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RESEARCH ARTICLE

Characterization of disease resistance in nine traditional rice (*Oryza sativa* L.) cultivars and expression of chennellu PR1 gene in response to *Xanthomonas oryzae* pv. *oryzae*

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Abstract
Bacterial blight (BB) is one of the most destructive diseases of rice, resulting in substantial economic losses. There is difficulty in controlling BB, so the molecular characterization of rice pathogenesis related (PR) genes may help in the selection of higher resistance rice plants against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), BB pathogen. The objective was to analyze the best resistant cultivar with the susceptible rice variety to BB as control and estimate the expression level of PR1 gene in resistant cultivar compared to susceptible variety. PR1 gene studies have been subject to extensive research in view of their possible role in resistance against pathogens. To assess the traditional resistant rice cultivars in response to BB, physiological and biochemical profiling (total chlorophyll, Proline, and total protein) was done in a set of nine traditional rice cultivars from Wayanad, northern Kerala using *IR24* (an improved Indian rice variety susceptible to rice blight) as a control. Less lesion length and increased biochemical parameters suggested that *Chennellu* is the most resistant against *Xoo* among the others. Expression study of PR1 gene was conducted using qRT-PCR in *Chennellu* as well as *IR24* as control. The results showed that PR1 gene was expressed at the highest level (7859.06 ± 116.5-fold up-regulated) during 48 hpi in *Chennellu* compared to *IR24*. Hence the analysis provides fundamental knowledge about the expression of PR1 genes in the interaction between rice and *Xoo* and can improve the agricultural economy by providing transgenic varieties using traditional cultivar *Chennellu*.

Keywords Rice · *Xanthomonas oryzae* pv. *oryzae* · PR1 gene · RT-PCR · qRT-PCR

Introduction

Rice is one of the prominent food crops in the world and feeds half of the global population. Bacterial Blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has been extensively studied as a model disease of rice to understand the host–pathogen interactions and defense responses in monocotyledonous plants (Grewal et al. 2012; Rumdeep et al. 2012). *Xoo* travels short distances from the infected plants and enter the rice leaf through hydathodes and also through wounds or openings caused by emerging roots at the base of the leaf sheath and gain access to the xylem (Ou 1985). *Xoo* multiplies in the intercellular spaces of the underlying epithelium and spread into the plant (Noda and Kaku 1999). After a few days the bacterial cells fill the xylem vessels and ooze out through hydathodes, forming beads or strands of exudates on the leaf surface. This is a characteristic sign of the BB disease called lesions, which may coalesce to form brown colour lesions or blighted portions on leaves.

Yield loss in rice due to BB can reach up to 50% during tillering stage amounting to the US $60 million even if 2% of total paddy affected by BB. The loss would be a staggering US $2.4 billion if the same quantity of paddy was affected globally (Swapan 2013). It badly affects the economy of India. Eradication of this disease by normal practices like crop rotation is not effective. Chemical control is an effective method to manage BB; which is unsustainable due to high cost, effects on soil fertility and environmental pollution. Identification of resistant rice varieties or development of transgenic plants can improve resistance against specific pathogens.

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Generally, the hybrid varieties persist in developing high yield but lose their disease resistance capacity. Unlike hybrid varieties, the traditional rice cultivars are endowed with tremendous disease resistant capacity, as they are not subjected to subtle selection over a long period of time. For proper utilization and incorporation of these indigenous rice lines from the natural crop resources, identification of the best resistant line is the utmost importance. Physiological performances under induced bacterial stress and quantity of bioactive compounds (chlorophyll, proline and total protein) have a significant role in stress can be used for the proper characterization of rice lines in relation to stress tolerance.

Host- pathogen interaction elicit specific responses in plants and its understanding is essential to control the plant diseases. The plant cell defends microbial attack by eliciting characteristic reaction which forms a part of the cellular defense mechanism. Innate immunity involved in the production of antimicrobial peptides by transcription and translation of a single gene (Boman 1995). Hence, the defense mechanism is affected by a set of defense genes that encode diverse proteins that elicit the SAR. Pathogenesis-related (PR) proteins are one such class of proteins that have played a vital role in activating disease resistance.

PR genes are one of the most important defense genes induced during the host–pathogen interactions. PR proteins are present at basal concentrations in healthy tissues, but hyper accumulated during pathogenesis or related stress situations in plant–pathogen interactions (Van Loon et al. 2006). PR proteins are first identified in tobacco, induced during TMV infection (Van Loon and Van Kammen 1970). These proteins are induced either by pathogens or by abiotic stresses like wounding, fungal cell wall elicitors, and secondary signals like ethylene, JA, salicylic acid, UV light, heavy metals, etc. (Grover and Gowthaman 2003).

PR1 proteins are the first pathogenesis-related proteins identified in the PR family (Stintzi et al. 1993). PR1 classes of proteins are commonly used as a marker gene for SAR and also for the activation of hypersensitive response (HR)-mediated defense pathways (Van Loon and Van Strien 1999). It is used as a characteristic marker for defense-related studies in rice since they are expressed in the early as well as late infection period (Mitsuhara et al. 2008). PR1 proteins are grouped as antifungal proteins however; the molecular mechanism of its antifungal activity is not clearly understood. PRs are represented in every plant species and homologues have been identified in animals, fungi and insects. However, the biological functions of the PR1 protein from plants are obscure in spite of extensive research (Lincoln et al. 2018).

Kerala harbors rich resource of indigenous rice varieties the ensuing agroclimatic changes resulted in the loss of wild germplasm. The disease resistance potential of most of these indigenous varieties is still obscure. Keeping this as one of the objectives of the present study, nine farmer varieties from the upland rice were tested for *Xoo* resistance by physiological and biochemical screening to elucidate a systematic and multi-informative profile of selected cultivar in relation to BB resistance. Molecular cloning and expression analysis of PR1 gene from the resistant cultivar infected with *Xoo* was performed.

Materials and methods

Plant material

Nine traditional rice cultivars (Adukkan , Ayiramkana , Chennellu , Chomala , Gandhakasala, Kayma , Valichoori, Veliyan and Thondi) were collected from Wayanad, North Kerala, India and IR24 , an improved rice variety susceptible to blight, procured from Punjab University was used as control for the study. Seeds were surface-sterilised with 0.1% (w/v) $HgCl_2$ for one min and washed with sterile distilled water. The surface-sterilized seeds were grown in disposable petriplates containing filterpaper discs moistened with distilled water. 7-day-old seedlings were planted in the field. The experiment was laid out in a Randomized Block Design (RBD) in plots of 6 × 4 m 2 size. Inoculation of Xoo was performed on the 45-day old rice plants.

Preparation of *Xoo* culture

Cultures of *Xoo* were procured from Kerala Agriculture University, Vellanikara, Kerala and maintained in nutrient agar medium. The inoculum was prepared by incubating two-day old cultures of *Xoo* at 28 °C on a rotary shaker (130–140 rpm) for 48 h in nutrient broth. Expanded leaves were inoculated with 5 µl of *Xoo* (10^8 CFU) to develop blight symptoms and control received distilled water in lieu to sample and the treated plants were examined daily for severity of infection up to three weeks.

Physiological and biochemical screening

Disease severity assessment

Leaf lesion length (cm) was used as criteria to assess the intensity of infection and assessed at weekly intervals. Rice cultivars were scored for BB disease severity using IRRI—standard evaluation system (SES) for rice (IRRI 1988; Sanchez et al. 2000). Mean leaf lesion length, Chlorophyll content, proline and total protein from infected leaf tissues were tested before treatment (BT), 7th day, 14th day and 21st day post inoculation (dpi).

Quantification of chlorophyll content

Chlorophyll content was quantified using 50 mg leaf tissue from the control and infected plants during the 7th, 14th and 21st dpi. Tissue samples were homogenized in a motor and pestle with 2 ml of 80% acetone and centrifuged at 5000 rpm for 10 min. Chlorophyll a, Chlorophyll b and total chlorophyll were quantified using a spectrophotometer according to Arnon (1949) and expressed as mg g^{-1} f. wt.

Determination of proline content

Free proline content was measured from fresh leaves of rice cultivars by a colorimetric method (Bates et al. 1973). Values were referred to L-proline (50 µg/5 ml) calibration curves. Three independent experiments were conducted to represent the average values and are expressed in mg g^{-1} f. wt.

Total protein estimation

Total protein from the leaf samples was measured using a gradient concentration of bovine serum albumin as the standard (Lowry et al. 1951).

Cloning and expression of PR1 gene

RNA extraction

Total RNA was extracted from the leaves of resistant and control samples during 0 h (control), 24, 48 and 72 h of treatment using a modified CTAB method (Bekesiova et al. 1999). The RNA was treated with 1U DNase 1 (Thermo Scientific, Japan) to remove the co-precipitated DNA. The purified RNA was quantified using UV scanning NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Germany). The ratio of absorbance at 260 and 280, and 230 nm were recorded to assess the purity of RNA. The RNA was run on a 1.5% (w/v) formaldehyde agarose gel and visualized using UV transilluminator. Good quality RNA was used as the template for One-step RT-PCR to amplify the PR1 gene. *O. sativa* PR1 gene sequence retrieved from GenBank (Accession No. AF306651.1) was used as the template to design PR1 primer using Primer-BLAST software of NCBI (Table 1).

The reverse transcription PCR reaction mixture contained 6 µl 5 × OneStep RT-PCR buffer, 1.0 µl 10 mM dNTP mix, 2.0 µl OneStep RT-PCR enzyme mix, 1.0 µl forward and reverse primer, < 300 ng template RNA and the final volume was adjusted to 30.0 µl by adding RNase-free water. Polymerase Chain Reactions (PCR) were carried out in a Mastercycler ProS (Eppendorf, Germany). Reverse transcription was performed at 50 °C for 30 min. followed by PCR cycles comprising denaturation at 95 °C for 15 min and 40 cycles of 95 °C for 20 s, gradient annealing temperatures (61.1, 62.4, 63.7, 64.9 and 65.9 °C) for 40 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. PCR products were fractionated on a 1% (w/v) agarose gel and 1 kb DNA ladder was used as the molecular marker. The amplified band corresponding to the product size of the primer was eluted and purified using MinElute Gel Extraction Kit (Qiagen, USA) as per the manufacturer's instructions. Purified PCR products were sequenced on charge basis from Scigenom Labs Private Ltd, Cochin, India. Homology of the cloned sequences was confirmed using BLAST and was submitted to NCBI.

Table 1 The primers for cloning the putative PR1 gene from the disease resistant chennellu variety designed using nucleotide sequences from the database, multiple sequence alignment and primer designing software

Name	Sequence	Tm (°C)	PCR product length (bp)
PR1-F	5′-GCATCG AAAATG GCAACC TCC-3′	69.6	766
PR1-R	5′-CGGCTG ACGGCTTTA TTCCC-3′	69.6	

cDNA synthesis

One microgram of total RNA was used for first-strand complementary DNA (cDNA) synthesis using reverse transcriptase and random primers obtained from PrimeScript™ first strand cDNA Synthesis Kit (Cat. #RR037A, Takara Bio Inc., Japan). A total of 10 µl reaction mixture was used to synthesize first strand cDNA, containiing 2.0 µl of 5 × primeScript buffer, 0.5 µl of PrimeScript RT enzyme mix, 0.5 µl oligo dT primer (50 µM), 2.0 µl random hexamers (100 µM), 1.0 µl (500 ng) template RNA and the final volume was adjusted to 10 µl using RNase-free water. cDNA synthesis was initiated at 37 °C for 15 min for reverse transcription, inactivation of reverse transcription at 85 °C for 5 s and hold at 4 °C. The quality of the synthesized cDNA was calculated from the 260/280 ratio and resolved on a 1% (w/v) agarose gel. The synthesized cDNAs were used as templates for determining the quantitative expression of PR1 gene.

Relative quantification of PR1 gene using Real-Time PCR

The relative expression of PR1 gene was studied using quantitative real-time PCR (qPCR) in *Chennellu*, the most resistant cultivar. Specific primers were developed from PR1 gene sequence of *Chennellu* (Accession No.

KP257093) using Primer 3.0 software (https://primer3.ut.ee/). Actin gene from *O. sativa* (Os03g0718100) was used as an internal control (Table 2).

Quantitative real-time PCR was carried out in a 20 µl reaction mixture, containing 10.0 µl of 2 × SYBRGreen PCR master mix, 0.4 µl ROX Reference dye (50 ×), 0.8 µl forward and reverse primer, 1.0 µl of template cDNA and 7.0 µl of sterile distilled water. The reaction conditions were: initial hold at 95 °C for 30 s, 40 cycles comprising a denaturation step at 95 °C for 5 s and annealing/extension at 61 °C for 30 s. All the reactions were performed in triplicate in a 48-well reaction plate using step one real-time PCR machine (Applied Biosystems, USA). Two biological replicates and three technical replicates were tested for each sample. No-template controls were included for each gene to detect any background signals by the DNA contamination or due to primer-dimer formation. The up/down regulation of mRNA was calculated based on the method of Livak and Schmittgen (2001). The threshold cycle (C_T) value of the internal control was subtracted from that of the gene of interest (PR1) to obtain the ΔC_T value. The $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T value for the control (untreated) from the ΔC_T value of the treated samples. The fold changes in the expression level compared to the control were determined using the equation $2^{-\Delta\Delta C}{}_T$.

Statistical analysis

Mean values of different parameters (leaf lesion length, total chlorophyll, proline and total protein) were subjected to analysis of variance (ANOVA) and correlation coefficients using Statistical Package for Social Sciences (SPSS) version 16.0 to test the significance at 5% level of probability ($P < 0.05$). Data are represented as mean ± standard error (SE). Regression analyses were performed using SPSS to assess the relationship between lesion length and the different parameters (Chl, Proline and total protein), and also between Chl and Pro under pathogen stress and chemical induction. The mean relative expression of PR1 gene during *Xoo* infection was calculated using SPSS 16.0 and represented as mean ± SE.

Results

Identification of resistant cultivar

The disease resistance potential of nine farmer varieties were compared to a susceptible variety. The mean lesion length of the farmer varieties was lesser compared to *IR24*. *Chennellu* showed least lesion length (2.40 cm ± 0.21 cm) followed *Kayma* (2.47 cm ± 0.26 cm), *Valichoori* (2.73 cm ± 0.1 cm), *Thondi* (2.87 cm ± 0.19 cm), *Adukkan* (4.31 cm ± 0.12 cm), *Veliyan* (6.07 cm ± 0.12 cm), *Ayiramkana* (6.53 cm ± 0.26 cm), *Chomala* (7.57 cm ± 0.15 cm), *Gandakasala* (9.0 cm ± 0.29 cm) and *IR24* (13.37 cm ± 0.41 cm) (Fig. 1).

Proline accumulation

Amongst all the treatments, *Chennellu* showed high proline content in (6.11 ± 0.05) followed by *Ayiramkana* (5.32 ± 0.03), *Kayma* (5.05 ± 0.06), *Thondi* (5.18 ± 0.03), *Chomala* (4.77 ± 0.02), *Veliyan* (3.93 ± 0.04), *Adukkan* (3.78 ± 0.03), *Gandakasala* (3.16 ± 0.02), *IR24* (3.14 ± 0.01), *Valichoori* (2.72 ± 0.1) compared to untreated control plants (Fig. 2). Proline quantity showed an increase during the 7dpi and accumulated until 14dpi, and declined during 21dpi, in all varieties. These results indicated that proline accumulation was elicited specifically on resistance response. Such enhanced proline content preceded the observation of HR lesions on the leaf tissues. *Chennellu,* accumulated highest quantity of proline during *Xoo* infection compared with the *IR24*.

Photosynthetic pigments Chl a and Chl b showed significant reduction in *Xoo* infected plants (Fig. 3). Chl a and Chl b quantity reduced with respect to the exposure period. The Chl a content in *Gandakasala* was highly reduced (3.15 ± 0.26) followed by *Chennellu* (5.74 ± 0.03) during 21dpi when infected with *Xoo*. The susceptible variety, *IR24* showed the highest reduction (3.28 ± 0.22). Chl b content in *Chennellu* showed a low reduction (2.27 ± 0.19) and *Gandakasala* showed higher reduction (0.59 ± 0.05), however, total Chl content was highest in *Chennellu* (9.25 ± 0.19) compared to the other varieties during 21dpi.

Table 2 The specific primers designed for quantities real-time PCR from the cloned putative PR1 sequence of chennellu rice variety treated with *Xoo*

Name	Sequence	Size (bp)	Tm (°C)	GC(%)	Reference
ACT-F	5′-GACCTTGCTGGGCGTGAT-3′	155	66.5	61.1	Ruan et al. (2011)
ACT-R	5′-GTCATAGTCCAGGGCGATGT-3′		63.9		
PR1-F	5′-ACTATCGTACGCTGATCCGG-3′	191	59.13	55	This study
PR1-R	5′-TTATTCCCTCCGGCACAAGT-3′		59.01		

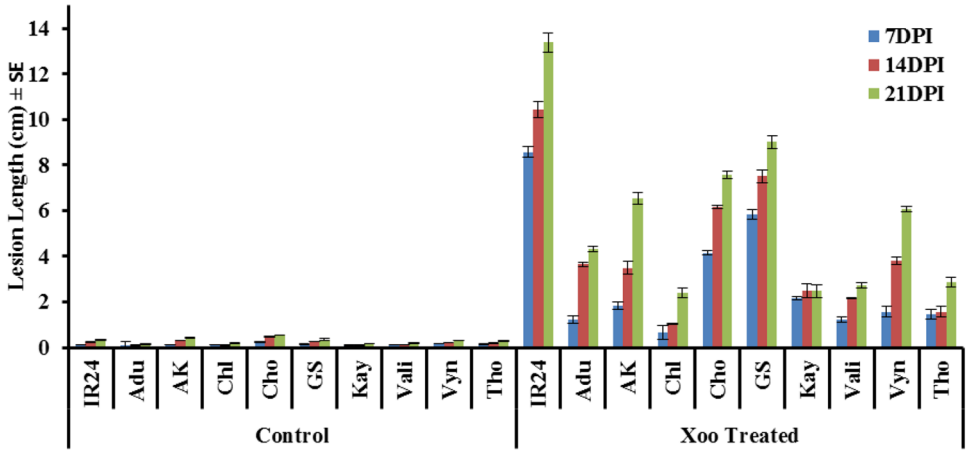

Fig. 1 Lesion length variations in rice cultivars on the 7th, 14th and 21st days in the control and *Xoo* treatments. The data collected was analysed statistically and the bars represent the values with SE for three replications

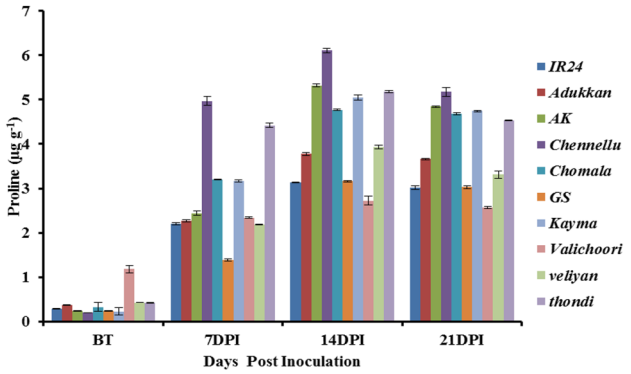

Fig. 2 The variations in proline content in the rice cultivars before treatment (BT) and during the 7th, 14th and 21st days post inoculation with *Xoo*

Chennellu showed highest protein content (0.22 ± 0.02 mg/ml) followed by *Kayma* (0.18 ± 0.01 mg/ml), *Thondi* (0.17 ± 0.02 mg/ml), *Valichoori* (0.15 ± 0.01 mg/ml), *Adukkan* (0.14 ± 0.02 mg/ml), *Veliyan* (0.12 ± 0.02 mg/ml), *Ayiramkana* (0.09 ± 0.04 mg/ml), *IR24* (0.06 ± 0.02 mg/ml), *Chomala* and *Gandakasala* (0.05 ± 0.03 mg/ml) during Xoo treatment (Fig. 4). Total protein content in the treated leaves of *Chennellu, Kayma, Thondi* and *Valichoori* showed slight increase compared to their respective controls while in susceptible *IR24* increased from 7 dpi and accumulated until 14 dpi and declined during 21 dpi. *Adukkan, Veliyan, Ayiramkkana, Chomala* and *Gandakasala* showed the same pattern in reduction of protein content at 21 dpi and categorized as susceptible to *Xoo*.

Xoo treatment substantially limited the length of the lesion coupled with a reduction in Chl and increase in proline and total protein content. Lesion length was negatively correlated with Chl (− 0.421**), Pro (− 0.243*) and total protein (− 0.635**). In addition, Chl had a significant

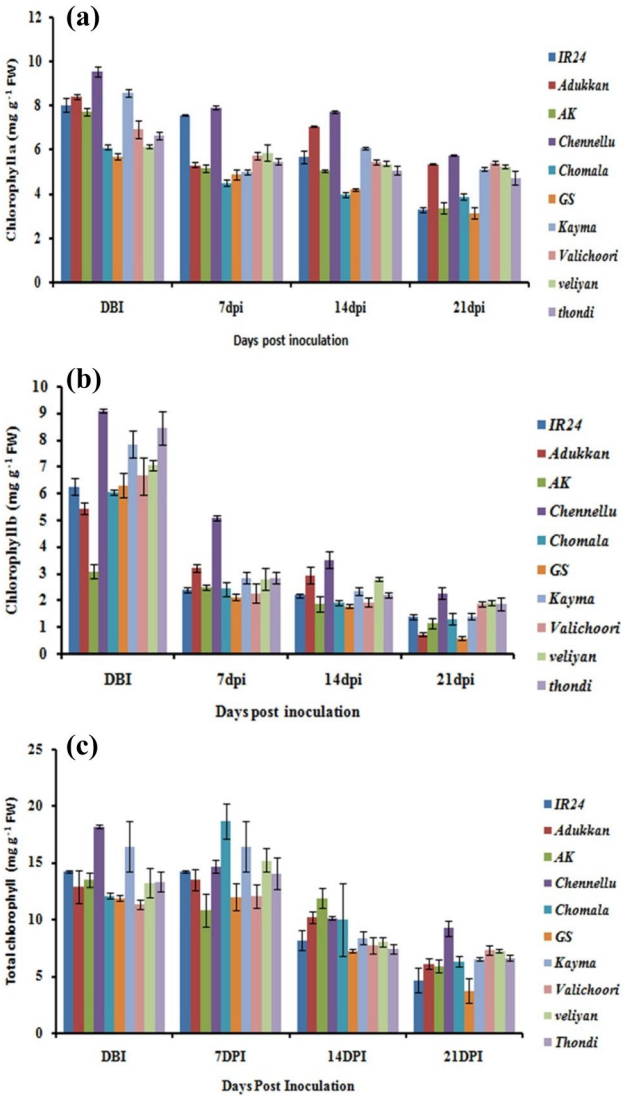

Fig. 3 Effects of Xoo treatment on the production of Chlorophyll a (**a**), Chlorophyll b (**b**) and Total chlorophyll (**c**) in different rice varieties on the day before inoculation (DBI), 7th, 14th and 21st days post infection

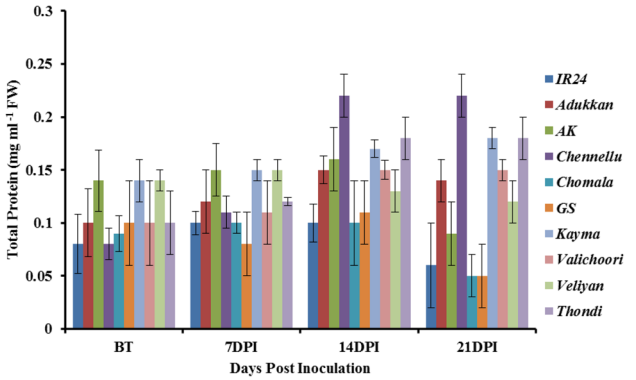

Fig. 4 Total Protein content in ten different rice cultivars treated with *Xoo*. The plant leaves were collected on the 0 day (BT), 7th, 14th and 21st days of inoculation and the total protein was quantified

Table 3 Correlation coefficients between all parameters under Xoo infection in different ricd varieties

	Lesion length	Total Chl	Proline	Total protein
Lesion length	–	−0.421**	−0.243*	−0.635**
Total Chl	–	–	0.243*	0.241*
Proline	–	–	–	0.342**
Total protein	–	–	–	–

Chl chlorophyll

*Correlation is significant at the 0.05 level

**Correlation is significant at the 0.01 level

positive correlation with Pro (0.243*) and total Protein (0.241*) (Table 3), (Fig. 5). There was a conversely high relation between Chl and Pro under *Xoo* treated condition (Fig. 6).

Cloning and expression of putative PR1 gene

Good quality RNA with a 260/280 value of 1.8—2.0 was isolated from the leaves of *Chennellu* and *IR24*. Reverse Transcriptase PCR using an annealing temperature 63.7 °C generated a high-intensity cDNA band and selected as annealing temperature for subsequent PCR. An intact cDNA band of 685 bp was obtained in *Chennellu* during 48 hpi of *Xoo* (Fig. 7).

Sequencing and annotation of putative PR1 gene

The cloned partial PR1 gene of 685 bp from *Xoo* treated *Chennellu* was sequenced using the dideoxy sequencing method. The raw sequence generated by PR1 forward and reverse primer was 719 and 716 bp and trimmed to 685 and 435 bp respectively. The sequences were merged by BioEdit tool. Homology analysis of the merged sequence showed 95% identity with *O. sativa* PR1 mRNA and with an E-value of 0.0 (Table 4). The sequence was deposited in Genbank (KP826795). Comparison of the translated amino acid sequence with the protein database (https://web.expasy.org/docs/swiss-prot_guideline.html) exhibited good homology with the primary structure of PR1 proteins from other species.

Relative expression of PR1-Xo gene

Relative expression of PR1 using quantitative real-time PCR for internal control gene actin showed a melting curve with a single peak with T_m of 81.24 °C. PR1-Xo gene expression in all the samples had a single peak melting curve at T_m of 82.29 °C. T_m varied for each amplicon with the sequence length and GC content (Fig. 8). Validation of assay specificity is completely dependent on the T_m and single melting curve of the amplicons.

The relative expression of *Xoo* induced putative PR1 gene progressed from 24 to 48 hpi and gradually declined at 72 hpi in both *Chennellu* and *IR24*. The PR1 gene expression in *Xoo* treated *Chennellu* showed 3118.99 ± 42.72 fold up-regulation during 24 hpi, 7859.06 ± 116.5 fold up-regulation during 48 hpi and 2079.97 ± 77.53 fold up-regulation during 72 hpi compared to the control (Fig. 9). However, the PR1 gene expression in *IR24* was 83.02 ± 1.26 fold up-regulated during 24 hpi, 165.35 ± 2.98 fold up-regulated during 48 hpi and 92.80 ± 1.14 fold up-regulated during 72 hpi of *Xoo*.

Discussion

PR1 genes are good candidate molecular markers for resistance since it is induced during biotic as well as abiotic stress conditions. The aim of the study was to characterize nine traditional upland varieties and identify the putative PR1 genes expression in rice during *Xoo* infection. Biochemical and physiological screening of the varieties were done to find out the resistance mechanism to adapt themselves to *Xoo* infection since disease resistance is the manifestation of morphological, physiological and biochemical adaptations of plants to thrive under stress conditions. These pathways lead to the production of stress-tolerant metabolites that converge and cross-talk to mitigate the abiotic stress (Xiao et al. 2013).

Pathogen infection induces stress responsive physiological activity of host plants. The resistance to blight generally does not affect plant height, heading date, and plant compactness (Ling et al. 2011). The manifestation of disease symptoms begin within 4–5 days of inoculation, but plants should attain physiological maturity of at least 30–90 days for BB infection (Noor et al. 2006). IRRI established an SES

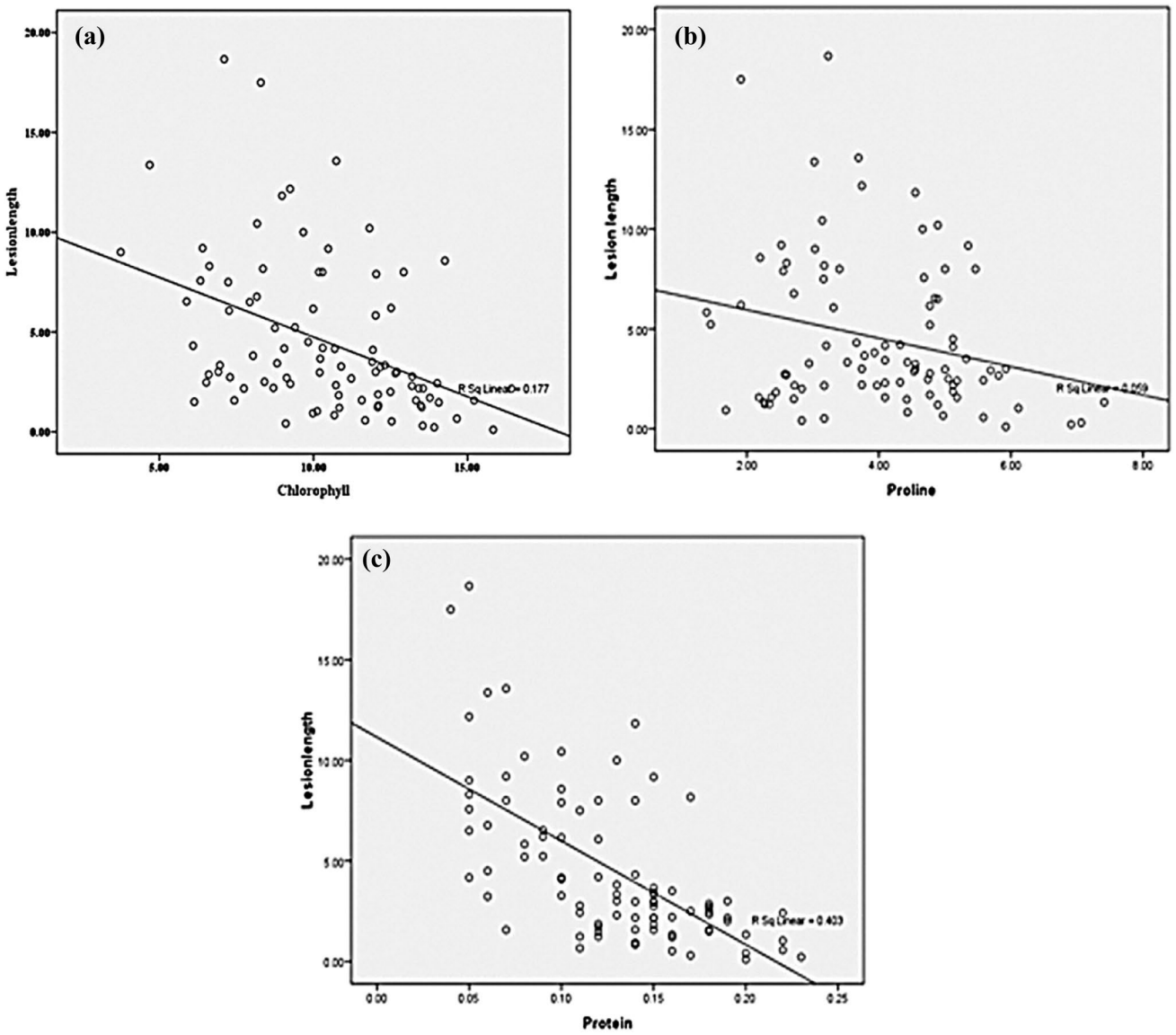

Fig. 5 Correlation analysis of lesion length with total chlorophyll (**a**), proline (**b**) and total protein (**c**) of the ten differetn rice cultivars treated with *Xoo*

scale system to evaluate BB disease severity and described lesion length ranging from 0 to 5 cm as resistant, 6–15 cm as moderately resistant to susceptible and > 15 as susceptible (IRRI 1996). However, some other methods mean lesion length of 0–6 cm classified as Resistance and > 6 cm as susceptible (Sanchez et al. 2000).

Proline is one of the stress-responsive aminoacid and the increase in the levels of free proline content in resistant rice cultivars is comparable with the levels reported for biotic and abiotic stresses (Fabro et al. 2004). The accumulation of proline was observed in rice during tungro virus infection which mimicked drought stress (Mohanty and Sridhar 1982). Proline accumulates in the leaf tissues of *Arabidopsis thaliana* infected with *Pseudomonas syringae* pv. *tomato* avirulent strains (*avrRpt2* and *avrRpm1*), that triggers HR by incompatible plant-pathogen interaction; but in contrast, proline accumulation was not increased in *Arabidopsis* in response to virulent pathogen infection (Fabro et al. 2004). The higher quantity of proline was also reported in tomato plants infected by *Meloidogyne javanica* or *Agrobacterium tumefaciens* (Meon et al. 1978). In our studies, the proline content was up-regulated in the resistant cultivars compared to *IR24*. All infected plants, in general, showed elevated proline content; however, the increase was fourfold higher in *Chennellu* after *Xoo* infection, similarly *Ralstonia solanacearum* infected resistant cultivar of *Lycopersicon esculentum* produced eightfold increase over control while

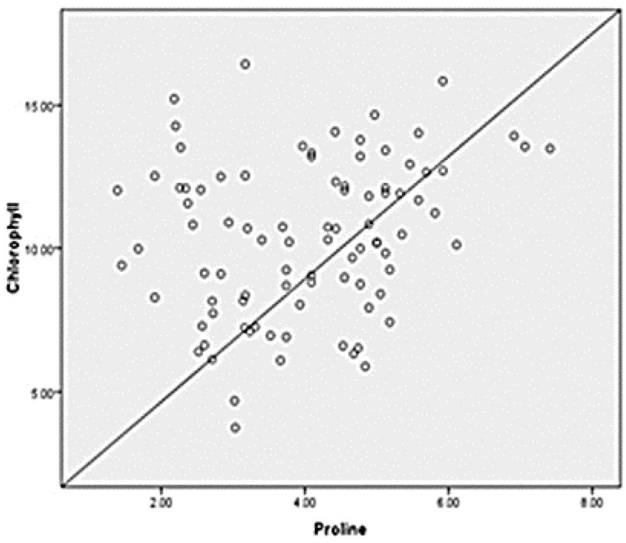

Fig. 6 Correlation analysis of total chlorophyll and proline of ten rice cultivars under treated with *Xoo*

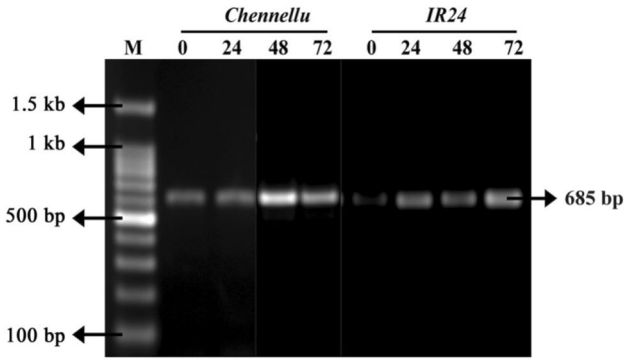

Fig. 7 PR1 cDNA band of size 685 bp obtained from RT-PCR in *Xoo* treated *Chennellu* and *IR24* during 0, 24, 48, 72 h. 100 bp ladder was used as marker

susceptible cultivar *Zingiber officinale* had only threefold increase (Sreedevi et al. 2013). In this study, proline content increased during 7 dpi, and 14 dpi and declined during 21 dpi. A time-dependent decrease in proline content was observed in many other species (Claussen 2005).

The concentration of total chlorophyll is one of the major factors affecting photosynthetic capacity (Zobayed et al. 2005). Total Chl content showed a gradual decrease in treated plants over control plants. The reduction in total Chl in *Chennellu* has supported the selection of resistant cultivar. Reports showed that leaf spot, blight and other diseases decreased the photosynthetic surface of the plant leading to leaf tissue destruction or defoliation. The resistant varieties developed a reduction in chlorophyll than the susceptible or high lesion length obtained cultivars. Long term stress exposure (days to weeks) reduced the chlorophyll content of leaves (Lichtenthaler and Miehe 1997). Advanced leaf symptoms caused by *Xanthomonas campestris* pv. *vesicatoria* on tomato leaves nullified the photosynthesis and virus-induced mosaic symptoms in cowpea reported drastically reduced chlorophyll content.

In our study, selected resistant cultivars (*Chennellu*, *Kayma,* and *Adukkan*) showed a meager increment in protein content whereas in susceptible cultivars the protein content was reduced drastically after inoculations. Reduction in protein content indicates the lack of signaling molecules to resist the pathogen infection. Similar effects on reduced protein levels were detected in healthy and inoculated plants as they became older and also leaves of infected plants contain a significantly low rate of proteins than the control during the first week after inoculation (Goicoechea et al. 2000). Reports suggested that amino acids were not related to BB resistance; however, the correlation between amino acid content and BB disease severity is not significant (Suryadi et al. 2011).

The cultivars with lesser lesion length and elevated level of total Chl, Pro, and total protein can be considered as resistant. Correlation coefficient suggests that lesion length is negatively correlated with Chl, Pro, and total protein content attributing the reduction in chlorophyll content to the resistant nature of the plant. However, under drought stress, Pro and total chlorophyll levels in leaves

Table 4 Homology of the cloned PR1-Xo gene sequence with other PR1 gene sequences

Plant species	Max. score	Query coverage (%)	E-value	Identity (%)	GenBank accession no
Oryza sativa Japonica PR1	1020	94	0.0	95	XM_015792496.1
Oryza sativa PR1 mRNA, complete cds	1014	94	0.0	95	AF306651.1
Oryza sativa Japonica PR1-3	682	65	0.0	94	XM_015789648.1
Oryza sativa indica PR1a, complete cds	682	65	0.0	94	EF061246.1
Zea mays subsp. parviglumis PR1 gene, complete cds	411	64	1e−110	84	DQ147150.1
Triticum aestivum PR1-9 gene, complete cds	333	62	3e−87	81	KF196283.1
Musa acuminata PR1 mRNA, partial cds	156	60	7e−34	74	EF055881.2

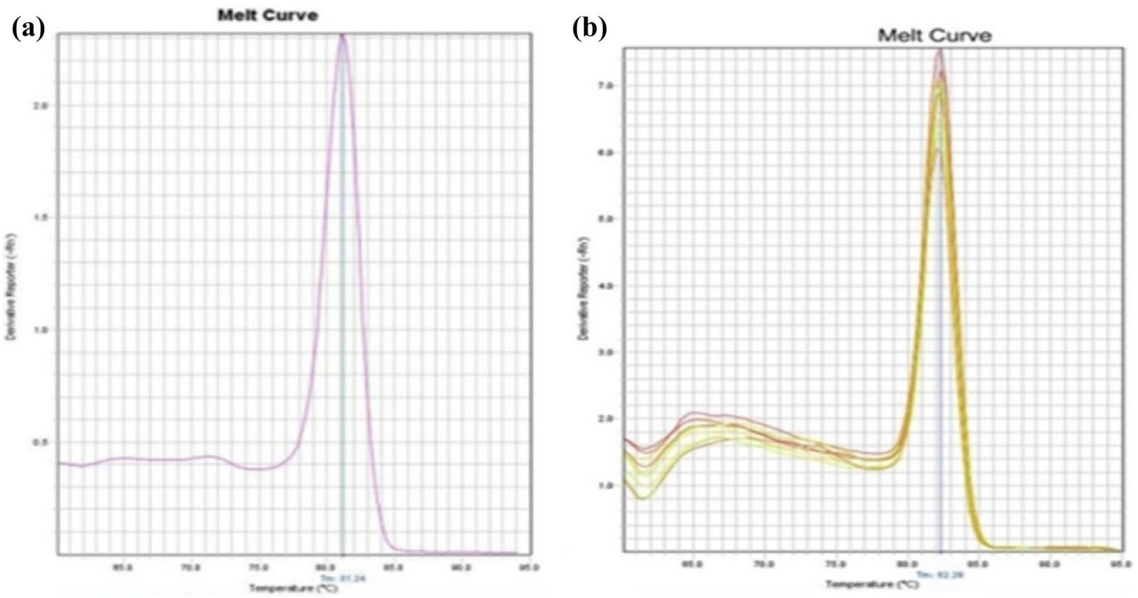

Fig. 8 Melting curve of **a** actin (Tm of 81.24 °C) and **b** PR1-Xo (82.29 °C) with single peak represents the amplicon specificity

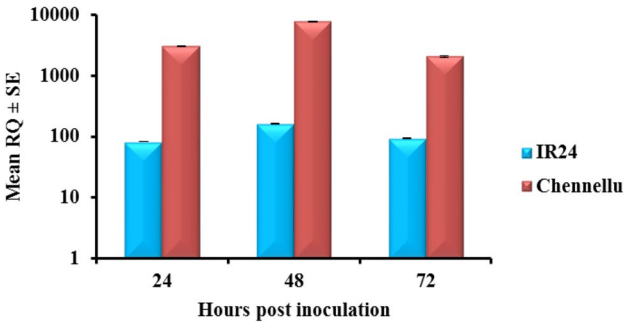

Fig. 9 Relative expression of putative PR1-Xo gene in *IR24* and *Chennellu* treated with *Xoo* for 24, 48 72 hpi and analyzed using quantitative real-time PCR

were increased (Canavar et al. 2014) along with increased chlorophyll content (Kraus et al. 1995).

The cDNA sequence of PR1 gene from *Chennellu* shared better sequence homology with other PR1 proteins, however, gene sequence size varied with respect to the organisms. The complete cloned PR1 mRNA of *O. sativa* (AF306651.1) was 864 bp in length (Park et al. 2012), parsley ranged from 750–900 nucleotides and PR1 mRNA of potato was 870 nucleotides (Taylor et al. 1990).

PR1 proteins were identified from the ascomycete fungus *Fusarium graminearum*, the primary agent of *Fusarium* head blight of wheat and barley (Lu and Edwards 2018). Studies showed that *OsPR1a* and *OsPR1b* played an important role in inducing resistance against *Xoo* and considered as potent resistance markers in rice (Ponciano et al. 2006). *OsAOS2*, a pathogen-induced gene and PR genes (such as *PR1a*, *PR3*, *PR5)* were expressed in the leaves of transgenic rice during *M. grisea* infection (Mei et al. 2006). The present study reported the upregulation of PR1 gene in the rice cultivar (*Chennellu)* compared to susceptible (*IR24).* The expression was studied in the early stage of infection since the expression was very low in untreated controls and in treated plants, it increased substantially from 24 to 48 hpi and declined at 72 hpi. Studies also supported that the transcripts were detectable only during 24 hpi and not in 12 hpi in the greenhouse grown plants (Wielgoss and Kortekamp 2006). Increase in the infection periods (one day, second day and third day post inoculations) influenced the gene expression, by up-regulating OsPR1 transcript levels during 3 dpi and maintained the same levels till 6 dpi (Jiang et al. 2015). The PR1 protein expression in early as well as late infection periods developed HR lesions during 2 dpi (Jiang et al. 2015). Higher levels of PR1 expression was observed in *Xa21*-mediated resistance at later stages after inoculation with *Xoo* (Hou et al. 2012). Actin used as the reference gene in qPCR analysis, based on its constitutive expression in plants (Sgamma et al. 2016). The relative expression analysis established that PR1 gene was upregulated in *Xoo* infected *Chennellu* leaves during 48 hpi compared to *IR24*. Recently, PR1 and other defense related genes were investigated in resistant rice variety against brown planthopper using qPCR (Jannoey et al. 2017). qPCR expression profiling of IET8585, an *indica* rice variety showed an increased expression of few defense-related genes after *Xoo* infection with a proven role in anti-bacterial pathogenicity (Kottapalli et al. 2007).

Earlier reports suggested that PR1 proteins are naturally occurring antifungal proteins with established anti-fungal activity in transgenic tobacco and tomato (Ulaganathan et al. 2002). 12 *OsPR1* genes were up-regulated in a compatible rice-blast fungus interaction, confirming the role of fungus in its induction. However, *Bacillus thuringiensis* suppressed bacterial wilt disease caused by *Ralstonia solanacearum* with systemic induction of PR1 gene in tomato (Hyakumachi et al. 2013). Our results are in congruence with earlier reports of PR1 expression, suggests that *Chennellu* PR1 has anti-microbial PR activity since its high up-regulation due to bacterial attack, contrary to the earlier reports that PR1 is an antifungal protein.

Conclusion

Understanding the basis of rice-blight disease resistance is crucial for the development of blight-resistant varieties in the future. The aims of the study were to screen the best resistant cultivar among the nine commonly used traditional rice cultivars in Wayanad region, North Kerala, India. *Chennellu* was identified as the best resistant cultivar against *Xoo*. The relative expression of PR1 genes during the *Xoo* infection in *Chennellu* and the control susceptible variety to BB *IR24* were studied. To achieve this objective, the PR1 genes were cloned and the relative expression from *Chennellu* and *IR24* during infection caused by *Xoo* was analysed. Quantitative expression of PR1 gene using qRT-PCR showed that PR1 gene was 7859.06 ± 116.5-fold up-regulated during 48 hpi in *Chennellu*, where as only 165.35 ± 2.98-fold up-regulated in *IR24* which indicated high induction of putative PR1 gene.

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