Theor Appl Genet (1995) 90: 969–981

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Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers

Received: 28 January 1994 / Accepted: 22 September 1994

Abstract This research was undertaken to identify and map quantitative trait loci (QTLs) associated with five parameters of rice root morphology and to determine if these QTLs are located in the same chromosomal regions as QTLs associated with drought avoidance/tolerance. Root thickness, root:shoot ratio, root dry weight per tiller, deep root dry weight per tiller, and maximum root length were measured in three replicated experiments (runs) of 203 recombinant inbred lines grown in a greenhouse. The lines were from a cross between *indica* cultivar Co39 and *japonica* cultivar Moroberekan. The 203 RI lines were also grown in three replicated field experiments where they were drought-stressed at the seedling, early vegetative, and late-vegetative growth stage and assigned a visual rating based on leaf rolling as to their degree of drought avoidance/tolerance. The QTL analysis of greenhouse and field data was done using single-marker analysis (ANOVA) and interval analysis (Mapmaker QTL). Most QTLs that were identified were associated with root thickness, root/shoot ratio, and root dry weight per tiller, and only a few with deep root weight. None were reliably associated with maximum root depth due to genotype-by-experiment interaction. Root thickness and root dry weight per tiller were the characters found to be the least influenced by environmental differences between greenhouse runs. Correlations of root parameters measured in greenhouse experiments with field drought avoidance/tolerance were significant but not highly predictive. Twelve of the fourteen chromosomal regions containing putative QTLs associated with field drought avoidance/tolerance also contained QTLs associated with root morphology. Thus, selecting for Moroberekan alleles at marker loci associated with the putative root QTLs identified in this study may be an effective strategy for altering the root phenotype of rice towards that commonly associated with drought-resistant cultivars.

Key words Drought · Rice · QTL analysis · Root morphology · Molecular markers

Communicated by G. E. Hart

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Introduction

Rice (*Oryza sativa* L.) is cultivated in a wide range of ecosystems under varying temperatures and water regimes. The majority of rice ecotypes are semiaquatic plants adapted to saturated soil conditions where it is difficult for other crop species to survive. Of the 148 million hectares of land planted to rice in 1991, about 79 million hectares (53%) were planted to irrigated rice and had assured availability of water (IRRI 1993). Another 28% of the world's ricelands (41 million hectares) are rainfed lowlands, characterized by alternating flooding and drying as the result of irregular rainfall patterns. In these areas, farmers generally do not have access to irrigation and yields may be seriously jeopardized by drought. In the rainfed lowland ecosystem of eastern India, farmers identified drought stress as the foremost constraint to higher yields (Widawsky and O'Toole 1990). About 11% of rice production is in soil with no surface water accumulation, and this "upland rice" is almost always exposed to moisture stress during part of the growing season. Water stress, therefore, is an important limitation to rice production, particularly among low income and subsistence farmers.

Root morphology and rooting patterns directly affect the amount of water available to a crop. In a number of cereal crops, including rice, sorghum and maize, increased

width, depth, and branching of root systems have all been shown to decrease plant water-stress (Mambani and Lal 1983; Wright and Smith 1983; Lorens et al. 1987). While measurements of rooting depth and root length density alone may not predict the ability of a genotype to extract soil water, computer simulations designed to assess the consequences of increasing the root zone depth in soybean, sorghum, and wheat have suggested that such a change was accompanied by increased leaf area, growth, photosynthesis, transpiration (Jones and Zur 1984), and yield (Jordan et al. 1983; Muchow and Sinclair 1986; Jones and O'Toole 1987). In rice, emphasis on root diameter, depth of rooting, and root:shoot dry weight ratio in relation to drought resistance appears justified based on the positive correlations between these characters and visual scores of plant vigor in upland field drought-screening trials both in the Philippines (O'Toole and Soemartono 1981; Yoshida and Hasegawa 1982) and in Bouake, Cote d'Ivoire (Reyniers and Binh 1977). Ahmadi (1983) reported that maximum root depth and the dry weight of roots below 30 cm measured on rice plants grown in aeroponic culture were good indicators of drought avoidance when the same genotypes were grown in upland fields.

Root length, thickness, dry weight, and root length density have been reported to be polygenic traits with a substantial proportion of additive variation and narrow-sense heritabilities greater than 50% for most traits (Bhaduri and Ghosh 1965; Armento-Soto et al. 1983; Ekanayake et al. 1985 a; Chang et al. 1987). Though there appears to be no genetic barrier to selection for deep and thick roots in rice, the trait is difficult to measure and has rarely been used in breeding programs. Molecular-marker technology makes it possible to investigate the inheritance of both single gene and polygenic traits and to locate and manipulate individual genetic factors associated with characters of interest (Tanksley 1993). The availability of a linkage map of rice containing over 700 markers (McCouch et al. 1988; Causse et al. 1994) provides a basis for this analysis.

The goal of our research was to elucidate the genetics of morphological traits commonly associated with drought avoidance in rainfed lowland rice. Specifically, our objectives were: (1) to gain insight into the genetic basis of root morphology under controlled conditions; (2) to identify and map the quantitative trait loci (QTLs) associated with rooting habit and drought avoidance on the molecular map of rice; and (3) to determine if there is a genetic relationship between root morphology and drought avoidance under both upland and rainfed lowland conditions. Our ultimate goal is to develop marker-based selection tools to facilitate selection for rice genotypes with desirable root systems related to drought avoidance.

Materials and methods

Plant population

A population of 203 recombinant inbred lines (RI lines), a subset of those originally developed to study the genetics of resistance to rice blast (*Pyricularia oryzae*) (Wang et al. 1994), was employed. The parents (both *Oryza sativa* cultivars) differed at the subspecies level. The maternal parent was CO39, a lowland *indica* cultivar developed in India, and it was crossed to Moroberekan, an upland *japonica* cultivar originally developed in Guinea. Moroberekan is drought tolerant and has a deep, thick root system. Conversely, CO39 is susceptible to drought and has shallow, thin roots. Recombinant inbred lines were developed by single-seed descent to the F_7 generation, and panicles were bagged in each generation.

RFLP analysis

The RFLP data set used to detect genes controlling resistance to rice blast (Wang et al. 1994) provided the basis for the present study. The parents were surveyed for polymorphism using five restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Sca*I) and 280 RFLP markers distributed throughout the 12 chromosomes of rice (clones were from both genomic and cDNA libraries from cv IR36, courtesy of Steve Tanksley, Cornell University). Based on segregation in the 281 RI lines, a map consisting of 127 RFLP markers was constructed. The DNA extraction, digestion, Southern blotting, labeling of probes, Southern hybridization, band detection, and mapping procedures were as described in Wang et al. (1994).

Map construction

Marker order and genetic distances between markers along chromosomes were determined using Recombinant Inbred Plant Manager (RIPM)/Skew software (Manly 1993). The RIPM/Skew software enabled us to calculate the recombination frequency between markers in a recombinant inbred population in which allele frequencies are skewed (80% CO39:20% Moroberekan throughout the genome). Recombination frequencies are expressed as Kosambi cM (Kosambi 1944). The marker order generated by RIPM/Skew was identical to that determined by Mapmaker/Exp 3.0 (Lander et al. 1987; Lincoln et al. 1992 b), but the genetic distances between markers were calculated to be slightly greater than those computed using the RI algorithm in Mapmaker 3.0, due to the adjustment for the skewed allele frequency in our RI population.

Evaluation of root characters

The evaluation of root morphology was conducted in three different greenhouse experiments in pots at IRRI. All experiments used a randomized complete block design with one plant per pot (rep). Pots 10-cm diameter and 1-m deep were lined with a plastic sleeve having two drainage holes in the bottom, and filled with soil to within 8 cm of the top. The soil was a nonagricultural sandy loam taken from a river bank near Pagsanhan, Laguna. The natural fertility of the soil was assessed and supplemented with full-strength nutrient solution (Yoshida et al. 1976). After filling and before planting, pots were watered and then refilled with soil until the saturated soil level was again within 8 cm of the top.

At 38 days-after-sowing (DAS) plant height was measured from the soil surface to the tip of the longest leaf, the tillers were counted, and the shoot was excised from the roots near the soil surface. The plastic bag containing the soil and roots was then laid out on a screen frame and separated into 15-cm sections, measured from the soil surface. The lowermost sections of soil were sequentially searched in order to identify the maximum rooting depth of any single nodal root (MaxRD). The thickness (THK) of six nodal roots at 2 cm below the stem base was measured to the nearest 100 microns with a graduated optical lens. The shoot and the roots from each 15-cm section were placed in envelopes and dried at 60C for 2 days. Dry weights were used to compute dry root weight/dry shoot weight (R/S), dry root weight tiller-1 (R/T), and deep root weight tiller-1 (DR/T), computed as dry root weight below 30 cm per tiller. The abbreviations for each root parameter are listed in Table 1 and will be used throughout the remainder of the text.

Identification of QTLs associated with root morphology experiment

The root morphology of the 203 RI lines and the two parents were evaluated in the first set of greenhouse experiments. The experiment was repeated three times (GH1, GH2, and GH3), with three replications of each genotype per run, for a total of nine replications. Experiment GH1 was seeded on 14 September, 1992, GH2 on 3 November, 1992, and GH3 on 15 January, 1993. Ten seeds of a single RI line or parental cultivar were sown in a pot and thinned to one plant per pot 10 days after sowing. All plants in the first and third runs were watered three times per week with 300 ml of nutrient solution. Plants in the second run of the experiment were watered daily with 150 ml of nutrient solution.

The three runs of the QTL identification experiment provided three different environments. The greenhouse temperatures were the highest for GH1, which coincided with an unseasonable period of high temperatures and cloudless days. Plants in GH2 and GH3 experienced much cooler temperatures and skies that were overcast most of the time. In addition, half of the third replication in each run was exposed to a window on two sides and subsequently to cooler temperatures than was experienced by the other replications. Significantly reduced root growth of the third replication in both GH2 and GH3 was observed (ANOVA, $P< 0.05$). These two replications were excluded from the analyses, leaving GH1 with three replications, and GH2 and GH3 with two replications each. Computations of mean values were therefore based on a total of seven replications in which no significant differences between greenhouse runs were observed (two-way ANOVA; $P< 0.05$).

Anaerobic conditions experiment

This experiment was done to determine if the expression of root phenotypic characters was consistent under flooded conditions (simulating the lowland ecosystem) and well-drained conditions (simulating the upland ecosystem). After plants had been scored for root characters in the QTL identification experiment (see above), a subset of 15 entries was chosen to represent three composite groups with five entries each, i.e., low, medium, and high scores for the five root traits combined. Entries were selected based on their overall phenotype, considering all traits simultaneously, rather than by considering scores for individual traits. The 15 entries were replicated five times. They were sown on 14 April, 1993, and grown in the greenhouse under the same experimental conditions as described for the QTL mapping experiment except that the soil was continuously flooded. Plants were harvested at 38 DAS and evaluated as previously described.

QTLs associated with the seedling early and late vegetative stage drought-avoidance experiments

The 203 RI lines grown in the greenhouse QTL-identification experiment were dry seeded in three different field experiments (FE1, FE2, and FE3) at the IRRI Central Research Farm on a well-drained site of Maahas clay loam soil. Plants were visually evaluated for leaf rolling two to four times during each experiment starting when the most susceptible entries had tightly rolled leaves in response to moisture stress. On evaluation days the degree of leaf rolling by each genotype was visually assessed and scored from "1" to "5" according to the scale of Turner et al. (1986). A score of "1" indicates completely unrolled leaves (tolerant phenotype), compared with "5" for plants with tightly rolled leaves forming a cylinder (susceptible). Evaluations were made between the hours of 10:00 and 14:30. The results of leaf-rolling evaluation on a single date were analyzed.

The 203 entries were planted at a rate of six seeds per hill in a randomized complete block design. Entries were seeded in 2- by 10-hill plots spaced 20 cm × 20 cm apart (0.4 m^{-2}). For comparison, Moroberekan and CO39 were grown side by side every ten plots in 1-hill by 10-hill plots. After seeding, Carbofuran was applied at 2.5 kg ha^{-1}. During periods of no moisture stress, sprinkler irrigation was applied three times per week until the soil was saturated.

The first field experiment (FE1) was designed to evaluate the effect of moisture stress in the late vegetative stage. The late vegetative-stage experiment consisted of four replications of the 203 RI lines seeded on 19 December, 1991. At 40 DAS, irrigation was terminated. Rain fell at 48 DAS, after which stress resumed. Due to the slow drying time for the soil in this experiment, 22% of the entries entered the reproductive phase and were omitted from further analyses.

The second field experiment (FE2), designed to evaluate stress in the early vegetative stage, consisted of three replications seeded on 18 January, 1992. Irrigation water was withheld beginning at 27 DAS. At 35 DAS, 2 cm of rain fell, temporarily alleviating moisture stress.

The third field experiment (FE3) was a four-replication seedling-stage stress experiment, seeded on 24 March, 1992. Irrigation was stopped at 21 DAS but this experiment received 2.5 cm of rainfall at 27 DAS and observations on leaf rolling were taken 10 days after the rain.

Soil water potential was taken on a subset of entries in each field experiment at the time of visual evaluation for leaf rolling using soil tensiometers placed at 15, 30, and 45 cm below the soil surface and located approximately every 250 m^{-2}. This data provided an estimate of the relative degree of moisture stress experienced by the plants just prior to data collection. Leaf water potentials of 24.1, 22.3, and 19.0 kPa for the late vegetative, early vegetative, and seedling-stage experiments, respectively, were recorded. These readings demonstrated that there were differences in the absolute degree of moisture stress experienced by the plants when leaf rolling was evaluated in the three experiments; the FE3 (seedling-stage) experiment received the least degree of stress. These differences were unavoidable because of the unpredictability of rainfall under field conditions and because plants were analyzed at different growth stages. As determined by these measurements, the relative degree of moisture stress within an experiment was approximately equivalent.

All three experiments received 30 kg ha^{-1} of NPK the day of seeding, and the late vegetative-stage experiment received an additional 30 kg ha^{-1} at 37 DAS. Throughout the experiments the water table was deeper than 1.5 m below the surface.

Statistical analysis

A standard analysis of variance (PROC GLM, SAS 1988) was used to evaluate mean differences between the two classes defined by each marker locus for THK, R/S, R/T, DR/T, and MaxRD in the greenhouse QTL identification experiment and leaf rolling was evaluated at the seedling stage, early vegetative stage, and late vegetative stage in the field experiments. This analysis is referred to hereafter as the single-marker analysis. Results were compared to those obtained using Mapmaker/QTL (Paterson et al. 1988; Lincoln et al. 1992 a), referred to as the interval analysis. Because Mapmaker/QTL does not have a specialized algorithm to analyze RI populations, the function for F_2 intercrosses was used and all genetic effects were constrained to be additive. Dominance effects cannot be analyzed in an RI population and, in this study, residual heterozygotes were coded as missing data. Both single-marker and interval analysis were performed on each trait for greenhouse and field experiments. In the greenhouse experiment, analyses were performed on the overall mean of each root trait and on the mean of each experiment separately. Each trait was also checked for genotype-by-experiment interactions. The significance threshold for identification of a QTL was set at $F>19.22$ ($P< 0.0001$) for single-marker analysis. This was equivalent to a LOD>4.0, which was used for interval analysis (see Appendix).

Two variable and higher-order regression models (PROC RSQUARE, SAS, 1988) were also used to assess the significance of combinations of putative QTLs. In addition, Mapmaker/QTL models were examined in which the variance associated with one variable was held constant while the remainder of a chromosome was scanned for significance of a second putative QTL.

Results

Frequency distribution of phenotypes

Figure 1 illustrates the distributions observed in FE1 and FE3 of the drought-avoidance QTL experiments. Though the mean visual score differs in the two experiments, the distribution of the lines is similar with transgressive variation toward the susceptible type in both cases. Similar frequency distributions were observed for the FE2 experiment and for the five root traits (THK, R/S, R/T, DR/T, and MaxRD) (data not shown). The lower leaf-rolling scores (indicating higher drought tolerance) in the seedling-stage experiment (FE3) are largely explained by the fact that the plants experienced less moisture stress in FE3 than in the other experiments at the time the data was recorded.

Table 1 summarizes the average phenotypic values of the two parents and the range of values observed in the RI population. The data indicate transgressive variation for all traits. The tendency toward smaller, shallower root systems is most obvious for R/S, R/T, SS, and LVS, but was also evident for THK. Our results are similar to those reported in the study by Ekanayake et al. (1985 b).

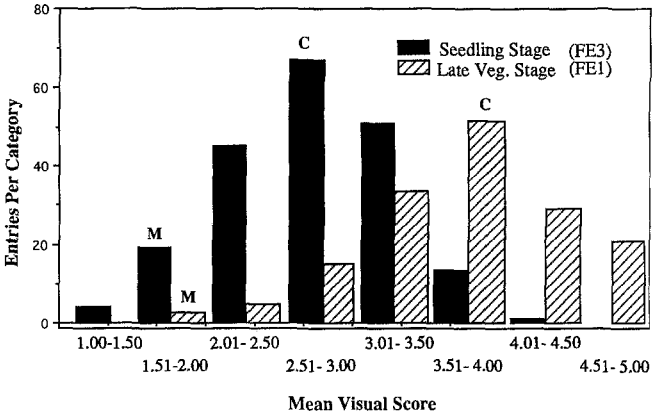

Fig. 1 The distribution of recombinant inbred lines for visual leaf rolling in two moisture-stress field experiments. *M*=mean visual score of Moroberekan; *C*=mean visual score of CO39. A score of 1.00 represents a low level of leaf rolling (high level of drought avoidance). Note the transgressive variation toward the susceptible type in both experiments

Correlation among root characters

A significant correlation was observed between most traits, suggesting that the parameters of root morphology as measured in our population are interrelated. The highest correlations were found between R/S and R/T (0.52) and R/T and DR/T (0.85) respectively. Relatively lower correlations of THK with other root traits may indicate greater genetic independence of thickness, though we were unable to identify individuals in this RI population to test this hypothesis (see section on pseudolinkage). Additionally, plant height was significantly and positively correlated with R/T, DR/T, and MaxRD (R=0.38, 0.30, and 0.20, respectively) in this study. A positive correlation between plant height and deep rooting has been reported previously by several researchers in soil (Chang et al. 1972; Yoshida and Hasegawa 1982) as well as in aeroponic culture systems (Armento-Soto et al. 1983). This character will be analyzed more fully in future experiments.

QTLs associated with the root morphology experiment

All QTLs which exerted a positive effect on root characters were derived from Moroberekan. The trait with the greatest number of QTLs and the highest level of significance was THK, followed by R/S, R/T, DR/T, and MaxRD, in descending order. The statistics presented in Tables 2 and 3 are based on a consensus set of markers derived from single-marker (F>19.22) and interval analysis (LOD>4.0). Values presented in parentheses are significant at F>11.0 (equivalent to LOD>2.4). We chose to present this data despite lower significance levels because it includes markers associated with DR/T that overlap with putative QTLs

Table 1 Parental values and range for each trait

Designation	Trait	CO39		Moroberekan		Range
		Mean	S.E.	Mean	S.E.	
THK	Root thickness (microns)[a]	710.0	(0.04)	890.0	(0.07)	650.0 – 990.0
R/S	Root/shoot ratio (mg/kg)[a]	157.0	(0.03)	267.0	(0.09)	120.0 – 280.0
R/T	Root dry weight per tiller (mg)[a]	71.0	(0.01)	206.0	(0.13)	45.0 – 206.0
DR/T	Root dry weight per tiller below 30 cm (mg)[a]	12.0	(0.01)	37.0	(0.03)	4.0 – 45.0
MaxRD	Maximum root depth (cm)[a]	85.0	(5.22)	89.4	(11.28)	70.0 – 98.0
PH	Plant height (cm)[a]	80.0	(9.5)	99.0	(18.8)	63.0 – 108.0
SS	Seedling stage leaf rolling[b]	2.8	(0.56)	1.9	(0.62)	1.0 – 4.0
EVS	Early vegetative stage leaf rolling[c]	4.0	(0.68)	2.5	(0.54)	1.0 – 5.0
LVS	Late vegetative stage leaf rolling[b]	3.7	(0.63)	1.9	(0.65)	2.0 – 5.0

[a] Average of seven replications from pot experiments
[b] Average visual rating of four replications from field experiments
[c] Average visual rating of three replications from field experiments

for other root characters. The R^2 statistic represents the proportion of phenotypic variation explained by each putative QTL (PROC GLM, SAS 1988).

The relationship between the F value and the LOD score is described in Appendix 1. This relationship suggests that in our study an F>19.22 is equivalent to a LOD>4.0, and that an F>11.0 is approximately equivalent to a LOD>2.4. Similar sets of markers were identified in most cases using single-marker and interval analyses. This was not surprising, given the inherent similarity of single-marker and interval analysis when a saturated genetic map is used as a source of markers. Where different results were obtained, the significance levels and linkage relationships among markers were examined in greater detail using multiple regression, Mapmaker/QTL scanning, and evaluation of pseudolinkage. The set of putative QTLs reported in this study are those that met the significance criteria of all the analytical procedures employed.

Root thickness (THK)

Eighteen marker loci were associated with THK and these results are summarized in Table 2, along with the RSq (% variance explained) and phenotypic effect (in microns) associated with each marker. The percent variance explained ranges from 33% for RG214 (chromosome 4) to 13% for RZ892 (chromosome 10), and differences of 57 to 86 microns were observed between groups of RI lines carrying Moroberekan or CO39 alleles at individual marker loci. Figure 2 illustrates the 1-LOD and 2-LOD support intervals for each putative QTL.

In order to determine the combination of markers that could explain the most variation for THK, a step-wise regression procedure was performed (PROC R-SQUARE, SAS 1988). The best three-variable model explained 56% of the phenotypic variation and included RG214 (chromosome 4), RG197 (chromosome 1), and RZ398 (chromosome 6). Significant two-way interactions were detected among the putative QTLs associated with THK (ANOVA, $P< 0.01$). If the three markers in the multivariate model had been additive, they would together have explained over 80% of the variance. Several three-variable models were capable of explaining 50–57% of the observed variation and consisted of various combinations of the following markers: RG214, RG197, RG811, and RG437.

The interaction among these loci could be explained as epistasis, or alternatively, as pseudolinkage, which might have been exaggerated by the skewed allele frequencies in the population. These two possibilities could not be distin-

Table 2 Marker loci associated with root characters based on a consensus between *single-marker* and *interval* analysis. Where several markers associated with putative QTLs fall in one chromosomal region, data from the most significant marker(s), and from those showing convergence among traits are presented

Marker	Chrom.	THK		R/S		R/T		DR/T	
		RSq[a]	Effect[b] (microns)	RSq	Effect (mg/kg)	RSq	Effect (mg)	RSq	Effect (mg)
RG197	1	0.25	72						
CDO920	1			0.10	31	0.13	28		
RG811	1	0.22	81						
RG140	1			0.12	30	0.13	25		
RG139	2			0.09	29	0.12	28	0.12	6.0
RG437	2	0.15	71			0.18	34	0.17	7.0
RG104A	3	0.17	73	0.17	37	0.10	23		
RZ576	3	0.19	74	(0.09	24)*				
RG910	3	0.15	71	0.17	38	0.18	32	(0.06	5.0)
RG214	4	0.33	85	0.12	27				
RG788	4	0.15	57			0.16	24	0.12	5.0
RG190	4			0.13	35				
waxy	6	0.29	84	0.19	36	0.15	26		
RG351	7					0.11	21	(0.06	3.0)
CDO533	7	0.22	67						
CDO405	7			0.18	32				
RG528	7	0.14	65			0.11	24		
RZ66	8	0.22	75	0.19	37				
RG136	8	0.21	86			0.16	31	(0.07	5.0)
RG553	9	0.19	77	0.22	42	0.12	26		
RZ12	9	0.19	80	0.14	35			(0.08	5.0)
RZ892	10	0.13	76	0.14	38				
CDO365	11	0.21	84	0.11	31				
RG181	12					0.14	29	(0.07	5.0)
RG9	12					0.11	26		
RZ397	12	0.15	74	0.15	37				

[a] "RSq" is the percent of phenotypic variance explained by individual markers
[b] "Effect" is the mean phenotypic difference between groups or RI lines carrying Moroberekan or CO39 alleles at individual marker loci for each root character evaluated
[c] Markers in parentheses are significant at F>11.0 and < 19.2 (equivalent to LOD >2.4 and < 4.0)

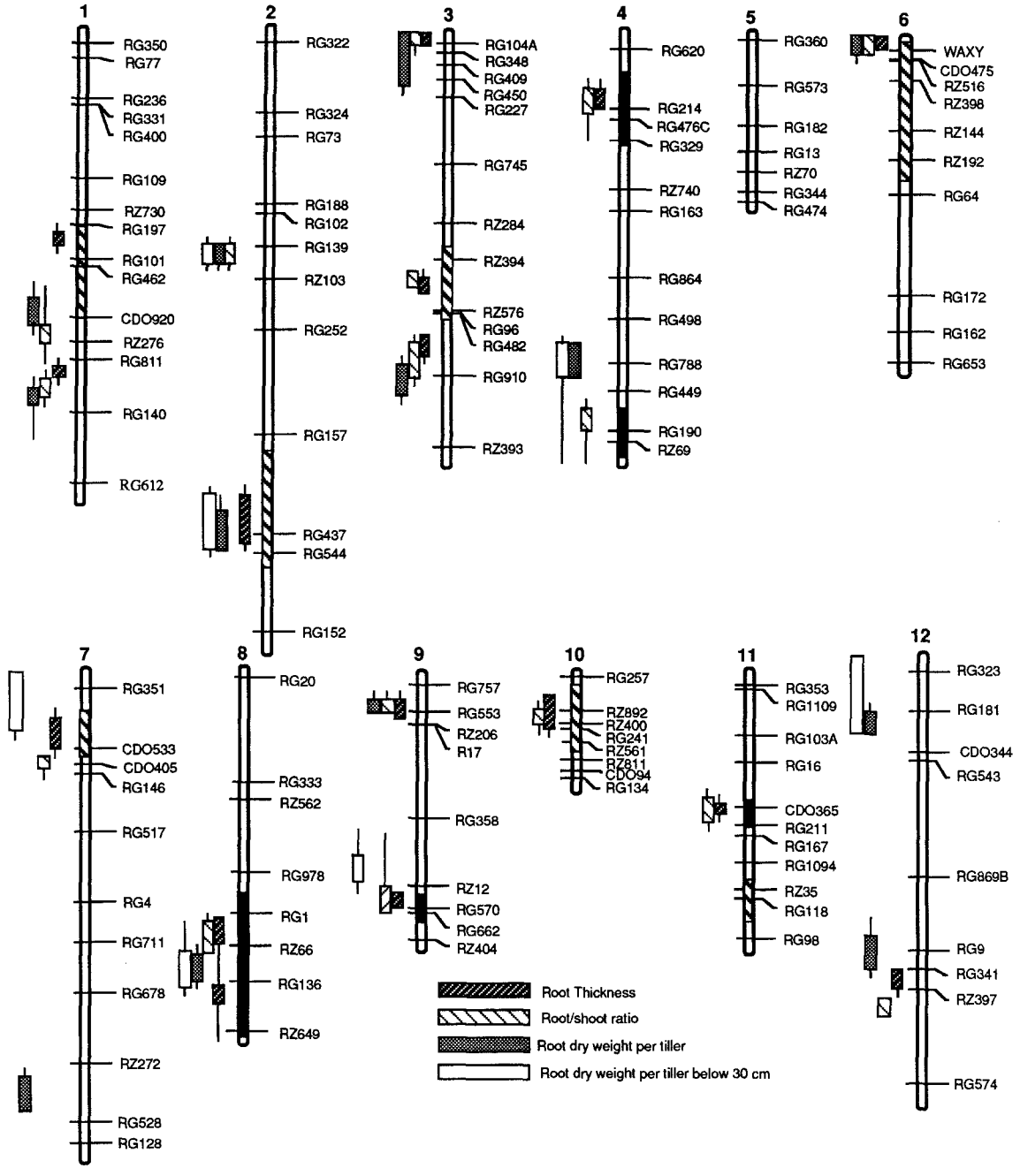

Fig. 2 Linkage map of CO39/Moroberekan F_9 recombinant inbred lines showing the putative location of QTLs associated with root characters from the greenhouse experiments. *Boxes* represent 1-LOD support intervals surrounding each putative QTL; whiskers illustrate the associated 2-LOD support intervals. *Darkened areas* within chromosomal bars represent putative QTLs associated with drought avoidance in all three field experiments; *cross-hatched* areas within chromosomal bars represent regions associated with drought avoidance in two of the three field experiments

guished statistically. However, upon further investigation, it was found that the RI lines that carried the Moroberekan allele at RG197 (chromosome 1) and the group that carried the Moroberekan allele at RZ398 (chromosome 6) were largely overlapping. This was determined to be the effect of pseudolinkage, and it was demonstrated that RG197 made a significant contribution to thicker roots whereas RZ398 did not. Similar analyses suggested that an association between THK and marker RZ272 on chromosome 7 was also due to pseudolinkage. Based on these results, RZ398 and RZ272 were eliminated from our summary of marker loci associated with THK, and the markers presented in Table 2 and Fig. 2 are those that showed no evidence of pseudolinkage.

Root/shoot ratio (R/S)

Sixteen putative QTLs were identified for R/S based on the overall-means analysis (Table 2, Fig. 2). These mark-

ers explained 9–22% of the observed variation. The best three-variable model included RG197 on chromosome 1, RG910 on chromosome 3, and RG553 on chromosome 9, and explained 38% of the phenotypic variation for R/S. No pseudolinkage was observed for R/S. It will be noted that RG197 was significant in the three-variable model but was just below the signficance threshold in single-marker analysis for R/S.

Environmental differences between runs in greenhouse experiments affected R/S, but the genotype × experiment interaction was not significant (ANOVA, $P>0.05$). Shoot dry weight averaged 4.5 g for GH1, versus 3.2 g for GH2 and 3.4 g for GH3. Mean root dry weights were, however, the greatest for GH3 at 0.82 g versus 0.69 and 0.43 for Runs 1 and 2, respectively. Analyses of GH2 did not reveal any significant markers for R/S, while ten were significant in both GH1 and GH3.

Root dry weight per tiller (R/T)

Fourteen QTLs were associated with R/T based on overall means (Table 2, Fig. 2), and nine of those loci were identified in all three runs. The best three-variable model included markers RG437 (chromosome 2), RG910 (chromosome 3), and RZ740 (chromosome 4), and explained 35% of the total phenotypic variation. It is noteworthy that the best three-variable models for THK, R/S, and R/T identify similar regions on chromosomes 1, 2, 3, and 4.

Deep root weight (DR/T)

Fewer significant QTLs were identified in association with DR/T (Table 2, Fig. 2) than with the previously discussed traits. This corresponded to a greater coefficient of variation, suggesting either sensitivity to environmental variation or else large experimental error in measuring this character. From the combined analysis, the coefficient of variation for DR/T was 48%, while it was less than 10% for THK, 33% for R/S%, 34% for R/T, and 8% for MaxRD.

RG437 (chromosome 2) was the most significant marker when overall means were analyzed for DR/T and, in analyses of individual runs, it was the only marker that was significant in all three runs. When combined in a two-variable regression model, markers RZ12 (chromosome 9) and RG139 (chromosome 2) accounted for 18.5% of the total variation. Adding a third and fourth locus to the model had minimal effect on the proportion of explained variation.

Maximum root depth (MaxRD)

A significant genotype-by-experiment interaction was detected for this variable (ANOVA, $P< 0.01$) and, therefore, no QTLs associated with the overall mean MaxRD are reported. However, based on analyses of individual runs, GH2 revealed the largest number of significant markers for MaxRD (RG324-chromosome 2; RG139-chromosome 2; RZ12-chromosome 9; and CDO365-chromosome 11), while only one putative QTL was detected in GH1 (RZ12), and no marker loci were significantly associated with MaxRD in GH3. These results suggested that this parameter was highly sensitive to environmental variation. The narrow diameter of the pots used in this study (10 cm) may also have interfered with our ability to accurately assess the genetic potential of the RI lines for MaxRD because roots from all RI lines were forced to grow downward after contacting the pot walls.

Anaerobic conditions experiment

The three groups of entries selected to represent high, medium, and low phenotypic expression for each root parameter under aerobic conditions were also tested under anaerobic conditions. With the exception of MaxRD, the high, medium, and low ranking of the three groups under well-drained conditions was maintained under flooded conditions for all other root characters measured. This result indicates stability of many root morphological characters under highly variable environmental conditions. MaxRD, however, is highly sensitive to environmental variation and for this character, the group with the shallowest roots under well-drained conditions had the deepest roots under flooded conditions.

QTLs associated with the drought avoidance field experiment

Table 3 lists the QTLs associated with increased drought avoidance in all the field experiments. Eight putative QTLs associated with drought avoidance were identified in FE3, 15 in FE2, and ten in FE1. Most of the QTLs controlling drought avoidance were significant in more than one experiment and demonstrate a degree of stability of response over environments (Table 3, Fig. 3). The level of stress for the seedling-stage experiment (FE3) was the least (see Field experiments section of Materials and methods) and may explain why the seedling-stage experiment identified the fewest QTLs. Drought avoidance expressed in terms of yield could not be measured in this experiment because of the poor adaptation of many of the lines and the high levels of sterility related to factors other than water stress.

For FE3, RG662 (chromosome 9) explained the largest proportion of the phenotypic variance (19%) and, when combined with RG544 (chromosome 2) and RG1 (chromosome 8) in a multiple regression model, accounted for 35% of the total variation for leaf rolling at the seedling stage.

In FE2, the markers with the largest effect were RG139 (chromosome 2) and RG1 (chromosome 8), both of which had an RSq of 18. The best three-variable regression model consisted of markers RG139, RG214 (chromosome 4), and RG462 (chromosome 1), and explained 40% of the total variation.

Table 3 Markers associted with drought avoidance evaluated visually at three different growth stages in the field

Marker	Chrom.	SS (FE3)[a]		EVS (FE2)[b]		LVS (FE1)[c]	
		RSq[d]	Effect[e]	RSq	Effect	RSq	Effect[d]
RG462	1	0.13	0.48	0.16	0.63		
RG324	2			0.11	0.57		
RG139	2			0.18	0.83		
RG437	2			(0.09	0.54)[f]		
RG544	2	0.15	0.66				
RZ394	3					(0.08	0.43)
RG910	3			(0.09	0.54)		
RG214	4	0.13	0.45	0.15	0.40	0.16	0.60
RG190	4	0.10	0.51	0.12	0.72	0.13	0.68
RZ516	6	0.10	0.43	0.10	0.53		
RZ192	6					0.11	0.48
CDO533	7			0.11	0.63	(0.09	0.42)
RG1	8	0.11	0.48	0.18	0.72	0.14	0.60
RG662	9	0.19	0.57	0.13	0.59	0.16	0.61
RZ892	10					0.11	0.58
RZ561	10			(0.09	0.62)		
CDO365	11	0.13	0.55	0.11	0.63	0.13	0.65
RZ53	11			0.11	0.74	0.13	0.81

[a] Evaluated at the seedling stage (SS) in field experiment 3 (FE3) for drought resistance
[b] Evaluated during early vegetative stage (EVS) in field experiment 2 (FE2) for drought resistance
[c] Evaluated during late vegetative stage (LVS) in field experiment 1 (FE1) for drought resistance
[d] "RSq" is the percent of phenotypic variance explained by individual markers
[e] "Effect" is the phenotypic difference between groups of RI lines carrying Moroberekan or CO39 alleles at individual marker loci for each root character evaluated
[f] Markers in parentheses are significant at F>11.0 and < 19.2 (equivalent to LOD >2.4 and < 4.0)

The two markers explaining the most variation in FE1 were RG214 (chromosome 4) and RG662 (chromosome 9), each accounting for 16% of the total based on one-variable models. A three-locus model containing RG214, RG662, and RG190 accounted for 35% of the total variation.

Correlations among drought-avoidance scores and root characters

Relative drought avoidance among genotypes was consistent across replications of each field experiment and across experiments. The correlation coefficient between the seedling-stage and early vegetative-stage experiments was 0.62 ($P< 0.0001$), compared to 0.57 ($P< 0.0001$) between the seedling-stage and late vegetative-stage experiments. The correlation coefficient between the early vegetative-stage and late vegetative-stage experiment was 0.60 ($P<0.0001$).

More importantly, significant correlations were found between means of the root characters measured on RI lines grown in the greenhouse experiments and corresponding mean drought-avoidance scores in the field (Table 4). The correlations between root parameters and visual drought-avoidance rating were low, but all were significant and negative, indicating that the greater the root parameter, the lower the drought-avoidance score. Given the highly variable nature of roots, the interaction of root characters with other facets of plant performance under water stress, and the crudeness of the visual evaluations used in this study, it is noteworthy that significant correlations were observed. However, they are not high enough to provide specific predictive ability with respect to field performance at this time. The putative associations between molecular markers and root parameters offers a way of further dissecting this association.

The relationship between a deep and extensive root system and drought avoidance was further investigated by comparing the field performance of a subset of RI lines selected to represent the extremes of the distribution for each root trait. Using the means of each root trait from the seven replications of greenhouse data the 203 RI lines were ranked for each root trait and the 25 entries at each extreme were grouped. The two groups were then analyzed in a standard analysis of variance to determine if there was a significant difference between groups for drought avoidance. Table 5 illustrates that for each root trait the 25 RI lines with the most extensive roots had increased drought avoidance over the 25 RI lines with the least extensive roots. This finding was the same in all field drought-avoidance experiments.

Markers for use in future genetic studies and in marker-aided selection for root morphology

Markers were combined in a multiple regression model in an attempt to identify a group of markers that could most nearly maximize the explained variation for each root morphological parameter and for each field study. It was found

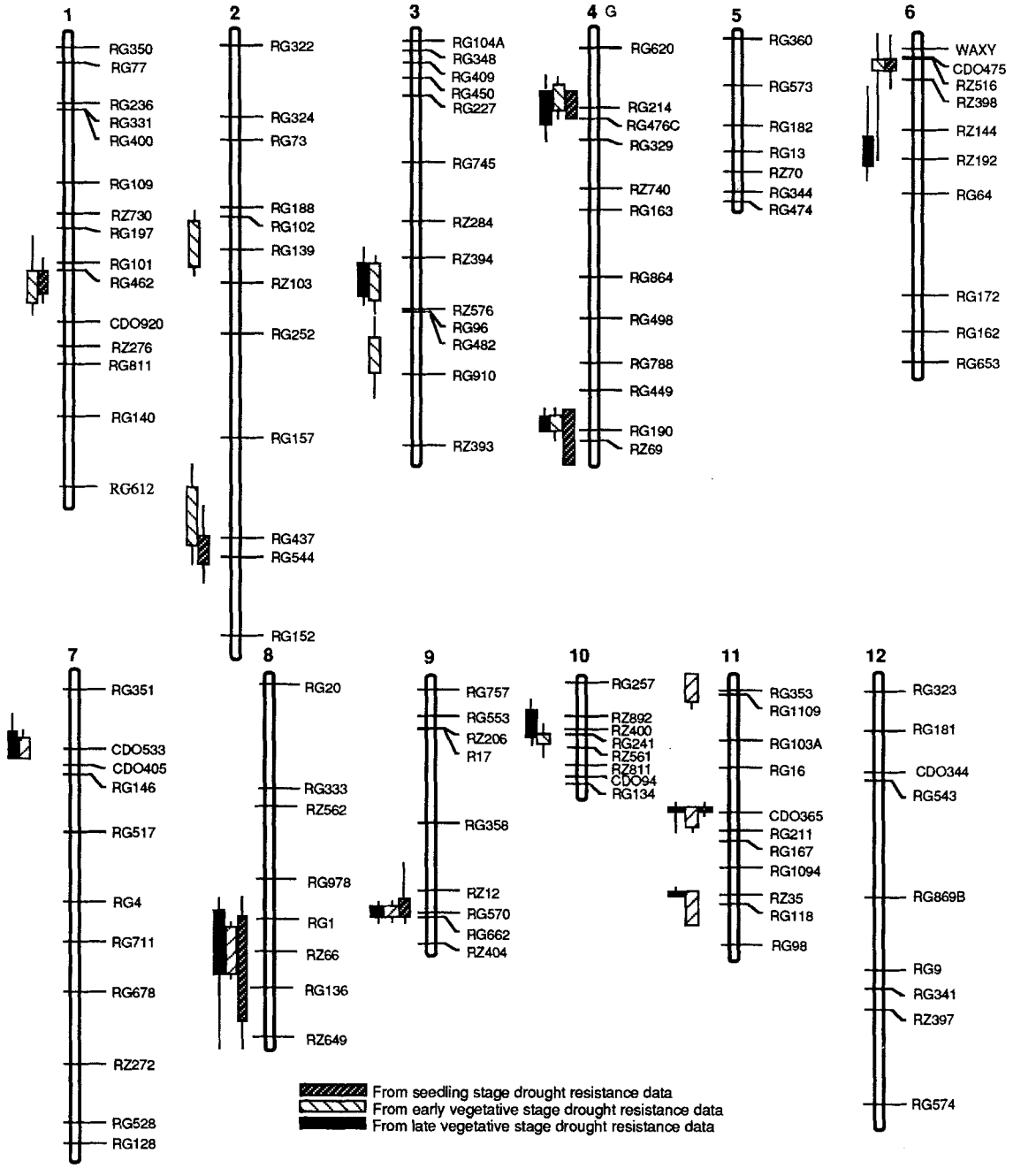

Fig. 3 Linkage map of the CO39/Moroberekan F_9 recombinant inbred lines showing putative locations of QTLs associated with leaf rolling as a measure of drought avoidance in field experiments. *Boxes* represent 1-LOD support intervals for each putative QTL; *whiskers* show the associated 2-LOD support intervals

that selecting various combinations of three to four markers among RG462, RG437, RG139, RG214, RG190, RZ516, CDO533, RG136, and RG570 could account for much of the observed variation for each of the phenotypes evaluated in this study. As can be seen in Fig. 2, many of these markers lie in regions associated with multiple characters evaluated in both greenhouse and in field studies. Significant two-way interactions (ANOVA, $P< 0.01$) were observed among most markers, suggesting epistasis. The best-performing lines always had a relatively larger number of random Moroberekan alleles than poorly performing lines (though not all of the Moroberekan loci were significantly associated with root characters or drought performance). This situation is summarized for THK in Fig. 4. It may be noted that all lines with roots thicker than 900 m contained greater than 25% Moroberekan alleles. A similar trend was observed for R/S and R/T (data not shown). Further crossing and marker-assisted selection would allow us to address the question of whether it is possible to break these linked and pseudolinked configurations of *japonica* alleles in an *indica* background.

Table 4 Simple correlations between mean root values measured in the greenhouse and visual drought rating of field-grown plants at three different growth stages

Variable	SS[a]	EVS[b]	LVS[c]
THK	-0.25*	-0.43**	-0.31**
R/S	-0.27*	-0.43**	-0.44**
R/T	-0.20*	-0.35**	-0.31**
DR/T	-0.26*	-0.33**	-0.24**
MaxRD	-0.31*	-0.29**	-0.23*

[a] Evaluated at the seedling stage (SS) in field experiment 3 (FE3) for drought resistance
[b] Evaluated during early vegetative stage (EVS) in field experiment 2 (FE2) for drought resistance
[c] Evaluated during late vegetative stage (LVS) in field experiment 1 (FE1) for drought resistance
** Significant at the $P< 0.0001$ level; * Significant at the $P< 0.001$ level

Table 5 Probability of obtaining a test statistic greater than F for a difference in the field performance (based on visual assessment of leaf rolling) of the 25 "best" lines and the 25 "worst" lines identified in greenhouse experiments for each root character. Plants were evaluated at three different growth stages

Variable	SS[a]	EVS[b]	LVS[c]
THK	0.002	0.0001	0.05
R/S	0.0002	0.0001	0.0006
R/T	NS[d]	0.0003	0.02
DR/T	0.004	0.0001	0.07
MaxRD	0.0001	0.0001	0.02

[a] Evaluated at the seedling stage (SS) in field experiment 3 (FE3) for drought resistance
[b] Evaluated at the early vegetative stage (EVS) in field experiment 2 (FE2) for drought resistance
[c] Evaluated at the late vegetative stage (LVS) in field experiment 1 (FE1) for drought resistance
[d] NS, not significant at $P<0.1$

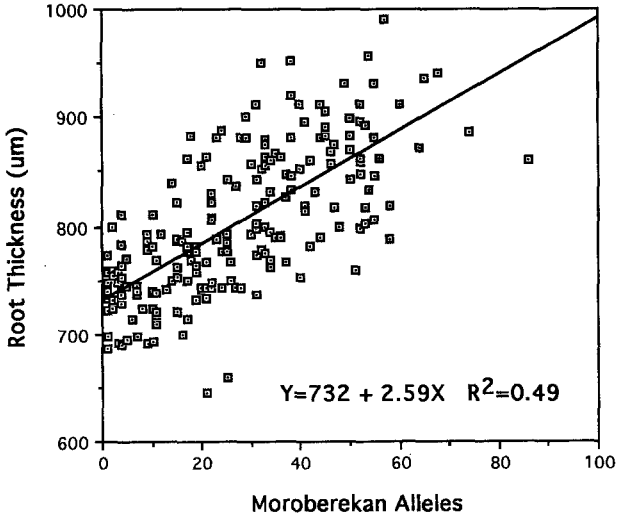

Fig. 4 Root thickness regressed on the number of Moroberekan alleles for each recombinant inbred line

Discussion

Upon inspection of putative QTL locations for all five root traits (Fig. 2), it can be seen that many of the support intervals overlap between traits. We suggest that these regions may contain genes that interact with each other to exert a general effect on root morphology in accordance with the physics of soil penetration and water uptake. This overlap offers insight into the correlations between root traits that have been observed by other researchers (Armento-Soto et al. 1983; Ekanayake et al. 1985 a, b; Ingram et al. 1990), helping to explain the commonly observed relationship between root thickness and depth of rooting. In the present study, THK was the most reliable variable measured and may serve as a predictor of other root characters favorably associated with pre-heading drought avoidance. This association will be further tested in future crosses. Figures 2 and 3 reveal that most of the support intervals for QTLs identified for drought avoidance in the field overlap areas declared significant for two or more of the root parameters identified in greenhouse studies. These regions are hypothesized to contain one or several genes that exert an influence over drought avoidance via root morphology. It is likely that some of these regions represent evolutionarily significant complexes of adaptive genes which tend to be inherited as a unit, providing the plant with a complex physiological strategy for survival under stress.

Two analytical approaches were employed in this study. QTLs with small effects, such as those for DR/T and MaxRD, tended to be more readily identified by single-marker analysis, in agreement with the simulations examined by Knott and Haley (1992). However, both approaches are equally vulnerable to Type-I errors when skewed allele frequencies occur throughout the genome. The most pragmatic remedy for this problem is to increase the significance threshold for the declaration of a QTL, as presented here.

Development of a deep and extensive root system is one adaptive strategy of plants for drought avoidance discussed by Ludlow and Muchow (1990). Moroberekan, like many upland *japonica* rice varieties, appears to rely heavily on its deep and extensive root system to achieve its demonstrated capacity for drought avoidance. Other adaptive strategies, more typical of the *indica* subspecies, include shortening of growth duration and tissue-level tolerance which Moroberekan lacks. Moroberekan is known to have a long growth duration, and recent studies have shown it to have very low tolerance to desiccation, and almost no detectable osmotic adjustment ability in response to moisture stress (M. Ludlow, CSIRO, personal communication). Whether a drought-avoidance strategy based almost entirely on a well developed root system can be combined with tissue-level tolerance and/or short growth duration to improve plant performance under water stress in specific environments is a question which is central to drought-resistance breeding in cereals, and would probably require selectively combining complex *japonica* and *indica* characteristics.

The phenomenon of "return to parental type" after repeated generations of selfing following *indica/japonica* hybridization is familiar to rice breeders and makes it difficult to obtain favorable recombinants through traditional means (Oka 1988; Sato et al. 1990). Both differential adaptation to edaphic factors, such as soil, water, and temperature regimes, and genetically controlled sterility barriers, separate these two major sub-species (Oka 1964). Nonetheless, mapping studies in F_2 generations by McCouch et al. (1988), Saito et al. (1991) and Kurata et al. (1994) suggest that recombination in *indica/japonica* crosses is abundant throughout the genome. Therefore, early, positive selection based on markers associated with QTLs offers the possibility of obtaining desirable recombinants, even in crosses of this kind. To investigate this possibility, further crossing and selection of specific, marker-defined "introgression lines" (Eshed et al. 1992) need to be undertaken.

Stable responses of QTLs over different environments have been reported in tomato and maize (Paterson et al. 1991; Stuber et al. 1992). Though the responsiveness of root characters to different environments was tested in the present study, based on differences in temperature and in watering regimes encountered in the three runs of the QTL experiment, the QTLs controlling THK and R/T were largely unaffected by these environmental differences. On the other hand R/S, DR/T, and MaxRD showed greater variation in response to environment. MaxRD was the root character most sensitive to environment evaluated in this study. The stability of all root characters except MaxRD under both aerobic and anaerobic conditions and under field conditions suggests the general adaptability of, and the usefulness of the QTLs associated with such characters.

Ludlow and Muchow (1990) have reported that one of the most important determinants of yield is the amount of water passing through a plant over the growing season. A root system that extends the root zone to more fully exploit available soil water thus has the potential to increase yield under drought (Mambani and Lal 1983). Having identified putative QTLs with major effects on both rice root morphology and vegetative-stage drought avoidance, our study provides an excellent starting point for follow-up studies to determine whether any of the Moroberekan-derived alleles identified here can be introgressed into an agronomically acceptable *indica* cultivar in an attempt to improve its drought-avoidance capability. Multiple regression models with this population suggest that selecting for Moroberekan alleles at three or four of the loci with the largest effects on root morphology and field drought-avoidance scores might substantially alter the root phenotype toward that commonly associated with drought-resistant cultivars. Though QTLs associated with water-use efficiency in tomato were reported by Martin et al. (1989), the present study provides the first report of specific chromosomal regions in any cereal which are likely to contain genes affecting root morphology. Futher, this work has identified a set of molecular markers that enable us to track these putative QTLs in future crosses. Based on recent studies of synteny among Gramineae species (Ahn et al. 1993), relationships between specific markers and root characters may provide clues as to the common genetic control of these drought-related characteristics in a range of cereal crops.

By observing the performance of selected lines in various drought-prone field locations, and by developing a set of introgression lines for further genetic analysis, we aim to clarify the effect of specific QTLs on root morphology and to determine how predictably these root characters are associated with drought avoidance under both upland and lowland field conditions.

Acknowledgements The authors wish to acknowledge Ken Manly for custom-designing the computer program RIPM/Skew specifically for this project and the Rockefeller Foundation for funding this research. We would also like to thank J. Olufowote and C. Nelson for helping with the analyses, K. Ingram, and M. Arraudeau for their critical reviews, and Mhelay Malabanan for her help in all aspects of this project.

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Appendix 1

The relationship between the LOD score and the analysis of variance F-statistic when detecting QTLs using single markers

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Detecting quantitative trait loci (QTLs) using single-marker data may be performed using LOD-score analysis at the marker, or by using one-way analysis of variance (ANOVA). This work shows that the LOD-score calculation is a simple function of the F-statistic that is achieved by using an analysis of variance (ANOVA) on single-marker data (Doerge 1993).

Under the assumption of normality for the distribution of the quantitative trait values in each of the genotype marker classes (say, M_1M_1 and M_1M_2) and equality of the sample variances (σ^2), define the individuals X to be in the M_1M_1 marker class, and individuals Y to be in the M_1M_2 marker class. The quantitative trait values in each marker class are then distributed as:

$$X \sim N(\mu_{M_1M_1}, \sigma^2)$$

$$Y \sim N(\mu_{M_1M_2}, \sigma^2)$$

where μM_1M_1, μM_1M_2, σ^2 are the means and variance within each marker-class. The hypothesis used when trying to detect QTLs using single-marker analysis are:

$$H_0: \mu_{M_1M_1} = \mu_{M_1M_2}$$

$$H_a: \mu_{M_1M_1} \neq \mu_{M_1M_2},$$

and the likelihood function is

$$L(\mu_{M_1\,M_1}, \mu_{M_1\,M_2}, \sigma^2, x_1, \ldots, x_{n_1}, y_1, \ldots, y_{n_2})$$
$$= \left[\frac{1}{2\pi\sigma^2}\right]^{\frac{n_1+n_2}{2}} \exp\left[-\frac{1}{2}\sum_{i=1}^{n_1}\left(\frac{x_i-\mu_{M_1\,M_1}}{\sigma}\right)^2\right]$$
$$\exp\left[-\frac{1}{2}\sum_{i=1}^{n_2}\left(\frac{y_i-\mu_{M_1\,M_1}}{\sigma}\right)^2\right].$$

Let Θ define the three-dimensional parameter space of the alternative hypothesis, $\mu_{M_1M_1}$, $\mu_{M_1M_2}$, σ^2, and let Θ_0 be the restricted parameter space $\mu_{M_1M_1}=\mu_{M_1M_2}=\mu$, σ_0^2.

Under Θ the maximum likelihood estimates are:

$$\hat{\mu}_{M_1\,M_1} = \bar{x}$$
$$\hat{\mu}_{M_1\,M_2} = \bar{y}$$
$$\hat{\sigma}^2 = \left[\frac{\sum_{i=1}^{n_1}(x_i-\bar{x})^2 + \sum_{i=1}^{n_2}(y_i-\bar{y})^2}{n_1+n_2}\right].$$

Substituting these estimates into the likelihood function we have,

$$\sup_{\Theta} L = \left[\frac{n_1+n_2}{2\pi\left[\sum_{i=1}^{n_1}(x_i-\bar{x})^2 + \sum_{i=1}^{n_2}(y_i-\bar{y})^2\right]}\right]^{\frac{n_1+n_2}{2}} \exp\left[-\frac{n_1+n_2}{2}\right] \quad (1)$$

Under Θ_0 the maximum likelihood estimates are:

$$\hat{\mu} = \frac{n_1\bar{x}+n_2\bar{y}}{n_1+n_2}$$
$$\hat{\sigma}_0^2 = \frac{1}{n_1+n_2}\left[\sum_{i=1}^{n_1}(x_i-\bar{x})^2 + \sum_{i=1}^{n_2}(y_i-\bar{y})^2 + \frac{n_1n_2}{n_1+n_2}(\bar{x}-\bar{y})^2\right].$$

Similarly, the likelihood function evaluated under the restricted parameter space gives

$$\sup_{\Theta_0} L = \left[\frac{n_1+n_2}{2\pi\left[\sum_{i=1}^{n_1}(x_i-\bar{x})^2 + \sum_{i=1}^{n_2}(y_i-\bar{y})^2 + \frac{n_1n_2}{n_1+n_2}(\bar{x}-\bar{y})^2\right]}\right]^{\frac{n_1+n_2}{2}} \times \exp\left[-\frac{n_1+n_2}{2}\right]. \quad (2)$$

The LOD score is defined (Morton 1955) as

$$LOD = \log_{10}\left[\frac{\sup_{\Theta} L}{\sup_{\Theta_0} L}\right].$$

Using (1) and (2)

$$\frac{\sup_{\Theta} L}{\sup_{\Theta_0} L} = \frac{2}{n_1+n_2}\left[\frac{\hat{\sigma}^2}{\hat{\sigma}_0^2}\right] = \left[1+\frac{\frac{n_1n_2}{n_1+n_2}(\bar{x}-\bar{y})^2}{\sum_{i=1}^{n_1}(x_i-\bar{x})^2 + \sum_{i=1}^{n_2}(y_i-\bar{y})^2}\right]^{\frac{n_1+n_2}{2}}. \quad (3)$$

Table 1 LOD score (LOD) as a function of the one-way ANOVA (T^2) and sample size (n_1+n_2)

n_1+n_2	T^2	P-value (ANOVA)	LOD
50	1.0	0.3223	0.2239
	3.0	8.97×10^{-2}	0.6582
	5.0	3.00×10^{-2}	1.0759
	10.0	2.70×10^{-3}	2.0547
	20.0	4.70×10^{-5}	3.7817
	40.0	8.00×10^{-8}	6.5810
100	1.0	0.3198	0.2205
	3.0	8.60×10^{-2}	0.6548
	5.0	2.80×10^{-2}	1.0806
	10.0	2.10×10^{-3}	2.1099
	20.0	2.10×10^{-5}	4.0328
	40.0	7.60×10^{-9}	7.4327
200	1.0	0.3188	0.2188
	3.0	8.40×10^{-2}	0.6531
	5.0	2.70×10^{-2}	1.0831
	10.0	1.80×10^{-3}	2.1398
	20.0	1.30×10^{-5}	4.1791
	40.0	1.70×10^{-9}	7.9912

It is well known from statistical theory that the quantity

$$T = \frac{\sqrt{\frac{n_1n_2}{n_1+n_2}(\bar{x}-\bar{y})^2}}{\sqrt{\frac{\sum_{i=1}^{n_1}(x_i-\bar{x})^2 + \sum_{i=1}^{n_2}(y_i-\bar{y})^2}{n_1+n_2-2}}}$$

has a t-distribution with n_1+n_2-2 degrees of freedom (Mood et al. 1974). Also well known is the relationship between the F-distribution and the t-distribution. Using this relationship, the statistic T^2 has an F-distribution with numerator degrees of freedom equal to 1, and denominator degrees of freedom equal to n_1+n_2-2 (Mood et al. 1974). T^2 is the F-statistic as calculated in a one-way ANOVA.

Therefore, (3) becomes

$$\frac{\sup_{\Theta} L}{\sup_{\Theta_0} L} = \left[1+\frac{T^2}{n_1+n_2-2}\right]^{\frac{n_1+n_2}{2}},$$

making the LOD score

$$LOD = \frac{n_1+n_2}{2}\log_{10}\left[1+\frac{T^2}{n_1+n_2-2}\right]$$

a simple function of the one-way ANOVA F-statistic, T^2. The relationship between the LOD score and the one-way ANOVA is demonstrated in Table 1.

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