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Identification and characterization of differentially expressed genes in the resistance reaction in taro infected with *Phytophthora colocasiae*

Kamal Sharma · Ajay Kumar Mishra · Raj Shekhar Misra

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Abstract Leaf blight disease caused by *Phytophthora colocasiae* represents a major constraint to the growth and yield of taro (*Colocasia esculenta* L.). Ongoing research on model plant systems has revealed that defense responses are activated via signaling pathways mediated by endogenous signaling molecule such as salicylic acid, jasmonic acid, and ethylene. Activation of plant defenses is associated with changes in the expression of large number of genes. To gain a better understanding of defense responses, virulent race of *P. colocasiae* was used to inoculate the taro cultivar UL-56 (compatible) and its nearly isogenic line Muktakeshi (incompatible). We have employed suppressive subtractive hybridization (SSH), cDNA libraries, Northern blot analysis, high throughput DNA sequencing, and bioinformatics to identify the defense-related genes in taro induced by *P. colocasiae* infection. Two putative resistance genes and a transcription factor were identified among the upregulated sequences. The expression of several candidate genes including lipid transfer proteins (LTPs), and other pathogenesis-related genes were evaluated following 8–48 h of appearance of symptom in compatible and incompatible interactions. Results confirmed the higher overall expression of these genes in Muktakeshi (resistant) compared to UL-56 (susceptible). This study constitutes the first attempt to characterize the taro differential transcriptome associated with host–pathogen interactions from different genotypes. All the generated ESTs have been submitted to GenBank for further functional studies.

Keywords *Colocasia esculenta* · Defense responses · Leaf blight · Suppression subtractive hybridization · Resistance gene

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]. The corms, leaves, and petioles are used as vegetable and considered as a rich source of carbohydrates, proteins, minerals, and vitamins. In India, two taro types viz, *C. esculenta* var. *esculenta* (Dasheen type) and *antiquorum* (Eddoe type) are commonly cultivated throughout the country. There are growing concerns over the narrow genetic base of taro cultivars particularly with 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% [2–4]. This has led to initiation of several breeding programs with the aim of broadening the genetic base of breeding populations. Lack of flowering, shy flowering, self- and cross-incompatibilities are the limiting factors for the breeding program to develop high yielding varieties resistant to leaf blight. Metalaxyl- and mancozeb-based fungicides method have been advocated to control disease. But waxy leaf surface and occurrence of disease during rainy season make fungicidal spray ineffective [5]. Furthermore, the fungicide sprays are too costly to be afforded

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K. Sharma · A. K. Mishra · R. S. Misra (✉)
Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala 695017, India
e-mail: rajshekharmisra@gmail.com

by marginal farmers, soil microorganism rapidly degrade metalaxyl and release into water and soil system and development of resistance against the fungicides is another major threat [6]. Thus, there is need to develop integrated management strategies to combat this disease using natural and environmental friendly mechanism.

Resistance responses to plant pathogens are the focus of intensive research, because current technologies offer the possibility of genetically engineering in the plants for broad-based effective resistance against pathogen [7]. Resistance responses can be divided into a series of inter-related stages [7–9]. There is an initial recognition of the pathogen by the host plant that frequently involves the interaction between host resistance (R) genes and pathogen avirulence (Avr) gene, which codes for specific elicitor. An incompatible interaction results in a triggering of the defense responses through signaling pathways, which activate broad series of defense responses that curb or eliminate the pathogen. These responses include the hypersensitive response (HR), upregulation of phenylalanine ammonium lyase (key enzyme in plant defense), deposition of cell wall reinforcing materials, and the synthesis of a wide range of anti-microbial compounds including pathogenesis-related (PR) proteins and phytoalexins [7].

The application of subtractive suppression hybridization (SSH) and expressed sequence tags (EST) cloning can be used to maximize the identification of genes involved in host responses to pathogen infection and disease development. The SSH technique has been used to isolate the plant genes that are expressed in response to infection [10, 11]. The cDNA library generated by molecular hybridization and subtraction techniques reduces the cloning of abundantly expressed housekeeping genes or genes commonly expressed in both control and treated plants and thereby normalize the cDNA expression profiles during library construction. As a result, it significantly enhances the chances of cloning of differentially expressed genes. This is particularly important because many pathogenesis-related genes are expressed at low levels and can be limited to a particular tissue or cell type [12]. These genes are less likely to be represented in a library if standard EST cloning methods are used.

The aim of this study was to characterize the molecular events in the taro–*P. colocasiae* interaction using the SSH technique to compare the populations of mRNA from resistant and susceptible taro genotypes. This report provides the transcriptome analysis of putative genes that are associated with host defense and/or resistance responses in taro in response to *P. colocasiae* infection. The identified putative genes can be used to develop molecular markers for leaf blight resistance genetic mapping project.

Materials and methods

Plant materials and *P. colocasiae* inoculation experiment

Two taro [*C. esculenta* (L.) Schott] lines, cv. UL-56 and its near-isogenic line cv. Muktakeshi were used as the leaf blight susceptible and resistant cultivars respectively. Both the cultivars were grown from tubers in pots containing soil and *Trichoderma* enriched compost. For infection, taro plants grown in pots were placed in an illuminated growth chamber (300 μE m^{-2} s^{-1}) with 12 h photoperiod at 28°C for 4 days. After 4 days, taro leaves of same age were inoculated on their abaxial surfaces with 50 μl of *P. colocasiae* sporangial suspension containing approximately 500 sporangia or with water as control. Treated leaves were further incubated at 27°C with 85% humidity in the same light condition to allow the symptom to develop. Leaves were thoroughly washed and leaf blight infected tissues consisting of a 1 cm section of leaf tissues were sampled, flash-frozen in liquid nitrogen and stored at −80°C until use.

RNA isolation and SSH cDNA library construction

To study the expression of differentially expressed defense-related genes, leaf blight-infected taro leaf tissues were collected after 8, 12, 24, 36, and 48 h after the appearance of symptom. All the samples were immediately stored at −80°C for later RNA extraction. Total RNA was extracted from infected tissues using Trizol (Invitrogen, San Diego, USA) at a ratio of 0.8 g fresh tissue per 10 ml of Trizol and stored at −80°C until use. Poly(A)$^+$ mRNA was purified from the total RNA according to the manufacturer's instructions (Oligotex^{TM} mRNA Kit, QIAGEN, Tokyo, Japan). This step eliminated the possibility of DNA contamination in the RNA samples used for library construction. Purified mRNA samples were checked by gel and further evaluated with a spectrophotometer reading at $A_{260/280}$ nm. Only high quality mRNA was selected for cDNA synthesis. The SSH approach, based on the Clontech PCR-Select cDNA Subtraction Kit (K1804-1) was used for construction of the cDNA library. The SSH process was carried out using a Clontech PCR-Select cDNA subtraction kit (Clontech Inc., CA, USA). A complete control subtraction was performed in parallel with the experimental subtraction using skeletal muscle cDNA provided as driver. The skeletal muscle tester cDNA was prepared by mixing control skeletal muscle cDNA with diluted ϕX 174/*Hae*III control DNA provided in the kit. For experimental subtraction, tester (Muktakeshi) and driver (UL-56) cDNAs were digested with restriction enzyme *Rsa*I to generate blunt-ended fragments. The tester cDNA was then

subdivided into two portions and each was ligated with a different cDNA adaptor provided in the kit. Adaptor ligation was confirmed by PCR followed by two rounds of hybridizations. In the first round, an excess of driver was added to each sample of tester, leading to the enrichment of differentially expressed sequences. During the second round of hybridization, the two primary hybridization samples were mixed together to form new double-stranded hybrids with different ends. Fresh denatured driver cDNA was added again to further enrich differentially expressed sequences. After ligating the ends with adapters supplied by manufacturer, primary PCR (30 cycles) and secondary PCR (15 cycles) amplification were performed using the primers that matched the different adaptors to the 5′- and 3′-ends according to the manufacturer's instructions for the PCR-Select. The PCR products of the subtraction were analyzed by gel electrophoresis. The bands were eluted using GFX Gel band purification kit (Amersham, NJ, USA) and cloned into the pGEM-T® vector (Promega, WI, USA). The cDNA clones were transformed and amplified in *Escherichia coli* DH5α cells and positive transformants were selected by 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.

Screening and sequencing of the subtractive clones

Transformants were randomly selected from the subtracted library to amplify the inserted sequences. Each reaction tube contained 2.5 μl 10X *Taq* buffer, 2 μl $MgCl_2$ (25 mM), 2 μl dNTP (2.5 mM each), 1 μl of nested primer 1 and nested primer 2R (10 μM), 16.4 μl of PCR-grade water and 0.1 μl *Taq* DNA polymerase. PCR was performed according to the following parameters: 95°C for 30 s and 25 cycles at 95°C for 10 s, 68°C for 30 s and 72°C for 1.5 min. PCR products were analyzed by electrophoresis on 1.2% agarose gel. Five μl PCR product of each positive clone was mixed with 5 μl 0.6 N NaOH. Then 1 μl of mixture was applied to a positively charged nylon membrane (Amersham, NJ, USA). PCR products of subtracted cDNAs were purified separately by a PCR Purification Kit (Watson Biotechnologies Inc., Shanghai, China) and cleaved with *Rsa*I and *Sma*I. The cleaved cDNAs were probed by DIG DNA Labeling (Roche Molecular Biochemicals). Two rounds of differential screening were performed to screen the subtracted library. Two copies of positively charged Hybond™ nylon membrane (Amersham, NJ, USA) were prepared for differential screening by subtracted probes. Hybridization and washing were carried out by conventional protocol [13]. Detection procedure was according to the recommendations of the manufacturer (Roche Molecular Biochemicals, Penzberg, Germany). The positive clones then were selected for further analysis and sequenced by using T7 or SP6 promoter primers.

Northern blot analysis

The gene expression profiles for defense-related genes in taro leaf tissues after 8, 12, 24, 36, and 48 h of leaf blight infection and the corresponding non-infected controls were investigated by Northern blot analysis. An aliquot of 20 μg total RNA (8–48 h) was fractionated on 1.2% agarose-formaldehyde gel and transferred by capillary action overnight to positively charged Hybond™ nylon membrane (Amersham Biosciences, NJ, USA) using 10X SSC [13]. The RNA on the membrane was fixed by baking at 80°C for 2 h. Hybridizations were performed by using 5 ng μl^{-1} DIG-labeled subtracted cDNAs as described by Southern [14]. Hybridization signals were detected using CDP-Star® as outlined by the manufacturer (Roche Diagnostics, Penzberg, Germany).

Sequence analysis for predicting gene function

BLASTn and BLASTx from NCBI and other bioinformatic tools were applied to analyze all sequence data. Gene annotations were done based on similarities to either known or putative ESTs in the public databases. All annotations were based on Blast searches with a score threshold of ≥200 for BLASTn. For tBLASTx a score threshold of >100 was set as these generally had e-values $<10^{-5}$ with a minimum of 50% identity over at least 30% of the length of the protein, which are the commonly used thresholds for reliable sequence annotation [15, 16]. All sequences were deposited to the National Center for Biotechnology Information (NCBI) GenBank database (USA).

Results

Qualitative and quantitative analysis of RNA

The RNA appeared as an undegraded on 1.2% agarose-formaldehyde gel. Typical $A_{260/280}$ absorbance ratios of the RNA range from 1.8 to 2.0 indicating that little or no protein contamination has occurred [17]. The $A_{260/230}$ ratios are >1 indicating that little or no polysaccharide or polyphenol contamination exists [17]. Yields were in the range of 0.7–1.6 mg g^{-1} fresh weight. The $A_{260/280}$ ratios of purified poly $(A)^+$ RNA of both materials were >1.9. A clear smear >0.5 kb was present on the 1% agarose gel with the area between 1.5 and 2.0 kb being most intense indicating that the quality of the obtained poly$(A)^+$ RNA was sufficient for further use.

Identification of subtraction efficiency

The subtracted products on gel analysis appeared as a smear ranging in size from 100 bp to 2 kb with 10–12 definite bands (Fig. 1, lane 4) clearly distinguish them from the unsubtracted sample control. The subtracted skeletal muscle sample (Fig. 1, lane 2) showed DNA fragment corresponding to the ϕX174/*Hae*III digest. The control-subtracted sample provided in kit (Fig. 1, lane 1) gave an identical pattern, which confirms the efficiency of the control subtraction experiment. Analysis of subtraction efficiency of experimental samples by PCR amplification of constitutively expressed actin gene primers revealed that it appeared after 23 cycles, when using the unsubtracted sample control as a template but did not appear until after 33 cycles, when using the subtracted cDNA as a template. This indicates that cDNA homologous to both tester and driver was eliminated by subtraction.

Identification of positive clones characterization of the SSH cDNA library

Infection levels with the 98–111 race of *P. colocasiae* in the inoculated Muktakeshi (incompatible) and UL-56 (compatible) reared to maturity were 5/30 plants (16.8%) and 28/31 plants (90.3%), respectively. This demonstrated that there was sufficient level of disease in the compatible interaction and an effective resistance response had occurred in the incompatible interaction to use the biologic material. Of the

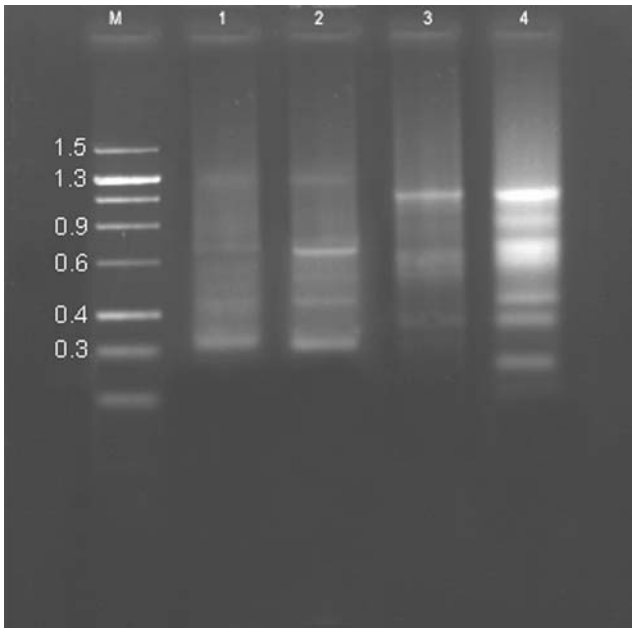

Fig. 1 PCR product after subtraction. Lane M, marker; lane 1, control subtracted product from kit; lane 2, subtracted control skeletal muscle cDNA; lane 3, subtracted sample after first PCR; lane 5, subtracted sample after second PCR

120 independent clones arrayed from the SSH cDNA library by blue–white spots from the Muktakeshi cultivar inoculated with the leaf blight pathogen approximately 95% of transformants contain inserts (ranging from 100 to 1,200 bp). Approximately 12% of the total arrayed clones were differentially regulated. Nine up-regulated clones were identified using the subtracted PCR product probes that were further sequenced and sequences have been deposited in the NCBI-EST database (Table 1). BLAST results for all up-regulated clones indicated that 77.8% of the identified genes had significant homology to genes of known function in available public databases. The homology search and function classification indicated that up-regulated sequences as a result of taro–*P. colocasiae* interaction appeared to function in cellular metabolism and development, abiotic/biotic stress responses, transcription and signal transduction responses. The remaining scored genes (22.2%) exhibited significant homology to rice or Arabidopsis sequences for which any functional roles remain to be established.

Time-course expression of defense-related genes

The infection levels in a portion of the *P. colocasiae* inoculated UL-56 (compatible) and Muktakeshi (incompatible) plants allowed to proceed to maturity were 10% and 90.3%, respectively, indicating that disease pressure was adequate to permit expression of defense-related responses in plants. The time-course Northern blot analysis demonstrated that the expression level of phospholipids transfer protein originating from the SSH library and two taro PR-proteins (peroxidase, endochitinase) were globally higher in resistant variety (data not shown). Additionally, the highest expression levels were observed following 16 and 32 h after appearance of disease (Fig. 2), while untreated control samples failed to exhibit detectable levels of expression.

Discussion

The present study constitutes the first genome wide effort to understand the molecular basis of a host–*P. colocasiae* interaction in taro. Suppression subtractive cDNA libraries from two genotypes (resistant and susceptible) from leaf tissues at the infection stage and leaf blight disease development stage were constructed to identify spatial and temporal transcriptional changes resulting from *P. colocasiae* infection. Because a whole taro genome sequence has not yet been completed, ESTs could serve as an efficient alternative approach to the discovery of novel genomic information.

Northern analysis of seven out of the nine selected ESTs from SSH confirmed their differential expression under the

Table 1 Annotations and BLAST scores of differentially up-regulated sequences from the leaf blight-infected taro SSH cDNA library

Clone No.	GenBank accession No.	BLAST matching accession No.	Gene description	e-value
pCE011_endchi	EU368044	X74919.1	*P. vulgaris* gene for endochitinase	0.0
		X57187.1	*P. vulgaris* mRNA for chitinase	0.0
		X88803.1	*V. unguiculata* mRNA for chitinase	5e-148
pCE023_chla/b	EU364507	X13908.1	Rice cab1R gene for light harvesting chlorophyll *a/b*-binding protein	1e-18
		X13908.1	*Hordeum vulgare* chlorophyll *a/b* binding protein precursor mRNA	5e-176
		AY389606.1	*Hyacinthus orientalis* chloroplast chlorophyll A–B-binding protein 40 mRNA, complete cds	0.093
pCE005_26Srib	EU364506	AF479225.1	*Neurada procumbens* 26S ribosomal RNA gene	1e-90
		AY189100.1	*Pimpinella saxifraga* 26S large subunit ribosomal RNA gene, partial sequence	1e-90
		AY727953.1	*Eurya japonica* 26S ribosomal RNA gene	4e-91
pCE004_peroxd	EU369669	AF014467.1	*Oryza sativa* peroxidase(POX22.3), mRNA	2e-12
		AY857759.1	*Triticum monococcum* peroxidase 5 (POX5) mRNA	0.035
		DQ317315.1	*Musa acuminata* putative peroxidase mRNA	9e-149
pCE006_gtp_bind	FD480279	AY114643.1	*Arabidopsis thaliana* ADP-ribosylation factor-like protein (At5g37680) mRNA	5e-167
pCE015_Phlip_bind	FD483997	DQ147179.1	*Zea mays* ssp. *parviglumis* isolate p13 phospholipid transfer protein 2 (plt2) gene	0.0
		DQ147190.1	*Zea diploperennis* isolate d5b phospholipid transfer protein 2 (plt2) gene, partial cds	6e-171
pCE001_DNA_bind	FD509778	NM_123399.4	*Arabidopsis thaliana* MYB24 (myb domain protein 24); DNA binding/transcription factor (MYB24) mRNA	0.0
pCE016_Unknown	FD509779	–	Unknown	–
pCE012_Unknown	FD509780	–	Unknown	–

tested conditions. Seven transcripts showed up-regulation in the tissue types and condition from which they were cloned. The present study identified a group of transcripts that are regulated in response to *P. colocasiae* infection

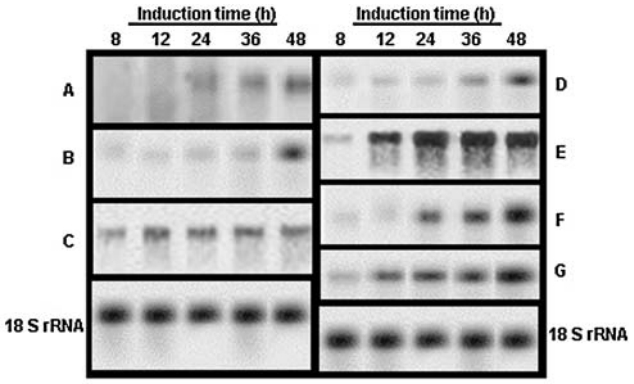

Fig. 2 Induction of defense-related genes in *P. colocasiae*-infected taro leaves (cv. Muktakeshi). Total RNAs (20 μg per lane) were isolated at the indicated time points. GTP binding protein (**a**), Chlorophyll *a/b* binding protein (**b**), Expression levels 26 ribosomal RNA (**c**), DNA binding transcriptional factor (**d**), Phospholipid transfer protein (**e**), Peroxidase (**f**), endochitinase (**g**), were monitored by RNA gel blot analysis as described in Materials and methods

and may represent the key elements in development of the defense response.

The up-regulated transcripts involved in energy, metabolism, transcription, and defense response among the annotated transcripts from the inoculated tissue also supports the presumption that many of these transcripts are specifically involved in the *P. colocasiae* resistance response. The predominant defense-related transcripts up-regulated during expression of the resistance gene in the taro SSH library was lipid transfer protein (LTP). Non-specific LTPs, are reported in various organs and tissues in many mono- and dicotyledonous species, which involve in the extracellular transport of lipids and are considered important in several types of stress responses including attack by plant pathogens [18, 19]. Their role in plant defense is not clearly understood but ns-LTPs have been implicated in plant defense against viral, bacterial, and fungal plant pathogens [18, 20]. Certain ns-LTPs appear to be involved in the formation of cutin and suberin layers in the plant epidermis, thereby strengthening structural barriers in organs against mechanical disruption and pathogen attack [19, 21]. Elevated ns-LTP transcript or protein levels have been observed following infection with the fungal

pathogens *Blumeria graminis*, *Rhynchosporium secalis* [22], and tobacco mosaic virus [23]. The consistently higher expression of this gene from 12 to 48 h after the appearance of symptom in the resistant cultivar compared to the closely related susceptible cultivar suggests that they may have a role in more generalized defense responses. Alternatively, it is possible that these genes or associated regulatory elements are physically linked to the other defense-related gene, resulting in the developmentally based constitutive expression of these genes.

Transcripts of the PR-proteins (endochitinase) exhibited a rapid up-regulation as early as 8 h after the appearance of symptom and reached maximum levels after 48 h. It was expressed at higher levels in the inoculated Muktakeshi compared to the non-inoculated treatment and both treatments in UL-56. Chitinases are reported to play a dual role in the host–parasite interaction; apoplastic chitinases degrade fungal chitin following initial penetration of the intercellular spaces by the pathogen [24, 25]. The released chitin may then trigger a more generalized defense response resulting in the up-regulation of both apoplastic and vacuolar chitinases and other defense responses including the hypersensitive response [25]. Plants also produce an array of PR-1 proteins that exhibit differential toxicities to various plant pathogens [26]. The expression of endochitinase after 8 h post-infection and rising to maximum levels after 36 h suggested that up-regulation of this defense-related protein is among the first defense response affected toward pathogen infection. None of the original PR-proteins were scored in the SSH library. Similar results were observed in other SSH libraries after plant exposure to low temperature and leaf rust [27]. This could be explained by a similar up-regulation in PR-protein transcripts occurred in inoculated UL-56 (compatible) treatment that served as the driver in the subtraction process. Because a 10X excess of driver cDNA was used in the SSH procedure, it is likely that differences in up-regulation in PR-proteins <10 times would not be detected. Even in susceptible reactions, up-regulation of PR-protein genes tend to occur later than in resistant reactions and at lower levels and delays in up-regulation of defense responses have been observed in compatible interactions involving other pathogens [28, 29]. Collectively, the up-regulation of defense-related proteins including the R genes, LTPs, transcription factor, reaching maximum expression levels 32 h after the appearance of symptom was expected, as the SSH cDNA library was constructed from RNA isolated at 10 days post-inoculation. The expression of the PR-proteins also followed a similar expression pattern.

In addition to genes with defense-related functions, defense signaling molecules induced several genes encoding proteins predicted to function in photosynthesis, such as ferrodoxin, chlorophyll *a/b* binding proteins, rubisco, and oxygen-evolving enhancer proteins. This is consistent with a previous report suggesting that there may be some cross-talk between defense pathways and the phytochrome A/red light-mediated signaling pathway [30]. In addition to their energy roles, some of these proteins may have defense functions. For example, Yang et al. [31] have showed that oxygen evolving enhancer protein (OEE) is phosphorylated by a protein complex containing Wall-associated kinase 1, a PR-protein required for survival of plants during the pathogen response [32]. Activation of OEE by Wak 1 is thought to modulate formation of reactive oxygen species (ROS), which could function in defense signaling, the induction of defense-related genes, and the regulation of the HR [31].

This study will undoubtedly shed light on defense signaling pathways in taro and identifying genes in disease resistance pathway. This is the first report of analysis of differentially expressed transcripts in *Colocasia* sp. by SSH technique. Clones from the enriched subtracted library will be particularly valuable because these genes play a role in defense and may be used in future as an important source of genes for improvement of high yielding variety of taro against leaf blight caused by *P. colocasiae*.

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