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Identification of *Phytophthora colocasiae* genes differentially expressed during infection on taro (*Colocasia esculenta*)

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ABSTRACT

Taro leaf blight caused by *Phytophthora colocasiae* presents the single biggest constraint for taro production globally. Understanding the molecular mechanisms underpinning infection will be of great asset in managing this devastating disease. Here, we used a suppression subtractive hybridization (SSH) approach to identify genes that are differentially expressed in *P. colocasiae* during a compatible interaction with taro. A cDNA library enriched for upregulated *P. colocasiae* genes were generated using a novel inoculation system that allowed the physical separation of the induced mycelium from the host. Reverse northern analysis of randomly selected clones revealed clear induction of these genes during infection. Sequence analysis of these genes classified them into various biological, molecular and cellular processes. Reverse transcriptase quantitative PCR (RT-qPCR) assay of selected *P. colocasiae* genes showed an increased expression of these genes during infection of taro. The results of the study provide valuable insights into the gene expression patterns in *P. colocasiae* during infection on taro, which could be important for pathogenicity.

Introduction

Taro [*Colocasia esculenta* (L.) Schott] is an important tropical tuber crop, used as a staple food or subsistence food by millions of people in the developing countries in Asia, Africa, and Central America [1]. Leaf blight caused by *Phytophthora colocasiae* Raciborski is the most destructive disease of taro [2]. The disease has become a major constraint for taro production globally, including India causing yield reduction up to 50% every year [3].

At present, metalaxyl based fungicides have been advocated to control the disease. However, the presence of waxy leaf surface and occurrence of disease during rainy season make this approach ineffective. Lack of flowering or shy flowering are the major limiting factors for the breeding program to develop high yielding varieties resistant to leaf blight. Although, some cultivars (Muktakeshi, Jhankri, Topi) are tolerant to leaf blight disease, they have been under looked due to lacking of other desirable market value traits. Therefore, finding an alternative disease management strategy in taro production system is acute.

P. colocasiae can infect the plant at any stage of growth throughout the growing season. Like other *Phytophthora* spp., *P. colocasiae* also has a lifestyle that features a biotrophic and necrotrophic phase [4–6]. In the early biotrophic phase, specialized infection structures (termed haustoria) are formed to breach the plant cell wall and interface with the host membrane [7]. The initial biotrophic phase is crucial for infection and disease establishment, after which the pathogen enters necrotrophic phase with rapid intracellular growth and colonization, ultimately leading to a host cell death and initiation of a new infection cycle [8].

To develop improved methods for the management of taro leaf blight caused by *P. colocasiae*, it is crucial to understand the mechanisms underpinning infection and disease establishment. One approach to identify important factors in the establishment of

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an infection is to examine pathogen gene expression patterns during the course of infection. This will enable the identification of essential transcriptional changes that occur in the pathogen during infection and its establishment. Components essential for pathogenesis could be involved in different phases of plant colonization, e.g. attachment, germination, appressorium formation, invasive growth, nutrient uptake and sporulation [9]. In addition, these factors could also play a role in protection against plant defense responses.

The aim of the present study was to provide helpful insights into the *P. colocasiae* genes that are differentially expressed during a compatible interaction with taro using a suppression subtractive hybridization (SSH) approach. It was anticipated that the results of this study will allow a better understanding of the mechanisms involved in the infection process which could lay foundation for future studies in taro-*P. colocasiae* interaction.

Materials and methods

Plant material and P. colocasiae strain

P. colocasiae strain PC2 was isolated from leaf blight infected samples and maintained on Carrot Agar (CA; 250 g l^{-1} carrot and 20 g l^{-1} agar) slants at 15 °C in the dark [10]. Virulence was maintained by infecting detached taro leaves and re-isolation every 2–3 months. To obtain mycelia for inoculation, isolate was grown on CA plates overlaid with sterile cellophane at 28 °C in the dark for 5–7 days. This allowed the easy recovery of the mycelia from the plate by gentle scraping.

The taro cultivar Sree Kiran susceptible to leaf blight was used for the study. Plants were grown axenically in a greenhouse under normal conditions.

P. colocasiae inoculation and sampling

The leaf inoculation procedure was developed in this study. Mycelia grown on CA plates were gently scrapped using a scalpel, washed with sterile water and blot dried on Whatman filter paper. First, small portions of mycelia were placed in Petri dish (200 mm) containing 50 ml of water (control) or on the abaxial side of a taro leaf disc (5 × 5 cm) floating in 50 ml water (tester). Next, 0.2 ml of water was added to the mycelia on the leaf disc and the leaf was covered with another taro leaf disc. Finally, plates were covered with a lid containing moistened filter paper to maintain high humidity and placed at 25 °C in the dark. The dishes were incubated for 4, 8, 15 or 24 h and the induced mycelia were harvested by carefully picking the mycelia from the infection site using forceps. The control sample was also handled in a similar manner. The samples were pooled to create one 'control' and one 'tester' sample. The mycelia was dried quickly on Whatman filter paper, flash frozen in liquid nitrogen and stored in −80 °C until further use.

Total RNA extraction

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and stored at −80 °C until use. The integrity of the RNA was confirmed by agarose gel electrophoresis. Poly (A) + mRNA was purified from the total RNA using an Oligotex™ mRNA Kit according to the manufacturer's recommendations (QIAGEN, Tokyo, Japan). This step eliminated the possibility of DNA contamination in the RNA samples used for library construction.

Suppression subtractive hybridization (SSH) and library construction

The SSH process was carried out using a Clontech PCR-Select™ cDNA subtraction kit (Clontech Inc., CA, USA). All PCR amplifications were carried out using the Advantage cDNA PCR kit (Clontech). A complete control subtraction was performed in parallel with the experimental subtraction using skeletal muscle cDNA provided in the kit. Briefly, cDNA was synthesized from mRNA using an oligo-dT primer that allowed the amplification of mRNA population contained in each sample. After adapter-ligation and two rounds of subtractive hybridizations, the PCR products were purified using a GeneJET™ PCR purification kit (Fermentas, EU). The purified products were cloned into the pTZ57 R/T vector (InsTAclone PCR cloning kit, Fermentas, EU) and transformed into competent *Escherichia coli*, DH5α (TaKaRa). Aliquots (100 μl) of the transformation mixture was spread on LB agar plates containing 50 μg ml^{-1} ampicillin, 40 μg ml^{-1} X-gal and 40 μg ml^{-1} IPTG and were incubated at 37 °C overnight for blue/white screening. Large white colonies were picked and used to regenerate single clone cultures in 96-well microtiter plates. After overnight growth at 37 °C, glycerol was added to a final concentration of 15% and cultures were stored at −80 °C.

Reverse northern hybridization

Individual white clones were randomly picked from the subtracted library and placed in LB medium containing ampicillin (50 μg ml^{-1}) and incubated at 37 °C for 8 h. One microliter of the medium was removed as a template and the inserts were amplified using the nested PCR primer1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and nested PCR primer2 (5′-AGCGTGGTCGCGGCCGAGGT-3′). PCR amplifications were performed in an Agilent sure cycler 8800 (Agilent Technologies, USA). The PCR products were electrophoresed on a 1.5% agarose gel to confirm their quality and also to identify their insert size. The amplification products from each clone of the library were denatured at 100 °C for 5 min and blotted onto positively charged Hybond-N+ nylon membrane (Amersham, GBR). Samples were cross-linked to the membrane by exposing the membrane to UV light for 66 s. The cDNA probes were synthesized from total RNA prepared from the mycelium of tester and control samples and were biotin labeled according to manufacturer's instructions (Thermo scientific, USA). The procedures of hybridization and detection were performed according to the Pierce North2South hybridization and detection kit (Thermo scientific, USA) following the manufacturer's instructions.

Sequencing and sequence analysis

Plasmids containing cDNA fragments that were differentially expressed were sequenced with universal M13 forward and reverse primers homologous to the vector sequence. The nucleotide sequences obtained were processed to remove vector sequence and transformed into contigs using Geneious Pro software version 5.6. The resulting high quality sequences were compared against the GenBank non-redundant (nr) database using the BLASTX algorithm from the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). For each contig, the nucleotide sequence with highest homology that had an annotated physiological role and an *E* value of less than 1e-4 were considered to be significant. The sequences were annotated using BLAST2GO (http://www.blast2go.com), a universal web-based annotation application.