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Mapping of Quantitative Trait Locus Related to Submergence Tolerance in Rice with Aid of Chromosome Walking

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Abstract

The major QTL for submergence tolerance was locate in the 5.9 cM interval between flanking RFLP markers. To narrow down this region, a physical map was constructed using YAC and BAC clones. A 400-kb YAC was identified in this region and later its end fragments were used to screen a rice BAC library. Through chromosome walking, 24 positive BAC clones formed two contigs around linked-RFLP markers, R1164 and RZ698. Using one YAC end, six BAC ends and three RFLP markers, a fine-scale map was constructed of the 6.8-cM interval of S10709–RZ698 on rice chromosome 9. The submergence tolerance and related trait were located in a small, well-defined region around BAC-end marker 180D1R and RFLP marker R1164. The physical-to-map distance ratio in this region is as small as 172.5 kb/cM, showing that this region is a hot spot for recombination in the rice genome.

Key words: rice (*Oryza sativa L.*); submergence tolerance; positional cloning; physical mapping; bacterial artificial chromosome (BAC); yeast artificial chromosome (YAC)

1. Introduction

Of 40 million hectares of rain-fed lowland rice (*Oryza sativa L.*) cultivation in South and Southeast Asia, approximately 50% are affected by flash flooding at various stages of growth. Submergence tolerance is characterized by the ability of rice plants to recover after flooding.[1] The *Sub1* was reported as a single locus controlling submergence tolerance.[2,3] To date, major QTL determining traits associated with submergence tolerance was coincidentally mapped in the same vicinity on rice chromosome 9 with minor and small-effect QTLs on rice chromosomes 2, 5, 7, 10, and 11.[4]

Positional cloning provides a promising method for isolating genes based on phenotypic variation and precise genomic locations. Two classic examples are the cloning of *Pto* in tomato and *Xa21* in rice.[5,6] These genes, which play roles in the signal transduction pathway, are responsible for resistance against bacterial pathogens. Cloning attempts of plant genes responsible for stress resistance to abiotic factors or natural occurrence have faced more challenges due to their complex interaction between genetic and environmental factors. To date, the major QTL controlling response to photoperiod in rice, *Hd1* that based on naturally occurring allelic variation had been cloned by means of positional cloning.[7] *Hd1* was speculated to affect gene expression at transcriptional level.[7] Submergence tolerance was thought to be mechanisms of reserving energy for rice plant to recover rapidly after desubmergence.[8,9] Because of the dramatic effect of the QTL, positional cloning is the method of choice for isolating genes associated with this major QTL.

The positional cloning strategy relies on an accurate position of the target gene on a genetic map. The tightly linked markers were used to isolate clones containing these markers from a large-insert genomic library, and finally to complement the recessive phenotype by transformation of candidate clones.[10] If the closest linked markers are located from a distance to the target gene, more markers are required to reach the target locus before chromosome walking steps begin.[11]

To facilitate a positional cloning strategy for submergence tolerance major QTL on chromosome 9, genetic mapping around these target genes was initiated. Unfortunately, only few markers were located in this region

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(S10709–R1687 region).[12] DNA markers needed to be filled for more precise analysis of the region. Enrichment of molecular markers to a specific chromosomal region has been done by several methods. The bulked segregant analysis (BSA) utilizes differential phenotypic pools to screen for tightly linked markers.[13,14] AFLP combined with phenotypic pooling also have been used successfully to isolate region-specific markers.[3,15,16] Furthermore, chromosome landing and pooled progeny techniques were used to enrich the DNA markers within the sub-cM region around the target gene.[17] Instead of segregating progenies, bulked line analysis (BLA) which using bulk of conventional lines sharing the same trait were developed to identify of DNA markers linked with the Rf gene of rice.[18] RFLP subtraction has been established to target a number of markers tightly linked to the *regA* locus of *Volvox carteri*.[19] Most of all, enrichment of markers near QTL that mapped to different locations using BSA is imprecise.

Recently, large-insert libraries developed for several species (http://www.genome.clemson.edu/groups/bac/) can be used to target markers to a specific region with more precision. Attempts to produce markers at chosen loci by subcloning YAC DNA have been reported for human, fungus, and rice.[20–24] Chromosome walking has been used to generate DNA markers from a specific genome region.[11,25,26] By using the closely linked marker to screen those large-insert libraries, contiguous clone formation around the genes can be done using available YAC, BAC or PAC clones. The end-probes from the physical map can provide more DNA markers for any chromosomal region. Here, we report genetic mapping of the major QTL controlling the submergence tolerance on chromosome 9 with the aid of chromosome walking. The fine-scale map was assembled by analyzing the RIL population consisting 313 individuals. The major QTL region was enriched for markers using BAC and YAC ends from the contiguous map.

2. Materials and Methods

2.1. Plant materials

Three mapping populations were used. The first population was derived from the cross IR49830-7-1-2-2 (submergence tolerant breeding line from IRRI) × CT6241-17-1-5-1 (upland submergence intolerant line from CIAT). A population of 265 F_1-derived doubled haploid lines (DHL) (accession series IR67709) was developed at IRRI (International Rice Research Institute), 65 DHLs were randomly selected for linkage map construction. The second population consisted of the 313 recombinant inbred lines (RILs) randomly selected from 405 lines (accession series IR67819) of a cross between FR13A (an Indian land race cultivar, recently known as one of the most submergence tolerant lines) and CT6241-17-1-5-1. This population was used for fine-scale mapping at the submergence tolerance genomic region. The 186 F_2 plants of Nipponbare × Kasalath[27] were used for genetic mapping of the YAC ends.

2.2. Submergence screening

The 65 F_1-derived DHLs and three checks (FR13A, IR49830-7-1-2-2 and CT6241-17-1-5-1) were tested for submergence tolerance under field condition in Ayuttaya, Thailand in 1994 and 1995.The 313 RIL population and the same set of checks were also tested at the same location in 1998. Four-week-old seedlings were completely submerged by keeping the water level at 30 cm above the tallest plants.

Plant recovery is described as the ability of the plant to overcome submergence stress by re-growth. Recovery score (RS) was visually scored 3–15 days after de-submergence by comparison with check cultivars using the 1 to 9 scale of Suprihatno and Coffman with modifications.[28] RS was scored at 0, 3, 5, and 8 days for the DHL population and at 0, 5, 10, and 15 days for the RIL population. The best recovery check, FR13A, was scored as 1. IR 49830 was scored as 2–3. CT 6241 and IR 42, the most susceptible checks, were scored as 9 (completely dead).

2.3. Genomic DNA isolation, Southern hybridization and AFLP analysis

Rice genomic DNA was isolated from fresh leaves of plant using the CTAB-NaCl method.[29] Southern-blotting analysis followed the standard procedure.[30] Total genomic DNA was digested with the respective restriction enzymes, separated by electrophoresis on a 0.6% agarose gel, and transferred onto a positively charged nylon membrane (Beohringer Mannheim). Labeling of the probes and hybridization were performed with a Digoxygenin (DIG) system. Banding was detected by X-ray film after 2–4 hr of exposure. The AFLP methodology was essentially as described by Zabeau and Vos.[31] Fourteen combinations of AFLP primers were used in polymorphism survey between two parents. AFLP was performed according to Vos et al.[32] The PCR products were fractionated on 4.5% denaturing polyacrylamide gel electrophoresis (PAGE) and detected by silver staining. One hundred and five DNA markers were used for linkage map construction in the DHL population.

2.4. YAC analysis

YAC library screening: The STS primers designed from sequence of R1164 were used to screen a total of 8000 YAC clones library of the Rice Genome Project (RGP), Japan[33] by the three-step PCR screening.[34] High molecular weight (HMW) DNA of YAC clones were prepared in agarose plug modified from the method of Wing

et al.[35] YAC-end fragments were generated by adapter mediated PCR.[36]

Restriction mapping of YAC clones: The agarose plugs contained HMW DNA of Y3143 and Y6653 were digested with restriction enzymes, *Not* I, *Sac* II, and *Sma* I, according to Wing et al.[37] The digested DNAs were fractionated by PFGE in a 1% agarose, 0.5 × TBE, electrophoresis for 14 hrs at 6 V/cm, 10–40 sec pulse time, and 12.5°C. The gels were blotted and hybridized with the BAC-end probes.

2.5. BAC contiguous map construction

BAC library screening: A BAC library constructed from cultivar Shimokita[38] was used for construction of a contiguous map. The library, equivalent to 7× genome coverage, consists of 21,504 clones. Seven high-density BAC filters (3072 BAC clones/filter) were used in colony hybridization for screening with the YAC-ends and RFLP markers located in the submergence tolerance QTL region. Labeling and hybridization were performed using the ECL system (Amersham). Pre-hybridization and hybridization reactions were carried out at 42°C as recommended by the manufacturer. The filters were exposed to X-ray film at a room temperature for 4 hr.

BAC DNA isolation: BAC-DNA was isolated using the *alkaline-lysis* procedure.[30] For small-scale (2–10 ml) preparations, a plasmid isolator (Kurabo Model PI 100, Sigma) was used after 18 hr of culture in LB (12.5 μg/ml chloramphenicol). BAC-insert DNA was released by *Not* I digestion and size-estimated by pulsed-field gel electrophoresis (PFGE).

Pulsed-field gel electrophoresis (PFGE): The digested BAC-DNA was loaded on a 1% agarose gel in 0.5 × TBE buffer. The sPFGE conditions were as follows: switch-time ramping from 2 to 10 sec, 12.5°C, pulse angle at 120 degrees, using 0.5 × TBE running buffer for 14 hr. The size of each BAC clone was estimated based on its migration and compared to MidRange PFG MarkerI (Biolabs).

BAC-end isolation: The end fragments of the BAC clones were isolated by either thermal asymmetric interlaced-PCR (TAIL-PCR)[39] or Vectorette-PCR[40]. Four arbitrary primers were employed for the TAIL-PCR.[39] Five restriction enzymes, *Avr* II, *Dra* I, *Eco*RV, *Nhe* I, and *Spe* I, were used to digest the BAC DNA before ligated to the vectorette duplex.[40] Vectorette-PCR were performed using BAC-L/BAC-R primer and 224 primer.[40,41] The repetitiveness of the BAC-end was checked by hybridization to genomic DNA of the parents. Only single-copy BAC-ends were used for chromosome walking.

Chromosome walking: All candidate BAC clones identified by colony hybridization were analyzed by both *Not* I-Southern-hybridization and *Hin*dIII fingerprinting to confirm their overlapping, and the contig map was constructed. The out-most ends of the contigs were used as probes in the next round of screening and walking.

2.6. Partial fine-scale mapping and QTL analysis

The left ends of YAC clone (30L), three flanking RFLP markers (RZ698, R1164, and S10709), and six BAC-end markers from chromosome walking were used for fine-scale mapping in the submergence tolerance region using 313 RILs. Linkage analysis was carried out using the program MAPMAKER/EXP 3.0.[42] Map distance was based on the Kosambi function.[43]

QTL analysis was performed with the software package MQTL.[44,45] Both simple interval mapping (SIM) and simplified composite interval mapping (sCIM) techniques were used for QTL detection. Each data set was analyzed with 1000 permutations, a 5 cM walking speed, and 5% Type I error rate. The significance threshold (a LOD score $\leq$ 2.4) was used to declare the presence of a QTL.

3. Results

3.1. Phenotypic distributions, QTL and genetic mapping of the DHL population

Phenotypic distribution of RS at 8 days measured in DHL populations (Fig. 1A) supported major gene with other modifying the effect or quantitative modes of inheritance. The two parents were at opposite ends of the frequency distribution.

QTLs controlled traits responsive to submergence stress were mapped coincidentally on chromosome 5 and 9. A small-effect QTL was detected on chromosome 5 by the SIM procedure (data not shown). The QTL on chromosome 5 accounted for 26%, 31%, and 27% of phenotypic variance explained (PVE) for RS at 3 days, 5 days, and 8 days, respectively. The IR49830 allele on chromosome 5 promoted more tolerance than CT6241.

The largest-effect QTL on chromosome 9 was first detected with both SIM and sCIM for RS (Fig. 1B). The DHL mapping population located major submergence tolerance QTL within the 5.9-cM interval flanked by three RFLP markers, R1164, S10709, and RZ698, and one AFLP marker, A331106 on the short arm of chromosome 9. This region accounted for 76%, 72%, and 72% of PVE in RS; at 3, 5, 8 days, respectively.

3.2. Enrichment of the 5.9 cM region by chromosome walking

To enrich molecular markers around the major QTL on chromosome 9, previous linked makers R1164 and S10709 were used for YAC library screening. Two YAC clones, Y3143 (30D7) and Y6653 were identified (data not shown). The end fragment of 30D7, 30L, was mapped 0.9 cM away from R1164 (data not shown) on the Nipponbare × Kasalath population while 30R was

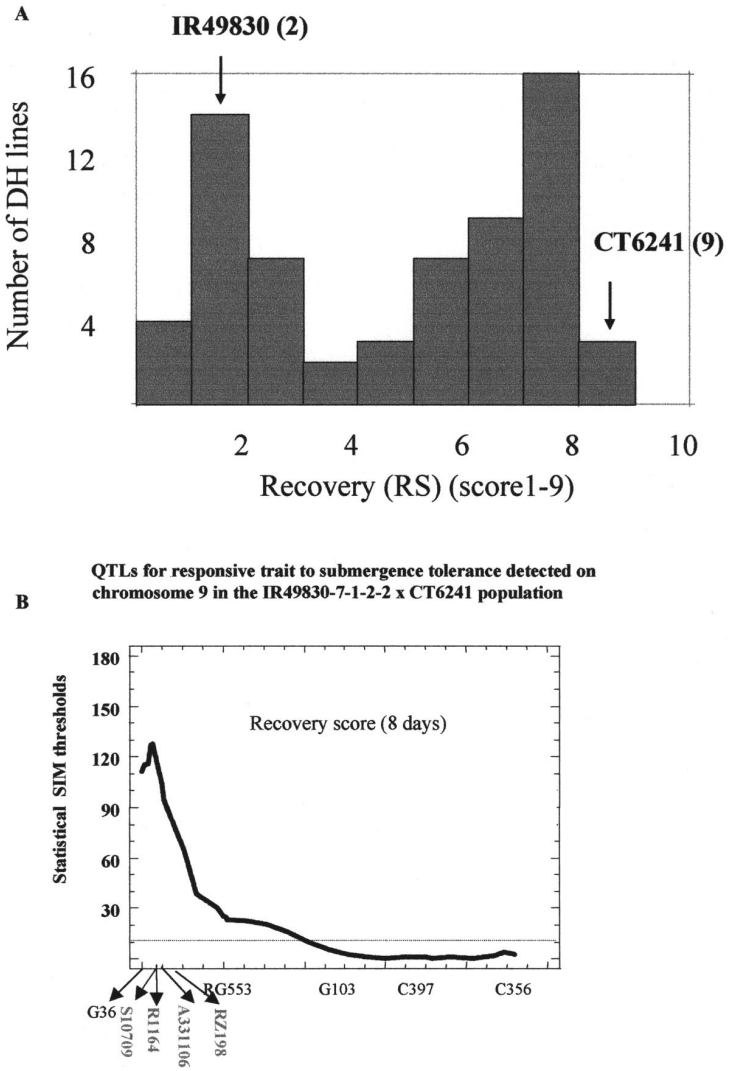

Figure 1. Frequency distribution of recovery score (RS) at 8 days of the 65 DHLs derived from the IR49830-7-1-2-2 × CT 6241-17-1-5-1 cross (A). B. show the SIM test statistics from the QTL analysis of recovery score at 8 days on chromosome 9 in the DHL population. The x-axis shows the ordering of markers. QTL peak intervals are shown as "gray bold." The horizontal bar indicates the maximum significant thresholds (P = 0.05).

monomorphic among the parents. In order to identify overlapping BAC clones, the three RFLP markers, R1164, RZ698, and S10709, together with 30L and 30R were used to screen the BAC library. Some false positives from colony hybridization were eliminated based on *Hin*dIII fingerprinting and the *Not* I hybridization pattern. Since 30R and S10709 detected the same four BAC clones (43F10, 147C2, 213H6, and 206E11) only 30R was used for the further analysis. The R1164 marker identified three clones: 180D1, 44A3, and 39B2. The 30L identified two clones: 74D12 and 124E10. Three overlapping clones were identified by RZ698: 148C7, 147G4, and 65E10. These positive BAC clones, which were assembled into four subcontigs, encompass the submergence tolerance region.

The orientation and overlapping of these clones were validated by BAC-end hybridization. The informative, single-copy end-probes were selected for chromosome walking. To validate the correct position of BAC-ends on the Nipponbare genome, 21 BAC ends were screened with 8000 YAC clones, 7 of which had their position confirmed by restriction mapping on the 30D7 YAC clone originally identified. To close the gap between the 30L and R1164 subcontigs, *Not* I shybridization performing using BAC ends as probe, 126G1 connected two subcontigs together (Fig. 2). Three BAC ends were used to confirm the connection between R1164 and 30R-S10709 subcontigs. Chromosome walking from S10709 was terminated at 147C2R. All subcontigs surrounding 30L, R1164, 30R, and S10709 were connected after one step of walking from

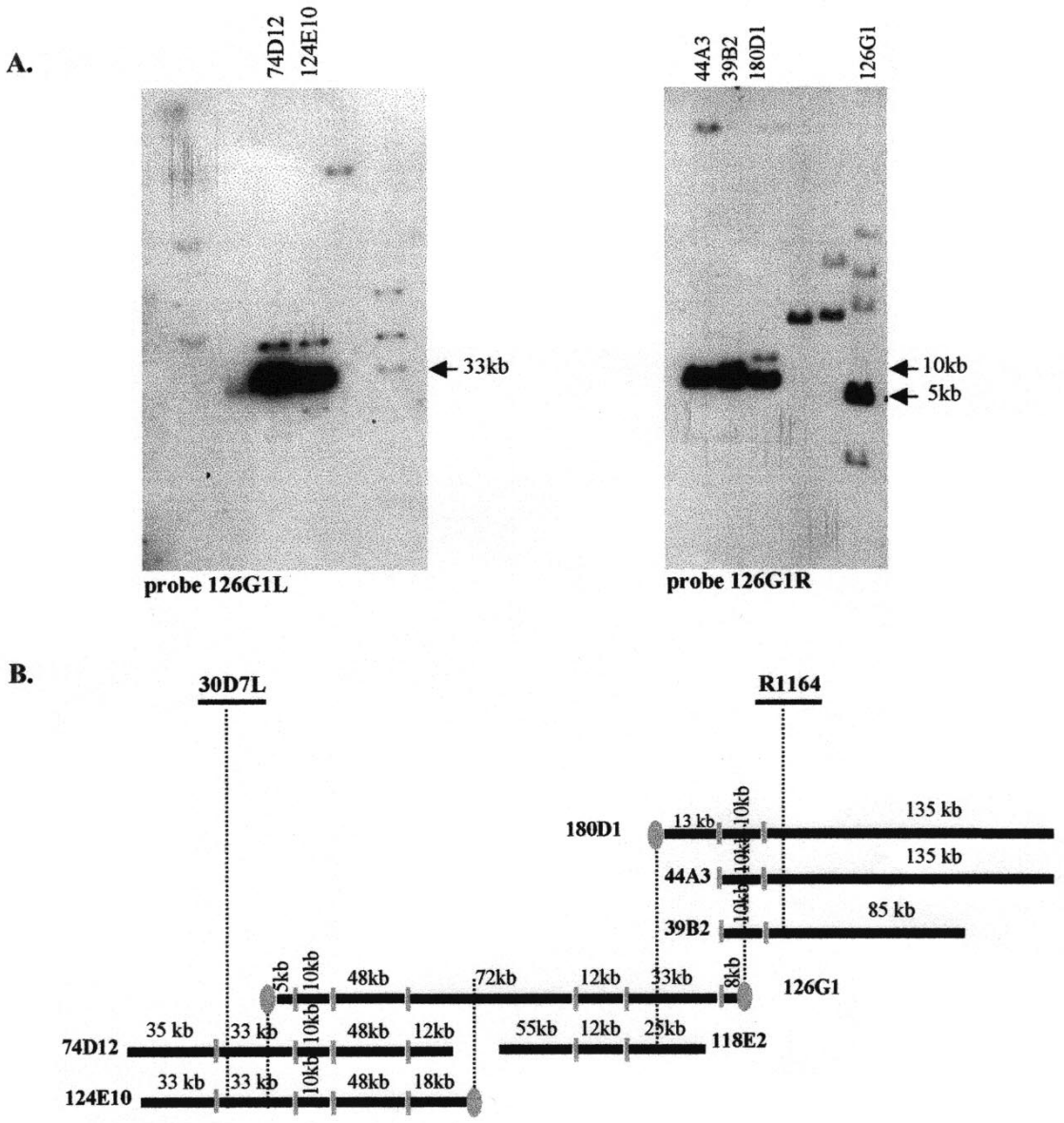

Figure 2. The BAC end 126G1L, generated from 126G1, was hybridized to a 33-kb band of 74D12 and 124E10 in the 30L subcontig and 126G1R was hybridized to a 10-kb band of 39B2, 44A3, and 180D1 in the R1164 subcontig (A). B shows the connection of 30L and R1164 subcontigs.

both directions from the four markers. For the subcontig surrounding RZ698, extension was made from 148C7R to new four clones, 66C5, 64B9, 46D2, and 54E9, while 147G4R identified three new clones, 36H11, 11E5, and 102F1. Connection between 30D7L and RZ698 could not be made because of the repetitiveness of 74D12R and 124E10L (data not shown). The final two contigs consisted of 24 BAC clones which span approximately 560 kb and 400 kb around markers R1164 and RZ698, respectively (Fig. 3).

3.3. Phenotypic distributions, fine-scale mapping and QTL analysis using RIL population

Phenotypic distributions of RS at 15 days for the RIL population are shown in Fig. 4. Again, this distribution supported quantitative inheritance for plant recovery. The two parents were at opposite ends of the frequency distribution. However, distribution was skewed toward a higher score.

Six ordered BAC-ends, one YAC-end and three RFLP markers are located on the RIL fine-scale map (Fig. 3). Ten markers were mapped to nine positions, which spans a 6.8-cM region. About half of the map distance can be

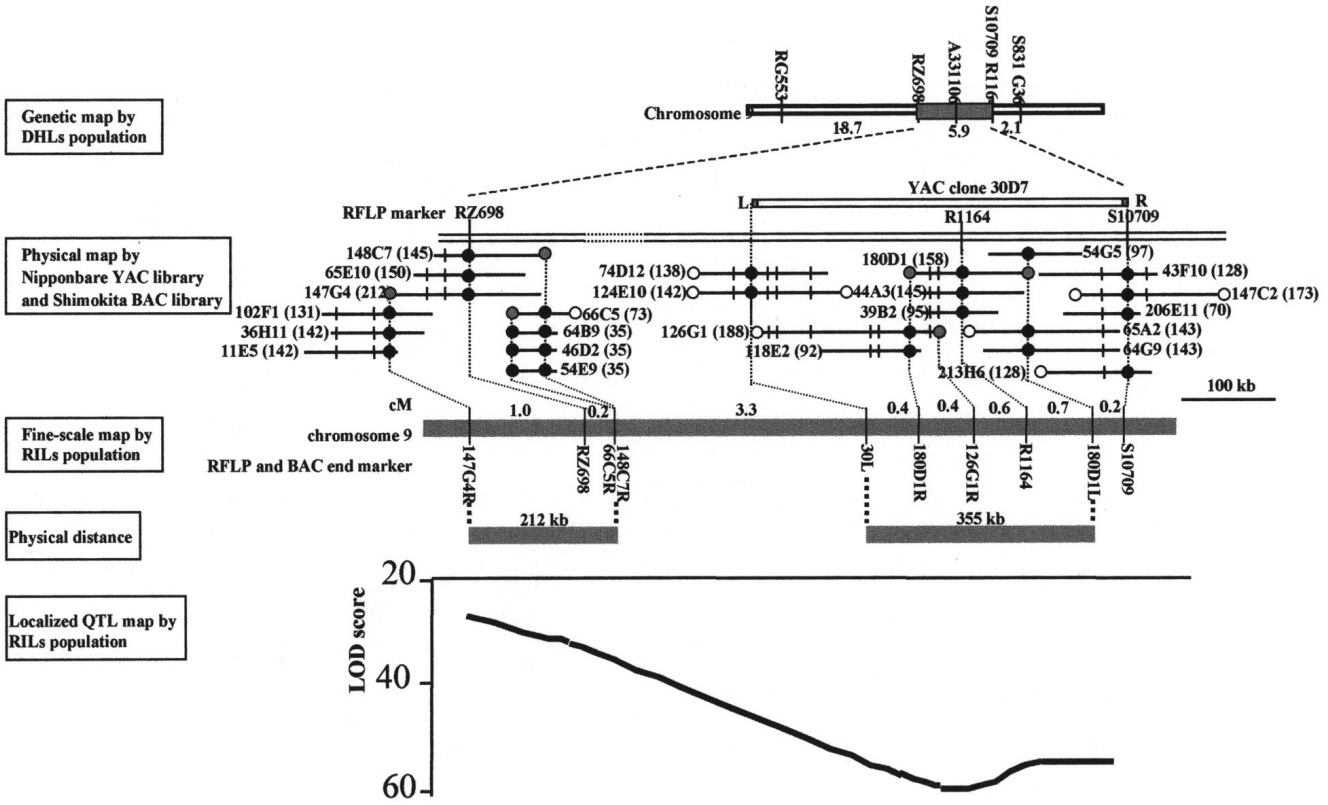

Figure 3. Alignment of the genetic, contiguous maps, and QTL analysis of the region controlling submergence tolerance. The genetic map of the submergence tolerance major QTL was constructed using data for 65 individuals of DHL population. The contiguous maps are shown as YAC and overlapping BAC clones. The horizontal bars accompanied by a combination of letters and numbers represent BAC clones. The numbers in parentheses indicate the size of the BAC clones in kb. The broken vertical lines indicate *Not* I sites. The gray circles represent the BAC end markers using in fine-scale mapping while empty circles represent multiple-copy BAC ends. The physical distance was estimated from the *Not* I-digested fragments of BAC clones fractionated by PFGE. The fine-scale map and localized genomic QTL analysis was constructed with 313 RIL individuals.

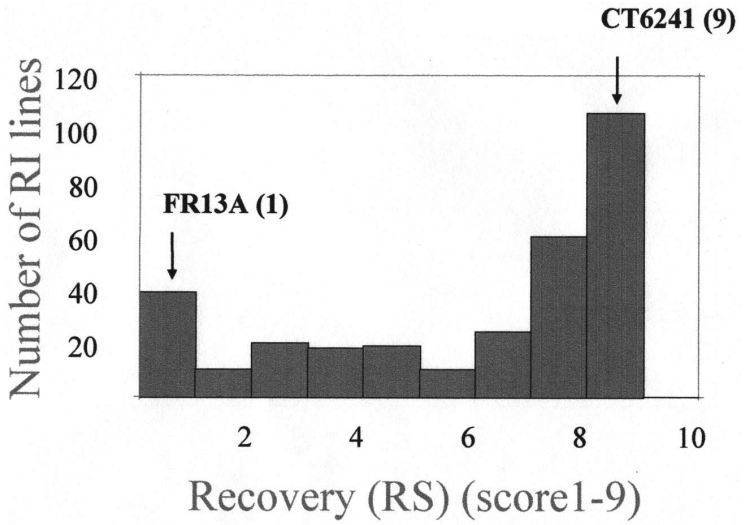

Figure 4. Frequency distribution of recovery score (RS) at 15 days of the 313 RILs derived from the FR13A × CT 6241-17-1-5-1 cross.

accounted for by the distance between 30L and 66C5R (3.3 cM) in which no BAC clones were identified. In contrast to the 30L-66C5R interval, six markers were clustered in a 2.3-cM subcontig around R1164 and four markers on the 1.2-cM of RZ698 subcontig.

QTL analysis with 313 RILs population (Fig. 3) revealed that the LOD value of the RS on chromosome 9 peaked in the interval around 180D1R-R1164 region. Both simple interval mapping (SIM) and simplified composite interval mapping (sCIM) pinpointed 180D1R as the most significant marker in the region where 58–63% of PVE of the trait was affected (data not shown).

4. Discussions

The position of major QTL in both DHL and RIL mapping populations were located nearby the *Sub1* gene previously identified using different donor.[2,3] When comparing submergence tolerance, IR49830 has less recovering ability than FR13A (Figs. 1A and 4). Because the FR13A × CT6241 map was partial, one possibility is that minor QTLs may exist on other chromosomes in FR13A. For the RIL population, the whole genome map needs to be explored in more detail for detection of minor QTLs on other chromosomes. In this study, the submergence tolerance still based on the statistics value, a progeny testing with more accurate measuring of submergence tolerance in a large population is needed for more precise identification of the major QTL genotype.

Construction of a fine-scale map for positional cloning requires a number of molecular markers in a particular chromosome interval.[5,46] In the present study, we used BAC-end markers to provide a number of polymorphic DNA markers for more precise linkage analysis of the submergence tolerance QTL region.

The efficiency of marker enrichment can be considered from the number of successful marker landings at the target chromosomal region and the number of false positives. The BSA combined with RAPD, performed by Giovannoni et al., need about 200 primers to screen four DNA pools in order to find only two tightly linked markers to *jointless* and another false positive marker located 45 cM away.[13] The AFLP analysis carried out by Cervera et al. required 144 oligonucleotide primers and careful analysis of 11,500 sets of bands.[16] Finally, only three AFLP markers were potentially mapped tightly to the target region.[16] BSA and AFLP have been used for high-resolution linkage mapping at the *Sub1* locus, by using 900 primer combinations with a large population of 2950 segregating F_2 plants, 12 markers were found within 2.2 cM and 2 of them cosegregated with this locus.[3] In our experiment, 14 combinations of AFLP primers were used to screen six pools of DNA, and only one marker, A331106, was found to locate in the submergence tolerance region (Fig. 1). This marker was used as a probe for screening a BAC library (data not shown). However more than 100 BAC clones were hybridized by this probe, possibly because of the nature of AFLP that usually band heterogeneity or repetitiveness.[47,48]

In the present study, about 60% of the BAC ends were repetitive (data not shown) and useless as probes in colony hybridization for chromosome walking. Six BAC ends could be selected as informative, single-copy DNA markers linked to the submergence tolerance region. These BAC ends were instantaneously converted into PCR-based markers for efficient marker-aided selection (MAS) in breeding program.

The relationship between physical and map distance in rice was estimated as 1 cM: 250 kb and 1 cM: 273 kb.[49,50] This ratio is not constant throughout the genome. The genomic regions containing crossover hot spots seem to have the smaller ratio. Ronald et al. reported the physical to map distance at the *Xa-21* locus was 265 kb/cM.[10] Yoshimura et al., on the other hand, reported the physical/genetic ratio at the *Xa-1* region was 130 kb per cM in the IR24/Kogyoku population.[51] Sanchez et al. showed that the physical to map distance at the *xa13* region was only 96 kb.[25] In the present study, the submergence tolerance QTL was mapped close to the centromeric region. However, the average physical to map distance ratio for the submergence tolerance QTL region estimated by PFGE was 172.5 kb/cM (Fig. 3). This ratio agrees with sequence data of PAC clones located in this region, PAC0645D04 and PAC0651G05 (accession nos. AC090054 and AC090055) (http://www.ncbi.nlm.nih.gov/), that estimate the distance from S10709 to R1164 as 121.5 kb. Therefore, it is interesting to understand the genomic structure that contributes to such a high recombination frequency near the centromeric region.

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