Microbiological Research ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Available online at www.sciencedirect.com
ScienceDirect

Microbiological Research

www.elsevier.de/micres

Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae* ☆

Ajay Kumar Mishra, Kamal Sharma, Raj Shekhar Misra*

Central Tuber Crops Research Institute, Thiruvananthapuram 695017, Kerala, India

Received 10 July 2008; received in revised form 25 October 2008; accepted 25 October 2008

KEYWORDS
Elicitor;
Phytophthora colocasiae;
Colocasia esculenta;
Cell suspension culture;
Plant defense

Summary

The rapid and effective activation of disease resistance responses is essential for plant defense against pathogen attack. These responses are initiated when pathogen-derived molecules (elicitors) are recognized by the host. A cDNA encoding elicitor, the major secreted extracellular glycoprotein of *Phytophthora colocasiae*, a pathogen of taro (*Colocasia esculenta*) plants, was isolated, sequenced and characterized. The expression of the corresponding elicitor gene during the disease cycle of *P. colocasiae* was analyzed. Elicitor was shown to be expressed in mycelium grown in culture media, whereas it was not expressed in sporangiospores and zoospores. *In planta*, during infection of taro, particularly during the biotrophic stage, expression of elicitor was down-regulated compared to *in vitro*. The highest levels of expression of elicitor were observed in *in vitro* grown mycelium and in late stages of infection when profuse sporulation and leaf necrosis occur. The elicitation of the suspension-cultured taro cells was effective in the induction of the enzyme activity of L-phenylalanine-ammonia lyase, peroxidase and lipoxygenase as well as the expression of defense-related endochitinase gene. All these biological activities were exerted within a low concentration range. The glycoprotein represents a powerful tool to investigate further the signals and their transduction pathways involved in induced disease resistance. It may also be useful to engineer broad disease protection in taro plant against *Phytophthora* leaf blight.

Introduction

As a result of host–pathogen coevolution, plants have developed sophisticated mechanisms to protect themselves from diseases. Pathogen specialization results when a complex set of preformed and

☆Nucleotide sequence data have been submitted to the NCBI Data Bank as accession number FE040598.

*Corresponding author. Fax: +91 471 2590063.
E-mail address: rajshekharmisra@gmail.com (R.S. Misra).

0944-5013/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2008.10.002

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

induced mechanisms is put into motion to defend a plant against invading pathogens. In some interactions, preformed physical barriers and antimicrobial compounds in the plant help to ward off pathogens (Osbourn, 1996). In other interactions, perception by the plant of signal molecules namely elicitors produced by the virulent pathogen leads to the induction of effective defense responses including a programmed cell death response termed the hypersensitive response (HR), that limits the spread of microorganisms (Lam et al., 2001). In addition, the distal uninfected parts of the plants usually develop systemic acquired resistance (SAR), which leads to a broad range of resistance against diverse pathogens (Wang et al., 2003). Both the HR and SAR are regulated by a complex network of signaling molecules, including AOS, salicylic acid, nitric oxide and jasmonic acid (Wendehenne et al., 2001).

Different classes of elicitor protein have been reported from various species of *Phytophthora* fungi: acidic-elicitins (capsicein and parasiticein) from *Phytophthora capsici* and *Phytophthora parasitica* respectively, and basic-elicitins (cryptogein and cinnamomin) from *Phytophthora cryptogea* and *Phytophthora cinnamomi*, respectively. 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). Many biochemical and physiological aspects of the defense response were studied in suspension-cultured plant cells. A 32 kDa glycoprotein purified from *Phytophthora megasperma* f.sp. *glycinea* was shown to stimulate various defense responses in cultured parsley cell suspensions including ion fluxes, oxidative burst, expression of defense-related genes and phytoalexin accumulation (Nürnberger et al., 1994). A glycoprotein of molecular mass 46 kDa isolated from *Phytophthora nicotianae* was shown to elicit phytoalexin accumulation in tobacco callus (Farmer and Helgeson, 1987). Treatment of suspension-cultured tobacco cells with elicitins from *P. megasperma* leads to rapid protein phosphorylation, Ca^{2+} influx, extracellular and transient H_2O_2 production, alkylinization of the extracellular medium, acidification of the cytosol, lipid peroxidation, gene expression, disruption of microtubular cytoskeleton, and cell wall modifications (Sasabe et al., 2000; Binet et al., 2001).

Phytophthora colocasiae, a hemibiotrophic oomycete plant pathogen, causes leaf blight, an economically devastating disease of taro (Raciborski, 1900). The life cycle and infection process of *P. colocasiae* are well known (Misra et al., 2008). The molecular basis of host specificity of *P. colocasiae* is poorly understood. To date, no race-specific avirulence gene of *P. colocasiae* has been isolated. In the course of our studies in *P. colocasiae*–taro interactions, we got the evidence of glycoprotein of 15 kDa in the 7 days old culture filtrate of *P. colocasiae*. It activates a qualitatively similar spectrum of defense responses as the well-characterized oligopeptide elicitor of molecular mass 14.9 kDa derived from *P. megasperma* (Baillieul et al., 2003). The present work describes the cloning of a cDNA encoding the protein moiety of the molecule. This elicitor can be used as a potential tool to engineer disease resistance against a broad spectrum of pathogens by manipulating the HR in transgenic taro plants.

Material and methods

Isolation of elicitor and N-terminal amino acid peptide sequencing

The elicitor protein was purified from the mycelium of *P. colocasiae* race 98–111 by anion exchange chromatography and gel filtration chromatography. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin (BSA) as standard protein. In order to sequence the N terminus of the protein moiety, the purified glycoprotein (2.8 μg) was subjected to SDS-PAGE before being transferred onto a PVDF Immobilon membrane (Bio-Rad), in 50 mM Tris base, 50 mM boric acid at 15 V overnight. After staining with a solution of 0.1% Amidoblack in 45% methanol, 1% acetic acid, the protein band was cut out and the protein was sequenced directly on the membrane. Sequencing of the amino terminus was performed by automated Edman degradation (Edman and Begg, 1967) at the Rajiv Gandhi Biotechnology Centre (Trivandrum, India). The sequenced peptide was reverse translated and for primer designing highest frequency codons were selected by using *S. cerevisiae* codon and standard genetic code table.

Nucleic acid extraction

Total RNA from *P. colocasiae* and from infected taro leaves was isolated using the guanidine hydrochloride extraction method (Logemann et al., 1987). Poly $(A)^+$ mRNA was purified from

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

the total RNA according to the manufacturer's instructions (OligotexTM mRNA Kit, QIAGEN). This step eliminated the possibility of DNA contamination in the RNA samples used for library construction. Purified mRNA samples were evaluated with a spectrophotometer reading at $A_{260/280\,nm}$. Only high-quality mRNA was selected for cDNA synthesis. Total DNA of *P. colocasiae* was isolated from mycelium grown in liquid culture as described (Mishra et al., 2008). A DNA concentration was measured spectrophotometrically at 260 nm.

cDNA library construction and screening

Double-stranded cDNAs were synthesized from 3 μg of poly(A)$^+$ RNA of *P. colocasiae* race 98–111 with a λZapII cDNA cloning kit (Stratagene, LaJolla, CA) and ligated to *Eco*RI linkers according to the instructions of the supplier. After kinasing of *Eco*RI ends and digestion with *Xho*I, the cDNAs were ligated to predigested λZapII vector arms, and further packaged into phage particles with an *in vitro* packaging kit (Gigapack II, Stratagene). The initial cDNA library, which consisted of 2×10^3 independent recombinant clones, was amplified in *Escherichia coli* XL1-Blue (Stratagene). PCR reaction was performed in a 25 μl volume, containing 5 μl aliquot of the cDNA library (initially treated at 70 °C for 5 min), 2.5 μl of 10 × reaction buffers, 4 μl of 25 mM $MgCl_2$, 2 μl of 2.5 mM dNTPs, 0.4 μM for universal T3 and T7, and 2 μM of designed primers. Amplification was performed in a thermal cycler (Techne progene). The reactions were heated in an initial step of 94 °C for 2 min and subjected to 30 cycles of the following program 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min. After the last cycle, the temperature was maintained at 72 °C for 8 min. Amplified DNA was electrophoresed in a 1.4% agarose gel containing 0.5 mg ml^{-1} ethidium bromide and photographed on UV transilluminator. The amplified cDNA library was screened with a PCR product that was labeled with DIG DNA Labeling (Roche Molecular Biochemicals). Two rounds of screening were performed to screen the cDNA library. In the first round, 2×10^3 clones were transferred to positively charged Hybond^{TM} nylon membrane (Amersham Biosciences, NJ, USA). Hybridization and washing were carried out by conventional protocol (Sambrook et al., 1989). Detection procedure was done according to the recommendations of the manufacturer (Roche Molecular Biochemicals, Penzberg, Germany). Positive plaques were selected and purified by two subsequent rounds of plating and screening, at low density, under the same conditions. The finally selected positive plaque was subjected to *in vivo* excision of the pBluescript recombinant plasmid from λZapII according to the instructions of the supplier. The pBluescript recombinant plasmid DNA was purified with a Qiagen (Hilden, Germany) plasmid preparation kit and digested with *Eco*RI+*Xho*I (Fermantas, Burlington, Canada), and the products were analyzed by electrophoresis on 0.8% agarose gel, subcloned into pGEM-T vector (Promega) and sequenced using T7 or SP6 promoter primers. The nucleotide and amino acid sequences were analyzed with a BLAST (NCBI) and the sequence was submitted to the Gene Bank under accession number FE040598.

RT-PCR analysis

To validate the elicitor gene, total RNA isolated from *P. colocasiae* was used for RT-PCR analysis. First strand cDNA was synthesized from 8 μg total RNA from each sample using MMLV reverse transcriptase (Promega, WI, USA) according to the supplier's manual. Forward (5′-CCGCACATGAACATTAAGAC-3′) and Reverse (5′-GCCGTGAGCTCATAAGGT-3′) primer for the candidate gene was designed by the Primer premier software. General PCR was conducted with the following program: an initial denaturation at 94 °C for 2 min, followed by 25–30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, a final extension at 72 °C for 6 min and held at 4 °C. RT-PCR experiments were repeated three times and the PCR products were detected by 1.2% agarose gel in 1 × TAE with EtBr.

Callus and suspension culture initiation

Epicormal tip adjacent to the corm was used as explant. Explant were washed in running tap water and were surface sterilized using ethanol (70% v/v) for 15 min followed by treatment with sodium hypochlorite (50% v/v) with 5 drops of Tween 20 for 15–20 min. After 3–4 washes with sterile distilled water, explants were trimmed and kept in sterile distilled water for inoculation. Friable calli were induced by culturing explants on MS (Murashige and Skoog, 1962) basal media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (6 mg L^{-1}), 6-benzyl adenine (BA) (6 mg L^{-1}), and sucrose (30 g L^{-1}). Calli were maintained under dark and light conditions at 25 ± 2 °C. The light intensity was 4.4117 J m^{-2} s^{-1} under the continuous mode. Cell suspension cultures were established from the callus cultures, and were maintained in 125 ml Erlenmeyer flasks with 50 ml of MS medium containing 2,4-D (6 mg l^{-1}), BA (6 mg l^{-1}), and

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

sucrose (30 g l^{-1}) by biweekly sub-culturing on a rotary shaker at 90 rpm maintained at 25 ± 2 °C. Growth was determined by measurement of packed cell volume (pellet volume after centrifugation at 2500*g* in 50 mL graduated conical centrifuge tubes). They were sampled for the experiment in the exponential phase of growth (every 4 d, 10 ml of cells were subcultured in 100 ml of same media).

Treatment of taro cell suspension with elicitors and enzyme extraction

The culture filtrates of *P. colocasiae* including the elicitors were precipitated with acetone (90%), the pellet was freeze-dried, resuspended in 1 ml of distilled water and stored at −20 °C. No further purification of these preparations was made to ensure retention of the elicitor activity. To test the activity and specificity of elicitor preparations, dilution series (1 ×, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128, where 1 × is the original concentration) were used. Each preparation of elicitor was injected into leaflets of resistant (Muktakeshi) and susceptible (Telia) cultivars of taro and the injected areas rated for the presence of necrosis or chlorosis. Concentrations of elicitor used in experiments with cell suspensions were based on the minimum dilution of elicitor that induced necrosis on leaves of Muktakeshi giving the most rapid necrotic response. A dilution of 1/16 was used in this experiment and concentration of this dilution found 1.20-μg protein L^{-1} as measured by the method of Bradford (1976). For elicitor treatment, 10 ml (4 d old) cells were transferred into 250-ml Erlenmeyer flasks containing 100 ml of fresh medium. Later (10 h), sterile elicitor (1/16 dilution) and water (control) was added to the cell suspension. After elicitation cell cultures were harvested at different time intervals for 4 days to monitor the influence of elicitor on defense responses in susceptible and resistant cultivar of taro. The harvested cells were washed twice with 100 mL water on a porous-glass funnel with filter paper (Whatman No. 1) and crude enzyme extracts were prepared according to method described by Hahlbrock and Ragg (1975) and stored at −80 °C until assay.

Measurements of defense reactions in taro cell suspension cultures

All measurements were made on taro suspension cells treated with elicitor or water after 4 d of subculture. Peroxidase (POD; E.C. 1.11.1.7) activity was assayed in the supernatant by the method of Van Gestelen et al. (1997). The activity of L-phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5.) was determined by the method of Zucker (1965). Lipoxygenase (LOX; E.C. 1.13.11.12) activity was determined by potassium iodide-starch method developed by Williams et al. (1986).

Northern blot hybridization

Suspension-cultured taro cells were collected by filtration after 12, 24, 36 and 48 h of elicitor (1.20-μg protein L^{-1}) or water (control) treatment and subsequently frozen in liquid N_2. Total RNA was isolated according to the protocol described by Verwoerd et al. (1989). About 10–15 μg of total extracted RNA was denatured at 50 °C in 1 M glyoxal, DMSO, and 10 mM sodium phosphate, electrophoresed, and transferred to positively charged Hybond™ nylon membrane (Amersham Biosciences) (Sambrook et al., 1989). The cDNAs corresponding to the coding regions of the endochitinase of taro (NCBI accession number EU368044) was labeled with DIG DNA Labeling kit (Roche Molecular Biochemicals) and transcript was detected according to the recommendations of the manufacturer (Roche Molecular Biochemicals). Equal loading of the gel was controlled by hybridization with a 200 bp fragment corresponding to the 18S rDNA of *P. colocasiae*.

Southern blot hybridization

Twenty micrograms of highly purified genomic DNA of *P. colocasiae* was digested with 5 units mg^{-1} of *Bam*HI and *Alu*I (Fermantas, India), electrophoretically separated on 0.8% agarose gels and after alkaline denaturation blotted to positively charged Hybond™ nylon membrane (Amersham Biosciences). Hybridization was performed in 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.1% SDS, and herring sperm DNA (100 mg mL^{-1}) at 65 °C with DIG DNA (Roche Molecular Biochemicals) labeled probe, corresponding to the *EcoR*I–*Xho*I fragment of the elicitor cDNA. Detection was done according to the recommendations of the manufacturer (Roche Molecular Biochemicals).

Plant assay

Inoculation of taro leaves (cv. Telia) with *P. colocasiae* in time course experiments was conducted by placing 20 μL containing approximately 2×10^3 spores on the abaxial surface of

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

detached taro leaves (cv. Telia). The leaves were placed in Petri dishes containing water agar (15 g/liter) to maintain high humidity at 28 °C. Leaf blight symptoms were scored daily, and leaf disks of similar sizes were dissected around the inoculated area and used for RNA extractions.

Results

Molecular cloning of elicitor gene

The molecular cloning of the peptide portion of elicitor gene was undertaken by a polymerase chain reaction (PCR)-based approach. The glycoprotein was purified to homogeneity and amino acid sequences of peptides derived from the molecule were obtained. The amino terminal sequence, established after electrophoresis of the glycoprotein and transfer to a polyvinylidene difluoride (PVDF) membrane, consists of 12 amino acids (E–T–C–S–P–T–D–E–T–T–A–Y). The amino terminus was used for the design of the oligonucleotides primers EP1 (5′-TGTTCGCCCACCGATGAAAC-3′), EP2 (5′-TGCTCACCTACCGACGAGAC-3′) and EP3 (5′-GTTCGCCGACTGACGAAAC-3′). A PCR with EP2 or EP3 primer and an aliquot of phage suspension containing a cDNA library of isolate 98–111 of the *P. colocasiae* did not result in a specific amplification product. However, when primer EP1 was used in combination with a primer corresponding to the T7 promoter of the λZapII vector near the 3′ end of the unidirectionally cloned cDNA, approximately 600-bp PCR product was obtained. This product was subcloned, sequenced and used as probe for screening the cDNA library of *P. colocasiae*. After three rounds of screening, 6 positive clones were analyzed by PCR with the primers T3+T7. Two of these clones resulted in a PCR product of approximately 750 bp with T3+T7 primers and 600 bp with primers T7 and EP1. Both of the clone of this homogenous group was subjected to *in vivo* excision of the pBluescript SKII (+) vector (Promega) and sequenced. The cloned cDNA has a size of 620 bp, of which 60 bp corresponds to the 5′ translated signal peptide region, 68 bp corresponds to the 3′ untranslated region and 510 bp corresponds to the unique open reading frame present in the cDNA. Translation of the open reading frame indicates the elicitor gene product has 170 amino acids with a predicted molecular mass 17 kDa and estimated isoelectric point of 3.83. The predicted protein contains all of the peptides sequenced from the purified protein demonstrating that the given gene is the elicitor gene. The integrity and congruity of the isolated fragment from phage plaques was verified additionally by RT-PCR analysis using primers spanning the ORF that yielded 560 bp amplification product. Comparison of the amino acid sequences of elicitor protein of *P. colocasiae* with sequences present in the database showed significant sequence similarity with other families of elicitor protein (Figure 1). Moreover, the high proportion of serine (15.3%) and threonine (18.3%), which are potential sites for *O*-glycosylation, together with the presence of a putative *N*-glycosylation site (Asn-X-Ser/Thr) located at position 103, accounting for high glycosylation level of the glycoprotein.

Occurrence of elicitor gene sequences in *P. colocasiae* genome

To determine the number of copies of the cloned elicitor gene sequences that occur in the *P. colocasiae* genome, Southern blot hybridizations were performed. *Bam*HI and *Alu*I digested DNA of *P. colocasiae* isolate 98–111 was hybridized with the

Figure 1. Alignment of elicitor glycoprotein sequence from *Phytophthora colocasiae* (1) with glycoprotein elicitor of *P. infestans* (2), elicitin-like protein of *P. brassicae* (3), putative elicitin protein RAM2A of *P. ramorum* (4) and putative elicitin protein SOJ2C of *P. sojae* (5). Shading indicates blocks of identical (gray) or similar (red) amino acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

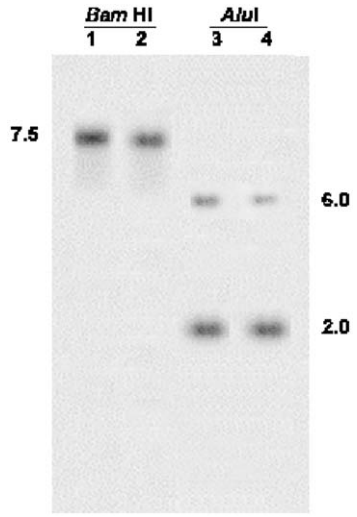

Figure 2. Southern blot analysis of *Phytophthora colocasiae* genomic DNA. Total genomic DNA (20 μg) of *P. colocasiae* isolate 98–111 was digested with *Bam* HI (lanes 1 and 2) and *Alu*I (lanes 3 and 4) and hybridized with cDNA (elicitor glycoprotein).

entire insert of elicitor cDNA. One *Bam*HI fragment of approximately 7.5 kb and two *Alu*I fragments of 6.0 and 2.5 kb were detected (Figure 2). Since there is one *Alu*I site and no *Bam*HI site in the cloned elicitor cDNA, this result clearly indicated the presence of only one elicitor gene in *P. colocasiae* genome. Southern blot analysis with four other *P. colocasiae* isolates, PC-53, PC-71, PC-Tvm and 98-35a, revealed the same hybridization pattern as *P. colocasiae* 98–111 with both the entire cDNA probe (data not shown) even though these isolates are polymorphic at other loci (data not shown). This suggests that elicitor gene sequences are conserved between the five tested *P. colocasiae* isolates.

Expression of elicitor during various developmental stages of *P. colocasiae*

To determine the patterns of expression of the elicitor gene, Northern blot analyses were performed, and accumulation of elicitor mRNA in various developmental stages of *P. colocasiae* 98–111 was determined. No elicitor mRNA was detected in RNA extracts of zoospores, germinating cysts and sporangiospores. However, elicitor mRNA was detected in extracts of mycelium obtained 24 h after germination of sporangiospores in defined liquid medium (Farmer and Helgeson, 1987) (Figure 3) suggesting that the elicitor gene is exclusively expressed in mycelium. Control hybridizations with a probe of the constitutively

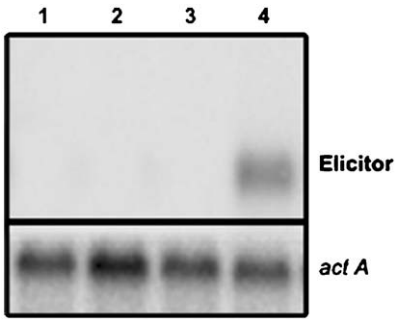

Figure 3. Expression of elicitor gene in various developmental stages of *Phytophthora colocasiae*. Total RNA from *P. colocasiae* zoospores (1), geminated cyst (2), sporangiospores (3) and (4) mycelium was sequentially hybridized with probes from the cDNA of elicitor and *actA* genes.

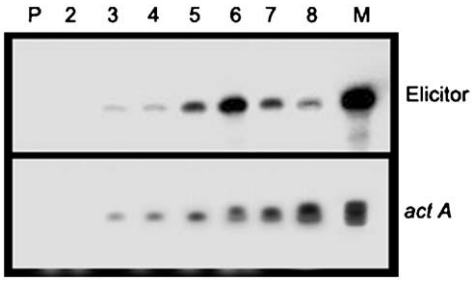

Figure 4. Time course of expression of elicitor and *actA* gene of *Phytophthora colocasiae* during infection of taro (cv. Telia). Total RNA isolated from infected leaves of taro at 2–8 days after inoculation of *P. colocasiae*, from non-infected leaves (P) and from *P. colocasiae* mycelium grown in potato-dextrose medium (M) was sequentially hybridized with probes from the elicitor and *actA* genes.

expressed *actA* gene showed that all lanes contained similar amounts of total RNA (Figure 3).

Expression of elicitor *in planta*

To analyze the expression of elicitor during the interaction of *P. colocasiae* with its host plant taro, total RNA was isolated from leaves of taro (cv. Telia) 1–8 days after inoculation with *P. colocasiae* and from *P. colocasiae* mycelium. A Northern blot containing these samples was hybridized with probes of the *P. colocasiae* elicitor cDNA and *actA* genes (Figure 4). Because the total RNA extracted from infected leaves consists of a mixture of *P. colocasiae* and plant RNA, the signals obtained on Northern blots with probes of differentially expressed genes was normalized to actual *Phytophthora* RNA levels as determined by the signals obtained with a probe of a constitutively expressed gene. Consistent with increases in *P. colocasiae* biomass during the infection, the

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

mRNA of the constitutively expressed *actA* gene expression level was increased in the following days until reaching a maximal level at days 6–8. In contrast, elicitor mRNA was first detected at day 3 after inoculation and reached the highest level at days 5 and 6, when extensive sporulation of *P. colocasiae* and leaf necrosis occurred. Subsequently, at days 7 and 8 when little additional sporulation occurred, the level of elicitor mRNA decreased. In contrast to the *actA* mRNA, the levels of the elicitor mRNA observed *in planta* were always lower than the levels observed *in vitro*,

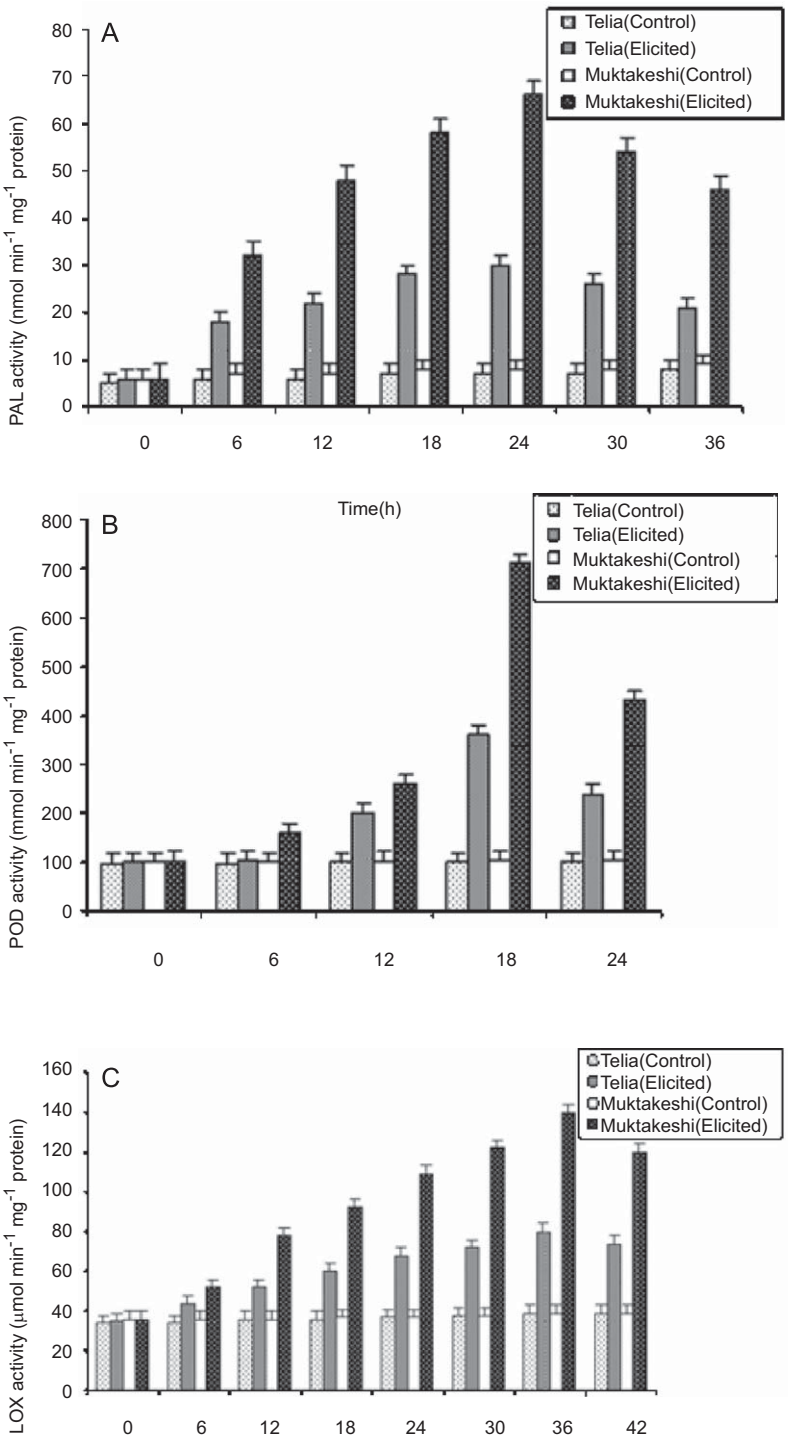

Figure 5. Levels of L-phenylalanine-ammonia lyase (A), peroxidase (B) and lipoxygenase (C) activities obtained in elicited and control suspension-cultured taro cells (cv. Telia and Muktakeshi). Results are the means ± SD of three replicates and representative of three experiments.

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

particularly during the early stages of infection (days 3 and 4) and the late postsporulation stages (days 7 and 8). These results suggest that expression of elicitor protein is down-regulated during infection of taro.

Elicitation of suspension-cultured taro cells

The obtained results indicate that except for the control treatment, there were differences in PAL activity between the two taro cultivars studied (Figure 5A) Muktakeshi cultivar showed a higher increase in the PAL activity with the concentrations of the 1.20 $\mu g\,L^{-1}$ eliciting solution in the cell suspension culture medium. The cultivar Telia responded moderately to the same concentration of elicitor solution in its cell suspension media. Some works (Campbell and Ellis, 1992) point the enzyme PAL as the precursor of the lignin biosynthesis, phenols, flavonoids and phytoalexines by plant tissues, related to the plant response system against microorganisms, insects and other stress factors. The observed results with two cultivars could reflect the cell response capacity due to the fungal glycoprotein applied.

Peroxidase induction was observed at the highest level of elicitor application in the Muktakeshi cultivar. As for Telia cultivar significant difference was observed compared to the control but comparatively lower than Muktakeshi cultivar (Figure 5B). The peroxidase activity, in general increases under different stress conditions like wounds, fungi infections, salinity, water stress and nutritional disorders, inducing the lignin increment and production of ethylene and phenols (Van Huystee, 1987). This activity suggests a cell effort for the establishment of a physiochemical barrier, able to isolate the infected area. The potential of the Muktakeshi cultivar is clear, presenting increase in peroxidase activity after the treatment of elicitor protein and it seems to be the most responsive genotype against *P. colocasiae*.

In recent years, an increasing body of information has tended to prove that metabolites generated by the lipoxygenase (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) or rice (Melan et al., 1993) or during treatment of plant cell cultures by fungal elicitors. We have now shown that the elicitor treatment was similarly effective in stimulating LOX activity in taro cell cultures (Figure 5C). The rapid and transient increase in enzyme activity peaked around 36 h after treatment of the cells with the elicitor. However, induction level was highest in Muktakeshi cultivar compared to Telia cultivar of taro.

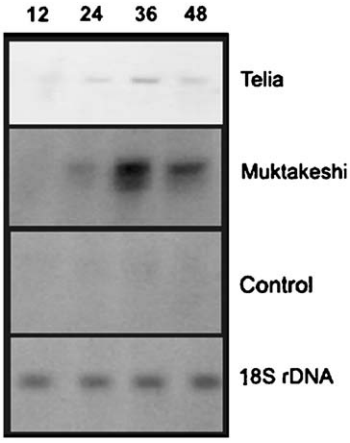

Figure 6. RNA was extracted from suspension-cultured taro cells after elicitor or water treatment at times (h) indicated. Total RNA (10 μg) was subjected to denaturing electrophoresis in a formaldehyde-agarose gel, transferred to a nylon membrane and hybridized with probe corresponding to taro endochitinase and 18S rDNA.

Collectively, the accumulation of PR proteins was maximum at 3 days and remained high for 5 days in taro cell suspension treated with the fungal glycoprotein, which also corresponded to the time point when the induced necrosis was well established.

The time course Northern blot analysis demonstrated that the expression level of endochitinase was higher in resistant variety compared to susceptible variety of taro. Additionally, the highest expression levels were observed following 36 h after elicitor treatment (Figure 6), while control cell suspension failed to exhibit detectable levels of expression.

Discussion

Ever since the taro leaf blight epidemics of the mid-nineteenth century, members of the genus *Phytophthora* have emerged as major pathogens of numerous crops (Erwin and Ribeiro, 1996). Despite the importance of *Phytophthora* species as devastating plant pathogens, little is known about the molecular mechanisms that determine the outcome of interactions between *Phytophthora* and plants (Judelson, 1997). 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. In this paper, we report the molecular

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

cloning of a *P. colocasiae* cDNA encoding a host-specific elicitor protein. The DNA sequence of the elicitor cDNA revealed a 510-bp ORF encoding a pre-elicitor protein of 170 amino acids. Processing of the 20 amino acid N-terminal signal peptide resulted in the mature 150 amino acid elicitor protein of molecular mass 15 kDa. Comparison of the amino acid sequences of elicitor protein with sequences present in the database showed significant sequence similarity with other families of elicitor protein. The pattern of expression of the elicitor gene was followed throughout the disease cycle and in various stages of the life cycle of *P. colocasiae*. During the early stages of infection, expression of elicitor does not occur prior to the penetration of plant cells. Expression of elicitor occurs later during infection although at a reduced level. The highest levels of elicitor expression occur at later stages concurrent with the onset of extended leaf necrosis, saprophytic growth and profuse sporulation. The expression of elicitor is then repressed in sporangiospores and zoospores until a new infection cycle is initiated. The elicitor gene reaches maximal levels of expression in sporulating mycelium whether growing *in vitro* or *in planta*. Comparison of the expression patterns of elicitor and *actA* indicate that elicitor is down-regulated during infection of taro. The products of elicitor or avirulence genes trigger defense responses in plants that are ultimately deleterious to the pathogen. Therefore these genes are under selective pressure to mutate to inactive forms leading the pathogen into an evolutionary race with the plant (Staskawicz et al., 1995). The down-regulation of the expression of the elicitor gene during infection of taro, noted in this study, could therefore be an adaptation of *P. colocasiae* to evade plant defense responses. Therefore, even though elicitor is produced during infection, it is not detected by compatible host plants and does not appear to play a role in restricting the disease process.

Expression of the various biochemical defense responses in plants is known to be coordinately regulated (Graham and Graham, 1991). For instance, in tobacco, PAL and OMT gene expression is induced about 24 h after TMV inoculation (Cordelier et al., 2003), in taro, transcripts of the PR-protein (endochitinase) exhibit a rapid up-regulation as early as 8 h after the appearance of symptom and reached maximum levels after 36 h (Sharma et al., 2008). PR-protein gene transcription starts only 2–3 days of post-infection (Ward et al., 1991). Similar differential kinetics of PR-protein activity was observed in the present study after treatment of taro cell suspension with the elicitor glycoprotein, indicating its function as classical elicitor molecules that trigger defense responses in taro plant. We observed that the elicitor protein of *P. colocasiae* does not induce a HR on other tuber crops. Even though the HR was always associated with the resistance response of taro to *P. colocasiae*, the timing, severity, and extent of the HR varied considerably, depending on the examined genotype. Similarly, *P. colocasiae* reached different levels of colonization on different taro plants. The data showed that the molecule is a potent elicitor that induces necrosis and defense gene expression in the taro–*P. colocasiae* interaction. The time course of gene induction is comparable to results described for defense gene induction in tobacco after leaf infiltration with an elicitor from *P. megasperma* (Baillieul et al., 2003) or treatment of a cell suspension with cell wall fragments from *P. parasitica* var. *nicotianae* (Rickauer et al., 1997). Considered as a whole, the results of the present study strongly suggest that the effect of elicitor glycoprotein on suspension-cultured taro cells mimics a typical HR, which is viewed as rapid plant cell death, coordinate expression of biochemical defence responses under the control of endogenous signal molecules. The glycoprotein may act as an avirulence factor in the *Phytophthora*–taro interaction contributing, with elicitins, to the incompatible interaction between these two species. It represents a molecular tool to understand the role of cell death in limiting a challenging pathogen and in providing the neighboring and distant zones undergoing systemic acquired resistance. Finally, cloning of the sequences coding for the fungal elicitor and their expression in transgenic plants under the control of promoters inducible by compatible pathogens could lead to manipulation of the HR and confer broad disease resistance in taro in a manner similar to the avr 9/Cf 9 cassette strategy proposed to confer broad disease resistance in tomato (De Wit, 1995). Since, constitutive elicitor production can be lethal to a plant, so that, elicitor activation should occur only at the time of pathogen challenge and not under other circumstances. Thus, the strategy described above requires a gene encoding a highly active protein elicitor and a functional promoter that is specifically inducible by a virulent pathogen *P. colocasiae*. The selected gene promoter will neither be developmentally nor tissue specifically regulated and will not respond to environmental stimuli. The promoter will be activated in taro only during interaction with *P. colocasiae* and other pathogens. Thus resistance response will appear only after the interaction with pathogens and spatially coordinate activation of defense mechanisms.

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002

Acknowledgments

The funding provided for conducting the research work by the Indian Council of Agricultural Research, New Delhi, is gratefully acknowledged. The authors thank Director, Central Tuber Crops Research Institute, Thiruvananthpuram, for providing the infrastructure facilities.

References

Baillieul F, Ruffray PD, Kauffmann S. Molecular cloning and biological activity of α-, β-, and γ-megaspermin, three elicitins secreted by *Phytophthora megasperma* H20. Plant Physiol 2003;131:155–66.

Binet M, Humbert C, Lecourieux D, Vantard M, Pugin A. Disruption of microtubular cytoskeleton induced by cryptogein, an elicitor of hypersensitive response in tobacco cells. Plant Physiol 2001;125:564–72.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye-binding. Anal Biochem 1976;72:248–54.

Campbell MM, Ellis BE. Fungal elicitor-mediated responses in pine cell cultures. Planta 1992;186:409–17.

Cordelier S, de Ruffray P, Fritig B, Kauffmann S. Biological and molecular comparison between localized and systemic acquired resistance induced in tobacco by a *Phytophthora megasperma* glycoprotein elicitin. Plant Mol Biol 2003;51:109–18.

de Wit PJGM. Fungal avirulence genes and plant resistance genes: unraveling the molecular basis of gene-for-gene interactions. Adv Bot Res 1995;21:147–85.

Edman P, Begg G. A protein sequenator. Eur J Biochem 1967;1:80–91.

Erwin DC, Ribeiro OK. *Phytophthora* diseases worldwide. St. Paul, MN: American Phytopathological Society; 1996.

Farmer EE, Helgeson JP. An extracellular protein from *Phytophthora parasitica* var. nicotianae is associated with stress metabolite accumulation in tobacco callus. Plant Physiol 1987;85:733–40.

Graham TL, Graham MY. Cellular coordination of molecular responses in plant defense. Mol Pl–Microbe Interact 1991;4:415–22.

Hahlbrock K, Ragg H. Light-induced changes of enzyme activities in parsley cell suspension cultures. Arch Biochem Biophys 1975;166:41–6.

Hammond-Kosack KE, Silverman P, Raskin I, Jones JDG. Race-specific elicitors of *Cladosporium fulvum* induce changes in cell morphology and the synthesis of ethylene and salicylic acid in tomato plants carrying the corresponding Cf disease resistance gene. Plant Physiol 1996;110:1381–94.

Judelson HJ. The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. Fungal Genet Biol 1997;22:65–76.

Kato T, Maeda Y, Hirukawa T, Namai T, Yoshioka N. Lipoxygenase activity increment in infected tomato leaves and oxidation product of linolenic acid by its in vitro enzyme reaction. Biosci Biotech Biochem 1992;56:373–5.

Lam E, Kato N, Lawton M. Programmed cell death, mitochondria and the plant hypersensitive response. Nature 2001;411:848–53.

Logemann J, Schell J, Willmitzer L. Improved method for isolation of RNA from plant tissue. Anal Biochem 1987;163:16–20.

Melan MA, Dong X, Endara ME, Davies KR, Ausubel FM, Peterman TK. An Arabidopsis thaliana lipoxygenase gene can be induced by pathogens, abscisic acid and methyl jasmonate. Pl Physiol 1993;101:441–50.

Mishra AK, Sharma K, Misra RS. Rapid and efficient method for the extraction of fungal and oomycetes genomic DNA. Gene Genome Genomics 2008;2(1): 57–9.

Misra RS, Sharma K, Mishra AK. *Phytophthora* leaf blight of taro (*Colocasia esculenta*)—a review. Asian Austral J PlantSci Biotech 2008;2(2):55–63.

Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Pl 1962;15:473–93.

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell 1994;78:449–60.

Osbourn AE. Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell 1996; 8:1821–31.

Peever TL, Higgins VJ. Electrolyte leakage, lipoxygenase, and lipid peroxidation induced in tomato leaf tissue by specific and nonspecific elicitors from *Cladosporium fulvum*. Plant Physiol 1989;90:867–75.

Raciborski M. Parasitische Algen und Pilze, Java's (Java's Parasitic algae and fungi). I. Batavia. (Cited in Waterhouse 1970a under *P. colocasiae*), 1900. 9pp.

Rickauer M, Brodschelm W, Bottin A, Véronési C, Grimal H, Esquerré-Tugayé MT. The jasmonate pathway is involved differentially in the regulation of different defence responses in tobacco cells. Planta 1997; 202:155–62.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

Sasabe M, Takeuchi K, Kamoun S, Ichinose Y, Govers F, Toyoda K, et al. Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. Eur J Biochem 2000;267: 5005–13.

Sharma K, Mishra AK, Misra RS. Identification and characterization of differentially expressed genes in the resistance reaction in taro infected with *P. colocasiae*. Mol Biol Rep 2008.

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JDG. Molecular genetics of plant disease resistance. Science 1995;268:661–7.

Van Gestelen P, Asard H, Caubergs RJ. Solubilization and separation of a plant plasma membrane NADPH–O_2-

synthase from other NAD(P)H-oxidoreductases. Plant-Physiol 1997;115:543–50.

Van Huystee RB. Some molecular aspects of plant peroxidase biosynthetic studies. Ann Rev PlantPhysiol 1987;36:205–19.

Verwoerd TC, Dekker BMM, Hoekema A. A small-scale procedure for the rapid isolation of plant RNA's. Nucleic Acids Res 1989;17:2362.

Wang YC, Hu DW, Zhang ZG, Ma ZC, Zheng XB, Li DB. Purification and immunocytolocalization of a novel *Phytophthora boehmeriae* protein inducing the hypersensitive response and systemic acquired resistance in tobacco and Chinese cabbage. Physiol Plant Pathol 2003;63:223–32.

Ward E, Uknes S, Williams S, Dincher S, Wiederhold D, Alexander D, et al. Coordinate gene activity in response to agents that induce systemic acquired resistance. PlantCell 1991;3:1085–94.

Wendehenne D, Pugin A, Klessig DF, Durner J. Nitric oxide: comparative synthesis and signaling in animal and plant cells. Trends PlantSci 2001;6:177–83.

Williams DC, Lim MH, Chen AO, Pangborn RM, Whitaker JR. Blanching of vegetables for freezing—which indicator enzyme to choose. Food Technol 1986;40: 130–40.

Zucker M. Induction of phenylalanine ammonia-lyase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol 1965;40:779–84.

Please cite this article as: Mishra AK, et al. Cloning and characterization of cDNA encoding an elicitor of *Phytophthora colocasiae*. Microbiol Res 2006; (2009), doi:10.1016/j.micres.2008.10.002