ISOLATION AND CHARACTERIZATION OF TWO NOVEL DIRIGENT-LIKE GENES HIGHLY INDUCED IN COTTON (*GOSSYPIUM BARBADENSE* AND *G. HIRSUTUM*) AFTER INFECTION BY *VERTICILLIUM DAHLIAE*

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SUMMARY

The Suppression Subtractive Hybridization (SSH) method was applied to construct a cDNA library from *Gossypium barbadense cv.* 'Pima 90'. Two dirigent-like (DIR) genes, Gbd 1 and Gbd 2, were isolated from the library. RT-PCR and Northern blot showed that they were induced after infection by *Verticillium dahliae*. The nucleotide sequences of *Gbd 1* and *Gbd 2* were 705 bp and 801 bp long with open reading frames of 531 bp and 525 bp, respectively. Sharing high homologies to disease resistance-responsive family proteins of *Arabidopsis thaliana*, *Gbd 1 and Gbd 2* shared a DIR-conserved domain and possessed a hydrophobic N-terminal signal peptide. *Gbd 1 and Gbd 2* encode a class of cell-surface proteins with signals for receptor-mediated plant defense/immunity activities. The possible function of the genes and the mechanism of disease-resistance of 'Pima 90' are discussed.

Key words: Verticillium wilt, *Gossypium barbadense*, *Gossypium hirsutum* DIR genes, disease-resistance response.

INTRODUCTION

Verticillium wilt, caused by fungi of the genus *Verticillium*, is widespread, affects over 400 different plants worldwide, and accounts for significant losses in many major crops. In China, for instance, nearly 100 million ha of cotton suffer yearly from this disease and the losses have increased significantly in recent years. Effective control measures are few, for instance, management of irrigation can reduce, but not eliminate yield and quality losses caused by this disease. Only relative tolerance (based on symptom severity) but no immunity has been found in a germplasm survey of upland cotton (*Gossypium hirsutum*) which is responsible for more than 90% of world cotton production (Jian *et al.*, 2003).

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Some cotton species like *G,. barbadense* L. (sea-island cotton) are believed to hold resistance (R) genes (Ma *et al.*, 2000). In 'Pima 90', a cultivar of sea-island cotton, resistance to Verticillium wilt is conferred by a single dominant resistance gene, and two major QTLs have been mapped (Gao *et al.*, 2003). Studies to identify R genes to this disease in plants have rarely been successful with the exception of tomato where two *Ve* genes have been isolated through positional cloning (Kawchuk *et al.*, 2001).

To disclose molecular interactions associated with resistance of 'Pima 90', a cDNA library was constructed utilizing the Suppression Subtractive Hybridization (SSH) method (Diatchenko *et al*, 1996) and some disease-resistance response genes were identified (Zhu *et al.*, 2005a). Among them, two genes (denoted *Gbd1* and *Gbd2*) share a dirigent conserved domain with the disease-resistance responsive family proteins of *Arabidopsis thaliana*. Dirigent proteins are considered to mediate the free radical coupling of monolignol plant phenols in plant to yield lignans and lignins (Davin *et al.*, 1997; Burlat *et al.*, 2001) and some dirigent proteins have been shown to be involved in the plant disease-resistance response (Fristensky *et al.*, 1988). To better understand the possible role of these two genes in disease-resistance response, we cloned these two genes and analyzed their expression in cotton.

MATERIALS AND METHODS

Plants, pathogen and infection. Seeds of 'Pima 90' (*G. barbadense*, resistant) and 'Ejing 1' (*G. hirsutum*, highly susceptible) were sown in pots (about 250 ml in volume) in the greenhouse under natural light at 23-28°C. An isolate of *Verticillium dahliae* Kleb., strain V_{991}, kindly donated by Guiliang Jian (Plant Protection Research Institute, CAAS, Beijing, China) was cultured in Czapek liquid medium for 10 days at 25°C. The conidial suspension obtained was diluted with Czapek solution to 10^6 conidia ml^{-1}. Seedlings of 'Pima 90' and 'Ejing 1' with two fully expanded leaves were inoculated with the pathogen according to Jian *et al.* (2001) by adding 10 ml conidial suspension into each pot. Control

seedlings were inoculated with water. The roots of 'Pima 90' were then collected for total RNA extraction at 2, 4, 8, 12, 24, 48, 72 and 96 h after inoculation.

Seedlings with roots removed were dipped in Czapek liquid medium containing 10^4 conidia ml^{-1} for further disease-resistance response investigation according to the method of Ma *et al*. (2004). Root-cut seedlings dipped in water without conidia were used as control.

RNA extraction. Total RNA was extracted in bulk from pooled roots from several seedlings according to a modified protocol (Zhu *et al.*, 2005b). The main steps included extraction of RNA by guanidinium thiocyanate, removal of proteins and other impurities by chloroform-isoamylalcohol without phenol, precipitation and resuspension of RNA, and purification of RNA with phenol and chloroform-isoamylalcohol.

Construction and differential screening of the cDNA library. Suppression Subtractive Hybridization (SSH), highly effective technique, was employed to isolate the differentially expressed cDNAs in 'Pima 90' in response to *V. dahliae* infection. We refer to the cDNAs from inoculated plants as tester and the reference cDNAs from uninoculated plants as driver. SSH was applied following the manufacturer's instructions (Clontech, Mountain View, USA). The resulting PCR product was enriched in tester-specific cDNAs and was then directly inserted into a T/A cloning vector. Plasmid clones were extracted and arrayed on Hybond N^+ membranes (Amersham Pharmacia, Little Chalfont, UK). Probes, cDNAs from inoculated and control 'Pima 90', respectively, were applied to screen the positive genes expressed differentially during inoculation.

The positive clones were sequenced with an ABI 3730 DNA analyzer (ABI, Foster City, USA).

Transcription analyses. Total RNA from roots was separated by gel electrophoresis, transferred to Hybond N^+ membranes (Amersham Pharmacia, Little Chalfont, UK) and hybridized with the reverse transcriptase PCR (RT-PCR) product obtained from *Gbd 1* as probe labeled with [α-^{32}P]dCTP by Prime-a-Gene Labeling system (Promega, Madison, WI, USA). Expression of dirigent-like genes in cotton was also detected by RT-PCR amplification. Primers were designed from the ORF of *Gbd 1*: *Gbd 1* F (5' GGTTCTGATTATCTGCCTCT 3') and *Gbd 1* R (5' CTTACCGGTATTGAATCCA 3'). The *histone3* gene of cotton was used as a control.

First-strand cDNAs were synthesized from total RNA by M-MLV reverse transcriptase (Promega Corp, Madison, WI, USA) and PCR was performed in 20 µl volumes containing the following reagents: 1µl 1x PCR buffer, 0.2 mM dNTPs, 50 ng cDNA, 25 nM each primer, 1 U Taq polymerase (Promega Corp, Madison, WI, USA). PCR conditions were: 1 step of 94°C for 1 min; 33 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s; final extension at 72°C for 5 min. The products were separated on a 1.5% agarose gel.

Sequence analysis. Blastn and tBlastx programs were used to search for similarity in NCBI databases, and Bl2seq was utilized to compare sequences of the two genes (Altschul *et al.*, 1997; Tatusova and Madden, 1999). Bioinformatic methods were employed to analyze the protein sequence for signal peptides, motifs, transmembrane helices, subcellular location, cellular role and gene ontology category (Emanuelsson *et al.*, 2000; Jensen *et al.*, 2002, 2003; Bendtsen *et al.*, 2004)

RESULTS

Screening cotton for genes responsive to Verticillium wilt. Cultivars 'Pima 90' and 'Ejing 1' reacted differently to infection by *V. dahliae*: wilting and chlorosis of leaves were found in 'Ejing 1', while 'Pima 90' showed no symptoms 15 days after inoculation (DAI). 'Ejing 1'root-cut seedlings dipped in the conidial solution died 30 DAI, whereas 'Pima 90' stayed green and regenerated fresh roots. All control plants without inoculation remained alive. These results confirmed that 'Pima 90' was highly resistant to *V. dahliae*. A total of 78 clones which were up-regulated and potentially involved in the disease-resistance response were isolated from the SSH library by array screening. Sequence similarity searches showed that a number of clones classified as up-regulated were analogous to genes in the disease resistance-responsive protein family and glutathione s-transferase. Genes with proposed roles in cellular metabolism or regulation were also identified, such as MYB transcription factor, transducin family protein/WD-40 repeat family protein and Cys2/His2-type zinc finger protein. Two genes sharing high homology to an auxin-repressed protein and an enzyme of gibberellin biosynthesis, copalyl diphosphate synthase, which participate in the metabolism of phytohormones, were also isolated.

Characterization of two novel dirigent-like genes in cotton. Two cDNAs of dirigent-like proteins were obtained and named *Gbd 1* (*G. barbadense* dirigent-like protein 1, AY560544) and *Gbd 2* (*G. barbadense* dirigent-like protein 2, DQ018709). *Gbd 1* contains a transcript of 705 bp, including a complete open reading frame (ORF) of 531 bp, which encodes a peptide of 176 amino acids, with a predicted molecular mass of 18.9 kDa and an isoelectric point of 6.34. The putative protein showed an identity of 46% with a disease resistance responsive family protein of *Arabidopsis thaliana*, and shared a conserved dirigent domain. It possessed a putative signal peptide cleavage site which immediately followed the part of the transmembrane helix predicat-

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Gbd1 mrgtlml swv liiclslvav  qsqYYSETLP  YRPRPVKVTN LHFFMLEFTG ITAVQVAQVN   [60]
Gbd2 .... sv...i .... vcq... r.....D... .Q....L... ...Y.H.... T...VLT.A.   [60]

Gbd1 ITSSDNNSSV PFASLVAVND PLRTGPEPDS ELIGNVQGIA LLAGINASST QYIDFGFNTG   [120]
Gbd2 ... --..... ...T..... ..........  .........S ....S..... ..........   [120]

Gbd1 KLN GSSLSVF SRGEPGLAVV GGRGRFMMAT GVALFNPILI NATNVIIEFN VTVIHY       [176]
Gbd2 .F........ ....A..... ....Q.A... .T.....L.. ..........  ......       [176]
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Fig. 1. Alignment analysis of the deduced amino acid sequence of *Gbd 1* and *Gbd 2*. Amino acid identity is indicated by dots and a gap is indicated by -. Amino acids constituting putative signal sequences are shown in lower case.

ed in its hydrophobic N-terminal. *Gbd 2* also encoded a membrane protein with a predicted molecular mass of 18.6 kDa and an isoelectric point of 6.59, consisting of an N-terminal transmembrane segment of 16 residues and a signal peptide of 23 residues, just like *Gbd 1*. A further 96-base sequence and two signals (AATAAA) for poly A were found in the 3' untranslated region of *Gbd 2*. ProtFun 2.2 predictions showed that the two proteins may play roles in the stress or immune response of plants. *Gbd 1* and *Gbd 2* shared identities of 82% in full length, while the 525 bp ORF of *Gbd 2* shared 90% identity to the ORF of *Gbd 1* with a 6-base gap. Moreover, the deduced proteins of *Gbd 1* and *Gbd 2* shared 84% identity and 91% positives (Fig. 1).

Expression analysis. Results of Northern blot analysis showed that in 'Pima 90', *Gbd 1* and *Gbd 2* were highly induced by the pathogen; expression of the two genes was induced at 4 h after infection with a peak at 12 h, and high levels persisting until 96 h from inoculation (Fig. 2A). A similar gene expression pattern was also found in 'Ejing 1' (Fig. 2B). Compared with 'Pima 90', expression was slightly delayed at 4 and 12 h after inoculation. RT-PCR showed there was low constitutive expression of the genes in plant roots and confirmed the induced expression pattern observed in the Northern blot analysis (Fig. 3).

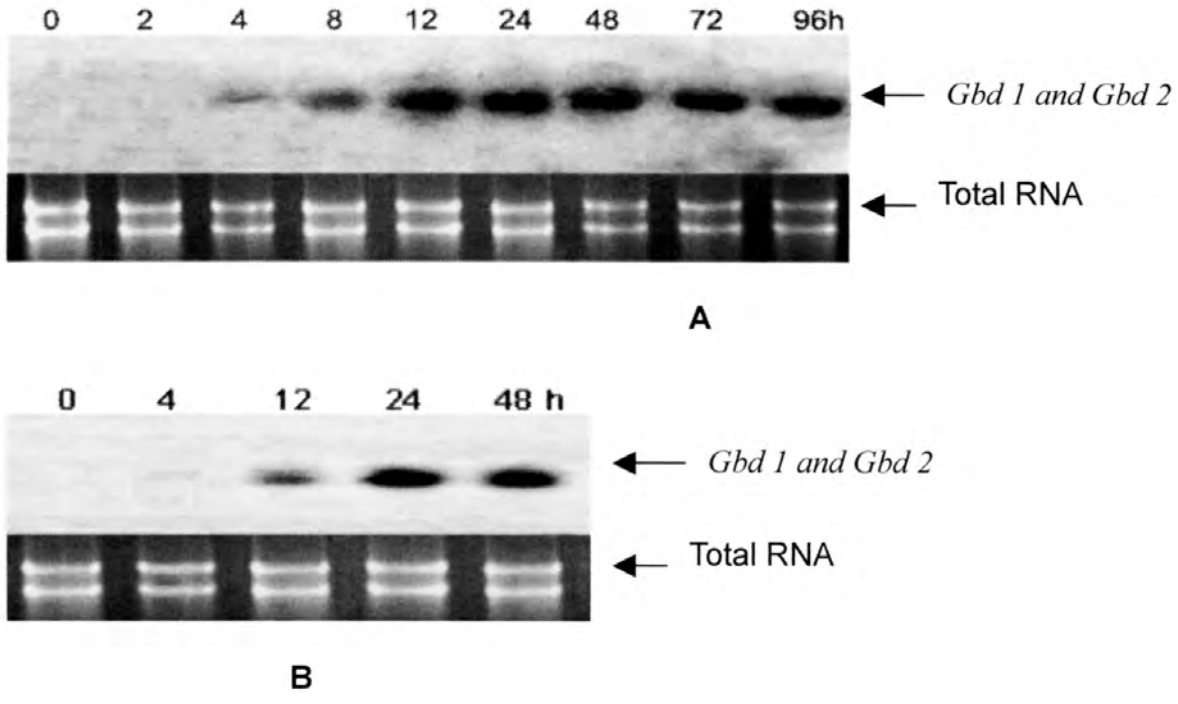

Fig. 2. Northern-blot analysis of *Gbd 1* and *Gbd 2* from RNA isolated from roots of cotton infected by *V. dahliae.* A: Northern-blot analysis of *Gbd 1* and *Gbd 2* in 'Pima 90' infected by *V. dahliae* after 0, 2, 4, 8, 12, 24, 48, 72 and 96 h, respectively. B: Northern-blot analysis of *Gbd 1* and *Gbd 2* in 'Ejing I' infected by *V. dahliae* after 0, 4, 12, 24 and 48 h, respectively. Each blot contained 10 µg of total RNA per lane and was probed with a random-primer-labeled, 0.35 kb fragment from the ORF of *Gbd 1* cDNA. Considering the high similarity of gene sequences between *Gbd 1* and *Gbd 2*, the hybridization result should represent both genes. Autoradiograph exposure time was 4 days.

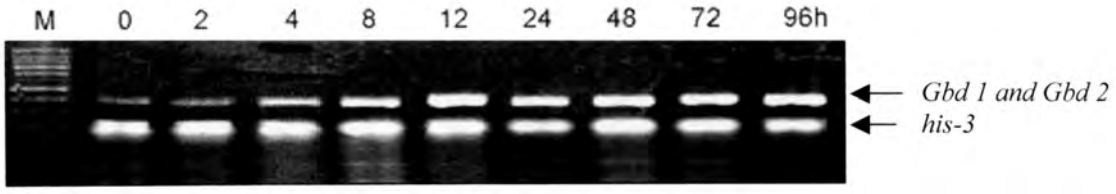

Fig. 3. RT-PCR analysis of *Gbd 1* and *Gbd 2* in 'Pima 90' following infection by *V. dahliae* after 0, 2, 4, 8, 12, 24, 48, 72 and 96 h, respectively. M: 100 bp DNA ladder. *his-3* of cotton as the control.

DISCUSSION

Genetic and molecular analysis of the resistance response to Verticillium wilt in cotton are recently reported, and most studies are focused on the gossypol pathway and phytoalexin biosynthesis (Joost *et al.*, 1995; Bianchini *et al.*, 1999; Luo *et al.*, 2001; Xu *et al.*, 2004).

Heterogeneous lignins are structural cell wall components of vascular tissues, and since cell wall reinforcement occurs in cotton hypocotyls in response to a *V. dahliae* elicitor, cotton stems with higher lignin content were thought to be more resistant or tolerant to this disease (Smit and Dubery, 1997; Davin and Lewis, 2000). The two novel cotton genes reported here have signal sequences associated with the secretory pathway and their deduced proteins possess a transmembrane domain; informatic analysis suggests location at the cell surface, like other dirigent protein genes (Gang *et al.*, 1999). The expressed proteins were present in the actively dividing cambial cell regions of stems and roots (Burlat *et al.*, 2001). The expression pattern of *Gbd 1* and *Gbd 2* indicates that they are constitutively expressed at low level and are induced quickly after *V. dahliae* infection in accordance with the need for overproduction of lignins for defense purposes. The induced expression of these genes could be helpful to improve lignin content at the infection site.

This appears to be the first report on the molecular cloning and characterization of cotton dirigent-like genes. Expression of such genes in other plants (e.g. *Arabidopsis*, sugarcane) during disease-resistant response suggests that they play important roles in plant-pathogen interaction. *G. barbadense cv* 'Pima 90' was more resistant to *V. dahliae* than *G. hirsutum cv* 'Ejing 1' in our infection experiments, and expression of dirigent-like genes in 'Pima 90' was also induced more quickly than in 'Ejing 1'. However, both cultivars made these proteins in response to infection, implying that lignins may act together in the plant immunity response, just like gossypol or phytoalexin, which are not specific to the Verticillium wilt system. Their highly induced expression could help to reinforce the cell wall, restricting or blocking pathogen invasion. Due to high similarity between *Gbd 1* and *Gbd 2*, the RT-PCR and Northern blot results should represent the expression pattern of both genes.

Analysis of the 5' or 3'-untranslated regions of these two genes may provide more information about their function in cotton. Some R genes are constitutively expressed independently of plant developmental stage, and R genes could encode enzymes able to neutralise fungal toxins, like the *Hm1* gene in maize (Meeley and Walton 1991; Century *et al.*, 1999). The different results obtained with inoculated root-cut seedlings of the two cotton cultivars may imply that resistance to Verticillium wilt could be partly due to a detoxification enzyme in 'Pima 90'. This hypothesis is under investigation.

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REFERENCES

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Bendtsen J.D., Nielsen H., Heijne G., Brunak S., 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340**: 783-795.

Bianchini G.M., Stipanovic R.D., Bell A.A., 1999. Induction of d-cadinene synthase and sesquiterpenoid phytoalexins in cotton by *Verticillium dahliae*. *Journal of Agricultural Food Chemistry* **47**: 4403-4406.

Burlat V., Kwon M., Davin L.B., Lewis N.G., 2001. Dirigent proteins and dirigent sites in lignifying tissues. *Phytochemistry* **57**: 883-897.

Century K.S., Lagman R.A., Adkisson M., Morlan J., Tobias R., Schwartz K., Smith A., Love J., Ronald P.C., Whalen M.C., 1999. Developmental control of *Xa21*-mediated disease resistance in rice. *The Plant Journal* **20**: 231-236.

Davin L.B., Lewis N.G., 2000. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. *Plant Physiology* **123**: 453-461.

Davin L.B., Wang H.B., Crowell A.L., Bedgar D.L., Martin D.M., Sarkanen S., Lewis N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* **275**: 362-366.

Diatchenko L., Lau Y.F., Campbell A.P., Chenchik A., Moqadam F., Huang B., Lukyanov S., Lukyanov K., Gurskaya N., Sverdlov E.D., Siebert P.D., 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Science USA* **93**: 6025-6030.

Emanuelsson O., Nielsen H., Brunak S., von Heijne G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300**: 1005-1016.

Fristensky B., Horovitz D., Hadwiger L.A., 1988. cDNA sequences for pea disease resistance response genes. *Plant Molecular Biology* **11**: 713-715.

Gang D.R., Costa M.A., Fujita M., Dinkova-Kostova A.T., Wang H.B., Burlat V., Martin W., Sarkanen S., Davin L.B., Lewis N.G., 1999. Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. *Chemistry and Biology* **6**: 143-151.

Gao Y.Q., Nie Y.C., Zhang X.L., 2003. QTL mapping of genes resistant to Verticillium wilt in cotton. *Cotton Science* **15**: 73-78.

Jensen L.J., Gupta R., Blom N., Devos D., Tamames J., Kesmir C., Nielsen H., Staerfeldt H.H., Rapacki K., Workman C., Andersen C.A.F., Knudsen S., Krogh A., Valencia A., Brunak S., 2002. Prediction of human protein functions from post-translational modifications and localization features. *Journal of Molecular Biology* **319**: 1257-1265.

Jensen L.J., Gupta R., Stærfeldt H.H., Brunak S., 2003. Prediction of human protein function according to Gene Ontology categories. *Bioinformatics* **19**: 635-642.

Jian G.L., Sun W., Ma C., 2001. A new method for identifying the Verticillium wilt resistance: sprinkle the strain suspension on root in non-bottom plastic film bottle. *Cotton Science* **13**: 67-69.

Jian G.L., Zhou Y.F., Ma C., 2003. Analysis and resolve of Verticillium wilt of cotton in China. *Chinese Cotton* **30**: 13-14.

Joost O., Bianchini G.H., Bell A.A., Benedict C.R., Magill C.W., 1995. Differential induction of 3-hydroxy-3-methylglutaryl CoA reductase in two cotton species following inoculation with *Verticillium dahliae*. *Molecular Plant Microbe Interactions* **8**: 880-888.

Kawchuk L.M., Hachey J., Lynch D.R., Kulcsar F., van Rooijen G., Waterer D.R., Robertson A., Kokko E., Byers R., Howard R.J., Fischer R., Prufer D., 2001. Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Science USA* **98**: 6511-6515.

Luo P., Wang Y.H., Wang G.D., Essenberg M., Chen X.Y., 2001. Molecular cloning and functional identification of (+)-d-cadinene-8-hydroxylase, a cytochrome P450 monooxygenase (CYP706B1) of cotton sesquiterpene biosynthesis. *The Plant Journal* **28**: 95-104.

Ma P., Huang H.C., Li S.Z., Tang W.H., 2004. A new inoculation method for Verticillium wilt on cotton and its application in evaluating pathogenesis and host resistance. *Acta Phytopathologica Sinica* **34**: 536-541.

Ma Z.Y., Wang X.F., Zhang G.Y., Liu S.Q., Sun J.Z, Liu J.L., 2000. Genetic studies of Verticillium wilt resistance among different types of sea island cottons. *Acta Agronomica Sinica* **26**: 315-321.

Meeley R.B., Walton J.D., 1991. Enzymatic detoxification of HC-toxin, the host-selective cyclic peptide from *Cochliobolus carbonum*. *Plant Physiology* **97**: 1080-1086.

Smit F., Dubery I.A., 1997. Cell wall reinforcement in cotton hypocotyls in response to a *Verticillium dahliae* elicitor. *Phytochemistry* **44:** 811-815.

Tatusova T.A., Madden T.L., 1999. Blast 2 sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiology Letters* **174**: 247-250.

Xu Y.H., Wang J.W., Wang S., Wang J.Y., Chen X.Y., 2004. Characterization of *GaWRKY1*, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-delta-cadinene synthase-A. *Plant Physiology* **135**: 507-515.

Zhu L.F., Tu L.L., Zhang X.L., Nie Y.C., Guo X.P., Xia Q.Z., 2005a. Construction and analysis of SSH library of *Gossypium barbadense* upon infection with *Verticillium dahliae*. *Acta Genetica Sinica* **32**: 528-532.

Zhu L.F., Tu L.L., Li H.Y., Liu D.Q., Zhang X.L., 2005b. An improved simple protocol for the isolation of total RNA from *Gossypium* spp. by guanidinium thiocyanate. *Acta Agronomica Sinica* **31**: 1657-1659.

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