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Mapping quantitative trait loci associated with regeneration ability of seed callus in rice, *Oryza sativa* L.

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Abstract Quantitative trait loci (QTL) controlling the regeneration ability of rice seed callus were detected using 245 RFLP markers and 98 BC_1F_5 lines derived from two varieties, 'Nipponbare' and 'Kasalath'. Regeneration ability was evaluated by two indices: average number of regenerated shoots per callus (NRS) and regeneration rate (RR). The BC_1F_5 lines showed continuous segregation for both indices. Five putative QTL for NRS (tentatively named *qRg1*, *qRg2*, *qRg4a*, *qRg4b* and *qRg4c*) located on chromosomes 1, 2 and 4 were detected. Digenic interaction among these detected QTL was not significant ($P < 0.01$). Among the five QTL detected, four 'Kasalath' alleles and one 'Nipponbare' allele increased NRS. According to an estimate based on the nearest marker loci, the five QTL accounted for 38.5% of the total phenotypic variation of the BC_1F_5 lines. For RR, four putative QTL were detected on chromosomes 2 and 4, and all of these were in the same chromosomal regions as the NRS QTL. The four RR QTL accounted for 32.6% of the total phenotypic variation.

Key words Regeneration ability · QTL · Rice · *Oryza sativa* L. · Seed callus

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Introduction

Many investigations have been performed on culture conditions and explant sources in attempts to improve plant regeneration systems. In cereals, explants containing immature meristematic tissues have been found to induce a callus that is competent to express totipotency. Callus cultures allow the establishment of highly efficient regeneration systems for some genotypes for which culture media and environmental conditions have been optimized (Bhaskaran and Smith 1990). Despite various efforts up to now, no culture condition has been found that induces high regeneration ability in all genotypes.

In alfalfa, high regeneration ability has been reported to be transferable from a parent with the higher ability to the other parent through backcrosses and selection (Bingham et al. 1975; Ray and Bingham 1989). Through many Mendelian and quantitative genetic studies, important knowledge has been obtained; there are genes for regeneration ability with additive gene action and genes controlling somatic embryogenesis that appear to be independent of those controlling microspore embryogenesis (Lazar et al. 1987; Agache et al. 1988; Taylor and Veilleux 1992). In wheat, genes for culture traits have been located on particular chromosomes or chromosome arms using addition, substitution and translocation lines (Henry et al. 1994). Recently, DNA markers have been used to locate those genes contributing to somatic embryogenesis in maize (Armstrong et al. 1992), tomato (Koornneef et al. 1993), alfalfa (Yu and Pauls 1993) and barley (Komatsuda et al. 1995; Mano et al. 1996) and genes contributing anther culture ability in maize (Cowen et al. 1992; Murigneux et al. 1994; Beaumont et al. 1995). The segregation distortion of restriction fragment length polymorphism (RFLP) markers occurs in maize plants regenerated from anthers of F_1 individuals (Wan et al. 1992; Bentolila et al. 1992) and rice (Yamagishi et al. 1996).

In rice (*Oryza sativa* L.), statistical analyses have been performed to clarify the mode of inheritance for the regeneration ability of seed callus (Abe and Futsuhara 1991; Tsukahara et al. 1995; Taguchi-Shiobara et al. 1997) and anther culture (Peng and Hodges 1989; Quimio and Zapata 1990). Several of the genes associated with regeneration ability showed significant additive and dominance effects in all of these investigations. Distorted segregation of RFLP markers occurs in plants regenerated from F_1 anthers (Yamagishi et al. 1996); however, no genes involved in the regeneration ability of somatic embryogenesis have yet been mapped. Thus, we performed an extended genetic analysis to detect the quantitative trait loci (QTL) conferring regeneration ability to rice seed callus using a hybrid population derived from a cross between a *japonica* variety, 'Nipponbare', and an *indica* variety, 'Kasalath'. These parents were chosen because of the many RFLP markers available and their different performance in tissue culture; 'Nipponbare' has a low regeneration ability compared to 'Kasalath'. Here we describe the successful detection of the RFLP markers linked to QTL associated with the regeneration ability of rice seed callus.

Materials and methods

Plant materials and linkage map construction

The 'Nipponbare' and 'Kasalath' varieties were crossed, and their F_1 plant was backcrossed with 'Nipponbare' to produce BC_1F_1 seeds. BC_1F_5 lines were developed from the 98 resultant BC_1F_1 plants by the single-seed descent method. One BC_1F_5 individual from each line was used to construct the linkage map. For the linkage map construction, 245 RFLP markers distributed over all 12 rice chromosomes were selected from a high-density linkage map (Kurata et al. 1994). The recombination frequency (r) of one meiosis between two markers was estimated by the following formula: $r = 2w/(3 - 4w)$, where "w" is the frequency of the observed recombination between two markers; this formula was developed by modifying the formula for recombinant inbred lines (Haldane and Waddington 1931). The linkage map of this population will be described in detail by Lin et al. (in preparation).

Culture procedures

Mature seeds of the 101 genotypes – 'Nipponbare', 'Kasalath', F_1 and 98 BC_1F_5 which were the siblings of the 98 BC_1F_5 individuals used in constructing the linkage map – were cultured. The procedures from callus induction to regeneration of the shoots have been described previously in detail (Taguchi-Shiobara et al. 1997). Briefly, about 20 seeds were incubated on solid callus-inducing medium for 4 weeks; calli derived from 5 independent seeds were then selected to initiate suspension cultures. Calli induced from 5 seeds were independently subcultured for 1 week. Ten calli, 1 mm in diameter and originating from 1 seed, were incubated on shoot-inducing medium. Five dishes, each of which had ten calli, were cultured for each experimental group. After incubation for 4 weeks, the average number of regenerated shoots per callus (NRS) and the regeneration rate (RR: the percentage of regenerated calli normalized to the total number of calli) were calculated for each dish, and the means of both indices for the five dishes were used to represent each of the 101 genotypes.

Statistical analysis

Prior to analysis, the data were transformed to normalize variances; $y = \ln(x + 1)$ for NRS, and $y = \arcsin \sqrt{x}$ for RR. In order to detect markers linked to loci for regeneration ability, we analyzed the data by analysis of variance (ANOVA). We employed a 0.01 probability level as the threshold to detect putative QTL for the two indices. Associations between markers and regeneration ability were determined using the General Linear Model (GLM) procedure in SAS programs (SAS Institute, 1989) to detect significant differences between the mean values of 'Nipponbare' and 'Kasalath' homozygotes at each marker locus. The data for heterozygotes, which were very few, were considered to be missing data. To evaluate two-locus interactions among putative QTL, we performed a two-factor analysis of variance using markers that were significant in single-point analysis. The MAPMAKER/QTL program (Paterson et al. 1988) was also used to confirm the existence of putative QTL detected by ANOVA and to estimate gene effects for each detected QTL.

Results

Phenotypic variation of the two parents, F_1 and 98 BC_1F_5 lines

'Nipponbare' showed a much lower regeneration ability than 'Kasalath' (Fig. 1). The NRS means were 1.3 for 'Nipponbare', 6.0 for 'Kasalath' and 5.5 for their F_1 progeny; the mean RR values were 72%, 88% and 96%, respectively. Continuous variation was observed both for NRS and RR among the 98 BC_1F_5 lines (Fig. 2), ranging from 0.0 to 7.4 and from 0% to 100%, respectively. Transgressive segregation was observed in the BC_1F_5 lines for RR.

QTL for NRS

Five putative QTL for NRS were identified and tentatively named *qRg1*, *qRg2*, *qRg4a*, *qRg4b* and *qRg4c* (Table 1). One QTL each was located on chromosomes 1 and 2, and the other three were on chromosome 4 (Fig. 3). The means for 'Kasalath' homozygotes were larger than those of 'Nipponbare' homozygotes at *qRg1*, *qRg2*, *qRg4a* and *qRg4b*; *qRg4c* was an exception. In other words, 'Kasalath' had positive alleles at four QTL, while 'Nipponbare' had a positive allele at one QTL. A multilocus model involving the five putative QTL explained 38.5% of the total phenotypic variance in the 98 BC_1F_5 lines. No digenic interaction was found among these five QTL through two-factor analysis of variance.

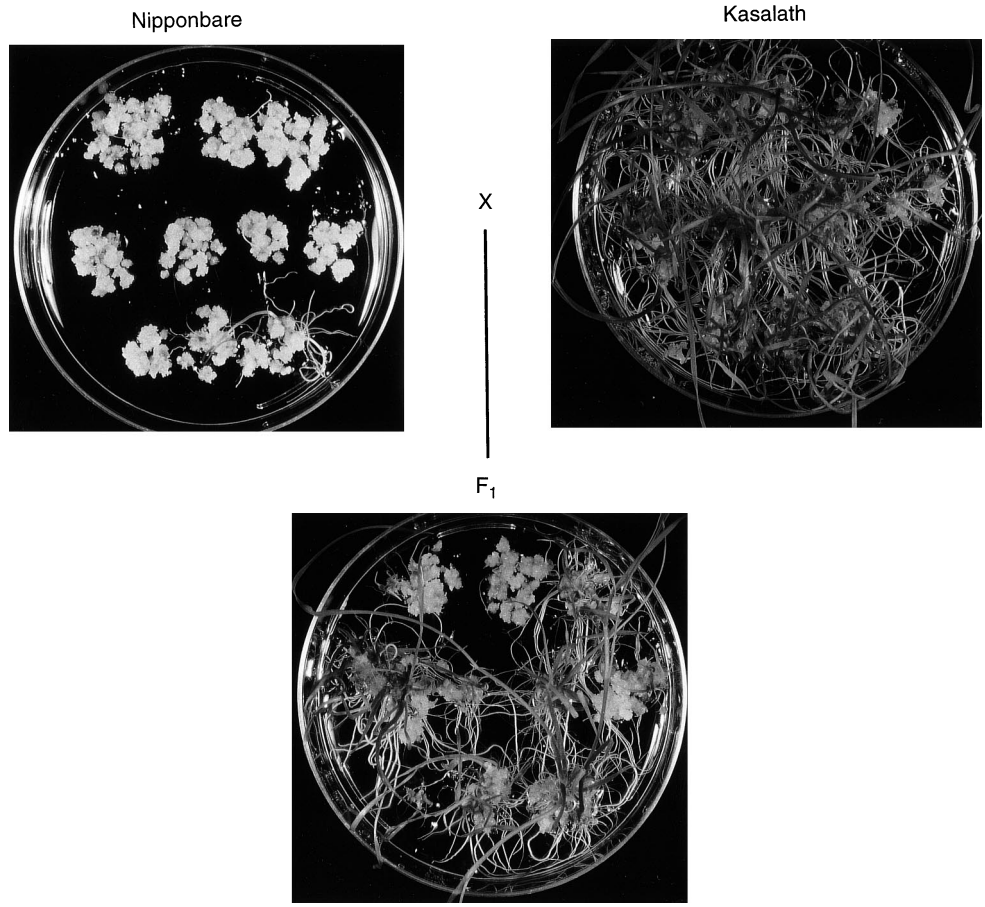

Fig. 1 Shoot regeneration from seed callus derived from 'Nipponbare' and 'Kasalath' parental varieties and their F_1 progeny

QTL for RR

Four putative QTL (*qRg2*, *qRg4a*, *qRg4b* and *qRg4c*) were detected (Table 1), one on chromosome 2 and the other three on chromosome 4 (Fig. 3). 'Nipponbare' had a positive allele at *qRg4c*, while 'Kasalath' had positive alleles for the other three QTL. These four QTL were mapped to the same chromosomal regions of four out of the five QTL for NRS. However, the variance accounted for by each QTL was not the same for the two indices. A multilocus model involving the four putative QTL accounted for 32.6% of the total phenotypic variance in the BC_1F_5 lines. No digenic interaction was found among these four QTL.

Discussion

QTL associated with regeneration ability were identified in rice for the first time. Five QTL for NRS were identified, four of which are likely to correspond to the four QTL for RR, as evidenced by their locations. Yamagishi et al. (1996) observed distorted segregation in regenerated plants derived from the anther culture of F_1 hybrids of rice on chromosomes 1, 2, 7, 10, 11 and 12. The distorted segregation in some of these areas might be associated with the efficiency of anther culture. These locations are different from those of the QTL identified in this report. Based on a comparison of these results, we suggest that genes controlling regeneration from somatic tissue culture might be independent of those controlling regeneration in anther culture; a conclusion which is consistent with previous reports (Agache et al. 1988; Lazar et al. 1987; Taylor and Veilleux 1992).

We used 'Nipponbare' and 'Kasalath' as parents to produce the study population because of their different phenotypes (Fig. 1) and genotypes (Taguchi-Shiobara et al. 1997) for regeneration ability. Because culture work is laborious and time-consuming, the number of seeds used in culture was small. When such seeds are selected to represent an individual, fixed lines are desirable in order to decrease sampling error. While doubled-haploid (DH) lines are fixed, they are produced by anther culture, in which differences in culture ability are observed among varieties. When DH lines are produced, some cross combinations show a low efficiency in anther culture and some show segregation distortion in their DH lines (Yamagishi et al.

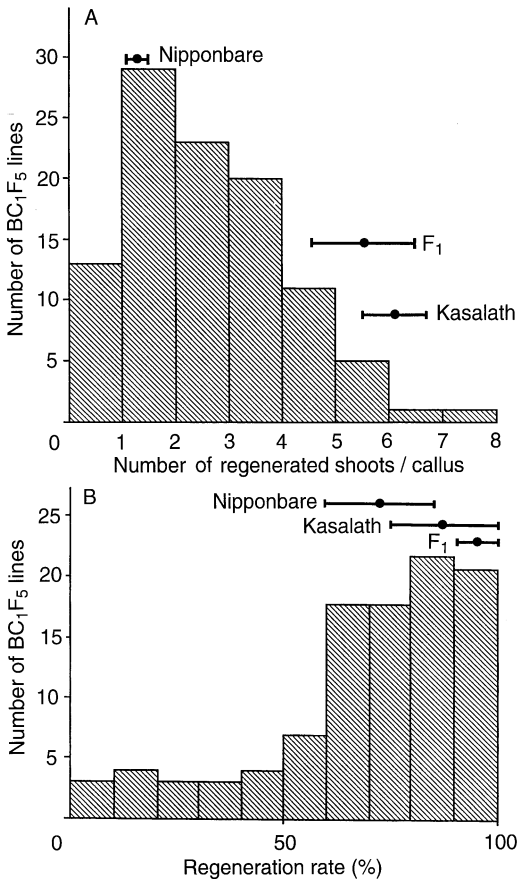

Fig. 2A,B Frequency distributions for BC_1F_5 lines of the average number of regenerated shoots per callus (**A**) and the regeneration rate (**B**), with the mean values and standard deviations for 'Nipponbare', 'Kasalath' and their F_1 progeny

1996). In rice, haploid production by interspecific hybrid systems, such as the *bulbosum* method of barley, have not been established yet; a population for analysis should be produced through conventional breeding to avoid selection by tissue culture. Harushima et al. (1996) reported distorted segregations in F_2 individuals between 'Nipponbare' and 'Kasalath'. The strongest distortion was observed on chromosome 3 with 3.2% of the homozygous genotype in 'Nipponbare', 49.5% in 'Kasalath' and 47.3% in the heterozygote. This distortion was recovered from by backcrossing an F_1 between 'Nipponbare' and 'Kasalath' with 'Nipponbare' to produce a BC_1F_1 plant, which was selfed four times. In the BC_1F_5 lines used in the present analysis, segregation at this point on chromosome 3 was observed with 55.7% of the homozygous genotype in 'Nipponbare', 37.1% in 'Kasalath' and 7.2% in the heterozygote. Of the BC_1F_5 lines, 95.4% had homozygous genomes (Lin et al. in preparation). Thus, the BC_1F_5 lines were considered to be almost fixed and to be a suitable population for our genetic analysis.

Austin and Lee (1996) compared QTL detected in F_2 individuals and those in F_6 lines and demonstrated that some of the QTL in the F_2 were dissected into multiple QTL in the F_6. In the present analysis, the use of nearly fixed lines produced through many recombination events was considered to increase the resolution of mapping putative QTL and led to the successful dissection of multiple QTL on chromosome 4 (Fig. 3B). A precise identification of QTL will enable a better understanding of the genetic basis of the regeneration process in plant tissue culture.

We adopted two indices, number of regenerated shoots per callus (NRS) and regeneration rate (RR).

Table 1 Association between QTL and regeneration ability, the average number of regenerated shoots per callus (NRS) and the regeneration rate (RR), based on a single-point analysis of variance and MAPMAKER/QTL

QTL	Nearest marker	Chromosome	SAS/GLM		MAPMAKER/QTL[a]			
			Probability	R^2	LOD	% Variation	AE[b]	DPE[c]
NRS								
qRg1	C1370	1	0.0035	0.09	1.2	6.2	0.147	K
qRg2	R418	2	> 0.0001	0.21	4.3	18.6	0.223	K
qRg4a	C445	4	> 0.0001	0.15	3.7	16.4	0.227	K
qRg4b	C1100	4	0.0004	0.14	2.6	11.6	0.223	K
qRg4c	C946	4	0.0007	0.12	2.5	11.2	− 0.174	N
					Total[d]	38.5		
RR								
qRg2	R3393	2	> 0.0001	0.13	3.1	14.0	0.139	K
qRg4a	C445	4	0.0004	0.13	3.1	14.1	0.151	K
qRg4b	R374	4	0.0065	0.08	1.3	6.4	0.121	K
qRg4c	R1854	4	> 0.0001	0.16	3.1	13.7	− 0.139	N
					Total[d]	32.6		

[a] MAPMAKER/QTL was used in the "bc1"setting
[b] Additive effect (1/2 weight): a 'Kasalath' allele effect compared with a 'Nipponbare' allele effect
[c] Direction of phenotypic effect. Parents having positive action are shown. *N* and *K* indicate 'Nipponbare' and 'Kasalath', respectively
[d] Estimates obtained from a multiple QTL model

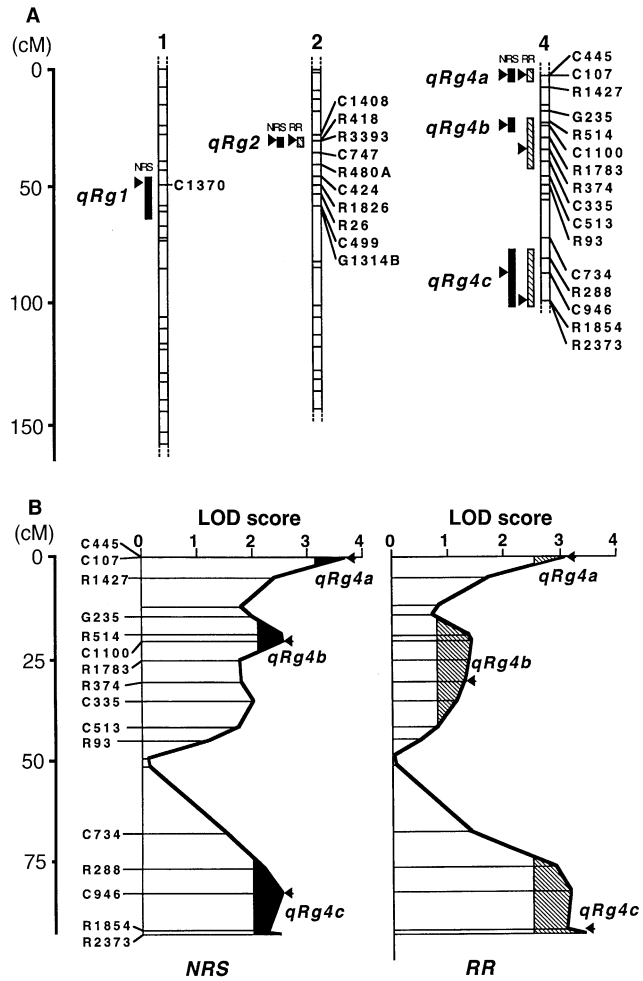

Fig. 3A,B Genetic linkage map showing locations of putative QTL associated with regeneration ability (**A**) and LOD scores of chromosome 4 obtained from the single-point analysis (**B**). *Black and striped bars* (**A**) or *areas* (**B**) show the QTL for the number of regenerated shoots per callus (*NRS*) and for the regeneration rate (*RR*), respectively. *Bar length* indicates a 0.5-LOD support interval for the most likely QTL positions shown in **B**. Names of the markers significant at the 0.01 probability level are shown, and *arrows* indicate the most significant marker in each QTL detected in single-point analysis of variance

RR is the index of callus regeneration used in most of the previous genetic analyses of regeneration ability of somatic cell cultures (Armstrong et al. 1992; Koornneef et al. 1993; Yu and Pauls 1993; Komatsuda et al. 1993; Mano et al. 1996). We adopted RR in this study to make our results comparable with those from these earlier studies. Among the previous papers in which RR was adopted, three reported locations of loci for regeneration ability and the percentages of phenotypic variance that these loci accounted for, although the explants used were not seed (mature embryo) but immature embryos. In maize, Armstrong et al. (1992) detected three loci ($P < 0.05$) which explained 82% of the total phenotypic variance. In barley, Komatsuda et al. (1993) identified two loci ($P < 0.01$), each of which explained 65.4% and 20.7% of the total phenotypic variance, respectively. Analyzing barley DH lines originating from different cross combinations, Mano et al. (1996) detected four QTL (LOD > 2.0) which explained 49.8% of the total phenotypic variance. The number of loci detected in these reports ranged from two to four, consistent with our results; however, the percentages of phenotypic variation accounted for by these loci were very different. One possible explanation for this difference is that each report employed a different threshold to detect loci. Another possible explanation is that there were differences between sets of genes detected in the various populations.

The two parents, 'Nipponbare' and 'Kasalath', have different phenotypes for regeneration ability (Fig. 1); however, the difference between the parents for RR is not clear (Fig. 2B). For NRS, the difference between the parents is clear (Fig. 2A), and NRS has a higher broad-sense heritability, 0.919, than does RR, 0.736 (Taguchi-Shiobara et al. 1997). We also adopted NRS because it reflects the genotype more accurately than RR, and we expected that NRS would be able to detect QTL which RR would not. In accordance with this expectation, using NRS we detected one more QTL, *qRg1*, which was not detected using RR (Fig. 3A). The four QTL for RR were mapped to the same chromosomal regions as the QTL for NRS, although most of the BC_1F_5 lines showed the same regeneration rates as their parents. NRS could explain more phenotypic variance (38.5%) than RR (32.6%), which is consistent with their broad-sense heritabilities.

BC_1F_5 lines having one or more alleles for increased regeneration ability had a higher regeneration ability in general. However, BC_1F_5 lines having positive alleles for all of the detected QTL did not always have the highest regeneration ability. These results suggest that some unknown genes with minor effect for this trait remain unknown and that the regeneration ability of BC_1F_5 lines could be enhanced further by the addition of such genes.

The detection of QTL contributing to plant regenerable callus formation has already been reported in several species. It is not yet clear whether there are any genes in common in different species. Comparative mapping of genera and/or families using common markers in the vicinity of such QTL is necessary to identify homologous QTL or genes which can work universally in plant tissue culture.

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