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ORIGINAL PAPER

A marker-assisted backcross approach for developing submergence-tolerant rice cultivars

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Abstract Submergence stress regularly affects 15 million hectares or more of rainfed lowland rice areas in South and Southeast Asia. A major QTL on chromosome 9, *Sub1*, has provided the opportunity to apply marker assisted backcrossing (MAB) to develop submergence tolerant versions of rice cultivars that are widely grown in the region. In the present study, molecular markers that were tightly linked with *Sub1*, flanking *Sub1*, and unlinked to *Sub1* were used to apply foreground, recombinant, and background selection, respectively, in backcrosses between a submergence-tolerant donor and the widely grown recurrent parent Swarna. By the BC_2F_2 generation a submergence tolerant plant was identified that possessed Swarna type simple sequence repeat (SSR) alleles on all fragments analyzed except the tip segment of rice chromosome 9 that possessed the *Sub1* locus. A BC_3F_2 double recombinant plant was identified that was homozygous for all Swarna type alleles except for an approximately 2.3–3.4 Mb region surrounding the *Sub1* locus. The results showed that the mega variety Swarna could be efficiently converted to a submergence tolerant variety in three backcross generations, involving a time of two to three years. Polymorphic markers for foreground and recombinant selection were identified for four other mega varieties to develop a wider range of submergence tolerant varieties to meet the needs of farmers in the flood-prone regions. This approach demonstrates the effective use of marker assisted selection for a major QTL in a molecular breeding program.

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Introduction

Flash floods or short-term submergence regularly affect around 15 million hectares of rice (*Oryza sativa* L.) growing areas in South and Southeast Asia. Even more favorable irrigated areas experience flooding problems during the monsoon season. An economic loss of up to one billion US dollars annually has been estimated (Herdt 1991; Dey and Upadhyaya 1996; Mackill et al. 1996). Submergence tolerant varieties have been developed (Mackill et al. 1993), but have not been widely adopted. One reason is that these tolerant varieties lack many of the desirable traits of the widely grown varieties, referred to as “mega varieties” that

are popular in major rice-growing areas of Asia, because of their high yield and grain quality (Mackill et al. 2006).

A major QTL (*Sub1*) explaining about 70% of phenotypic variation in submergence tolerance has been identified and fine mapped on chromosome 9 in the submergence tolerant cultivar FR13A (Xu and Mackill 1996; Nandi et al. 1997; Xu et al. 2000). Three related ethylene response factor (ERF)-like genes at this locus were identified, *Sub1A*, *B* and *C*, although japonica cultivars and some indicas do not have the *Sub1A* gene (Xu et al. 2006). *Sub1A* and *Sub1C* were up-regulated by submergence and ethylene (Fukao et al. 2006). *Sub1A* was strongly induced in the tolerant cultivars in response to submergence, whereas intolerant cultivars had weak or no induction of the gene. Overexpression of *Sub1A* conferred submergence tolerance in an intolerant japonica cultivar and down-regulation of *Sub1C* (Xu et al. 2006). Even though a single gene controlling tolerance has been identified, the transfer of this gene through conventional breeding is still the most effective way to develop submergence tolerant cultivars (Xu et al. 2006).

The basis of a marker-assisted backcrossing (MAB) strategy is to transfer a specific allele at the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome. The use of molecular markers, which permit the genetic dissection of the progeny at each generation, increases the speed of the selection process, thus increasing genetic gain per unit time (Tanksley et al. 1989; Hospital 2003). The main advantages of MAB are: (1) efficient foreground selection for the target locus, (2) efficient background selection for the recurrent parent genome, (3) minimization of linkage drag surrounding the locus being introgressed, and (4) rapid breeding of new genotypes with favorable traits. The effectiveness of MAB depends on the availability of closely linked markers and/or flanking markers for the target locus, the size of the population, the number of backcrosses and the position and number of markers for background selection (Frisch et al. 1999a; Frisch and Melchinger 2005). MAB has previously been used in rice breeding to incorporate the bacterial blight resistance gene *Xa21* (Chen et al. 2000, 2001) and *waxy* gene (Zhou et al. 2003) into elite varieties.

The availability of the large-effect QTL *Sub1* for submergence tolerance, a theoretical frame-work for MAB and the existence of intolerant varieties that are widely accepted by farmers provided an opportunity to develop cultivars that would be suitable for larger areas of submergence-prone rice (Mackill 2006). The objectives of our project were (1) to develop a submergence-tolerant version of the widely grown cultivar Swarna within a 2–3 years time-frame through a targeted MAB approach for the *Sub1* QTL and (2) to develop a panel of tightly linked and flanking markers for converting other mega varieties to submergence tolerant varieties.

Materials and methods

Plant materials and crossing scheme

IR49830-7-1-2-2 (IR49830-7), one of the FR13A-derived submergence-tolerant breeding lines (Mackill et al. 1993), was used as the donor of *Sub1*. The recipient variety was Swarna, a widely grown cultivar in India and also in Bangladesh. This variety was derived from the cross Vasista/Mahsuri. Vasista is from the cross of the indica High Yielding Variety (HYV) IR8 with the Indian cultivar SLO 13. Mahsuri is from the cross Taichung 65/2*Mayang Ebos 80, the first parent being a japonica cultivar. Other mega varieties used for marker polymorphism studies included Samba Mahsuri and CR1009 (Savitri) from India, IR64 from IRRI-Philippines, and Thadokkham 1 (TDK1) from Laos. An additional indica donor for submergence tolerance IR40931-33-1-3-2 (IR40931-33) also inherited its *Sub1* gene from FR13A (Mackill et al. 1993).

For the MAB scheme, Swarna was crossed with IR49830-7 to obtain F_1 seeds (Fig. 1). F_1s were backcrossed with Swarna to obtain a large number of BC_1F_1 seeds. In the BC_1F_1 generation, individual plants that were heterozygous at the *Sub1* locus were identified reducing the population size for further screening (foreground selection). From the individual plants that were heterozygous for *Sub1*, those that were homozygous for the recipient allele at one marker locus (RM219) distally flanking the *Sub1* locus (i.e. recombinant) were identified. We termed this as “recombinant selection” (Collard and Mackill 2006). From these recombinant plants, individuals with the fewest number of markers from the donor genome were selected (background selection).

In the second BC generation the same strategy was followed for selection of individual plants with the desired allele combination at the target loci including selection for recombinants between *Sub1* and the nearest proximal marker locus (RM316) and suitable genomic composition at the non-target loci and crossed with the recipient parent to develop the next generation. Selected BC_2 and BC_3 plants were self-pollinated for further analysis.

Molecular marker analysis

DNA was extracted from young leaves of 2-week-old plants using a modified protocol as described by Zheng et al. (1995). PCR was performed in 10 µl reactions containing 5–25 ng of DNA template, 1 µl 10× TB buffer (containing 200 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 µl of 1 mM dNTP, 0.50 µl each of 5 µM forward and reverse primers and 0.25 µl of *Taq* DNA polymerase (4 U/µl) using an MJ Research single or dual 96-well thermal cycler. After initial denaturation for 5 min at

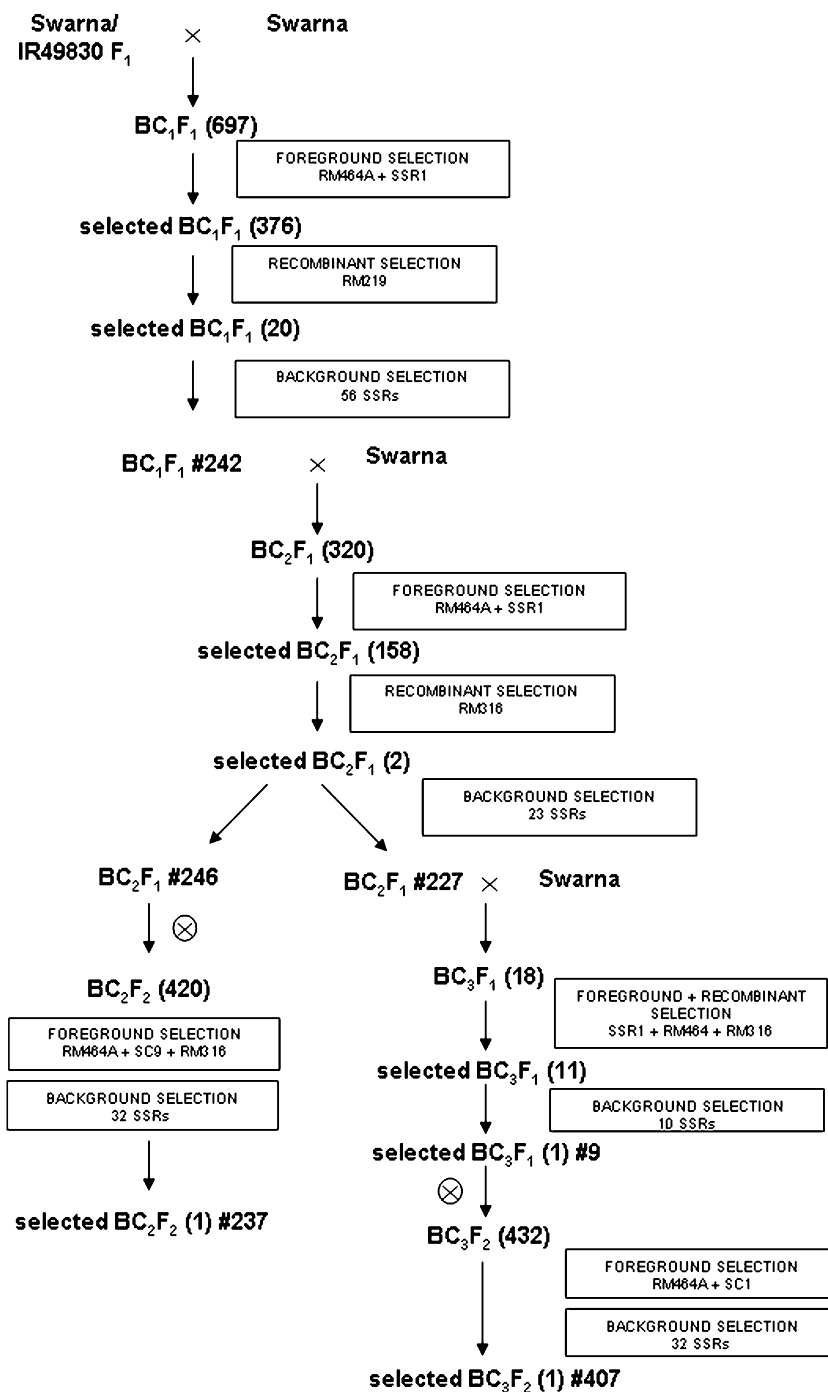

Fig. 1 Development of the submergence-tolerant Swarna-*Sub1* with details of markers used for foreground, recombinant, and background selection. The numbers of plants selected in each generation are indicated in parentheses

94°C, each cycle comprised 1 min denaturation at 94°C, 1 min annealing at 55°C, and 2 min extension at 72°C with a final extension for 5 min at 72°C at the end of 35 cycles. The PCR products were mixed with bromophenol blue gel loading dye and were analyzed by electrophoresis on 8% polyacrylamide gel using mini vertical polyacrylamide gels for high throughput manual genotyping (CBS Scientific Co. Inc., CA, USA). The gels were stained in 0.5 mg/ml ethidium bromide and were documented using Alpha Imager 1220 (Alpha Innotech, CA, USA). Microsatellite or Simple sequence repeat (SSR) markers were used for selection (Temnykh et al. 2001; McCouch et al. 2002; IRGSP 2005).

Foreground and recombinant selection

At the initial stages of the experiment, for selection of the *Sub1* locus (foreground), the reported rice microsatellite (RM) markers RM219 and RM464A, which were found to be linked to *Sub1* by 3.4 and 0.7 cM were used (Xu et al. 2004), and RM316 was also used for foreground selection,

which was reported to be a distance of 1.5 cM from RM464A according to the published map (Temnykh et al. 2001). Based on the fine mapping of the *Sub1* locus and sequence information (Xu et al. 2006), four Bacterial Artificial Chromosome (BAC) clones (AC090056, AP005705, AP005907 and AP006758) of japonica Nipponbare (IRGSP 2005) corresponding to the *Sub1*-linked marker (RM464A) were identified. The sequences of BACs were obtained from GenBank (http://www.ncbi.nih.gov/Genbank/). Motifs of SSRs in the BAC clones were identified using the Simple Sequence Repeat Identification Tool (SSRIT) from http://www.gramene.org/db/searches/ssrtool (Temnykh et al. 2001). Six microsatellite and two indel (insertion–deletion) primers were designed using Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). In order to know whether our new SSR primers have RM numbers associated with them, we used a new tool, named CHARM (http://www.charm.plbr.cornell.edu/comm_identify_ssr.php). Two primer pairs, SC3 (RM8300) and SC9 (RM23887) have been found very useful as recombinant or foreground markers. Since the reported microsatellite markers were class 1 SSRs comprising perfect nucleotide motifs more than 20 nucleotides in length (Temnykh et al. 2001), either new primer pairs were designed targeting the reported microsatellite motif or new microsatellite markers targeting the other types of complex repeats were designed (see Electronic Supplementary Material, ESM).

Based on the sequence information from the BAC clones of IR40931-26 and Teqing (a submergence sensitive variety), two single nucleotide polymorphisms (SNPs) were identified for the *Sub1A* gene (Xu et al. 2006). One SNP was targeted for a cleaved amplified polymorphic sequence (CAPS) marker development with a restriction site for *Alu*I (AG↓CT) or *Pvu*II (CAG↓CTC) (New England Labs), named GnS2 (see ESM). The sequence of the susceptible Teqing was CGCGCATACA**G**CTGGAGTGCT and that of the tolerant IR40931-33 was CGCGCATACA**A**CTGGA GTGCT. *Alu*I and *Pvu*II targeted the same site AG↓CT and CAG↓CTG, respectively. PCR was performed in 20 μl reactions containing 50 ng of DNA template, 2 μl 10× TB buffer (containing 200 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2 μl of 1 mM dNTP, 1 μl each of 5 μM forward and reverse primers and 0.5 μl of *Taq* DNA polymerase (4 U/μl) using an MJ Research 96-well thermal cycler. After initial denaturation for 5 min at 94°C, each cycle comprised 30 s denaturation at 94°C, 30 s annealing at 55°C, and 1 min 30 s extension at 72°C with a final extension for 7 min at 72°C at the end of 35 cycles. After determining the amplification success, the PCR product was used for restriction digestion. A total reaction volume of 20 μl with 2 μl restriction buffer, 0.3 μl (10 U/μl) of *Alu*I or *Pvu*II restriction enzyme (10 U/μl; New England Labs) and 10 μl of PCR product was incubated from 2 h to overnight at 37°C. The restriction products were separated by 2% agarose gel electrophoresis and the gels were stained in 0.5 mg/ml ethidium bromide and were documented using Alpha Imager 1220 (Alpha Innotech, CA, USA).

For flanking markers used for recombinant selection, about 5 Mb region on each side of the *Sub1* region was targeted. Microsatellite markers were identified from the reported 20 BACs flanking the *Sub1* locus (IRGSP 2005). New primer pairs for the reported microsatellite motifs and new microsatellite markers were designed for the flanking regions that were not represented in the reported microsatellite markers database. Polymorphism between the two donors and five recipients was surveyed using these markers.

Background selection

Microsatellite markers unlinked to *Sub1* covering all the chromosomes including the *Sub1* carrier chromosome 9, that were polymorphic between the two parents, were used for background selection to recover the recipient genome (Fig. 2). Based on the polymorphism information, initially evenly spaced microsatellite markers were selected per chromosome. At least three polymorphic microsatellite markers per chromosome were used. The microsatellite markers that revealed fixed (homozygous) alleles at non-target loci at one generation were not screened at the next BC generation. Only those markers that were not fixed for the recurrent parent allele were analyzed in the following generations. For the selected plants from BC_2F_2 and BC_3F_2, an additional 32 microsatellite markers were used to check the fixation of the recipient genome.

Screening for submergence tolerance

Submergence screening was performed in the greenhouse at the International Rice Research Institute, Los Baños, Philippines, following standard protocols (Xu et al. 2000). Seeds of the selected plants of BC_1F_2, BC_2F_2 and BC_3F_2 along with parents and susceptible check IR42 were germinated in rows in 20 cm × 15 cm × 10 cm trays. Fourteen-day-old seedlings were submerged for 14 days. The survival of plants was scored 14 days after de-submergence (calculated as a percentage) for confirmation of the presence of the *Sub1* locus.

Results

Foreground and recombinant selection

In each backcross generation (BC_nF_1), the target locus *Sub1* was monitored by markers linked to the *Sub1* genes (Fig. 1). Individual BC_nF_1 plants were first selected based

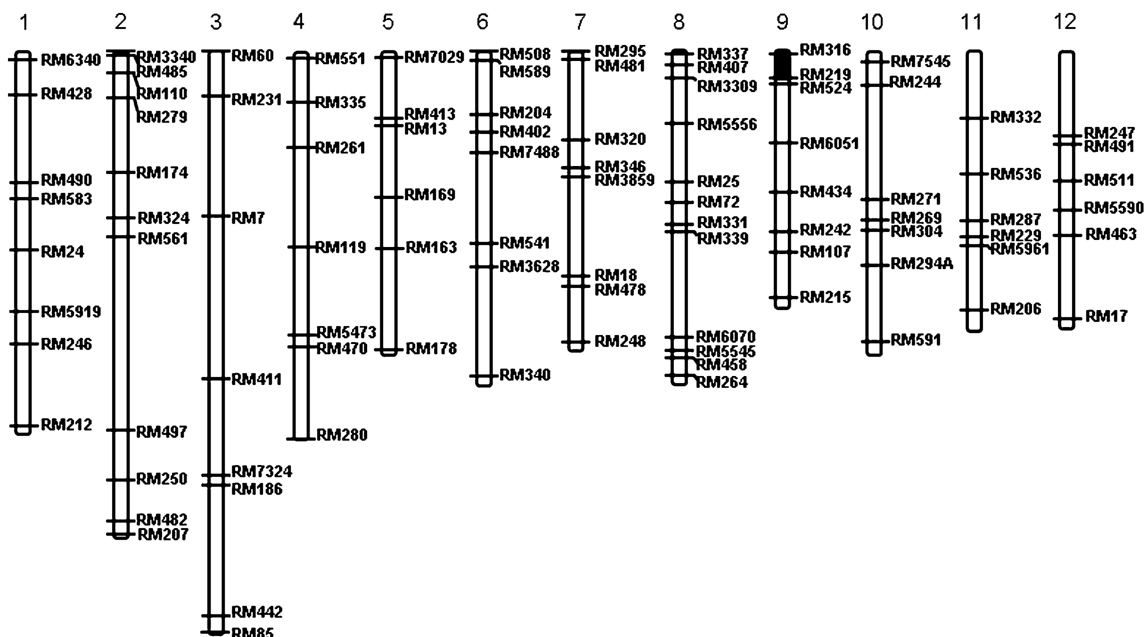

Fig. 2 Graphical genotype of the Swarna-*Sub1* (BC_2F_2) plant that is homozygous for the recipient genome except for the *Sub1* region on chromosome 9. The tip of chromosome 9 containing *Sub1* introgressed from IR49830-7 is shown in *black*. The distances were represented based on published map of Temnykh et al. (2001)

on the heterozygous nature of all the target loci at *Sub1* region. Only a few such selected individuals (Fig. 1) that had the least donor alleles of the background markers were chosen to be backcrossed with Swarna. In advanced backcrosses and selfed generations, marker RM7481/RM23887 with newly developed primers tightly linked with *Sub1* was also used.

In the cross of Swarna with IR49830-7, nine markers on one side (with average distance between two markers of 493.3 kb) and nine markers (with average distance between two markers of 403.6 kb) on the other side were used as flanking markers (Fig. 3). In conjunction with background section (see below), the *Sub1* carrier chromosome 9 of a few selected individuals, including plants Nos. 227 and 246 in BC_2F_1, plant No. 246-237 in BC_2F_2, plant No. 227-9 in BC_3F_1 and plant No. 227-9-407 in BC_3F_2, was characterized with three markers for foreground selection (RM464A, RM7481/RM23887 and SSR1 and 18 flanking markers; Fig. 3). When the selected plants of BC_2 (plants Nos. 246 and 246-237) were screened with these 21 markers, the alleles of markers from RM23668 (600952bp) through RM7481/RM23887 (6540629bp) were of the donor (IR49830-7) type, and the alleles of all the remaining markers from RM23917 (7259947bp) to RM24070 onwards were of Swarna origin (Fig. 3), indicating that these plants were single recombinants. However, screening of the selected BC_2F_1 plant No. 227 and BC_3F_2 plant No. 227-9-407 with the 18 markers showed that the alleles of markers in an interval from RM23668 (600952bp) to RM23778 (3912968) were of Swarna origin, and that the alleles of markers from RM23788 (4235905bp) to RM7481/RM23887 (6540629bp) including SSR1 and RM464 were of IR49830-7 type, and that the alleles of the remaining markers from RM23917 (7259947bp) to RM24070 onwards were the same as those in the BC_2 plants, i.e. of Swarna origin (Fig. 3), indicating that the selected BC_3 plants were double recombinants. Based on physical positions of these markers in the Nipponbare genome, the segment size of introgressed donor *Sub1* region into Swana was estimated to be 2.3–3.4 Mb for the selected double-recombinant plants (plant No. 227-9-407 and its descendents) in BC_3F_2 and BC_3F_3 generations, and 6.5 Mb for the selected plant (plant No. 246-237) in BC_2F_2 (Fig. 3).

Background selection

A total of 56 microsatellite markers (in addition to the flanking marker RM219 and others from chromosome 9) were used for background selection in 20 BC_1F_1 plants resulting from foreground and recombinant selection (Figs. 1, 2). The average distance between adjacent markers ranged from 11.6 cM (chromosome 12) to 33 cM (chromosome 3; Fig. 2). Among these the percent of markers homozygous for the recipient allele ranged from 39.3 to 67.9% (data not shown). In the BC_1F_1 plant No. 242 (Fig. 1), 38 out of 56 markers (67.9%) were of recipient type. The contribution of the recipient genome to the 11 individual

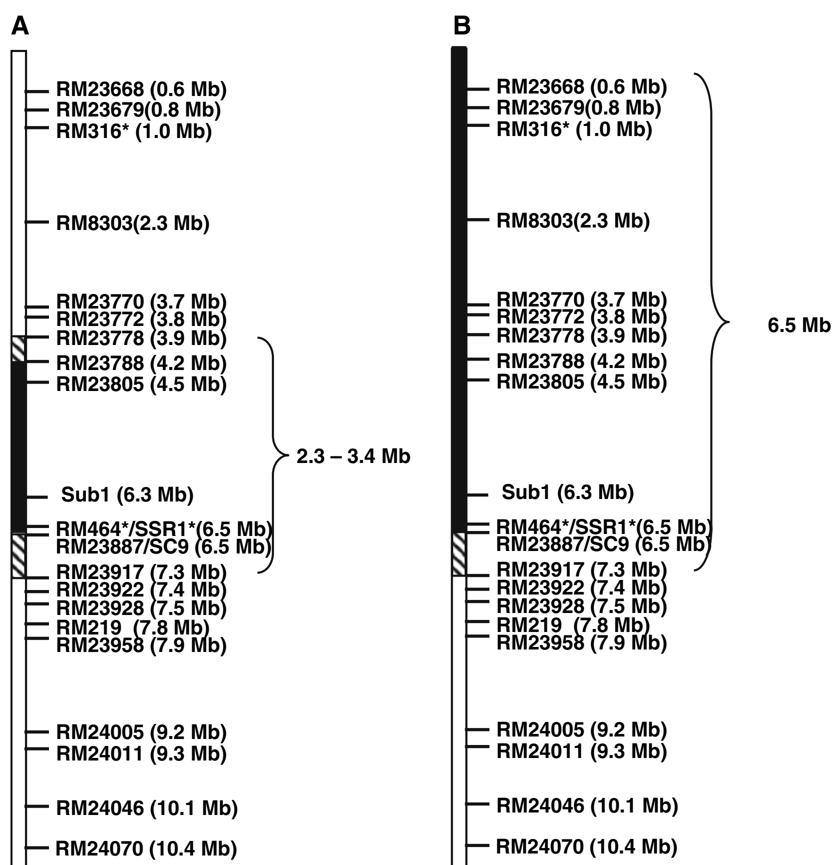

Fig. 3 Details of the introgressed fragment containing *Sub1* in: **a** selected BC_3F_2 plant (No. 227-9-407) and **b** selected BC_2F_2 plant No. 246-237. Marker position is represented in Mb according to Nipponbare sequence information are given in parentheses. The markers used initially are marked by an asterisk (*). The region containing *Sub1* introgressed from IR49830-7 is shown in *black*. The *diagonal boxes* indicate where recombinant breakpoints occurred

non-*Sub1*-carrier chromosomes varied widely in this selected plant. Chromosomes 1, 8 and 11 were completely of the recipient type, whereas the remaining ranged from 0% (chromosome 12) to 83.3% (chromosome 3).

In BC_2, five new markers (RM428, RM551, RM304, RM591 and RM491) were added to the 18 heterozygous markers of BC_1. Based on the foreground and background selection, two BC_2F_1 plants (Nos. 246 and 227) were selected. For plant No. 246, chromosomes 2, 5, 6, 10 and 12 were of complete recipient type. In the remaining non-carrier chromosomes the recipient genome ranged from 80 (chromosome 4) to 83.3% (chromosome 3). Overall, 94.2% of the markers of this plant were from the recurrent parent. The second plant (No. 227) had the complete recipient genome origin for chromosomes 2, 5, 6, 8, 11 and 12. In the remaining non-*Sub1*-carrier chromosomes the recipient genome ranged from 66.6% (chromosome 7) to 83.3% (chromosome 3). Overall, 87.2 % of the genome of this plant was from the recipient. For the selected plant of the BC_2F_2, No. 246-237, all the non-*Sub1*-carrier chromosomes were of the recipient type.

In the BC_3, for the selected plant No. 227-9, all the non-carrier chromosomes were completely of the recipient type except for chromosomes 7 and 10. In these two chromosomes, the recipient genome ranged from 75 to 83.3%. The overall recipient genome percentage for non-carrier chromosomes in this BC_3F_1 plant was 96.2%. In a selected BC_3F_2 plant 227-9-407, all the non-carrier chromosomes were of the recipient type (Fig. 3). Thirty-two more microsatellite markers were used to fill the gaps between the selected markers in the BC_2F_2 and BC_3F_2 generations, and all these markers showed the recipient type in the selected plants No. 246-237 (BC_2F_2) and No. 227-9-407 (BC_3F_2).

Seed from the BC_2F_2 selection was available earlier and most of the characterization of submergence tolerant plants was done with this selection, which we designated "Swarna-*Sub1*". Fourteen-day-old seedlings of selected plants of BC_1F_2, BC_2F_2 and BC_3F_2 were scored as tolerant and survived the 14 days submergence confirming the introgression of the *Sub1* locus whereas the susceptible check IR42 and non-introgressed Swarna showed zero survival (data not shown). In a replicated test, Swarna-*Sub1* showed a survival percentage slightly below that of the tolerant cultivar FR13A but significantly higher than the intolerant parent and check (Table 1). The Swarna-*Sub1* plants appear identical to the recurrent parent Swarna in important characteristics, such as grain quality and yield. In non-submerged experiments both Swarna and Swarna-*Sub1* showed no significant difference in grain yield (5011 and 5285 kg/ha, respectively). Agronomic traits measured from an unreplicated test were very similar (see ESM). Data from both cultivars showed similar grain yield (four replicated trials)

Table 1 Submergence tolerance of Swarna-*Sub1* (BC_2 progeny) compared to that of the donors FR13A and IR49830-7, recipient variety Swarna, susceptible check IR42, and several widely grown cultivars

Variety or line	*Sub1* present	Submergence tolerance (percentage survival)[a]		
		Test 1	Test 2	Test 3
IR64	–			36.1
CR1009	–			27.3
TDK1	–			24.3
Samba mahsuri	–			20.1
IR42	–	3.1	11.8	5.6
Swarna	–	8.4	32.8	15.6
Swarna-*Sub1* (BC_2F_2 No. 237)	+	41.9	69.9	70.9
FR13A	+	49.9	81.2	
IR49830-7	+			85.7
LSD (0.05)		6.3	15.9	15.7

[a] Tests 1 and 2 based on five replications of screening of single row plots (7–40 plants per row) using 14-day-old seedlings (randomized complete block design). Seedlings were submerged for 10 days and scored for survival after 21 days. Test 3 based on three replications with an average of 43 seedlings per plot (minimum of 18 seedlings); 19-day-old seedlings were submerged for 14 days and scored 6 days after desubmergence

under normal conditions in a preliminary field evaluation in a separate experiment (Sarkar et al. 2006). However, one significant difference is the distinctive gold coloration of the hull in Swarna that is absent in the Swarna-*Sub1* plants of both BC_2F_2 (supplementary materials) and BC_2F_2 lines. This appears to be the result of linkage between the *Sub1* locus and the gene *Inhibitor of Brown furrows* (*IBf*) located in the same region on chromosome 9 (Ideta et al. 1995). We suspect that the *Sub1* donor (FR13A) also possesses the *IBf* gene. Fortunately, this gene did not affect the agronomic or quality characteristics of the advanced backcross lines.

Developing markers for converting other mega varieties

Out of six microsatellite markers designed that were tightly linked to the target locus, only two, RM8300 (SSR motif ACCATT) and RM7481/RM23887 (SSR motif ACCATTAT)' showed clear codominant pattern and differentiated all the recipients from the donors (see ESM). A number of the markers that were tightly linked to the target gene were null alleles, suggesting the possibility of deletions in the region (Xu et al. 2006). The CAPS marker, GnS2, designed based on the SNP in the *Sub1A* gene between the tolerant IR40931-26 and intolerant Teqing, amplified the expected fragment of 242 bp in both the donors IR49830-7, IR40931-33, and recipient varieties except for Swarna, which does not have *Sub1A*. On restriction with *Alu*I or *Pvu*II, the PCR products of Samba Mahsuri, CR1009, and IR64 were cleaved into two fragments 132 and 110 bp, whereas the products of IR49830-7, IR40931-33, and Thadokkham 1 were not cleaved. Therefore, this CAPS marker could be used for monitoring the introgression of *Sub1* from the donors into most of the selected mega variety recipients, with the exception of Thadokkham 1.

Swarna has Taichung 65, a japonica variety, in its pedigree. The high level of polymorphism between Swarna and the other mega varieties for the *Sub1*-linked markers (see ESM) and the absence of the *Sub1A* fragment in Swarna unlike the other indica type mega varieties of the present study, suggests that Swarna probably contains a japonica type chromosomal segment (Xu et al. 2006) in this region of chromosome 9.

Many hypervariable polymorphic microsatellite markers comprising both class1 repeats and complex repeats were either identified from the microsatellite database or designed from the genome sequence that can be used to monitor donor introgressions across various recipients (see ESM).

Discussion

The present study clearly demonstrated that the conversion of the mega variety Swarna, which is grown on around 5 million ha in India and on additional areas of Bangladesh, to submergence tolerant within a two year time span for the BC_2 and 2.5-year-time span for the BC_3. To the best of our knowledge, this is the first report of the introgression of a QTL with the specific aim of reducing the size of a donor segment using tightly linked flanking markers (i.e. recombinant selection). Takeuchi et al. (2006) introgressed different heading date QTLs into the cultivar Koshihikari; however, no details were provided on recombinant selection and the sizes of the donor regions were not clearly reported. Previous research studies have reported the introgression of QTLs associated with submergence tolerance (Siangliw et al. 2003; Xu et al. 2004; Toojinda et al. 2005); however, the extent of the size of the donor chromosomal segment was not monitored. In addition, this study demonstrates the practical use of molecular markers for the introgression of the *Sub1* QTL into important varieties grown in areas prone to submergence stress. The MAB strategy has thus been shown to be an effective means of utilizing QTLs with large effects in rice breeding programs.

Initially the *Sub1* locus was monitored by markers shown to be closely linked with the gene (Xu et al. 2000, 2004). Using tightly linked (RM464A) and flanking (RM219, RM316) markers, as suggested by Hospital and

Charcosset (1997), ensured efficient foreground and recombinant selection. In advanced backcrosses and selfed generations, newly developed markers from the *Sub1* region were used for the target locus. With the availability of the rice genome sequence, polymorphic microsatellite markers were designed from the same BAC clone (AP005907) harboring the *Sub1* genes (Xu et al. 2000, 2006), leading to the development of six markers for target locus (RM444, RM464, RM464A, SSR1 (Xu et al. 2006), RM8300 and RM7481/RM23887. These microsatellite markers could be very useful in addition to gene-based markers for the introgression of the *Sub1* locus in a wide range of recipients.

Realizing the importance of the flanking markers for recombinant selection in the reduction of the linkage drag, a series of microsatellite markers were either identified from reported microsatellite marker databases or designed from the sequence information with average distance between two markers ranging from 561 to 1,250 kb (IRGSP 2005), so that the transfer of the donor segment was precisely monitored. Particular emphasis was placed on recombinant selection in this study because of the desire to recover all the important traits of the recipient variety, and minimize the effects of linkage drag from the *Sub1* donor, which possesses several undesirable agronomic characters. With the identification of closely linked markers in the target region, recovery of double recombinants with a relatively small donor fragment was possible. Most strongly submergence tolerant cultivars possess this same major gene (*Sub1A)* at this locus (Xu et al. 2006). Therefore, the series of markers identified in the *Sub1* region can be used across different donors and recipients as a Marker-Assisted Selection (MAS) 'kit' for the submergence locus.

A donor fragment of 6.5 Mb was introgressed in the BC_2F_2 and 2.3–3.4 Mb in BC_3F_2 (Fig. 3) of the Swarna crosses. From 389 Mb of Nipponbare sequence (IRGSP 2005), this represented 1.67 and 0.59% of the genome, respectively. In the selected plant of BC_2F_2, the tip of chromosome 9 was introgressed. This was because the population size of the BC_2F_1 was perhaps too low (320) to obtain the recombinant and eliminate background segments at the same time. Even with an introgressed fragment of less than 3 Mb there is potential for linkage drag to affect the phenotype of the resulting plants. A chromosome fragment of 2 Mb could contain 200 genes. However, the result must be compared to conventional backcrossing where introgressed fragments can easily be 50 cM or more (Young and Tanksley 1989; Salina et al. 2003). Reducing the length of the introgressed fragment further than achieved here would be expected to reduce the number of introduced genes. This is a bit costly in terms of labor to produce more backcross seeds and expense of genotyping. However, a reduction in the cost of marker genotyping would make it feasible to screen a larger population size for identifying the desired recombinant. The grain quality parameters in the *Sub1* lines were on par with the non-introgressed Swarna (see ESM), but there was inhibition of brown furrows in the seed coat of *Sub1* introgressed Swarna, yielding plants with straw-colored hulls instead of the golden hull color of Swarna. This hull coloration difference has an advantage in allowing researchers and farmers to easily distinguish the submergence-tolerant version of Swarna from the original. Nevertheless, this clearly illustrates the potential effects of linkage drag since the phenotype was very prominent. Young and Tanksley (1989) first proposed the idea of reducing the size of donor fragments containing target loci: they suggested that, using 1 cM flanking markers on each side of a target locus, the size of the introgressed segment could theoretically be 2 cM in two generations, in comparison with traditional backcross breeding where it would be expected to require 100 BC generations to obtain such a small segment.

By using markers for background selection, there was a great acceleration of recipient genome recovery in the present study. The general conclusion was that a few well-placed markers (two to four markers on a chromosome of 100 cM) provide adequate coverage of the genome in backcross programs (Visscher et al. 1996; Servin and Hospital 2002). For Swarna, an average of eight markers was used per chromosome and the average distance between two markers was 19.3 cM. The best plant had 67.9% of the recipient genome by BC_1F_1, 87.2-–94.2% by BC_2F_1 and 96.2% by BC_3F_1. Therefore, an average distance between markers of 20 cM and a minimum of four markers per chromosome as recommended from MAB simulation studies (Hospital et al. 1992; Hospital 2003; Servin et al. 2004) appears to be sufficient for the accelerated recovery of the recipient parent genome, although the use of a higher density of markers would ultimately confirm this prediction.

In recent years, considerable research in developing and optimizing MAB schemes has been investigated by using computer simulations. Particular attention has been given to reducing marker data points (MDPs) by determining minimum population sizes required for recombinant selection (Frisch et al. 1999b; Hospital 2001) and appropriate population sizes, ratios and selection strategies for background selection (Hospital et al. 1992; Visscher et al. 1996; Frisch et al. 1999a). The results in this study, which are actual experimental data, are consistent with the results from simulation studies that two or three BC generations may be saved by using markers compared to conventional backcrossing (Frisch et al. 1999a).

Frisch et al. (1999b) indicated that relatively small population sizes can be used for recombinant selection. However, in this study relatively large BC_1 and BC_2 populations were produced because of the small distances between target locus and tightly linked flanking markers and the desire

to have a higher probability of identifying recombinants between *Sub1* and flanking markers at the early BC generations. Simulation results from Hospital (2001) indicated that MDP for recombinant selection could be reduced by using additional BC generations. This approach was not adopted because the main objective of the project was to reduce the timeframe of the backcrossing scheme.

The number of major QTLs that have been identified for agronomically important traits is growing rapidly and many of them are being fine mapped (Ashikari et al. 2005; Ren et al. 2005). With the availability of large numbers of microsatellite markers, high throughput genotyping and fine mapping of the major QTLs, virtually any major QTL can now be introgressed into a variety without changing the desirable agronomic characteristics. Thus, the present study also serves as a case study of a strategy for introgression of a major QTL into a set of popular varieties.

Breeding improved submergence tolerant cultivars has been going on for more than three decades (HilleRisLambers and Vergara 1982; Mackill 1986; Mohanty and Chaudhary 1986). However, improved cultivars have generally not been adopted by the farmers in submergence-prone areas. The development of submergence-tolerant versions of popular varieties with high grain quality and wide adaptability offers a model for an alternative to the time-consuming, labor-intensive task of developing new varieties in a conventional crossing program. Adoption of a completely new variety could take considerable time, whereas the chances of acceptability of converted popular varieties are relatively higher (Mackill 2006). The evaluation of these new varieties should be streamlined because the comparison is between the new version and the original mega variety, which is already accepted widely by farmers.

In summary, we have developed an enhanced mega variety of rice by using a marker assisted backcrossing approach to incorporate submergence tolerance, which was controlled by a major QTL. The recovery of the recipient parent genome was greatly accelerated emphasizing the increased efficiency of using markers to assist selection of backcross lines. More importantly given the agronomic characteristics of the donor parent, the size of the donor chromosomal segment containing the target locus was reduced to ensure that there were minimal changes to the genetic composition of the recipient mega variety. This practical example of marker-assisted selection clearly illustrates the superiority of using MAB compared to conventional backcrossing because obtaining such a small donor region within only a few backcross generations would be impossible using conventional methods. This approach is currently being used to enhance several other rice mega varieties for submergence tolerance in rice breeding programs.

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