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NO and H_2O_2 induced by *Verticillium dahliae* toxins and its influence on the expression of *GST* gene in cotton suspension cells

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Nitric oxide (NO) and hydrogen peroxide (H_2O_2) have been shown to be important signaling molecules that participate in the regulation of several physiological processes. In particular, they have significant role in plant resistance to pathogens by contributing to induction defense genes. Here, whether NO and H_2O_2 participate in the resistance responses against *Verticillium dahliae* toxins (VD-toxins) and their effects on the expression of *GST* gene are studied. The results reveal that NO and H_2O_2 are produced as part of a complex network of signals that respond to VD-toxins and may converge to function both synergistically and independently by inducing resistant responses. *GST* gene is potentially involved in the resistance mechanism in the cotton suspension cells. NO induces the expression of *GST* gene independently of H_2O_2. H_2O_2 may be a more potent signal in the resistance responses against VD-toxins.

cotton suspension cells, hydrogen peroxide, *GST* gene, nitric oxide, *Verticillium dahliae* toxins

Verticillium dahliae Kleb. is a fungal pathogen that can induce *Verticillium* wilt and cause serious damage to cotton. The infection of the pathogen can induce many reactions in the cotton cells, including the production of antibiotic terpenoid phytoalexins, and structural defences such as vascular occlusion[1]. The pathogen or pathogen-derived elicitors/toxins infection can also induce active expression of some disease response genes[2−6] and production of the antifungal compounds, such as pathogenesis-related (PR) proteins and lignin[7−10]. Our recent research data demonstrated that the pathogen-derived toxins induced alteration of cytoskeletons and nucleoli in *Arabidopsis* suspension cells[11]. However, little is known of the molecular basis of the defense response of the cotton cell and the genetic determinants of resistance remain elusive.

It has been reported that the interaction of plant cells with pathogens or pathogen-derived elicitors does result in the generation of hydrogen peroxide (H_2O_2), which is one of the earliest cellular responses to potential pathogen or elicitor molecules. H_2O_2 appears to be a signaling intermediate, resulting in gene expression and programmed cell death in plant defense[12−17]. Desikan et al.[18] showed that H_2O_2 regulates at least 175 genes in *Arabidopsis*, some of which are up-regulated and others are down-regulated, implying that H_2O_2 does have multiple roles in plant responses to stress.

Nitric oxide (NO) has also been identified as an essential molecule that mediates defense gene activation in plants[19−26]. It has been demonstrated that NO cooperates with reactive oxygen species (ROS) to promote host cell death[23,27,28] and activates hypersensitive reaction (HR)[29,30]. NO generation might be important in the initiation and development of HR, and suppression of NO production increases susceptibility to attacks[31]. It was shown that NO and H_2O_2 could act either individually or

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in partnership in regulating gene expression[32].

In a previous study, we have shown that H_2O_2 and NO play an important role in the induction of resistance to *V. dahliae* toxins in *Arabidopsis*[33]. However, the versatility of H_2O_2 and NO-signaling mechanisms have not been well defined in cotton cells.

Glutathione S-transferases (GSTs) play an important role in the response of plants to changing environmental conditions, and their expressions are induced by a large range of factors. It has been proposed that these factors activate *GST* expression by inducing conditions of oxidative stress[34]. GSTs have been found to function in numerous cellular processes[35,36] and act as potential regulators of apoptosis[37]. In animals, this function is well established i.e. GSTs regulate kinase activity during oxidative stress[38]. Recent evidence suggests that the GSTs appear to act as binding proteins for bioactive ligands, and some act as transport protein. It is probable that GSTs fulfil their essential roles in intracellular transport. Therefore GSTs are considered to have the same potential in cell signaling in plants[39].

In this study an attempt was made to account for the observed differences in a resistant cultivar and a susceptible cultivar of cotton (*Gossypium hirsutum* L.) treated by *V. dahliae* toxins. The primary focus was on the differences in the timing and level of production of NO and H_2O_2, and their effects on the expression of *GST* gene. Analysis revealed that NO and H_2O_2 may be part of a complex network of signals that respond to VD-toxins and induce the expression of *GST* gene. *GST* gene is potentially involved in the resistance mechanism in the cotton suspension cells.

1 Material and methods

1.1 Plant material

The two cotton varieties used in the study were BD18, a *G. hirsutum* L. cultivar resistant to the wilt pathogens *V. dahliae*, and Simian 3, a susceptible *G. hirsutum* L. cultivar.

The seedling and the callus were cultured as described previously[9]. The resulting calluses were transferred into liquid suspension culture medium containing inorganic and organic nutritions as with the case of the callus culture medium besides agar. Two-d-old cell cultures were used in the experiments.

1.2 Treatments of cotton suspension cells

The suspension cells were transferred to a buffer (containing 50 mmol/L morpholino ethane sulphonate (MES), 75 mmol/L sucrose, 1 mmol/L $CaCl_2$, 1 mmol/L K_2SO_4. pH 5.8), and were supplemented with (1) 25 μg/mL VD-toxins; (2) 0.5 mmol/L sodium nitroprusside (SNP, an NO donor); (3) 0.5 mmol/L H_2O_2; (4) 1000 U/mL catalase (CAT, a scavenger for H_2O_2); (5) 0.4 mmol/L 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, a scavenger for NO); (6) 25 μg/mL VD-toxins and 0.4 mmol/L cPTIO; (7) 25 μg/mL VD-toxins and 1000 U/mL CAT. The cells treated by the buffer served as a control. The cells were harvested at different time intervals (0, 1, 2, 4, 8 h) after treatment by the above solutions. Materials not used immediately were frozen in liquid N_2 and stored at −20℃ for later usage.

1.3 Preparation of crude VD-Toxins from *V. dahliae*

V. dahliae have been reported to produce phytotoxins implicated in symptom development[1,10,40,41]. In the present study, a highly infectious and non-defoliating strain of *V. dahliae* Kleb (V229) was used. The fungus was cultured as described previously[10]. The fungus culture was filtered through filter paper; the filtrate was centrifuged at 10000 *g* for 30 min to remove the spores. The supernatant was frozen (−20℃) for 24 h, lyophilized for 36 h and dissolved in distilled water to make a 0.5 mg/mL solution. And then the solution was dialysed by 1 kD of Dialysis Membranes (MWCO) in 4℃ for 24 h. The concentrate solution was frozen and lyophilized again and the dust was dissolved in distilled water. The solution was then refiltered through a 0.45 μm pore size Millipore filter. The resulting filtrate was used as a crude VD-toxins extract for further experiment. Protein contents in the crude extracts were used to represent the concentrations of VD-toxins and determined for all further experiments according to Bradford[42]. Bovine serum albumin (BSA, Sigma) was used as a standard.

1.4 Detection of H_2O_2 accumulation

H_2O_2 production was assayed by the loss of scopoletin (Sigma) fluorescence at 460 nm following excitation at 350 nm. Five microliter (5 μL) of scopoletin solution (2 mmol/L) was added to 1 mL of cotton cell culture (final concentration: 10 μmol/L). After 5 min incubation on a shaker, the cells were filtered by centrifugution at 10000 *g* for 30 s. The supernatant (800 μL) was assayed by a fluorescence spectrophotometer F-4500 (HITACHI). H_2O_2 production was calculated by $(A_{control}-A_{samples})$/

$A_{control}$·10 μmol/L[12,19].

1.5 Detection of NO accumulation

Preparation of oxyhemoglobin (HbO_2) stock solution. 25 mg of hemoglobin (Sigma) was dissolved in 1 mL of phosphate buffered saline (PBS) buffer (pH 7.2). 4 mg of $Na_2S_2O_4$ powder was then added. A light stream of O_2 was blown into the solution or in the surrounding air (the hemoglobin will convert to HbO_2 form). The resulting HbO_2 solution was desalted and purified by passing it though a Sephadex G-25 column. The HbO_2 was stored on ice in dim light. The concentration of the desalted HbO_2 stock solution can be checked at 415 nm (ε = 131 (mmol/L)$^{-1}\cdot$cm^{-1}) by a spectrophotometer.

NO assay. Cotton suspension cells were incubated with 100 U catalase and 100 U superoxide dismutase for 5 min to remove ROI. The maximal changes in absorbance at 401 and 421 nm were measured, and the NO levels were calculated by using an extinction coefficient of 77 (mmol/L)$^{-1}\cdot$cm$^{-1}\cdot$[A_{401}(metHb)−A_{421}(HbO_2)][43].

1.6 Total RNA extraction and RT-PCR

Total RNA from cotton cells was extracted using the CTAB-acidic phenolic method[44].

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) were used to analyze the expression of *GST* (Accession No. CD485867) gene with *histon3* (Accession No. AF024716) gene as the internal standard. 1 μg of total RNA was reversely transcribed for the first-strand cDNA synthesis with M-MLV reverse transcriptase (Promega) according to product instructions, using specific downstream primers of *histon3* and *GST*. Then 3 μL of cDNA solution was taken as the template for RT-PCR analysis. PCR amplification was carried out with the upstream primer for *GST* being 5′-GAACCGATGGCGAAGAACA-3′ and the downstream primer being 5′-GATTTTAGGCCCTGCAAAGACT-3′. The amplified product of PCR was 188 bp. *Histon3* upstream primer was 5′-CGTAAATCTGCCCCAACCAC-3′ and the downstream primer was 5′-CACATTGAACCTACCACTACCATC-3′. The amplified product of PCR was 454 bp. The PCR conditions were as follows: initial denaturation was at 95℃ for 3 min, followed by 24 cycles of 94℃ for 30 s, 57℃ for 30 s, and 72℃ for 1 min, again with a final extension cycle at 72℃ for 10 min. The PCR products were run on a 1% agarose gel, and stained with ethidium bromide. Gel pictures were obtained using a u.v. transilluminator (Alphamager™ 2200, Alpha Innotech com) and then photographed. The intensity of the bands was quantified using a digital image analysis programme (ALPHA2200 Image Analysis Software).

Each reaction was performed three times independently.

1.7 Statistical analysis

The data presented are the mean ± SE of three independent experiments. Statistical differences between measurements at different times or on different treatments were analyzed following the two-tailed *t*-test. Differences were considered significant at a probability level of $P<0.05$.

2 Results

2.1 VD-toxins induced NO and H_2O_2 accumulations in cotton suspension cells

To determine whether cotton cells respond to VD-toxins by accumulation of NO, cells were treated with VD-toxins and the kinetics of NO accumulation were determined. The results showed that VD-toxins (25 μg/mL) stimulated NO accumulation in a rapid and transient manner. The peak NO accumulation was detected approximately 30 min post treatment in suspension cells (Figure 1), with peak height elevated approximately 4-fold in BD18 cells before gradually decreasing out, while it was only 1.5-fold in Simian 3 cells.

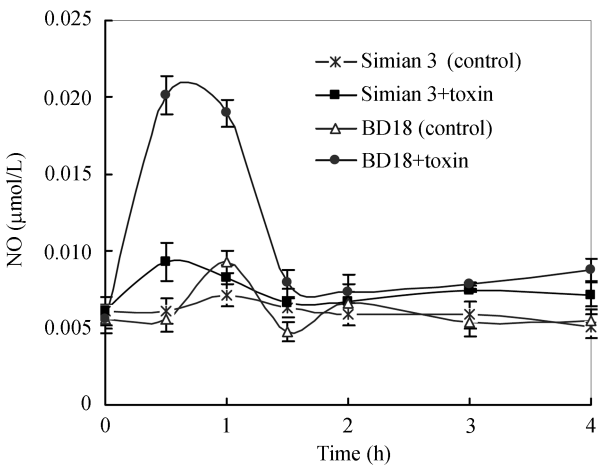

Figure 1 The kinetic analysis of the accumulation of NO induced by VD-toxins treatment in cotton suspension cells. ●, BD18 cells treated with VD-toxins (25 μg/mL); △, BD18 cells without treatment (control); ■, Simian 3 cells treated with VD-toxins (25 μg/mL); ∗, Simian 3 cells without treatment (control). The experiments were repeated 3 times. The error bars indicate ± SE.

Similarly, the kinetics of H_2O_2 production was measured over time (Figure 2). Treatment with VD-toxins (25 μg/mL) induced rapid accumulation of H_2O_2 typical of oxidative burst. A large and sustained burst peak appeared at 30 min post treatment in BD-18 cells and maintained at the level for 2 h, and then it gradually disappeared. The increase in H_2O_2 production in Simian 3 cells was only about half of the peak in BD18 cells and remained at the level for 4 h period.

Thus, it was clear that the accumulation of NO and H_2O_2 were at higher levels in resistant cultivars than in susceptible cultivar.

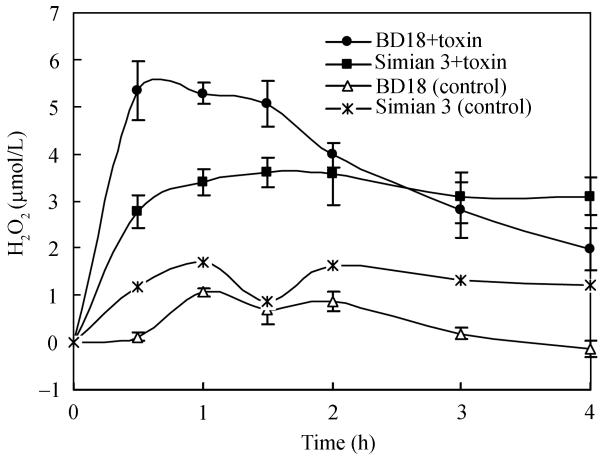

Figure 2 The kinetic analysis of the accumulation of H_2O_2 induced by VD-toxins treatment in cotton suspension cells. ●, BD18 cells treated with VD-toxins (25 μg/mL); △, BD18 cells without treatment (control); ■, Simian 3 cells treated with VD-toxins (25 μg/mL); ✳, Simian 3 cells without treatment (control). The experiments were repeated 3 times. The error bars indicate ± SE.

2.2 Effects of SNP, cPTIO, H_2O_2, CAT and VD-toxins on the induction of NO and H_2O_2 accumulation in cotton suspension cells

In order to investigate the possibility of the interaction between NO and H_2O_2 in VD-toxins induced responses in cotton suspension cultures cells, the effects of the CAT (1000 U/mL), H_2O_2 (0.5 mmol/L), SNP (0.5 mmol/L) and cPTIO (0.4 mmol/L) on the accumulation of NO and H_2O_2 were assessed.

In resistant cultivar, VD-toxins and exogenous H_2O_2 significantly increased NO accumulation. The accumulation was inhibited slightly by CAT, albeit statistically not significant (Figure 3(a)). In susceptible cultivar, NO accumulation was induced only by VD-toxins and the increase was not significant. Moreover, there were no significant differences in NO accumulation affected by H_2O_2 and CAT compared with the control.

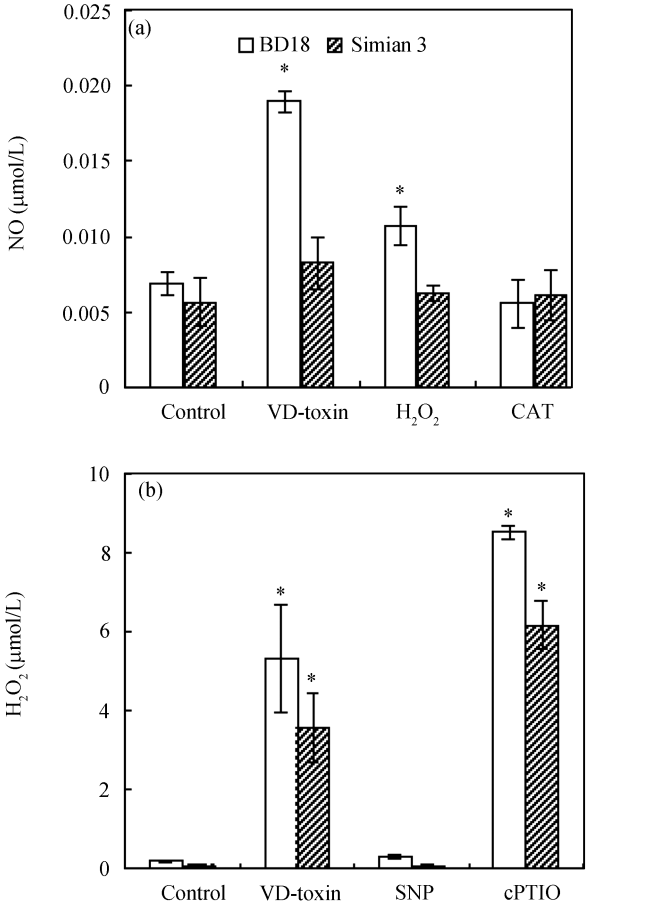

Figure 3 Effects of SNP, cPTIO, exogenous H_2O_2, CAT and VD-toxins on inducing NO and H_2O_2 accumulation in cotton suspension cells. (a) NO accumulation was monitored 1 h after treatment with exogenous H_2O_2 (0.5 mmol/L), CAT (1000 U/mL) and VD-toxins (25 μg/mL) in BD18 cells (open bar) and Simian 3 cells (shaded bar). (b) H_2O_2 accumulation was monitored 1 h after treatment with SNP (0.5 mmol/L), cPTIO (0.4 mmol/L) and VD-toxins (25 μg/mL) in BD18 cells (open bar) and Simian 3 cells (shaded bar). *, significant differences at the 5 % level between cells treated by different chemicals and untreated controls.

However, H_2O_2 accumulations could be affected by VD-toxins, SNP and cPTIO, and they were similar between two cotton cultivars. VD-toxins and cPTIO significantly increased the H_2O_2 accumulation. Conversely, H_2O_2 levels did not increased when cells were treated by SNP; therefore, H_2O_2 accumulation was not induced by SNP (Figure 3(b)).

These results indicate that H_2O_2 has trivial effects on NO accumulation, whereas NO plays an important role in the modulation of H_2O_2 accumulation. NO may act as an anti-oxidant to inhibit H_2O_2 accumulation.

2.3 Effects of H_2O_2, NO and VD-toxins on the expressions of GST gene in cotton suspension cells

To assess the effects of VD-toxins on the transcription of

specific genes involved in defense responses, the expression of *GST* was determined. High levels of *GST*-mRNA were rapidly induced in the cotton cells treated by VD-toxins. Induction was more rapid in BD18 than in Simian 3 (Figure 4). Treatment of culture cells of BD18 with VD-toxins resulted in a significant increase in *GST* mRNA within 2 h, and the level *GST* gene expression increased by 71.5% compared with the control. The maximal accumulation of *GST* mRNA appeared within 4 h and increased by about 185 %. The largest relative increase for *GST* mRNA in cells of Simian 3 treated by VD-toxins within 4 h was about 69.2 % not as large as that in cells of BD18.

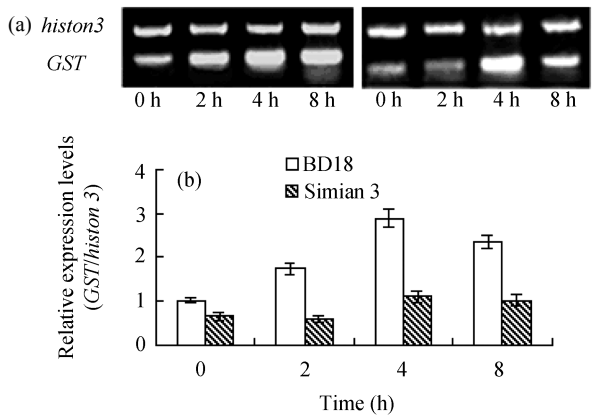

Figure 4 RT PCR analysis of *GST* gene expression in cotton cells treated with VD-toxins. (a) *GST* gene expression in cotton cells after different time treated with VD-toxins (25 μg/mL). Left, BD18; right, Simian 3. (b) The relative expression levels of *GST* gene in BD18 cells (open bar) and Simian 3 cells (shaded bar) treated with VD-toxins.

The ability of exogenous H_2O_2 (0.5 mmol/L) and NO donor, SNP (0.5 mmol/L) to affect *GST* mRNA expression was also assessed. Treatments of exogenous H_2O_2 stimulated the accumulation of *GST* mRNA as shown in Figure 5. The levels of *GST*-mRNA in BD18 increased faster than in Simian 3. The time-course data indicated that *GST* mRNA concentration increased significantly within 1 h of exposure to H_2O_2 in BD18, while it took 2 h in Simian 3. The increases of *GST* mRNA accumulations were about 47.7 % and 61.3 %, respectively. The accumulation of *GST* mRNA went on for up to 4 h in both varieties. The results suggest that the increase of *GST* gene expression was stimulated by H_2O_2.

SNP (0.5 mmol/L) treatment resulted in smaller and more transient increases in *GST* mRNA accumulations than the exogenous H_2O_2 (0.5 mmol/L) did (Figure 6). The maximal accumulation of *GST* mRNA occurred 1 h in resistant cultivar and 2 h in susceptible cultivar after

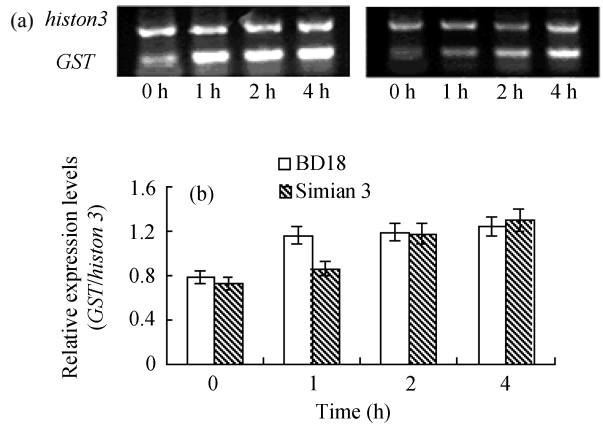

Figure 5 RT PCR analysis of *GST* gene expression in cotton cells treated with H_2O_2. (a) *GST* gene expression in cotton cells after different time treated with H_2O_2 (0.5 mmol/L). Left, BD18; right, Simian 3. (b) The relative expression levels of *GST* gene in BD18 cells (open bar) and Simian 3 cells (shaded bar) treated with H_2O_2.

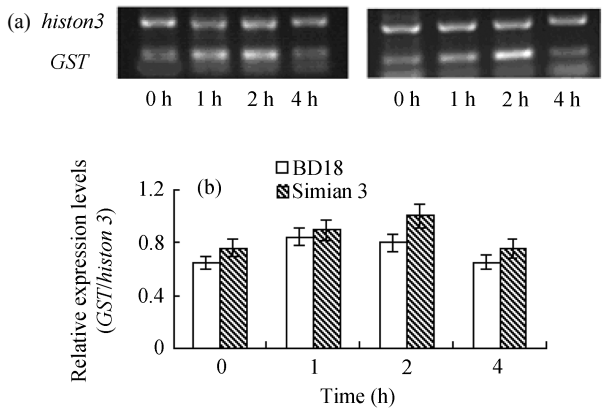

Figure 6 RT PCR analysis of *GST* gene expression in cotton cells treated with SNP. (a) *GST* gene expression in cotton cells after different time treated with SNP (0.5 mmol/L). Left, BD18; right, Simian 3. (b) The relative expression levels of *GST* gene in BD18 cells (open bar) and Simian 3 cells (shaded bar) treated with SNP.

treatment, the increases of *GST* mRNA accumulations being about 29.6 % and 31.9 %, respectively. The accumulation returned to basal levels by 4 h post treatment. This indicated that NO also increased *GST* expression. Because SNP at the concentration did not induce H_2O_2 production, it was possible that NO increased *GST* expression by signaling pathway that is independent of H_2O_2. It appears that H_2O_2 is more capable of stimulating the expression of *GST* gene than NO is.

To further investigate the potential role of endogenously generated H_2O_2 and NO in mediating the effects of VD-toxins on *GST* gene expression, cells were treated with VD-toxins in the absence and presence of CAT (1000 U/mL) and cPTIO (0.4 mmol/L). As shown in Figure 7, the presence of CAT attenuated VD-toxins in-

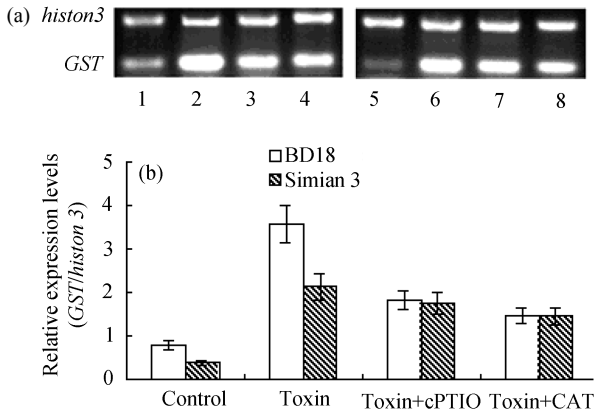

Figure 7 RT PCR analysis of *GST* gene expression in cotton cells treated with VD-toxins, cPTIO and CAT. (a) *GST* gene expression in cotton cells 4 h after treated with VD-toxins (25 μg/mL), VD-toxins plus cPTIO (0.4 mmol/L), VD-toxins plus CAT (1000 U/mL), respectively. Left, BD18; right, Simian 3. Lane 1, control; lane 2, cells treated with VD-toxins; lane 3, cells treated with VD-toxins plus cPTIO; lane 4, cells treated with VD-toxins plus CAT; lane 5, control; lane 6, cells treated with VD-toxins; lane 7, cells treated with VD-toxins plus cPTIO; lane 8, cells treated with VD-toxins plus CAT. (b) The relative expression levels of *GST* gene in BD18 cells (open bar) and Simian 3 cells (shaded bar) treated with VD-toxins, VD-toxins plus cPTIO or CAT.

duced *GST* mRNA accumulation by about 59.4 % in resistant cultivar and 31.8 % in susceptible cultivar, while the presence of cPTIO attenuated *GST* mRNA accumulation by about 49.6 % and 17.8 % in resistant and susceptible cultivar respectively. From this data it was clear that the effect of CAT on attenuating *GST* mRNA accumulation was larger than that of cPTIO. This suggests that H_2O_2 played a major role in stimulating *GST* gene expression.

3 Discussion

A deep-going understanding of the molecular mechanisms underlying plant defense responses to *V. dahliae* is a major prerequisite for effective control of *Verticillium* wilt. Although previous studies have revealed that NO and H_2O_2 have a crucial role in the induction of defense responses, and the signal molecules may be involved in the induction of a variety of defense-related genes[12,45 − 51], little is known about the molecular mechanisms by which NO and H_2O_2 participate in the defense responses against *V. dahliae* or VD-toxins in cotton cells.

In the present study, we observed that NO and H_2O_2 were responding to VD-toxins induction rapidly. VD-toxins triggered stronger NO and H_2O_2 bursts in resistant cultivars than in susceptible cultivar, and NO and H_2O_2 generation occurred in parallel. This may indicate that NO and H_2O_2 may act both synergistically and independently as the signaling molecules involved in the resistant responses against VD-toxins in cotton cells. That is, the simultaneous production of NO and H_2O_2 is required to induce the resistant responses against VD-toxins.

It has been reported that NO acts synergistically with ROS to potentiate cell death during the HR[19,52]. One possible mechanism for this cooperation can be that NO ensures maintenance of high, persistent H_2O_2 levels necessary to trigger the HR[27,32]. Others, however, have reported that there is no synergy between NO and ROS in inducing cell death in *Arabidopsis* suspension cultures. According to Clarke et al.[53], cell death was induced by NO independent of ROS. Beligni and Lamattina[54,55] suggested that NO acted as an anti-oxidant that scavenges ROS to inhibit H_2O_2 signaling pathways leading to cell death. Other finding indicated that NO and H_2O_2 had complementary functions in the activation of transcription dependent defenses[29]. Recently, the results are further supported by the reports that NO modulates H_2O_2-mediated defenses in the *Colletotrichum coccodes*-tomato interaction[51].

Our data indicated that NO donor alone could inhibit H_2O_2 accumulation in two cotton cultivars. This suggests that NO may act as an anti-oxidant to inhibit H_2O_2 accumulation in cotton cells. In order to confirm the hypothesis, cotton cells were treated with the NO scavenger cPTIO. It showed that H_2O_2 production increased significantly. The results indicated that the concentration of NO exerted an antioxidative effect to protect against oxidative processes. In accordance with previous reports[54,55], our data reveal that NO possibly functions in cotton cells resistant responses against VD-toxins by inhibiting the oxidative machinery, and ensuring lowered H_2O_2 levels. These findings are in agreement with the result that NO serves to modulate H_2O_2 accumulation and down-regulates its effects on defense-related gene expression[56−58].

H_2O_2-induced expression of *GST* gene has been demonstrated in *Arabidopsis* and soybean[12,14]. Increased expression of a homologue of a GST-like gene was observed in cotton root and hypocotyls tissues infected with *Fusarium oxysporum* f. sp. *vasinfectum*[59]. In this present work, we demonstrated that the *GST* gene was up-regulated in response to VD-toxins in cotton suspension cells. The *GST* gene expression was induced

more rapidly and stronger in resistant cultivar than susceptible cotton cultivar. This may indicate that the differences in resistant response against VD-toxins can be related to the differential expression of *GST* gene. The data confirmed our hypothesis that the expression of *GST* gene can provide effective resistant response against VD-toxins; that is, *GST* gene is potentially involved in the resistance mechanism against VD-toxins in cotton suspension cells. H_2O_2 may function downstream of VD-toxins and it is a major signal molecular in the pathway leading to *GST* gene expression. NO is also involved in the signaling pathway leading to expression of *GST*, albeit the effect is smaller, and it may be independent of H_2O_2. Different from our results, NO can induce the expression of *GST* gene depending on H_2O_2 in *Arabidopsis*[26,60], and NO is not involved in the pathways leading to cryptogein-induced expression of *GST* gene in tobacco[61]. Thus, the relationship between NO, H_2O_2 and *GST* gene could differ depending on plant−elicitor/pathogen interactions.

In conclusion, the results presented in this work for the first time suggest that NO and H_2O_2 are produced as part of a complex network of signals in cotton-*V. dahliae* toxins interactions and they may converge to act both synergistically and independently by inducing resistant responses against VD-toxins. *GST* gene is potentially involved in the resistance mechanism. However, NO induces the expression of *GST* gene independently of H_2O_2. H_2O_2 may be the more potent signal in the resistance responses against VD-toxins. The exact mechanism of action of NO and H_2O_2 in the regulation of resistant responses against VD-toxins remains to be elucidated in future studies.

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