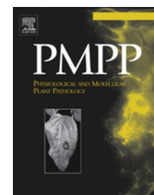
Physiological and Molecular Plant Pathology 80 (2012) 50–57

Contents lists available at SciVerse ScienceDirect

Physiological and Molecular Plant Pathology

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



Comparative expression analysis in susceptible and resistant *Gossypium hirsutum* responding to *Verticillium dahliae* infection by cDNA-AFLP

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ARTICLE INFO

Article history:
Accepted 17 September 2012

Keywords:
Verticillium dahliae
Gossypium hirsutum
cDNA-AFLP
Transcript-derived fragments
Quantitative real-time PCR

ABSTRACT

Verticillium wilt caused by the soil-borne fungus *Verticillium dahliae* (*V. dahliae*), is a devastating disease of cotton, leading to serious loss of lint yield worldwide. To study its resistance responses in both susceptible (Ejing No. 1) and resistant (NJ0703 and NJ0705) upland cotton cultivars (*Gossypium hirsutum*), cDNA-AFLP analysis was used to identify differentially expressed transcripts from resistant and susceptible cultivars that were infected with *V. dahliae* strain V991. A total of 83 transcript-derived fragments (TDFs) were obtained using 64 pairs of primer combinations. Interestingly, none of the differentially expressed fragments identified from susceptible cultivar Ejing No. 1 was found from resistant cultivars (NJ0703 and NJ0705). However, there were some similarities between NJ0703 (R) and NJ0705 (R), and 10 differentially expressed fragments were identified from both two resistant cultivars. The results indicated that the susceptible and resistant upland cottons responded differently to Verticillium infection. Moreover, the expression of transcripts was further validated through quantitative real-time PCR. Data showed that the activation of the transcripts was rapid and transient upon *V. dahliae* infection.

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1. Introduction

Verticillium wilt is caused by the soil-borne fungus *Verticillium dahliae* (*V. dahliae*). It is a devastating disease of cotton, leading to serious loss of lint yield worldwide [4,37]. Typical symptoms of Verticillium wilt in cotton include marginal chlorosis or necrosis in leaves, discoloration of stem vascular bundles, defoliation and terminal dieback [5]. In China, more than 200 million hectares of cotton planting areas are subjected to Verticillium wilt, resulting in a tremendous economic loss every year [27]. However, no efficient chemical control is available against this pathogen [14,23]. Therefore, the usage of wilt-tolerant/resistant cultivars becomes the primary method to manage this disease. Our laboratory is committed to breed and cultivate wilt-tolerant/resistant upland cotton cultivars, and several wilt-tolerant/resistant cultivars have been successfully developed. We performed the resistance evaluation in the field using 10 upland cotton cultivars from 2006 to 2008. Among these cultivars, the relative disease indexes (RDI) of NJ0703 and NJ0705 are 7.00 ± 0.38 and 4.97 ± 0.39,respectively. Therefore, these two cultivars are deemed to be highly resistant cultivars to Verticillium wilt due to their RDI values lower than 10.0. In the field, resistant cultivars showed delayed symptoms and retarded disease progression. Resistant cultivars can also be colonized by the fungus, but they demonstrate quicker induction of a series of basal defense responses than susceptible ones [10,16,42]. With the rapid development of molecular biological technology, it is possible to better understand the molecular mechanism of plant–pathogen interaction. Plants can protect themselves against pathogen attack by inducing sophisticated defense mechanisms through a complex perception, transduction and exchange of signals [3,43].

During the past decades, different methods have been made in the molecular characterization of defense responses in cotton plants upon *V. dahliae* infection. Hill et al. (1999) [18] constructed a cDNA library from root tissues of upland cotton after inoculation with *V. dahliae*, and they screened several genes responsible for defense response. Suppression subtractive hybridization technique has been used to isolate differentially expressed ESTs from *Gossypium barbadense* [45,49] and *Gossypium hirsutum* [47] during Verticillium wilt defense process. Wang et al. (2011) [44] and Zhao et al. (2012) [48] studied defense responses in *G. barbadense* and *Gossypium thurberi* upon *V. dahliae* infection using proteomic analysis, respectively. Among these, many genes that participate in a complex molecular network of regulation have been identified.

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http://dx.doi.org/10.1016/j.pmpp.2012.09.001

These include components of defense responses and other various stresses, transcriptional regulation factors, signal transduction components, regulators of primary and secondary metabolisms, lipid transport components and cytoskeleton reorganization components, and most of these genes are identified from *G. barbadense*. Moreover, the cotton genome is complicated, and the comprehensive understanding of the cotton defense response to *V. dahliae* still remains limited.

The complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) technique is a valid method for sorting out differentially expressed genes. It has been successfully employed in various pathogen-host systems for identification of differential gene expression, and it helps clarify the molecular mechanism of interaction [13,15,38]. In cotton, cDNA-AFLP is mainly used to study transcriptome profiling of cotton fiber [9,28,35] and identify genes involved in cotton yield [25]. This method has several advantages, such as its lower false positive rate, effectiveness in detecting even the poorly expressed genes and quick comparison of a variety of materials. Therefore, we employed cDNA-AFLP to identify the specifically expressed transcripts from root tissues of susceptible and resistant *G. hirsutum* after inoculation with *V. dahliae*. In the present study, we aimed to investigate whether the mechanism against Verticillium was different between susceptible and resistant *G. hirsutum*. Our work addressed three major issues as follows: i) a general screening of gene expression was performed to identify differentially expressed genes in susceptible and resistant *G. hirsutum*; ii) susceptible and resistant *G. hirsutum* had different mechanisms against Verticillium infection, while resistant cultivars might have similar mechanism against Verticillium infection; iii) those genes in resistant *G. hirsutum* were involved in cotton defense responses upon Verticillium infection, which had variations in the gene expression rather than at the sequence level.

2. Materials and methods

2.1. Plant material

Three upland cotton (*G. hirsutum*) cultivars were used in this study, including NJ0703, NJ0705 (Verticillium wilt-resistant upland cotton) and Ejing No. 1 (Verticillium wilt-susceptible upland cotton). As mentioned above, NJ0703 (R) and NJ0705 (R) were wilt-resistant cultivars. Resistance evaluation showed that the RDI of Ejing No. 1 was 51.27 $\pm$ 2.92, therefore, it was deemed to be a susceptible cultivar to Verticillium wilt. In order to remove the fuzz on the surface, the seeds were treated with H_2SO_4 (98%). Subsequently, they were immersed in 70% ethanol for 5 min and 5% H_2O_2 for 2 h to sterilize the surface, followed by the rinse with sterile distilled water for three times. Seed germination and seedling growth were conducted with 1/3 MS media [31] in sterile culture pots under long-day conditions (16-h photoperiod) with 26/20 °C day/night temperatures until the second true leaves appeared.

2.2. Fungal strains and inoculation procedure

The highly toxic and defoliant wild type pathogenic *V. dahliae* strain (V991) was isolated from an infected upland cotton and used for inoculations [47]. For conidial production, V991 was subcultured from potato dextrose agar plates onto Czapek's medium (containing 2 g $NaNO_3$, 1 g K_2HPO_4, 1 g $MgSO_4 \bullet 7H_2O$, 1 g KCl, 2 mg $FeSO_4 \bullet 7H_2O$ and 30 g/L sucrose) and incubated at 25 °C for 3–5 days. Fungal cultures were filtered through sterile gauze to retain the mycelia. The inoculum suspension was adjusted to a final concentration of 10^7 conidia/mL, and the inoculation was performed by root dipping for 30 min. Moreover, additional seedlings were mock-inoculated appropriately with sterile distilled water as non-inoculated controls. The root tissues from the inoculated and non-inoculated plants were harvested at various time points and immediately frozen in liquid nitrogen.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the non-inoculated roots and inoculated roots collected at different V991 inoculation time points (6 h, 12 h, 24 h, 36 h and 48 h) by a modified guanidinium thiocyanate method [47]. The RNA integrity was assessed using agarose gel electrophoresis and spectrophotometrically examined according to its A260/A280 absorption (Perkin–Elmer, USA). The Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, US) was used to separate mRNA. Poly$(A)^+$ RNA was used as the template for double-stranded cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, US).

2.4. cDNA-AFLP reaction and PAGE analysis

cDNA-AFLP and silver-staining processes were performed as described by [2]. First, cDNA was digested by *Eco*R I and *Mse* I, and then ligated by *Eco*R I and *Mse* I double-stranded adapters (Table 1). Pre-amplification primers corresponding to *Eco*R I and *Mse* I adapters were used in the amplification. Briefly, the pre-amplification reaction was carried out with 30 cycles at a melting temperature of 94 °C for 30 s, an annealing temperature of 56 °C for 30 s, and an extension temperature of 72 °C for 1 min. Subsequently, the amplicons from the pre-amplification were used for selective amplification. A total of 36 cycles were performed in the selective amplification, including 11 touchdown cycles with a reduction of the annealing temperature from 65 to 56 °C, 0.7 °C per cycle, and then the reaction was maintained at 56 °C for 22 cycles. Table 1 shows the 64 primer combinations used for the selective amplification. The amplicons from the selective amplification were separated on a 5% PAGE at 60 W and 50 °C until bromophenol blue reached the bottom. The bands were stained with silver nitrate.

2.5. Isolation and sequencing of cDNA-AFLP fragments

Fragments corresponding to differentially expressed transcripts on PAGE were cut out using a clean razor blade, and then they were

Table 1
Adapters and primers used in AFLP analysis.

Name	Oligonucleotide sequence (5′ to 3′)
EcoR I adaptor (forward strand)	CTCGTAGACTGCGTACC
EcoR I adaptor (reverse strand)	AATTGGTACGCAGTC
Mse I adaptor (forward strand)	GACGATGAGTCCTGAG
Mse I adaptor (reverse strand)	TACTCAGGACTCAT
EcoR I pre-amplification (E01)	GACTGCGTACCAATTCA
EcoR I amplification (E32)	GACTGCGTACCAATTCAAC
EcoR I amplification (E33)	GACTGCGTACCAATTCAAG
EcoR I amplification (E35)	GACTGCGTACCAATTCACA
EcoR I amplification (E36)	GACTGCGTACCAATTCACC
EcoR I amplification (E37)	GACTGCGTACCAATTCACG
EcoR I amplification (E38)	GACTGCGTACCAATTCACT
EcoR I amplification (E40)	GACTGCGTACCAATTCAGC
EcoR I amplification (E41)	GACTGCGTACCAATTCAGG
Mse I pre-amplification (M02)	GATGAGTCCTGAGTAAC
Mse I amplification (M47)	GATGAGTCCTGAGTAACAA
Mse I amplification (M48)	GATGAGTCCTGAGTAACAC
Mse I amplification (M49)	GATGAGTCCTGAGTAACAG
Mse I amplification (M50)	GATGAGTCCTGAGTAACAT
Mse I amplification (M59)	GATGAGTCCTGAGTAACTA
Mse I amplification (M60)	GATGAGTCCTGAGTAACTC
Mse I amplification (M61)	GATGAGTCCTGAGTAACTG
Mse I amplification (M62)	GATGAGTCCTGAGTAACTT

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