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Full Length Research Paper

Systemic induction of pathogenesis related proteins (PRs) in *Alternaria solani* elicitor sensitized tomato cells as resistance response

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Induction of acquired resistance and pathogenesis related proteins in intact plant leaves and the potentiation of pathogen signals in plant cell cultures by elicitors is mediated by different signaling pathways. We show here, the induction of PR proteins, the most widely used marker of acquired resistance, in tomato cells in response to *Alternaria solani* elicitors. The crude glycoprotein elicitor preparation from A*. *solani* was used to detect the possible induction of pathogenesis - related proteins chitinase (PR-3), thaumatin like proteins (PR-5) and β- 1,3 glucanase in suspension - cultured cells and leaves of tomato cultivar CO 3. Western blot analysis revealed the presence of 23 kDa thaumatin like protein and 44 kDa chitinase in elicitor treated suspension - cultured cells and leaves of tomato. The biochemical approach described in this paper with tomato should provide the basis for further efforts concentrating on the isolation and characterization of elements involved in perception and in the early steps of intracellular signal transduction.

Key words: *Alternaria solani*, elicitors, pathogenesis - related proteins, tomato, suspension-culture.

INTRODUCTION

Plants evoke a series of general defense reactions, including the production of phytoalexins and antimicrobial proteins, upon sensing invading microorganisms. Many of these biochemical reactions are caused by the activation of defense-related genes. The likelihood that early events in plant pathogenesis are initiated at the cell surface level has lead to the search for the active components - "elicitors" at the host-pathogen interface. Several elicitors have been isolated from various pathogens (Linden et al., 2000; Vidhyasekaran, 1997; Christopher et al., 2000; Schweizer et al., 2000). For several of these general elicitors, specific binding sites residing in the plasma membranes of plant cells (Nurnberger, 1999). Host responses to pathogens are very complex and difficult to interpret due to the spatial and temporal heterogeneity of defense responses in the infected tissues, as well as in the cells surrounding diseased tissues (Graham and Graham, 1991). Defense reactions that result from pathogen attack depend on the timing or rapidity and location of these individual defense responses (Dixon and Harrison, 1990).

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In order to facilitate biochemical and physiological studies on induced defense reactions of plants to pathogenic organisms, several workers have successfully used cell suspension cultures instead of whole plants and replaced pathogens by elicitors, the isolated compounds responsible for inducing defense responses in plants (Ebel et al., 1986; Edwards et al., 1985; Dalkin et al., 1990; Lange et al., 1994; Wojtaszek et al., 1995 Walkes and O' Garro, 1996). It is relatively easy to control the cell condition and apply the substances homogenously to each cell.

The present investigation was undertaken to study the induction of pathogenesis related proteins in cell cultures and leaves in response to treatment combined with elicitors isolated from *A. solani*, the early blight pathogen.

MATERIALS AND METHODS

Fungal culture

A. solani isolated from infected tomato (cv.CO-3) plants was obtained from the culture collection of the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India and maintained on potato dextrose agar (PDA) medium.

Elicitor extraction

A. solani was isolated from infected CO-3 cultivar of tomato and grown in Czapek's-Dox broth at 27 ± 2°C for 10 days. The mycelial mats were harvested and rinsed with sterile distilled water several times. The mycelia were resuspended in sterile distilled water (20/25 ml g^{-1} mycelium) and ground with a pestle and mortar. The mycelial slurry was filtered through 2 layers of muslin cloth and the residue obtained on the muslin cloth was homogenized 3 more times in water, once in a mixture of chloroform and methanol (1:1) and finally in acetone. This preparation was air dried and this fraction was considered as mycelial wall (Anderson-prouty and Albersheim, 1975). Elicitor was extracted from the mycelial walls by suspending 1 g of mycelial wall in 100 ml of distilled water and autoclaving for 20 min. The autoclaved suspension was filtered through 2 layers of muslin cloth clarified by centrifugation at 10,000 rpm for 20 min, concentrated to 10 ml using Buch EL 141 Rotavaporator at 50°Cand used as crude elicitor. The crude elicitor was assayed for carbohydrate and protein.

Determination of carbohydrate and protein

The elicitor solution was assayed for hexose by the anthrone method (Dische, 1962). Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of ice cold 95% H_2SO_4. 1 ml of elicitor was hydrolysed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5N HCl and cooled to room temperature. The solution was neutralised with solid sodium carbonate. The volume was then make up to 50 ml and centrifuged at 10,000 rpm for 20 min. To 1 ml of the supernatant, 4 ml of anthrone reagent was added and the solution was heated for 8 min in a boiling water bath. The tubes were then cooled rapidly in a running tap water and the colour was read in a Beckman DU 64 spectrophotometer at 630 nm. Glucose was used as a standard.

Protein was determined by the method of Bradford (1976). 10 mg of coomassie brilliant blue G-250 was dissolved in 4.7 ml of absolute ethanol and 10 ml of concentrated phosphoric acid and the volume was make up to 100 ml with distilled water. A sample of 50 µl elicitor was added to 950 µl of dye solution and the mixture was incubated for 5 min at room temperature. The absorbance was recorded at 595 nm using a Beckman DU-64 spectrophotometer. Bovine serum albumin was used as a standard.

Elicitor active moiety

To identify the elicitor-active moiety of the glycoprotein isolated from *A. solani*, the elicitor was treated with sodium periodate ($NaIPo_4$) which oxidizes or cleave polysaccharides and with pronase E enzyme which digests proteins (De Wit and Roseboom, 1980). 1 ml of elicitor solution containing glucose was treated with 1 ml of 20 mM $NaIPo_4$ and incubated in dark for 22 h at 5°C. To remove the excess of $NaIPo_4$ the sample was dialyzed against distilled water for 24 h with 4 changes. The elicitor solution was reduced to dryness in vacuo and redissolved in 1 ml of distilled water.

Sensitivity of the elicitor to enzymatic breakdown by treating the elicitor with 1ml of pronase (0.2 mg/ml) in 0.25 M potassium phosphate buffer, pH 7.8 for 22 h at 37°C (De Wit and Roseboom, 1980). 1 ml of treated and untreated elicitors were tested for their ability to induce phenolic synthesis after 24 h treatment in suspension-cultured cells and leaves of tomato.

Elicitor treatment

Tomato cv CO 3 were grown for 14 - 16 days under a 17 h, 7 h light-dark cycle (28°C). 2 expanded leaves and a small apical leaf were wounded at the edges and applied with 1 ml elicitor solution (236 µg protein/ml) (Stratmann and Ryan, 1997). Pathogen inoculation has been done with spraying of leaves with a conidial suspendsion of *A. solani* (5 x 10^{-6} conidia/ml) (De Wit et al., 1982). Analogous treatments with water alone served as control. The treated leaves of four plants were excised at the petiole and immediately frozen in liquid nitrogen to assay PR proteins.

Embryogenic calli were initiated from 7 day old cotyledonary young leaves of tomato (cv. CO 3) on MS basal medium (Murashige and Skoog, 1962). The suspension cultured cells were obtained by transferring friable calli (2g) to 250 ml conical flasks containing 50 ml MS liquid medium supplemented with 2, 4-D (2 mg/l) and shaken in a rotary shaker at 120 rpm at 25°C under continuous darkness. Suspension cultures were subcultured every 7 days by transferring 10 ml of culture to 40 ml of fresh medium. After transfer to fresh medium, tomato cell cultures were incubated for 6 days prior to treatment with 1 ml of filter sterilized crude cell wall elicitor and conidial suspension of *A. solani* (5 x 10^{-6} conidia/ml). Control was treated with 1 ml of sterile distilled water. Samples were collected at 0, 24, 48 and 72 hours after treatment and analyzed for induction of PR proteins.

Assay of chitinase

Leaves and suspension cells (1 g) were homogenized in 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 X g at 4°C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared according to Berger and Reynolds (1958) from carb shell chitin (Sigma). The commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was desalted as described by Boller and Mauch (1988). For the colorimetric assay of chitinase, 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted out into a 1.5 ml Eppendorf tube. After 2 h at 37°C and centrifuged at 1,000 x g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme for 1 h. The resulting monomeric N-acetylglucosamine (GlcNAc) was quantified according to Ressig et al. (1959) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as n mol GlcNAc equivalents min^{-1} mg^{-1} protein.

Assay of β-1,3- glucanase activity

β-1-3- Glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan et al. 1991). Leaves and suspension cells (1 g) were extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4°C using a pestle and mortar. The extract was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. Enzyme activity was expressed as nmo min^{-1} mg^{-1} protein. Protein concentrations were determined by the Bradford assay (Bradford, 1976).

Western blot analysis

Protein extracts were prepared by grinding 1 g of leaves and suspension cells with 5 ml of 0.1 M phosphate buffer, pH 6.5 containing 0.5 mM phenylmethylsulphonyl fluoride as described by Velazhahan et al. (1998). Proteins (100 µg) in aliquots of extracts were separated on 12 % SDS-PAGE (Lammli, 1970) and electroblotted

Table 1. Effect of sodium periodate (NaIPO4) and Pronase-E treatments on *A. solani* elicitor to induce phenolics in suspension cells and leaves of tomato

Treatment	Phenol (µg g^{-1} fresh weight)	
	Suspension cultured cells	Leaves
Without elicitor	311[c]	260[c]
NaI PO_4 (0.02 M)	317[c]	271[c]
Pronase – E (0.02 mg/l)	404[b]	271[c]
Untreated elicitor	518[a]	453[a]

(Means followed by a common letter are not significantly different at the 5% level by DMRT)

mass of proteins was determined by comparison with molecular weight standards (Rainbow markers, Amersham pharmacia, USA). Protein concentrations were determined by Bradford assay (Bradford, 1976).

RESULTS AND DISCUSSION

Systemic acquired resistance is one of the better understanding signaling pathways in plant defense leads to the expression of PR proteins (Delaney, 1997; Dorey et al., 1997; Sticher et al., 1997). Suspension cultured cells have been widely used by many workers to study the host-pathogen interaction at molecular and cellular level (Kessmann et al., 1990; Felix et al., 1991; GrossKopf et al., 1991; Vidhyasekaran et al., 1994; Velazhahan et al., 1994). Tomato leaves and suspension - cultured cells were used in the present study to investigate the induction of pathogenesis related proteins involved in systemic acquired resistance in response to *A. solani*. *A. solani* elicitor was found to be glycoproteinacious in nature (236 µg bovine serum albumin equivalent ml^{-1} of protein and 128 µg glucose equivalents ml^{-1} of carbohydrate). Tomato plants were sensitized with crude elicitor preparations induced phenols as a defense compound against *A. solani* with minimum PDI (18.513) compared to control (50.017). A study conducted with selectively masking either of carbohydrate or protein moieties (De Wit and Roseboom, 1980), the results indicated that treatment of elicitor with sodium periodate resulted in complete loss of phenol inducing activity (whereas pronase treatment reduced phenolic synthesis to a lesser extent. The result suggested that carbohydrate moiety may have more function and the intact glycoprotein was more active than the degraded products suggesting that the whole glycoprotein unit is required for the elicitor activity (Table 1). The results are in agreement with the finding obtained by Velazhahan and Vidhyasekaran (2000) in which they isolated and partially purified an elicitor from the mycelial walls of *R. solani* Osman et al. (2001) and Buhot et al. (2001) reported a proteinaceous elicitor (Elicitin) isolated from *Phytopthora* species, molecular weight 10 kDa triggering defense reactions in tobacco plants. Several glycoprotein elicitors have been identified and reported from *P. cinnamomi* (Bartnicki - Garcia, 1966), *Cladosporium fulvum* (De Wit and Roseboom, 1980), *C. lindemuthianum* (Coleman et al., 1992), C. capsici (Chawla and Wagle, 1997), C. falcatum (Ramesh Sundar, 1998) and R. solani (Velazhahan et al., 2000).

PR-proteins are individually encoded by single genes. Hence, these defense systems are highly amenable to manipulation by gene transfer (Lamb et al., 1997). β -1, 3 glucanases and chitinases play multiple roles in plant self-defense (Ham et al., 1991). In the present study, induction of glucanase was higher in suspension -cultured cells and leaves of tomato upon inoculation with *A. solani* and its elicitor. Similar trend was also observed in chitinase enzyme. The induction of these PR proteins was noticed from 0 (immediately after inoculation) hrs to 48 h (Figures 1 and 2). The duration of response in plants depends on continuous activation of receptors and continuous presence of respective ligands (Felix et al., 1991; Basse et al., 1993). In the tomato cells used in this study, continuous presence of *A. solani* elicitor stimuli has been found to be required for continuous stimulation of responses.

A 44 kDa chitinase was found to be induced in elicitor treated suspension cells as well as in leaves of tomato. Induction of 23 kDa TLP was also observed in tomato leaves and cells. There won't be any isoforms of these proteins were identified while performing the western experiment. An important understanding from this study is that the application of elicitors induces signaling process that begins upstream activation of PR proteins. Expression of elicitor inducible PR proteins has been well correlated with disease resistance (Vidhyasekaran, 1997). Expression of PR-proteins viz., chitinase, β-1, 3-glucanase exhibited antifungal activity against *A. solani* in response to the release of hypersensitive response elicitors (HR) from fungal cell wall in tomato (Christopher et al., 2000; Lawrence et al., 1996). *R. solani* elicitor treated pearl millet leaves were found to produce a 45 kDa chitinase (Radhajeyalakshmi et al., 2000 and Radhajeyalakshmi et al., 2004). A soluble carbohydrate elicitor from *Blumeria graminis* f. sp. *tritici* induced the accumulation of thaumatin like proteins in barley, oat, rye, rice and maize reported by Schweizer et al. (2000). These findings lead us to conclude that the induction of PR proteins upon inoculation of *A. solani* derived glycoprotein elicitor is due to the stability in the appearance and the binding sites may be protected from degradation for several hours.

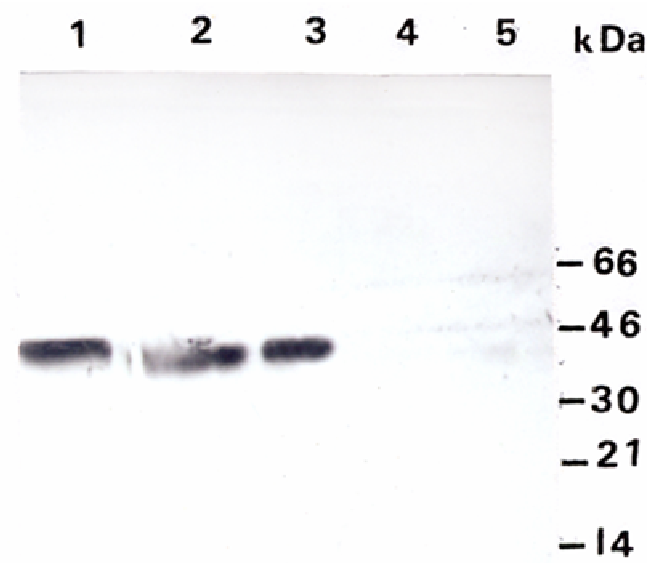

Figure 1. Western blot showing the recognition of 44 kDa chitinase against barley chitinase antiserum.
Lane 1- Positive control, 2 - Elicitor + Pathogen inoculated (suspension cells), 3- Elicitor + Pathogen inoculated (leaves), 4 - Control (suspension cells), 5- Control (leaves).

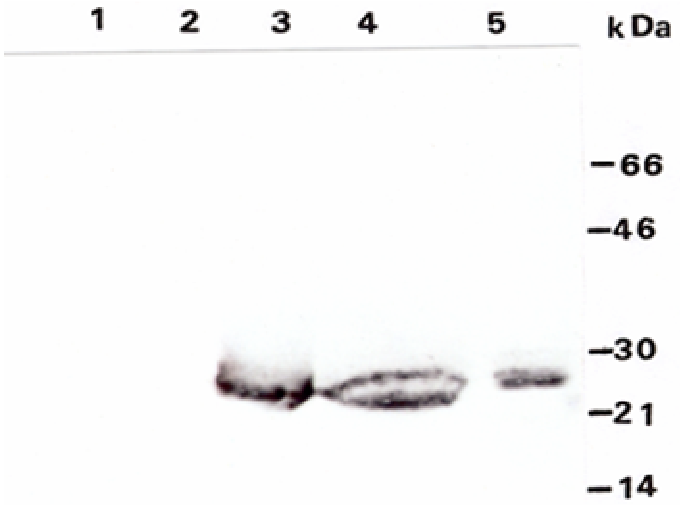

Figure 2. Western blot showing the recognition of 23 kDa thaumatin like protein against tobacco TLP antiserum.
Lane 1 - Control (leaves), 2-Control (suspension cells), 3-Positive control , 4- Elicitor + Pathogen inoculated (leaves), 5 - Elicitor + Pathogen inoculated (suspension cells).

Thus, a “hit and stick rather than a “hit and go” mechanism could cause the prolonged activation of elicitor.

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