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Mapping of quantitative trait loci controlling low-temperature germinability in rice (*Oryza sativa* L.)

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Abstract Low-temperature germination is one of the major determinants for stable stand establishment in the direct seeding method in temperate regions, and at high altitudes of tropical regions. Quantitative trait loci (QTLs) controlling low-temperature germinability in rice were identified using 122 backcross inbred lines (BILs) derived from a cross between temperate *japonica* varieties, Italica Livorno and Hayamasari. The germination rate at 15°C was measured to represent low-temperature germination and used for QTL analysis. The germination rate at 15°C for 7 days of Italica Livorno and Hayamasari was 98.7 and 26.8%, respectively, and that of BILs ranged from 0 to 83.3%. Using restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers, we constructed a linkage map which corresponded to about 90% of the rice genome. Three putative QTLs associated with low-temperature germination were detected. The most effective QTL, qLTG-3-1 on chromosome 3, accounted for 35.0% of the total phenotypic variation for low-temperature germinability. Two additional QTLs, qLTG-3-2 on chromosome 3 and qLTG-4 on chromosome 4, were detected and accounted for 17.4 and 5.5% of the total phenotypic variation, respectively. The Italica Livorno alleles in all detected QTLs increased the low-temperature germination rate.

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Introduction

Low-temperature-induced retardation of seedling growth is a common problem in temperate rice growing areas, at high altitudes of tropical and sub-tropical areas, and in areas with a cold irrigation water supply. In such areas, water temperatures at sowing are frequently below 15°C, whereas the optimum range for germination and early seedling growth of rice is 25 to 35°C. Delayed emergence of the rice seedlings from water greatly increases seedling mortality (Peterson et al. 1978), and causes serious decreases of yields and increases of weed competition. In many Asian countries, the direct seeding method has become increasingly important (Dingkuhn et al. 1992). Therefore, vigorous germination at low temperature is an important character for stable establishment of seedlings in the direct seeding method, in which rice is sown directly into flooded fields.

A wide range of variation in the low-temperature germinability of rice has been observed in more than 700 varieties from Japan, Europe, China, Russia and other regions (Kotaka and Abe 1988). However, despite the substantial amount of genetic variation for low-temperature germination in rice, it has not been easy to improve

the level of low-temperature germinability in rice breeding programs because the genetic basis of low-temperature germinability has not yet been clarified, due to its quantitative inheritance. Evaluation of varietal differences of low-temperature germinability revealed that Italica Livorno had the most vigorous low-temperature germination (Kotaka and Abe 1988). Although Italica Livorno has been used as a donor parent in several studies, many undesirable characteristics, such as poor straw strength, maturity, shattering and poor kernel, hindered the introduction of a high level of low-temperature germination into elite cultivars (McKenzie et al. 1994).

In spite of the significance of low-temperature germinability in rice cultivation, the genetic mechanism of the trait is not well understood. Low-temperature germinability is considered to be a quantitative trait under the control of several genes. The linkage relationships between the genes for low-temperature germination and several morphological marker genes were examined using *japonica* linkage testers (Sasaki et al. 1973). A phenotypic correlation between low-temperature germinability and d_2, *wx*, d_6 and *I-Bf*, which are located on chromosomes 1, 6, 7 and 9, respectively, was reported (Sasaki et al. 1973). Five QTLs for low-temperature germination located on chromosomes 2, 4, 5 and 11 were detected by crossing between *japonica* Nipponbare and *indica* Kasalath (Miura et al. 2001).

The identification of the number and magnitude of gene effects would contribute to a better understanding of the genetic control of low-temperature germinability, and hence might facilitate the rapid development of vigorous low-temperature germination cultivars. Italica Livorno has been classified as a temperate *japonica* based on molecular analysis (Mackill 1995; Mackill et al. 1996). In order to perform QTL analysis for low-temperature germinability using Italica Livorno as a donor, it is necessary to construct a molecular linkage map from the cross between temperate *japonica* varieties. However, there is an especially low level of polymorphism of DNA markers among *japonica* varieties (Zhang et al. 1992; Mackill 1995). When using *japonica* varieties, simple sequence repeat (SSR) markers generally show relatively higher levels of polymorphism than RFLP markers (Akagi et al. 1997; Kono et al. 2000).

The objective of the present study was to identify QTLs for low-temperature germinability using backcrossed inbred lines (BILs) with Italica Livorno as a donor. A molecular linkage map constructed with RFLP and SSR markers, which covered about 90% of the rice genome, was developed. Three putative QTLs for low-temperature germinability were detected.

Materials and methods

Plant materials

Hayamasari, one of the temperate *japonica* varieties adapted in the northernmost island (Hokkaido) in Japan, was crossed with Italica Livorno, one of the temperate *japonica* varieties in Italy with vigorous low-temperature germinability. The resultant F_1 plants were crossed with Hayamasari to produce BC_1F_1 seeds. One hundred and twenty-two BC_1F_5 lines (BILs) were developed from the resultant BC_1F_1 plants by the single-seed descent method. The 122 BILs and their two parental lines were grown under natural field conditions in Naganuma, Hokkaido, Japan in 1998. Germinated seeds were sown in nursery beds in a greenhouse, and 4-week-old seedlings were then transplanted in paddy fields with a spacing of 35×12.5 cm. One plant from each line was selected and leaf tissue was collected for the extraction of total DNA. Low-temperature-germinability was evaluated for self-pollinated seeds of the selected plants.

Evaluation of low-temperature germinability

Thirty seeds per line were placed on a filter paper in a 9-cm Petri dish, and 10 ml of distilled water was added. The dishes were placed in an incubator at 15°C. The number of germinated seeds was counted daily. The data of the germination rate for 7 days after incubation were used for QTL analysis when large parental differences were observed. Also, the germinability of parental varieties were tested under 30°C, the optimum temperature for germination in rice.

Marker analysis

Total DNA of the 122 BILs and their parental lines was extracted from leaves by the CTAB method (Murray and Thompson 1980). Southern blotting was performed according to the method described by Kurata et al. (1994). Total DNA of the 122 BILs and their parental lines was digested with eight restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RV, *Hind*III, *Apa*I, *Dra*I, *Eco*RI and *Kpn*I. We selected 1,063 DNA clones as probes that covered the entire 12 chromosomes from a high-density genetic map (Harushima et al. 1998). We surveyed these clones for parental polymorphism and used the clones that showed such polymorphisms for the analysis of the BILs. Southern hybridization and detection were carried out using the ECL direct labelling and detection system (Amersham Pharmacia Biotech, UK).

To amplify the genomic DNA to detect SSRs, we used 273 primers of the International Rice Microsatellite Initiative (IRMI) (McCouch et al. 2002) that were mainly located in chromosomal regions, in which RFLP markers did not show polymorphism in this study. The PCR reaction mixture consisted of 25 ng of total DNA, each dNTP at 200 mM, 20 pmol of primer, and 2 units of *Taq* DNA polymerase with PCR buffer (Promega, USA) in a 15.0 µl volume. The thermocycler was programmed for 30 cycles of 94°C (1 min), 55 or 50°C (2 min) and 72°C (3 min), and a final cycle of 72°C for 7 min. Amplified DNA products were electrophoresed on 3 or 4% agarose gels in 0.5 × TBE buffer and stained with SYBRGold (Molecular Probes, USA), or electrophoresed on 6% polyacrylamide denaturing gels and stained with Silver Sequence (Promega, USA). Amplified DNA fragments showing clear polymorphism were used for the analysis of BILs and linkage mapping.

Four additional SSR markers were developed and used for the analysis of BILs and linkage mapping, because no parental polymorphism was observed in the regions using the IRMI SSR markers (McCouch et al. 2002). The four BAC/PAC sequences of AC97627, AC98695 and AP000615 on chromosome 3, and AP004619 on chromosome 8, were obtained from IRGSP (http://rgp.dna.affrc.go.jp/). SSR motifs were detected using 'SSRIT' (http://www.gramene.org/gramene/searches/ssrtool). The four SSR markers obtained thereby were GBR3001 of AC97627 (U: 5′-CCTCTTCCCTTCTTGTGTCA-3′ and L: 5′-GGGATTTTTTC-ATCGAAATT-3′), GBR3002 of AC98695 (U: 5′-AGAGCATAA-CATCAAAGCCA-3′ and L: 5′-ATAGCTCCAATTCGATCTTC-3′), GBR3003 of AP000615 (U: 5′-AGCCAGGTATGTCATA-AATGATAATAACAA-3′ and L: 5′-TGCTATTAAACAAGCG-GTTTTTTCGTTTCG-3′) and GBR8001 of AP004619 (U: 5′-

CCATAGAGGCCTACAAGTAT-3′ and 5′-CCAGATGATAGAA-GAGGTGT-3′).

Linkage and QTL analysis

Linkage analyses were performed with MAPMAKER/EXP 3.0 (Lander et al. 1987). The frequencies of observed recombinations between two markers were converted to genetic distance, using the map function of Kosambi (1944). Chromosome assignment was performed based on the map location of the RFLP markers corresponding to the chromosomes in a high-density linkage map of rice (Harushima et al. 1998).

Composite interval mapping was performed to identify QTLs by using the software package QTL CARTOGRAPHER with forward-backward regression (Zeng 1993; Zeng and Weir 1996; Basten et al. 1998). The experiment-wise LOD (log of the odds ratio) threshold significance level was determined by computing 1,000 permutations (Churchill and Doerge 1994), as implemented by the QTL CARTOGRAPHER. These permutations can account for non-normality in marker distribution and trait values. The level of significance for QTLs in this study was determined to be $P<0.05$: LOD 3.09.

Results

Phenotypic variations

The gemination rate at 30°C for 3 days after incubation of Italica Livorno and Hayamasari was 100%. They hardly differ in their germinability. A clear varietal difference was observed in the germination rate at 15°C (low temperature germinability) (Fig. 1). In Italica Livorno, germination began 3 days after the start of incubation, and 3 days thereafter almost all of the seeds were germinated. In contrast, Hayamasari began to germinate 6 days after the start of incubation, and 5 more days were needed for near-completion of all germination. The germination rate at 15°C for 7 days of Italica Livorno and Hayamasari was 98.7 and 26.8%, respectively. The germination rates of BILs varied continuously from 0 to 83.3% at 7 days after incubation (Fig. 2).

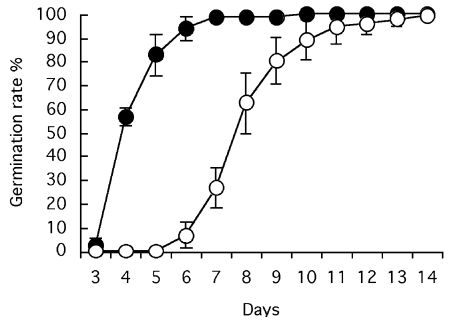

Fig. 1 Changes in the germination rate of two varieties, Italica Livorno and Hayamasari, at 15°C. *Closed* and *open circles* show the means for Italica Livorno and Hayamasari, respectively. *Bars* show the standard deviation

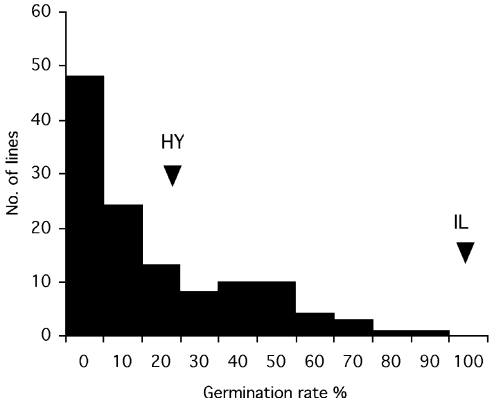

Fig. 2 Frequency distribution of germination rate at 15°C for BILs at 7 days after the start of incubation. *Arrows* indicate the means of Italica Livorno (IL) and Hayamasari (HY)

Linkage mapping

The percentage of DNA clones showing polymorphism with at least one restriction enzyme between Hayamasari and Italica Livorno is summarized in Table 1. We

Table 1 Number of RFLP and SSR markers surveyed for parental polymorphism

Chromosome	RFLP markers			SSR markers			Coverage[a] %
	No. of DNA clones tested	No. of polymorphisms	%	No. of SSR primers tested	No. of polymorphisms	%	
1	148	27	18.2	42	13	31.0	94.2
2	119	25	21.0	21	8	38.1	92.5
3	167	13	7.8	33	10	30.3	93.4
4	79	17	21.5	16	11	66.7	64.0
5	93	11	11.8	17	3	17.6	87.0
6	72	24	33.3	19	9	47.4	95.5
7	73	21	28.8	19	6	27.8	98.5
8	73	12	16.4	21	6	25.0	99.6
9	52	10	19.2	26	5	19.2	65.2
10	63	16	25.4	19	3	15.8	85.7
11	67	13	19.4	23	5	21.7	88.9
12	57	12	21.1	17	5	29.4	88.7
Total	1,063	201	18.9	273	84	30.8	87.8

[a] The genome coverage was estimated by assignment with a rice high-density linkage map (Harushima et al. 1998)

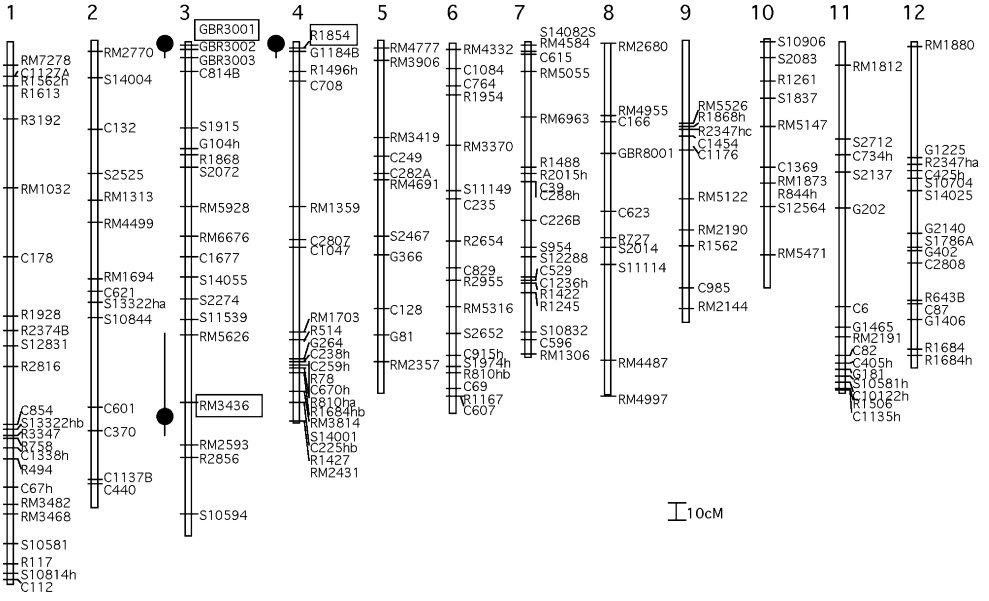

Fig. 3 Chromosomal locations of QTLs for low-temperature germinability in rice. The mapped markers are positioned by the chromosome assignment with the high-density RFLP linkage map, the *white bars*, (Harushima et al. 1998). *Solid symbols* represent the putative QTLs with *lines* bracketing the QTL symbols denoting the 2-LOD support limits. *Boxes* indicate the nearest marker loci to the QTLs

Table 2 Chromosome location, estimated position, flanking markers, coefficient of determination (R^2) and additive effects(AE) of the QTLs for low-temperature germinability

QTL	Chromosome	Marker interval	Interval (cM)	LOD	R^2	AE[a]
qLTG-3-1	3	GBR3001*-GBR3002	3.9	15.7	35.1	12.4
qLTG-3-2	3	RM3436*-RM2593	9.4	8.8	19.3	10.2
qLTG-4	4	R1854*-G1184	1.4	3.2	5.5	5.7

* Indicates nearest marker to the QTL
[a] Additive effects of the Italica Livorno allele based on arc sine transformation of germination rate

surveyed a total of 1,063 DNA clones for RFLP analysis, and 201 DNA clones (18.9%) showed polymorphism. The frequency of polymorphism varied among the chromosomes, ranging from 7.8 to 33.3%. For parental polymorphism screening with SSR primers, 84 of 273 primers (30.8%) amplified scoreable and reproducible polymorphic bands (Table 1). The frequency of polymorphism varied among the chromosomes, ranging from 15.8 to 66.7%.

We constructed 13 linkage groups with 139 RFLP and 47 SSR markers; these groups were anchored to all 12 rice chromosomes (Fig. 3). The order of most of the RFLP markers coincided with the rice high-density linkage map (Harushima et al. 1998). The estimated genome coverage of the map developed in this study was about 87.9% of the rice high-density linkage map (Harushima et al. 1998). Map positions of 108 RFLP markers were in good agreement with the rice high-density linkage map (Harushima et al. 1998), but 31 RFLP markers that showed multiple banding patterns between the parents did not map to the same position as the rice high-density linkage map (Harushima et al. 1998). Thus, these new fragments were named by the original name followed by 'h'.

The segregation ratios of the two genotype classes in most loci fit the expected Mendelian ratio of 3 (Hayamasari): 1 (Italica Livorno). Segregation distortion was observed for four loci on chromosomes 3 and 9, at $P<0.01$. The frequency of Hayamasari alleles increased in these loci on chromosomes 3 and 9.

QTLs for low-temperature germinability

Three putative QTLs associated with low-temperature germinability were detected (Fig. 3, Table 2), and the Italica Livorno alleles in all detected QTLs increased the low-temperature germination rates. The QTL with quite a large effect, qLTG-3-1, was mapped at the marker GBR3001 on the most distal end of the short arm of chromosome 3 (Fig. 3). The phenotypic variation explained by qLTG-3-1 was 35.0%. The additive effects of the Italica Livorno alleles in qLTG-3-1 increased 12.4% in the arc-sine transformation of the germination rate (Table 2). Two additional QTLs, qLTG-3-2 near the marker RM3436 on chromosome 3 and qLTG-4 at the marker R1854 on the most distal end of the short arm of chromosome 4, were detected and accounted for 17.4 and 5.5% of the total phenotypic variation, respectively.

Discussion

Low-temperature germinability is one of the major determinants for stable stand establishment in the direct seeding method in temperate regions and at high altitudes

of tropical regions. Previous genetic analyses have revealed that low-temperature germination is controlled by several genes (Sasaki et al. 1973; Miura et al. 2001). However, the genes responsible for the high level of low-temperature germinability in Italica Livorno remain to be analysed. In this study, we found new loci for low-temperature germination. We demonstrated that a QTL, qLTG-3-1 on chromosome 3, explains a large part of the total phenotypic variation in the BILs population (Table 2).

The results revealed that low-temperature germination in Italica Livorno was controlled by one gene with a major effect, qLTG-3-1, and other genes with minor effects, qLTG-3-2, qLTG-4 (Table 2). The major gene, qLTG-3-1, was localized to a 3.7-cM interval between the markers GBR3001 and GBR3002 on chromosome 3. This QTL, qLTG-3-1, and the nearest markers linked to qLTG-3-1, will be useful for manipulating low-temperature germinability in rice breeding programs.

Five QTLs for low-temperature germination have been identified by studies of crosses between *indica* Kasalath and *japonica* Nipponbare (Miura et al. 2001). One QTL near the marker C946 located on chromosome 4, and the Kasalath allele in this QTL, increased low temperature germination (Miura et al. 2001). Based on the chromosomal location, this QTL appears to correspond to the qLTG-4 detected in this study. Further analysis will be required to prove the allelic relationship between these QTLs. The other QTLs detected in this study appear to be different from those identified in the *japonica/indica* population (Miura et al. 2001).

The low level of DNA polymorphism makes molecular mapping difficult in closely related rice varieties. To identify most of the QTLs, a molecular linkage map that covers the whole genome is necessary. Italica Livorno has been classified as a temperate *japonica* based on molecular analysis (Mackill 1995; Mackill et al. 1996). As expected, low polymorphism was observed between Hayamasari and Italica Livorno using RFLP markers (Table 1). Although we surveyed more than 1,000 DNA clones, the linkage map constructed with RFLP markers corresponded to only 40% of the rice genome. Because of low polymorphism between the Japanese temperate *japonica* cultivars, Akihikari and Koshihikari, the map could not cover the whole rice genome even when using more than 1,200 RFLP markers (Takeuchi et al. 2001). For *japonica* varieties, SSR markers generally show relatively higher levels of polymorphism than RFLP markers (Akagi et al. 1997; Kono et al. 2000). SSR markers showed a higher level of polymorphism between Hayamasari and Italica Livorno than the RFLP markers (Table 1). In the present study, using RFLP and SSR markers we were able to construct a molecular linkage map that covers about 90% of the rice genome (Harushima et al. 1998) (Fig. 3). This is the largest coverage of the rice genome for a molecular map derived from crosses between temperate *japonica* varieties.

It is quite difficult to introduce low-temperature germinability into commercial rice cultivars due to the large effort required for reliable evaluation of the trait and to undesirable traits or genes introduced from the donor parent. The SSR markers developed in the present study that are closely linked to qLTG-3-1 for low-temperature germinability will be useful for introducing a high level of low-temperature germination by marker-assisted selection (MAS) in rice breeding programs.

More comprehensive characterization of the QTLs detected here can be performed to identify the manner of genic interaction for QTLs using nearly isogenic lines (Lin et al. 2000, 2002, 2003). Genes at QTLs have been isolated for the heading date in rice by the positional cloning strategy (Yano et al. 2000; Takahashi et al. 2001; Kojima et al. 2002). It should be possible to clone the QTL with quite a large effect, qLTG-3-1, using a map-based strategy. To elucidate the biological functions of qLTG-3-1, fine mapping and development of nearly isogenic lines for this gene, using molecular markers, are now in progress.

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