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Molecular-marker-facilitated investigation on the ability to stimulate N_2 fixation in the rhizosphere by irrigated rice plants

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Abstract An F_2 population, consisting of 231 individuals derived from a cross between rice cultivars with a similar growing duration, Palawan and IR42, was utilized to investigate the genetic nature of rice varietal ability to stimulate N_2 fixation in the rice rhizosphere. To assess rhizospheric N_2 fixation, an isotope-enriched ^{15}N dilution technique was employed, using ^{15}N-stabilized soil in pots. IR42, an indica variety, had 23% higher N derived from fixation (Ndfa) than Palawan, a javanica genotype. Normal segregation of atom% ^{15}N excess was obtained in the F_2 population, with an average of 0.218 with 8% of plants below IR42 (0.188) and 10% of plants above Palawan (0.248). One-hundred-and-four RFLP markers mapped on 12 chromosomes were tested for linkage to the putative QTLs. Significant ($P<0.01$) associations between markers and segregation of atom% ^{15}N excess were observed for seven marker loci located on chromosomes 1, 3, 6 and 11. Four QTLs defined by the detected marker loci were identified by interval-mapping analysis. Additive gene action was found to be predominant, but for at least one locus, dominance and partial dominance effects were observed. Significant ($P<0.01$) epistatic effects were also identified. Individual marker loci detected between 8 and 16% of the total phenotypic variation. All four putative QTLs showed recessive gene action, and no phenotypic effects associated with heterozygosity of marker loci were observed. The results of this study suggest that rice genetic factors can be identified which affect levels of atom% ^{15}N excess in the soil by interacting with diazotrophs in the rice rhizosphere.

Key words *Oryza sativa* L. · Atom ^{15}N% excess · N_2 fixation · Restriction fragment length polymorphism (RFLP)

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Introduction

Considerable variation for rice varietal ability to stimulate N_2 fixation in the rhizosphere has been recognized over the past two decades (App et al. 1986; Ladha et al. 1988). Varieties with higher biological nitrogen fixation (BNF) ability are desirable because such varieties would enhance BNF without requiring a change in current practices and would not impose major socio-economic or environmental constraints (Ladha et al. 1993). Before initiating a breeding program to develop a rice variety which can support more N_2 fixation, it is advisable to study the genetic background of the trait. Using a diallel-cross analysis and the acetylene-reducing assay (ARA), Iyama et al. (1983) demonstrated that this ability is heritable and that the activity may be considered as a quantitative trait controlled by recessive genes. ARA values found in rice mutants may be twice that of their parents (Dommergues 1978). However, ARA is not a time-integrated method and also does not measure the incorporation of fixed N_2 into the plant because the measured rates cannot be easily or accurately extrapolated to obtain a quantitative estimation of the amount of N_2 fixed (Roger and Ladha 1992).

The ^{15}N isotope dilution technique has been accepted as the most accurate time-integrated way of estimating the contribution of legumes and non-legumes alike to N fixation. The method assumes that the $^{15}N/^{14}N$ ratio of N absorbed from the soil is the same for the plants under comparison, which is met when the ^{15}N enrichment of soil N is stabilized (Pareek et al. 1990; Rarivoson and Ladha 1992). The reliability of the ^{15}N dilution method using soil-available-N as a reference (Wu 1993) has made it possible to quantitatively analyze the genetic background of the trait for the first time. The application of the method in large-scale screening programs is limited because of the time involved and other technical limitations. Sophisticated procedures are required for preparing ^{15}N-enriched and -stabilized soil and sample preparations for ^{15}N analysis. For this reason, the availability of molecular marker(s) linked to QTLs for the trait would provide a valuable tool for breeders.

The advent of methods for detecting DNA polymorphisms has made possible the development of molecular maps for many plant genomes, thus permitting the localization of quantitative trait loci (QTLs) and the determination of the relative magnitude of their effect on traits of interest. The well-developed rice RFLP map (Causse et al. 1994) allows genetic evaluation of QTLs associated with quantitative traits of interest in rice (Champoux et al. 1994; Wang et al. 1994). The objective of the present study was to identify RFLP marker(s) associated with QTLs affecting rice varietal ability to stimulate associative N_2 fixation in the rhizosphere, and to estimate the relative effects and types of gene action exhibited by the putative QTLs.

Materials and methods

Plant materials and ^{15}N-labelled soil preparation

Two long-duration (125 to 130) rice varieties, IR42 (indica, IRRI release) and Palawan (javanica, Philippine native variety), were previously identified as having higher and lower ability, respectively, to stimulate N_2 fixation in the rhizosphere (Ventura and Watanabe 1983; App et al. 1986). These varieties were crossed in a greenhouse at the International Rice Research Institute (IRRI), Los Banos, Philippines, in 1992. The resultant F_1 hybrid was selfed and 250 F_2 plants were used in a ^{15}N-dilution pot experiment during 1993. The first tiller of each of the F_2 plants was separated and replanted, and later used for DNA extraction.

The pot experiment was conducted with 10 kg of air-dried soil per pot. The soil [Isohyperthermic Andaquepic Haplaquoll (Maahas series)] was collected from the IRRI farm, and contained about 0.15% total N and 31.2 ppm of 2 M KCl-extractable NH_4^+-N (KCl:soil=3:1). The soil was air-dried and sieved through a 1-cm screen before being incubated in a concrete pool under water (5 cm water above the soil surface). Seventy-five grams of ^{15}N-labelled (75%) $(NH_4)_2SO_4$ was dissolved in about 5 l of tap water and applied uniformly to the pool. The soil was incubated for 6 months with a thorough weekly mixing. Four replications of the soil sample were randomly collected from the incubation pool at 30-day intervals to examine the stabilization of ^{15}N NH_4^+-N (Table 1). The soil NH_4^+-N was extracted from 50 g of a fresh soil sample using 150 ml of 2 M KCl (Buresh et al. 1982). An amount of extract sufficient to obtain 1.2 mg N in the distillate was distilled with 5% MgO into a saturated boric acid solution and was titrated with an automatic titrator (Model AT-117, Kyoto Electronics, Japan). The distillate was acidified with one or two drops of 0.1 N H_2SO_4, and the volume reduced to near dryness in a glass vial on a hot plate to recover the $(^{15}NH_4)SO_4$ salt. Complete drying of the sample was avoided. The ^{15}N analyses were carried out on a mass spectrometer (VG MM 622, England) connected with Rittenberg setup at the IRRI Analytical Service Laboratories and results were expressed in terms of atom ^{15}N% excess (Rennie et al. 1978), as:

$$\text{Atom } ^{15}N\% \text{ exc.} = {}^{15}N \text{ abundance (sample)} - 0.3663 \text{ (natural } ^{15}N \text{ abundance)}$$

$$^{15}N \text{ abundance} = 100/(2R+1),$$

where R is the M/e 28 and M/e 29 ratio from the mass-spectrometer ion current.

The difference in relative percent N derived from air (Ndfa%) between the parents was calculated using Palawan as a reference plant according to the formula (Rennie et al. 1978):

Ndfa% of IR42=[1–(atom ^{15}N% exc. of IR42/atom ^{15}N% exc. of Palawan)]×100

Experimental procedure and plant ^{15}N analysis

The pot experiment was conducted in a greenhouse at IRRI using the stabilized ^{15}N-enrichment soil; 15-day-old seedlings of two parents and 250 F_2 plants were transplanted from seedling trays into pots. F_2 plants and 20 replications of the two parents were individually potted. Five grams of P and K per pot were applied to the soil as $CaHPO_4$ and KCl. No N-fertilizer was applied but phosphorus and potassium were supplemented for the experiment. The pots were randomly arranged and maintained under flooded conditions during the course of the experiment.

To investigate the growth pattern of F_2 plants, maximum tillering and heading stages were recorded for each of the F_2 plants. Whole plants were harvested at maturity. After the plants were carefully washed and rinsed with distilled water, they were oven dried at 70°C for 3 days and ground to a powder capable of passing completely through a 1-mm sieve. A subsample was ground by a vibrating mill (Heiko TI-100, Heiko Seisakusho Ltd. Japan). Ten milligrams (±0.1) of fine sample was prepared for ^{15}N analysis by the mass spectrometer as above.

Constructing the RFLP map

Rice genomic clones (RG), rice cDNA (RZ) and oat cDNA (CDO) were kindly provided by Dr. S. Tanksley, Cornell University. A subset of 200 markers spaced at less than 20-cM intervals throughout the 12 chromosomes, based on the published rice RFLP map (Causse et al. 1994), was used to survey the parents for polymorphisms. Leaf tissue from each parent was collected and ground in liquid N_2. DNA was isolated, digested with one of six restriction enzymes (*Eco*RI, *Eco*RV, *Sca*I, *Hin*dIII, *Xba*I and *Dra*I) according to the manufacturer's recommendations (Boehringer Mannheim), electrophoresed overnight at 20–25 V, blotted in 0.4 M NaOH solution onto N^+-Hybond membranes (Amersham Corp., Chicago), and probed with [^{32}P]dCTP-labelled probes (Feinberg and Vogelstein 1984). Autoradiographs were made by exposing Kodak X-ray film to the membrane at –80°C with intensifying screens for 2–4 days depending on the radioactivity. One-hundred-and-nine RFLP markers were polymorphic between the parents. Two-hundred-and-thirty-one F_2 plants were scored with the polymorphic markers. The scores were analyzed with MAPMAKER (Mapmarker ver./1.0, Macintosh) to construct a genetic map. A minimum LOD of 3.0 was used to place a marker in a linkage group and the genetic distance in cM was estimated using the Kosambi function. The order of the markers was established using multiple-point analysis at a LOD of 3.0. Of the 109 markers, five could not be associated with any linkage groups. Thus, the RFLP map was constructed with 104 markers.

Statistical analysis

The effects of individual marker loci and all possible digenic epistatic interactions were tested on the segregation of atom ^{15}N% ex-

Table 1 Atom ^{15}N% excess in 2 M KCl-extracted NH_4^+-N (KCl:soil=3:1) of the incubated soil at different sampling times (n=4)

Time lapse (days)	0	30	60	90	120	150	180
Atom ^{15}N% exc.	0.547	0.418	0.366	0.310	0.292	0.289	0.294
CV%	2.14	2.09	1.37	1.41	1.12	1.26	1.18
NH_4^+-N (ppm)	26.83	31.82	29.75	32.91	30.21	30.06	29.73
CV%	4.01	5.54	3.74	5.43	2.04	3.11	2.88

cess using analysis of variance (SAS GLM procedure). Two orthogonal contrasts (linear and quadratic) at each locus, and for significant epistatic interactions detected, were tested for detecting gene actions (Edwards et al. 1987). Marker loci were considered to be significantly associated with a QTL when the F-test for a contrast exceeded an F-value with a probability level less than 0.01. Interval-mapping analysis using Mapmaker/QTL (v/1.1) (Lander and Botstein 1989) was also carried out to identify putative QTLs. The dominance effect was expressed as the ratio of dominance effect to additive effect according to the suggestion of Stuber et al. (1987). A χ^2 test was used for testing marker genotype frequency. To determine whether heterozygosity per se was predictive of atom $^{15}N\%$ excess performance, the percent heterozygosity of the 104 marker loci was regressed against the value of the atom $^{15}N\%$ excess of each of the 231 plants.

Results

Phenotypic variation in atom $^{15}N\%$ excess

A significant difference in atom $^{15}N\%$ excess was found between the two parents. The relative%Ndfa of IR42, estimated in terms of the difference in atom $^{15}N\%$ excess between the parents (IR42: 0.189; Palawan: 0.246), was about 23% higher, which is consistent with previous observations (Wu 1993). Normal segregation of atom $^{15}N\%$ excess was observed in F_2 plants with an average of 0.218±0.019, though some transgressive variation was observed. About 8% of the plants fell below IR42 and 10% of the plants were higher than Palawan (Fig. 1). The growing durations of the parents and the F_2 plants were found to be similar with 125 to 130 days, and no clear differences in maximum tillering and heading stages were found in the F_2 plants.

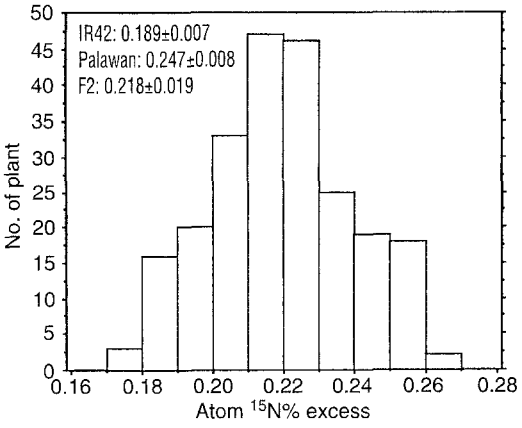

Fig. 1 Distribution of atom $^{15}N\%$ excess in the F_2 population with 231 individuals derived from Palawan/IR42

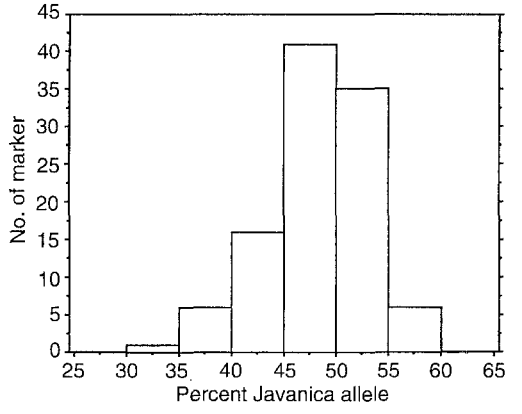

Fig. 2 Frequency of Javanica allele of 104 markers in an F_2 population with 231 individuals derived from Palawan/IR 42

Segregation and linkage of RFLP marker loci

The majority of RFLP loci fit the expected 1:2:1 segregation ratio (Fig. 2). Fourteen percent of marker loci exhibited segregation distortion toward the indica parent with 6% skewed toward the javanica parent. The linkage map obtained from the 104 markers was similar to the linkage map developed at Cornell University (Causse et al. 1994). However, the recombination fraction between linked markers was generally larger, possibly due to the fact that the population was derived from an inter-subspecific, rather than an inter-specific, cross (Fig. 3). Sixteen percent of marker loci showed a dominant/recessive segregation pattern such that only two genotypes could be identified. Multiple-copy fragments were observed for some markers. Generally only a single polymorphic fragment was scored and mapped among the copies.

Identification of putative QTLs

Linear and quadratic contrasts among three genotypic classes for 104 marker loci were examined to identify the marker loci associated with the expression of the atom $^{15}N\%$ excess, as well as the gene action at each locus. Highly significant ($P<0.01$) differences between marker classes were presumed to be due to linkage to QTLs affecting the ability to stimulate N_2 fixation in the rhizosphere. Eight marker loci located on four chromosomes had a significant linear contrast. A significant quadratic contrast was found for marker RG247 (Table 2). No significant ($P<0.01$) digenic epistatic interactions were observed between the significant marker loci detected.

Four intervals with significant LOD scores ≥3.0 were observed and the intervals were flanked by RG381 and RZ730 on chromosome 1, RG944 and RZ329 on chromosome 3, RZ588 and RG213 on chromosome 6, and RG1094 and RG247 on chromosome 11 (Table 3 and Fig. 3). This result is consistent with that of single-marker analysis (Table 2). Among the four putative QTLs, segregation distortion was only detected for RG944 ($P<0.01$ in χ^2 test) with skewness toward the Indica genotype (Table 4). It is noteworthy that this marker falls in a region previously reported to contain a gametophyte gene that systematically favors indica alleles in indica/japonica crosses (Nakagahra 1972; Causse et al. 1994).

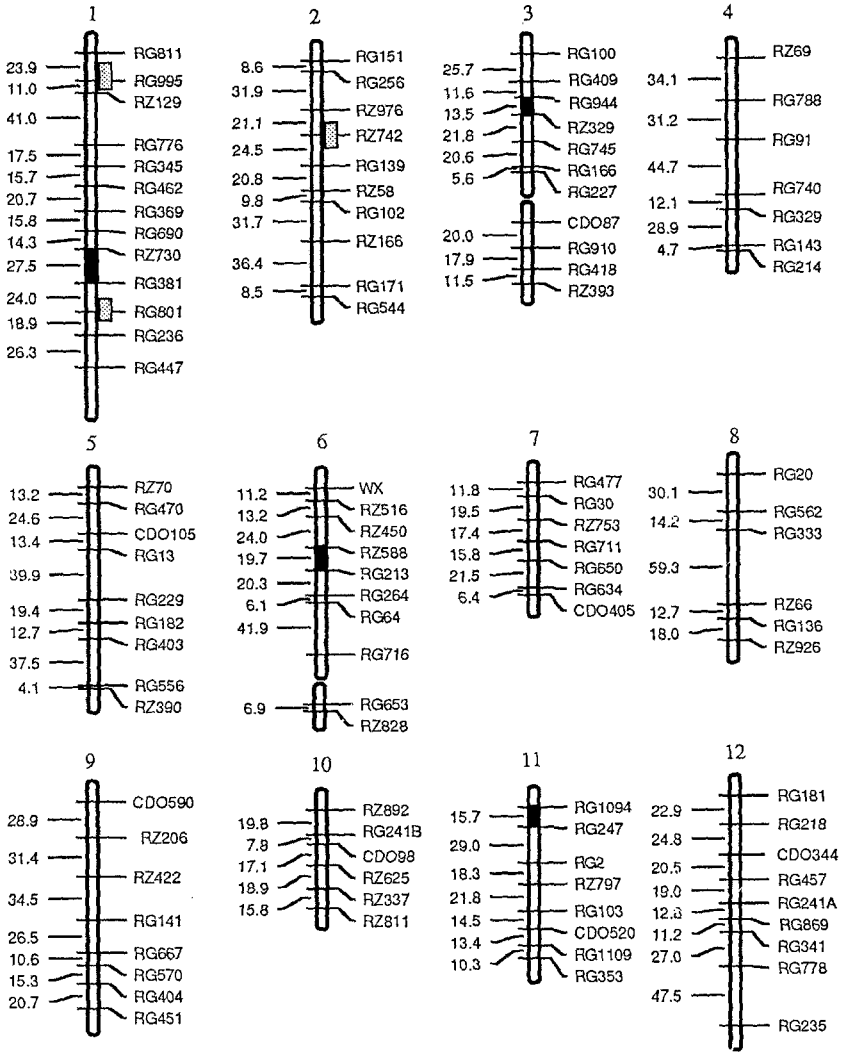

Fig. 3 RFLP map derived from the segregation data of 231 F_2 individuals obtained from Palawan/IR42. The map distances depicted here are calculated in centiMorgans using Mapmaker/ Version 1.0 (Macintosh). The *solid-filled* areas on chromosomes 1, 3, 6 and 11 represent supporting intervals containing QTLs underlying atom N% excess variation. The *stippled bars* on the right indicate the marker loci with non-allelic interaction effects between RG801 and two other loci. Designations to the right represent marker names and to the left represent map distances in cM on the Kosambi function

Table 2 Orthogonal contrast for detecting single marker loci significantly ($P<0.01$) associated with atom ^{15}N% excess variation

RFLP marker	Chromosome number	Linear[a]		Quadratic	
		MS[b]	F-value	MS	F-value
RG381	1	0.005	14.63***	2.95E-4	0.96
RZ730	1	0.003	9.99**	0.001	3.69
RG944	3	0.006	20.03***	1.60E-4	0.50
RZ329	3	0.003	9.62**	4.87E-4	1.42
RG409	3	0.003	8.47**	5.08E-5	0.14
RZ588	6	0.006	13.23***	2.81E-4	0.48
RG247	11	0.002	6.71**	0.002	5.64**

, * Significant at the 1% and 0.1% level, respectively
[a] Linear orthogonal coefficients are 1, 0, −1; quadratic coefficients are 1, −2, 1
[b] MS = mean square

Types of gene action

Although the results of contrast analysis (Table 2) showed that gene action was predominantly additive for atom ^{15}N% excess, the ratio of dominance to additive effects (D/A) for the putative QTLs indicated that there may be some partial dominance and that dominant gene action may be important for at least one locus (Table 3). A mean comparison of the four marker loci putatively associated with QTLs showed that the genotypic class consisting of IR42 alleles was superior (i.e., had a lower atom ^{15}N% excess) to the genotypic class with Palawan alleles and that the effect was due to recessive genes (Table 4). Significant additive×additive and dominant×dominant non-allelic interaction effects (epistasis) were observed in two allele combinations, RG801 with RG995 and RG801 with RZ742, respectively (Table 5). The mean comparison results shown in Table 6 indicate that the recombinant genotypes, consisting of individuals that are homozygous for the Indica allele at RG801 and Javanica alleles at RG955 and RZ742, showed a greater ability to dilute ^{15}N abundance. The individual marker loci detected explained up to 16% of the total phenotypic variation (Table 3). Collectively, the four marker loci that are closely linked to the four QTLs detected were able to account for nearly 36% of the observed phenotypic variation (multiple R^2=0.36). A regression analysis between the percent heterozygosity of 104 marker loci and the atom ^{15}N% excess value of each of the 231 plants was

Table 3 Markers bracketing four QTLs underlying atom $^{15}N\%$ excess variation

QTLs	Chromosome	LOD[a]	Add.[b]	Dom.[c]	% Variation[d]	D/A[e]
RG381-RZ730	1	4.7	0.010	0.007	15.7	0.70
RG944-RZ329	3	5.7	0.009	0.004	13.5	0.45
RZ588-RG213	6	4.5	0.008	0.003	8.9	0.33
RG247-RG1094	11	3.0	0.006	0.006	7.8	1.00

[a] LOD scores (log10-likelihood ratio)
[b] Additive effect
[c] Dominance effect
[d] The fraction of the total variation in the trait across the population explained by the QTL
[e] The ratio of dominant effect to additive effect

Table 4 QTL effects on atom $^{15}N\%$ excess variation as determined by a means comparison (LSD) of closely linked RFLP markers

QTL	RFLP marker	Geno-type[a]	Mean[b]	Frequency (%)	χ^2
anf-1q	RG381	I/I	0.207A	23.1	
		I/J	0.218B	50.4	
		J/J	0.223B	27.0	0.32
anf-2q	RG994	I/J	0.209A	37.5	
		I/J	0.220B	49.1	
		J/J	0.227B	13.4	11.73**
anf-3q	RZ588	I/I	0.209A	29.3	
		I/J	0.219B	46.8	
		J/J	0.224B	23.9	0.95
anf-4q	RG247	I/I	0.209A	25.7	
		I/J	0.220B	55.4	
		J/J	0.219B	18.9	1.74

** Significant ($P<0.01$) deviation from the expected frequency
[a] I=Indica, J=Javanica
[b] A significant difference ($P<0.01$) among genotypic classes is indicated by different letters (A, B)

Table 5 Orthogonal contrast for detecting non-allelic interaction significantly associated with atom $^{15}N\%$ excess variation

RFLP marker	Chrom. No.	*df*	MS	F-value
RG801 × RG995	1, 1	4		
Add. × Add.		1	0.006	17.58***
Add. × Dom.		1	5.45E-5	0.13
Dom × Add.		1	4.51E-4	1.14
Dom × Dom.		1	2.52E-6	0.01
FG801 × RZ742	1, 2	4		
Add. × Add.		1	0.002	4.13*
Add. × Dom.		1	0.001	1.76
Dom × Add.		1	0.001	3.17
Dom × Dom.		1	0.004	11.55***

*, *** Significant at 5% and 0.1% levels, respectively

conducted. Heterozygosity per se failed to predict the phenotypic performance for atom $^{15}N\%$ excess ($R^2<0.02$).

Discussion

It has been reported that ^{15}N abundance in soil-available N decreased sharply after the growth of rice and a slight difference in growing pattern may result in the apparent difference in ^{15}N contents at harvest (Watanabe 1991). Therefore, a similar growing pattern of the parents and the F_2 plants is desirable to reduce the error of estimating the difference in atom $^{15}N\%$ excess of the plants. This requirement was met in the present study. In addition, soil already stabilized with ^{15}N was used in the present study. Therefore the requirement of the ^{15}N dilution method that the $^{15}N/^{14}N$ ratio of N absorbed from the soil is the same for N_2-fixing and reference varieties was met. The normal segregation pattern of atom $^{15}N\%$ excess observed in the F_2 population, and the appearance of some transgressive variants, support the suggestion of Iyama et al. (1983) that this character, which is related to associative N_2 fixation in the rhizosphere, is a quantitatively inherited trait. Seven marker loci were identified that were significantly associated with atom $^{15}N\%$ excess in this cross. Four intervals on four different chromosomes were identified as containing putative QTLs for this trait (Table 2 and Fig. 3).

Using a diallel cross and the acetylene-reducing assay (ARA), Iyama et al. (1983) investigated the genetic background of N_2-fixing ability in the rice rhizosphere. He reported that N_2-fixing ability in rice is likely to be governed by recessive alleles and that non-allelic interaction existed. The findings in the present study support this suggestion. Though the gene action of the significant QTLs was mainly

Table 6 Means comparison of atom $^{15}N\%$ excess for combinated genotypes of significant non-allelic interaction gene actions

RFLP marker	Genotype[a]	Mean ± SD
RG801 × RG995		
Add. × Add.	II/JJ	0.196 ± 0.015
	II/JJ	0.231 ± 0.015
	JJ/II	0.222 ± 0.018
	JJ/JJ	0.215 ± 0.024
RG801 × RZ742		
Dom. × Dom.	(II + JJ)/(II + JJ)	0.212 ± 0.020
	(II + JJ)/JJ	0.232 ± 0.015
	IJ/(II + JJ)	0.221 ± 0.018
	IJ/IJ	0.218 ± 0.019
Add. × Add.	II/JJ	0.218 ± 0.028
	II/JJ	0.201 ± 0.017
	JJ/II	0.210 ± 0.015
	JJ/JJ	0.219 ± 0.020

[a] II = Indica homozygosity, JJ = Javanica homozygosity, IJ = heterozygosity

additive (Table 2), recessive effects were important for at least one of the marker loci (Table 3).

Of the four independent QTLs detected, marker locus RG381 on chromosome 1 explained the largest percent of the phenotypic variation for $^{15}N\%$ excess. The contribution of RG381 was twice that of the marker loci RG247 and RG588, on chromosomes 11 and 6, respectively. This suggests that although the varietal effect on the rhizosphere condition is controlled by multiple factors, some genetic factor(s) have a greater effect than others.

The possible mechanisms by which rice varietal differences associated with $^{15}N\%$ excess may affect diazotrophs in the rhizosphere that are responsible for biological nitrogen fixation remain a mystery. It has been hypothesized that the diazotrophs become associated with roots due to a specific attractant excreted by the roots, and/or by the establishment of favorable environmental conditions in the rhizosphere. Malic acid in the root exudates has been identified as the most important diazotrophs attractant associated with C_4 crops (Neyra and Hagerman 1978). In the case of rice, a C_3 crop planted under flooded conditions, the main organic acids in root exudates are citric acid and oxalic acid (Vancura 1964; Lin and You 1989). This suggests that the main attractant in the chemotaxis of diazotrophs in the rice rhizosphere is different from that in C_4 crops. Although it has long been reported that most of the bacteria (80%) found in rice roots are N_2-fixing bacteria (Watanabe et al. 1981), the physiological and biochemical mechanisms operative in highly efficient associations between rice roots and diazotrophs have not so far been elucidated (Ladha 1986). The questions that must be addressed if we are to understand the mechanisms leading to a more efficient association between diazotrophs and rice roots of specific genotypes are: (1) what component(s) of rice-root exudates are key attractants to the chemotaxis of diazotrophs, (2) what genotypic variation exists in the attractants, (3) what genera of diazotrophs proliferate in the rhizosphere and histosphere, and (4) what is the relationship between the rhizosphere environmental condition that favour N_2 fixation and plant agronomical and physiological traits. The difficulties encountered in investigating these questions reside in the complexity of analyzing root exudates from plants grown under natural conditions in soil. The use of molecular markers to identify genetic factors underlying these mechanisms may help us to understand and manipulate the associative N_2 fixation in the rhizosphere of rice.

Although the factors that affect the rhizosphere are complex, the identification in this study of at least four QTLs associated with the ability to stimulate associative N_2 fixation provide useful tools for further investigation. Near-isogenic lines containing QTLs of interest would provide valuable material for investigating under what conditions these genetic factors are associated with higher N_2 fixation and, specifically, whether they are responsible for the production of substances that interact with diazotrophs in the soil. The availability of molecular markers associated with $^{15}N\%$ excess opens up a new approach to the study of associated N_2 fixation, and paves the way for using these markers to assist in the development of rice varieties capable of stimulating biological nitrogen fixation in the rhizosphere.

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