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CELL WALL REINFORCEMENT IN COTTON HYPOCOTYLS IN RESPONSE TO A *VERTICILLIUM DAHLIAE* ELICITOR

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Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton; lignin; phenylalanine ammonia-lyase; cinnamyl alcohol dehydrogenase; peroxidase.

Abstract—Cotton (*Gossypium hirsutum*) hypocotyl tissue responded with increased lignification following treatment with a protein–lipopolysaccharide elicitor from *Verticillium dahliae*, the causative agent of vascular wilt disease in cotton. The induction of defence reactions was investigated over a period of 0–35 hr. Following exposure to the elicitor, increased synthesis and deposition of lignin and lignin-like phenolic polymers occurred. The induced phase of active lignification correlated with and was preceded by a transient increase in levels of enzyme activities; phenylalanine ammonia-lyase, cinnamyl alcohol dehydrogenase and cell wall associated peroxidase. The defence responses in two cultivars of *G. hirsutum* (cv. OR_{19} and $Acala_{1517-70}$, resistant and susceptible to *V. dahliae*, respectively) were compared. The resistant cultivar exhibited higher and earlier induced levels of enzyme activity and lignin-like polymers compared to the susceptible cultivar. This indicates that the effectiveness of induced defence responses depends on their rapid initiation, development and accumulation and suggests a possible correlation between the timing and intensity of lignin-like polymer accumulation and resistance/tolerance of *G. hirsutum* seedlings against *V. dahliae*. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Plants elaborate a number of inducible defence responses following elicitor treatment, mechanical damage or microbial attack. These responses include *inter alia* the synthesis of antifungal proteins and phytoalexins, the synthesis and accumulation of pathogenesis-related proteins including lytic enzymes, such as chitinases and β-1,3-glucanases, as well as the synthesis of hydroxyproline-rich glycoproteins and the reinforcement of cell walls through the deposition of lignin and lignin-like phenolic polymers [1]. The incorporation of phenylpropanoid derived material in lignin and lignin-like polymers following infection or elicitation, results in reinforcement of cell walls and structural rigidity which can lead to resistance against enzymic degradation and the restriction of diffusion of enzymes and toxins from the fungus to the host [2].

Lignin is an aromatic polymer composed mainly of cinnamyl alcohols which originates from t-cinnamic acid, the product of phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5). Cinnamyl alcohol dehydrogenase (CAD, E.C. 1.1.1.95) catalyses the second reductive step of the lignin committed branch, leading to hydroxycinnamyl alcohols, the monomeric precursors of lignin [3]. These precursors are enzymically dehydrogenated in the cell walls to phenoxy radicals, which then polymerize spontaneously to yield a complex net of crosslinks among monolignols, proteins and polysaccharides. Cell wall associated peroxidases (POD, donor:H_2O_2 oxidoreductase, E.C. 1.11.1.7) have been implicated in these cross-linking reactions [4].

Verticillium dahliae is a destructive soil-borne fungus that penetrates the host, cotton (*Gossypium hirsutum*), through the roots and spreads systematically through the xylem, leading to the appearance of wilt disease symptoms. An extracellular protein–lipopolysaccharide (PLP)-elicitor from *V. dahliae* was purified and characterized [5] and it was shown that binding sites for the complex occur in the plasma membranes of hypocotyl tissue [6] and cotyledon protoplasts [7].

The present paper deals with a comparison of the biochemical defence responses, related to cell wall strengthening, in two cotton cultivars (cv. OR_{19} and $Acala_{1517-70}$, resistant and susceptible to *V. dahliae*, respectively) induced by the fungal elicitor in order to investigate the possible correlation between higher levels of resistance, and a more rapid and intense defence response.

RESULTS AND DISCUSSION

Inducible defence responses may proceed from the same recognition event(s) or be interlinked through

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common precursors and may work together in a complementary manner towards resistance [2]. The strengthening of cell walls through the deposition of phenylpropanoid materials in the cell wall is an important aspect of the disease-resistance response of all plants [8]. Accumulation of lignin and lignin-like polymeric phenolic material in plant tissue has been associated with attempted fungal infection [9], but it is unclear whether lignification is a primary or secondary determinant of compatibility [2]. It has been hypothesized that the deposition of lignin and the covalent cross-linking with carbohydrate and protein during lignin polymerization interfere with the enzymic hydrolysis and mechanical penetration of plant tissue by fungal pathogens and may also impair the movement of water and diffusible molecules between plant and fungus.

When applied to seedlings in concentrations between 2.5 and 25 μg/ml the PLP preparation resulted in interveinal flaccidity and chlorosis, followed by interveinal and then general desiccation and necrosis. These symptoms are all characteristic of those on cotton plants naturally infected with severe strains of *V. dahliae*. The sensitivity of the seedlings to the PLP-elicitor correlated with the susceptibility of cotton cultivars to *V. dahliae*, in that the resistant cultivar OR_{19} exhibited a hypersensitive response when 4 h of 10 μg/ml PLP application. Less severe symptoms were noticed in the susceptible cultivar $Acala_{1517\text{-}70}$, and only from 10 to 12 hr onwards. Cultivars such as Letaba, Deltapine and Sicala exhibited an intermediate sensitivity towards the elicitor [5].

Esterification of phenylpropanoic acids to the cell wall precedes the polymerization towards true lignin, and the two processes may be regarded as contiguous, rather than separate, that gradually integrate and overlap [8]. The time dependent deposition of lignin and lignin-like phenolic polymers, determined as the thioglycolic acid derivatives, in both *G. hirsutum* cultivars are shown in Fig. 1 and indicate that the measured amounts increase 4–5 times and that it is significantly higher in the resistant cv. The timing of the response seems to be of greater importance as increased lignification was measurable after 6 hr of elicitation and reached a maximum at 22 hr in the resistant cv., while in the susceptible cv. these points were reached at 12 and 32 hr, respectively.

The timing and extent of the response are comparable with other plant–pathogen interactions where lignification has been studied. A rapid increase in lignin biosynthesis after the elicitation of *Pinus banksiana* cell suspensions with an elicitor isolated from *Thelephora terrestris* was reported [10], with maximum concentrations at 48 hr after elicitation. Elicitation of tobacco cells in suspension by elicitor molecules from *Phytophthora nicotianae* led to the accumulation of lignin-complexes in cell walls 4 hr after elicitation and increased ten-fold up to 96 hr [11]. The cell wall glucan elicitor of *Phytophthora sojae* (*megasperma* Drechs f. sp. *glycinae*) [12], was shown to induce a rapid and massive accumulation of phenolic polymers in *Glycine max* cotyledon cells proximal to the point of elicitor application. Deposition of phenolic polymers was 10 times more in elicitor-treated tissue than control-tissue, and reached maximum values after 24 hr.

The enzymes involved in the synthesis of lignin precursors and the polymerization thereof, PAL, CAD and POD were transiently induced by the PLP-elicitor produced by *V. dahliae* (Figs 2, 3 and 4). Maximum levels of PAL activity occurred 4 hr after elicitation in the resistant cv. but in the case of the susceptible cv. only at 11 hr. CAD-activity reached maximum levels at 7 and 15 hr, respectively. A similar pattern was also observed for POD where maximum activity could be measured at 8 hr and 16 hr, respectively. Electrophoretic separation followed by enzyme staining allowed the individual detection of specific isoforms. Figures 5 and 6 show the induction patterns of CAD and POD following anodic polyacrylamide gel electrophoresis. In both cases only major isoform was located. By preparing intercellular fluid from hypocotyl tissue segments with the inclusion of 0.3 M KCl, it was confirmed that the inducible POD isozyme

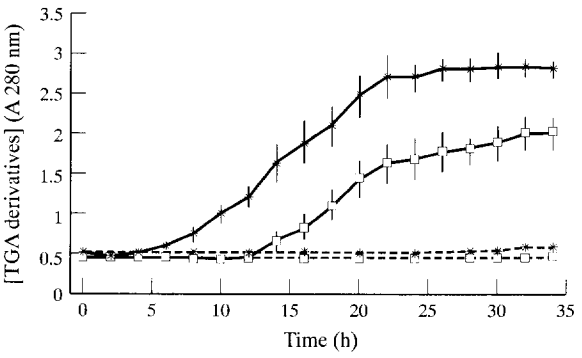

Fig. 1. The induction of phenolic polymer/lignin deposition in hypocotyls from *G. hirsutum* cv. OR_{19} (*) and cv. $Acala_{1517\text{-}70}$ (□) after treatment with 15 μg/ml PLP elicitor from *V. dahliae*. Dashed lines indicate water-treated controls. Error bars indicate standard deviation.

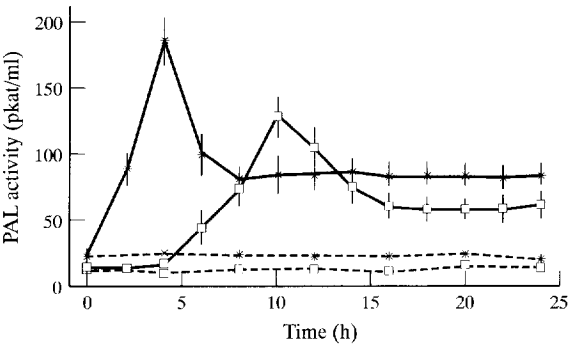

Fig. 2. The induction of phenylalanine ammonia-lyase from *G. hirsutum* cv. OR_{19} (*) and cv. $Acala_{1517\text{-}70}$ (□) after treatment with 15 μg/ml PLP elicitor from *V. dahliae*. Dashed lines indicate water-treated controls. Error bars indicate standard deviation.

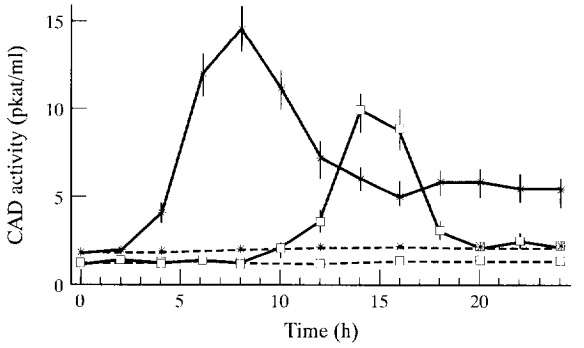

Fig. 3. The induction of cinnamyl alcohol dehydrogenase from *G. hirsutum* cv. OR_{19} (*) and cv. $Acala_{1517\text{-}70}$ (□) after treatment with 15 μg/ml PLP elicitor from *V. dahliae*. Dashed lines indicate water-treated controls. Error bars indicate standard deviation.

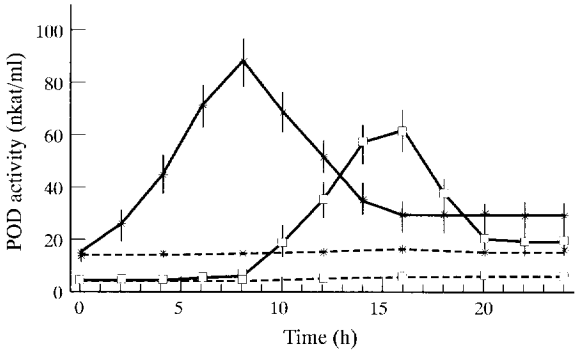

Fig. 4. The induction of peroxidase from *G. hirsutum* cv. OR_{19} (*) and cv. $Acala_{1517\text{-}70}$ (□) after treatment with 15 μg/ml PLP elicitor from *V. dahliae*. Dashed lines indicate water-treated controls. Error bars indicate standard deviation.

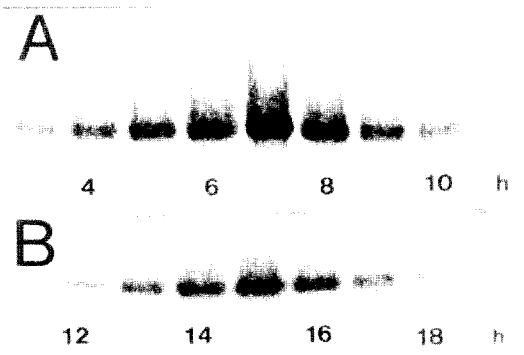

Fig. 5. Electrophoretic induction patterns of cinnamyl alcohol dehydrogenase from *G. hirsutum* cv. OR_{19} (A) and cv. $Acala_{1517\text{-}70}$ (B) after treatment with 15 μg/ml PLP elicitor from *V. dahliae*.

observed in the hypocotyl homogenate was located in the apoplast and probably cell wall associated.

Levels of these enzymes decreased and stabilized after reaching maximum, but never returned to control-values. This is an indication that sustained higher levels of these enzymes are maintained in response to elicitation following initial induction.

As also observed with the lignification response, the most significant differences between the two cvs with

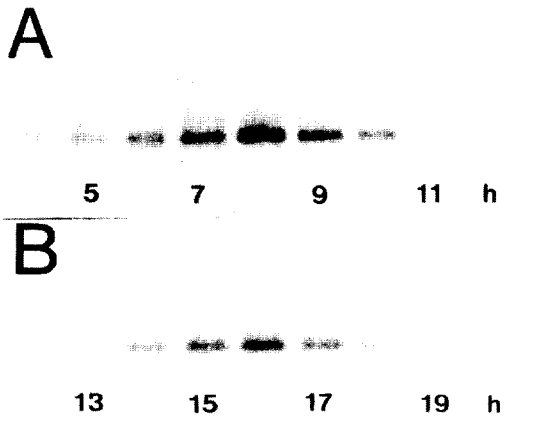

Fig. 6. Electrophoretic induction patterns of peroxidase from *G. hirsutum* cv. OR_{19} (A) and cv. $Acala_{1517\text{-}70}$ (B) after treatment with 15 μg/ml PLP elicitor from *V. dahliae*.

regard to induced enzyme activities were found in the timing rather than the extent of the responses.

The sequential pattern of induction of the enzymes was the same in both cultivars. PAL was induced first, followed by CAD and then POD. CAD and POD reached maximum levels 3–4 hr and 4–5 hr after PAL, respectively. The induction of phenylalanine ammonia-lyase, CAD and POD, in this order, probably confirms the positions of the enzymes in the common phenylpropanoid and the specific lignin pathways [3].

PAL is an extraordinarily sensitive indicator of the physiological state of the plant, and its elevation, which leads to increased synthesis of defence-related phenylpropanoids and phenolics, has often been favoured as an indicator of resistance [13]. Similarly, the role of CAD and POD in lignin synthesis has been correlated with resistance in different plant–pathogen systems [14]. Exposure of *Phaseolus vulgaris* cell cultures to elicitor molecules from *Colletotrichum lindemuthianum* led to a marked, but transient increase in the rate of synthesis of PAL [15] and CAD [16, 17]. Similar results were obtained with other plant models [18].

Changes in the pattern of POD isozymes have been demonstrated at the local site of pathogen invasion as well as in systematically responding tissues [19]. Although the role of POD enzymes in defence responses is not clear, POD activity is described as a biochemical event which accompanies resistance in some instances and in other cases the accumulation of POD isozymes *per se* can be linked to race-specific resistance [19, 20]. Studies on POD activity support the view that the timing and intensity of activation of the defence mechanisms are critical for the expression of resistance [21, 22].

Plant defence is dependent on the efficiency of the rapid initiation and development of defence responses, and the expression thereof in biochemical and physical changes which the plant undergoes. The effectiveness of induced defence responses often depends more on

their rapid initiation, development and accumulation than on the ability of the plant to synthesize defence metabolites and proteins.

It has been suggested that resistance to wilt pathogens such as *Verticillium* depends primarily on the mechanical isolation of the pathogen during the determinative phases of colonization and response within and outside the vascular system [13]. Lignin probably forms a structural barrier that limits the spread of pathogen and prevents the diffusion of extracellular enzymes and toxins. Reinforcement of the cell wall could confer resistance only if lignification occurred rapidly and in advance of the ingressing fungal hyphae [8]. Cell wall strengthening, through the deposition of lignin and lignin-like polymers, preceded by the induction of the synthesizing enzymes which are responsible for the formation thereof, consequently appears to play an important role in the defence response of *G. hirsutum* seedlings against *V. dahliae*.

EXPERIMENTAL

Buffers and chemicals. The following buffers were used: (A) 0.1 M sodium borate, pH 9.0; (B) 0.1 M sodium borate, pH 9.0, containing 0.1 M L-phenylalanine; (C) 0.1 M Na–Pi, pH 5.0; (D) 0.1 M Na–Pi, pH 5.0, containing 50 mM guaiacol; (E) 0.1 M Na–Pi, pH 5.0, containing 10 mM H_2O_2; (F) 0.1 M Tris–HCl, pH 8.8; (G) 0.1 M Tris–HCl, pH 8.8, containing 3 mM coniferyl alcohol; (H) 0.1 M Tris–HCl, pH 8.8, containing 6 mM NADP; (I) 0.1 M Na–Pi, pH 5.0 containing 0.03% H_2O_2 and 0.1% 4-chloro-1-naphthpl; (J) 0.1 M Tris–HCl, pH 8.8, containing 0.025% coniferyl alcohol, 0.05% NADP, 0.015% nitroblue tetrazolium and 0.001% phenazine methosulphate.

Preparation of pathogen and plant material. The fungus was grown in 2 l flasks containing 200 ml growth medium at 25° in the dark for 7 days [5]. After 7 days the culture was filtered and the filtrate centrifuged at 10 000 *g* for 10 min to remove spores. The supernatant was then concentrated by means of rotary evaporation and subjected to 80% Me_2CO fractionation at −18°. The resulting ppt., containing the protein–lipopolysaccharide elicitor, was dried and then redissolved in distilled water.

The cotton cv. OR_{19}, resistant to *V. dahliae*, was developed through pedigree selection from the susceptible *Acala* cvs [23]. Seedlings were grown in vermiculite at 25° in continuous light for 7 days after which they were rinsed with distilled water and placed in a 15 μg/ml solution of PLP [5]. After 1 hr the seedlings were removed from the solution, rinsed and placed in distilled water. Three samples of five hypocotyls each were homogenized, at 2 hr intervals, by using a microsintered glass homogenizer. The resulting suspensions were centrifuged at 10 000 *g* for 5 min and the supernatants assayed for enzyme activity and protein concentration. All experiments were repeated three times.

Preparation of apoplast fluid. The intercellular fluid present in the hypocotyls was extracted using the vacuum infiltration/centrifugation method [24] with 50 mM NaOAc buffer, pH 5.0, containing 0.3 M KCl.

Determination of enzyme activities. PAL activity was measured spectrophotometrically [25]. Activity was expressed as pkat/ml from A_{290} values using a molar extinction coefficient for cinnamate in toluene of 20 000 [25].

The determination of the dehydrogenation of coniferyl alcohol was performed by measuring the production of coniferyl aldehyde at 400 nm [26, 27]. The reaction mixture contained 2.5 ml buffer F, 200 μl buffer G, 200 μl buffer H and 100 μl enzyme. The assay was carried out for 30 min at 30° and enzyme activity was determined over the linear part of the reaction. CAD-activity was expressed as pkat/ml from A_{400} values using a molar extinction coefficient for coniferyl aldehyde of 18.5×10^6 [16].

The conversion of guaiacol to tetraguaiacol by POD was assayed at 470 nm. The reaction mixture contained 2.295 ml buffer C, 500 μl buffer D, 200 μl buffer E and 5 μl enzyme. The assay was carried out for 5 min at 25° and enzyme activity was determined over the linear part of the reaction. POD-activity was expressed as pkat/ml from A_{470} values using a molar extinction coefficient of 26 600 for tetraguaiacol [28].

Protein was determined by the dye-binding method of Bradford [29], with bovine serum albumin as a standard.

Determination of lignin-like polymers. Cotton seedlings were grown, elicited and harvested as described above. The homogenate was filtered through Whatman no. 4 filter paper and the residue dried at 60° for 24 hr. The dried alcohol insoluble residue, containing both true lignin and phenolic acids esterified to cell walls, was used for the lignin determination. HCl (5 ml, 2 N) and 0.5 ml thioglycolic acid (TGA) were added to 100 mg residue and the mixture was placed in boiling water for 4 hr. The mixture was then centrifuged at 10 000 *g* for 10 min and the ppt. was washed with 5 ml distilled water and again centrifuged. The ppt. was suspended in 5 ml 0.5 N NaOH, shaken at room temperature for 2 hr and then centrifuged at 10 000 *g* for 10 min. Conc HCl (1 ml) was added to the supernatant and the lignin-thioglycolic acid was allowed to ppt. at 4° for 4 hr. After centrifugation at 10 000 *g* for 10 min the ppt. was dissolved in 0.5 N NaOH, again centrifuged and the absorbance of TGA derivatives in the supernatant measured at 280 nm [9].

Gel electrophoresis and enzyme staining. Native anodic PAGE for the visualization of CAD and POD activities was performed in vertical slabs [30]. Gels, 0.75 mm thick (5% gel and 4–20% gradient gel, respectively, for CAD and POD), were prepared. Protein (20 μg) was applied to each well for each time point in the induction range. Electrophoresis was performed at a constant 20 mA and the process was terminated as soon as the marker dye reached the bottom of the gels.

The gels were briefly washed with distilled water and placed, respectively, in buffer F (the 5% gel for CAD staining) and buffer C (the 4–20% gradient gel for POD staining) for 15 min. The gels were placed, respectively, in buffer J (the 5% gel at 30°C for CAD staining) and buffer I (the 4–20% gradient gel at room temperature for POD staining). The formation of coloured bands was monitored until sufficient intensity was obtained. The gels were washed with distilled water, dried and photographed.

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