

Marker Assisted Backcross Breeding of Bg 358 (*Oryza sativa* L.) for the Anaerobic Germination Tolerant QTL *AG1*

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ABSTRACT: Sustainability of transplanting systems for rice crop establishment in Sri Lanka is threatened due to climatic changes and heavy rains. Tolerance to anaerobic germination (AG) stress is a key trait that will ensure good crop establishment in direct seeded rice (DSR) farming systems. In this study Marker Assisted backcross breeding was carried out to transfer the quantitative trait loci - QTL *AG1* (*qAG-9-2*), in to the local variety Bg 358 for the development of AG stress tolerant elite lines by *AG1* donors IR64-*AG1* and Cihorang-Sub1*AG1*. Marker assisted selection (MAS) was carried out through foreground and recombinant selection. Molecular marker optimization for RM24161, RM8300 and RM553 to the QTL *AG1* was carried out using BC1F1 populations. DNA was extracted and PCR was performed using simple sequence repeat (SSR) markers and visualized on 2.5% agarose gel. Based on polymorphism exhibited, RM24161 was chosen as the foreground selection marker, RM553 and RM8300 chosen as proximal and distal markers for recombinant selection. It was suggestive through this study to choose flanking markers RM105 & RM219, closer to the *qAG-9-2* locus for recombinant selection as the selected recombinant makers were ineffective and exhibited marker segregation distortion. RM24161 was found to be a tightly linked marker to the AG tolerant QTL *AG1* (*qAG-9-2*) and was successfully used for selection of lines by MAS in generation of anaerobic germination tolerant elite lines.

Keyword: Anaerobic germination, Backcross breeding, Foreground selection, Flanking markers, Marker assisted selection.

INTRODUCTION

Abiotic stresses including flooding, drought and salinity limit crop production worldwide. The development of cultivars with enhanced tolerance to abiotic stresses has therefore been advocated as a low-cost means to improve productivity in stressful environments (Ismail *et al.*, 2009). In the past, efforts to develop such varieties have typically relied entirely on phenotypic selection in target environments, but with the advances made in marker assisted selection (MAS) development of varieties for abiotic stress conditions have being made easier due to the numerous advantages associated with MAS, including rapid identification of quantitative trait loci (QTL) introgression, selection independent to environmental variation and accuracy. Thus, MAS can be used as a potential method of choice in breeding for abiotic stress tolerance.

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Modern rice varieties are sensitive to flooding during germination and early growth, a problem commonly encountered in rain-fed areas, but few landraces including *KhaoHlan On*, *Ma-Zhan* (red), *Khaiyan* and *Kalongchi* are capable of germination under abiotic stress conditions, enabling research in to anaerobic stress tolerance mechanisms (Angaji *et al.*, 2010). Major QTLs for anaerobic germination (AG) including *AG1* and *AG2* have being identified, and are being targeted for molecular breeding and cloning (Septiningsih *et al.*, 2009; Angaji *et al.*, 2010). In Sri Lanka 95% of rice is grown under direct seeding (Weerakoon *et al.*, 2011). Sustainability of transplanting systems for rice crop establishment in both irrigated and rain-fed conditions in Sri Lanka is threatened due to increasing rainfall, flooding and labor scarcity for rice cultivation. Thereby adaption to mechanization by DSR farming systems and development of rice varieties tolerant to AG stress is a key trait that will ensure good crop establishment. Thus the availability of anaerobic germination stress tolerant varieties will help increase mechanization in farming systems in Sri Lanka. The *AG* trait can be combined through gene pyramiding with the *SUB1* QTL conferring resistance to submergence, to ensure additive tolerance to flooding during the vegetative stage of the rice crop development.

Among the tolerant landraces and the corresponding QTLs for AG stress tolerance identified at IRRI, the *AG1* QTL is located in the chromosome 9 (*qAG-9-2*) having a logarithm of the odds (LOD) score of 20.3 and explaining 33.5% of the phenotypic variation for the trait of anaerobic germination (Angaji *et al.*, 2010). This QTL was found to be a promising target for marker-assisted selection (MAS) to develop varieties with anaerobic germination tolerance and thus suited for direct seeded rice (DSR). Most of the donors for *AG1* QTL are old landraces, with poor phenotypic features such as susceptibility to lodging, awns and poor yields thus, marker assisted back-crossing (MABC) can be effectively used to transfer the tolerant QTL to popular varieties, hence introgression of *AG1* QTL in to local rice varieties with desirable characters will have a major impact on DSR and improved rice yield.

The objective of the present study is the introgression of the *AG1* QTL into local rice variety Bg 358 by crossing with *AG1* donor parents- IR64-*AG1* and *Ciherang-Sub1AG1* and subsequent selection through MABC, with foreground and recombinant selection, in the effective identification of the AG tolerant QTL, in a genetic background of the local, recurrent parent Bg 358, which will result in the generation of elite lines tolerant to AG stress.

METHODOLOGY

Anaerobic germination tolerant donor variety IR64-*AG1* and *Ciherang-Sub1AG1* were acquired from the International Rice Research Institute (IRRI) and was used for introgression of the anaerobic germination QTL *AG1*, in to local variety Bg 358. Production of F₁ generations was carried out by crossing the respective parents as shown in Table.1 and Fig.1. Subsequently the BC₁F₁ backcross populations were produced by hot water treatment method of emasculation (Gracia-Yzaguirre and Carreres, 2008) at the Rice Research and Development Institute Bathalagoda (RRDI) and transplanted under field conditions.

Table 1. Plant materials and crossing scheme

Recurrent Parent	Donor Parent	F ₁	BC ₁ F ₁ Population
Bg358	IR64 - <i>AGI</i>	Bg 358 / IR64 <i>AGI</i>	Bg 358 / IR64 <i>AGI</i> //Bg 358
Bg 358	<i>Ciharang-Sub1 AGI</i>	Bg 358 / <i>Ciharang Sub1 AGI</i>	Bg 358 / <i>Ciharang Sub1 AGI</i> //Bg 358

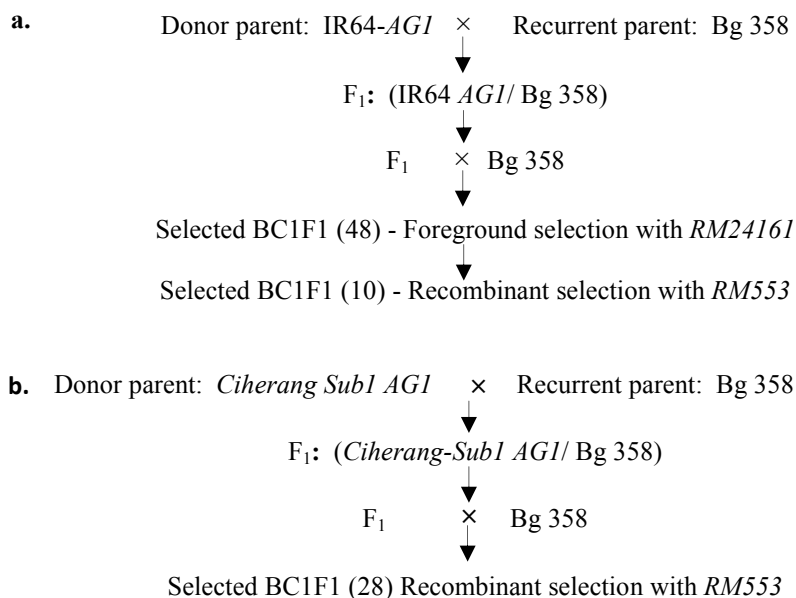


Fig.1 Crossing scheme for the populations a. Bg 358/ IR64-*AGI* b. Bg 358/ *Ciharang-Sub1AGI*; details of markers used for foreground & recombinant selection and the numbers of plants selected in each generation for MAS

Molecular Marker Analysis

Genomic DNA was extracted from a total of 90 BC₁F₁ plants, 48 plants from Bg 358/ IR64-*AGI*//Bg 358 and 38 plants from Bg 358/*Ciharang-Sub1AGI*//Bg 358 and the parental varieties IR64-*AGI*, *Ciharang-Sub1AGI* and Bg 358. DNA extraction was carried out using leaves of 4weeks old rice plants transplanted in the field using a simple method described by Kottearachchi *et al.*, 2008. Three pairs of simple sequence repeat (SSR) markers mapped to the *AGI* QTL (*qAG-9-2*) region (Angaji *et al.*, 2010) were selected based on the mapped positions of the markers to perform foreground and recombinant selection.

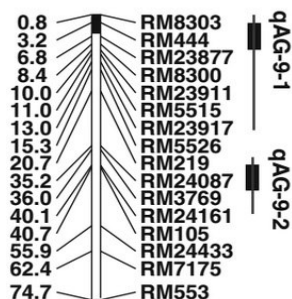


Fig.2. A genetic linkage map of rice chromosome 9 showing the mapped positions of the QTLs associated with tolerance to flooding during germination extracted from Angaji *et al.*, 2010

For foreground selection marker *RM24161*, a marker tightly linked to (*qAG-9-2*) and for recombinant selection markers *RM8300* and *RM553* was used as the proximal and distal markers respectively. The Primer sequences, annealing temperatures and expected product size of each primer pair are given in Table 2.

Table 2. Primer sequences, melting temperatures and annealing temperatures of the SSR markers used for genotyping

Primer Name	Primer Sequence 5'→ 3'	Annealing Temperature (°C)	Expected PCR product size (bp)
RM 8300 F	GCTAGTGCAGGGTTGACA CA	51.7	215
RM 8300 R	CTC TGGTCGTTT CAT GGTATG		
RM24161F	GTATGGCGAGACCCTACAGAC C	52.3	279
RM24161R	GACCCACTTAATGTGTCACAAGG		
RM 553 F	AACTCCACATGATTCCAC CCC	50.6	162
RM 553 R	GAG AAGGTGGTTGCAGAA GC		

(Source: www.gramene.org)

The SSR markers *RM24161*, *RM553* and *RM800* were assayed on the parents IR64-*AG1*, *Ciherang-Sub1AG1* and Bg 358 and the markers showing polymorphism among the parental combinations was assayed on the respective BC₁F₁ progeny lines. PCR was performed in 15µl reaction mixture volumes containing 3µl of 50 ng template DNA, 3µl of 10X PCR buffer, 0.9µl of 25 mM MgCl₂, 0.15µl of 2.5 mM Deoxynucleoside triphosphate mixture, 1µl each of 10µM forward & reverse primers and 0.2µl *Taq* DNA polymerase (5units/µl). The PCR programme consisted of an initial denaturation at 94°C for 3 minutes; followed by 35 cycles denaturation at 94°C for 2 minutes, primer annealing at a primer specific temperature given in Table 2 for 1 minute and an extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes on a thermal cycler (MyCycler, Biorad Inc., USA). Amplified PCR products and 100bp DNA marker ladder (Promega, USA) were separated using agarose gel electrophoresis on a 2.5% agarose gel separated at a voltage of 60V for 2 hours and stained using 0.5 mg/ml ethidium bromide and visualized with a UV trans-illuminator gel documentation system (Euduro, labnet.Int.Inc, USA).

RESULTS AND DISCUSSION

Molecular Marker Analysis

The *AGI* QTL is located in the long arm of the chromosome 9 (*qAG-9-2*) which has a LOD score of 20.3 explaining 33.5% of the phenotypic variation of the trait as explained by Angaji *et al.* (2010), therefore, having a major contribution in conferring tolerance to anaerobic germination.

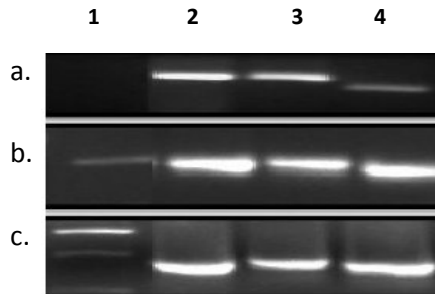


Fig.3. Parental polymorphism of markers a. *RM 24161*, b. *RM 553*, c. *RM 8300*

Thereby, foreground and recombinant selection markers were selected and used effectively to screen for the presence of the QTL *AGI* assisting the selection of elite lines with tolerance to anaerobic germination and for detection of additional recombinants for fine mapping of the QTL. The marker *RM24161* showed length polymorphism between the respective parents, Bg 358 and IR64-*AGI* and was chosen as the foreground selection marker to genotype the population Bg 358 / IR64-*AGI*. For recombinant selection *RM553* was selected for genotyping the population Bg 358 / IR64-*AGI* and *RM8300* was chosen to genotype the population Bg358 / *Ciherang-Sub1AGI*.

Genotyping of BC₁F₁ Bg 358/ IR64-*AGI* with *RM24161* - Foreground selection

Foreground selection was utilized to select for the presence of the target *AGI* QTL which enabled easy selection of the AG trait that would otherwise be difficult to select through laborious and time-consuming phenotypic screening procedures. The marker *RM24161* was selected based on the location of the marker on the long arm of the chromosome 9, as the closest linked marker to *AGI* (*qAG-9-2*), for foreground selection as carried out by Septiningsih *et al.*, 2009. Subsequent screening with *RM24161* resulted in generating good quality genotyping results validating the marker to be used in marker assisted selection of the *AGI* QTL in future backcross programs and gene pyramiding.

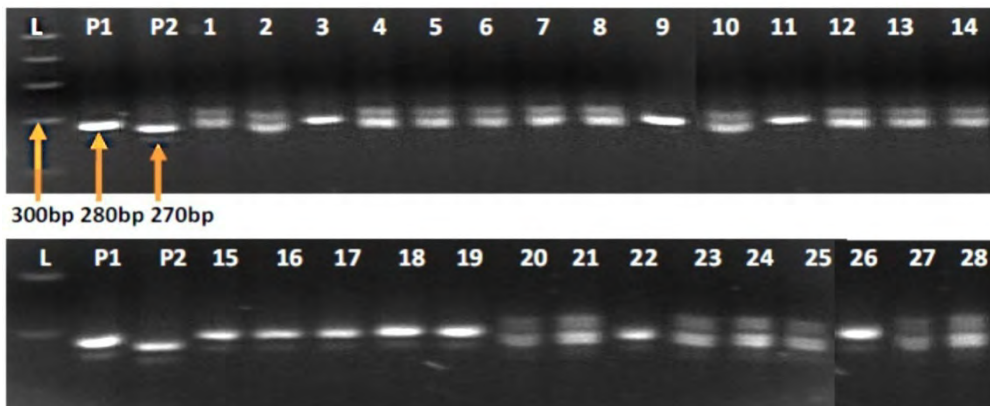


Fig.4. Genotyping of BC₁F₁ Bg 358 / IR64-*AGI* with marker *RM24161*: L-100bp Ladder, P1- Bg 358 , P2- IR64-*AGI*, 1-28 BC₁F₁ population

A total of 48 BC₁F₁ plants were genotyped and results showed segregating banding patterns (Fig.4), 29 heterozygous double bands were observed possessing both the respective alleles from the donor (*AGI* allele) and recurrent parent (Bg 358) at the expected band size of 279 bp (www.gramene.org). A total of 16 homozygous single bands (280bp) were observed corresponding to the recurrent parent Bg 358 and 3 homozygous single bands (270bp) were observed corresponding to the donor IR64-*AGI*. Occurrence of homozygous single bands corresponding to the donor parent in a BC₁F₁ population is not expected according to the expected Mendelian segregation ratio of 1:1 of the heterozygous DR : Recurrent parent RR respectively; the 3 plants that resulted in donor parent genotype was due to selfing that occurred during back crossing with hot water method of emasculation. The 29 individual plants which showed heterozygous double bands will be selected for generation of BC₂F₁ progeny aiming at increasing the recurrent parent characters by backcrossing with Bg 358, while possessing the QTL *AGI* which confers resistance to anaerobic germination.

Genotyping of BC₁F₁ Bg 358 / IR64-*AGI* with *RM553*-Recombinant selection

The second level of molecular selection is recombinant selection, which involves selecting backcrossed progeny with the target QTL and recombination events between the target locus and flanking markers. Recombinant selection is done to reduce the size of the donor chromosome segment containing the target locus, thus reducing the background effect, which contributes immensely to minimizing linkage drag (Hospital, 2005). Markers selected for recombinant selection included, *RM8300* as a proximal marker and *RM553* as a distal marker targeting the QTL region *AGI* (*qAG-9-2*), in order to select recombinants for fine mapping of the region.

A sum of 10 individual samples were selected based on foreground selection with the molecular marker *RM24161*; as individuals possessing heterozygous bands to be genotyped with *RM553* for recombinant selection. Upon carrying out PCR reactions clear banding patterns were not observed and proper amplification was not seen. It was inferred that further optimization in PCR reactions and cycling time had to be carried out to.

Genotyping of BC₁F₁ Bg 358 / *Ciherang-AGI Sub1* with marker *RM8300*

A total of 28 BC₁F₁ plants were genotyped with the molecular marker *RM8300*, banding patterns did not follow an expected Mendelian segregating pattern. Higher proportion of the

genotyped plants expressed the homozygous dominant bands-[16 plants] corresponding to the donor parent *Ciherang-AG1Sub*, 8 plants expressed homozygous recessive bands corresponding to the recurrent parent Bg 358 and one individual showed heterozygous double bands. A chi-square value of 18.64 was observed, $p=0.103$. Therefore, the marker showed apparent segregation distortion and was not suitable for screening the selected population for the QTL *AG1*.

Due to the ineffectiveness of the proximal and distal markers chosen, it was suggestive through this study to choose flanking markers *RM105* and *RM219* respectively, which are closer to the *AG1* (*qAG-9-2*) locus (Fig.1) for recombinant selection for fine mapping in the future, which will be used to effectively to select lines with reduced background effect from the donor parent.

CONCLUSION

The molecular marker *RM 24161*, a tightly linked marker to the anaerobic germination tolerant QTL *AG1* (*qAG-9-2*) was successfully used as a foreground selection marker. The 29 plants that were selected from the foreground marker will be used for backcrossing resulting in BC_2F_1 generation, which will be used in the development of elite lines tolerant to anaerobic germination. The proximal and distal markers used for recombinant selection was ineffective, thus it was also suggestive through the study to select flanking markers *RM219* and *RM105* for recombinant selection.

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