Journal of Root Crops, 2018, Vol. 44 No. 2, pp. 3-11
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

Development of an Efficient Real-time PCR Assay to Accurately Quantify Resistant Gene Analogue Expression in Taro (*Colocasia esculenta*)

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Abstract

Taro (*Colocasia esculenta* (L.) Schott.), an important tropical tuber crop with high nutritive, and medicinal potential, is ranked fourteen among the most consumed vegetable worldwide. Leaf blight caused by *Phytophthora colocasiae*, is one of the most destructive diseases of taro which leads to severe yield loss up to 50%. The objective of this study was to standardize the quantification of RGA (resistant gene analogues) expression in resistant and susceptible taro varieties. For isolating the taro RGAs, PCR-based strategy with degenerate primers was used and the obtained sequences showed similarity with other RGA sequences in the NCBI database, which categorised them into the NBS-LRR class of gene family. The conserved domain search has proved the presence of Nucleotide Binding-ARC domain in all the sequences. RGA specific primer was designed based on sequence information, which is the first report in taro. The expression of RGAs in Muktakeshi and Sree Kiran genotypes was determined by the SYBR green PCR assay with actin as reference gene. The target gene was up-regulated during the course of infection in both the resistant and susceptible varieties, but the difference was that the hike in expression upon pathogenwas found earlier in resistant variety than the susceptible variety and the level of expression (fold change) was also more in the resistant variety. The found results should be used as a good start point for further studies such as candidate gene mapping for taro leaf blight.

Key words: Taro, leaf blight, *Phytophthora colocasiae*, resistant gene, real time PCR

Introduction

Taro (*Colocasia esculenta* (L.) Schott.), an important tropical tuber crop, a member of the monocotyledonous family Araceae with nearly 1000 cultivars grown for its edible corms, and leaves, serves as a staple food or a subsistence crop for millions of people in developing countries in Asia, Africa, Caribbean and Pacific Islands. It is the fourteen most consumed vegetable worldwide with about 10.2 million tonnes of cormels produced globally from about 1.7 million hectares with an average yield of 6 t ha^{-1} (FAOSTAT, 2017). Taro cormels are considered as a rich source of carbohydrates, minerals, proteins and vitamins. Taro is also used as a traditional medicine with root extract used to treat rheumatism and acne, while leaf extract is used for blood clotting at wound sites, neutralizing snake poison and as a purgative medicine.

Taro leaf blight caused by *Phytophthora colocasiae*, an oomycete water mould, is one of the most destructive diseases of taro resulting in severe yield reduction and plant death. The disease is prevalent in all taro growing regions of the globe including India causing yield reduction to the magnitude of 30-50% (Misra et al., 2008). Taro leaf blight (TLB) is a threat to food security

and economy in those countries where taro is a major staple and an export commodity. Several approaches have been advocated to manage the disease. Cultural practices like crop rotation and shifting of planting time have been unsuccessful in protecting the crop. Metalaxyl and mancozeb based fungicides have proved effective in controlling the disease but waxy leaf surface and occurrence of disease during rainy season makes fungicidal spray ineffective (Jakson, 1999). Moreover, the fungicide sprays are too costly to marginal farmers while the development of resistance against the fungicides is another major threat. Therefore, exploiting the natural host resistance is thought to be the most promising, non-conventional and ecologically friendly approach to mitigate leaf blight disease. For the fulfillment of this approach, a clear understanding of the molecular mechanisms involved in host resistance to pathogen and about the high genetic diversity existing among the various taro genotypes with respect to their susceptibility or resistance to leaf blight is required.

The molecular communication between plant and pathogen commence almost immediately after the pathogen makes contact with the plant surface, which suggest that resistance (R) gene product recognises a specific avirulence gene product specified by the pathogen (gene-for-gene principle) (Flor et al., 1971). During the last 15 years, a total of 70 different resistance genes showing resistance to major plant pathogen had been isolated, cloned and characterised in different plants. Approximately 75% of these resistance genes belong to NBS-LRR gene family. The NBS domain consists of around 3000 amino acids and four conserved motifs named as P loop (kinase 1 a), kinase 2, kinase3a, and GLPLAL motif. The LRR region consists of many leucine rich sequences which promotes recognition of a virulence gene product (Jia et al., 2000). A key feature of these classes of genes is that, they are involved in gene-for-gene resistance towards a wide array of plant pathogens viz. fungi, virus, bacteria or nematodes (Grant et al., 1995).

The PCR-based strategies has proven as an excellent tool for identification, cloning and mapping of resistance gene family members analogues with the use of degenerate or specific primers for conserved motifs of different NBS–LRR genes. Through this approach, successful cloning of putative NBS–LRR resistance gene analogues (RGA) from many crops like soybean, wheat, barley, rice, sugar cane, maize, ginger, taro and greater yam was possible. RGA strategy is very useful in marker development and identification of quantitative trait loci for plant disease resistance genes, and for diversity and evolutionary studies of R genes.

Identification of genes whose expression is evoked or inhibited in response to pathogen infection can provide an improved understanding of the molecular mechanisms related to resistance and susceptibility and provide a foundation for biotechnology approaches to improve disease resistance in plants (Casado-Diaz et al., 2006). The NBS-LRR disease resistance genes are basally expressed in plant tissues but upon pathogen detection, the expression of these genes is up-regulated in order to initiate defense responses. Gene expression of NBS-LRR genes were successfully studied in citrus plants infected by *Leiberibacter asiaticus* (Maryam et al., 2007). The result of the study showed increased gene expression in infected plants. Hu et al. (2017) has studied about the expression of 12 NBS-encoding genes in response to pathogen infection by *A. alternata* in two apple cultivars and found that all the genes were involved in the host response against pathogen infection. The gene expression profile showed that, 3 out of 12 NBS-encoding genes were observed to be highly expressed and can be used as valuable candidates for breeding resistant apple cultivars through genetic engineering. In order to identify resistance gene for TLB, a study was conducted to standardize the quantification of resistant gene expression in different taro cultivars/lines upon leaf blight infection.

Materials and Methods

DNA isolation and PCR amplification

DNA was isolated from young leaves of healthy taro plants, Sree Kiran (susceptible) and Muktakeshi (resistant) grown in ICAR-CTCRI fields. Hundred mg fresh leaf tissue was ground in liquid nitrogen and DNA was extracted using CTAB method (Sharma et al., 2008). The quantity and quality of DNA isolated was measured by 0.8% agarose gel electrophoresis followed by an ethidium bromide visualisation using 1Kbp plus DNA ladder (Fermentas) as DNA size marker.

Two sets of degenerate primers reported earlier were used for the amplification of RGAs in *Colocasia esculenta*, which targets the conserved region between P-loop

and GLPL of R-genes. The sequences of the primers were RGA 1F: 5'GGIGGIGTIGGIAAIACIAC 3', RGA 1R: 5' ARIIGCTARIGGIARICC 3', RGA 2F: 5'GGTGGGGTTGGGAAGACAACG 3', RGA 2R sequence: 5'CCACGCTAGTGGCAATCC 3'(Aswati Nair and Thomas, 2007). PCR reaction was carried out in a 50 µl reaction mixture with 50 ng template DNA, 200nM of each forward and reverse primer, 200 µM of Deoxyribonucleotide triphosphate mix, 10X PCR buffer (16 mM $(NH)_2SO_4$, 67 mM Tris-HCl, 15 mM $MgCl_2$) and 1U Taq DNA polymerase (Merck Genie, India). PCR was performed in a Agilent Technologies Sure Cycler 8800 (Agilent Technologies, USA) using the following cycling conditions: initial denaturation at 94°C for 5min followed by 42 cycles each consisting of denaturation at 94°C for 1min, annealing at 50/60°C for 1min and extension at 72°C for 1min. The amplification was concluded with a final extension step at 72°C for 8 min. PCR products were subjected to electrophoresis on a 1.5% agarose gel containing 0.5 µg/ ml ethidium bromide and gel photographs were scanned through the Gel Doc System (Alpha Imager, Alpha Innotech, USA).

The reamplified gel elutes were sequenced (Genetic Analyzer ABI 3500) at the AgriGenome Labs Pvt. Ltd., Kochi, Kerala. The sequences obtained were edited with BioEdit Sequence Alignment Editor Programme version 7.2.5 and was compared to known RGA sequences using BLASTX algorithms against the NCBI database (http://www.ncbi.nlm.nih.gov).

Designing and validation of taro RGA specific primers

Specific primers has to be designed for studying the gene expression and this was done by using Primer 3 plus software based on the obtained sequence information. They were designed for the amplification of the NB-ARC conserved domain of the resistance gene analogues in taro varieties

T RGA F (Forward primer) sequence :
5'CTGAGCCTT TCCTCATCTGC 3'

T RGA R (Reverse primer) sequence :
5'CCA AA CCT TCACCATGACT3'

The annealing temperature of the specific primer was optimized by using gradient PCR set from 50-60°C, and the optimum annealing temperature for better amplification was observed at 55°C.

Artificial inoculation of disease for studying gene expression

Virulent *P. colocasiae* culture (CTCRI-PC) being maintained at ICAR- CTCRI was used for the study. *P. colocasiae* culture was inoculated into the leaves of susceptible variety (Sree Kiran) by placing 5 mm disc on different positions on the leaf. Ten days old inoculated leaves were crushed in sterile water and kept for 15 min in 4°C.This is filtered through a clean cheese cloth and the filtrate was used as the spore suspension. The zoospore suspension (25µl) was applied on the abaxial and adaxial surface of the leaves and for control plants sterile water was sprayed.

Total RNA extraction and cDNA synthesis

Plants raised from uninfected and surface sterilized tubers, maintained under controlled conditions were used for the study. Leaf samples collected after 12, 24, 36 and 48 hours of pathogen inoculated and control plants of both varieties, were taken for RNA isolation. The RNA was isolated using TRIzol based method and stored at -80°C. The purity and density of the extracted RNA was assessed on a NanoDrop spectrophotometer using RNase-free water as blank. Single-stranded cDNA was synthesized using Revert Aid FIRST strand cDNA synthesis kit (Thermo scientific, USA) with oligodt primers as described by the manufacturers protocol. Approximately 2 µg of total RNA in a single 20 µL reaction was converted to single stranded cDNA using standard thermal conditions.

Real – time quantitive PCR (RT-qPCR) analysis

The cDNA of control and test samples from resistant and susceptible varieties were diluted to a final concentration of 100 ng ml^{-1} with sterile water. The real-time quantitative PCR (qPCR) was performed with the Applied Biosystems PCR system in a total volume of 20 ml. The reaction volume contained 10 mL of 2X SYBR Master Mix, 2 mL of diluted cDNA, 0.4 mL of each primer (10 mM), and additional ddH2O to bring the total volume to 20mL. The thermal profile was as follows: 95°C for 30 s for initial denaturation, 40 cycles of 95°C for 5s for denaturation of template, and 60°C for 20s for annealing and extension. Three replicates were used for each sample. A melting curve analysis was conducted for all of the primers that were used. Expression levels were calculated by the method and actin was used as a reference gene for normalizing the expression.

Results and Discussion

PCR amplification and sequence analysis

PCR amplification of taro genomic DNA using the two degenerate RGA primers resulted in amplicons of 500 and 620 bp size respectively (Fig. 1 and Fig. 2). The BLAST analysis of the four obtained sequences showed maximum similarity to NBS-LRR disease resistance protein of *Panax notoginseng* (a medicinal plant) other than NBS LRR disease resistance protein of *Colocasia esculenta* (Table 1). The NBS-LRR proteins by one of the biggest and significant gene family engaged with disease resistance. Defense response by NBS-LRR proteins is a sophisticated method which induces effector-triggered immunity (ETI). Conserved Domain search tool at NCBI (http://www.ncbi.nlm.nih.gov Structure/cdd/wrpsb.cgi) was used to confirm the presence of NB-ARC domain in all the obtained sequences of both the varieties.

The PCR-based strategies by using degenerate primers designed from the NBS region of already reported disease resistance genes has been proved as an excellent tool for cloning many resistance gene like sequences in several plant species (Mago et al., 1999, Joshi et al., 2010, Liu et al., 2003, Nath et al., 2013). The degenerate primers reported by Aswati Nair and Thomas (2007), which

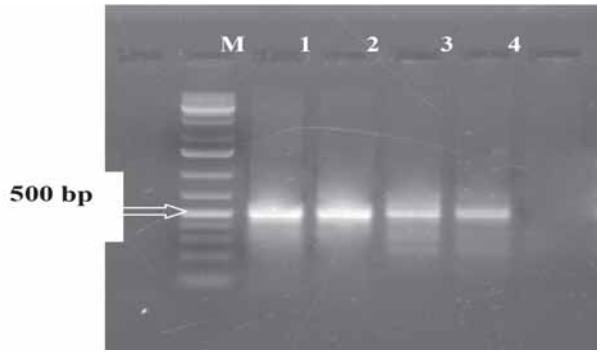

Fig. 1. PCR product of Muktakeshi (Lane 1 and 2) and Sree Kiran (Lane 3 and 4) using degenerate RGA 1 primer separated on agarose gel. M: 1kb plus DNA marker

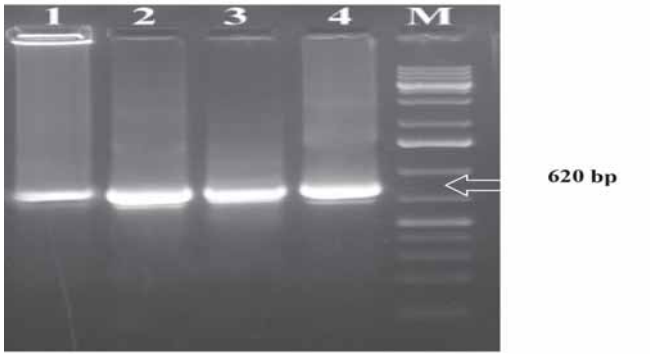

Fig. 2. PCR product of Sree Kiran (Lane 1 and 3) and Muktakeshi (Lane 2, 4) using degenerate RGA 2 primer separated on agarose gel. M: 1kb plus DNA marker.

Table 1. Amino acid sequence identity between obtained RGAs and other plant species

Primer	Variety	Similar sequences identified	Accession No	RGA sequence identity (%)
RGA 1	Muktakeshi	NBS LRR disease resistance protein (*Colocasia esculenta*)	AOD75225.1	98.24
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ9633O.1	77.91
		Disease resistant protein homolog (*Arabidopsis thaliana*)	AAB61691.1	45.93
		Disease resistant protein product (*Vitis vinifera)*	CB127879.1	47.65
	Sree Kiran	NBS LRR disease resistance protein (*Colocasia esculenta*)	AOD75222.1	97.65
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ96329.1	46.63%
		Disease resistant protein homolog (*Arabidopsis thaliana*)	AAB61689.1	46.51%
		RCa9 (*Manihot esculenta*)	AA038220.1	47.06%
RGA2	Muktakeshi	NBS LRR diseaseresistance protein (*Colocasia esculenta*)	AOD75225.1	59.28%
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ9633O.1	51.79%
		NBS/LRR Resistance protein-like protein (*Theobroma cacao*)	AAL009991	40.48%
		Putative disease resistance protein isoform XI (*Cinnamomum micranthum*)	RWR90802.1	42.51%
	Sree Kiran	NBS LRR diseaseresistance protein (*Colocasia esculenta*)	AOD75225.1	60.45%
		NBS LRR disease resistance protein(*Panax notoginseng*)	QBZ9633O.1	51.69%
		CC-nbs-lrr-Resistance protein –like protein (*Theobroma cacao*)	EOY25574.1	41.01%
		Disease resistance protein At5g63020 isoform XI (*Citrus sinensis*)	XP00648460.1	38.33%

targets the P-Loop and GLPL conserved domains of NBS-LRR gene are used for this study. A common feature of degenerate primers is the co-amplification of non specific DNA fragments along with desired fragment and is observed in many plant species.

Two sets of degenerate primer pairs (RGA1 and RGA2) were used in the present study which resulted in amplicons of size 500 and 620 bp. The amino acid sequence analysis of the obtained four sequences showed the presence of RGAs when compared with other known sequences in the database. The specific primer was designed for the amplification of Nucleotide Binding – ARC Domain in the taro varieties and the optimun annealing temperature was 55.5°C (Fig. 3).

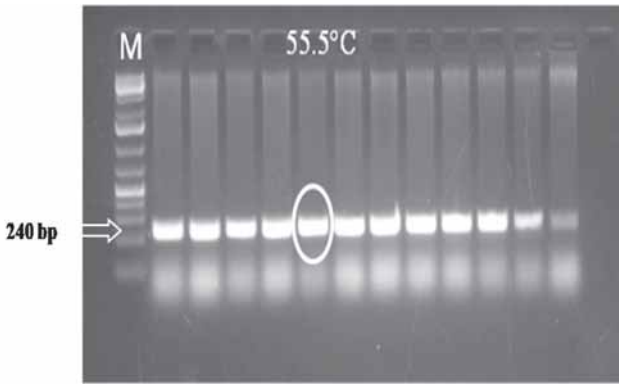

Fig.3. Gradient PCR (50°C - 60°C) to optimize annealing temperature of newly designed primer T RGA. Optimum annealing temperature – 55.5°C.

Disease development in two cultivars

Symptoms initiated within 24 h of inoculation in susceptible variety and in resistant variety it took 48 h. The initial symptoms were the occurrence of light water soaked brown lesions which later developed into circular, purplish brown lesions. Bright orange coloured plant exudate oozing from the infected sites was also observed on the lower surface. The development of symptoms in the susceptible variety (Sree Kiran) was faster and severe compared to the resistant variety (Muktakeshi). The presence of yellow tissue around the lesions were also observed. As the infection spreads, the lesions coalesce and leads to the destruction of entire leaf. On the seventh day of inoculation, severity occurred in the susceptible variety (Fig 4). The appearance of leaf blight symptom was delayed in resistant cultivars and the rate of infection spread was also slow as compared to susceptible cultivars (Misra and Singh, 1991; Misra and Chowdhury, 1997).

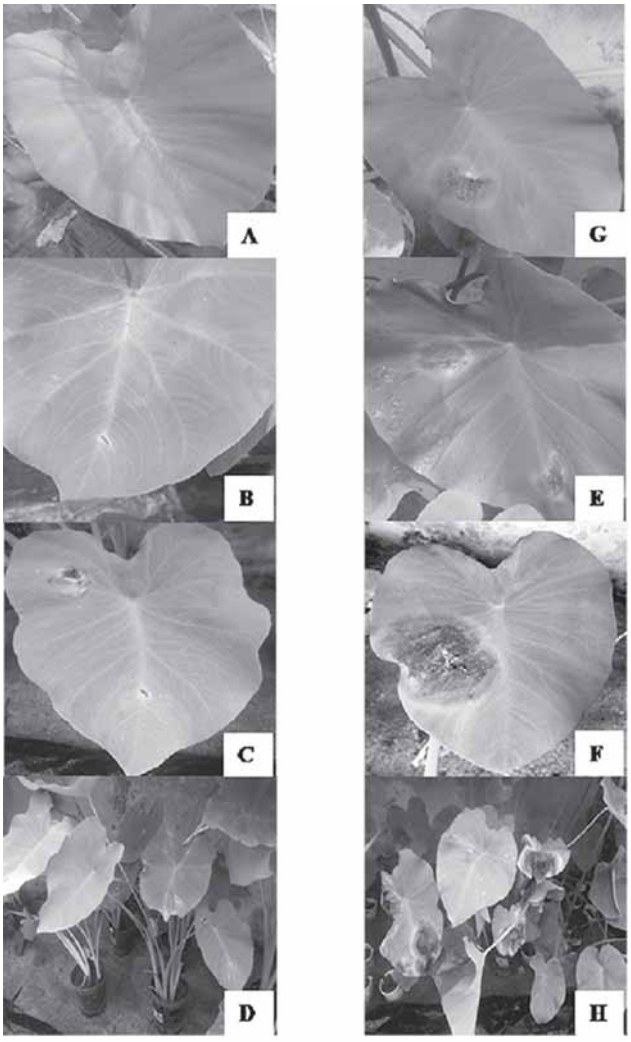

Fig. 4. (A) Artificial inoculation of *P.colocasiae* on two taro varieties, Muktakeshi and Sree Kiran. (A to D): Symptoms in Muktakeshi (resistant variety) on 1st, 3rd, 5th and 7th day. (E to H): Symptoms in Sree Kiran (susceptible variety) on 1st, 3rd, 5th and 7th day.

Expression analysis of resistant gene analogues

The results of real time PCR showed the presence of target gene in both the resistant and susceptible cultivars. The reference gene (Actin) had a low ΔCt value than that of the target gene, which means that the target gene was expressed in low concentration. The melting curve analysis at the end of the cycling reactions revealed single dissociation peak at 75°C (T RGA F and T RGA R) and 78°C (ACT F and ACT R) indicating the specific binding of the designed primers (Fig. 5). The difference in gene expression in the resistant taro cultivar (Muktakeshi) was compared with that of susceptible taro cultivar (Sree

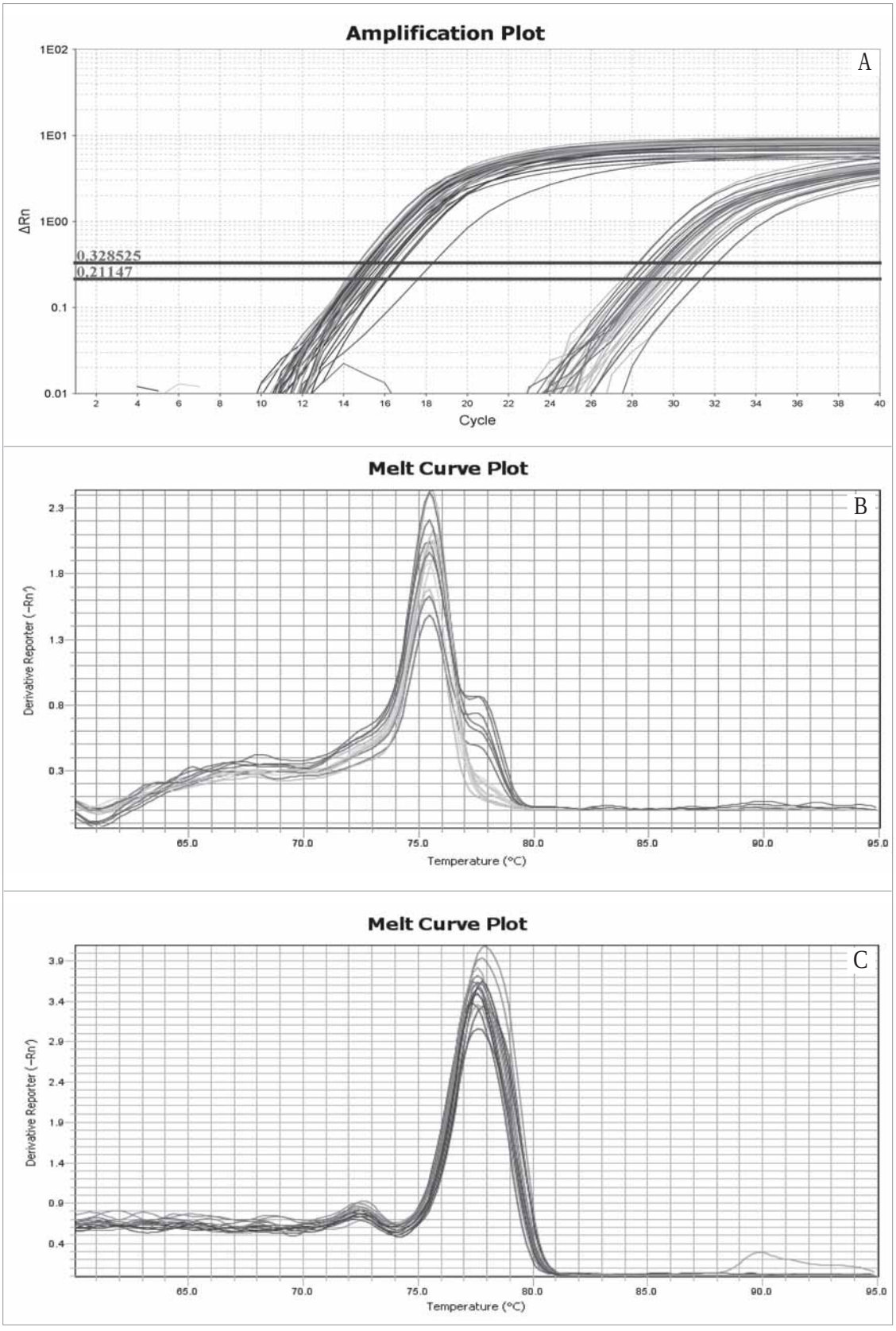

Fig. 5. Amplification plot (A) and melt curve analysis of the Real-time PCR assay using the designed primer pairs (B) and reference gene Actin (C).

Kiran). Whole plants raised from surface sterilized tubers were used for the assays, where as similar studies were done in detached leaves by other researchers (Wang et al., 1999; Tian et al., 2006).

The plants were maintained under sterile conditions in order to avoid interaction with other pathogens and was also monitored to avoid wound responses not coupled with the infection progress. These are taken into special care, since many pathogenesis or defense related genes are also induced followed by abiotic stress like wounding (Vignutelli et al., 1998; Bertini et al., 2003).

The standard fluorescent amplification representing exponential growth of PCR products was observed in each cycle, yielding threshold cycle (Ct) values that ranged from 15 – 30.2 for the target (T RGA F and T RGA R) and reference (ACT F and ACT R) primers. In the resistant variety (Muktakeshi), the sample collected after 12 h of pathogen inoculation had a 1.42 fold change which rapidly increased in the following samples and the surge in the expression was maintained till the sample collected after 48 h with 3.14 fold change. In the susceptible variety (Sree Kiran), the sample collected after 12 h of pathogen inoculation had 0.8 fold change which increased to 2.15 fold change at 36 h, but it decreased to 1.86 fold change in the samples collected after 48 h of pathogen inoculation (Fig 6).

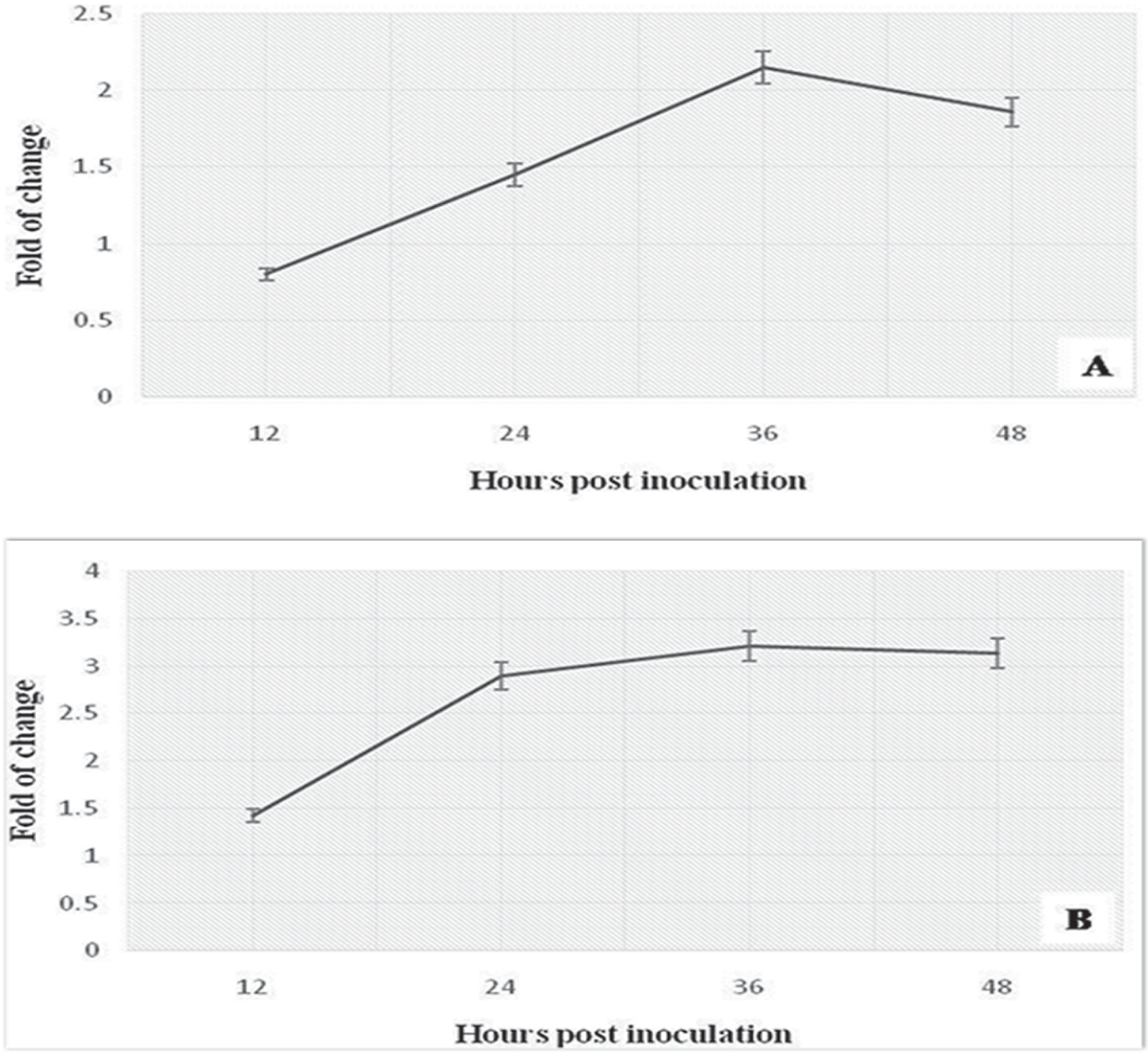

Fig. 6. Relative gene expression of RGA in samples of Sree Kiran (A) and Muktakeshi (B).

In short, the results of real time PCR showed that the target gene was up-regulated during the course of infection in both the resistant and susceptible varieties. The normalized gene expression was increased with a noticeable hike in the sample collected after 24 h of pathogen inoculation of the resistant variety and a gradual increase in the sample collected at 36 h of pathogen inoculation in the susceptible variety.

In tobacco, many RGAs was found to show induced expression after infection with either *Tobacco mosaic virus* (TMV) or the tobacco black shank pathogen (*Phytophthora parasitica var. nicotianae*) (Gao et al., 2009). Expression of RGAs in other species had also shown a similar expression pattern i.e. low gene expression prior to pathogen attack and induced expression subsequent to pathogen attack (Hulbert et al., 2001; Wang et al., 1999; Yoshimura et al., 1998). A similar up-regulated gene expression pattern was observed in chickpea RGAs due to *Fusarium* wilt (Gutierrez et al., 2012). The increase in transcript level observed after 24 h of pathogen inoculation on Muktakeshi seems to indicate their role in early pathogen recognition, thereby makes it resistant variety. The results could be utilized to study the resistance in different taro lines after validation with more cultivars or lines with known resistance.

Conclusion

In the present study the candidate gene tested behaved differently in the resistant and the susceptible genotype in response to *P. colocasiae*, which indicates the role of RGAs in the resistance response against the pathogen. The identified and quantified RGA could be used as a good start point for further studies such as candidate gene mapping for taro leaf blight.

Acknowledgement

The support provided for the research work by the College of Agriculture, Kerala Agricultural University,Vellayani, is gratefully acknowledged. The authors are grateful to Dr. Archana Mukherjee, Director, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram for providing the facilities and support.

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