Increase of β-1, 3-Glucanase and Chitinase Activities in Cotton Callus Cells Treated by Salicylic Acid and Toxin of Verticillium dahliae

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Abstract: The different resistance of cotton (Gossypium hirsutum L.) cultivars to crude toxin of Verticillium dahliae (VD) was correlated with the activities of chitinase and β-1, 3-glucanase in callus cells. The activities of chitinase and β-1, 3-glucanase in the callus cells treated with the VD-toxin were increased to the higher level at earlier time point in resistant cultivars than these in the susceptible cultivars. Exogenous salicylic acid (SA) induced the accumulation of chitinase and β-1,3-glucanase, which resulted in the resistance of callus cells to the VD. toxin. Western blot using a polyclonal antibody against β-1,3-glucanase identified 28 kD protein that was induced by VD-toxin, SA, or VD-toxin plus SA.

Key words: Gossypium hirsutum; toxin of Verticillium dahliae; salicylic acid; chitinase; β-1,3-glucanase

In compatible and incompatible interactions between plants and pathogens, pathogenesis-related (PR) proteins are often induced in response to infection. The synthesis and accumulation of these PR-proteins have long been thought to play an important role in the plant defense response. Among these PR proteins, hydrolases such as β-1, 3-glucanase and chitinase have been suggested to be involved in plant resistance against fungal pathogens (Boller, 1985; Kombrink et al., 1988; Joosten and de Wit, 1989; Cote et al., 1991; Kim and Hwang, 1994; Lozovaya et al., 1998; van Loon and van Strien, 1999). These enzymes can be induced by pathogens or exogenous chemicals (Boller, 1985; Cote et al., 1991; Simmons, 1994), and release oligosaccharide elicitor inducing the production of phytoalexins. These enzymes have been reported to be related with defense against pathogens in several plants, including maize, pepper, barley, pear, millet and wheat (Kim and Hwang, 1994; Cordero et al., 1994; Caruso et al., 1999; Kini et al., 2000). Multiple forms of chitinase and β-1,3-glucanase have been characterized in different plants. The different forms have different enzymatic activities and physiological properties.

Salicylic acid (SA) has been proposed as a systemic signal, inducer, or a required factor in resistance mechanisms in tobacco, cucumber, bean, pea, Arabidopsis and tomato (Malamy et al., 1990; Metraux et al., 1990; Uknes et al., 1992; Conti et al., 1996; Dann et al., 1996; Lennon et al., 1997; Frey and Carver, 1998; Spletzer and Enyedi, 1999). SA could act as a systemic signal that triggers a local and systemic resistance response (Metraux, 2001). White (1979) showed that exogenous application of SA induced resistance against TMV. Exogenous SA was found to induce PR-protein accumulation in tobacco, and this accumulation correlated with increased TMV resistance. SA could induce resistance against Pythium root disease on tobacco (Chen et al., 1996). But exogenous SA did not induce resistance to cucumber root rot caused by P. aphanidermatum (Chen et al., 1999). Seah et al (1996) also failed to induce resistance against take-all in wheat with exogenous SA. SA may not be involved in all cases of systemic resistance and may not act as an exogenous inducer against all plant pathogens (Silverman et al., 1995; Penninckx et al., 1996). It is not clear that what role SA plays in resistance against cotton Verticillium wilt. In this study we designed experiments to test whether exogenous SA could induce systemic resistance against cotton Verticillium wilt caused by toxin of V. dahliae Kleb. We compared the difference of the susceptibility in the resistant and susceptible cultivars of cotton (G. hirsutum) to V. dahliae, by measuring the activities of β-1, 3-glucanase and chitinase during a timing course.

1 Materials and Methods

1.1 Plant materials and tissue-culture conditions

Seeds from three Gossypium hirsutum L. varieties BD18 (resistant), Zhong 12 (tolerant), Simian 3 (susceptible) were surface sterilized in 15% H2O2 for 2-4 h, then washed thoroughly with sterile distilled water and germinated on 1/2 MS medium. The seedlings were grown at 25 °C with 1300
µmol·mm⁻²·s⁻¹ illumination for 12 h per day. The hypocotyls from 5-day-old seedlings were cut to approximately 0.5 cm. The explants were cultured on 1/2 MS medium supplemented with 0.2 mg/L IAA, 0.1 mg/L 2, 4-D, 0.1 mg/L KT for inducing the callus. Twenty-day-old calli were transferred to the same medium as above supplemented with: (1) 15 µg/L Verticillium dahliae (VD)-toxin; (2) 1 mmol/L SA; (3) 15 µg/L VD-toxin and 1 mmol/L SA. Calli with no VD-toxin or SA were used as control. Calli were harvested at different time intervals (0, 1, 2, 5, 8, 12, 16, 20 d) after co-cultured with VD-toxin or SA and used for enzyme extraction. For enzyme extraction, 0.5 g of tissue was used.

1.2 Preparation of crude VD-toxin from V. dahliae

The fungus V. dahliae were cultured in Czapek’s hydroponis medium (Meyer et al., 1994) in a shaker with 120 r/min at 28°C for 14 d. The cultured medium was filtrated and centrifuged at 10 000g for 30 min, and supernatant was used as the crude VD-toxin extracts (Davis et al., 1998). Protein content in crude extracts was determined for all experiments by using the method of Braford (1976). Bovine serum albumin (Sigma) was used as a standard.

1.3 Protein preparation

For enzyme studies, calli were homogenized in 0.05 mol/L sodium acetate buffer (pH 5.2) using a pro-chilled pestle and mortar at 4°C. The homogenate was centrifuged at 12 000g for 20 min at 4°C and the supernatant was used as crude extract.

1.4 β-1, 3-Glucanase activity

β-1, 3-Glucanase activity was estimated according to the rate of reducing-sugar production with laminarin reduced by NaBH₄ treatment as the substrate (Felix and Meins, 1985). The assay mixture consisted of the enzyme, 500 µg reduced laminarin, and 10 µg BSA in 100 µL of 50 mmol/L sodium acetate (pH 5.2). Incubation was carried out for 30 min at 40°C in a flat-bottom microwell plates. To measure the reducing sugar, 100 µL of Somogyi’s reagent (Somogyi, 1952) was added to each microwell, and the plate was incubated for 45 min in H₂O-saturated air at 90°C. After cooling, 50 µL of Nelson’s chromogenic reagent (Nelson, 1944) was added to each well and the absorbance measured at 620 nm. Glucose was used as a standard. One unit of activity is defined as the amount of enzyme producing 1 mmol/min of glucose equivalents at 40°C.

1.5 Chitinase assay

Endo- and exochitinase activities were measured by a colorimetric assay. The reaction mixture (0.5 mL) containing enzyme, 1 mg colloidal chitin, 0.3 µmol sodium azide, and 14 µmol sodium acetate buffer (pH 4.5) was incubated at 37°C for 2-4 h. After the addition of 0.1 mL 0.8 mol/L sodium borate buffer (pH 9.1), the mixture was centrifuged (1 000g for 5 min), and 0.3 mL of the supernatant was used for the colorimetric determination of GlcNAc (Reissig et al., 1955). For the colorimetric determination of endochitinase, the reaction mixture was the same as above. After 2 h at 37°C, the reaction was stopped by centrifugation (1 000g for 2 min), and 0.3 mL of the supernatant were incubated at 37°C with 0.02 mL 3% (W/V) desalted snail gut enzyme and 0.03 mL 1 mol/L potassium phosphate buffer (pH 7.1) to hydrolyze the chitin oligomers. The resulting GlcNAc was determined according to Reissig (1955), using internal standards of GlcNAc (50 and 100 mmol) in the assay mixtures for the calculations. Enzyme and substrate blanks were included. Formation of a reaction product was non-linearly related to the enzyme concentration. Therefore, a dilution series of the enzyme was prepared and the activity was determined for enzyme concentrations approaching zero. The amount of enzyme producing 1 mmol/s GlcNAc equivalents at infinite dilution was defined as one unit of activity.

1.6 SDS-PAGE, Western blotting immunodetection

Total protein (40 µg) extracted from callus was separated on 12% SDS-polycrylamide gels (Hames and Rickwood, 1990). Following electrophoresis, gels were either stained for total protein using Coomassie brilliant blue or blotted to nitrocellulose membranes using a semidry transfer system (Towbin et al., 1979). Membranes were blocked, probed with primary antibody (a gift from Dr. Bernard FRITIG; the antibody had been raised against the β-1,3-glucanase from tobacco) at a dilution of 1:500, and then with goat-antirabbit alkaline phosphatase conjugated secondary antibody. Alkaline phosphatase was detected using the alkaline phosphatase conjugate substrate kit following manufacturer’s instructions.

2 Results

2.1 The response of callus cells to VD-toxin and SA

After adding the VD toxin to the culture medium, cotton callus cells produced marked symptoms of tissue browning and wilt. The symptoms at different levels were observed among the three different varieties. The resistant variety BD18 showed less disease symptoms than susceptible variety Simian 3 did.

The symptoms of cell necrosis were reduced to a lower degree in both resistant variety BD18 or susceptible variety Simian 3 calli treated with VD-toxin and SA simultaneously comparing with VD-toxin treatment alone. The disease reaction of calli treated with the combination of VD-toxin and SA happened 1-2 d later than that treated with the VD-toxin alone. The 90% of Simian 3 calli showed the
disease symptoms when treated with VD-toxin, but it was reduced to 50% - 60% when treated with combination of SA and VD-toxin or to 40% with SA alone. In BD18, 50% of calli showed the disease symptoms with treatment of VD-toxin, and less than 20% with the treatment of both SA and VD-toxin or SA alone. These results suggest that application of SA increases the resistance of calli to VD-toxin (Fig. 1).

2.2 VD-toxin and SA treatments increase the chitinase, β-1,3-glucanase activities in callus cells

The activities of chitinase at different time intervals are shown in Fig.2A. The chitinase activity was increased to a higher level in all three cultivars after being treated with VD-toxin. As control, the chitinase activity did not show apparent change over 20 d with no VD-toxin treatment (data not shown). The highest chitinase activities were reached on the 5th day after being treated with VD-toxin in all three cultivars. But the intense of chitinase activities increased was larger in resistant, tolerant cultivar BD18 and Zhong 12 than that in susceptible cultivar Simian 3.

The activities of β-1,3-glucanase in the callus of three cultivars are shown in Fig.3A. The highest activity of β-1, 3-glucanase was induced 1 d after being treated with VD-toxin in the resistant, tolerant cultivar BD18 and Zhong 12, but 2 d in the susceptible cultivar. Interestingly, the β-1,3-glucanase activity in the resistant, tolerant cultivar BD18 and Zhong 12 showed the second peak in 5 d.

The treatments of the callus cells with SA plus VD-toxin, or SA increased the activities of chitinase and β-1,3-glucanase (Figs.2B, 3B). However, there are the similar increase levels for all treatments, indicating that the combination of VD-toxin with SA did not have an added induction for these two enzymes. As control with no treatment, the chitinase and β-1,3-glucanase activities did not show a clear change during the timing course.

After induced to the highest levels, the enzymes activities of treated callus cells were decreased to the levels that are still higher than that of control during the 20 d treatments. This indicates that sustained higher levels of these enzymes are maintained during the treatments.

The significant differences between the resistant, tolerant cultivar BD18 and Zhong 12 with the susceptible cultivar Simian 3 and the different treatments with regard to the increase of the chitinase and β-1,3-glucanase activities were found in the time and the extent of responses (Tables 1, 2).

2.3 A polyclonal antibody against β-1, 3-glucanases identified a 28 kD protein that was induced by VD-toxin, SA or VD-toxin plus SA

Because the β-1, 3-glucanase activities in callus cells

Fig.1. Increase of the callus resistance to Verticillium dahliae (VD)-toxin in resistant variety BD18 and susceptible variety Simian 3 at 8 d after being treated with salicylic acid (SA). The hypocotyls from five-day-old seedlings were cut to 5 cm and transferred to the medium containing VD-toxin, SA, or VD-toxin + SA as treatment. The medium with no treatment was used as control. A. Resistant variety BD18 control with no treatment. B. BD18 treated by SA. C. BD18 treated by VD-toxin. D. BD18 treated by SA +VD-toxin. E. Susceptible variety Simian 3 control with no treatment. F. Simian 3 treated by SA. G. Simian 3 treated by VD-toxin. H. Simian 3 treated by SA +VD-toxin.
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were induced by VD-toxin, SA, or SA+VD-toxin, we analyzed the induction pattern of β-1,3-glucanase protein by Western blotting using a polyclonal antibody against β-1,3-glucanases from tobacco. Total protein extracts from callus of resistant cultivar BD18 and susceptible cultivar Simian 3 were treated with VD-toxin or SA+VD-toxin or untreated, and separated on SDS-PAGE (Fig.4A). The β-1,3-glucanase was homogeneous by SDS-PAGE with an apparent molecular weight of 28 000 and cross-reacted with the antibody of tobacco β-1,3-glucanase. The antibody recognized a β-1,3-glucanase in cotton callus. The intensity of the band was greater when callus was induced with SA+VD-toxin (Fig.4B).

Fig.2. The chitinase activity increased in the cotton callus under different treatments. A. The chitinase activity increased in the callus cells of three cultivars, resistant (BD18), tolerant (Zhong12) and susceptible (Simian 3), treated with VD-toxin. B. The chitinase activity in the callus of Zhong12 increased after being treated with VD-toxin, SA, SA+VD-toxin, respectively. Callus cells without any treatment were used as control. Abbreviations are the same as in Fig.1.

Fig.3. The β-1,3-glucanase activity was increased in the cotton callus under different treatments. A. The β-1,3-glucanase activity was increased in the callus cells of three cultivars, resistant (BD18), tolerant (Zhong12) and susceptible (Simian 3), treated with VD-toxin. B. The β-1,3-glucanase activity in the callus of Zhong12 was increased with VD-toxin, SA, SA+VD-toxin, respectively. Callus cells with no any treatment were used as control. Abbreviations are the same as in Fig.1.

Table 1 Chitinase and β-1,3-glucanase activities in callus cells of different cultivars of Gossypium hirsutum after being treated with Verticillium dehliae (VD)-toxin

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Chitinase activities* (U/mg protein)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD18</td>
<td>5.550 ± 0.046</td>
<td>6.893 ± 0.091</td>
<td>7.713 ± 0.191</td>
<td>8.786 ± 0.132</td>
<td>8.200 ± 0.109</td>
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<tr>
<td>Zhong 12</td>
<td>5.508 ± 0.087</td>
<td>7.120 ± 0.090</td>
<td>7.503 ± 0.103</td>
<td>8.638 ± 0.142</td>
<td>7.990 ± 0.132</td>
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<tr>
<td>Simian 3</td>
<td>6.340 ± 0.040</td>
<td>7.222 ± 0.081</td>
<td>7.972 ± 0.110</td>
<td>8.342 ± 0.140</td>
<td>8.112 ± 0.085</td>
<td></td>
</tr>
<tr>
<td>Days after treatment</td>
<td>β-1,3-glucanase activities* (U/mg protein)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>BD18</td>
<td>0.109 ± 0.002</td>
<td>0.209 ± 0.005</td>
<td>0.198 ± 0.008</td>
<td>0.232 ± 0.007</td>
<td>0.220 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>Zhong 12</td>
<td>0.097 ± 0.002</td>
<td>0.222 ± 0.004</td>
<td>0.189 ± 0.008</td>
<td>0.227 ± 0.007</td>
<td>0.223 ± 0.015</td>
<td></td>
</tr>
<tr>
<td>Simian 3</td>
<td>0.101 ± 0.003</td>
<td>0.189 ± 0.006</td>
<td>0.214 ± 0.009</td>
<td>0.207 ± 0.008</td>
<td>0.190 ± 0.006</td>
<td></td>
</tr>
</tbody>
</table>

* average of two independent experiments with four replicates. The values followed by the same letters are not significantly different from each other in same column according to Fischer’s least significant difference. Abbreviations are the same as in Fig.1.
Glucanase and chitinase are thought to play multiple roles in higher levels of resistance. Connect with a more rapid and intense enhance activity in higher levels of resistance. Compared to V. dahliae, cultivars exhibited higher and earlier induced levels of PAL, V. dahliae responses in two cultivars of G. hirsutum. Elicitors by plant, and synthesis of PR proteins (van Loon, et al., 1997). In compatible interactions, the virulent pathogen, and the reaction of the plant (Metraux, 2001). Plant defence is dependent on the efficiency of the invading pathogen and the reaction of the plant (Metraux, 2001). The results indicate that the callus cells of all three varieties produced a hypersensitive reaction to VD-toxin, and which differed in the speed and the extent of the responses (Figs. 1-3; Tables 1, 2). This confirmed the system of interaction response callus of cotton and VD-toxin is suitable and convenient as a model system to research the defensive mechanism of cotton to the V. dahliae. Generally, the success of the induced defense mechanism depends on the outcome of the race between the invading pathogen and the reaction of the plant (Metraux, 2001). Plant defence is dependent on the efficiency of the rapid initiation and development of defence responses, and the expression in biochemical and physiological changes which the plant undergoes. The effectiveness of induced defence responses often depends more on their rapid initiation, development than on the ability of the plant to synthesize defence proteins (Wubben et al., 1996; Smit and Dubery, 1997). In compatible interactions, the virulent pathogen is often recognized too late and the plant will be infected. In the case of avirulent pathogens, plants rapidly recognize the microbe and induce resistance mechanisms which act very efficiently against the invader. Induced mechanisms include the detection of signal molecules or elicitors by plant, and synthesis of PR proteins (van Loon, 1997; van Loon and van Strien, 1999; Chen et al., 2000; Egea et al., 2001). Smit and Dubery (1997) compared the defence responses in two cultivars of G. hirsutum hypocotyls to a V. dahliae elicitor, demonstrating that the resistant cultivar exhibited higher and earlier induced levels of PAL, CAD, POD activities and lignin-like polymera compared to the susceptible cultivar. Our results demonstrate that the higher levels of resistance connect with a more rapid and intense enhance activity in β-1,3-glucanase and chitinase.

In the interaction between plant and fungus, β-1,3-glucanase and chitinase are thought to play multiple roles in plant self-defense (Cote et al., 1991; Kim and Hwang, 1994; Cordero et al., 1994; Caruso et al., 1999). The results of the present study substantiate this in vitro that the ability of toxin of V. dahliae by increased chitinase and β-1,3-glucanase activities induce defence responses in callus from cotton cultivars. Similarly, the activities were also markedly increased in callus treated with SA. The results of the labeling with the polyclonal antibody against β-1,3-glucanase.

### Table 2  Chitinase and β-1,3-glucanase activities in callus cells of Zhong 12 after being treated with VD-toxin, SA, SA+VD-toxin, respectively

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Chitinase activities* (U/mg protein)</th>
<th>β-1,3-glucanase activities* (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
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<tr>
<td>VD-toxin</td>
<td></td>
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<tr>
<td>SA</td>
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<tr>
<td>SA+VD-toxin</td>
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</table>

*; the same as in Table 1.

### Discussion

The results indicate that the callus cells of all three varieties produced a hypersensitive reaction to VD-toxin, and which differed in the speed and the extent of the responses (Figs. 1-3; Tables 1, 2). This confirmed the system of interaction response callus of cotton and VD-toxin is suitable and convenient as a model system to research the defensive mechanism of cotton to the V. dahliae.

Generally, the success of the induced defense mechanism depends on the outcome of the race between the invading pathogen and the reaction of the plant (Metraux, 2001). Plant defence is dependent on the efficiency of the rapid initiation and development of defence responses, and the expression in biochemical and physiological changes which the plant undergoes. The effectiveness of induced defence responses often depends more on their rapid initiation, development than on the ability of the plant to synthesize defence proteins (Wubben et al., 1996; Smit and Dubery, 1997). In compatible interactions, the virulent pathogen is often recognized too late and the plant will be infected. In the case of avirulent pathogens, plants rapidly recognize the microbe and induce resistance mechanisms which act very efficiently against the invader. Induced mechanisms include the detection of signal molecules or elicitors by plant, and synthesis of PR proteins (van Loon, 1997; van Loon and van Strien, 1999; Chen et al., 2000; Egea et al., 2001). Smit and Dubery (1997) compared the defence responses in two cultivars of G. hirsutum hypocotyls to a V. dahliae elicitor, demonstrating that the resistant cultivar exhibited higher and earlier induced levels of PAL, CAD, POD activities and lignin-like polymera compared to the susceptible cultivar. Our results demonstrate that the higher levels of resistance connect with a more rapid and intense enhance activity in β-1,3-glucanase and chitinase. In the interaction between plant and fungus, β-1,3-glucanase and chitinase are thought to play multiple roles in plant self-defense (Cote et al., 1991; Kim and Hwang, 1994; Cordero et al., 1994; Caruso et al., 1999). The results of the present study substantiate this in vitro that the ability of toxin of V. dahliae by increased chitinase and β-1,3-glucanase activities induce defence responses in callus from cotton cultivars. Similarly, the activities were also markedly increased in callus treated with SA. The results of the labeling with the polyclonal antibody against β-1,3-glucanase.

![Fig. 4.](image-url)
also indicated the increases in activity of $\beta$-1,3-glucanase in callus induced by VD-toxin and VD-toxin + SA. A similar pattern of protein production was observed in cultivar BD18 and Simian 3, but the resistant reaction of toxin of VD resulted in the accumulation of a greater intensity of $\beta$-1,3-glucanase than the susceptible reaction with toxin of VD, indicating that increased activity of $\beta$-1,3-glucanase was related directly to induced resistance. Studies on $\beta$-1,3-glucanase and chitinase activity support the view that the timing and intensity of activation of the defence mechanisms are critical for the expression of resistance.

The results here suggest that SA plays an important role in the induction of resistance to toxin of V. dahliae in cotton. SA is most likely to be a signal molecule involved in the induced resistance response and exogenous application of this compound also stimulates resistance to toxin of V. dahliae by induction increasing chitinase and $\beta$-1,3-glucanase activities. However, it is not yet fully ascertained whether salicylic acid is the primary mobile signal or is simply involved in a cascade of events which culminates in the expression of systemic resistance response. The role of SA requires further investigation.

References:


Kombrink E, Schroder M, Hahlbrock K. 1988. Several “pathogenesis-related” proteins in potato are 1,3-$\beta$-glucanases and chitinases. Proc Natl Acad Sci USA, 85:782-786.


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