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Gene expression studies in bacterial leaf blight resistant and susceptible rice (*Oryza sativa*) lines

N M PRAVEEN[1], A RAMANATHAN[2], A BHARANIDEEPAN[3], R MANASA[4], S MONISHA[5], S NAKKEERAN[6] and N KUMARAVADIVEL[7]

Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu 641 003, India

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ABSTRACT

Bacterial leaf blight of rice is a major constraint in tumbling productivity of the crop which shows the precarious nature of the disease. Host resistance is an effective strategy for the management of bacterial leaf blight disease over the cultural, chemical and biological management methods. Glass house and field studies were performed during 2017–19 at Paddy Breeding Station, TNAU, Coimbatore and also the molecular laboratory facility at Department of Plant Pathology was utilized. The objective of the study was to perform gene expression studies resistance genes (*Xa21*, *xa5*, *xa13* genes) and defense genes (*LOX* and *PAL*) in bacterial leaf blight resistance (IRBB 60) and susceptible rice line (TN1) after application of *Xanthomonas oryzae.* pv. *oryzae* and/or riboflavin. Genotyping of IRBB 60 which showed lowest lesion length validated the presence of *Xa21, xa5* and *xa13* resistance genes using corresponding gene specific primers. Application of riboflavin (0.5mM) along with the *Xanthomonas oryzae.* pv. *oryzae* suspension resulted in the highest expression level of these R genes and defense genes except *xa13* gene which showed its highest expression level with the inoculation of *Xoo* alone. Expression of *Xa13* gene was meagre with the treatment of riboflavin where rest of the gene expression was noticeably enhanced when compared to the control. In brief, study performed showed that application of abiotic agent (Riboflavin) enhanced the gene expression of both the resistance and defense gene in rice line when compared to the susceptible variety.

Key words: Bacterial leaf blight, Riboflavin, Rice, RT-PCR

Rice plays a crucial role in agricultural system by serving as caloric intake to half of the world population. In crop production scenario, biotic and abiotic stress to the host are the major threat. Among the biotic agents, bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* bring about serious economic damage to food crop rice. The vascular disease, during epiphytotic seasons, due to genetic susceptibility of the cultivars lead to an erratic rice cultivation even from the early stage of the crop which sum up to a yield loss of 65-95% across the world (Redd 1980). In India, the extent of yield loss is up to 81% (Rao and Kauffman 1971, Kumar *et al.* 2012). Genetically, disease resistance is controlled by one, a few, or many genes for resistance (R genes) in the plant and is termed as true resistance (Agrios 2005). Resistance to bacterial blight is regulated by two classes of genes—major disease resistance (R) genes, and defense-related or defense-responsive genes. Disease resistance (R) genes employed in plant disease management consists of both dominant and recessive allelic genes which recognize specific pathogen effectors either directly (Flor 1946) or indirectly (Van der Hoom 2008) and trigger downstream induction of molecular signal cascades to initiate rapid disease resistance in the host (Belkhadir *et al*. 2004). Forty resistance genes against bacterial leaf blight of rice, *Xa1* to *Xa39* have been identified that could induce defense response (Kim *et al.* 2015). Effective gene combinations of *Xa21+xa13+xa5* and *Xa21+xa13* were successfully pyramided into Samba Mahsuri (Sundaram *et al.* 2008), Triguna (Sundaram *et al.* 2009) and Pusa Basmati (Joseph *et al.* 2004) in India. Apart from R genes that confer disease against *Xoo*, defense responsive genes, are also triggered constitutively after bacterial leaf blight pathogen infection. The proteins encoded by defense-responsive genes are components of the signal transduction pathways that lead to defense responses of the host plant after the recognition event triggered by an R gene product. Hence, elucidation of expression analysis of R genes and defense genes present in highly resistant varieties or near isogenic lines of rice are important to use it as a donor parent for

[1,3,4]Ph D Scholar (pn40785@gmail.com, bharanideepan10@gmail.com, manasaramaraju3@gmail.com), [5]M Sc Student (monishasakthi1996@gmail.com), [6]Professor (nakkeeranayya@gmail.com), Department of Plant Pathology, [7]Professor & Head, (kumaravadivel.n@tnau.ac.in), Department of Plant Molecular Biology and Bioinformatics, Tamil Nadu Agricultural University; [2]Professor(nathanram@rediffmail.com), Department of Plant Pathology, Tamilnadu Rice Research Institute, Aduthurai.

resistance breeding. In the above context, objective of the study was screening of near isogenic lines against bacterial leaf blight disease and to compare the expression pattern of resistance and defense genes in bacterial leaf blight resistant and susceptible rice lines

MATERIALS AND METHODS

The study was conducted during the period of 2017–19 where the field experiments were carried out in Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore (10.9955182 N; 76.9164959 E; MSL: 1395 ft). Bacterial leaf blight symptom in rice was collected from wetlands of Tamil Nadu Agricultural University, Coimbatore, India and the samples were subjected for isolation of pathogen. The present research work was conducted during the period of 2017-19. Field experiments were carried out in Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. Under *in vitro* condition, the collected leaves were dissected such that it containing both blightened and healthy portions. The leaf bits were then transferred into Eppendorf tube containing sterile water and crushed using sterile rod and kept for 10 min such that bacteria ooze out into the sterile water. Later, loopful of bacterial suspension were streaked over the Petri plates mediated with autoclaved and solidified peptone sucrose agar and incubated at 25°C. Pathogenicity of the isolate was proved after artificial inoculation (Kauffman *et al.* 1973) of the isolate into bacterial leaf blight susceptible variety, ADT 38 and re-isolated the organism from the lesion produced. Twenty eight rice differentials which were gene pyramided with combinations of *Xa1* to *Xa21* resistant genes (IRBB 1, IRBB 3, IRBB 4, IRBB 5, IRBB 7, IRBB 8, IRBB 10, IRBB 11, IRBB 13, IRBB 14, IRBB 21, IRBB 50, IRBB 51, IRBB 52, IRBB 53, IRBB 65, IRBB 66, DV-85, ISM, TN1) were collected from IIRR, Hyderabad were subjected for screening against bacterial leaf blight pathogen and garnered the best line based on Standard Evaluation System for Rice (SES scale) (Table 1).

Genomic DNA of the bacterial pathogen was extracted by the protocol of Chen and Kuo (1993). To assess the genetic identity of the pathogen, PCR was performed using 16s rRNA primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') which shows the amplicon size near to 1400 bp. The PCR product was outsourced for sequencing to identify up to the species level of bacteria. Hence, the bacteria identified as *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) through molecular tools was used for the further studies.

Rice differentials were grown in pots and maintained in glass chamber at 25ºC with relative humidity (RH) ranges from 85 to 90%. Rice lines after 40 days of sowing were used for screening against bacterial leaf blight disease where the lines were inoculated following the method of Kauffman *et al*. (1973). Disease scoring was performed after 14 days of inoculation where the disease reaction was measured as lesion length from the basipetal length of clip inoculated leaves by following Standard Evaluation System for Rice (SES scale) (Table 1). Validation of genotypes in resistant line (IRBB 60) was confirmed using gene specific primers designed (Primer3 Plus Software) for *xa5, xa13* and *Xa21* genes (Table 2).

Among the 28 differential lines, the best resistant line (IRBB 60) along with susceptible line (TN1) were selected for analyzing their quantitative expression of *Xa21, xa13, xa5*, *PAL* and *LOX* genes in quantitative RT-PCR (Table 3). The experiment was laid out under glass house (25ºC, 85-90% RH) where triplicates of IRBB 60 and TN1 (Susceptible line) at booting stage were inoculated with *Xoo* alone (T_2) (Kauffman *et al.* 1973), riboflavin (0.5mM) followed by inoculation of *Xoo* by an interval of 3 days (T_3), riboflavin alone (T_4) onto 40 days old plants to compare the fold change of expression of resistant genes (*Xa21*, *xa13* and *xa5*) and defense genes (*LOX* and *PAL* genes) in the host system. Control lines (T_1) were maintained separately for each line where sterile water was applied instead of *Xoo* or riboflavin. Leaf tissues (100mg) of treated lines and control were collected 24 h after application and were immediately frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted from the samples and followed by cDNA conversion were carried out (ThermoScientific). Relative quantification of resistant and defense genes were assessed by quantitative PCR, Applied Biosystem® (Singapore) using

Table 2 Primer sequences of resistant genes

Gene	Nucleotides	Product size (bp)	Annealing temperature (°C)
Xa21	GCAGCACCAGGTTAATCCTAA AGACTCTTTGATGGCACGCA	651	53
xa13	GCCAGAATTTAGCAGTGTGGA TCCTAGACTGAACATCAACATGGA	456	54
xa5	CGTAACTGATACGGGGGAGC ACAGGCTCACAGCATCTCAC	518	54

Table 3 Primer sequences of resistance, defense and reference genes for quantitative RT-PCR analysis

Gene	Nucleotides	Product size (bp)
Xa21	CTCCACTCGCTGAACCTTTC AAGGAACACGTCGGAAGATG	146
xa13	CCCCTCTCTCCATCATCGTA ATGAACTCGACGCTCTTGGT	146
xa5	CGCTGGGTTCGTACGATAAT AAACGATTCACCCGAACTTG	151
LOX	CGACGACCGTGTCTACGACTA GAGGGTAGGGGAACTGCTTG	110
PAL	GGACTACGGGTTCAAGGGC ACGAGACCCAGCGAGTTCA	135
Actin	GAGCTACGAGCTTCCTGATGGA CCTCAGGGCAGCGGAAA	65

Table 1 Score chart of Standard Evaluation System for Rice (SES scale) for bacterial leaf blight

Resistant standard	Lesion length
Highly resistant (HR)	>1
Resistant (R)	>1-3
Moderately resistant (MR)	>3-6
Moderately susceptible (MS)	>6-10
Highly susceptible (HS)	>10

SYBR Green (Takara, Japan) as the detection system along with housekeeping gene (*Actin* gene). Primers for *Xa21, xa13, xa5*, *LOX* and *PAL* genes were designed using Primer 3 software. Rate of change of expression of transcripts of genes were analyzed by calculating $e^{-\Delta\Delta CT}$ from the Ct mean value obtained after analysis using StepOne software v2.3. Formula is depicted below:

$\delta C_{T, Target} = C_{T, control} - C_{T, Treatment}$; $\delta C_{T, Reference} = C_{T, control} - C_{T, Treatment}$; $\delta\delta CT = (\delta C_{T' Target} - \delta C_{T'} Reference) - (\delta C_{T' Target} - \delta C_{T' Reference})$

RESULTS AND DISCUSSION

Isolation of bacterial leaf blight pathogen: Diseased samples of bacterial leaf blight of rice were subjected for isolation of incitant pathogen on autoclaved PSA media under *in vitro* condition. The isolate was inoculated onto the susceptible variety (ADT 38) using the method of Kauffman *et al.* (1973) showed the characteristic symptom development of bacterial leaf blight from the tip of the plant within 4 days of inoculation. Re-isolation of the organism from the lesion showed resemblance with that of the isolate obtained before and hence proved the pathogenicity of the isolate. The pathogen was stored in double autoclaved 70% glycerol stock at -80ºC for further studies.

Molecular characterization of bacterial leaf blight pathogen: Bacterial genomic DNA was isolated and PCR amplification of the nucleic acid with 16sr RNA primers revealed the amplification of DNA at 1400 bp and the PCR product after partial sequencing of both forward and reverse sequences, nucleotides were obtained. BLAST analysis of the nucleotides showed that the sequence showed 100% identity to *Xanthomonas oryzae* pv. *oryzae.* Thus isolated and characterized bacterial leaf blight pathogen (Accession no: MH464904) was employed to phenotypic studies of rice differential lines.

Phenotypic studies: Rice differential lines inoculated at booting stage with *Xoo* suspension showed resistant reaction against bacterial leaf blight disease. IRBB 53, IRBB 58, IRBB 59 and IRBB 60 showed highly resistant reaction to the pathogen with 0.9, 0.9, 0.7 and 0.8 cm of lesion length, respectively. Moreover, the remaining lines also showed comparatively better resistant reaction showing an average lesion length of 1.4 cm. Noticeably, no rice lines showed moderately susceptible or susceptible reaction, eventually, it is evident that the lines were embedded with some resistant source that resist the multiplication of bacterial leaf blight pathogen. The highly resistant lines obtained from the experiment were employed for the further studies. In conformation to the above study, Bharathkumar *et al.* (2014) had screened NIL lines that conferred resistance against the disease and selected the line with more than two gene combination. Similarly, here, line carrying four R genes (*Xa4, xa5, xa13* and *Xa21*) showed highly resistant reaction. It is imperative that the use of gene-combination, *Xa21 + xa13 + xa5* is widely arrayed by many rice breeding groups (Huang *et al.* 1997, Sanchez *et al.* 2000, Singh *et al.* 2001, Joseph *et al.* 2004) and functional specific markers to identify the same have been classified (Hajira *et al.* 2016). Hence such identified lines were subsequently selected for the further studies.

Expression analysis of resistance and defense genes: Gene pyramiding with multiple R genes specific to *X. o.* pv. *oryzae* has significance to resist the occurrence of bacterial leaf blight to large extent (Hajira *et al.* 2016, Arunakumari *et al.* 2016). IRBB 60 showed highly resistant reaction against *Xoo* among the 28 rice lines screened and which was validated with the presence of *xa5, xa13* and *Xa21* gene. Hence, the line was subjected to quantitative RT-PCR analysis to identify the gene expression pattern of the R genes and defense genes (*LOX* and *PAL* gene) with the application of T_2, T_3, T_4. Riboflavin is renowned for its nutritional value and as an enzyme cofactor, recent studies revealed that riboflavin could induce resistance response in plant (Zhang *et al.* 2009). Gangbeomyong *et al.* (2016) has reported riboflavin based product could induce defense response against rice blast and bacterial leaf blight pathogen. Thus confirmed the role of riboflavin in inducing defense response and hence riboflavin and/or *Xoo* were used in the study to analyze the fold of expression of transcripts of R genes and defense genes by quantitative real time PCR technique. Furthermore, there are evidence from other studies such that, riboflavin induced resistance are dependent on H_2O_2 and a functional *NPR1* gene in Arabidopsis (Kachroo *et al.* 2003, De Jong *et al.* 2004) and the signaling messengers are upregulated prior to the expansion of local lesion (Ahn e*t al.* 2005, Ahn e*t al.* 2007). Moreover, the expression of most of the rice defense-responsive genes was induced by 24 hours after inoculation with the pathogen (Wen *et al*. 2003). Hence the riboflavin and/or *Xoo* treated samples were collected after 24 hours of application to assess the intact expressional pattern of R genes and defense genes. Melt curve of the respective genes showed the accuracy of the expression pattern progressed during the run. The study revealed that *Xa21* was expressed to the maximum (287.14 fold) with T_3 where the remaining treatments were failed to express even basal level of expression to impart resistance. Similarly, Bimolata *et al.* (2015) analyzed allelic expression of *Xa21* and *xa5* in IRBB 21 and IRBB 5 respectively where *Xa21* was not expressed or detected with the inoculation of *Xoo* pathogen. Similar observation was obtained in the present study where the expression of *xa5* gene was highest (9.85 folds) when T_3 was used when compared to the other treatments. In contrary to the

expression of other genes with the application of T_3, *xa13* gene has showed highest expression with T_2 and T_4 (2.06 folds). T_3 has showed a declined expression (0.42 folds). Hence, riboflavin failed to trigger the induction of *xa13* gene transcripts once the pathogen initiates infection process. Considering the expression of defense gene, *LOX* and *PAL* gene expressed their maximum respectively up to 5.41 and 5.74 folds after the application of T_3.

The study revealed that among the 28 rice lines screened, IRBB 60 showed lesser infection with the inoculation of bacterial leaf blight pathogen. Genotyping of IRBB 60 revealed the presence of resistance genes, viz. *Xa21*, *xa5* and *xa13* and was subjected to quantitative RT-PCR to analyze the expression pattern of these genes along with defense genes (*LOX* and *PAL* gene) after the application of T_3. Enhanced gene expression of R genes and defense genes in IRBB 60 when compared to susceptible variety, TN1 revealed the potent nature of the former to contain the disease by triggering the defense related genes.

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