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Development of InDel marker for rice blast resistance gene *Pi9*

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ABSTRACT

Pi9 is one of the major blast resistance genes which encodes a nucleotide-binding site-leucine-rich repeat (NBS-LRR) domain-containing protein. This gene was observed to show resistance against many pathotypes of the blast pathogen in Malaysia. Resistance allele *Pi9* from rice variety 75-1-127 had previously been cloned using map-based cloning strategy. The gene sequence was used to design specific primers to amplify susceptible *Pi9* allele from MR219 rice variety prior to cloning. The resistance and susceptible allele of *Pi9* were 8.587kb and 8.785kb in length respectively. Allele mining was carried out by comparing between the susceptible and resistance allele of *Pi9*. One potential InDels polymorphism at position 590bp and 920bp was identified. Primer named as *Pi9*_InDel was designed targeting this region in such a way that the resistance and susceptible genotypes yielded 327 bp and 438 bp amplicon respectively.

Key words: Blast, *Magnaporthe oryzae*, *Pi9*, Rice, Specific marker.

INTRODUCTION

Rice blast disease, caused by *Pyricularia grisea* is one of the serious constrains to rice production worldwide (Helliwell and Yang, 2013). The disease reduces annual rice productivity by 10-30% and have obvious negative consequences on economy (Miah *et al*., 2012; Imam *et al.*, 2013; Sharma *et al.*, 2012). Most rice farmers are usually with limited resources and therefore rice cultivars with disease resistance particularly to Blast disease could increase their income through better rice disease management. GM rice carrying genes that impact resistance to blast could overcome the problem but commercialization of GM crops still an issue as people are yet to accept them globally (Dutta *et al*., 2016). Indeed, breeding for disease resistance is a cost-effective, labor-saving and environmentally sound crop protection strategy.

Cultivar conferred resistance to disease by major genes has been extensively used for breeding in many crop species (Singh and Singh, 2003; Heath, 1981). A commonly used strategy for rice blast disease management was to develop durable blast resistance varieties by breeding for *R*-genes (Dangl and Jones, 2001). By developing varieties with broad-spectrum and durable resistance to blast disease, this could help to increase rice production and improve sustainability (Pradhan *et al.*, 2015; Jeung *et al.*, 2007). The advantage of broad-spectrum resistance can be seen as a variable of resistance level to the majority of geographically different isolates of the same pathogen or the resistance to two or more unrelated pathogens. More recently, a broad spectrum *R* gene known as *Pi9* has been reported to exhibit durable resistance against a diverse of blast pathotypes in Malaysia (Siti Norsuha *et al.*, 2012). This *Pi9* gene is originated from a wild rice species called *Oryza minuta* (Amante-Bordeos *et al.*, 1992; Sitch *et al.*, 1989) and was later introgressed into near-isogenic lines (NIL), IRBL22 cv IRTP21683 (Telebanco-Yanoria *et. al.,* 2008b). The availability of a set of near-isogenic lines (NILs) provides an opportunity to characterize resistance genes in a common genetic background (Mackill and Bonman, 1992). This has led us to the identification of a near isogenic line IRTP21683 containing *Pi9* gene, which can be used to generate durable rice varieties resistant to blast disease (Dr. Habibuddin *personal communication*). Utilization of DNA markers in plant breeding is called marker-assisted selection (MAS) technique utilizes DNA markers to indirectly select the phenotype in which its efficiency is highly dependent on the strength of association between the selected DNA markers and genes responsible for certain phenotypes (Biswas and Bhattacharya, 2013; Koide *et al.*, 2009). Although many markers had been identified markers and available for rice blast resistance genes, most of them are known to be linkage markers (markers that are linked to the resistance genes with variable genetic distance), whilst only a few of them are specific markers also known as functional markers (Jayawardana *et al.*, 2014; Singh *et al.*, 2015). The major limitation of applying linkage markers to MAS is this type of markers is weakly associated with phenotypes as compared to functional markers. Inheriting accuracy of these markers could be low in progenies due to a possible genetic recombination between the marker and the target gene (Koide

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Table 1: List of primers used for *Pi9* gene isolation/Allele mining and for *Pi9*_InDel.

Primer	SequenceForward/Reverse	Purpose	Expected PCR product (bp)
Pi9	5'-ATG GCG GAG ACG GTG CTG AGC ATG-3'/5'-TCA GCC AGC TT G AGC TGT GCC TAT-3'	Pi9 gene isolation/Gene mining	8.587kb for R/ 8.785kbFor S
Pi9-InDel	5'-ATC CAC GAA ACA TCC ACC AT-3' /5'-ACA GCC GGA TTC GAC AGA-3'	Allele-specific marker	327bp for R/ 438bp for S

R resistance, *S* susceptible.

et al., 2009). Thus, the functional markers derived from polymorphic loci within the genes contributing to phenotypic variation, could overcome the constrain by the linkage-markers. Indeed, functional markers have strong association with phenotype and able to distinguish polymorphisms underlying the phenotypic effect of a gene (Tian *et al.*, 2016; Hua *et al.*, 2015; Mc Couch *et al.*, 2007), thereby these markers facilitate an efficient selection of favorable alleles in breeding population. The functional marker can also be identified within the important genes contributes to certain agronomic traits. This requires the mining of the targeted alleles between distinct parental populations in order to develop high accuracy functional markers. In this study, the major *Pi9* gene which has been introgressed in IRTP21683 was chosen for study and to be used in developing an InDel functional marker.

MATERIALS AND METHODS

Plant material: Rice isogenic line IRTP21683 and MARDI rice variety MR276 were used for identification of resistance and susceptible alleles of *Pi9* respectively. Both plant materials were obtained from Rice Genebank located at MARDI Seberang Prai, Malaysia. F2 population was derived from a cross between IRTP21683 and MR276 rice varieties. MARDI's varieties consist of MR219, MR211, MR272 and MRQ76 were used in markers validation.

Pi9 gene isolation and InDel identification: Genomic DNA was extracted from IRTP21683 and MR276 using plant DNA extraction kit (QIAGEN, USA). The gene sequence of *Pi9* from an *indica* rice variety 75-1-127 (GeneBank; DQ285630) (Qu *et al.*,2006) was used to design primers for amplification of *Pi9* allele. Fifty microliter of PCR reaction mixture containing 1X PCR buffer, 2.5mM $MgCl_2$, 0.3µM each of forward and reverse primer, 0.4µM dNTPs mix and 1 unit Taq polymerase was added to 100ng DNA prior to DNA amplification with the following PCR conditions: 94°C/ 2min for 1 cycle and proceed to 30 cycles of 98°C/10sec, 60°C/30sec, 68°C/9.5min and a final extension at 68°C for 10 mins. The PCR products were cloned into pJET1.2 (Life Technologies, USA) and sequence was confirmed using automated sequencer based on Sanger methods (Sanger and Coulson, 1975).The sequence alignment of *Pi9* alleles was performed using the online Clustal W sequence alignment tool (www.ebi.ac.uk/Tools/clustralw/) to identify potential InDel. Primer was designed targeting an insertion/deletion (InDel) polymorphism using the online tool, Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR cycling protocol was optimized for the generated primers in thermal cycler (Bio Rad, USA) before the primers were further use (Table 1).

Validation of functional markers: Young leaves were harvested from randomly selected 190 individual plants of F2 population and selected MARDI's varieties namely MR276, MR219, MR211, MR272 and MRQ76. Genomic DNA of each sample was extracted using plant DNA extraction kit (QIAGEN, USA). InDels have been amplified using optimized PCR protocol. Ten microliter of total PCR reaction mixture containing 1X PCR buffer, 1mM $MgCl_2$, 0.2mM dNTP mix, 0.2µM of each forward and reverse primer and 0.5 Unit Taq Polymerase was prepared and added to DNA for PCR amplification with the following conditions: 94°C/3min for 1 cycle, 30 cycles of 94°C/45sec, 52°C/30sec, 72°C/1min and a final extension at 72°C for 10 mins. PCR products were separated by electrophoresis on a 2% agarose gel using 0.5XTBE buffer stained with ethidium bromide.

RESULTS AND DISCUSSION

Development of functional marker targeting *Pi9* gene: The allele mining strategy has been widely used to develop functional or closely-linked molecular markers. Costanzo and Jia (2010) have developed an InDel-based marker for blast resistance gene *Pi km* using similar approach; Hayashi *et al.* (2006) developed PCR-based InDel markers for 9 blast resistance genes based on reported sequence information on the candidate genes. In addition, InDel markers were previously applied in PCR based amplicon length polymorphism (ALP) by Amarawathi (2008). However, this approach used was not suitable for routine genotyping work as poly-acrylamide gel electrophoresis (PAGE) is required for DNA resolution. Interestingly, primers designed targeting the flanking sequence of InDel has given an advantage as it can detect the polymorphism in a co-dominant fashion (Ramkumar *et al.*, 2011; Sakthivel *et al.*, 2009). We also applied the allele mining strategy in this study to search for the variations within *Pi9* alleles. Phenotypic study previously has shown two rice varieties IRTP21683 and MR276 are highly resistant and susceptible respectively. These varieties were selected to analyze *Pi9* gene allelic variation. The *Pi9* alleles from each variety were amplified, cloned and sequenced. The size of resistance and susceptible allele of

Pi9 were 8.587kb and 8.785kb respectively. Sequence comparison at nucleotide level of both *Pi9* alleles revealed the presence of 15 single nucleotide polymorphisms (SNPs) and two significant InDels observed between the resistant and susceptible genotype. The larger InDel at position 590 bp to 920 bp was selected to be the marker for further analysis in this study (Fig 1) and named as *Pi9*_InDel. The amplicon sizes for resistant and susceptible genotypes were estimated as 327 bp and 438 bp amplicons respectively which could be resolved in a low percentage agarose gels within a short time span (Fig 2).

Reliability test of newly developed marker: PCR genotyping of a total 190 F_2 mapping population individuals derived from crossing between IRTP21683 and MR276 by using *Pi9*_InDel marker allowed clear distinction between the homozygotes and heterozygous F_2 plants. The segregating of newly developed InDel marker followed the 1:2:1 ratio of F2 population with the observed Chi square value of 2.6

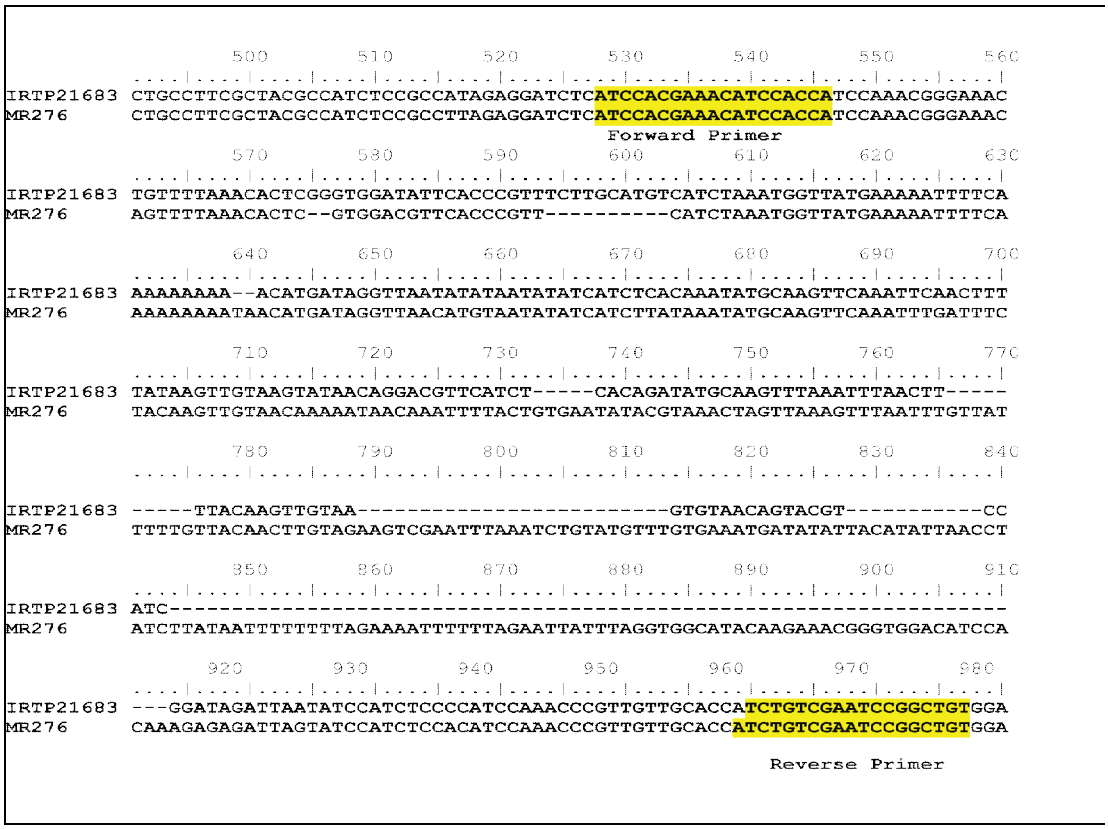

Fig 1: InDel detected based on sequence alignment between the resistance and susceptible alleles of *Pi9* obtained from IRTP21683 and MR276 rice genotype respectively. Short sequences marked with yellow indicate the positions where the forward and reverse primer of *Pi9*_InDel marker were designed.

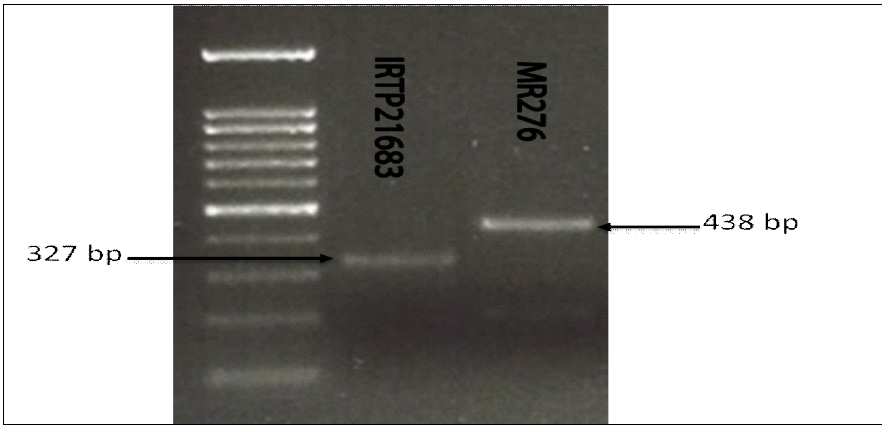

Fig 2: Functional validation of *Pi9*_InDel marker. PCR products resulted from these primers are able to differentiate between *Pi9* alleles from resistance (IRTP21683) and susceptible (MR276) rice genotypes indicating their use as functional markers.

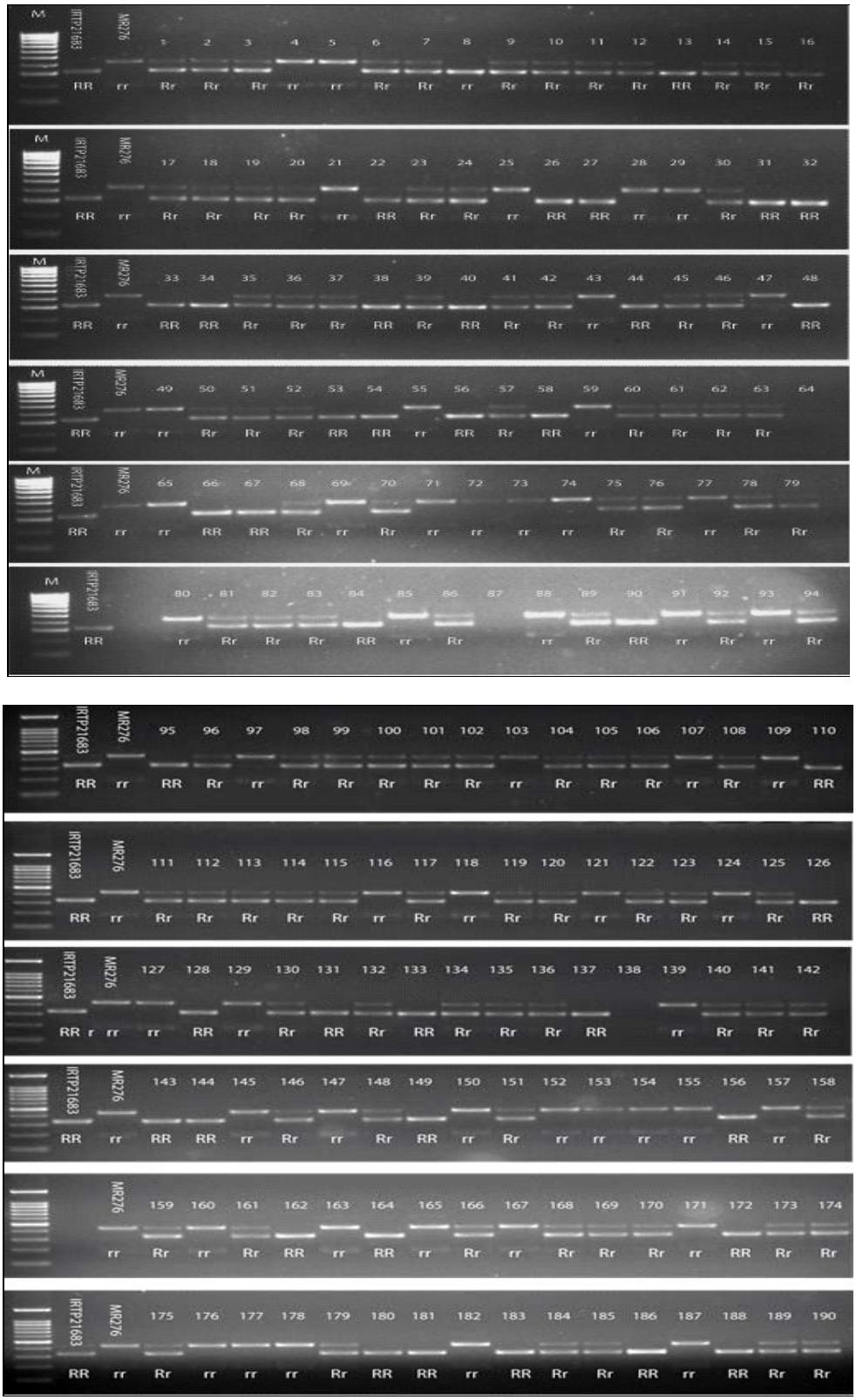

Fig 3: Genotyping of F2 population using *Pi9*_InDel marker. A total of 190 F2 progeny derived from a cross between IRTP21683 and MR276 were genotyped and parental lines were used as positive and negative control, respectively. The amplicon size for resistance and susceptible alleles were at 327 bp and 438 bp respectively. 1-190 individual F2 mapping progeny; RR- homozygous resistant; rr- homozygous susceptible; and Rr- heterozygous resistant; M-100bp ladder.

which lower than tabulated Chi square which 3.841 at 0.05 confidences level with 3 degree of freedom. (Fig 3 and Table 2). This mean the marker is suitable to be applied for tagging the *Pi9* gene in marker assisted breeding program. Due to its co-dominant nature, suggesting that this marker is potentially to be used in testing a large set of segregating progenies to facilitate the blast disease resistance breeding improvement program through marker assisted selection (MAS). To further validate the performance of this marker, we further analyzed 4 notified MARDI's release varieties namely MR219, MR211, MR272 and MRQ76 which IRTP21683 and MR276 were included as a control for resistance and susceptible allele respectively. Phenotypic screening of foliar blast and panicle blast for these varieties were carried out to identify the resistance status. Foliar blast screening was done under induced natural infection condition in a blast nursery meanwhile panicle blast screening was done in a field condition where the test lines were inoculated with dominant pathotype of *P. oryzae*. The resistance status showed that MR211 was moderately susceptible and highly susceptible for foliar blast and panicle blast screening respectively, meanwhile MR219 was moderately susceptible for both screening (Table 3). During field trial, under favourable environment, blast incidence was occurred for variety MR272, MR276 and MRQ76. Pathotypes isolated from diseased sample of these varieties indicated other pathotypes than the dominant pathotype used in the blast screening. Therefore, MR272 and MR276 were not released for farmers. The result showed that Pi9_InDel unambiguously classified the resistance and susceptible varieties, suggesting that a good potential for this marker to be applied in *Pi9* derived blast resistance rice breeding programme in Malaysia or elsewhere (Fig 4). Reliability binding positions of *Pi9*_InDel has been confirmed by cloning and sequencing of PCR amplicon derived by this marker. DNA sequences were aligned together with resistance *Pi9* allele of indica variety 75-1-127. The result showed that the DNA sequences obtained from susceptible lines share a similar sequence with control susceptible variety MR276 whereas resistance allele of IRTP21683 has shown sequence similarity with resistance allele of 75-1-127 (Fig 5). Thus, these results confirmed the consistent performance of *Pi9*_InDel as a reliable functional marker for rice blast disease breeding application.

Table 2: Chi square analysis for 1:2:1 ratio for F2 population.

State	Observed	Expected	Chi square value
Homozygote (RR)	38	46.75	1.64
Heterozygote (Rr)	97	93.5	0.13
Homozygote (rr)	52	46.75	0.59
Total	187	187	2.36

Table 3: Phenotypic screening results of foliar and panicle blast for MARDI rice released varieties.

Variety	Foliar blast	Panicle blast
MR211	Moderately Susceptible	Highly Susceptible
MR219	Moderately Susceptible	Moderately Susceptible
MR272	Moderately Resistant	Resistant
MR276	Moderately Resistant	Moderately Resistant
MRQ76	Moderately Susceptible	Moderately Resistant

Although InDel functional marker has been thought to possibly alter the protein structure or mRNA splicing and hence its function (Lin *et al.*, 2017; Zhang *et al.*, 2012), however, the effect of InDel mutation of NBS-LRR domains cannot be assessed to determine its role in pathogen recognition (Sharma *et al.*, 2005). This newly developed *Pi9*

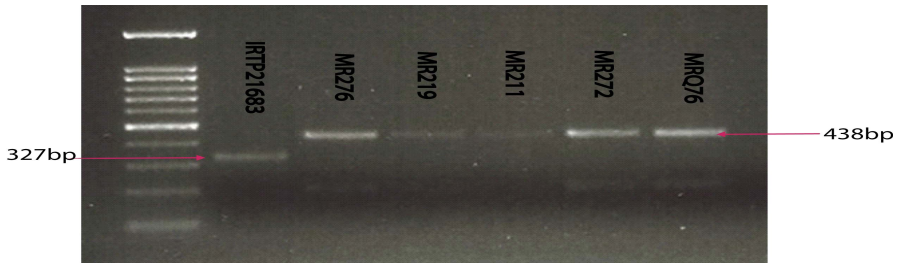

Fig 4: Amplification pattern of *Pi9* allelle on 6 different rice cultivars by *Pi9*_InDel. MR219, MR211, MR272, MRQ76 - MARDI rice varieties with susceptible *Pi9* allele; MR276 - as negative control with susceptible *Pi9* allele; IRTP21683 - as positive control with resistance Pi allele.

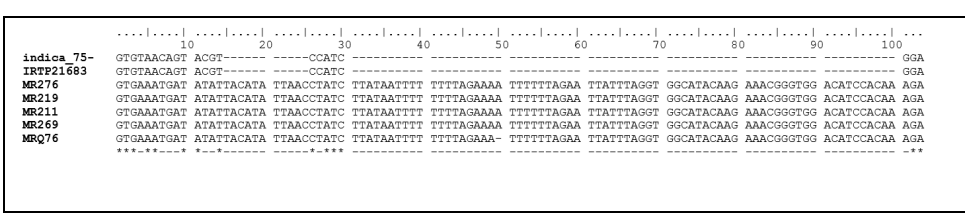

Fig 5: Sequence alignment between two resistant genotypes (Indica line 75-1-127 and IRTP21683) and susceptible genotypes (MR276, MR219, MR211, MR269 and MRQ76) conform the existing of 81bp insertion and deletion in susceptible and resistance *Pi9* alleles respectively. Result shows reliability of the primers to bind at right binding position prior to amplify the amplicon.

marker is co-dominant in nature, which is differed from the previously reported blast resistance functional markers that are dominant (Hayashi *et al.*, 2006; Jia *et al.*, 2002) and thus it could provide a more practical application in MAS.

CONCLUSION

Pi9 is one of the important genes being used in rice breeding programs at MARDI. Although functional markers used in molecular breeding for blast resistance genes such as *Pita* (Jia *et al.*, 2002) and *Pi54* (Ramkumar *et al.*, 2011), however the use of *Pi9* as an InDel marker has not yet been reported. The marker provides high accuracy for genotyping a segregating population and efficiently predicts the allelic status in many rice cultivars. Hence, this newly developed *Pi9* InDel-based functional marker will be highly useful in marker-assisted breeding programs eventually to improve blast resistance in elite rice cultivars.

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