

Original Research Article

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Expression of *Xa21* Allele Resistant to Bacterial Blight under Artificial Epiphytic Condition in Indian Basmati rice (*Oryza sativa* L.)

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ABSTRACT

A bacterial blight (BB) resistance gene *Xa21* linked to pTA248 molecular marker was transferred into Pusa Basmati-1 (IPB-1) to develop improved Pusa Basmati-1 (IPB-1). The bacterial culture *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was used to create artificial epiphytic condition. The leaf samples were taken from PB-1 and IPB-1 treated with bacterial blight culture at different time interval (0 hour, 6 hour, 12 hour, 24 hour, 36 hour, 48 hour, 60 hour and 72 hour) to observe level of expression. RNA isolation and purification was carried out by TRIzol method and analyzed quantity of the total RNA for cDNA preparation. The 2- $\Delta\Delta$ CT method was used to analyse the relative changes in gene expression from real-time quantitative PCR. The normalized expression indicated that the variety IPB-1 carrying gene *Xa21* expressed higher fold changes than that of susceptible variety PB-1 and control (0 hour) at tillering as well as adult stage. The adult stage expressed higher fold change in 2- $\Delta\Delta$ Ct values than that of the tillering stage. However, the 2- $\Delta\Delta$ Ct value of 72 hour at adult stage indicated that the expression of *Xa21* allele is down-regulated after 60 hours in artificial epiphytic condition.

Keywords

Xanthomonas oryzae pv. *oryzae*, BB resistance, Quantitative real time PCR, *Oryza sativa* var. *indica*

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Introduction

Rice is the major staple food crops to feed the people all over the world. Its genome is used as reference genome for most of the genomic studies for improvement of cereal crops. Rice crop is adversely affected by several biotic and abiotic stresses. Bacterial blight (BB) caused by the Gram-negative proteobacterium *Xanthomonas oryzae* is major devastating rice

disease and reduce the yield up to 80 % depending on the stage of the crop, cultivar susceptibility and the environmental conditions (Perez *et al.*, 2008; Leung *et al.*, 2004). Bacterial blight appears on leaves of young plants, as pale-green to grey-green water-soak streaks near the leaf tip and margins. Thirty seven genes that confer resistance against various races and pathotypes of *Xoo* causing the disease have

been identified (Cheema *et al.*, 2008). The genes *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *xa13* and *Xa21* have been incorporated in rice germplasm using marker assisted breeding procedures (Sanchez *et al.*, 2000; Davierwala *et al.*, 2001; Singh *et al.*, 2001; Rajpurohit *et al.*, 2011).

The knowledge of the pathogen population structure, virulence characteristics and expression pattern of important genes responsible for the bacterial blight is essential for a successful breeding program designed for development of improved varieties with durable resistance. It has been observed in many cases that the rice lines having more than one BB resistance gene showed a wider resistance spectrum and manifested increased levels of resistance to the *Xoo* races, as compared with those having a single BB resistance gene. The broad spectrum bacterial blight resistance gene *Xa21* was introgressed from a wild species *O. longistaminata* onto *O. sativa* chromosome 11 (Khush *et al.*, 1989). BB resistance dominant gene *Xa21*, has been found to confer resistance to diverse BB pathotypes (Khush *et al.*, 1990; Ikeda *et al.*, 1991). Marker-assisted selection for pyramiding important genes along with rapid background recovery of the recurrent parent (Xu and Crouch, 2008), while maintaining the exquisite quality characteristics of basmati rice could be more effective approach for its improvement.

However, the pyramiding of resistant genes using marker assisted selection may be takes up to enhance the functional expression of effective resistant genes in the breeding program. Therefore, it is felt necessary to study functional expression of every resistance genes before we release the genotype as a variety for commercial production. The article reports the resistance pattern and level of expression of gene *Xa21* under induced conditions

Materials and Methods

Pusa Basmati-1 (PB-1) null for *Xa21* and IPB-1 introgressed with *Xa21* gene were selected to analyse expression pattern of *Xa21* allele. Three replications of these lines were transplanted in the field conditions during *kharif* crop season. PB-1 and IPB-1 isogenic lines were treated by BB inoculums at tillering and adult plant stage and samples were taken at different time intervals (0 hour, 6 hour, 12 hour, 24 hours, 36 hours, 48 hours, 60 hours and 72 hours after inoculation).

Preparation of bacterial culture and artificial epiphytotic condition

The *Xanthomonas oryzae* pv. *Oryzae* was used in the present study. The culture of strain of BB (*Xoo*) pathogen was multiplied and maintained on *Wakki Motto* media. Inoculum of BB was used to create artificial epiphytotic condition. Bacteria was collected in sterile distilled water and adjusted to a concentration of an optical density at 600 nm = 1 (Iyer and McCouch, 2004). An average of five leaves per plant were inoculated with bacterial suspension of BB inoculum using scissor tillering stage and adult plant stage by clipping method. Tissues from the sample leaves (middle portion of leaf leaving 2 cm from the base and tip) were used. The samples were taken at different time intervals from PB-1 and IPB-1 which was treated by BB inoculums. The samples were flashed frozen in liquid nitrogen and stored at -80oC.

Isolation of RNA and preparation of cDNA

Molecular marker pTA248 F (5'-GCGCGTAGTGCGCATTT-3'); R (5'-CGCGCAAGCAAGTCAAGA-3') linked to *Xa21* was used to screening PB-1 and IPB-1. Total RNA was extracted from approximately 10 mg leaf tissue using TRIzol method. RNA Samples were treated with RNase free DNase

followed by a heat-inactivation step (75°C for 5 min). Reverse transcription for each sample was carried out in a 20 ml reaction using a first strand cDNA synthesis kit (M-MULV RT-PCR KIT Bangalore genei). Second-strand synthesis was carried out with the *Xa21* forward primer and PCR was performed on 100 ng of each cDNA.

Real Time RT-PCR assay

PCR amplification was performed in a real-time PCR Detection System. The RT-PCR was carried out by using RT-PCR Kit with SYBR Green Supermix (Bangalore genei). Quantitative gene expression was measured by real-time RT-PCR using One Step RT – PCR Kit with SYBER Green (Bio–Rad) in three replications. Molecular marker pTA248 F(5'-GCGCGTAGTGCGCATTT-3'); R(5'-CGCGCAAGCAAGTCAAGA-3') and *actin* primer F(5'-CTCCCCCATGCTATCCTTCG-3');R (5-TGAATGAGTAACCACGCTCCG-3') was used for PCR amplification in a real-time PCR Detection System.

PCR cycling and detection for 35 cycles (10 seconds at 95°C; 45 seconds at 55 - 60°C, 72° at 45 seconds). The observations were recorded in all three replication and mean was used. The relative values are normalized to the rice actin level.

Data analysis using 2- $\Delta\Delta$ CT method

The 2- $\Delta\Delta$ CT method was used to analyse the relative changes in gene expression from real-time quantitative PCR. 2- $\Delta\Delta$ CT is the amount of target, where $\Delta\Delta$ CT = (CT.Target – CT.Actin) Time x – (CT.Target – CT.Actin) Time 0. Time x is any time point and Time 0 represents the 1x expression of the target gene normalized to actin. The mean CT values for both the target and internal control genes were determined at time zero. The fold change in the target gene, normalized to actin and

relative to the expression at time zero, was calculated for each sample (Livak and Schmittgen, 2001).

Results and Discussion

BB resistance

Infection of *Xoo* evidently differentiated resistant and susceptible varieties on the basis of intensity of lesions averaging from 8.2 cm to 14.6 cm (PB-1) and 0.55 cm to 1.27 cm (IPB-1). This phenotypic evaluation demonstrates that IPB-1 is resistant to *Xoo* while PB-1 is susceptible. PB-1 and IPB-1 expressed their phenotypic expression after application of inoculums for pathotype *Xoo* infection (Fig. 1). Response of both PB-1 and IPB-1 exhibited the expected phenotypes with disease severity at tillering stage (51.33% and 3.89%, respectively) and at adult stage (85 % and 4.44%, respectively). The amplified product with marker pTA248 from the resistant donor line was of 1,000 bp while that from the susceptible parent PB-1 it was about 650 bp which could be easily resolved on 1% agarose. It indicated that IPB-1 was resistant against bacterial blight due to the introgression of *Xa21* gene (Fig. 2).

Relative quantification of gene expression

The expression of *Xa21* gene was analysed at tillering as well as adult plant stage in PB-1 (susceptible) and introgressed line IPB-1 (resistant). BB inoculated plants of PB-1 and IPB-1 were used to isolate RNA for cDNA construction. The samples were taken at 0 hour, 6 hour, 12 hour, 24 hour, 36 hour, 48 hour, 60 hour and 72 hour at tillering as well as adult plant stage. The sample taken at 0 hour was used as control. To analyse the expression of *Xa21* gene in the Indian basmati varieties, quantitative real-time RT-PCR was carried out, qRT-PCR analysis is represented as the ratio between the samples taken at

different time intervals which was calculated after normalization with housekeeping gene actin which was used as an internal control. At tillering stage, the $2^{-\Delta\Delta CT}$ values of the samples of IPB-1 at different time interval from 0 h 72 hours were observed and the highest $2^{-\Delta\Delta CT}$ value (6.453) was at 72 hours after inoculation. However, $2^{-\Delta\Delta CT}$ values of the sample of PB-1 ranged from 1.00, to 0.8467. At adult stage, the $2^{-\Delta\Delta CT}$ values of the IPB-1 at different time intervals were obtained and it ranged from 1.00 to 13.3614. However, the highest $2^{-\Delta\Delta CT}$ value was at 60 hours after inoculation. In PB-1, the range of $2^{-\Delta\Delta CT}$ value was from 1.00 to 0.7219. At

adult plant stage, the $2^{-\Delta\Delta CT}$ values indicated that the expression of *Xa21* allele is higher in IPB-1 than control and susceptible variety PB-1 but at 72 hour ($2^{-\Delta\Delta CT}$: 9.7811) the expression was down regulated than at 60 hour ($2^{-\Delta\Delta CT}$ 13.3614) and 48 hour ($2^{-\Delta\Delta CT}$: 11.3924). Variation in $2^{-\Delta\Delta CT}$ values at different time intervals reflects higher fold-change in resistant variety IPB-1 as compared to susceptible variety PB-1 in both tillering and adult plant stage (Fig. 3). The higher fold change in $2^{-\Delta\Delta CT}$ values indicated that the expression of *Xa21* was higher at adult stage than the tillering stage (Fig. 4-5).

Fig.1 Introduction of a *Xa21* gene into a susceptible line Pusa Basmati-1 conferred resistance to bacterial blight treated with *Xoo* (A) Pusa Basmati – 1 (PB-1) and (B) Improved Pusa Basmati-1 (IPB-1)



Fig.2 PCR analysis of PB-1 and IPB-1 using pTA248 molecular marker linked to *Xa21* gene

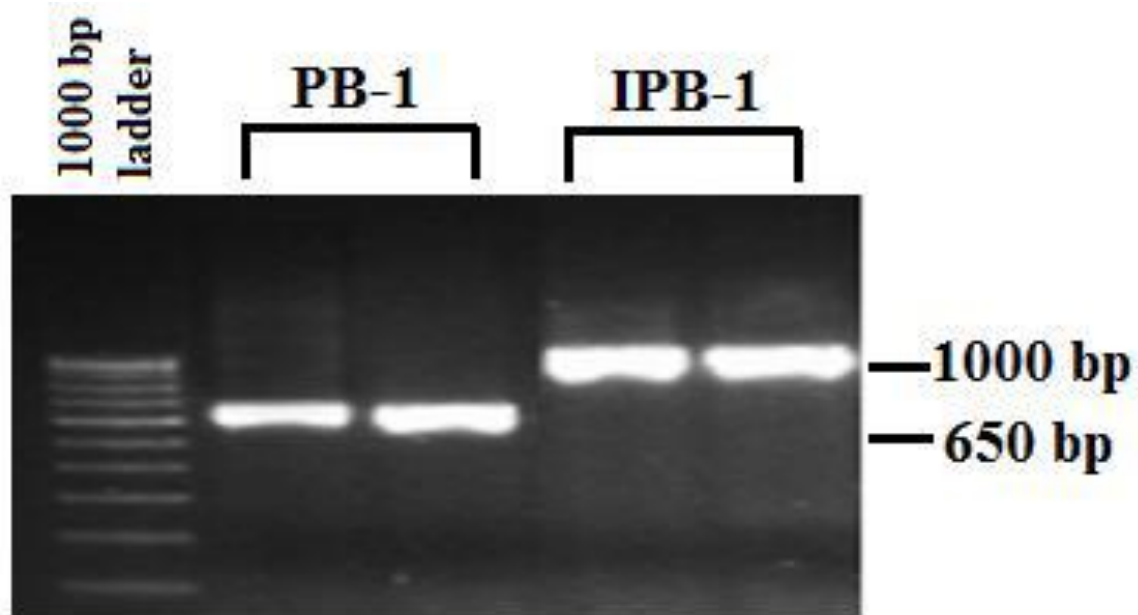


Fig.3 Expression of *Xa21* in improved Pusa Basmati-1 (IPB-1) at tillering (blue bars) and adult stage (red bars) in artificial epiphytic condition. Error bars represent the standard deviation of the mean

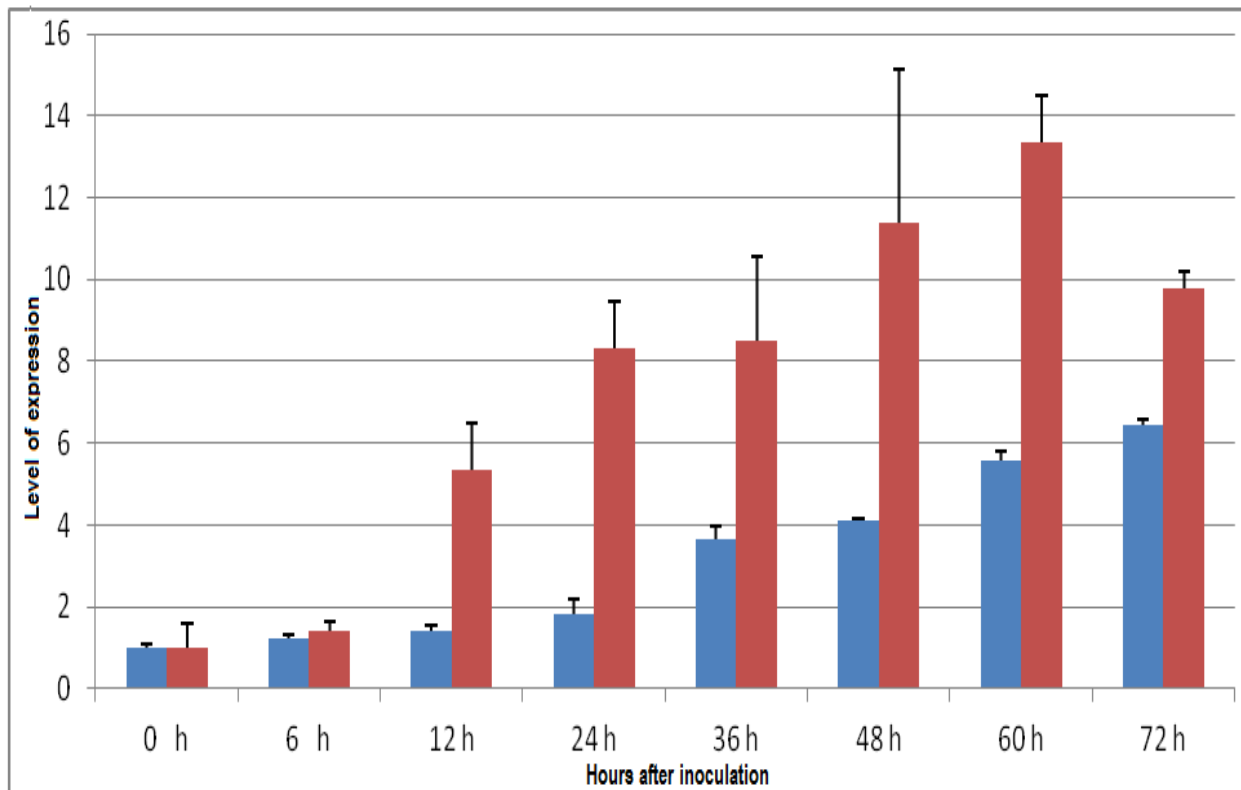


Fig.4 Expression of *Xa21* gene at tillering stage in PB-1 and IPB-1 at different time interval

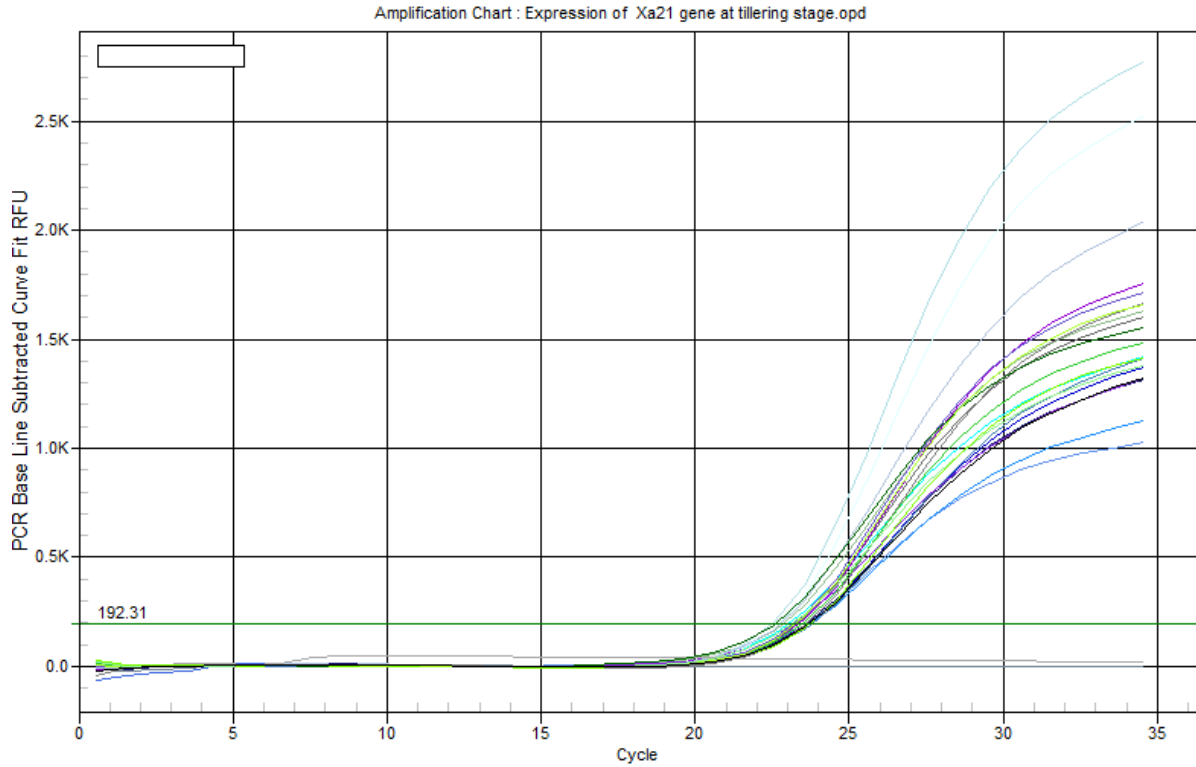
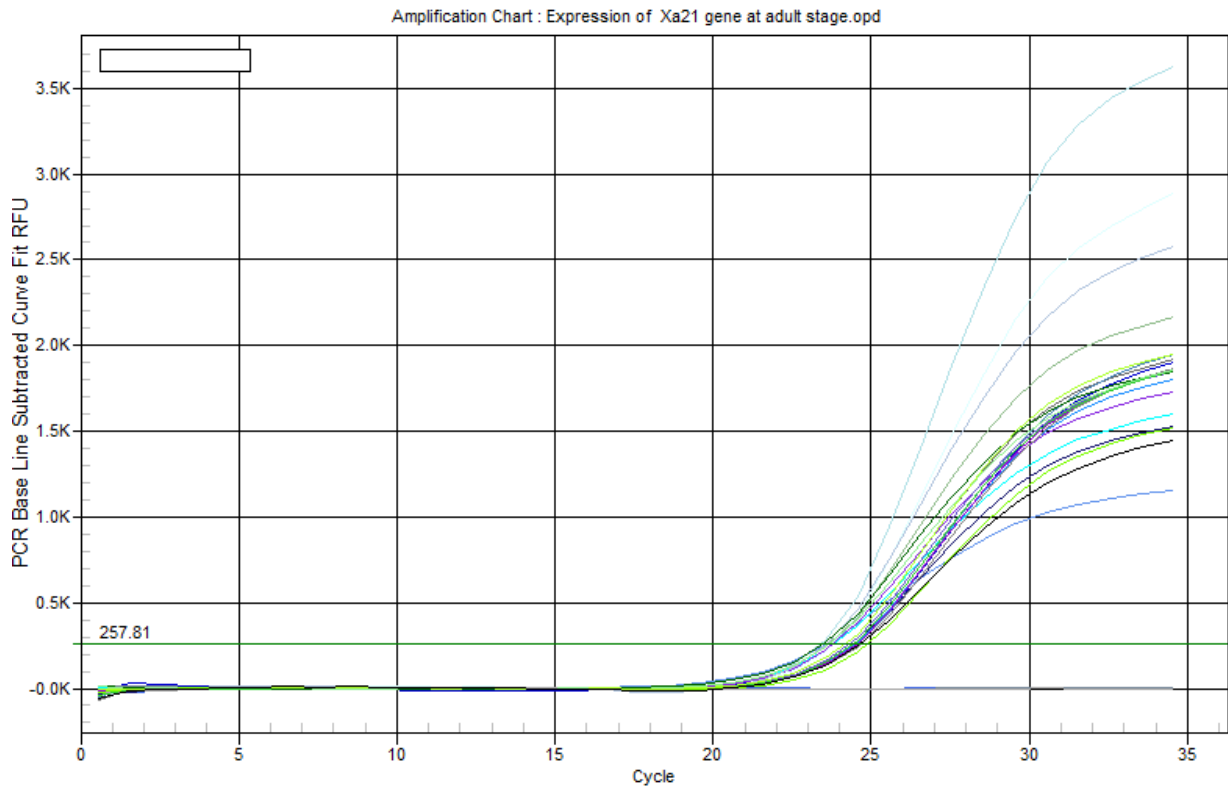


Fig.5 Expression of *Xa21* gene at adult plant stage in PB-1 and IPB-1 at different time interval



Development of broad-spectrum resistance to bacterial blight in the Indian subcontinent is major challenge due to the diverse agro-climatic zones as well as the number of genetically distinct virulent *Xoo* strains from different geographical areas of the region. The *Xa21* allele found to confer resistance to all known *Xoo* races in India and Philippines. The locus (*Xa21*) has been cloned which is the member of a small multigene family of kinases. Most of these family members are linked, suggesting that *Xa21* is part of a complex locus. The use of molecular marker pTA248 linked to *Xa21* confirmed the BB resistance in improved line of Pusa Basmati (IPB-1). The differential expression of *Xa21* allele in PB-1 and IPB-1 implicated its association with the BB resistance. The 2- $\Delta\Delta$ CT values indicated that *Xa21* allele was expressed at tillering as well as adult plant stage. However, the expression of *Xa21* allele was much higher at adult plant stage than at the tillering stage. The higher fold change in transcripts number at adult plant stage also indicates that the disease resistance mediated by the resistance gene *Xa21* is developmentally controlled in rice (Mazzola *et al.*, 1994, Century *et al.*, 1999). Recently, it was discovered that the developmental control point may be a proteolytic cleavage of the *Xa21* protein (Xu *et al.*, 2006). The structure of *Xa21* represents a previously uncharacterized class of cloned resistance genes. The deduced amino acid sequence of *Xa21* encodes a receptor kinase-like protein carrying leucine-rich repeats (LRR) in the putative extracellular domain, a single pass transmembrane domain, and a serine-threonine kinase intracellular domain. It is assumed that the extra-cellular LRR of *Xa21* constitute the most probable domain that could participate in the protein-protein interactions for pathogen recognition (Song *et al.*, 1995, Boyes *et al.*, 1996). It has been observed from the previous studies that the *Xa21* allele expressed durable resistance

against bacterial blight. It was not easily broken down by several pathotypes (Suwarno *et al.*, 2004). The introgression of *Xa21* allele in IPB-1 line contributed to resistance against bacterial blight for various races at different growth stages under field condition. The pyramiding of the *Xa21* gene with the other BB resistant genes is a useful approach to achieve durable resistance (Zhang *et al.*, 2006). The expression of *Xa21* is induced by interaction *Xa21* product with avirulent (*Avr*). The interaction *Xa21-Avr* activates the signal transductions involved in *Xa21*-mediated BB resistance (Yoshimura *et al.*, 1998). Moreover, the expression of the *Xa21* gene may use to predict potential of disease resistance under stress conditions.

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