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# A high-resolution linkage map of the vicinity of the rice submergence tolerance locus *Sub1*

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**Abstract** Resistance to submergence stress is an important breeding objective in areas where rice cultivars are subjected to complete inundation for a week or more. The present study was conducted to develop a high-resolution map of the region surrounding the submergence tolerance gene *Sub1* in rice, which derives from the Indian cultivar FR13A. Submergence screening of 8-day-old plants of $F_3$ families kept for 14 days submerged in 60 cm of water allowed an accurate classification of *Sub1* phenotypes. Bulked segregant analysis was used to identify AFLP markers linked to *Sub1*. A population of 2950 $F_2$ plants segregating for *Sub1* was screened with two RFLP markers flanking the *Sub1* locus, 2.4 and 4.9 cM away. Submergence tolerance was measured in the recombinant plants, and AFLP markers closely linked to *Sub1* were mapped. Two AFLP markers cosegregated with *Sub1* in this large population, and other markers were localized within 0.2 cM of *Sub1*. The high-resolution map should serve as the basis for map-based cloning of this important locus, as it will permit the identification of BAC clones spanning the region.

**Key words** Submergence tolerance · Flooding · Amplified fragment length polymorphism (AFLP) · Fine-scale mapping · Rice

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## Introduction

In contrast to other crop species, rice is well known for its ability to grow in flooded soil. However, most rice cultivars cannot survive if the plants are completely submerged for more than 7 days (Palada and Vergara 1972; Adkins et al. 1990). At least 16% of the world's rice area, or over 22 million ha (excluding China), is frequently subjected to short-term submergence (Mackill et al. 1996a; Setter et al. 1997). An estimated 1 million ha of rice are lost every year due to submergence stress (Setter et al. 1989), and the total economic loss in South and Southeast Asia may exceed $600 million per year (Herdt 1991). Because of the sensitivity of rice and the prevalence of the stress, submergence tolerance has been an important breeding objective for decades in rain-fed lowland areas of Asia (Richharia and Misro 1960; Mackill 1986). The use of deep-water strategies for weed control in direct-seeded rice has also generated interest in submergence tolerance for rice production in temperate zones (Williams et al. 1990).

Submergence tolerance was previously considered to be a quantitative trait under the control of several genes (Suprihatno and Coffman 1981; Mohanty and Khush 1985; Haque et al. 1989). The use of molecular markers, however, indicated that a single submergence tolerance locus, *Sub1*, controls most of the phenotypic variation in an $F_2$ population derived from a submergence-tolerant *indica* line, IR40931-26 (which derived its tolerance from the Indian cultivar FR13A), and a susceptible *japonica* line, PI543851 (Xu and Mackill 1996). This locus was mapped to rice chromosome 9, in an interval spanning approximately 11 cM between two RFLP markers, RZ698 and C1232. Nandi et al. (1997) confirmed the importance of *Sub1* for submergence tolerance in FR13A, and identified four loci with much smaller effects.

Recent positional cloning experiments have been highly successful in the model plant species *Arabidopsis thaliana* (Giraudat et al. 1992; Chang et al. 1993; Leyser

et al. 1993; Bent et al. 1994; Mindrinos et al. 1994), and in model crop species such as tomato (Martin et al. 1993; Loh and Martin 1995; Milligan et al. 1998; Ling et al. 1999) and rice (Song et al. 1995; Yoshimura et al. 1998; Ashikari et al. 1999; Wang et al. 1999). The approach has been successfully applied in non-model crops, such as wheat (Lagudah et al. 1997), barley (Buschges et al. 1997; Lahaye et al. 1998; Schwarz et al. 1999), sugar beet (Cai et al. 1997) and lettuce (Meyers et al. 1998). Most of the genes cloned in this way confer pest resistance, and the detection of common motifs has allowed additional resistance-gene analogs to be mapped in many species (Kanazin et al. 1996; Chen et al. 1998; Collins et al. 1998; Spielmeyer et al. 1998). On the other hand, positional cloning of genes that confer resistance to abiotic stresses has not been successful, owing to the more complex inheritance of these traits and the difficulties encountered in accurately measuring them. It is therefore not as easy to use a candidate gene approach for identifying such genes.

A high-resolution genetic map is essential for the successful isolation of a gene of interest through map-based cloning. Characterization of the *Sub1* gene(s) should lead to a better understanding of the mechanism of rice submergence tolerance, and facilitate introduction of *Sub1* into susceptible rice cultivars through transformation. The cloned *Sub1* gene would also allow its introduction into other crop species that are grown in areas where flooding causes yield loss. In our previous work (Xu and Mackill 1996), *Sub1* was reported as a major QTL that was localized to a 11-cM interval, but individual plants could not be classified as being tolerant or susceptible. Here we report the construction of a high-resolution linkage map in the vicinity of this locus. The map was assembled by analyzing an $F_2$ population comprising 2950 individuals. The 11-cM region in the vicinity of *Sub1* was enriched for markers using the AFLP technology (Vos et al. 1995) and the bulked segregant analysis (BSA) method (Michelmore et al. 1991).

## Materials and methods

### Plant materials

Two $F_2$ populations segregating at the *Sub1* locus were developed for this study: The first, as described previously, was a small population (169 individuals) derived from a cross between IR40931-26, a tolerant *indica* line, and PI543851, a susceptible *japonica* line, in which *Sub1* was initially mapped to chromosome 9 (Xu and Mackill 1996). IR40931-26 derived its tolerance from the Indian cultivar FR13A. The second mapping population comprised 2950 individuals obtained from a cross between DX18-121 (a tolerant $F_3$ plant from the first population) and M-202 (a submergence-susceptible *japonica* cultivar that is widely used in California). Tillers of a single $F_1$ plant from this cross were split to produce 20 propagules for increased seed production. These plants were grown under isolated conditions to prevent outcrossing. The genotype of the $F_1$ plant was verified using *Sub1*-linked RAPD markers found in our previous studies. The 169 plants of the first population and a random subset (178 plants) of the large population were used to saturate the target region with AFLP markers. The approximate map position of the AFLP markers identified was determined using the subset, and the high-resolution map was developed with the entire large population.

### Submergence screening

Because of its destructive nature, submergence screening was performed on the $F_3$ families rather than on the $F_2$ individuals. Submergence tolerance for the first population was scored quantitatively as means of individual $F_3$ plants (Xu and Mackill 1996). A total of 565 $F_3$ families, including 178 families from each $F_2$ plant in the subset of the large population and 387 recombinants identified from the large population using the two RFLP markers RZ698 and C1232, were screened under complete submergence as described by Xu and Mackill (1996), with some modifications. Briefly, 40–60 pre-germinated seeds per $F_3$ family were sown in four replications (pots). Plants were completely submerged in water (to a depth of approximately 60 cm) for 14 days, beginning 8 days after seeding, in tanks in a greenhouse. The plants were then allowed to recover for 7 days. Submergence tolerance for each $F_3$ family was scored twice: once immediately after the water was drained off and once after the 7-day recovery period, and classified into three categories: S (susceptible), T (tolerant) and H (segregating). For any families with ambiguous results, submergence screening was repeated at least one more time. A series of seven submergence experiments were carried out to complete the classification of the 565 $F_3$ families in the large population in the summers of 1996 and 1998. Three parental lines, IR40931-26, DX18-121 and M-202, were used as controls throughout the experiments.

### Bulked segregant analysis (BSA) and AFLP analysis

DNA was isolated from ground fresh leaves as described by Redoña and Mackill (1996). Four DNA bulks, two for each phenotype consisting of the first and second five most (or least) tolerant lines, were formed from the first population as well as the subset. This slight modification of BSA, i.e. using four bulks instead of two, is based on our previous experience: too many false positive bands were generated in the course of identifying RAPD markers linked to *Sub1*, where only two bulks were used.

The procedure of Vos et al. (1995) was followed to assay AFLPs in the two tolerant and two intolerant bulks in order to identify markers linked to the *Sub1* locus. The DNA bulks (around 100 ng from each plant) were cleaved with rare-cutter (*Eco*RI) and frequent-cutter (*Mse*I) restriction endonucleases, then ligated with an *Mse*I adapter and an *Eco*RI adapter. Selected *Mse*I-*Eco*RI fragments were amplified with primers that matched the adapter and contained an additional three nucleotides at the 3′ end, and the fragments were separated on denaturing polyacrylamide gels. Polymorphic AFLPs between the tolerant and the susceptible bulks were verified using the individuals contributing to the bulks in each population. After the linkage between individual AFLPs and *Sub1* was confirmed, the AFLPs were applied to the entire subset, and some of them were used to test all the recombinants found in the large population.

### Cloning of AFLP markers

AFLP markers that were tightly linked to the *Sub1* locus were cloned into the pCR2.1 vector (Invitrogen). Each target AFLP band from at least eight lanes loaded with the same sample (3 μl per lane) was identified and excised from a 4.5% polyacralamyde gel stained using a silver staining kit (Promega). The gel slices containing the bands were then chopped into small pieces and eluted overnight in 500 μl of water in a 1.5-ml tube at 4 °C. An aliquot (5 μl) of the eluted solution was used as template DNA and amplified using its corresponding selective AFLP primers (+3) in a 20-μl PCR. The resulting PCR products were directly ligated into

the pCR2.1 vector, following the manufacturer's protocol, and used to transform One-Shot competent cells (Invitrogen), which were plated on LB medium containing kanamycin (50 μg/ml) to select for the plasmid. At least 10 white colonies for each insert-ligation were selected and their plasmids were prepared using alkali lysis as described in Sambrook et al. (1989). The plasmids were digested with *Eco*RI and the digests were run on a 2% agarose gel. Positive clones bearing inserts as expected were then identified by sizing the insert bands stained with ethidium bromide.

### Development of RFLP markers from AFLP bands and their use in RFLP analysis

RFLP markers were derived from the cloned AFLP bands. Each AFLP fragment insert was amplified from the recombinant pCR2.1 plasmids using its corresponding AFLP primers, and run on a 1.2% LMP agarose gel (Gibco-BRL). Probe DNA samples were purified from gel slices containing the AFLP fragments. If the probe DNA fragments were larger than 200 bp, they were purified using Wizard PCR Preps (Promega) following the supplier's instructions. Otherwise, they were collected with an Ultrafre-MC 0.45 μm filter unit (Millipore) after centrifugation, and used without further purification. The gel slices could be repeatedly frozen, thawed and centrifuged to obtain more DNA solution.

Membranes were prepared as described in Redoña and Mackill (1996). A total of 20 restriction endonucleases were used to digest the DNA samples from the parents (DX18-121 and M-202) of the large population to prepare screening membranes. Progeny membranes were prepared when a polymorphic probe-enzyme combination was found. Hybridization was performed in a hybridization incubator (Model 310, Robbins Scientific), and detected using the procedure supplied with the ECL direct nucleic acid and labeling detection system (Amersham), modified as follows. Approximately 50 ng of probe DNA in 10 μl of TE or water was boiled for 6 min. and cooled immediately on ice. Then 10 μl of Labeling Reagent (NIF819) and 10 μl of glutaraldehyde (NIF820) was added to the denatured probe and mixed well. The labeling reaction was carried out for 20–30 min at 37 °C in a water bath. Prior to hybridization, a membrane (10 × 20 cm) was prehybridized in a glass bottle (35 × 300 mm, Robbins Scientific), with the non-DNA side of the membrane against the bottle wall, at 42 °C for about 1–3 h in 8 ml of ECL gold hybridization buffer (Amersham), prepared according to the supplier's instructions. Calculation of the ratio of membrane area to the amount of buffer was found not to be necessary. The labeled probe, in a volume of 30 μl, was than directly added to the bottle containing the prehybridized membrane. Hybridization was allowed to proceed overnight in the incubator at 42 °C with rotation. The membrane was washed using primary wash solution without urea (0.4% SDS, 0.5 × SSC) and secondary wash solution (2 × SSC), and hybridized bands were detected using the mixture of Detection Reagent 1 and Detection Reagent 2 as recommended in the manufacturer's protocol. However, a large volume (20 l) of a 2× concentrated stock of the primary wash solution without urea was prepared, which could be stored for up to 3 months at room temperature. A 30-ml volume of the fresh mixture of Detection Reagent 1 and Detection Reagent 2 could be used to detect bands on more than five membranes (10 × 20 cm). As before, it was not necessary to calculate the ratio of membrane area to the amount of detection reagent as specified in the protocol. Hybridization signals were detected using either Fuji or Kodak films that were usually exposed for 10 min to 6 h depending on the strength of the signal. Normally, two films were exposed for each membrane or a set of membranes to obtain an optimal image.

### Linkage analysis

Segregation data for submergence tolerance score, AFLP and RFLP markers in the subset of the large population were analyzed using MAPMAKER 3.0 (Lander et al. 1987). In the entire large population, linkage analysis was performed only for markers closely linked to *Sub1*. As the size of the data set was above the limit for MAPMAKER 3.0, the map distances between markers and *Sub1* were calculated using Kosambi's mapping function (Kosambi 1944). The recombination values were estimated using either maximum-likelihood estimation (Allard 1956) when the recombination frequency was more than 1%, or the actual recombination frequency when the frequency was less than 1%. The number of recombinants was also used if the interval between two markers was extremely small. Crossover interference was ignored in this calculation.

## Results

### Screening of the large population for submergence tolerance

Two groups of $F_3$ families from the cross DX18-121 × M-202 were submerged – those derived from the subset of 178 $F_2$ individuals, and those from the 387 $F_2$ plants that were recombinant between the two RFLP markers RZ698 and C1232 in the large population. In the subset, 50, 79 and 49 $F_3$ families were classified as tolerant (T), segregating (H) and susceptible (S) types, showing a good fit to the expected ratio of 1:2:1 ($\chi^2 = 2.258$, $P = 0.32$). In the recombinants, the number of families in each class was 92, 200 and 95, also showing a good fit with 1:2:1 ($\chi^2 = 0.483$, $P = 0.79$). These results showed that the effect of *Sub1* was sufficient to allow scoring as a simple Mendelian factor. In nearly all cases, the classification into the three genotypes was obvious. Only 30 families gave slightly ambiguous results. Upon re-screening, they received the same score given during the first screen.

### Identifying AFLP markers closely linked to *Sub1* using BSA

A total of 900 AFLP primer combinations (+3/+3; 484 for the first population, 416 for the subset) were applied to the four bulks for each cross, and 106 positive bands were identified in BSA (Table 1). All the 106 positive bands were examined to confirm their linkage to *Sub1* using the individuals forming the bulks. This resulted in the identification of 24 that were closely linked to *Sub1*. These 24 bands were mapped in the subset using the computer program MAPMAKER 3.0 (Fig. 1A). The genetic length of the interval between RZ698 and C1232 in the subset was 7.3 cM, in contrast to 11 cM for the same interval in the first population. Of these 24 markers, three cosegregated with the RFLP marker RZ698, 10 cosegregated with *Sub1*, seven were located between C1232 and *Sub1*, and one lay between *Sub1* and RZ698 in the subset. Primer sequence and linkage phase of the 10 AFLP markers cosegregating with *Sub1* are presented in Table 2.

Ten bands not linked to *Sub1* were also found, which is a much lower percentage of false positives than the 95% observed in our previous work with RAPD markers (Xu and Mackill 1996). This indicated the superiority of AFLPs over RAPDs, as well as the increased

**Table 1** Numbers of AFLP bands associated with *Sub1* found using BSA analysis in two segregating populations

| AFLP parameter | Population | | Total |
|---|---|---|---|
| | DX18 (169 individuals) | DX202 (178 individuals) | |
| Primer combinations used | 484 | 416 | 900 |
| Approximate number of bands obtained | 14,500 | 12,500 | 27,000 |
| Positive bands in BSA | 47 | 69 | 116 |
| Tightly linked to *Sub1* | 3 | 8 | 11 |
| Linked | 24 | 27 | 51 |
| Linked, but not closely | 16 | 28 | 44 |
| Not linked | 4 | 6 | 10 |

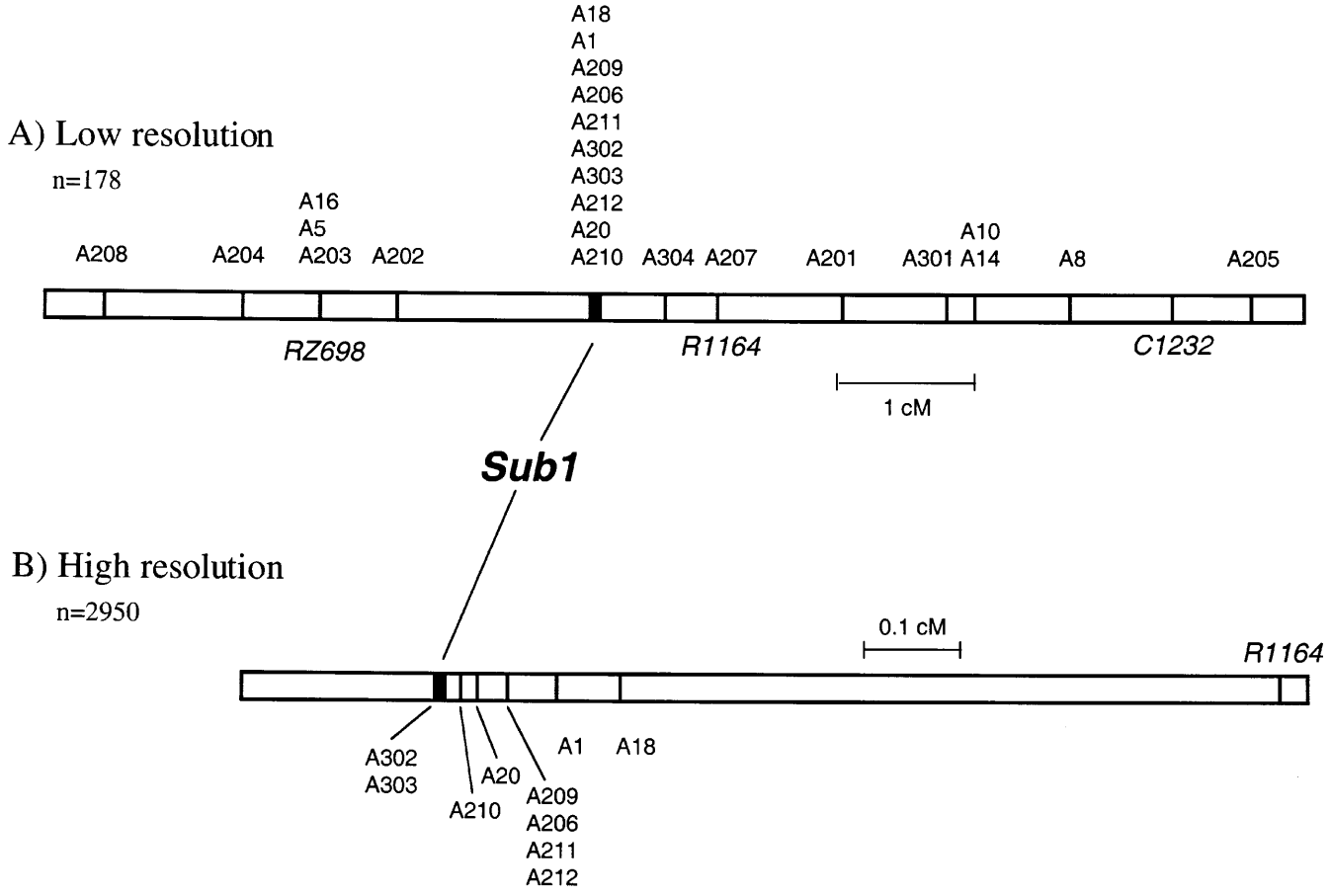


**Fig. 1** **A** Low resolution map of the *Sub1* region on rice chromosome 9, constructed using data for 178 individuals. **B** High-resolution map constructed with 2950 individuals. RFLP markers are shown *above* each map and AFLP markers *below*

reliability associated with the use of four bulks instead of two.

## Developing RFLP markers from AFLP bands

The 10 AFLP markers that cosegregated with *Sub1* on the low-resolution map (Fig. 1A) were cloned. Four of them were successfully converted into RFLP markers and designated AFLP20rf, AFLP209rf, AFLP211rf and AFLP303rf (other markers were named in a similar way). AFLP209rf, AFLP211rf and AFLP303rf were all low-copy-number, codominant markers, whereas AFLP20rf was a single-copy dominant marker. AFLP20rf, AFLP209rf and AFLP211rf were mapped at the same locations as their corresponding AFLP markers. However, AFLP303rf mapped at a distance of 1.4 cM from *Sub1* on the RZ698 side (Fig. 1A). This could have resulted if AFLP303rf was actually different from AFLP303 in DNA sequence. However, an alignment of the AFLP303 data set with that for AFLP303rf strongly suggested that AFLP303rf was the correct fragment from the corresponding AFLP (data not shown). Individuals without the AFLP303 band were completely identical to those tolerant parental types at the AFLP303rf locus, and those with the AFLP303 band were either susceptible parent types or heterozygotes at the AFLP303rf

**Table 2** AFLP primer sequences and fragment sizes for bands cosegregating with *Sub1* in the low-resolution map

| Name[a] | Selective sequence for *Eco*RI ends at 3′ end | Selective sequence for *Mse*I ends at 3′ end | Fragment size (bp) | Linkage phase to tolerant *Sub1* allele |
|---|---|---|---|---|
| AFLP1 | 5′-AAC | 5′-AGC | 137 | Coupling |
| AFLP18 | 5′-TAA | 5′-GTA | 150 | Coupling |
| AFLP20 | 5′-ATC | 5′-GTA | 124 | Coupling |
| AFLP206 | 5′-AAT | 5′-GAC | 161 | Coupling |
| AFLP209 | 5′-ATA | 5′-TGC | 151 | Coupling |
| AFLP210 | 5′-GAA | 5′-TAG | 230 | Coupling |
| AFLP211 | 5′-ATC | 5′-GTG | 343 | Coupling |
| AFLP212 | 5′-GTA | 5′-TAG | 205 | Coupling |
| AFLP302 | 5′-TAT | 5′-ACA | 75 | Repulsion |
| AFLP303 | 5′-CAC | 5′-GAT | 140 | Repulsion |

[a] The *Mse*I-end adapter was 5′-GACGATGAGTCCTGAG annealed to TACTCAGGACTCAT-5′; the *Eco*RI-end adapter was obtained by annealing 5′-CTCGTAGACTGCGTACC to CTGACGCATGGTTAA-5′. Primers for the first PCR amplification were 5′-GTAGACTGCGTACCAATTC (for *Eco*RI ends) and 5′-GACGATGAGTCCTGAG (for *Mse*I ends). Primer sequences in common for the second PCR amplification: 5′-GACTGCGTACCAATTC- (for *EcoRI* ends, and 5′-GATGAGTCCTGAGTAA- (for *MseI* ends)

locus. For this particular RFLP there may have been some difficulty in classification because many plants designated as heterozygous had one weak band.

AFLP210rf has not been remapped due to lack of polymorphism between the two parents, although there were only two copies of the sequence in the rice genome. Identical DNA sequences from five of the six AFLP210rf clones indicated that the cloned AFLP210 (AFLP210rf) was the true AFLP210 fragment (data not shown). Markers AFLP1rf, AFLP18rf, AFLP206rf, AFLP212rf and AFLP302rf showed multiple DNA sequences, however, and could not to be remapped to the *Sub1* region.

## High-resolution mapping of *Sub1*

Construction of a high-resolution map of *Sub1* required identification of individuals that were recombinant between two flanking markers linked to *Sub1* from the large population, because only such plants would be informative in orienting other markers closer to the gene. Our initial intention was to convert the flanking AFLP markers (AFLP202 and AFLP204, Fig. 1A) into STS (sequence-tagged site) markers, but our attempts to do so were not successful. Eventually, the two RFLP markers RZ698 and C1232, which flank *Sub1* and are 2.4 and 4.9 cM away, respectively (Fig. 1A), were used to identify the recombinants in the large population. A total of 393 recombinants (including those from the subset), representing 407 recombination events, were identified by comparing the two marker genotypes for each plant (Table 3), and submergence tolerance was assessed for 387 of these. The marker segregation at both C1232 and RZ698 loci departed significantly from the expected ratio of 1:2:1 in the large population, although the distortion at RZ698 was not significant at $P = 0.01$ (Table 3). This result was mainly due to an excessive number of homozygous plants at the two loci carrying M-202 alleles found in a subgroup of 400 plants. These 400 plants were planted in the field and then transferred into the greenhouse, where they were maintained for a period of 8 months. Approximately 50 of these plants died before DNA could be extracted. The death of these 50 plants may explain the segregation distortion. With the 400 plants excluded, no segregation distortion was observed (data not shown).

**Table 3** Number of recombinants between the markers RZ698 and C1232

| C1232[a] | RZ698[a] | | | Total |
|---|---|---|---|---|
| | $A_1A_1$ | $A_2A_2$ | $A_1A_2$ | |
| $B_1B_1$ | 595 | 7* | 94* | 696 |
| $B_2B_2$ | 7* | 704 | 108* | 819 |
| $B_1B_2$ | 90* | 87* | 1258 | 1435 |
| Total | 692 | 798 | 1460 | 2950 |

[a] $A_1$ and $B_1$ indicate alleles from the DX18-121 line (tolerant); $A_2$ and $B_2$ alleles from the M-202 parent (susceptible). Recombinants are indicated by the *asterisks*. Marker segregation at both loci departed significantly from the expected 1:2:1 ratio, as indicated by the $\chi^2$ test. For C1232, the difference is significant at $P = 0.01$ [$\chi^2_{(1:2:1)} = 12.43 > P_{(0.01,df=2)} = 9.21$], for RZ698, the difference is significant at $P = 0.05$ [$\chi^2_{(1:2:1)} = 7.92 > P_{(0.05,df=2)} = 5.99$]

The 10 AFLP markers tightly linked to *Sub1* were assayed in the 393 recombinants. The resulting high-resolution map of the *Sub1* locus shows that eight of the 10 markers mapped to the C1232 side, two mapped with *Sub1*, and none mapped on the RZ698 side (Fig. 1B). The eight AFLP markers on the C1232 side were mapped within a distance of 0.2 cM (12 recombinants) from *Sub1* (Fig. 1B). The four RFLP markers derived from AFLP markers were also investigated using the 393 recombinants. AFLP20rf, AFLP209rf and AFLP211rf were again mapped to the same locations as their corresponding AFLP markers, and AFLP303rf showed the same trend as on the low-resolution map. In addition, R1164, an RFLP marker in the *Sub1* region from the Japanese map (Harushima et al. 1998), was also assayed on these recombinants and mapped at a distance of 1.2 cM from *Sub1* on the same side as C1232 (Fig. 1B).

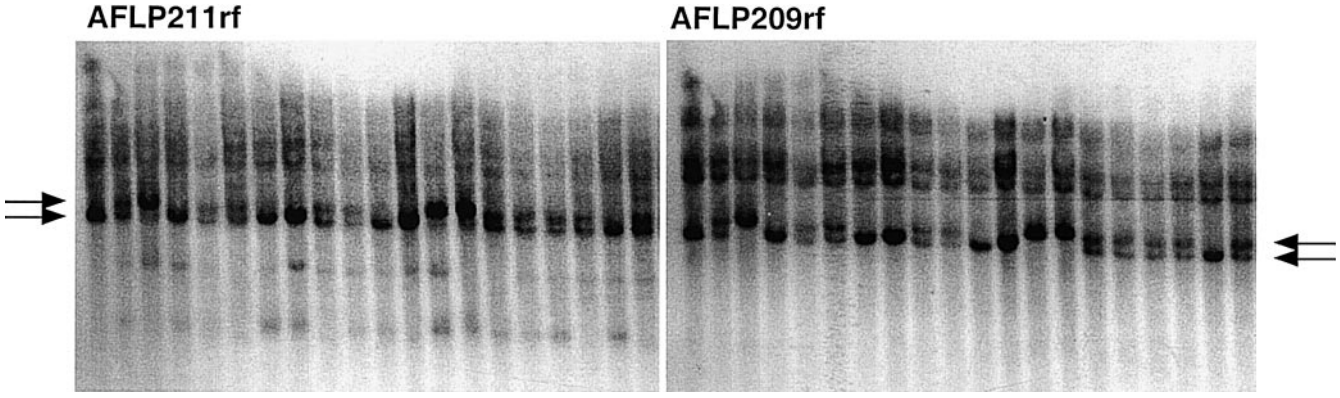


**Fig. 2** Estimate of the physical distance between the two RFLP markers AFLP209rf and AFLP211rf, converted, respectively, from AFLP209 and AFLP211, two cosegregating AFLP markers that are closely linked to *Sub1*. The *left panel* shows the results of a RFLP analysis, using the marker AFLP211rf, for recombinants between C1232 and RZ698. The DNAs were digested with *Xba*I. The *right panel* shows the results for the RFLP marker AFLP209rf on the same blot. The *arrows* show the two pairs of polymorphic bands detected by the two RFLP markers. The sizes of the corresponding bands were identical in both hybridizations, and were estimated at no larger than 5 kb

It is interesting to point out that AFLP209rf and AFLP211rf were linked very tightly not only in terms of genetic distance but also of physical distance. When they were surveyed using the same screening membrane, on which two parent DNA samples digested with 20 different restriction endonucleases were blotted, the band profiles detected by the two probes were quite similar. Two pairs of such bands with the same size, generated by the same restriction endonuclease, *Xba*I, were fortunately polymorphic for AFLP209rf and AFLP211rf, respectively. These two pairs of bands were all mapped to the same position in the large population as their corresponding AFLP markers. As the smaller band mapped was only approximately 5 kb long, the physical distance between the two markers may actually be less than 5 kb (Fig. 2).

## Discussion

In the past, submergence tolerance has been treated as a quantitative trait. In our previous work, we measured submergence tolerance quantitatively, but found that only one QTL explained nearly 70% of the phenotypic variation for the trait (Xu and Mackill 1996). A subsequent study by Nandi et al. (1997) confirmed the importance of *Sub1*, but identified four additional QTLs. Therefore, precise identification of the *Sub1* genotypes of the recombinants from the large population was a crucial requirement in developing the high-resolution map. This was accomplished by optimizing the submergence screen to emphasize the effect of *Sub1*, and making an additional cross to a susceptible *japonica* parent similar to the original susceptible parent used, equivalent to a backcross. In our screen we employed very young seedlings submerged in 60 cm of water. Shade cloth was used to increase the severity of the stress. Submergence tolerance could be assessed at two stages. Immediately after desubmergence, susceptible genotypes were highly elongated and whitish in color, while tolerant plants had moderate or no elongation and were green. After 7 days, susceptible genotypes had either turned white and died, or recovered very slowly, while tolerant plants recovered rapidly and continued to grow.

The use of a genetically more uniform backcross is thought to have removed or reduced the influence of loci with smaller effects, resulting in an unambiguous assessment of tolerance. This new population offered other advantages relative to the one previously used. The fertility of most $F_2$ plants in the first population was so low that it was difficult to generate sufficient $F_3$ seeds for submergence screening. In addition, transfer of the *Sub1* gene into the background of a widely grown California *japonica* cultivar facilitates evaluation of its effectiveness in weed suppression schemes in California (Williams et al. 1990). Distinguishing the homozygous tolerant and homozygous susceptible families was straightforward. Occasionally it was difficult to distinguish heterozygotes from the tolerant homozygotes. To eliminate this difficulty, at least 40 seedlings per $F_3$ family were screened. In heterozygous families, approximately one-fourth of the plants were expected to be susceptible. These plants could be easily observed among the 40 seedlings. A few families with an ambiguous score were re-screened to make sure all phenotypes were accurately recorded.

Based on our experience, $+3/+3$ AFLP primer combinations can generate an average of 30 bands among rice cultivars (Mackill et al. 1996b). Approximately one-third of the bands are polymorphic between *indica* and *japonica* cultivars. Presumably, therefore, a total of some 27,000 loci ($900 \times 30$), including 9000 polymorphic loci, throughout the rice genome were screened in this experiment. This would give an average of six polymorphic AFLPs per cM on the basis of a total length of 1500 cM for the rice molecular map (Harushima et al. 1998). Thus, we would expect to observe approximately 12 AFLP markers in the 2-cM interval

adjacent to *Sub1*. In this marker search process, 12 AFLP markers were indeed found within a 2-cM interval (AFLP304–A202) around *Sub1* (Fig. 1A). These results indicate that AFLP marker technology will be very useful in saturating or filling gaps in maps of specific regions of the rice genome.

The fragment size of the AFLP markers found in this experiment ranged from 75 to 343 bp. Such a small size limits the opportunities for developing STS markers, which are preferred in marker-assisted selection and other screening experiments. In fact, none of the 10 AFLPs were successfully converted into STS markers, because no polymorphic restriction endonucleases could be found. Some of the AFLPs cloned into vectors showed multiple sequences (data not shown). A possible reason for this observation is that a specific target band isolated from a silver-stained gel was actually a collection of multiple fragments. If this were true, we could not explain why the collection of multiple fragments were all segregating like a single band and linked to *Sub1* so tightly. Such multiple sequences of an AFLP band probably explained the low rate (40%) of successful development of codominant markers from the 10 AFLP markers. Out of the 10 AFLP markers, eight were linked to *Sub1* in coupling phase. There seemed to be a preference for amplification from the DNA sample of the tolerant parent. A similar phenomenon was also reported in a study that mapped the *R1* locus in potato (Meksem et al. 1995). This interesting observation regarding the AFLP technique remains to be explained.

The high-resolution map of *Sub1* was constructed using a large $F_2$ population (2950 plants, 5900 gametes), giving an average resolution for a genetic analysis of approximately 0.017 cM for codominant markers and 0.034 cM for dominant markers linked in coupling. Based on the size estimates for the rice genome of 1500 cM and 450,000 kb (Arumanagathan and Earle 1991; Harushima et al. 1998), two crossover breakpoints at a distance of 5 kb for codominant markers or 10 kb for dominant markers would be detectable with such a resolution. Based on the data presented here, we cannot draw any conclusions about the physical distance between these markers. It was observed, however, that the physical distance between AFLP209rf and AFLP211rf was less than 5 kb, while the genetic distance was zero. In addition, the accuracy of the map was strengthened by the codominant nature of *Sub1* attained through the submergence tolerance scoring system. The high-resolution map should, therefore, be suitable for choosing markers with which to screen BAC libraries for chromosome landing or chromosome walking. Clearly, to search for positive BAC clones, AFLP20rf, AFLP209rf and AFLP211rf would be the best candidates.

The region encompassing *Sub1* (from R1164 to R1687) on the most recent RGP map, where 2275 markers were included, harbors one of the largest gaps within the map (20.7 cM; Harushima et al. 1998). Such a gap could have resulted from a crossover hot spot underlying the region. If this were true, the ratio of physical distance to genetic distance in the *Sub1* region would be smaller than the average for the genome (300 kb per cM), and chromosome walking would also be easier in the *Sub1* region. The 10 AFLP markers closely linked to *Sub1* were, however, distributed unevenly in the 2-cM interval encompassing *Sub1*. On the RZ698 side, no markers have yet been found. This lack of markers closely linked to *Sub1* on one side might cause difficulties in constructing a BAC contig surrounding the gene. BAC clones mapping to this region, however, would be expected to provide markers closely flanking the *Sub1* locus. Provided crossovers are observed in this region, it should be possible to construct a contig spanning the locus.

Our recent studies indicate that the rice submergence tolerance locus is associated with inhibited leaf elongation (unpublished data of M. Carriere and K. Xu). This is thought to act by conserving carbohydrate reserves so that plants can rapidly recover upon desubmergence (Setter 1993; Setter and Laureles 1996). Submergence-tolerant cultivars exhibit markedly different patterns of gene expression following submergence compared to susceptible types (Umeda and Uchimiya 1994). Also, the map position of *Sub1* does not correspond to that of mapped enzymes associated with alcohol fermentation, such as PDC (Huq et al. 1999). The dramatic effect of this gene on what is essentially a quantitative trait suggests that it is a transcription factor or is involved in signal transduction in the response to submergence stress (Setter et al. 1997). For these reasons, the positional cloning approach is the ideal choice for isolation of this important gene.

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