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Expression analysis of defense response genes upon Sigatoka infection in *Musa paradisiaca* cultivar Kachkal

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Abstract

The Sigatoka disease is an economically important disease of banana as it causes tremendous yield losses all over the world. In the present study, the defense related gene expression implicated in an incompatible interaction between a resistant banana cultivar Kachkal and the Sigatoka pathogen was analyzed. The initial changes in the expression of some selected defense related genes after infection by the invading pathogen *Pseudocercospora musae* were studied. Semi-quantitative RT-PCR studies indicated a basal level of expression of the selected genes prior to infection in the host plant, while upregulation of some of the important defense response genes coding for PR4, osmotin, LTP, UGPase and JAR1 was observed after the infection by *P. musae*. The findings of the study would be useful in designing a precise breeding strategy for genetic control of the disease in banana.

Keywords: Host-pathogen interaction, resistance, Sigatoka, Semi-quantitative RT-PCR, *Musa paradisiaca*

Introduction

Bananas (*Musa* spp.) are grown all over the world in all tropical and sub-tropical regions (Soares et al. 2021). In terms of production, banana is the fourth most important crop after rice, wheat and maize worldwide. It is one of the most important and popular fruit crops in terms of production, nutritional value as well as international trade. India is the largest producer of banana in the world where it is mostly grown in North Eastern and Southern parts of the country. Various biotic factors including fungal, bacterial and viral diseases are limiting the global production of bananas. Of all the foliar diseases of banana, Sigatoka leaf spot disease is the most devastating fungal disease. The causal agent of sigatoka comprises of three species of *Pseudocercospora* (previously known as *Mycosphaerella*). Black Sigatoka, (caused by *P. fijiensis*), yellow Sigatoka (caused by *P.musae*) and eumusae leaf spot (caused by *P. eumusae*) (Carlier et al. 2000; Arzanlou et al. 2007; Churchill 2011). In India, Sigatoka has been a serious constraint to banana production which results in tremendous yield losses every year (Nwauzoma et al. 2011; Noorulla et al. 2013). Symptoms of the disease first appear as tiny, light yellowish spots parallel to leaf veins which later enlarge and coalesce leading to premature death of leaf tissues. The disease can be very destructive if timely control measures are not adopted. With the spread of the pathogen, the lesions and turn necrotic ultimately leading to premature death of large areas of leaf tissue thus, reducing the photosynthetic capabilities of the plant. It can also result in defoliation which can seriously reduce yields when less than ten viable leaves remain on the plant at the time of harvesting. Management of Sigatoka in commercial banana plantations is highly dependent on the use of fungicides, along with cultural control methods like desuckering, drainage, weed control etc. to reduce sources of inoculum and prevent favourable conditions for pathogen development. Chemical control is widely practiced to combat the disease but the use of fungicides brings collateral problems such as environmental pollution, human health hazards and casual fungus resistance to fungicides. Therefore, the use of resistant or tolerant varieties is the best possible alternative control measure for the disease.

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A number of studies have been carried out in several genotypes of *Musa* spp. to unravel host responses to various biotic stresses. A study by Portal et al. (2011), on a compatible *Musa- M. fijiensis* interaction revealed several genes involved in phenylpropanoids and detoxification compounds synthesis, pathogenesis-related (PR) proteins, jasmonic acid (JA), ethylene (ET) signaling molecules, GDSL-like lipase, UDP-glucose pyrophosphorylase etc. Later a genome-wide analysis and differential expression study of chitinases in *M. eumusae* challenged banana reported that three chitinase isoforms were overexpressed in the resistant genotype (Manoranjitham-AAA) compared to the susceptible genotype (Grand Naine-AAA) (Backiyarani et al. 2015). Later, a study by Sun et al. (2019) revealed that several genes associated with defense signaling (including mitogen associated protein kinase, calcium, salicylic acid (SA), JA and ET pathways), NB-LRR proteins, calmodulin-binding protein and phenylpropanoids biosynthesis were significantly upregulated in a resistant banana cultivar in response to Foc-TR4 infection. Furthermore, Zhang et al. (2019) reported that defense responses are induced earlier in the resistant genotype Pahang at the initial Foc TR4 infection stage compared to the susceptible genotype. A recent report by Rodriguez et al. (2020) on differential regulation of JA pathways in the resistant cultivar Calcutta 4 and susceptible cultivar Williams in response to *P. fijiensis* infection, states that signaling *via* JA/ET is the key to activating defense response in the resistant cultivar. Thus, an understanding of plant defense responses to a disease and identification of potential candidate genes from a resistant host will provide new opportunities for genetic improvement. Investigating the molecular basis of host-pathogen interactions reveals novel aspects of defense related signaling mechanisms and resistance-related gene expression, which in turn, assists in the development of new control strategies utilizing host resistance. With this rationale, the investigation was carried out to study the changes in gene expression patterns of some important defense related genes in response to infection by *P. musae* in a resistant banana cultivar. The study could predict the initial changes taking place in the host plant banana, after infection by the invading pathogen in the form of changes in gene expression patterns of some selected defense-related genes.

Materials and methods

Plant materials

A popular banana cultivar of Assam, Kachkal (*Musa paradisiaca*; Genome ABB) was chosen for the present study as it has been reported to be resistant to Sigatoka disease in Assam (Mishra 2000). Healthy and disease free suckers were collected from the Horticulture Orchard of Assam Agricultural University, Jorhat, and planted in pots of 40 cm height and 35 cm width containing a mixture of vermi-compost and autoclaved soil in the ratio 1:2, and grown inside shade net house till emergence of two to three leaves.

Isolation, purification and detection of the pathogen

The pathogen *P. musae* was isolated from infected leaves of banana identified by typical symptoms following methodology described by Selvarajan et al. (2001). Leaves with typical lesions of Sigatoka were collected, cut into small sections of 2 cm^2 size and dipped in sterile distilled water for 15 minutes. The sections were then stapled to sterile filter paper and placed on the lid of a petri-plate facing 3% water agar. The plates were incubated at room temperature and the release of ascospores was monitored regularly under a microscope. The ascospores identified based on their morphology were then transferred to potato dextrose agar (PDA) medium and then sub-cultured and incubated in PDA slants for 15 days. For molecular detection of the pathogen, genomic DNA of the fungus was isolated by a modified Cetyl Trimethyl Ammonium Bromide (CTAB) based method followed by PCR amplification using universal primers for ITS region (ITS1/ ITS4). PCR was performed using 50 ng fungal genomic DNA as template in a 20 µl reaction volume in a thermal cycler (Applied Biosystems 2720). The reaction mixture consisted of 1X PCR buffer, 2.5 µM dNTPs, 0.6 µM of forward and reverse primers each, 1U Taq DNA polymerase and nuclease free water to make up the volume. The reaction profile consisted of 35 cycles of denaturation at 94°C (1 min), annealing at 65°C (1 min) and extension at 72°C (1 min) with initial temperature for denaturation at 94°C (5 min) and final extension at 72°C (7 min). Detection of PCR amplified products was done by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Artificial inoculation of banana plants and sample collection

Young plants of Kachkal with atleast 3-4 opened leaves were artificially inoculated using *P. musae* spore suspension (10^6 spores mL^{-1}). The suspension was inoculated into the plants by spraying on both the upper and lower leaf surfaces. The plants be used as control were mock inoculated by spraying with sterile distilled water. The plants were then covered with polypropylene bags till 72 hours and monitored regularly for development of symptoms. Leaf tissue samples were collected from the pathogen inoculated (infected) as well as mock inoculated (control) plants at two different time-points *viz*. 24 and 72 hours post inoculation (hpi) and stored at -80 °C for subsequent semi-quantitative RT-PCR assays.

Isolation of RNA and cDNA synthesis

Total RNA extraction was done from the collected plant tissues using the RNeasy Plant Mini kit (Qiagen) following manufacturer's instructions with modification in the

form of addition of polyvinylpyrrolidone (PVP) to the lysis buffer at the time of extraction. The RNA samples were quantified and the purity was estimated with a NanoDrop spectrophotometer (Thermo Fisher Scientific). First strand cDNA synthesis was done by using ProtoScript First Strand cDNA Synthesis kit (New England BioLabs inc.) following manufacturer's instructions from the total RNA extracted from the leaf tissues.

Semi quantitative RT-PCR analysis

The first strand cDNA synthesized from control and artificially infected leaf tissues of Kachkal at 24 and 72 hpi were subjected to semi-quantitative RT-PCR analysis for nine selected defense response genes in order to study their expression patterns in these time points (Table 1). The reference gene *tubulin* was taken as endogenous control for the RT-PCR study. Out of the nine selected genes, three were chosen from a study by Portal et al. (2011), *viz*. the genes coding for gdsl like lipase, jasmonate- amino-acid-conjugating protein and osmotin-like protein. The primers for the rest of the defense response genes were designed from sequences available in the databases using primer designing software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The semi-quantitative RT-PCR reactions were performed in total reaction volume of 10 µl which comprised of 10X PCR buffer, 25 mM $MgCl_2$, 400 µM dNTPs, 0.6 µM forward and reverse primers, 1U Taq polymerase and 20 ng cDNA as template. The thermal cycler conditions followed were: 2 min. of initial denaturation at 94°C; followed by 25 cycles of amplification at 94°C for 30 sec, primer specific annealing temperature for 30 sec, at 72°C for 3 min and; final extension at 72°C for 5 min. The RT-PCR products were detected by running on a 1% agarose gel stained with ethidium bromide and visualized on a gel documentation system (Bio-Rad).

Results

The investigation started with isolation, purification and identification of the pathogen causing Sigatoka disease in banana. For molecular detection of the isolated pathogen, genomic DNA of the pure fungal culture was extracted using CTAB method with modifications. Good quality fungal genomic DNA was obtained upon extraction which was then used as template for PCR amplification using the ITS primers for molecular detection of the fungus. A single band of approximate size 650 bp was observed after amplification confirming the isolate to be a fungus (Fig.1a). After confirmation, the pathogen was used for artificial inoculation in Kachkal plants to study the disease progression and to obtain samples for semi-quantitative RT-PCR analyses. Artificial inoculation with the *P. musae* isolate did not lead to the development of typical Sigatoka symptoms (Fig. 2) but, very few tiny brown necrotic spots, 0.5 mm in diameter, were found to appear on the lowermost leaves of infected plants at around 20 dpi (days post inoculation). At 40-50 dpi, the spots slightly increased in size (0.8-1 cm) and turned necrotic towards the centre. After 60-65 dpi, the spread of the necrotic spots halted completely. Later on it was observed that, the new leaves which emerged out of the artificially infected plants, did not

Table 1. Genes selected for expression analysis along with the sequences of primers used for semi-quantitative RT-PCR analysis

S. No.	Primer Id/ Gene description	Forward and Reverse Primer Sequence (5'-3')	Annealing temperature (°C)
1	ITS1/ITS4	TCCGTAGGTGAACCTGCGG/ TCCTCCGCTTATTGATATGC	54
2	Tubulin	TGTTGCATCCTGGTACTGCT (F) GGTTTCTTGCATGGTACAC (R)	50
3	Osmotin-like protein	GGCCGAGGTACTGTTGC (F) CAGGTGAAGGTGCTCGT (R)	50
4	Jasmonate- amino-acid-conjugating protein (JAR1)	TCAACATCGACAAGAACACC (F) CACAACTCCCAGAAGATCAC (R)	50
5	Gdsl like lipase-1 (GDSL)	GCGCATCACAAAAGAAGAAG (F) CCCCGTCAACATTCATCAG (R)	50
6	PR1	CCGCTGGTTGGCGTAGTTCT (F) ACTTCGTGAGCCCCCACAAC (R)	54
7	Chitinase type II (PR4)	TCTTTACCCTGGCCTTCGTCG (F) CACCAGCCGTACTGGCTACA (R)	54
8	Lipid Transfer Protein (LTP)	CAGTGCGGCATCTCCGTTTC (F) GGGATGGGATGGAGGAGCAC (R)	54
9	Germin like protein (GLP)	TGCTGTTCTTGCCCTGGCTT (F) GCAGGCGAATCCGTTTCACAA (R)	54
10	UDP-glucose pyrophosphorylase (UGPase)	TGCACGGGGCCTAAATCTGT (F) AGCAGAGGGACATTGCATTCC (R)	52
11	Isoflavone reductase-like protein (IFR)	AGAGGGTCTACGTCCCGGAG (F) CTTCACAAAGGCGGAGTGGC (R)	54

show the development of any such necrotic spots or lesions.

To study the expression patterns of defense response genes in Kachkal following infection by the Sigatoka isolate, total RNA was extracted from leaf tissues collected from plants inoculated with *P. musae* spore suspension (as well as from the control plants) (Fig. 1b) at both the time points followed by first strand cDNA synthesis. Then, a semi-quantitative RT-PCR based expression analysis was carried out for nine selected defense related genes. The selected genes are those coding for pathogenesis related (PR) proteins such as PR1 and PR4, osmotin-like protein or osmotin, lipid transfer protein (LTP), germin like protein (GLP), UDP-glucose pyrophosphorylase (UGPase), gdsl like lipase (GDSL), jasmonate- amino-acid-conjugating protein (JAR1) and isoflavone reductase-like protein (IFR). All these genes have been reported to be involved in defense response to pathogen infection in plants (Ebrahim et al. 2011; Portal et al. 2011). The housekeeping gene tubulin was used as endogenous control for the expression analysis.

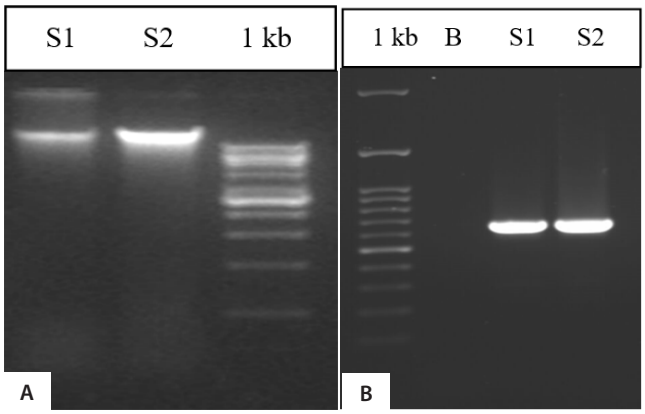

Fig. 1a. (A) Genomic DNA extracted from *P. musae* cultures isolated from infected banana leaf tissues (from two sample replicates S1 and S2); (B) PCR product obtained after amplification of fungal genomic DNA using ITS1/ITS4 primer pairs (run in 1% agarose gel). A single band of ~650 bp was obtained after amplification for two sample replicates S1 and S2 (B- blank).

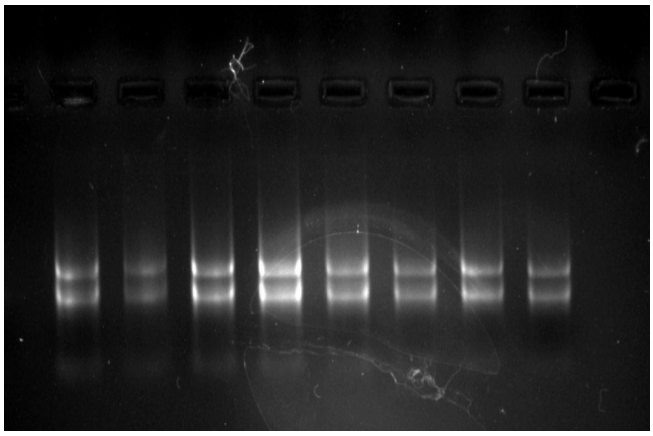

Fig. 1b. Total RNA isolated from control and infected leaf tissues of Kachkal. Lane 1-4: RNA from control and inoculated tissue at 24 hpi; lane 5-8: RNA from control and inoculated at 48 hpi (samples are in duplicate)

The expression pattern of tubulin was found to be consistent across the two time points in both control and infected tissues (Fig. 3). The semi-quantitative RT-PCR study revealed that all the genes were having a basal level of expression prior to infection, as observed in the control sample (mock inoculated). Upregulation in the expression of some of the important defense response genes such as PR4, osmotin, LTP, UGPase JAR1 was observed after infection either at the early or late time point as compared to the expression in control. The gene for PR1, showed a consistent expression pattern after infection at both the time points. But, in case of PR4, although no change in expression was observed at 24 hpi, it was found to be upregulated towards the later time point at 72 hpi. The expression of osmotin-like protein was observed to be slightly increased at 24 hpi. Its expression also increased considerably at 72 hpi. In case of lipid transfer protein, no change in expression was observed immediately after infection at 24 hpi but was upregulated at the later time point. However, no change was observed in the expression pattern of germin like protein after infection at both the time points.

In case of UGPase, expression remained the same at 24 hpi but an upregulation was observed at 72 hpi. Gdsl like lipase also showed a slight increase in expression at 72 hpi. However, the JAR1 was found to be upregulated immediately after infection at 24 hpi. A similar pattern was also seen at 72 hpi for this important defense signaling related protein. Lastly, for IFR, no change in the expression was seen even after infection at either of the time points.

Discussion

The objective of the present investigation was to study

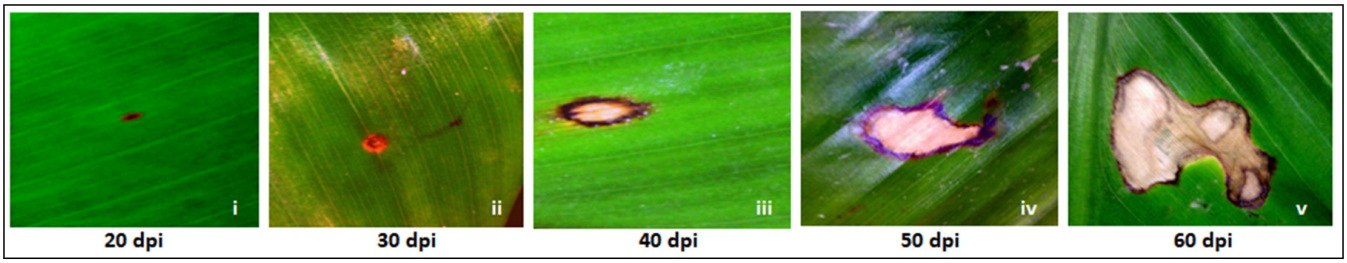

Fig. 2. Development of necrotic spots on leaf of the resistant cultivar Kachkal artificially inoculated with *P. musae* suspension, from 20 days post inoculation (dpi) till 60 dpi (figures i-v), with restricted spread and death of tissue indicating a hypersensitive response like reaction.

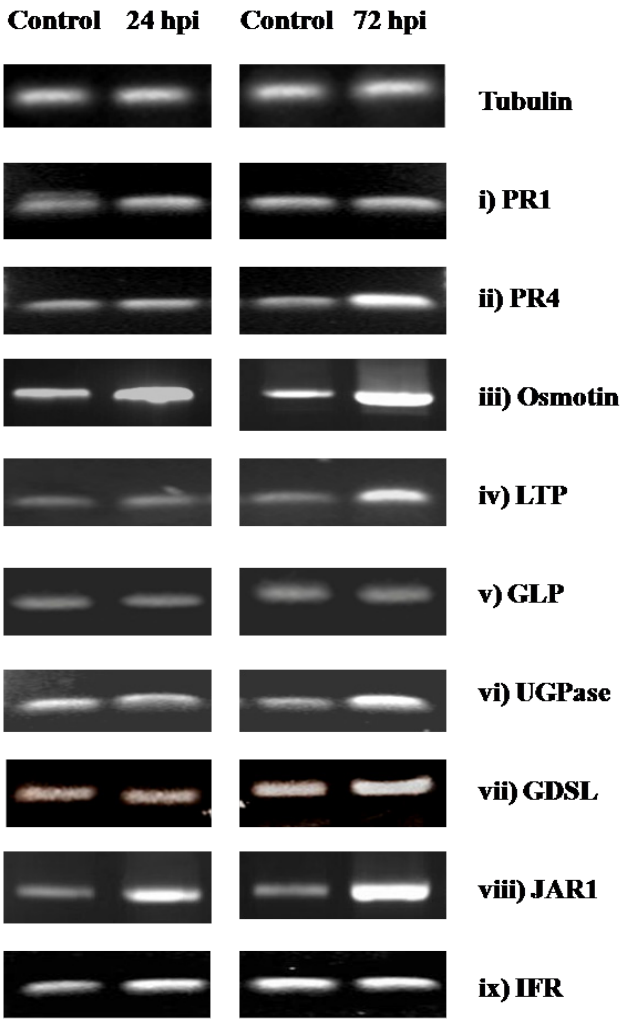

Fig. 3. Semi quantitative RT-PCR analysis showing expression patterns of nine selected defense related genes: i) PR1 ii) PR4 iii) Osmotin iv) LTP v) GLP vi) UGPase vii) GDSL viii) JAR1 and ix) IFR, in control (mock inoculated) and infected banana *M. paradisiaca* cv. Kachkal, at two time points *viz*. 24 and 72 hpi. Tubulin was used as the reference gene for the analysis

disease progression and expression of selected defense related genes involved in an incompatible interaction between the Sigatoka pathogen and banana cultivar Kachkal, which is known to be resistant to the disease. The investigation started with isolation, purification and detection of the causal fungus. Further, to study the disease progression and to obtain samples for gene expression analyses, banana plants raised and maintained under a shade net house were artificially inoculated using the fungal isolate. In the cultivar Kachkal, no typical Sigatoka symptom development was observed after infection although a few tiny brown necrotic spots appeared on the leaves at around 20 dpi. These spots slowly grew into small necrotic lesions with a dry central region but the spread was restricted by 60-65 dpi. No further spread of lesion was observed and the surrounding tissues remained completely green. This study substantiates the fact that, being a resistant cultivar, Kachkal responds to pathogen infection with a hypersensitive response (HR) to restrict the pathogen growth at the point of infection. Thus, well-timed recognition of the pathogen as well as quick and effective manifestation of host defense mechanisms is a significant feature of a resistant plant. A resistant plant limits pathogen spread by not only activating the localized HR but also triggers defense in uninfected parts of the plant by activating systemic acquired resistance (SAR), by way of inducing the expression of an array of genes in response to infection. To study the expression patterns of selected defense response genes in Kachkal upon infection by *P. musae*, a semi-quantitative RT-PCR analysis was carried out. The RT-PCR analyses revealed that all the genes were having a basal level of expression prior to infection, as observed in the control samples and some important defense response genes such as PR4, osmotin, LTP, UGPase and JAR1 were found to be upregulated upon pathogen inoculation.

Plants have developed several defense cascades against pathogens with the blend of some versatile molecules or phytohormones like SA, JA and ET, whose expression significantly differ within a plant species and depend on the type of pathogen. Several defense related molecules and signaling pathways are associated with defense in a resistant plant against fungal attack. The present study has shown that the genes associated with an array of defense related pathways in *M. paradisiaca* cultivar Kachkal are involved in conferring resistance to Sigatoka infection. An increased expression of the PR genes, which are considered as the signature genes of plant defense induction, represents the activation of SA signaling pathway (Ali et al. 2017). The PR proteins have been characterized from many plant species and classified into 17 different families (Van Loon and Van Strien 1999). The expressions of the PR genes have been found to be induced or upregulated particularly during biotic stresses like pathogen infection. The PR protein PR4, codes for chitinase type II protein, which is reported to have antifungal activity (Ebrahim et al. 2011). In the present investigation, PR4 was observed to be considerably upregulated at 72 hpi in Kachkal, while no change in expression was found in case of PR1 (known to be involved in SAR) in both the early as well as late time points. Thus, PR4 must have had a major role in providing antifungal activities against the invading pathogen by acting upon the structural component chitin of the fungal cell wall.

Another important defense related protein, osmotin-like protein, was upregulated at both the time points. Osmotin not only acts as an osmoregulator, but also provides protection from pathogens. The osmotin induced proline accumulation has been reported to confer tolerance against both biotic and abiotic stresses in plants (Abdin et al. 2011). Upregulation of the osmotin-like protein after infection

reflects that the protein is used by the plant in desiccating the area of infection to reduce availability of moisture in the tissue so that fungal proliferation is prohibited. Further, lipid transfer protein was found to be slightly upregulated in Kachkal after infection towards the late time point. LTPs are small, cationic, cysteine-rich peptides found in various plant species that facilitate the transfer of phospholipids between membranes and play a role in plant defense signaling upon pathogen attack. LTP-like genes, when overexpressed endogenously in *A. thaliana*, resulted in enhanced tolerance to pathogen (Sels et al. 2008). Thus, this gene might also have a role in contributing towards resistance in Kachkal. Germin like protein on the other hand, did not seem to have any significant role in defense against the pathogen as no change was observed in its expression after infection.

UGPase enzyme is involved in the biosynthesis of trehalose. Again, trehalose induces in response to infection prior to activation of plant antifungal genes and may act as osmoprotectant against membrane damage (Elbein et al. 2003). UDP-Glucose is supposed to be involved as an extracellular signaling molecule in damage-associated molecular mechanism in plants which may function as potential intracellular mediator of ROS signalling and PCD (Van Rensburg and Van den Ende 2017). Upon Sigatoka infection in Kachkal, the expression of UGPase was observed to be increased at 72 hpi, which demonstrates its potential role in defense related response against the pathogen.

Gdsl like lipase-1 is an ET responsive secreted protein which has been reported to play important role in plant immunity through feedback regulation of ET signaling in *Arabidopsis thaliana* (Kim et al. 2013). In the infected banana plant in our case, ET signalling cascade is apparent in defense as Gdsl like lipase-1 was found to be upregulated after infection at 72 hpi. Likewise, the expression of another plant defense related signaling component, jasmonate-amino-acid-conjugating protein (JAR1), was observed to be immediately upregulated after infection at 24 hpi and also at 72 hpi. Phytohormones like JA, SA and ET are important regulators in plant signal transductions pathways (Lorenzo and Solano 2005; Pozo et al. 2005; Grant and Lamb 2006). JA-Ile (the JAR1 product) mediated defense response has been demonstrated against blast fungus in rice and *Arabidopsis* (Okada 2015). This gene is probably induced at an early stage to facilitate or initiate the defense response. Thus, the expression analysis establishes the role of signaling pathways and associated components in defending the plant against the pathogen.

Isoflavone reductase-like protein (IFR), an enzyme involved in the biosynthetic pathway of phytoalexin in plants, plays significant roles in plant's response to various biotic and abiotic stresses. Several reports have established the involvement of IFR in conferring resistance to pathogens (Daniel et al. 1990; Chang et al. 2015), but in the present study, IFR was not found to be a major player involved in imparting resistance in Kachkal. Infection by the Sigatoka pathogen led to only a slight increase in expression of IFR at the early time point.

Advances in genomics are providing breeders with new tools and resources which have been leading plant breeding to a new era (Perez-de-Castro et al. 2012). The utilization of advanced resources such as the next generation sequencing technology, genome wide association studies, CRISPR/cas9 editing of the genomes, expression studies and functional characterization of candidate genes for introgression of plant defense-related traits into host are the golden future of breeding (Kankanala et al. 2019). Moreover, investigation of plant-pathogen interactions at molecular level facilitates identification of novel defense-related genes that could be incorporated into breeding programmes. Hence, expression studies can throw light on the prospects of such candidate genes towards developing disease resistant varieties using biotechnology combined with empirical breeding programs.

Authors' contribution

Conceptualization of research (PB); Designing of the experiments (PB, SB, BS); Contribution of experimental materials (PB); Execution of field/lab experiments and data collection (SB, BS); Analysis of data and interpretation (PB, SB, DB); Preparation of the manuscript (SB, PB, DB).

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