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SHORT COMMUNICATION

Purification and characterization of elicitor protein from *Phytophthora colocasiae* and basic resistance in *Colocasia esculenta*

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Elicitor;
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Summary

An elicitor was identified in the fungus *Phytophthora colocasiae*. The molecular weight of the purified elicitor was estimated by means of gel filtration chromatography and SDS-PAGE and was estimated as 15 kDa. Protease treatment severely reduced its activity, allowing the conclusion that the elicitor is proteinaceous. Infiltration of a few nanograms of this proteinaceous elicitor into taro leaves caused the formation of lesions that closely resemble hypersensitive response lesions. The elicitation of the cells was effective in the induction of the activity of lipoxygenase. Cellular damage, restricted to the infiltrated zone, occurred only several hours later, after the infiltration of the elicitor protein. After few days, systemic acquired resistance was also induced. Thus, taro plant cells that perceived the glycoprotein generated a cascade of signals acting at local, short, and long distances, and causing the coordinate expression of specific defence. The obtained results give important information regarding the plant–pathogen interactions, mainly as subsidy for taro improvement against *Phytophthora* leaf blight.

Introduction

Taro [*Colocasia esculenta* (L.) Schott] is a member of the Araceae family and one of the oldest cultivated crops grown for its edible corms and leaves (Coates et al., 1988). Taro corms and leaves are also accredited to have medicinal value and are used to reduce tuberculoses, ulcers, pulmonary congestion and fungal infection (Misra and Sriram, 2002). The Food and Agriculture Organization estimates that 9.1 million Mt of corms are produced annually on a surface of 2 million ha, but this largely underestimates production as few countries keep reliable figures (http://faostat.fao.org/).

There are growing concerns over the narrow genetic base of taro cultivars, particularly with

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reference to taro leaf blight caused by *Phytophthora colocasiae*. Leaf blight has become a limiting factor for taro production in all taro-growing countries including India, causing yield loss of 25–30% (Thankappan, 1985). Metalaxyl- and mancozeb-based fungicides have proved effective in controlling the disease, but waxy leaf surface and the occurrence of the disease during the rainy season make fungicidal spray ineffective (Misra, 1999). Furthermore, fungicide sprays are too costly for marginal farmers, and soil microorganisms rapidly degrade metalaxyl, which is released into the water and soil system, and development of resistance against the fungicides is another major threat (Cohen and Coffey, 1986). Thus there is a need to develop integrated management strategies to combat this disease using natural and environmentally friendly mechanisms.

Induced resistance exploiting the natural defense machinery of plants could be proposed as an alternative, non-conventional and ecologically friendly approach for plant protection. Its introduction into agricultural practice could minimize the scope of chemical control, thus contributing to the development of sustainable agriculture. Induced resistance can be defined as an increased expression of natural defense mechanisms of plants against various types of pathogens, provoked by a range of factors: pathogens causing hypersensitive necrotic reaction, avirulent or attenuated pathogenic strains, elicitors of pathogenic origin (glucans, proteins, lipids, etc.).

Different classes of elicitor protein have been reported from various species of *Phytophthora*. Glycoproteins of molecular mass 42 and 32 kDa, secreted by *Phytophthora sojae* and *Phytophthora megasperma* respectively, have been described, which induce defense reactions in the non-host plants parsley and tobacco, respectively (Baillieul et al., 1995). These various types of elicitor molecules induce biochemical changes as part of the resistance response. Electrolyte leakage, oxidative burst, production of phytoalexins and PR proteins, and increased biosynthesis of ethylene have been described in leaf tissue treated with non-specific elicitors (Peever and Higgins, 1989) and with specific elicitors (Hammond-Kosack et al., 1996).

In this communication, we describe the isolation and characterization of a new proteinaceous elicitor from the culture filtrate of *P. colocasiae*, and its biological effects on taro plant to induce the following responses of a typical HR: (i) induction of rapid plant cell death, (ii) induction of defense responses and (iii) production of endogenous signal(s) that are known to participate in the triggering of defense responses.

Materials and methods

Chemicals

All chemicals were of analytical grade and were purchased from Sigma, Merck and Bangalore Genei (India).

Biological materials

Taro (*C. esculenta*: cv. Telia & Muktakeshi) plants were grown from tubers. They were planted in pots containing soil and *Trichoderma*-enriched compost. Isolates of *P. colocasiae* (98–111) used in this study were isolated from mature leaves of taro showing typical symptoms of taro blight. *P. colocasiae* was confirmed by comparing their morphology with several other accessions of *P. colocasiae* maintained in the Indian Institute of Spices Research (IISR, Calicut, India) collections. For isolation, leaf tissue segments of 2–3 cm from leaf-blight-infected area were excised from lesion margins. The leaf segments were sterilized in 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water and placed onto *Phytophthora* selective media (rye agar amended with 20 mg L^{-1} rifamycin, 200 mg L^{-1} vancomycin, 200 mg L^{-1} ampicillin, 68 mg L^{-1} pentachloronitrobenzene and 50 mg L^{-1} 50% benlate). Segments were incubated in Petri dishes for 4–5 days at 20 °C and mycelia were then transferred and maintained on potato dextrose agar medium (PDA; 250 g L^{-1} potato, 20 g L^{-1} dextrose and 20 g L^{-1} agar). Sporangia were induced by transferring two pieces of culture blocks ($10 \times 10 \times 3$ mm) in 10 mL sterile distilled water in Petri dishes for 2 days under white fluorescent light (Aragaki et al., 1967). Zoospores were released from sporangia by chilling at 5 °C for 30 min. After filtration through muslin, the zoospore concentration was adjusted to 10^5 spore mL^{-1} after measurement with a haemocytometer.

Elicitor production and purification

P. colocasiae isolate (98–111) was grown in defined liquid medium as described by Farmer and Helgeson (1987) under continuous stirring (50 rpm) at 27 °C for 7 days. The colourless spent culture filtrates (100 mL) were filtered through Whatman No. 1 qualitative filter paper, 1.2-μm (RA) and 0.45-μm (HA) Millipore filters and finally through a sterile 0.22-μm (GS) Millipore filter. The filter-sterilized culture filtrate was concentrated by lypholization to about 2 mL and dialyzed for 24 h against cold distilled water (Sigma, dialysis bag,

molecular cut-off 8000). The obtained powder was solublized in 2 mL of 50 mM sodium acetate, pH 4.0, and subjected for purification by using anion exchange chromatography on a column (2 × 50 cm) of carboxymethyl–cellulose (CM-Cellulose, Bangalore Genei, India) equilibrated in the same buffer. Elution was achieved with 100 mL of linear gradient of 0–1 M NaCl. The column was monitored at 280 nm and recovered in a fraction of 1.8 mL. The fractions corresponding to the absorption peaks were pooled and tested for elicitor activity. Fractions corresponding to the third absorption peak were lypholized and the obtained powder was dissolved in 2 mL of sterile water. Furthermore, the dissolved sample was loaded on a gel filtration column (Sephadex G-50, Sigma), and equilibrated with sterile distilled water at 4 °C. The column elute was monitored at 280 nm, and fractions corresponding to the first and second peak were assayed for elicitor activity and stored at −20 °C for further analysis.

Elicitor activity

The chromatographic fractions of interest were pooled and dialyzed against distilled water at 4 °C for 16 h and subsequently lypholized, dissolved in 2 mL of water and injected (2.0 μg protein L^{-1}) into the main leaf vein of taro leave (cv. Muktakeshi). After 1–2 days the infiltrated areas appeared shiny. After 2–3 days, these zones became necrotic and fluoresced under UV light, suggesting the accumulation of phenolic compounds. Leaf tissues corresponding to the infiltrated area were excised and ground in liquid nitrogen and the powder was suspended in 3 mL g^{-1} solution composed of 250 mM sodium phosphate buffer, pH 6.5, and 5% polyvinylpyrrolidone. The crude extract was centrifuged at 2000*g*, and the supernatant was assayed for lipoxygenase (LOX) by the method developed by Williams et al. (1986). Protein concentration was measured by the method of Bradford (1976).

Determination of molecular weight

The molecular weight of the elicitor protein was determined by using gel filtration on Sephadex G-100 and by using polyacrylamide gel electrophoresis (SDS-PAGE). A column (2 × 50 cm) was evenly packed with Sephadex G-100, equilibrated with 0.05 M Tris–HCl, pH 7.5. The flow rate through the columns of approx. 30 mL h^{-1} was maintained throughout the experiment. The standard proteins used, such as cytochrome c-551(9 kDa), ribonuclease (13.7 kDa), trypsin inhibitor (21.5 kDa) and chymotrypsinogen (25 kDa), were dissolved in the equilibration buffer and were applied to the column and flow of column effluent was monitored at 280 nm. The elicitor protein was run through the column under the same condition and its molecular weight was estimated from a standard protein graph. Electrophoresis under denaturing condition (SDS-PAGE) was performed by the method of Laemmli (1970) with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Gels were stained with silver nitrate according to Oakley et al. (1980). Low molecular mark standard proteins (Bangalore Genei, India, and Sigma) were used for molecular mass determination.

Induction of systemic acquired resistance

Taro leaves (cv. Telia and Muktakeshi) of the same age were infiltrated with the elicitor (2.0 μg protein L^{-1}) or water (control) and after 2 days 50 μL of sporangial suspension containing approximately 500 sporangia were inoculated on the abaxial surfaces of the elicitor or water-treated leaves. Treated leaves were incubated at 27 °C with 85% humidity in an illuminated growth chamber (300 μE m^{-2} s^{-1}) to allow the symptoms to develop. Evaluation of disease development on taro leaves was assessed at 2 days' interval by careful examination of the increase in the lesion size. Disease growth rate was calculated using the following equation (Melinda and Stevenson, 1991):

$$R = \{[(D/2)^2 - (d/2)^2] \times p\}/T$$

where R is the mycelium growth rate; D the average diameter of the colony (cm); d the disc diameter (cm); $p = 3.14$ and T the incubation time (day). From the total leaf area and blight-damaged area, the percentage leaf area damaged due to blight was calculated. Experimental design was randomized and consisted of three independent experiments. All tests for significance were conducted at the $p \leqslant 0.05$ level.

Results

Generation of elicitor protein

A chemically defined growth media was used that allowed the rapid and uniform growth of the elicitor. Shaking condition at 50 rpm resulted in clumping and rapid growth rate of mycelia. It was observed that the elicitor protein at the 7-day time of harvesting resulted in the rapid development of necrosis and defense response. By examining protein profiles from gel electrophoresis, it was

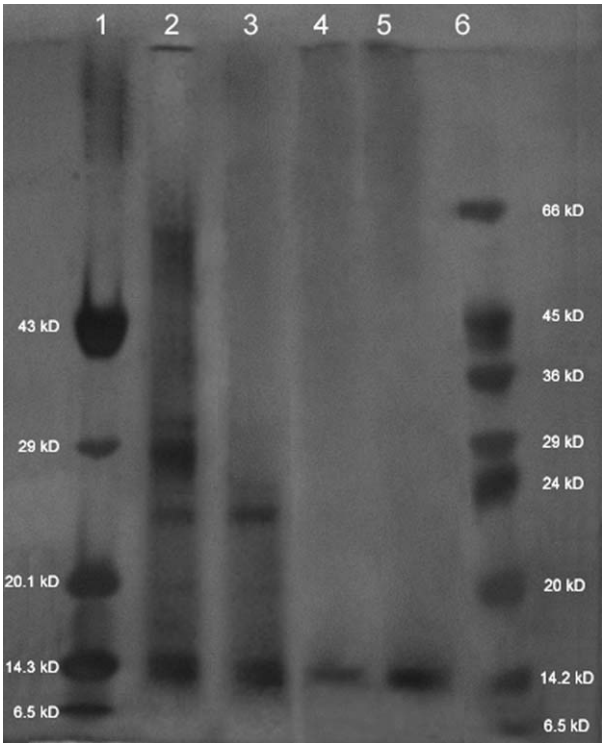

Figure 1. Silver-stained SDS-PAGE (12% of polyacrylamide) of protein. Lanes 1 and 6: molecular weight marker; Lane 2: elicitor protein in crude extract, harvested after 7 days; Lane 3: fraction II and III of CM-Cellulose chromatography; Lanes 4 and 5: fraction recovered from Sephadex G-50.

apparent that the elicitor protein comprises 30–35% of extracellular protein at the stage of harvesting (Figure 1).

Purification of the elicitor protein

Lypholized culture filtrate was applied on carboxymethyl–cellulose (CM-Cellulose, Bangalore Genei, India). Most of the material did not bind the column and was recovered in fraction I. Subsequent elution of the retained compounds with a linear gradient of NaCl resulted in two peaks of UV-absorbing material. The fractions corresponding to peaks II and III were pooled and analyzed by SDS-PAGE (Figure 1) and further analyzed for elicitor activity. The fraction corresponding to peak III showed significant increase of LOX activity upon injection into the main leaf vein of taro leaf (data not shown) and retained further for elicitor purification. Purification of protein corresponding to fraction III by the gel filtration column (Sephadex G-50, Sigma) resulted in one diffused and one sharp peak. Both of the fractions were lypholized separately and analyzed for elicitor activity. Fractions

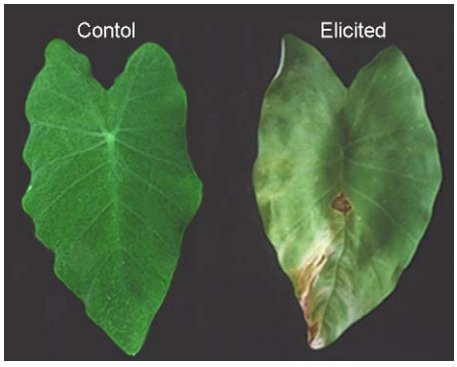

Figure 2. Hypersensitive reaction (necrosis) in resistant variety after treatment with elicitor protein compared to control.

corresponding to peak II showed necrotic area (Figure 2) and significant increase of LOX activity (data not shown) upon injection into the main leaf vein of taro leaves. The final preparation was homogeneous on SDS-PAGE, indicating that the method of purification was effective.

Molecular weight and properties of elicitor protein

The subunit number of elicitor protein was determined by SDS-PAGE. The result showed a single band and the apparent molecular band was estimated as 15 kDa (Figure 1). From a plot of log (Mr) versus the R_f value of standard proteins, molecular mass of the elicitor protein was estimated as 15 kDa. The elicitor protein was TCA precipitable and was stained by both Coomassie blue and silver stain. The elicitor is stable at 4 °C indefinitely when dissolved either in sterile distilled water or in 10 mM sodium phosphate buffer (pH 7.5). It is also stable to freeze-drying. It looses its activity at a temperature of 60 °C or higher. The periodate oxidation and non-specific protease treatment resulted in the loss of elicitor activity.

Induction of systemic acquired resistance

We tested whether infiltration of the glycoprotein into taro leaves would result in the establishment of systemic acquired resistance (SAR) in tissues free of glycoprotein and at a distance from the infiltrated site. When the taro leaves were infiltrated with elicitor followed by pathogen inoculation, there is no infection up to 2 weeks in the tolerant Muktakeshi cultivar, while the infection started in susceptible Telia cultivar within a week. However, the development of disease was less in Telia leaves treated with elicitor compared

to control leaves. In the untreated control leaves, the percentage infection reached 100% in susceptible cultivar within 14 days while in tolerant cultivar 40% infection was observed after 35 days. In the elicitor-treated tolerant cultivar, the level of infection reached a maximum of 22% in 35 days, while in the elicitor-treated susceptible cultivar the maximum level of infection was 44% in 14 days.

Discussion

An extensive analysis of the *P. colocasiae* 98–111, culture filtrate for compounds inducing rapid tissue necrosis after infiltration of taro leaves led to the isolation of a 15 kDa elicitor protein. The new protein was highly active, a few nanograms were sufficient to induce rapid leaf tissue necrosis in the resistant variety of taro, while susceptible variety showed no or late response. The heat inactivation profile of the elicitor activity is similar to that expected for denaturation of a protein; the treatment of the most purified elicitor with a non-specific protease preparation (pronase) greatly reduced the elicitor activity (data not shown). The major piece of evidence supporting the involvement of carbohydrate in the active molecule is the fact that periodates treatment completely inactivates the elicitor. These observations most easily explain that the elicitor is a glycoprotein in which both moieties are essential for activity. The observations made in the course of the purification step that elicitor protein is voided from a Sephadex G-50 column equilibrated and eluted with water, but is included when the column is equilibrated and eluted with dilute phosphate buffer, led us to propose that the elicitor molecules form aggregates with lower specific activity when the elicitor present in distilled water or in the presence of large amounts of contaminating carbohydrates material and the presence of low concentrations of salts in elicitor solutions favors disaggregation and a relatively higher elicitor specific activity. Rapid plant cell death is one aspect of the defence mechanisms that plants have against incompatible pathogens. They also include the induction of a large set of biochemical defence responses (Lamb et al., 1989).

In recent years, increasing information has tended to prove that metabolites generated by the LOX pathway play a pivotal role in the reactions of defence of higher plants to pathogen attack. LOX activity was found to be stimulated during pathogen attack of tomato (Kato et al., 1992), *Arabidopsis* (Peng et al., 1994) or rice (Melan et al., 1993) or during treatment of plant cell cultures by fungal elicitors (Fournier et al., 1993). We have observed that the elicitor treatment was similarly effective in stimulating LOX activity in taro leaf after infiltration. The rapid and transient increase in enzyme activity (about 15-fold) peaked around 20 h after treatment of the taro leaf tissues with the elicitor.

Application of the glycoprotein to taro plants also led to the induction of SAR, which results from the production and activity of a plant signal moving systemically. Salicylic acid was shown to be a key signal molecule involved in the phenomenon of SAR (Gaffney et al., 1993). Therefore, the consequence of infiltration of the glycoprotein into taro leaves is the production of such an endogenous signal and acquired resistance that developed after the application of the elicitor in the taro leaves.

Overall, the results of the present study strongly suggest that the effect of the 15 kDa glycoprotein on taro plants mimics a typical HR, which is viewed as rapid plant cell death and coordinate expression of biochemical defence responses under the control of endogenous signal molecules.

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