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

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ABSTRACT

Bacterial leaf blight of rice is a major constraint in tilling 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...
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, Coimbatore. Under glass chamber at 25°C 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 (25°C, 85-90% RH) where triplicates of IRBB 60 and TN1 (Susceptible line) at booting stage were inoculated with Xoo alone (T2) (Kauffman et al. 1973), riboflavin (0.5mM) followed by inoculation of Xoo by an interval of 3 days (T3), riboflavin alone (T4) 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 (T1) 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 was 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     | GCAGCACCAAGGTTAATCCTAAG | 651           | 53           |
|          | AGACTCTTTGATGGCAAGGCA  |               |              |
| xa13     | GCCAAGATTTAGCATGTTGGA  | 456           | 54           |
|          | TCTTAGACTGAAACTCAACATGGA |              |              |
| xa5      | CGTAACTGATACGGGGGAGC   | 518           | 54           |
|          | ACAGGCTCACAGCATTCA     |               |              |

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

| Gene     | Nucleotides          | Product size (bp) |
|-------------------------------|-------------------|
| Xa21     | CTCACCTGCTGAACCTTTCC | 146              |
|          | AAGGAACACGTCGGAAGATGA |                |
| xa13     | CCTCTCTTTACATCATGTA  | 146              |
|          | ATGAACTCGACGCTCTTGGT |                |
| xa5      | CGCTGGTTGCTTAGCAATAAT | 151             |
|          | AAACGATTCCACCGAACTTG |                |
| LOX      | CGACGACCGGTGTCAGACTA | 110              |
|          | GAGGTTAGGGGAACTGCTTG |                |
| PAL      | GACACCTGGCTCAAGGCG   | 135              |
|          | AGCGGACCCAGCAGGATCA  |                |
| Actin    | GAGCTACGAGCTCTGATGGA | 65               |
|          | CCTACGGCGCAGCGGAAA   |                |
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 $e^{-ΔΔCT}$ from the Ct mean value obtained after analysis using StepOne software v2.3. Formula is depicted below:

$$\delta C_T\text{, Target} = C_T\text{, control} - C_T\text{, Treatment}\quad \delta C_T\text{, Reference} = C_T\text{, control} - C_T\text{, Treatment}. \quad \delta ε C T = (\delta C_T\text{ Target} - \delta C_T\text{ Reference}) - (\delta C_T\text{ Target} - \delta C_T\text{ Reference})$$

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 -80°C 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 X. 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. 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 T2, T3, T4. 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). Gangbeomjong 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 H2O2 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 et al. 2005, Ahn et 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 T3 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 T3 was used when compared to the other treatments. In contrary to the
expression of other genes with the application of T3, xa13 gene has showed highest expression with T3 and T4 (2.06 folds). T4 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 T3.

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 T3. 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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REFERENCES

Agrios G N. 2005. Plant Pathology, 5th edn, 948p. Elsevier Academic Press.

Ahn I P, Kim, S, Lee, Y H and Suh, S C. 2007. Vitamin B1-induced priming is dependent on hydrogen peroxide and the NPR1 gene in Arabidopsis. Plant Physiology 143: 838–48.

Ahn I P, Kim S and Lee Y H 2005. Vitamin B1 functions as an activator of plant disease resistance. Plant Physiology 138:1505–15.

Arumukumari K, Durgarani C V, Satturu V, Sarikonda K R, Chittoor P D R, Vutukuri B, Lahaa G S, Nelli A P K, Gattu S, Jamal M, Prasadababu A, Hajira S, Sundaram R M. 2016. Marker-assisted pyramiding of genes conferring resistance against bacterial blight and blast diseases into Indian rice variety MTU1010. Rice Science 23(6): 306–16.

Belkhadir Y, Nimchuk Z, Hubert D A, Mackey D and Dangl J L. 2004. Arabidopsis RIN4 negatively regulates disease resistance mediated by RPM2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. Plant Cell 16(10), 2822–35.

Bharathkumar S, Gnanamanickam S S, Jitendra K, Archana B, Yasin B S K, Amit D and Deepak K N. 2014. Differential disease reaction of rice pathogen Xanthomonas oryzae pv. oryzae prevailing in india on rice cultivars. The Bioscan 9(3): 1257–62.

Bimolata W, Kumar A, Reddy S K, Sundaram R M, Lahaa G S, Qureshi I A and Ghazi I A. 2015. Nucleotide diversity analysis of three major bacterial blight resistance genes in rice. PLoS ONE 10(3): e0120186. doi:10.1371/journal.pone.0120186.

Chen W P and Kuo T T. 1993. A simple and rapid method for the preparation of Gram negative bacterial genomic DNA. Nucleic Acids Research 21(9), 2260–4.

De Jong C F, Laxalt A M, Bargmann B O, De Wit P J, Joosten M H and Mumnik T. 2004. Phosphatidic acid accumulation is an early response in the Cf4-Avr4 interaction. Plant Journal 39:1–12.

Flor H H. 1946. Genetics of pathogenecity of Melampsora lini. Journal of Agricultural Research 73: 335–57.

Gangbeomyong, Hanshongui, Gimcheolhong and Chu, K Y. 2016. Riboflavin-based BioDoctor TM induced disease resistance against rice blast and bacterial leaf blight diseases. Research Plant Disease 22(3): 202–7.

Hajira S K, Sundaram R M, Lahaa G S, Yugander A, Balachander S M, Virakatham B C, Sujatha K, Balacharanjevi C H, Pranathi K, Anila M, Bhaskar S, Abhilash V, Mahadevawamy H K, Kousik M, Dilip Kumar T, Harika G and Rekha G. 2016. A Single-Tube, functional marker-based multiplex PCR assay for simultaneous detection of major bacterial blight resistance genes Xa21, xa13 and xa in rice. Rice Science 23(3): 144–51

Huang N, Angeles E R, Domingo J, Magpantay G, Singh S, Zhang G, Kumaravadiel N J Bennett J and Khush G S. 1997. Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theoretical and Applied Genetics 95: 313–20.

Joseph M S, Gopalakrishnan R K, Sharma V P, Singh A K, Singh N K and Mohapatra T. 2004. Combining bacterial blight resistance and Basmati quality characteristics by phenotypic and molecular markerassisted selection in rice. Molecular Breeding 13: 377–87.

Kachroo A, He Z, Patkar R, Zhu Q, Zhong J, Li D, Ronald P, Lamb C and Chattoo B B. 2003. Induction of H2O2 in transgenic rice leads to cell death and enhanced resistance to both bacterial and fungal pathogens. Transgenic Research 12: 577–86.

Kauuffman H E, Reddy A P K, Hsieh S Y P and Merca S D. 1973. An improved technique for evaluating resistance of rice varieties to Xanthomonas oryzae. Plant Disease Reports 57: 537–41.

Kim S M, Suh J P, Qin Y, Noh T H, Reinke R F and Jen A K K. 2015. Identification and fine-mapping of a new resistance gene, Xa40, conferring resistance to bacterial blight races in rice (Oryza sativa L.). Theoretical Applied Genetics 123(10): 1933–43.

Kumar P N, Sujatha K, Lahaa G S, Sriniyasa Rao K, Mishra B, Virakatham B C, Hary Y, Reddy C S, Balachandran S M, Ram T, Sheshu Madhav M, Shobha Rani N, Neeraja C N, Ashok Reddy G, Shaik H and Sundaram R M. 2012. Identification and fine-mapping of Xa33, a novel gene for resistance to Xanthomonas oryzae pv. oryzae. Phytopathology 102(2): 222–8.

Rao P S and Kauuffman H E. 1971. New Indian host of Xanthomonas oryzae, incitant of bacterial leaf blight of rice. Current Science 40: 271–72.

Sanchez A C, Brar D S, Huang N, Li Z and Khush G S. 2000. Sequence tagged site markers assisted selection for three bacterial blight resistance genes in rice. Crop Science 40(3): 792–7.

Singh S, Sidhu J S, Huang N, Vikal Y, Li Z, Brar D S, Dhalaiwal H S and Khush G S. 2001. Pyramiding three bacterial blight resistance genes (xa-5, xa-13 and Xa-21) using marker-assisted selection into indica rice cultivar PR-106. Theoretical and Applied Genetics 102(6): 1015–11.

Sundaram R M, Priya M R V Laha G S, Shobha R N, Sriniyasa R P, Balachandran S M, Ashok R G, Sarma N P and Soni R V. 2009. Introduction of bacterial blight resistance into Triguna, a high yielding, mid-early duration rice variety by molecular marker assisted breeding. Biotechnology Journal 4: 400-7.
Sundaram R M, Vishnupriya M R, Binadar S K, Laha G S, Reddy A G, Rani N S, Sarma N P and Sonti R V. 2008. Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite indica rice variety. *Euphytica* **160**: 411–22.

Van der Hoorn R A and Kamoun S. 2008. From guard to decoy: a new model for perception of plant pathogen effectors. *The Plant Cell* **20**(8), 2009–17.

Wen N, Chu Z and Wang S. 2003. Three types of defense-responsive genes are involved in resistance to bacterial blight and fungal blast diseases in rice. *Molecular Genetics Genomics* **269**: 331–9.

Zhang S J, Yang X, Sun M W, Sun F, Deng S and Dong H S. 2009. Riboflavin-induced priming for pathogen defense in *Arabidopsis thaliana*. *Journal of Integrative Plant Biology* **51**(2): 167–74.