A novel intragenic marker targeting the ectodomain of bacterial leaf blight resistance gene Xa21 in rice

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Abstract

Background Among the diseases in rice (Oryza sativa L.), bacterial leaf blight (BLB) caused by Xanthomonas oryzae pv. oryzae results in devastating economic losses, both in terms of yield and quality. The Xa21 mapped to the rice chromosome 11, is known to convey resistance against BLB by its involvement in plant pathogen recognition and immunity responses. The closely-linked sequence-tagged site marker pTA248 is frequently used for marker-assisted selection (MAS) of Xa21. However, lower precision and linkage drag of linked-markers hinders the reliability and effectiveness in MAS compared to the use of flanking or intergenic markers. Results In the current study, a diagnostic intragenic marker was developed for MAS of Xa21 in rice. The new marker ABUOP0001 targets a 19-bp insertion/deletion (InDel) on the ectodomain of the Xa21, and amplifies a 200-bp amplicon from the rice line IRBB 62, which is known-to-carry the 'resistance allele', and a 181-bp amplicon from IRBB 7, which is known-to-carry the 'susceptible allele'. The resistance allele was identified in ten IRBB lines, and five newly improved Sri Lankan rice accessions through ABUOP0001 marker assay. In a field study, 14 of these accessions conveyed highly resistant BLB disease responses, and one conveyed an intermediate response. While carrying the susceptible allele at Xa21, 30 accessions conveyed a resistance response to BLB, and it could be due to the contribution of other Xa resistance alleles in their genetic background. Further, the Xa21 resistance allele was identified in 1,675 rice accessions through an in silico analysis of genomic sequences available through the 3K Rice Genomes Project. The intragenic marker ABUOP0001 performs equally to that of the linked-marker pTA248 anchored 224 kbp upstream of Xa21, in detecting resistant and susceptible BLB phenotypes. The marker ABUOP0001 is compatible for high-throughput screening with high resolution melting (HRM) and can be multiplexed effectively with an intragenic marker of BLB resistance gene Xa4. Conclusion The marker ABUOP0001 is a diagnostic intragenic marker that can be recommended for MAS of Xa21 in rice. The marker can be assayed as a high-throughput marker using HRM and multiplexed PCR.

Background

Rice (*Oryza sativa* L.) is an important cereal crop that has been cultivated for over hundreds of years under different agro-ecological conditions, feeding populations across the globe. Biotic stresses such as bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has affected the world rice production drastically leading to significant crop losses, both in terms of quality and quantity. In a severe BLB epidemic rice yield losses up to 50 - 80% has been reported [1,2,3].

Genetic mapping has succeeded in identifying nearly 40 *Xa* genes, which confer resistance against BLB [4]. One of the major BLB resistance genes in rice is *Xa21* and it is known to convey the highest stable resistance against multiple *Xoo* races [2,5,6]. The resistance allele of *Xa21* was originally introgressed into *O. sativa* from *Oryza longistaminata,* and was subsequently cloned and characterized [7]. The *Xa21* belongs to a receptor kinase-like gene family, and is physically mapped to the rice chromosome 11 [8,9]. The *Xa21* encodes for a pattern recognition cell-surface receptor-like kinase that recognizes conserved microbial signatures that activates defense responses to confer resistance against *Xoo* pathogens [10].

While the mechanism of conferring *Xa21*-mediated resistance is not exactly deduced, several studies have added to the current understanding [6,11,12,13,14,15,16,17,18,19,20,21,22,23].

The resistance against *Xoo* is driven by cell-surface receptor-like kinase XA21, that recognizes the sulfated-required for activation of *Xa21*-mediated immunity X (RaxX) protein expressed by *Xoo* pathogens in resistant rice plants [19]. For pathogen recognition and activation of immune responses, specific domains such as ectodomain, endodomain, and intracellular kinase domain on the XA21 peptide chain are important [16,21,22,23]. Several studies have shown the importance of XA21 ectodomain for recognition of pathogens [22,24,25,26]. With constructs of chimeric proteins, Thomas et al. [22] reported that ectodomain of XA21 is critical for the resistance responses against the *Xoo* strain PXO99A, where the protein chimera with XA21 ectodomain expressed resistance, and the reciprocal chimera was susceptible. Hence, the specificity of the XA21 ectodomain in mediating immune responses in rice against *Xoo* strains *via* rice specific signaling attributes that it is a critical domain for the resistance against BLB.

As *Xa21* conveys the highest contribution towards BLB resistance, the use of a reliable diagnostic molecular marker to screen *Xa21* resistance alleles in marker-assisted selection (MAS) is important for plant breeders. The most commonly used molecular marker for MAS of *Xa21* is the sequence-tagged site (STS) marker *pTA248* [27,28], however, compared to linked-markers the reliability of flanking or intragenic markers is greater, and are more likely to be diagnostic for a trait of interest [15]. Given the importance of the ectodomain for *Xa21*-mediated resistance responses for BLB, the current study was conducted targeting a 19-bp insertion/deletion (InDel) at the *Xa21* ectodomain with the objective of developing a diagnostic intragenic marker for the use in MAS of *Xa21* in rice.

Methods

Plant materials

In the current study, 21 IRBB lines (reference lines released by the International Rice Research Institute, Philippines known to carry specific *Xa* genes), 42 traditional and newly improved Sri Lankan rice varieties and Nipponbare as the *Oryza sativa* subsp. *japonica* genome reference (Additional File 2) was used. All seed stocks were sourced from the Plant Genetic Resources Centre (PGRC), Gannoruwa, Sri Lanka and Regional Rice Research and Development Institute (RRDI), Bombuwala, Sri Lanka. The IRBB lines were sourced from RRDI, Bombuwala, Sri Lanka. The DNA was extracted from the rice accessions using a modified CTAB method [49].

In silico sequence retrieval, alignment and annotation

A 3910-bp genomic sequence of the *Xa21* gene of the *japonica* genomic reference Nipponbare (*Os11g0569733;* synonym *LOC_Os11g36180*) and the sequence of the *Xa21* ortholog of *Oryza*

longistaminata (KN543217.1_FG001) was retrieved from Gramene Database [50] (http:// www.gramene.org/; Gramene Search UI Build #705.1 accessed on 12/27/2017). Using the locus position of *Os11g0569733* (Chr11: 21273533..21277443), the genomic sequence of *Xa21* was retrieved for IRBB 62 (IRGC 135947; known-to-carry the resistance allele at *Xa21*; [51,52] and IRBB 7 (IRGC 135948; known-to-carry the susceptible allele at *Xa21*; [52,53,54] from the Rice SNP-Seek Database [55] (http://oryzasnp.org/iric-portal/).

The retrieved genomic sequences were aligned with multiple sequence alignment function in Geneious v7.1.3 [56]; Biomatters Ltd., New Zealand) on a ClustalW platform with a gap open cost of 15 and a gap extend cost of 6.66. The structural features of *Xa21* and the functional domains were annotated based on the sequences retrieved from the Gramene Database [50] (http:// www.gramene.org/; Gramene Search UI Build #705.1 accessed on 12/27/2017) and Uniprot Database (The UniProt Consortium, 2017; http://www.uniprot.org/ accessed on 12/27/2017) for *Os11g0569733*.

Similarly, using the locus position of *Os11g0569733* (Chr11: 21273533..21277443), the genomic sequence of *Xa21* was retrieved for 2,897 rice accessions from Rice SNP-Seek Database (Alexandrov *et al*. 2015; http://oryzasnp.org/iric-portal/; Additional File 1; Table S2), and was aligned according to the methods described above (hereinafter referred to as *Xa21 in silico* sequence alignment).

Assaying and anchoring of *pTA248* to Rice Genome Assembly

The marker *pTA248* was used to amplify Nipponbare genomic sequence in the Rotor-Gene Q thermal cycler (Qiagen, Germany) using a 15 µl final PCR reaction volume that includes 50ng/µl template DNA, 1× GoTaq® Green master mix (Promega, USA), 0.66 µM of each primer and 1mg/µl bovine serum albumin (Promega, USA). In the no-template control, the template DNA was replaced by nuclease-free water (Promega, USA). The template DNA was initially denatured at 94 °C for 5 min followed by 35 cycles of PCR amplification with: 30 s denaturation at 94 °C, 30 s primer annealing at 58 °C and 30 s primer extension at 72 °C and an additional primer extension for 5 min at 72 °C. The amplified products were electrophoretically resolved on a 3% agarose gel (BIORON, Germany), 1× TAE buffer and was visualized under UV after post-staining with 0.5 µl/ ml ethidium bromide.

The PCR product was sequenced bidirectionally at GeneTech, Sri Lanka. The forward and reverse sequences were assembled, curated and trimmed using *de Novo* assemble feature of Geneious v7.1.3. [56] (Biomatters Ltd., New Zealand), and the consensus sequence generated was used in a BLAST homology search in Gramene (http://www.gramene.org/). The sequence was submitted to the GenBank sequence repository (http://www.ncbi.nlm.nih.gov; [57].

Attempts were made to anchor the *pTA248* sequence on to the Rice Genome Assembly (IRGSP-1.0) with a BLAST homology search at the Gramene Database (http://gramene.org/), and based on the regions corresponding to HSP the position of *pTA248* on the Rice Genome Assembly (IRGSP-1.0) was deduced.

The marker *pTA248* was assayed on Nipponbare (*japonica* genomic reference) and 63 rice accessions including 21 IRBB lines, 15 traditional and 27 newly improved Sri Lankan rice accessions/varieties) to determine the allele-call using the PCR conditions mentioned above.

Development and assaying of a novel molecular marker

A new primer pair *ABUOP0001_F* and *ABUOP0001_R* was designed using the Primer3 feature [58] in Geneious v7.1.3 to amplify the region spanning over the 19-bp InDel reported by Nanayakkara et al. [29] on *Xa21*. The PCR amplification of *ABUOP0001_F/R* was carried out in Rotor-Gene Q thermal cycler (Qiagen, Germany) using a 15 µl final PCR reaction volume containing 50 ng/µl template DNA (IRBB 62 and IRBB 7), 0.15 µM of each primer, 1× of GoTaq® green master mix (Promega, USA) and 1 mg/µl of bovine serum albumin (Promega, USA). In the no-template control, the template DNA was replaced by nuclease-free water (Promega, USA). The PCR amplification and visualization was carried out using the methods described for the marker *pTA248.*

Field evaluation of bacterial leaf blight disease responses

The field evaluation was conducted at the RRDI, Bombuwala, Sri Lanka for a panel of 63 rice accessions based on a completely randomized complete block design with three replicates. The seeds were germinated in nursery and was transplanted at the age of three weeks. At each replicate three plant lines were maintained with seven plants per line. The plants were fertilized and watered as per the recommendation of the Department of Agriculture, Sri Lanka. At the early booting stage, the middle three plants in a plant line were inoculated. In each plant the first three leaves were artificially inoculated using the clipping method, using a freshly made bacterial broth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) made out of diseased leaves as described in SES of IRRI. The BLB disease response of the inoculated plants were assessed based on the length of the lesion after 21 days from the date of inoculation, according to the SES of IRRI, Philippines based on a scale of 1, 3, 5, 7 and 9.

The estimated median of the disease score for each accession were calculated using Friedman test in Minitab 11.12 (Minitab LLC, USA). The accessions were categorized as highly resistant (score of ≤ 3), intermediate (score of > 3 and < 7) and highly susceptible (score of ≥ 7) according to Koch [30]. The disease scores of the highly resistant and highly susceptible groups and their respective allele call at the marker *ABUOP0001* and *pTA248* were analyzed using cross tabulation in Minitab 11.12 (Minitab LLC, USA) to evaluate the comparative performance of the two markers against each other in detection of resistance and susceptible disease responses.

High throughput screening assays

Multiplexed PCR was carried out to assay alleles of the BLB resistance genes *Xa21* and *Xa4* in a single tube PCR using the new primer pair *ABUOP0001_F/R*, and the marker *Xa4_F/R* [31], respectively. Of the IRBB lines, IRBB 21 (known-to-carry the resistance allele at *Xa21*; known-to-carry the susceptible allele at *Xa4*), IRBB 62 (known-to-carry the resistance allele at both *Xa21* and *Xa4*) and IRBB 4 (known-to-carry the susceptible allele at *Xa21*; known-to-carry the resistance allele at *Xa4*), IRBB 7 (known-to-carry the susceptible allele at both *Xa21* and *Xa4*) were used for the optimization of the multiplexed PCR. The PCR reaction was carried out in the Rotor-Gene Q thermal cycler (Qiagen, Germany) using a final PCR reaction volume of 25 µl with a template DNA concentration of 50 ng/µl, 2× PCR Sens Taq HotSart master mix (Applied Biological Materials, Canada), 0.15 µM each of the reverse and forward primers of both *ABUOP0001* and *Xa4* markers, and 1 mg/µl of bovine serum albumin (Promega, USA). In the no-template control, the template DNA was replaced by nuclease-free water (Promega, USA). The PCR amplification and visualization was carried out using the methods described for the marker *ABUOP0001.*

The HRM was performed in a Rotor-Gene Q thermal cycler (Qiagen, Germany) with a final PCR reaction volume of 15 µl containing 50 ng/µl template DNA (IRBB 62 and IRBB 7), 0.15 µM of each forward and reverse primer, 1× of GoTaq® colourless master mix (Promega, USA) and 1 mg/µl of bovine serum albumin (Promega, USA) and 2 µM of SYTO® 9 green fluorescent nucleic acid stain (Invitrogen, USA). In the no-template control, the template DNA was replaced by nuclease-free water (Promega, USA). The HRM-PCR program was as follows; initial denaturation at 94 °C for 5 min followed by 35 cycles of PCR amplification with: 30 s denaturation at 94 °C, 30 s primer annealing at 58 °C, 30 s primer extension at 72 °C and 5 min additional primer extension at 72 °C followed by a melt curve analysis at 65 °C to 95 °C with a ramping rate of 0.05 °C. The HRM analysis was carried out using Q-Rex v1.0.0 plugin (Qiagen, Germany).

Results

A novel high throughput marker targeting a 19-bp InDel within the *Xa21* ectodomain

The marker *pTA248* has been frequently used for MAS of *Xa21* to screen for lines carrying the allele conveying BLB resistance. Here we report that the sequence of the *pTA248* amplicon (GenBank accession: MK341051) could not be anchored on to the rice *Xa21* gene (*Os11g0569733*), however, based on the high-scoring segment pairs (HSPs) resulted from a BLAST homology search, *pTA248* amplicon was anchored on to the *Wall-associated kinase gene 120* (Chr 11: 21,047,440..21,049,536: *Os11g0565300*) in IRGSP 1.0 Rice Genome Assembly (Fig. 1; Additional File 1 Table S1). The marker *pTA248* is therefore only a closely-linked marker to *Xa21,* and amplifies a fragment from a gene that is 224 kbp upstream of the target *Xa21* gene (Fig. 1). Between the *Xa21* and the anchor position of the marker *pTA248*, 41 predicted genes were identified in the IRGSP 1.0 Rice Genome Assembly (Additional File 1 Table S2).

A 19-bp InDel was identified in the sequence alignment of *Xa21* (*Os11g0569733*) mapped to chromosome 11 at the position 21,276,906..21,276,924 in IRGSP 1.0 Rice Genome Assembly in the accessions Nipponbare (genomic reference of *O. sativa* subsp. *japonica*), IRBB 62 (known-to-carry the resistance allele at *Xa21*), IRBB 7 (known-to-carry the susceptible allele at *Xa21*), and *Oryza longistaminata* (wild relative of *O. sativa* from which the *Xa21* resistance allele was first introgressed into cultivated rice).

The primer pair *ABUOP0001_F/R* (*ABUOP0001_F* - 5$TC\forall GCTGGCA\mathbb{C}\forall TCTCA3$; *ABUOP0001_R* - 5$\mathbb{C}TCAGCGAC\forall CTA\mathbb{C}TCT3$**)** was designed to flank the said 19-bp InDel identified on the ectodomain of *Xa21* [29]. Unlike the previously used linked-marker *pTA248*, *ABUOP0001* amplifies an intragenic region of the *Xa21* (Fig. 2). The marker *ABUOP0001* amplified a 200-bp amplicon from IRBB 62 (known-to-carry the resistance allele at *Xa21*) and a 181-bp amplicon from IRBB 7 (known-to-carry the susceptible allele at *Xa21*), indicating that the 200-bp amplicon is diagnostic for the resistance allele and 181-bp amplicon is diagnostic for the susceptible allele of *Xa21* (Fig. 2).

The marker *ABUOP0001* was assayed on 63 rice accessions (21 IRBB lines, 15 traditional Sri Lankan rice accessions, and 27 newly improved Sri Lankan varieties), and 15 accessions carried the resistance allele at *Xa21* (Additional File 2). Further, based on an *in silico* sequence analysis targeting the 19-bp InDel of *Xa21* sequence in 2,899 rice accessions retrieved from genomic sequence repositories, 1,675 rice accessions including the *japonica* genomic reference Nipponbare and *O. longistaminata* was identified as carrying the resistance allele at *Xa21*, and the remaining 1,224 rice accessions were identified as carrying the susceptible allele at *Xa21* (Additional File 3).

Validation of the marker *ABUOP0001*

Based on the Standard Evaluation System for rice (SES) of the International Rice Research Institute (IRRI), Philippines a total of 63 rice accessions were evaluated for BLB resistance responses in a replicated field trial over the period through May to September of 2017. In the said field experiment, the disease response score ranged in a scale of one to nine. Based on the categorization of Koch [30], 44 accessions with an estimated median score of less than three were categorized as highly resistant and five accessions with an estimated median score of more than seven were categorized as highly susceptible (Fig. 3; Additional File 2). The remaining 14 accessions with an estimated median score of more than three and less than seven were categorized as carrying intermediate responses given that they could not be grouped into neither the highly resistant nor the susceptible groups with confidence (Fig. 3; Additional File 2).

Based on the estimated median score, all IRBB lines, newly improved varieties At 307, At 308, At 354, Bg 250, Bg 251, Bg 310, Bg 352, Bg 357, Bg 360, Bg 366, Bg 369, Bw 14-509, Bw 267-3, Bw 363, Bw 367, Ld

253, Ld 371, and traditional accessions Kuru wee, Mahakuru wee, Mada el, Pachchaperumal, Podi hatatha, and Murungakayan could be identified as highly resistant to BLB (Additional File 2).

The 14 rice accessions (IRBB lines IRBB 21, IRBB 52, IRBB 54, IRBB 57, IRBB 58, IRBB 59, IRBB 60, IRBB 64, IRBB 65 and IRBB 66, and the newly improved rice accessions At 354, Bg 250, Bg 251, and Ld 371) that carried the resistance allele at *Xa21* identified *via* the marker *ABUOP0001* (Additional File 2) conveyed a highly resistant BLB disease response in the field trial, however, Bw 372 carrying the resistance allele showed an intermediate BLB response. A total of 30 accessions that carried the susceptible allele at *Xa21* conveyed highly resistant BLB responses. The remaining accessions carried susceptible allele at *Xa21,* and conveyed intermediate (13 accessions) or highly susceptible (five accessions) BLB responses.

To compare the suitability of *ABUOP0001* over the previously used marker *pTA248*, the 63 rice accessions were genotyped with marker *pTA248*, where three previously reported allele types (1000 bp resistance allele, and 750 bp and 650 bp susceptible alleles) were detected in the current rice panel (Additional File 2). The allele calls of *ABUOP0001* were almost identical to *pTA248* except in five accessions (hereinafter referred to as 'recombinants'; Additional File 2). The detection of recombinants between the markers *ABUOP0001* and *pTA248* indicated that the *Xa21*-intragenic marker *ABUOP0001,* and the marker *pTA248* is not in complete linkage. According to the cross tabulation analysis both the markers *ABUOP0001* and *pTA248* showed no significant difference ($p > 0.05$) in detecting the resistant phenotypes from susceptible phenotypes ($p = 0.656$) in the field trial. However, if the marker *pTA248* was used instead of marker *ABUOP0001*, the recombinant accessions would have been misidentified as carrying the susceptible allele at *Xa21.* This provides additional evidence to indicate that the newly developed marker *ABUOP0001* is equally or better suited for MAS of *Xa21* resistance allele compared to the marker *pTA248.*

Compatibility of *ABUOP0001* for high throughput screening

In order to see the compatibility of the marker *ABUOP0001* in high throughput screening, the marker was tested in a multiplexed PCR assay with a marker amplifying alleles of BLB resistance gene *Xa4,* and on a high resolution melting (HRM) platform. The products of the marker *ABUOP0001* (200 bp and 181 bp) could be successfully binned with that of marker *Xa4* (198 bp and 217 bp) of [31] enabling multiplexed PCR to be carried out to enhance throughput on gel-based screening for the major BLB resistance genes *Xa21* and *Xa4*, respectively (Fig. 4a). Further, the alleles amplified from accessions known-to-carry the resistance allele at *Xa21* and known-to-carry the susceptible allele at *Xa21* for the marker *ABUOP0001* could be resolved through HRM enabling high throughput genotyping (Fig. 4b).

Discussion

Bacterial leaf blight is one of the most destructive biotic stresses affecting rice cultivations around the world, both in terms of yield and quality losses. Due to the poor efficiency of chemical control against

BLB, tapping genetic resistance is the most effective, economic, and environmentally-friendly control strategy against BLB [7,32]. Therefore, mining for resistance alleles that convey durable resistance against BLB has become a priority in the rice breeding programs worldwide. Hence, introgression of multiple resistance alleles covering a broad-spectrum of genes conveying BLB resistance into popular elite rice varieties can be considered as a sustainable way forward [33]. The genetic resistance in rice against *Xoo* strains are been regulated by nearly 40 genes, among which *Xa21* is a dominant gene that convey durable resistance against a wide range of *Xoo* strains [34]. Here we report the development of a novel intragenic marker for the use in MAS of *Xa21*, targeting a 19-bp InDel at the ectodomain, a region on the *Xa21* that impacts its disease responses against BLB.

The linked-marker *pTA248* was widely used in MAS of *Xa21* [1,17,33,35,36,37,38,39,40,41]. Here we deduced its position on to the *wall-associated kinase gene 120* (*Os11g0565300*), a gene mapped 224 kbp upstream of *Xa21* (Fig. 1). For MAS, intragenic or flanking markers are preferred over linked-markers as they carry minimum linkage drag, and ensure diagnostic detection of the desired trait through genotyping [15]. In the current study, a novel intragenic marker *ABUOP0001* was designed to amplify over a 19-bp InDel in the ectodomain of *Xa21*. The marker *ABUOP0001* amplifies a fragment of 200-bp that is diagnostic of the BLB resistance allele in rice.

Through phenotyping 21 IRBB lines, 17 newly improved and six traditional Sri Lankan rice accessions were identified as highly resistant to BLB. Among these, all IRBB lines and four newly improved varieties (Bg 250, At 354, Bg 251, and Ld 371) carried the *Xa21* resistance allele when screened with marker *ABUOP0001*. Only Bw 372 carrying the *Xa21* resistance allele was categorized in intermediate response category. The remaining accessions though they carried the susceptible allele at *Xa21,* they conveyed a resistance response, probably due to the presence of resistance alleles from other *Xa* genes in their genetic background. *In silico* sequence analysis revealed that 1,675 rice accessions including the *japonica* genomic reference Nipponbare and *O. longistaminata* carried the resistance allele at *Xa21.* These represented 73 countries around the globe. In countries where BLB is prevailing, development of rice varieties that carry genetic resistance to BLB is essential. An assessment of the *Xa21* alleles carried by a rice accession could be conducted either through *ABUOP0001* marker assay or through an *in silico* sequence analysis of the target region. The genotypic information on the *Xa21* resistance loci, could be utilized by rice breeders 1) for selection of parental lines for performing crosses to develop new varieties, and 2) to introduce *Xa21*-mediated resistance from traditional germplasm to elite varieties.

The new intragenic marker performed equally or better as the marker *pTA248* in detecting the resistant phenotypes from susceptible phenotypes. The marker *ABUOP0001* could identify five recombinants (Bg 250, At 354, Bg 251, Ld 371 and Bw 372) carrying the resistance allele at *Xa21*, which was misidentified as carrying the susceptible allele at *Xa21* by linked-marker *pTA248.* Therefore, of the two markers, the intragenic marker *ABUOP0001* is more suitable for MAS of the *Xa21* resistance alleles over the linked-marker *pTA248* as 1) it assays a polymorphism at the ectodomain of *Xa21,* a region assumed to be important for the *Xa21*- mediated disease responses, 2) it reduces the possibilities of inaccurate selection due to occurrence of recombination and 3) reduces chances of linkage drag. The said marker can be

recommended for tracking of the resistance allele of *Xa21* in MAS, gene pyramiding and in marker-assisted backcross breeding to enrich the breeding lines in order to release varieties with *Xa21*-mediated genetic resistance, efficiently and effectively.

With the advancement of technology, cost and time effective screening of germplasm is utmost important in rice breeding programs. The current trend in molecular breeding is towards the use of high-throughput markers which enables the genotyping of a large number of samples in a fraction of time. For the routine screening of germplasm for multiple genes in a single assay, multiplexing is a cost and time effective approach [42]. Here, the assaying of multiple markers in a single PCR is facilitated by picking markers with amplicon sizes that can be binned together without an overlap in the fragment sizes. This expedites genotyping in laboratories which has minimal access to high throughput marker technologies such as HRM, capillary gel electrophoresis, end-point genotyping or sequencing. The marker *ABUOP0001* can be effectively multiplexed with an intragenic marker assaying the BLB resistance gene *Xa4* (*LOC_OS11g45740*) reported in Yap et al. [31]. This enables the detection of the resistance/ susceptible alleles at the two major BLB resistance genes *Xa21* and *Xa4* in one PCR. The multiplexed PCR put forth a cost and time effective screening assay for rice breeders in technology-limited countries to genotype rice germplasm for desirable alleles for two major BLB resistance genes.

High resolution melting technology is an alternate high throughput marker assay option, where the samples are clustered according to the allele they carry based on the differences in the melt curves that occur as a result of variations in the length, composition and complementarity of the amplified sequences [43]. The HRM-based screening has effectively been used for screening many molecular markers in cereals [44,45,46,47,48]. In the current study, we report that the novel marker *ABUOP0001* can be successfully used on an HRM-based high throughput platform for genotyping *Xa21*, reliably and effectively.

Conclusions

Based on a 19-bp InDel at the ectodomain, a novel intragenic molecular marker *ABUOP0001* was developed to screen rice *Xa21* resistance alleles in MAS. Using the marker *ABUOP0001,* we have identified 15 rice accessions that carry the resistance allele at the *Xa21.* In addition, based on *in silico* sequence analysis of the target region, 1,674 rice accessions with diverse geographic distribution were also identified as carrying the resistance allele at *Xa21.* The marker *ABUOP0001* is compatible for high throughput HRM-based screening and can be effectively multiplexed with BLB resistance gene *Xa4*.

Abbreviations

BLB: Bacterial leaf blight

***Xoo*:** *Xanthomonas oryza*e pv. *oryzae*

RaxX: Required for activation of *Xa21*-mediated immunity X

MAS: Marker-assisted selection

STS: Sequence-tagged sites

InDel: Insertion/deletion

HSPs: High scoring segment pairs

HRM: High resolution melting technology

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its Additional information files].

Competing interests

The authors declare that they have no competing interests.

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Author contributions

DN designed the *ABUOP0001* marker assay, genotyped the rice accessions with the two marker assays and based on *in silico* analysis, wrote the manuscript; IE designed, implemented and analyzed the field experiment; LD optimized the high throughput assays; DW facilitated and provided the expertise for the field experiment; LS provided expertise on statistical analysis; VH provided guidance on high throughput assays; CP designed, facilitated and led the field trial and DJ developed the concept, provided expertise on marker development and high throughput assays and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

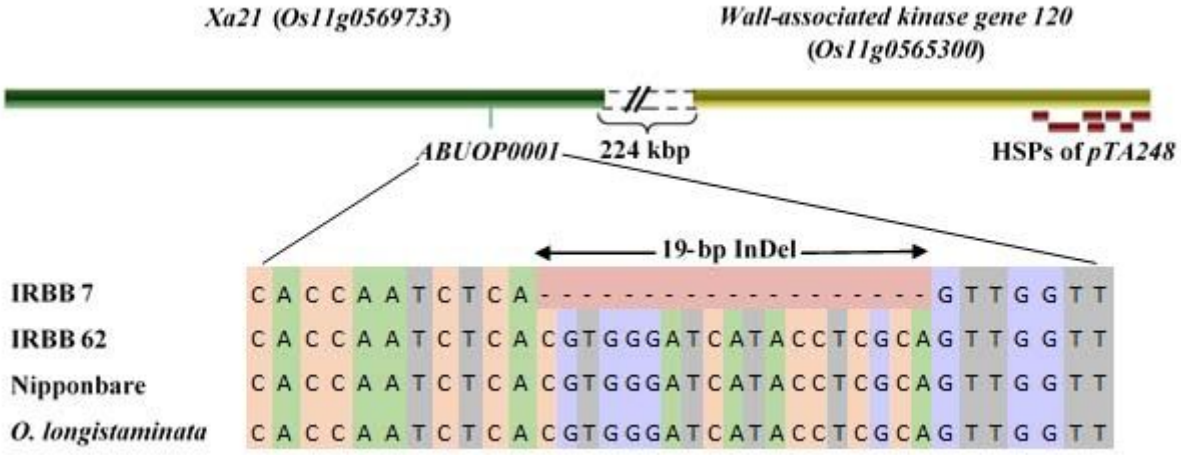

Figure 1

Illustration of anchored positions of pTA248 and ABUOP0001 on the rice genome assembly. The BLAST homology search high scoring segment pairs (HSPs) of marker pTA248 from Nipponbare on the rice genome assembly, relative to the position of Xa21 (Os11g059733) and the marker ABUOP0001 assaying the 19-bp insertion/deletion (InDel) on the ectodomain of the Xa21 are illustrated

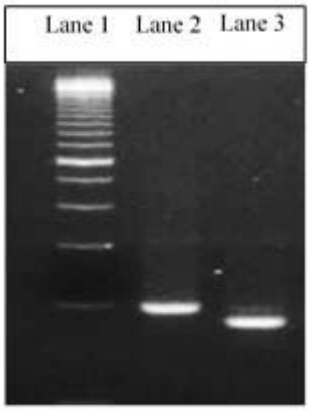

Figure 2

Visualization of the PCR amplicons of ABUOP0001. The Lane 1: 100 bp Ladder (Applied Biological Materials-Canada), Lane 2: the amplicon of IRBB62 sized 200-bp and Lane 3: the amplicon of IRBB7 sized 181-bp

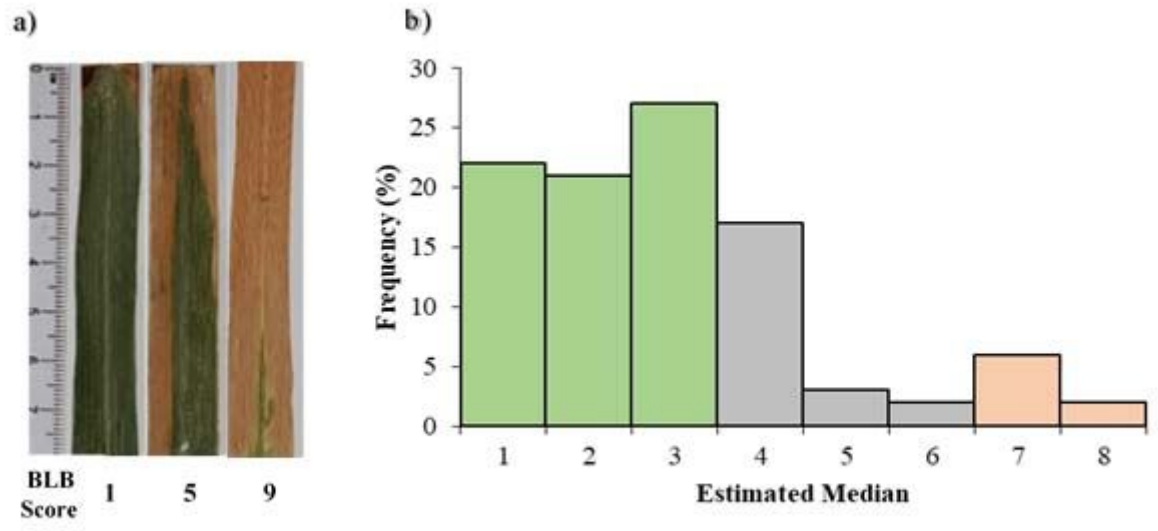

Figure 3

a) Lesion development of bacterial leaf blight and b) histogram of disease responses in 63 rice accessions used in the field trial. The estimated median of the Friedman test was used for categorizing the accessions according to the BLB response. Categories with estimated median score of less than three are identified as highly resistant (shaded in green), from four to six as intermediate response (shaded in blue) and more than seven as highly susceptible (shaded in pink) according to the SES of the International Rice Research Institute, Philippines

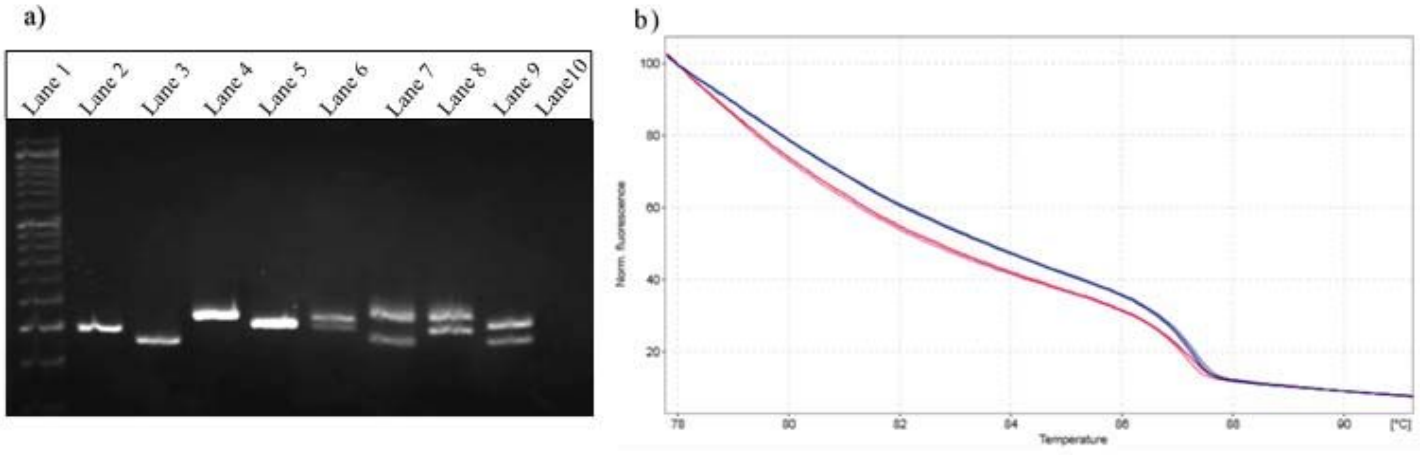

Figure 4

High throughput assaying of marker ABUOP0001 a) Multiplex PCR of Xa21 and Xa4 b) high resolution melting (HRM) technology. In a) the Lane 1: 100 bp Ladder (Applied Biological Materials-Canada), Lane 2: amplicon of IRBB 21 (known-to-carry the resistance allele at Xa21; known-to-carry the susceptible allele at Xa4), Lane 3: amplicon of IRBB 7 (known-to-carry the susceptible allele at both Xa21 and Xa4), Lane 4:

amplicon of IRBB 4 (known-to-carry the susceptible allele at Xa21; known-to-carry the resistance allele at Xa4), Lane 5: amplicon of IRBB 7 (known-to-carry the susceptible allele at both Xa21 and Xa4), Lane 6: amplicon of IRBB 21(multiplexed), Lane 7: amplicon of IRBB 4 (multiplexed), Lane 8: amplicon of IRBB 62 (multiplexed), Lane 9: amplicon of IRBB 7 (multiplexed) and Lane 10: non-template control. In b) blue colour: normalized HRM curve represents IRBB 62 (known-to-carry the resistance allele at Xa21, red colour: normalized HRM curve represents IRBB 7 (known-to-carry the susceptible allele at Xa21)

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