SHORT COMMUNICATION

VERTICILLIUM DAHLIAE TOXINS INDUCE CHANGES IN EXPRESSION OF A PUTATIVE HISTONE H2B AND A PUMILIO/PUF RNA-BINDING PROTEIN IN *ARABIDOPSIS THALIANA*

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SUMMARY

To gain insight into the molecular mechanisms of plant defence response to *Verticillium dahliae*, we identified the genes whose expression patterns were altered during early response to *V. dahliae* toxins (VD-toxins) in *Arabidopsis* seedlings using the mRNA differential display approach. Four true positive bands (DD1, DD2, DD3 and DD4) were confirmed from the 30 differentially displayed bands. The deduced nucleotide sequence of the DD1 and DD2 clones showed strong homology with genes encoding a putative histone H2B and a pumilio /Puf RNA-binding protein, respectively. Quantitative real-time RT-PCR assays suggested that the expression pattern of genes of DD1 and DD2 in two ecotypes of *Arabidopsis* were clearly different. The results indicate that the putative histone H2B and pumilio /Puf RNA-binding protein encoded by DD1 and DD2 genes may be involved in regulation of the defence responses against VD-toxins in *Arabidopsis* and the pumilio/Puf RNA-binding protein plays a more important role in the defence responses.

Key words: *Arabidopsis*, *Verticillium dahliae* toxins, mRNA differential display, histone H2B, pumilio /Puf RNA-binding protein, quantitative real-time RT-PCR.

Verticillium dahliae is a soilborne pathogen that causes Verticillium wilt in important plant species worldwide (Bhat and Subbarao, 1999). Although the physiology of plant defence against *Verticillium* infection is well established, comprising the production of the pathogenesis-related (PR) proteins, phytoalexins and phenolic compounds (Williams *et al.*, 2002; Zhen and Li, 2004) and active expression of some plant disease response genes (Fradin and Thomma, 2006), the molecular mechanisms involved in plant defence responses to *Verticillium* remain largely unknown.

Interactions between plant and pathogens induce a series of plant defence responses. Changes in gene expression are important for activation of defence mechanisms and transcriptional activation or repression of genes, which has been reported in several plant-pathogen systems (Kazan *et al.*, 2001). Identification of genes whose expression is changed in response to pathogens, in turn, will suggest functional involvement.

It had been reported that *Arabidopsis thaliana* was susceptible to *V. dahliae* (Veronese *et al.*, 2003). Recently, we showed that the symptoms induced by *V. dahliae* toxins (VD-toxins) were similar to those induced by the fungus itself. We partially purified the VD-toxins and found that they include a glycoprotein of 35.8-83.2 kDa. The phytotoxic activity of VD-toxins remained after treatment with high temperature, conA, and proteinase (Jia and Li, unpublished). We also showed that VD-toxins altered the cytoskeletons and nucleoli in suspensions of *Arabidopsis* cells (Yuan *et al.*, 2006), and induced NO and H_2O_2 production in *Arabidopsis* and cotton (Jia *et al.*, 2007; Shi and Li, 2008). These data provide a very useful experimental access to the study of molecular mechanisms that control the *Arabidopsis* defence response against *V. dahliae*. In this report, we used an *Arabidopsis*-VD-toxins system to investigate the changes in genes expression in the early response to VD-toxins.

Two *A. thaliana* ecotypes, Columbia (Col-0, relatively susceptible to *V. dahliae*) and C24 (relatively tolerant to *V. dahliae*) were used. Seeds were sterilized and grown on MS medium at 25°C with 12 h light (100 mmol·m^{-2}·s^{-1}) and 12 h dark. After culture for 8 days, the seedlings were transferred to MS medium supplemented with 150 µg ml^{-1} VD-toxins and grown for 7 more days.

A highly infectious and non-defoliating strain of *V. dahliae* Kleb (V229) was used to extract VD-toxins. The *Verticillium* culture filtrate was purified as described previously (Jia *et al.*, 2007; Shi and Li, 2008).

Total RNA preparations from seedlings treated with VD-toxins for 1 day were obtained using the Trizol regent and used as PCR template. PCR amplification of the synthesized first strand cDNA was carried out in duplicate with 1.2 µl (10 mmol l^{-1}) dNTP and 0.7 µl *Taq* DNA polymerase (Tiangen, Beijing, China) in a typical 50 µl reaction, and with the following parameters: 94°C for 5 min, for 1 cycle, 94° for 1 min, 40°C for 4 min,

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72ºC for 4 min, for 1 cycle, 94ºC for 45 sec, 60ºC for 1 min, 72ºC for 2 min, for 35 cycles, and a final elongation step at 72ºC for 10 min. The products were separated in 4% polyacrylamide gel and silver-stained. The differential display cDNAs were eluted from the bands and used as a template for reamplification using the original corresponding primers. Re-amplified cDNAs were analyzed on a gel.

Purified re-amplified DNA was loaded onto HybondN filter (Sagon, Shanghai, China). The first strand cDNAs, were labeled with digoxigenin (DIG), using DIG high prime DNA labeling and detection starter kit (Roche Diagnostic, Indianapolis, IN, USA). The hybrids were detected by colour development (NBT/BCIP) with the DIG-detection kit (Roche). The purified PCR products were cloned into the *Eco*RV restriction site of the pMD-18T vector (Takara Bio Inc., Dalian, China). Blue/white selected colonies were screened by PCR for plasmids with inserts. Sequences were analysed with the BLAST program in the TAIR and GenBank database.

Two differentially expressed genes (DD1 and DD2) obtained by the DDRT-PCR were analyzed by real-time PCR using SYBR Premix Ex Taq ^{TM} Kit (Takara, China) on an ABI 7500 Sequence Detector (Applied BioSystems). Primer pairs for each gene were designed by ABI program "Primer Express" to amplify fragments of approximately 65 bp. cDNAs were amplified in a reaction volume containing SYBR premix Ex Taq^{TM}, PCR forward primer, PCR reverse primer and ROX reference dye, according to the manufacturer's instruction (Takara, China). The PCR was carried out at 95ºC for 10 sec, followed by 40 cycles at 95ºC for 5 sec (denaturation), and annealing at 60ºC for 20 sec.

Thirty differentially displayed bands were identified during the early response to VD-toxins in *Arabidopsis* seedlings using mRNA differential display approach. The differentially displayed bands were divided into two groups: up-regulated and down-regulated (Fig. 1).

Four true positive bands (DD1, DD2, DD3 and DD4) were confirmed from the 30 differentially displayed bands using reverse Northern dot blot hybridization. DD1 was generated with the H-T_{11}-G anchor primer and DD2, DD3 and DD4 were generated with the H-T_{11}-A anchor primer (Fig. 2). The results also revealed that DD1, DD3, DD4 gene expressions were up-regulated and DD2 down-regulated.

DD1 and DD2 were selected for further sequence analysis. The cDNA sequences of DD1 and DD2 were found to be 373 bp and 343 bp, respectively. The de-

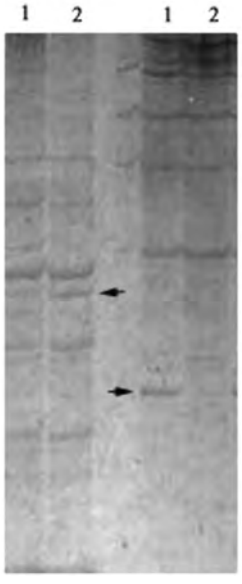

Fig. 1. The differential display gel shows the amplified products obtained with H-T_{11}-G (left) and H-T_{11}-A (right) anchor primers and random primers using as template cDNA derived from mRNA extracted from *Arabidopsis* seedlings (Col-0) treated by VD-toxins. Lane 1, control; lane 2, *Arabidopsis* seedlings treated with VD-toxins. Arrows indicate differentially expressed fragments. A typical result from three independent experiments is shown.

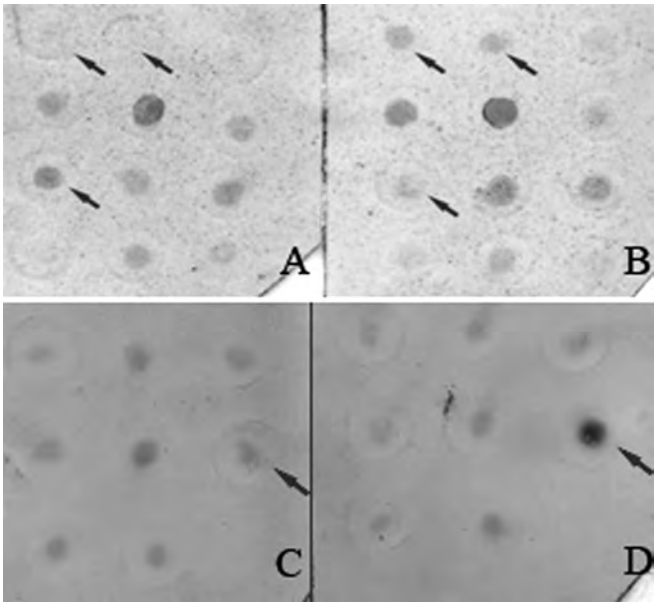

Fig. 2. Selection of clones with cDNA inserts from differentially expressed genes by reverse Northern dot blot analysis, utilizing DDRT-PCR products as hybridization probes. The probes were labeled with DIG. Total RNA was extracted from *Arabidopsis* Col-0 seedlings of control (A, C) and from seedlings treated with VD-toxins (B, D). In Fig 2A and B, the differentially expressed bands were generated with the H-T_{11}-A anchor primer. In Fig 2C and D, the differentially expressed bands were generated with the H-T_{11}-G anchor primer. Four true positive bands are indicated by arrows. Arrows on the upper line of Fig 2A and B indicate the fragments of DD3 and DD4, respectively and arrows on Fig 2C and D indicate the fragments of DD1, which were up-regulated after treatment with VD-toxins. Arrows on the lower line Fig 2A and B indicate the fragments of DD2 down-regulated after treatment with VD-toxins. A typical result from three independent experiments is shown.

duced nucleotide sequence of the DD1 and DD2 clones showed 99% identity with genes encoding a putative histone H2B (AT3G09480) and a pumilio /Puf RNA-binding protein (AT2G29104), respectively.

The quantitative real-time RT-PCR analysis showed that DD1 was up-regulated about 1.5-fold in both ecotypes of *Arabidopsis* at an early stage (6 h post-treatment). At later stages (24-48 h) after VD-toxins treatment, however, DD1 was regulated with different trends in each ecotype. In Col-0, 1.41 fold up-regulation was observed at 24 h, while a 1.39 fold up-regulation was seen in C24 at 48 h (Fig. 3A).

The differences in DD2 gene expression between Col-0 and C24 were also significant. DD2 was significantly down-regulated in Col-0 with a maximum down-regulation at 48 h of 0.5-fold compared to control. However, DD2 was up-regulated in C24 and showed a maximum up-regulation at 12 h of 1.5-2.0 fold compared to control (Fig. 3B).

Histones are small, abundant basic proteins most commonly found in association with DNA in the chromatin. Four histones, H2A, H2B, H3 and H4 are important for chromosome organization in the nucleosome. Moreover, studies have suggested that histones have additional functions, including antimicrobial defence in animals (Bergsson *et al.*, 2005).

However, few studies have shown that histones are involved in plant defence. Histone gene repression is reportedly correlated with repression of cell division, and repression of cell division would be the full requirement for transcription of defense-related genes (Logemann *et al.*, 1995). The generality of stress responses at the transcriptional level was time dependent (Swindell, 2006). Histone H2B gene expression is reduced to very low levels at 24 h after elicitor treatment in *Petroselinum crispum* (Logemann *et al.*, 1995). Similarly, histone gene families are expressed at raletively higher levels over 24-36 h after *Agrobacterium* infection, while the levels of expression of host defence response genes are significantly repressed (Jiang *et al.*, 2003). Our results indicated that the differential expression of histone H2B gene between *Arabidopsis* C24 and Col-0 ecotypes appeared mainly at a later stage (24-48 h) after VD-toxin treatment, at which time induction of the hypersensitive response (HR) and synthesis of antifungal proteins occurs (Hohl *et al.*, 1990). Therefore, it is plausible that the histone H2B protein involvement in activation of defence responses against VD-toxins and the lower expression level of histone H2B gene at 24 h after VD-toxin treatment in ecotype C24 may contribute to induce expression of defence-related genes.

Members of the Puf family of RNA-binding proteins regulate mRNA activity at the level of maturation, transport, translation and stability in a wide variety of eukaryotes (Crittenden *et al.*, 2002; Mazumder *et al.*, 2003). Processing, transport, translation and stability of

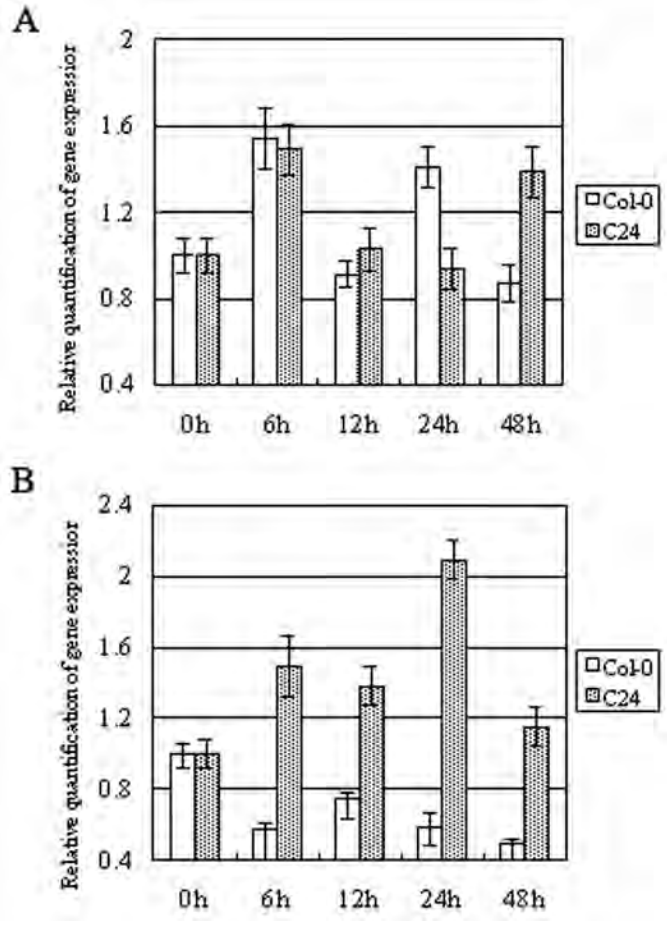

Fig. 3. Quantitative real-time RT-PCR shows the expression level of DD1 (A) and DD2 (B) in the tolerant ecotype (C24) and the susceptible ecotype (Col-0) of *Arabidopsis* seedlings treated with VD-toxins at 0, 6, 12, 24 and 48 h. The relative amounts of transcripts were normalized with respect to the level of the *actin2* gene. Two independent replicates were done and each cDNA was measured in triplicate. The columns show the mean of the two replicates and the bars show the result of each of these experiments.

mRNA can all be controlled to determine how much protein is produced from a gene, in which cell and at what time. Proteins of the pumilio family bind selectively to specific mRNAs and so repress specific genes at the mRNA level (Wickens *et al.*, 2002). Our present results show that expressions of the DD2 gene were significantly different in the two ecotypes. The higher DD2 transcription level was significantly more induced in C24 than in Col-0.

The higher level of DD2 gene expression may contribute significantly to its ability to bind selectively to specific mRNAs to regulate the defence response against VD-toxins. As suggested by Gerber *et al.* (2004), combinatorial binding of mRNAs by pumilio family proteins could allow rapid and efficient reprogramming of gene expression in response to changing physiological conditions. Our results suggest that the

pumilio /Puf RNA-binding protein could be a more important regulator to induce the defence-specific response against VD-toxins.

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