2.2. Cotton Verticillium dahliae

V. dahliae isolates (W strain) were provided by Dr. Min Wang (College of Plant Protection, Henan Agricultural University). After in-plate activation, *V. dahliae* was transferred to liquid medium and cultured for 10 days (200 rpm, 25 °C). Then, after filtration through 4 layers of sterile gauze, *V. dahliae* was diluted to a 1.2×10^7 spores/mL suspension with sterile water.

2.3. Inoculation and sampling

A total of 12 bowls (54 seedlings) were selected and the bottoms of the roots were ground moderately to ensure consistent inoculation. Seedlings were then placed in sterile dishes. *V. dahliae* suspension was inoculated immediately into six bowls and sterile water into the other six bowls. Inoculation method: 15 mL of spore suspension or sterile water was injected into each dish with disposable medical syringes for natural absorbance. The indoor conditions were as follows: day temperature, 25 ± 2 °C; night temperature, 22 ± 2 °C; relative humidity, 80%. These conditions were chosen to facilitate spore germination and mycelial infection. After 24 h of inoculation, the hypocotyl between the seedling root and cotyledons was cut open, rinsed in sterile water quickly, and the stem surfaces were dried with absorbent paper. The stems were then immediately frozen in liquid nitrogen and transferred to an −80 °C refrigerator for protein extraction.

2.4. Protein extraction

A total of 20 mg of inoculated or control cotton stem was ground to powder in a mortar with quartz sand in liquid nitrogen. Then, 4 mL of TCA/acetone was added for homogenization before the content was centrifuged at 15,000 *g* for 3 min at 4 °C. The supernatant was discarded, and the precipitate was washed two to three times with cold 80% acetone and centrifuged at 15,000 *g* at 4 °C for 3 min. Next, 1 mL of 1% SDS was added to dissolve the precipitate on ice, followed by centrifugation at 15,000 *g* at 4 °C for 3 min. The supernatant was then transferred. An equal volume of Tris–phenol was added to the supernatant before centrifugation at 15,000 *g* at 4 °C for 3 min. Five volumes of 0.1 M ammonium acetate/methanol solution were added and gently mixed with the transferred upper phenol phase before incubation for 30 min (or overnight) at −20 °C. The phenol phase was then centrifuged at 15,000 *g* at 4 °C for 10 min, and the precipitate was washed twice with cold alcohol. After air-drying, the precipitate was dissolved in the hydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, and 1% IPG buffer). The protein concentration was measured using the Bradford method, and an ND-2000 spectrophotometer (Thermo Scientific, U.S.A.). The protein samples were stored at −20 °C for future use.

2.5. Two-dimensional electrophoresis

Two hundred and fifty microliters of solution (700 μg) was loaded into the IPG strips pre-hydrated overnight (11 cm, pH 4–7, GE Healthcare, OK, U.S.A.) for isoelectric focusing using the Ettan III system (GE Healthcare) (20 °C, 300 V 1 h, 3000 V 1 h, 6000 V 10 h). After focusing, the strips were balanced with buffer (0.1 M Tris–HCl (pH 8.8), 2% SDS, 6 M urea, 30% glycerol, and 0.1 M DTT) for 15 min, followed by another 15 min of balancing after the addition of 0.25 M iodoacetamide. Then the proteins were separated on 12.5% SDS-PAGE gel (20 cm × 15 cm × 0.1 cm). The gels were stained with 0.1% (w/v) Coomassie brilliant blue (CBB) for 24 h and washed in 10% ammonium acetate (v/v) for bleaching. The images were processed using BioRad PDQuest software. Protein spots that showed no less than onefold of change in expression were selected for MALDI-TOF analysis.

2.6. Tandem mass spectrometry

Protein spots were cut by hand. They were then reduced in 10 mM of DTT, alkylated in 50 mM iodoacetamide, digested in 10 μg/μL trypsin (Promega, Madison, WI, U.S.A.), and digested in 50 mM ammonium bicarbonate at 37 °C for 16 h. The supernatant was vacuum-dried and resuspended in 10 μL of 0.1% trifluoroacetic acid. Then 0.5 μl of matrix (5 mg/mL 2-5-dihydroxy benzoic acid/(water:acetonitrile 2:1)) was added to 0.5 μl of dissolved substance. The protein fragments were analyzed using the Ettan MALDI-TOF Pro mass spectrometer (GE Healthcare, U.S.A.). The particle acceleration voltage was 20 kV. Two automatic enzymatic products of trypsin were used for internal controls in each mass spectrometry analysis. The mass and sequences of the obtained peptide were matched automatically using the Mascot algorithm in the NCBI non-redundant database (http://www.matrixscience.com). The search parameters were as follows: all amide methylation of cysteine, partial oxidation of methionine, peptide molecular weight tolerance (±1.2 Da), fragment mass tolerance (±0.9 Da), and gaps of the wrong match (2) were taken into account. Molecular weight (Mr) and isoelectric point (pI) were searched with full coverage across species. All