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Comparative Phylogenetic Analysis Of Orthologues Of Bacterial Leaf Blight Resistance Gene *Xa27* In Rice

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Abstract

Bacterial blight (BB), caused by *Xanthomonas oryzae pv. oryzae* (*Xoo*) has been one of the most serious diseases in rice. The present investigation was done to find out novel alleles of *Xa27* gene from rice. Twenty nine orthologs of bacterial blight resistance genes *Xa27* were used for comparative analysis. The phylogenetic analysis of cultivated and wild species of rice indicated clear phylogenetic relationship and *Oryza alta* was the farthest relative among all. Twenty nine orthologs have been divided into six groups. All twenty nine alleles studied in present investigation, have maximum ten motifs such as Protein kinase C phosphorylation site and N-myristoylation site indicating its role in signal transduction process. Motif distribution shows high degree of conservation among *Xa27* alleles. This study will help to understand molecular mechanism of Xa27 gene and its evolution in rice and related species. Besides, various resistant alleles cloned from wild species can be exploited in the effective management of bacterial blight disease after transferring these alleles either alone or in combination in the susceptible genotypes.

Keywords: Phylogenetic analysis, bacterial blight, *Xa27,* Rice

Introduction

Rice (*Oryza sativa L.)* is one of the most important crops being cultivated and consumed worldwide. Rice production is constrained by various biotic and abiotic stresses. Among biotic stresses, diseases of fungal, bacterial and viral origin cause a heavy annual yield loss.

Bacterial blight (BB), caused by *Xanthomonas oryzae pv. oryzae* (*Xoo*) has been one of the most serious diseases in rice. Bacterial blight

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was first noticed by the farmers of Japan in 1884 [Tagami et al. 1962]. The pathogen enters rice leaves through water pores or wounds and moves by invading the xylem, causing the disease. The severity of damage caused by infection of *Xoo* has necessitated the development of strategies to control and manage this disease, so as to reduce crop loss and to avert an epidemic. Use of pesticide is costly as well as environmentally undesirable [Song and Goodman 2001]. Thus, there is need to develop strategies providing durable resistance, giving protection for a long time and over a broad geographic area. Among such new strategies, the exploitation of host resistance appears to be the only reliable method of disease management.

Numerous major genes with resistance to various strains of *Xoo* have been identified, which have been named in a series from *Xa1* to *Xa34*. Till date 34 bacterial blight *R*-genes have been found on different chromosomal loci [Singh et al. 2011]. Thus far, six *R*-genes (*Xa1, Xa5, Xa13, Xa21, Xa26* and *Xa27*) have been isolated using map-based cloning strategies [Song et al. 1995; Yoshimura et al. 1998; Gu et al. 2005; Chu et al. 2006; Jiang et al. 2006]. The dominant R genes included *Xa1 to Xa28*. Six of the recessive R-genes*, xa5, xa8, xa13, xa24, xa26* and *xa28*, occur naturally and confer race-specific resistance; the other 5, *xa15, xa19, xa20, xa32* and *xa34* are created by mutagenesis and each confers a wide spectrum of resistance to *Xoo* [Ogawa 1996; Lee et al. 2003; Chen et al. 2011]. *Xa27* is one of the important BB resistant genes in rice and has been cloned [Gu et al. 2005]. Its cloning from wild species may give rise to novel alleles, which can be used for plant breeding.

After decoding of rice genome in 2005 by International Rice Genome Sequencing Consortium [IRGSP. 2005], huge sequence information is available in public databases. These sequences can be used for mining novel alleles for disease resistance. There are reports about the allele mining strategy in several cereal species to isolate alleles of target genes. Latha et al. [2004] used the rice calmodulin genes and a salt inducible rice gene for allele mining of stress tolerance genes on identified accessions of rice and related germplasm. Sharma et al (2009) analyzed 19 rice lines for mining alleles of three blast resistance genes([*Pi-ta, Pi5(Pi-k^h) and piz-(t)*. Allele mining for blast resistance genes*, Pi54(Pi-k^h) [*Kumari et al. 2013; Thakur et al. 2015*], Pi-ta* [Singh et al. 2014], *piz-(t)* [Thakur

et al. 2012] has been done for rice accessions. Thus in rice, genetic diversity analysis has been done mainly for blast resistance genes. Till date, single report on allele mining of bacterial blight resistance gene has been done [Bimolata et al. 2013]. Thus, allele mining supports the discovery of new alleles of target genes. However, the limitation of this approach in rice lies in the fact that very few genes of agronomical importance have yet been cloned. This is particularly true for genes involved in disease resistance.

A bacterial blight resistant gene *Xa27* isolated from rice variety IRBB27 has been mapped and cloned [Gu et al. 2005]. It is intronless gene and encodes a protein of 113 amino acids. Its recessive allele *xa27* from IR24 also codes for the same protein without any change in the protein sequence. *Xa27* , unlike other R-genes do not code for any known conserved domains like NBS-LRR or LRR receptor kinase and it has been predicted to encode a protein with a trans-membrane domain [Gu et al. 2005]. The sequence information of this gene can be used for allele mining. In view of above present investigation has been planned for PCR amplification, sequence analysis, molecular evolution of *Xa27* allele from wild species and cultivated rice lines.

Materials and methods

Plant Material and phenotypic analysis

Wild species of rice, *Oryza rhizomatis,* nucleotide sequence of *Xa27* gene isolated from rice line IRBB27, IR24, *Orya nivara, Oyza officinalis* and ecotypes of them were obtained from the public database and used in this analysis (Accessions no.s AY986491, AY986492, JN601064, JF304302, JF304301, HQ888857, HQ888856, HQ888855, HQ888854, HQ888853, HQ888852, JN016521, JN016520, JN016519, JN016518, JN016517, JN016516, JN016515, JN016514, JN016513, JN016512, JN016511, JN016510, JN016509, JN016508, JN016507, JN016506, HE858441). A virulent strain of *Xanthomonas oryzae pv oryzae* has been used for phenotypic analysis. The wild species of rice was inoculated with this bacterial suspension and disease reactions were observed after 15 days. Five emerging young leaves in each plant were inoculated with the *Xoo* through clip inoculation method [Kauffman et al. 1973]. The plant reaction to the pathogen was scored 15 days after inoculation following the scale proposed by Ogawa [1996].

Designing gene specific primer

The *Xa27* allele (NCBI Acc. No AY986492) was downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/), Coding DNA Sequence (CDS) of *Xa27* IRBB27 allele was retrieved. A primer pair was designed by using the Primer 3 software (http://frodo.wi.mit.edu) in such a way that complete CDS of *Xa27* allele can be amplified.

DNA isolation, gene amplification, sequencing and anlysis

DNA was extracted from rice leaves by modified CTAB method [Murray and Thompson 1980]. The PCR reactions were performed in 60 μl reaction volume containing 150 ng of template DNA, 0.2 μM both forward and reverse primers, 1X buffer, 1X enhancer, 0.2mM dNTP mix and 1 unit *Taq* polymerase enzyme mix (MBI, Fermentas). The cycling conditions involved initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55-60°C for 1 min, and primer extension at 72°C for 1 min. A final extension at 72°C for 7 min was done and products stored at 4°C until electrophoresis. The PCR products were resolved on 2% agarose gel. The amplified PCR products were purified by using the instructions for QIAEX II gel extraction kit (Qiagen, USA). The PCR amplicon was sequenced directly by using Sanger's dideoxy method on an automated capillary based DNA sequencer, Mega BACE 4000 (Amersham Biosciences, NJ, USA) in both forward and reverse direction twice using M 13 specific primers. All the sequence reads generated were assembled by using Phred/Phrap/Consed Software Package (http://www.phrap.org/phredphrapconsed.html) and saved in fasta format for further analysis. The gene prediction for the allele was performed by using FGENESH gene prediction tool (www.softberry.com) trained for monocots. Functional motifs in amino acid sequence of *Xa27* alleles were predicted using ExPASy tools (http://www.expasy.ch/tools/). Phylogenetic analysis among *Xa27* allele of rice species along with downloaded orthologs was done using MEGA 4.1 software [Tamura et al. 2007]. For multiple sequence alignment, various parameters like gap open penalty-15, gap extension penalty- 6.66 and weight matrix- IUB (for DNA) were used. The tree was viewed and edited using an online tool named iTOL (Interactive Tree Of Life) [Letunic et al. 2011]. We carried out the motif finding analysis to correlate motif with the phylogenetic analysis

using MEME 4.6.1 software [Bailey et al. 1994]. The width of a block shows the width of the motif relative to the length of the sequence.

Results

Phenotypic analysis of *Oryza* species with *Xanthomonas oryzae*

The rice species *Oryza rhizomatis* was classified resistant to the virulent strain of *Xanthomonas oryzae*.

Amplification and sequencing of *Xa27* alleles

The primer pair amplified 457 bp PCR product from the genomic DNA of *Oryza* species. The sequences were assembled to get consensus and the sequence was submitted to EMBL [Acession no. HE858441].

DNA sequence and structural organization of *Xa27* allele

The CDS region of *Xa27* allele isolated from rice variety IRBB27 was aligned with the nucleotide sequence of same allele isolated from 28 different rice lines and wild species by using clustalX Software [Thompson et al. 1997]. Structural analysis of *Xa27* alleles was performed to find number of exons and introns in the ORF region using FGENESH software (www.softberry.com). *Oryza rhizomatis* studied in present investigation, have only one exon in its ORF region from 67 to 408 bp.

Evolutionary relationship among *Xa27* alleles

Phylogenetic analysis of *Xa27* alleles from different rice lines and species along with downloaded sequence of *Xa27*-IRBB27 allele, *Xa27*-IR24 allele was performed to study their evolutionary relationship. The tree was dived into six distinct groups. In the gene tree, 8 *Oryza* species and 6 rice lines along with CDS sequence of *Xa27* allele, complete gene sequence of *Xa27*-IRBB27 allele and complete gene sequence of *Xa27*-IR24 allele were clustered in the group along with wild ecotypes from *Oyza nivara, Oryza rhizomatis* and *Oryza officinalis*, whereas only *Oryza alta* clustered in independent group I (Figure. 1). The *Xa27* allele of IRBB27 showed more genetic similarity with the alleles isolated from all wild species and *Oryza rhizomatis*. Thus phylogenetic tree reveals that resistant and susceptible alleles belong to two different groups.

Figure1: Phylogenetic relationship among Xa27 alleles isolated from lines and wild species of rice.

The motif analysis revealed that all selected twenty nine species have maximum ten motifs [Figure 2]. The distribution of motifs is seen in Figure 3. Motif search revealed 6 to 50 residues in all the alleles. A correlation between the motif pattern and the phylogenetic tree was found, since each cluster shared the same motif pattern. This study focuses on the fact that if genes are phylogenetically in same clade then they share common motifs. Motif finding and phylogenetic analysis of the *Xa27* alleles in our investigation clearly depicted deep evolutionary origin of R-genes. The motif distribution indicated that the genes containing the same motifs might arise from gene expansion within the same class.

Figure 2: Pattern of discovered Motifs in Xa27 Alleles.

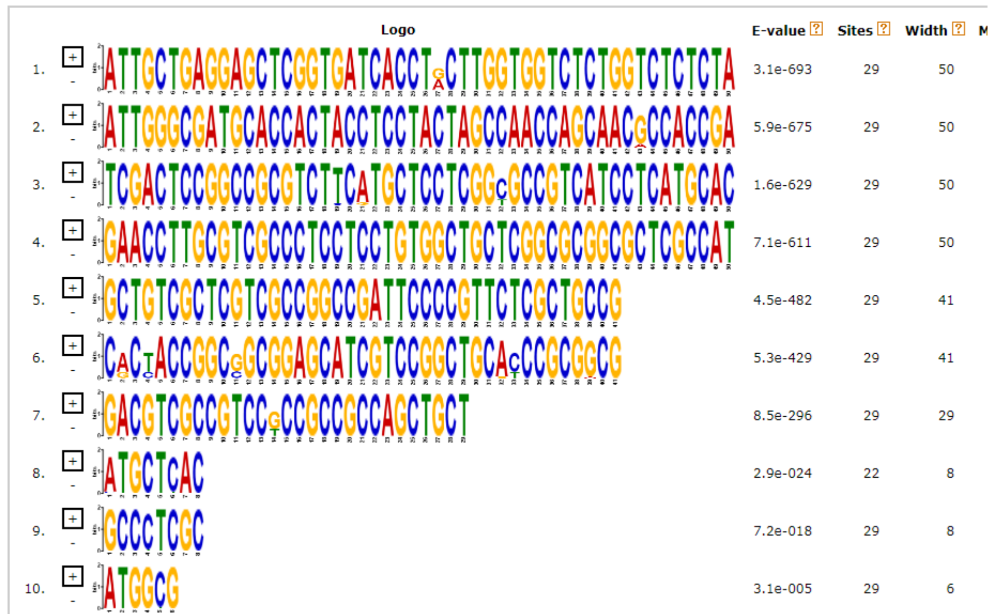
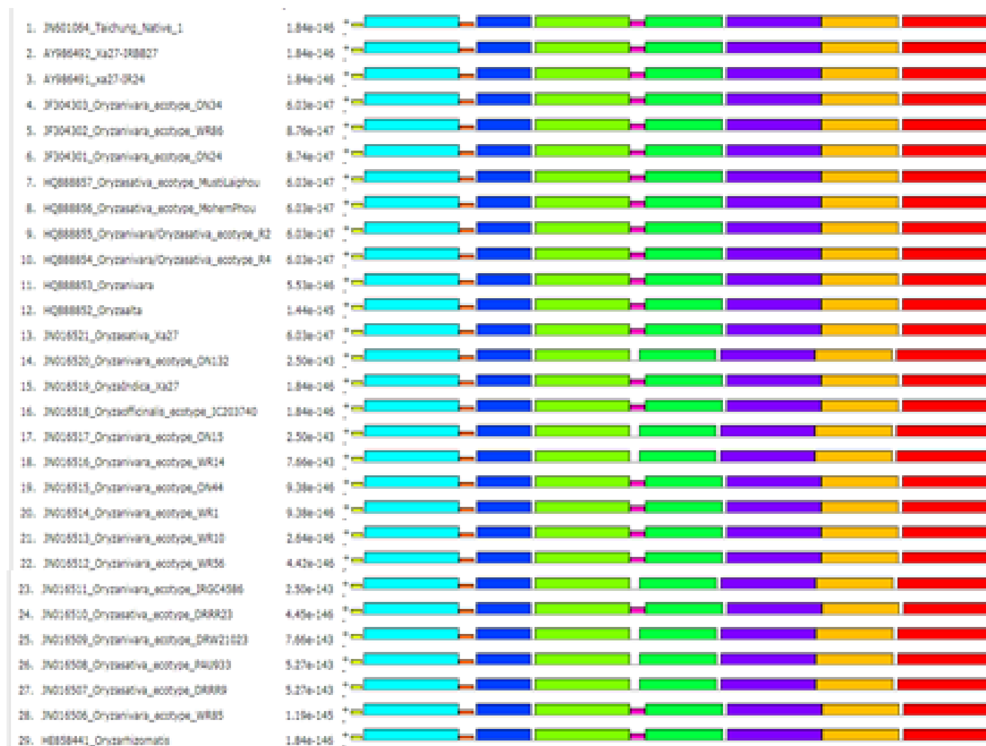


Figure 3: Distribution of Motifs in allels of Xa27 gene.



Discussion

Wild species and land races of rice could be useful for fishing out agriculturally important genes resistant to various diseases. One of the recent approaches used for cloning new and better forms of disease resistance gene is allele mining. However, the basic pre-requisite of using this strategy is that the target gene should be cloned, characterized and functionally validated. A bacterial blight resistant gene *Xa27,* isolated from rice line IRBB27 confers broad resistance and is representative of an unusual class of dominant R genes in rice [*Gu* et al. 2005]. Thus allele mining for *Xa27* gene from wild species of rice can be a potential tool for finding alternate and better forms of *Xa27* gene which can be used for bacterial blight management. Using PCR based approach, 19 lines of *O. sativa* were analyzed for mining alleles of three blast resistant genes (*Pi-ta, Pi54*(*Pi-K^h*) and *Pi-z(t)* [Sharma et al. 2009]. *Pi54*(*Pi-K^h*) and *Pi-z(t)* alleles were more variable than *Pi-ta* allele in Indian land races of rice. However there are very few attempts on the mining of rice bacterial blight resistance alleles from different wild species. Therefore, efforts were made, in present investigation to use the sequence information of bacterial blight resistant gene *Xa27* for the hunting of *Xa27* alleles in wild and cultivated species of rice.

Two motifs identified were Protein kinase C phosphorylation site and N-myristoylation site. Presence of phosphorylation site in the predicted protein indicates its involvement in signal transduction by activating/inactivating itself or by phosphorylation/dephosphorylation. The presence of N-myristoylation site in the predicted protein showed its role in membrane anchoring.

The phylogenetic relationship of *Xa27* alleles would be helpful in understanding its origin and evolution. In the gene tree constructed, 8 *Oryza* species including CDS sequence of *Xa27* allele, complete gene sequence of *Xa27*-IRBB27 allele and complete gene sequence of *Xa27*-IR24 allele were clustered in one group indicating that these were descended from the same ancestral sequence with little variation at nucleotide levels. All other groups are mixed type with ecotypes and species, showing close relationship of *O. nivara* and *O. sativa* species of *Xa27* gene. Group I is independent with only *O. alta* showing its most evolved evolution. The *Xa27* allele of IRBB27 showed more genetic similarity with the alleles isolated from wild species of *O.rhizomatis*.

In present investigation, the resistant nature of *Oryza rhizomatis* was confirmed phenotypically and genotypically indicating its role in counteracting pathogen attack under natural conditions. Phylogenetic analysis also confirmed the close relationship of *Xa27* gene with resistant *O. rhizomatis*. Also confirmed the darwinian pattern of selection which leads to divergent evolution of *Xa27* gene in nature. Finding of motifs confirmed the conserved nature of *Xa27* genes. The resistant alleles isolated in present investigation can be confirmed by complementation tests. These alleles can further be used for the gene pyramiding against rice pathogen *Xanthomonas oryzae*. This study is useful for marker-assisted-selection after validation in the mapping population.

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References

1. **Tagami Y. and Mizukami T. [1962]** Historical review of the researches on bacterial leaf blight of rice caused by *Xanthomonas.* Spec. Rep. Plant Dis. Insect Pest Forecasting Serv. Minist. Agric. Jpn. 10: 1-112.
2. **Song F and Goodman RM [2001]** Molecular biology of disease resistance in rice. Physiol Molec Plant Pathol 59: 1-11.
3. **Singh AK, Gopalkrishnan S, Singh VP, Prabhu KV, Mohapatra T et al [2011]** Marker assisted selection: a paradigm shift in Basmati breeding. Indian J Genet 71[2]: 1-9.
4. **Song WY, Wang GL, Chen LL, Kim HS, Pi LY et al [1995]** A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270: 1804-1806.
5. **Yoshimura S, Yamanouchi U, Katayose Y, Toki S, Wang ZX et al [1998]** Expression of *Xa1*, a bacterial blight resistance gene in rice, is induced by bacterial inoculation. Proceedings of the National Academy of Sciences, USA 95: 1663-1668.

6. **Gu K, Yang B, Tian D, Wu L, Wang D et al [2005]** R gene expression induced by a type-III effector triggers disease resistance in rice. Nature 435: 1122-1125.

7. **Chu Z, Yuan M, Yao J, Ge X, Yuan B et al [2006]** Promoter mutations of an essential gene for pollen development result in disease resistance in rice. Genes Dev 20: 1250-1255.

8. **Jiang GH, Xia ZH, Zhou YL, Wan J, Li DY et al [2006]** Testifying the rice bacterial blight resistance gene *xa5* by genetic complementation and further analyzing *xa5 [Xa5]* in comparison with its homolog TFIIAgamma 1. Mol Fenet Genomics 275: 354-366.

9. **Ogawa T [1996]** Monitoring race distribution and identification of genes for resistance to bacterial leaf blight. Rice Genetics III, Proceedings of the Third International Rice Genetics Symposium, IRRI, P.O. Box 933, Manilla, Philippines 456-459.

10. **Lee KS, Rasabandith S, Angels ER, Khush GS [2003]** Inheritance of resistance to bacterial blight in 21 cultivars of rice. Phytopathology 93[20]: 147-152.

11. **Chen S, Liu X, Zeng L, quyang D, Yang J et al [2011]** Genetic analysis and molecular mapping of a novel recessive gene *xa34[t]* for resistance against *Xanthomonas oryzae pv. oryzae.* Theor. Appl. Genet. 122: 1331-1338.

12. **IRGSP, International Rice Genome Sequencing project [2005]** The map based sequence of the rice genome. Nature 436: 793-800.

13. **Latha R, Rubia L, Bennett J and Swaminthan MS [2004]** Allele mining for stress tolerance genes in *Oryza* species and related germplasm. Molecular Biotechnology 27: 101-108.

14. **Sharma TR, Gupta YK, Thakur S, Singh PK, Upreti HC et al [2009]** Allele mining for important blast resistance genes from landraces of rice. In: 6th Int Rice Genet Symp, Manila Hotel, Manila, Philippines 3-59.

15. **Kumari A, Das A, Devanna BN, Thakur S, Singh PK, Singh NK, Sharma TR [2013]** Mining of rice blast resistance gene Pi54 shows effect of single nucleotide polymorphisms on phenotypic expression of the alleles. Eur J Plant Pathol 137: 55-65.

16. **Thakur S, Singh PK, Das A, Rathour M, Variar M, Prashanthi SK, Singk AK, Singh UD, Chand D, Singh NK, Sharma TR [2015]** Extensive sequence variation in rice blast resistance gene Pi54 makes it broad spectrum in nature. Front. Plant Sci. 6:345. doi:10.3389/fpls.2015.00345

17. **Singh PK, Thakur S, Rathour R, Variar M, Prashanthi SK, Singh AK, Singh UD, Sharma V, Singh NK, Sharma TR [2014]** Transposon-based high sequence diversity in Avr-Pita alleles increases the potential for pathogenicity of Magnaporthe oryzae populations. Funct Integr Genomics 14[2]: 419-29. doi: 10.1007/s10142-014-0369-0.

18. **Thakur S, Singh PK, Rathour M, Variar M, Prashanthi SK, Singk AK, Singh UD, Chand D, Singh NK, Sharma TR [2012]** Positive selection pressure on rice blast resistance allele Piz-t makes it divergent in Indian land races. DOI: 10.1080/17429145.2012.721523.

19. **Bimolata W, Kumar A, Sundaram RM, Laha GS, Qureshi IA, Reddy GA, Ghazi IA [2013]** Analysis of nucleotide cultivars of rice [*Oryza sativa*] and its wild relatives. Planta 238: 293-305.

20. **Kauffman HE, Reddy APK, Hsieh SPY, Merca SD [1973**] An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis Rep 57: 537-541.

21. **Murray MG and Thompson WF [1980]** Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res 8: 4321-4325.

22. **Tamura K, Dudley J, Nei M, Kumar S [2007]** MEGA4: molecular evolutionary genetics analysis [MEGA] software version 4.0. Mol Biol Evol 24: 1596–1599.

23. **Letunic I, Bork P. Interactive Tree Of Life v2:** online annotation and display of phylogenetic trees made easy. Nucleic Acids Research 2011;1–4.

24. **Bailey TL, Elkan C.** Proceedings International Conference on Intelligent Systems for Molecular Biology ISMB 1994; 2:28-36.

25. **Thompson JD, Gibson TJ**, Plewniak F, Jeanmougin F, Higgins DG. Nucleic acids research 1997; 25(24):4876-4882.