Theor Appl Genet (2003) 107:1505–1515
DOI 10.1007/s00122-003-1390-1

ORIGINAL PAPER

B. S. Zheng · L. Yang · W. P. Zhang · C. Z. Mao · Y. R. Wu · K. K. Yi · F. Y. Liu · P. Wu

Mapping QTLs and candidate genes for rice root traits under different water-supply conditions and comparative analysis across three populations

Received: 15 March 2003 / Accepted: 30 June 2003 / Published online: 15 August 2003

Abstract To investigate the genetic factors underlying constitutive and adaptive morphological traits of roots under different water-supply conditions, a recombinant inbred line (RIL) population derived from a cross between the lowland rice variety IR1552 and the upland rice variety Azucena with 249 molecular markers, was used in cylindrical-pot experiments. Eighteen QTLs were detected for seminal root length (SRL), adventitious root number (ARN), and lateral root length (LRL) and lateral root number (LRN) on the seminal root at a soil depth of from 3 to 6 cm under flooding and upland conditions. One identical QTL was detected under both flooding and upland conditions. The relative parameters under the two water-supply conditions were also used for QTL analysis. Five QTLs for upland induced variations in the traits were detected with the positive alleles from Azucena. A comparative analysis was performed for the QTLs detected in this study and those reported from two other populations with Azucena as a parent. Several identical QTLs for root elongation were found across the three populations with positive alleles from Azucena. Candidate genes were screened from ESTs and cDNA-AFLP clones for comparative mapping with the detected QTLs. Two genes for cell expansion, *OsEXP2* and endo-1,4-β-D-glucanase *EGase*, and four cDNA-AFLP clones from root tissues of Azucena, were mapped on the intervals carrying the QTLs for SRL and LRL under upland conditions, respectively.

Communicated by H.C. Becker

B. S. Zheng · L. Yang · W. P. Zhang · C. Z. Mao · Y. R. Wu · K. K. Yi · F. Y. Liu · P. Wu (✉)
The State Key laboratory of Plant Physiology and Biochemistry,
College of Life Science,
Zhejiang University,
310029 Hangzhou, P. R. China
e-mail: docpwu@cls.zju.edu.cn
Fax: 86-0571-86971323

Present address:
B. S. Zheng, College of Life Science,
Zhejiang Forestry University,
311300 Lin An, P. R. China

Key words Rice (*Oryza sativa* L.) · QTLs · Root morphology · Candidate genes · Flooding conditions · Upland conditions

Introduction

With the increase in population, the shortage of water available for irrigation and the need to reduce environmental pollution, future increases in rice production will rely on rainfed ecosystems (Garrity et al. 1986). Drought is an important limiting factor for rice productivity under rainfed ecosystems. Therefore, increasing drought tolerance is one of the major objectives of rice breeding programs for rainfed ecosystems.

Plant roots play an important role in water and nutrient acquisition. Both constitutive and adaptive root growth have been implicated in the improved performance of rice under rainfed lowland conditions (Kamoshita et al. 2002). Root growth and development under flooding conditions contributes to water extraction under water-limited stress (Hoque and Kobata 1998). The development of a deep and extensive root system is a drought-adaptation strategy for plants. Upland rice usually has a deep and thick root system, which allows the crop to satisfy its water requirements under upland conditions (Nguyen et al. 1997). Therefore, it may be possible to improve the drought tolerance of lowland rice by introducing such root traits to increase rice production under upland conditions.

It is difficult to measure root traits under field conditions and thus these have rarely been used as parameters in breeding programs. Marker-aided selection (MAS) based on the accurate mapping of root traits may make it possible to develop a "water-stress tolerant root system"-oriented breeding program. Many quantitative trait loci (QTLs) associated with root characteristics under different water conditions have been reported based on genetic populations derived from crosses between lowland *indica* rice and upland *japonica* rice varieties, or between upland *indica* rice and upland *japonica* rice varieties (Champoux et al. 1995; Price and Tomos 1997;

Yadav et al. 1997; Price and Courtois 1999; Hemamalini et al. 2000; Price et al. 2000, 2002a, b; Zheng et al. 2000; Zhang et al. 2001).

One of the objectives of molecular genetics is to identify and isolate genes for important traits (Pflieger et al. 2001). A candidate gene approach is an alternative strategy to cloning genes, and has been successfully used in plant genetics (Byrne and McMullen 1996). Due to the multiplicity of genes and their partial effects on phenotypic variation, the candidate gene approach is more suitable for QTL characterization than positional cloning or insertional mutagenesis (Pflieger et al. 2001). Rice high-density linkage maps and the positions of many genes or expressed sequence tags (ESTs) in the rice genome are available for the candidate-gene approach to clone QTLs for root traits under different water-supply conditions and different genetic backgrounds (Harushima et al. 1998; Wu et al. 2002).

In this study, we used a recombinant inbred line (RIL) population from a cross between the lowland *indica* variety IR1552 and the upland *japonica* variety Azucena to examine QTLs for root growth under flooding and upland conditions at the early seedling stage. Together with two other genetic populations with Azucena as a parent (Price and Tomos 1997; Yadav et al. 1997; Price and Courtois 1999; Hemamalini et al. 2000; Price et al. 2000, 2002a, b; Zheng et al. 2000; Zhang et al. 2001), a comparative mapping analysis was conducted based on the common molecular markers mapped across these three genetic populations. ESTs for cell expansion and elongation, and cDNA-AFLP clones from root tissues of Azucena, were also used for candidate gene-mapping analysis.

Material and Methods

Plant material

A recombinant inbred line (RIL) population composed of 96 lines from a cross between IR1552, an irrigated lowland *indica* variety, and Azucena, an upland tropical *japonica* variety, was used to detect QTLs for root traits under flooding and upland conditions. The population was developed by single-seed descent in the F_{10} generation, using bagged panicles. The parents showed contrasting root morphology, notably in the ability of seminal and adventitious roots to reach deep layers, based on our previous work (Zhang et al. 2001).

Pot experiments

Sandy soil culture experiments using cylindrical pots under flooding and upland conditions were conducted in a controlled environment room at Zhejiang University, China. Seeds of each RIL line and the parents were germinated at 37°C for 2 days. Germinated seeds were planted directly in cylindrical polyvinyl chloride (PVC) pots (15.5 cm inner-diameter, 55 cm height) filled with uniform sandy soil (3.5% clay, 2.5% silt, 93.5% sand; $pH_{water1:1}$ 6.0) to 5.0 cm from the top. The cylindrical pots were sealed at the bottom with a stainless steel screen.

Three replications were made for flooding and water-deficit conditions. For each replication, three plants for each RIL line of three lines were grown per pot. Six plants of each parent were also grown. The cylindrical pots were arranged randomly. Throughout the entire experiment, half-strength Yoshida's nutrient solution (Yoshida et al. 1976) was used. For the flooding condition, the cylindrical pots were placed in a plastic tank with the water level 2-mm above the sandy soil surface, while the upland condition pots were placed in plastic trays with the solution level kept 45 cm below the sandy soil surface by means of a drainage hole on the bottom of the pot. The top of the pot was sealed with polyethylene film for 3 days after sowing (DAS) to maintain a stable saturated humidity condition. For the upland condition, the water content at 0–15 cm, 15–30 cm and 30–45 cm below the sandy soil surface was determined at various times over 14 days using a gravimetric analysis. The temperature of day/night was 30/24°C and the relative humidity was 65%–70% with a 12-h photoperiod of approximately 300–320 μmol. m^{-2}. s^{-1} provided by 20 400-W sodium and metal haloid lamps.

Root parameter measurements

Fourteen days after sowing, the sandy soil was carefully washed with tap water and roots were sampled. Seminal root length (SRL) was measured as the length of the longest root. Adventitious root numbers (ARN) were counted for each plant. The lateral root length (LRL) and lateral root number (LRN) on the seminal root at a soil depth of from 3 to 6 cm were measured using a scanner connected to an image-analysis system (WinRhizo, Regent Canada). Relative parameters were defined as the ratio of the parameters under upland and flooding conditions, and used to assess drought tolerance.

Statistical analysis and QTL identification

The data were subjected to ANOVA analysis using the PROC GLM in SAS. Corrected means for the RILs under flooding and upland conditions were calculated, and relative parameters were obtained for each line. The broad-sense heritability (h^2_B) of root traits was calculated as 1-mean variance of (Azucena+IR1552)/variance of their progeny. QTL mapping was performed using the program QGene version 3.06 (Nelson 1997), which is based on interval-mapping analysis. Map units (cM) were derived using the Kosambi function. A $\log_{10}$ likelihood ratio (LOD) value of 2.4 was selected as the threshold for QTL detection to reduce the experimental false-positive rate to $P<0.005$ (Lander and Bostein 1989). Estimates of the additive effect and phenotypic variation (R^2) explained by each QTL were computed.

Comparative mapping analysis

The characteristics of three genetic populations are shown in Table 1. The construction of the molecular maps for the RIL and DH populations derived from IR1552/Azucena (Zhang et al. 2001) and IR64/Azucena (Liao et al. 2001), respectively, has been described previously. Twenty seven and 76 microsatellite (SSR) markers were added to the two maps, respectively, using MapMaker 3.0 (Lander 1993). Polymorphic SSR markers were screened with publicly available primer pairs (http://www.gramene.org). Amplifications were performed according to Chen et al. (1997). The silver-staining protocol described by Bassam et al. (1991) was used to visualize amplification products. According to QTL information from another RIL population derived from Bala/Azucena (Price and Tomos 1997; Price and Courtois 1999; Price et al. 2000, 2002a, b) and from the DH population derived from IR64/Azucena (Yadav et al. 1997; Hemamalini et al. 2000; Zheng et al. 2000), six bridge RFLP markers, C86, G45, RG409, R3166, R2232 and G1085, were mapped to our RIL population and/or the DH population using MapMaker 3.0 (Lander 1993) and six restriction enzymes, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*I, respectively, for a comparative mapping analysis.

Table 1 Characteristics of the three genetic populations with the upland rice Azucena as a parent used for detecting QTLs for root traits

Population name	Population size	Marker number	Population type	Reference
IR1552/Azucena	96	249	RI	The present study
	150	207	RI	Zhang et al. 2001
IR64/Azucena	135	175	DH	Yadav et al. 1997
	109	175	DH	Zheng et al. 2000
	56	175	DH	Hemamalini et al. 2000
Bala/Azucena	178	79	RI	Price and Tomos 1997
	205	215	RI	Price et al. 2000
	205	142	RI	Price et al. 2002b

Table 2 EST clones and genes for cell expansion and growth, and cDNA-AFLP clones screened from root tissues regulated by water deficit

Clone name	Size (bp)	Sequence similarity	Accession no.	Chromosome
S1746	1,500	Expansin (Os-EXP2)	D40027	1
S2106	1,300	Alpha-expansin (Os-EXP1)	D40257	4
E1328	1,100	Endo-beta-1,4-glucanase (Egase)	C72268	5
S3412	800	Endo-xyloglucan transferase (Xet)	D41124	8
S2134	1,400	Endoxyloglucan transferase (Xet)	D40273	11
S3187	1,500	Endoxyloglucan transferase (Xet)	D40972	11
S10410	1,500	Extensin-like gene	D46025	2
OsEXP4	1,200	Expansin	U85246	5
T82	318	bHLH protein	CB238451	2*
T17	225	Nickel-binding protein 2A	AU093103	3
T59	289	Similar to CLIP-associating protein 2	CB238466	2*
L16	234	Similar to SART-1 protein	CB238430	2

* No polymorphism in the RIL population (IR1552/Azucena)

cDNA-AFLP analysis

The upland tropical *japonica* variety Azucena was raised under both flooding and upland conditions according to the pot experiments. Seminal root tips (1 cm), adventitious root primordia and lateral roots were harvested ten DAS. Total RNA was extracted from seminal root tips, adventitious root primordia and lateral roots, respectively, using Trizol reagent (GIBCO, Germany), and poly $(A)^+$ RNA was isolated from RNA with an Oligotex mRNA Mini Kit (QIAGEN, Germany). Double-stranded cDNA was synthesized using a SMART cDNA Library Construction Kit (Clontech, USA) according to the manufacturer's instructions. LD-PCR products were purified by a QIAquick PCR Purification Kit (QIAGEN, Germany) and digested by the *Taq*I/*Ase*I enzyme combination. AFLP reactions were performed according to published methods (Bachem et al. 1996). Selective amplification products were separated on a 6% polyacrylamide gel run at 60 W until the xylene cyanole reached the bottom. DNA fragments were visualized by silver-staining according to the Silver Sequence DNA Sequencing System Technical Manual (Promega, USA). The cDNA-AFLP clones were recovered by PCR under the same conditions as used for pre-amplification. Purified PCR products were ligated to the pUCm-T vector. Sequencing of the cDNA clones was performed using MegaBASE 1000 (Amersham Pharmacia Biotech, USA). Nucleotide sequences and translated sequences were compared with sequences in GenBank by using the BLAST sequence alignment program.

Mapping candidate genes

Candidate genes were collected by two strategies: (1) EST clones and genes for cell expansion and growth were colleted from the Ministry of Agriculture, Forestry and Fisheries (MAFF, Japan), and (2) cDNA-AFLP clones were screened from root tissues regulated by water stress. A total of 20 EST clones and genes were used as probes, and screened for polymorphism between IR1552 and Azucena. Polymorphic information was found for one gene for α-expansin (*OsEXP4*) and seven ESTs, including S2106 for α-expansin (*OsEXP1*), S1746 for α-expansin (*OsEXP2*), three clones for xyloglucan endotransferase (*Xet*) genes (S3412, S2134 and S3187), E1328 for endo-1,4-β-D-glucanase (*Egase*) and S10410 for an extensin-like gene (Table 2). Rice bacterial artificial chromosome (BAC) clones were found based on sequence information or the accession number for 50 cDNA-AFLP clones, and were shown to be anchored to the Rice Genetic Map *in silico* (http://www.tigr.org/tdb/e2k1/osa1/sequencing.shtml). Four clones near QTLs for root traits in this case were used for polymorphism analysis between IR1552 and Azucena. Two polymorphic cDNA-AFLP clones (*T17* and *L16*) were mapped to the RIL population using MapMaker 3.0 (Lander 1993).

Northern analysis

Total RNAs (20 μg) of Azucena root tissues under flooding and upland conditions, respectively, were isolated using Trizol (GIBCOBRL), separated on 1.2% formaldehyde-agarose gels and transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, USA) according to the manufacturer's protocol. Hybridization was performed as described previously (Cho and Kende 1997). Hybridized membranes were scanned by a Typhoon 8600 scanner (Molecular Dynamics, USA).

Results

Water content at different soil layers

After drainage for 24 h, the water content in the first (0–15 cm) and second (15–30 cm) layers of sandy soil in the cylindrical pots was dramatically decreased from about 30% to 10% and 20%, respectively. Over the next 13 days, the water content continued to decrease slightly in both layers. On the 14th day, the water content in the first and second layers was about 5% and 15%, respectively. In

Table 3 Percentage of water content of sandy soil in three layers from the soil surface to the water level during 14 days under water-limiting treatment

Soil height from surface	Day after sowing (d)				
	0	1	5	10	14
0–15 cm	28.8±0.6	10.4±0.1	9.3±1.0	6.5±0.4	5.4±1.5
15–30 cm	28.8±0.6	19.3±0.1	17.4±0.6	17.0±0.7	15.3±1.5
30–45 cm	28.8±0.6	26.8±0.5	26.3±0.2	26.3±0.8	25.1±1.0

Table 4 Mean values of the four root parameters of the two parents (Azucena and IR1552) and the RILs population under flooding (F) and upland (U) conditions, and of the relative parameters (R). SRL: seminal root length. ARN: adventitious root number. LRL: lateral root length on the seminal root at a soil depth of from 3 to 6 cm. LRN: lateral root number on the seminal root at a soil depth of from 3 to 6 cm

Trait	Azucena	IR1552	RIL population	*t* value	*P*	h^2_B
Flooding condition (F)						
FSRL(cm)	37.7±0.9	29.5±4.5	23.1–43.0	4.40	0.0013	0.26
FARN(number)	5±1	9±2	2–11	5.78	0.0002	0.39
FLRL(cm)	20.4±5.8	36.1±4.9	6.8–74.5	4.64	0.0017	0.80
FLRN(number)	38±10	80±13	19–127	5.42	0.0010	0.83
Upland condition (U)						
USRL(cm)	38.7±7.5	20.5±5.7	7.4–44.9	5.17	0.0001	0.50
UARN(number)	4±1	6±1	1–9	3.55	0.0023	0.73
ULRL(cm)	170.7±17.2	71.1±7.0	14.0–216.8	11.92	0.0000	0.92
ULRN(number)	294±21	131±24	84–465	10.63	0.0000	0.91
Relative Parameter (R)						
RSRL	1.1±0.2	0.6±0.2	0.2–1.4	4.77	0.0044	0.43
RARN	0.9±0.1	0.6±0.2	0.4–1.6	3.92	0.0056	0.67
RLRL	8.7±2.2	2.0±0.2	0.3–15.0	6.03	0.0046	0.61
RLRN	8.5±2.9	1.8±0.4	1.3–16.0	4.95	0.0079	0.40

the third layer (30–45 cm), the water content decreased by less than 5% over the whole 14 days (Table 3).

Phenotypic performance

The mean values of seminal root length, adventitious root number, lateral root length and lateral root number in the defined region of the seminal root of the parents and the population under flooding (hereafter called FSRL, FARN, FLRL and FLRN) and upland (USRL, UARN, ULRL and ULRN) conditions, and the relative parameters (RSRL, RARN, RLRL and RLRN), are shown in Table 4. The parameters showed normal distributions, and transgressive segregations were observed among the RIL population. Highly significant ($P<0.01$) differences in all of the parameters were found between the two parents. The relative parameters indicate that the seminal root growth of upland rice Azucena was induced, while that of lowland rice IR1552 was suppressed. While both Azucena and IR1552 showed a reduced adventitious root number, the decrease in IR1552 was much greater than that in Azucena. The lateral root length and number both increased by more than 8-fold under upland conditions in Azucena, while they only doubled in IR1552 (Table 4). These results indicate that the change in the root system in response to water-limited stress is genotype-dependent. Broad-sense heritabilities (h^2_B) varied from 0.26 to 0.83 for the root traits under flooding, from 0.50 to 0.92 under upland, and from 0.40 to 0.67 for the relative parameters. The high h^2_B values of the relative parameters suggest that it may be possible to exploit the genetic variation in water-deficit-induced root systems in breeding programs.

QTL analysis

A total of 23 QTLs for the 12 root parameters were detected (Table 5; Fig. 1). Under flooding conditions, two QTLs for FSRL were found, one of which is identical to that for USRL on chromosome 2 between CDO718 and a cDNA-AFLP clone (L16) which encodes SART-1 protein. Another QTL for FSRL was detected on chromosome 7 flanked by RG650 and CDO497. Three QTLs for USRL were found on chromosome 1 between RG109B and an *OsEXP2* (S1746), chromosome 2 between CDO718 and L16, and chromosome 9 between RG570 and RG667, respectively, under upland conditions. Two QTLs for relative seminal root length (RSRL) were detected on chromosomes 1 and 9, respectively, which were identical to those for seminal root length under upland conditions (USRL). The positive alleles for all of the seven QTLs for seminal root length are from the upland rice Azucena.

Eight QTLs were found for adventitious root number (ARN) on five chromosomes, with four for FARN, three for UARN and one for RARN. No identical QTL was found among the eight QTLs. Two QTLs for FARN were found on chromosome 3 on the intervals flanked by RZ399/RZ448 and RG104/ACC-CTG2, respectively. Two other QTLs for FARN were found on chromosomes 4 and 9, respectively. The QTL on chromosome 9 flanked by RM328/RG570 accounted for about 20% of the total

Table 5 Detected QTLs for root traits of rice grown under flooding and upland conditions, and of the relative parameters

Chrom.-interval		Flooding			Upland			Relative parameter		
		LOD[b]	A[c]	R^{2d} (%)	LOD	A	R^2 (%)	LOD	A	R^2 (%)
SRL[a]										
1	S1746/RG109B				2.50	−2.48	11.3	2.53	−0.07	11.8
2	L16/CDO718	2.57	−1.51	12.0	2.58	−3.12	11.6			
7	RG650/CDO497	2.46	−1.38	11.5						
9	RG570/RG667				2.99	−2.84	13.4	3.02	−0.09	13.9
ARN										
1	RG109B/RM315				2.41	0.52	12.0			
1	CDO920/BCD134				2.71	0.82	13.4			
2	AGG-CAG13/G45				3.22	0.80	18.2			
3	RM282/RZ574							2.72	−0.14	15.0
3	RZ399/RZ448	3.01	0.72	13.7						
3	RG104/ACC-CTG2	2.46	0.75	11.4						
4	AAG-CAA4/RG396	3.08	0.93	14.0						
9	RM328/RG570	4.56	0.87	20.0						
LRL										
1	AAG-CAG1/RG462	2.43	4.82	11.8						
3	RG409/T17				2.94	−16.25	13.4			
5	RG313/E1328				3.18	−20.20	14.4			
6	AAC-CTT10/RZ140							2.42	−1.93	11.9
6	RZ588/RG472	2.42	−7.40	11.8						
LRN										
3	RG191/AAC-CAG5				2.48	−41.83	11.7			
4	RM252/AGG-CAG7							2.48	−1.12	12.3
6	AAC-CAG7/AAC-CTT10	2.78	−11.96	13.4						

[a] SRL: seminal root length; ARN: adventitious root number; LRL: lateral root length on the seminal root at a soil depth of from 3 to 6 cm; LRN: lateral root number on the seminal root at a soil depth of from 3 to 6 cm
[b] LOD=$\log_{10}$-Likelihood value
[c] Effects of substituting a single allele from one parent to another. Positive values show that allelic contribution is from IR1552 and negative values from Azucena
[d] Phenotypic variation explained by a single QTL

variation in FARN. Two QTLs for UARN were detected on chromosome 1 flanked by RG109B and RM315, and by CDO920 and BCD134, respectively. The third QTL for UARN was detected on chromosome 2 between AGG-CAG13 and G45. One QTL for RARN was detected on chromosome 3 flanked by RM282/RZ574. The positive alleles for the QTLs for FARN and UARN are from IR1552, while that for the QTL for RARN is from Azucena (Table 5).

Two QTLs for FLRL on chromosomes 1 and 6, two for ULRL on chromosomes 3 and 5, and one for RLRL on chromosome 6 were detected. Only one QTL each for FLRN, ULRN and RLRN was detected on chromosomes 6, 3 and 4, respectively. The positive alleles for all of the QTLs for FLRL, ULRL, RLRL, FLRN, ULRN and RLRN are from the upland rice Azucena, except for that for FLRL on chromosome 1.

The identification of QTLs for parameters from upland conditions indicates that the water-limited stress-induced changes in root traits are controlled by some genetic factors that respond to such stress. Except for two identical QTLs for USRL and RSRL, no identical QTLs were found for other parameters under upland conditions or the relative parameters, which suggests that the relative parameters should be used to evaluate root adaptation to water-limited stress in rice.

Comparison of QTLs in three genetic populations

The QTLs for the root traits detected here were compared with those reported based on a double-haploid (DH) population and a recombinant inbred-line (RIL) population derived from crosses between IR64 and Azucena (Yadav et al. 1997), and between Bala and Azucena (Price et al. 2002b), respectively. The seminal root is usually the longest root at the early seedling stage. Identical QTLs for maximum root length under upland conditions were found on chromosome 1 in all three populations, as supported by the bridge molecular markers RZ730 and C86 (Yadav et al. 1997; Zhang et al. 2001; Price et al. 2002b) (Fig. 2A). This is a region in which the *sd-1* gene, a major gene that controls plant height, was mapped (Huang et al. 1996). The QTLs for maximum root length under upland conditions mapped on chromosomes 2 and 9, respectively, may also be identical across the three populations based on the common molecular markers G45 and RG157 on chromosome 2 (Fig. 2B), and RG667 and G1085 on chromosome 9 (Fig. 2F). One identical QTL on chromosome 7 based on the bridge markers RG650 and CDO497 was found for SRL under flooding in this study and maximum root length under upland conditions in the other two populations (Fig. 2E) (Yadav. et al. 1997; Price et al. 2002a, b). QTLs for root

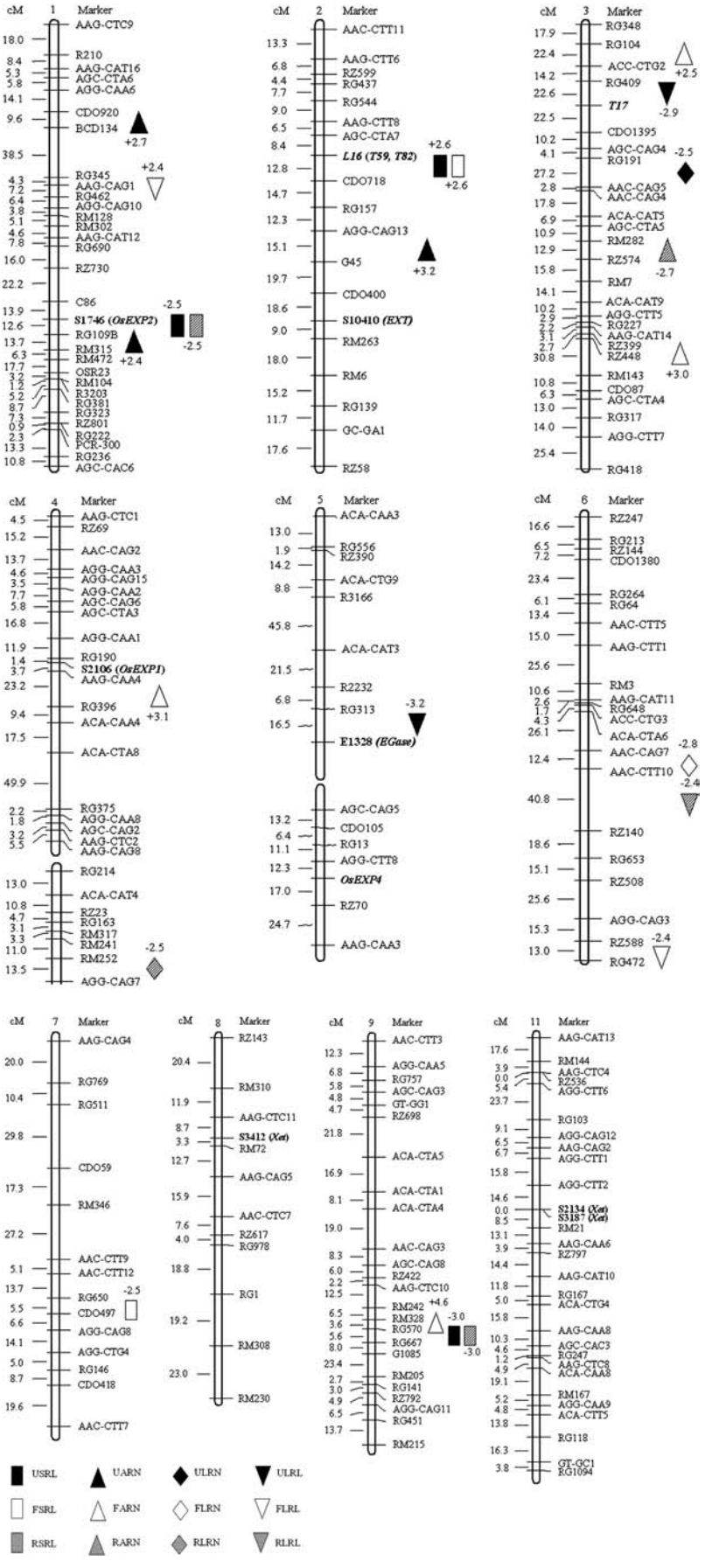

Fig. 1 QTLs for SRL, ARN, LRL and LRN detected based on the RIL population derived from a cross between IR1552 and Azucena. Those under flooding or upland conditions and of the relative parameters are identified by *empty*, *black* or *gray symbols*, respectively. The *symbols* for different root traits are shown below and the *number* above or below the symbol indicates the LOD score (LOD>2.4) for the QTL. A positive LOD value indicates that the allelic contribution is from IR1552 and a negative LOD value indicates that the allelic contribution is from Azucena. The marker name is shown on the *right* and the genetic distance between markers based on the Kosambi function is shown on the *left*. The markers in *bold characters* indicate the locations of genes

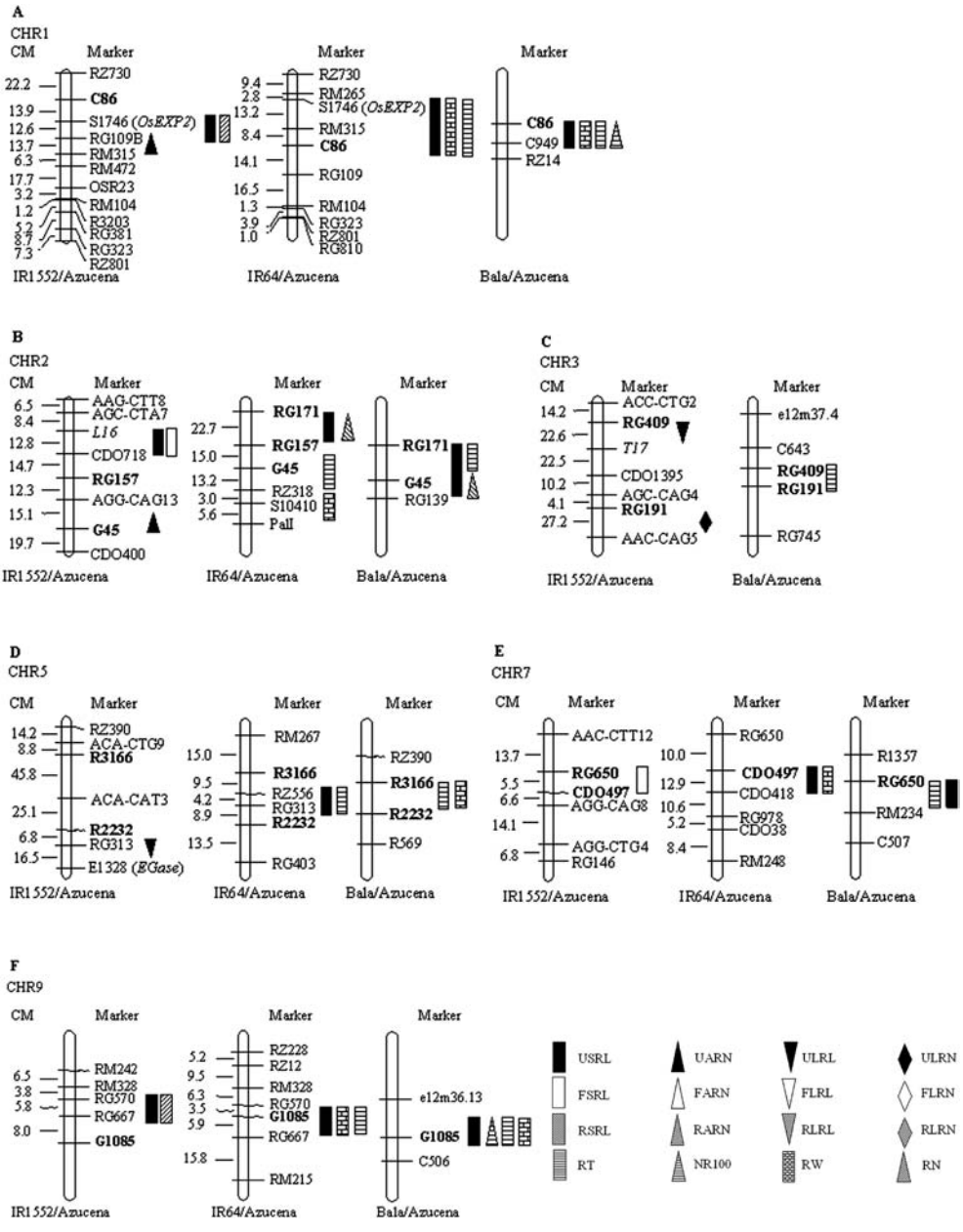

Fig. 2A–F Comparison of QTLs across three mapping populations. (**A–F**) indicate the same molecular markers linked with root traits on chromosomes 1, 2, 3, 5, 7 and 9, respectively. The symbols for different parameters are shown below. The *marker name* is shown on the right of the chromosome, and the genetic distance is shown on the left. The markers in *bold characters* indicate bridge markers. RT: root thickness; RW: root dry weight; RN: root number; NR100: number of root past 100 cm

thickness (RT) and root dry weight (RW) detected in the RIL population derived from Bala/Azucena and in the DH population on chromosomes 1, 2, 7 and 9 are also linked to QTLs for SRL (Fig. 2A, B, E, F).

The QTLs for UARN on chromosomes 1 and 2 may be identical to the QTL for total root number below 100 cm (NR100) on chromosome 1, based on the population derived from Bala/Azucena under early water-deficit treatment (WD0) (Price et al. 2002b) and to the QTLs for penetrated root number (PRN) on chromosome 2 based on the population from Bala/Azucena (Price et al. 2000), and total root number (RN) on chromosome 2 based on the DH population (Hemamalini et al. 2000) based on the bridge markers C86 and G45 (Fig. 2A, B). The QTLs for ULRL mapped on chromosomes 3 and 5, respectively, in this study may be identical to the QTL for RT on chromosome 3 (Price and Tomos 1997; Price et al. 2002b) and the QTLs for maximum root length (MRL), RW and RT on chromosome 5 detected in the other two populations (Yadav et al. 1997; Hemamalini et al. 2000; Price et al. 2002b) (Fig. 2C, D). The QTL for ULRN mapped on chromosome 3 may be identical to the QTL for RT in the population from Bala/Azucena based on the common marker RG191 (Price and Tomos 1997; Price et al. 2002b) (Fig. 2C).

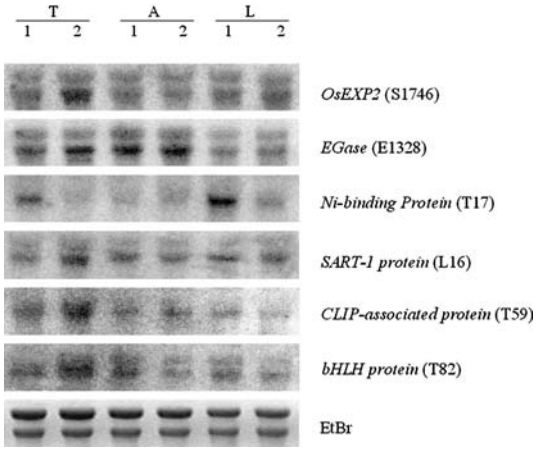

Fig. 3 Northern-blot analysis of the EST and cDNA-AFLP clones with 20 μg of total RNA of Azucena seminal root tip (*T*), adventitious root primordia (*A*) and lateral root (*L*) under flooding (*1*) and upland conditions (*2*). Each gel labeled with the name of the EST and cDNA-AFLP clones. Ethidium-bromide staining

Candidate gene mapping and Northern analysis

One gene for *OsEXP4* and seven ESTs for *OsEXP*, *EGase* and *Xet* were mapped on six chromosomes (Fig. 1). Among them, two EST clones for *OsEXP2* and *EGase*, respectively, were linked to the QTLs for USRL and ULRL on chromosomes 1 and 5, respectively. Northern-blotting analysis indicated that both genes were upregulated by water-deficit stress in the seminal root tip (Fig. 3).

Based on *in silico* mapping, three cDNA-AFLP clones, *L16, T59* and *T82*, for squamous cell carcinoma Antigen Recognised by cytotoxic T lymphocytes gene-1 (SART-1) protein, the protein associated with the cytoplasmic linker protein (CLIP) and the basic helix-loop-helix (bHLH) protein, respectively, were clustered at the locus on chromosome 2 where the QTL for USRL was mapped. The clone *L16* is polymorphic between the parents and was mapped (Fig. 1). Another cDNA-AFLP clone *T17* (the nickel-binding protein) was mapped and linked with CDO1395 on chromosome 3, where the QTL for ULRL was located (Fig. 1). Northern blotting results indicated that the clones for SART-1, the CLIP-associated protein and the bHLH protein were upregulated in the seminal root tip. The gene for the nickel-binding protein (*T17*), however, was induced by submergence in both the seminal root tip and the lateral root (Fig. 3).

Discussion

The tropical upland rice Azucena has been used as a parent to develop different genetic populations for the investigation of QTLs for root characteristics under different water-supply conditions (Table 1). This makes it possible to perform a comparative analysis of the genetic factors in upland rice for root adaptation to water-limited stress under different genetic backgrounds. In this study, a recombinant inbred line population derived from a cross between the lowland rice IR1552 and Azucena was used in experiments with flooding and upland conditions, while considering the highly significant differences in several root traits between the parents under both flooding and upland conditions. The maximum root length of Azucena seedling reached to a depth of 45 cm after 2 weeks of growth. Therefore, the water level at 45 cm below the soil surface was maintained in cylindrical pots to create an upland condition. The water-content data in different sandy soil layers indicate that, after 1 day of drainage, the water content was about 10% in the first layer (0–15 cm), less than 20% in the second layer (15–30 cm) and about 25% in the third layer (30–45 cm) (Table 3). While the water contents in the second and third layers remained relatively stable over the next 2 weeks, the water content in the first layer decreased to as little as about 5% after 2 weeks of drainage. Based on these results, growth during 2 weeks of drainage could be used as water-limited stress under upland conditions.

Upland rice Azucena was characterized by longer seminal and lateral roots, and fewer adventitious and lateral roots, compared to lowland rice IR1552 under flooding (Table 4). Under upland conditions, the growth and development of the lateral root number and length in Azucena increased by about 8-fold, while those in IR1552 only doubled. The seminal root length was induced by upland conditions in Azucena, while that in IR1552 was suppressed. The adventitious root number was decreased in both parents under upland conditions. These results indicate that upland rice has a stronger-root response to a limited water supply as evidenced by an increase in root length and the development of many more lateral roots. However, extreme drought stress can reduce root initiation and growth, as in the first layer. The phenotypic performance of Azucena in this study is consistent with that in earlier reports (Price and Tomos 1997; Yadav et al. 1997; Hemamalini et al. 2000; Price et al. 2000, 2002b; Zheng et al. 2000; Zhang et al. 2001). The broad-sense heritability analysis indicated that the genetic factors for root traits from upland rice have greater heritability under upland conditions.

Five limited-water-induced QTLs for four root traits were identified. Among them, two identical QTLs were found for both seminal root length under upland conditions and relative seminal root length (Table 5), which indicates that the seminal root length under upland conditions could be used to evaluate the water-deficit-inducible root length. No QTLs were detected for either parameter under upland conditions or for the relative parameters of adventitious root number, lateral root length and lateral root number, which suggests that the relative parameters may be used to evaluate the inducible genetic factors for these traits; although it is possible that some QTLs for the variations in the traits under upland conditions and the relative changes were missed in this study. The positive allelic effects for all of the QTLs

detected are consistent with the parental root characteristics (Tables 4 and 5).

Three populations with Azucena as a parent were used to detect QTLs for root traits under upland and lowland conditions (Table 1). The comparison of QTLs across the three genetic populations may provide a better understanding of the genetic factors in upland rice involved in the root response to water stress. Twenty three QTLs were found for the four root traits (Table 5). Several of the QTLs detected were identical across the three genetic populations based on bridge molecular-markers (Fig. 2). The seminal root is usually the longest root before the three-leaf stage. The maximum root length (MRL) was measured under different experimental conditions in the three populations. One QTL for MRL was detected on chromosome 1 linked with the bridge marker C86, based on an RI population from Bala/Azucena after growth for 49 days under sandy soil culture (Price et al. 2002b). In our case, the QTL for SRL at this locus is water-deficit-inducible, which is consistent with the result reported in a DH population from IR64/Azucena after growth for 35 days under upland conditions (Yadave et al. 1997). The QTL for MRL under solution culture was not detected before 28 days (Price and Tomos 1997), which supports our finding that at an earlier seedling stage the QTL was not detected under flooding conditions. Using the same population as used here, Zhang et al. (2001) reported a QTL for SRL linked to the bridge marker C86 in paper culture under aerobic conditions for 15 days. These results suggest that the QTL linked with C86 on chromosome 1 is water-deficit-inducible at an earlier seedling stage in rice.

Using the DH population (IR64/Azucena) grown for 85 days in soil-containing tubes with low moisture, Hemamalini et al. (2000) identified a QTL for root length (RL) on chromosome 2 linked with bridge markers RG157, RG171 and G45. On the same locus of chromosome 2, Yadav et al. (1997) also identified a QTL for MRL under aerobic upland conditions after growth for 35 days using the same DH population. Price and Tomos (1997) also reported two QTLs for MRL in 14- and 21-day-old seedlings under solution culture at this locus. In our case, the QTL was detected under both flooding and upland conditions, but was not detected using the relative parameter. Since relative parameters were not used in the earlier reports (Price and Tomos 1997; Yadav et al. 1997; Hemamalini et al. 2000), the results suggest that the QTL at this locus should be for a constitutive trait and should affect root growth at different growth stages under different genetic backgrounds. On chromosome 7 linked to the bridge marker CDO497, Yadav et al. (1997) reported a QTL for MRL under upland conditions after grown for 35 days using the DH population. Price et al. (2002b) also identified a QTL for MRL under sandy soil culture after growth for 49 days using the RIL population (Bala/Azucena) linked to the bridge marker RG650. In our case, the QTL for SRL after growth for 14 days was detected only under flooding conditions (Fig. 2E). These results indicate that the QTL at this locus should also be for a constitutive trait at the early seedling stage. One identical QTL for MRL under upland conditions across the two populations was also reported on chromosome 9 based on the bridge marker G1085 (Yadave 1997; Price 2002a, b). Our result suggests that this QTL is water-deficit-inducible (Table 5 and Fig. 2). All of the positive alleles for the reported QTLs are from Azucena, which is consistent with our results.

The QTLs for root number were linked to the common marker G45 on chromosome 2 in the two RIL populations, and the alleles at the QTLs are from the *indica* rice parents IR1552 and Bala (Price et al. 2000) (Fig. 2). Hemamalini et al. (2000) found a QTL for total root number (LOD=1.8) on the interval flanked by RG171 and RG157, which is close to G45 in the DH population. These results indicate that the QTLs for root number may be identical across the three populations.

Two QTLs for LRL linked to RG409 on chromosome 3 and to R2232 on chromosome 5 were detected under upland conditions in this study. QTLs for root thickness (RT), which is related to penetration ability under upland conditions, were reported at the above two loci based on the bridge markers RG409 and R2232 in the IR64/Azucena population and the Bala/Azucena population (Yadav et al. 1997; Hemamalini et al. 2000; Price et al. 2002b) (Fig. 2). MRL and root weight (RW) were also reported on chromosome 5 linked to R2232 in the IR64/Azucena population and the Bala/Azucena population (Yadav et al. 1997; Price et al. 2002b) (Fig. 2). These results indicate that these traits may be biologically related or may be, at least partially, controlled by loci with clustered genes.

Comparison of QTLs for root length with candidate genes

Stimulated cell expansion and elongation should contribute to stimulated root elongation under water-limited stress. Cell expansion and elongation are initiated by relaxation of the cell-wall (Lee et al. 2001). Genes for cell-wall loosening and expansion are involved in cell expansion and elongation. Expansins can be classified into two multigene families, the α- and β-expansins. There are at least 26 α-expansins and 14 β-expansins in the rice genome (Lee et al. 2001). Many plant endo-1, 4-β-D-glucanases (*Egase*) for cell-wall loosening have been cloned, and these belong to the group of family 9 *Egases* (Cosgrove 2000). In this study, two genes for cell-wall-loosening proteins, an α-expansin (*OsEXP2*) and an *EGase*, were found to be linked to the QTLs for SRL and LRL under upland conditions, respectively (Fig. 1). These two genes were upregulated by water-limited stress in the seminal root tip (Fig. 3), where cell-wall extensibility is entirely confined (Brummell et al. 1997; Cho and Kende 1997). *OsEXP2* functions as a primary cell wall-loosening agent, and its activity is rate-limiting for the enlargement of cells, whereas *EGase* may act as a secondary cell wall-loosening agent which makes the cell wall more responsive to the action of *EXPs* (Cosgrove

2000). Recent experiments using transgenic plants have provided strong evidence that *α-EXPs* play an endogenous role in cell growth and cell-wall modification (Lee et al. 2001). Our results suggest that the enhanced expression of *OsEXP2* and *EGase* in the root tip under water-limited stress may be related to the stimulation of root elongation.

LRL was greatly increased, especially in the upland rice Azucena under upland conditions (Table 4). The gene-encoding nickel-binding protein (*T17)* was mapped at the locus on chromosome 3 where the QTL for ULRL was detected (Fig. 1). The major function of the nickel-binding protein is to deliver the nickel to the appropriate enzyme and to protect the cell from free nickel (Watt and Ludden 1999). *T17* was repressed by water-limited stress in both the seminal root tip and the lateral root (Fig. 3). These result suggests that *T17* may negatively regulate lateral root elongation, if it plays any role at all.

Three genes for SART-1 protein (*L16*), a transcription factor bHLH protein (*T82*) and the CLIP-associated protein (*T59*), respectively, are clustered at the locus on chromosome 2 where one QTL for SRL was located (Fig. 1). The expression of these three genes was upregulated by water-limited stress in the seminal root tip (Fig. 3). Makarova et al. (2001) reported that SART-1 protein is essential for the assembly of mature splicesomes. It has been reported that transcriptional activators with a bHLH domain regulate the expression of drought-induced genes (Abe et al. 1997). CLIP-associated protein has been shown to play a crucial role in the regulation of microtubule dynamics and / or the interaction of microtubules with the cellular cortex, as documented by over-expression and antibody inhibition analyses (Akhmanova et al. 2001). This is an interesting locus with the QTL for SRL linked to three water-stress-upregulated genes in the seminal root tip.

QTL positions are quite imprecise because the associated confidence interval covers several megabases (Dupuis and Siegmund 1999). A QTL may cover a region containing several-hundred to several-thousand genes, which means that other factors are present in addition to these candidate genes. Fine-mapping experiments are necessary, based on the development of near-isogenic lines that segregate for shorter chromosome segments and that have homogenous genetic backgrounds (Touzet et al. 1995). The tight genetic linkage between these candidate genes and the QTLs for root traits does not definitively demonstrate a causal relationship. The further investigation of these genes for stimulated root elongation under water-limited stress in rice is needed.

Acknowledgements This research was supported by National Key Basic Research Special Foundation of P. R. China, No.G1999011700.

References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosakawa D, Shinozaki K (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9:1859–1868

Akhmanova A, Hoogenraad CC, Drabek K, Stepanova T, Dortland B, Verkerk T, Vermeulen W, Burgering BM, Zeeuw CID, Grosveld F, Galjart N (2001) CLASPs are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. Cell 104:923–935

Bachem CW, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J 9:745–753

Bassam BJ, Caetamo-Anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196:80–83

Brummell DA, Catala C, Lashbrook CC, Bennett AB (1997) A membrane-anchored E-type endo-1,4-β-glucanase is localized on Golgi and plasma membranes of higher plants. Proc Natl Acad Sci USA 94:4794–4799

Byrne PF, McMullen MD (1996) Defining genes for agricultural traits: QTL analysis and the candidate gene approach. Probe 7:24–27

Champoux MC, Wang G, Sarkarung S, Mackill DJ, O'Toole JC, Huang N, McCouch SR (1995) Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. Theor Appl Genet 90:969–981

Chen M, Temnykh S, Xu Y (1997) Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). Theor Appl Genet 95:553–567

Cho HT, Kende H (1997) Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell 9:1661–1671

Cosgrove DJ (2000) Expansive growth of plant cell walls. Plant Physiol Biochem 38:109–124

Dupuis J, Siegmund D (1999) Statistical methods for mapping quantitative trait loci from a dense set of markers. Genetics 151:373–386

Garrity DP, Oldeman LR, Morris RA (1986) Rainfed lowland rice ecosystems: characterization and distribution. In: Progress in rainfed lowland rice. IRRI, Manila, The Philippines, pp 3–23

Harushima Y, Yano M, Shomura A, Sato M, Shimano T, Kuboki Y, Yamamoto T, Lin SY, Antonio BA, Parco A, Kajiya H, Huang N, Yamamoto K, Nagamura Y, Kurata N, Khush GS, Sasaki T (1998) A high-density rice genetic linkage map with 2,275 markers using a single F2 population. Genetics 148:479–494

Hemamalini GS, Shashidhar HE, Hittalmani S (2000) Molecular-marker assisted tagging of morphological and physiological traits under two contrasting moisture regimes at peak vegetative stage in rice. Euphytica 112:69–78

Hoque MM, Kobata T (1998) Growth responses of drought-resistant rice cultivars to soil compaction under irrigated and succeeding non-irrigated conditions during the vegetative stage. Plant Prod Sci 1:183–190

Huang N, Courtois B, Khush GS, Lin H, Wang G, Wu P, Zheng K (1996) Association of quantitative trait loci for plant height with major dwarfing genes in rice. Heredity 77:130–137

Kamoshita A, Wade LJ, Ali ML, Pathan MS, Zhang J, Sarkarung S, Nguyen HT (2002) Mapping QTLs for root morphology of a rice population adapted to rainfed flooding conditions. Theor Appl Genet 104:880–893

Lander ES (1993) Mapmaker/EXE 3.0 and Mapmaker/QTL 1.1 tutorial and reference manual. Whitehead Institute, Cambridge, Massachusetts

Lander ES, Bostein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199

Lee Y, Choi D, Kende H (2001) Expansins: ever-expanding numbers and functions. Curr Opin Plant Biol 4:527–532

Liao CY, Wu P, Hu B, Yi KK (2001) Effects of genetic background and environment on QTLs and epistasis for rice (*Oryza sativa* L.) panicle number. Theor Appl Genet 103:104–111

Makarova OV, Makarova EM, Luhrmann R (2001) The 65- and 110-kDa SR-related proteins of the U4/U6·U5 tri-snRNP are essential for the assembly of mature spliceosomes. EMBO J 20:2553–2563

Nelson JC (1997) QGENE: software for marker-based genomic analysis and breeding. Mol Breed 3:239–245

Nguyen HT, Chandra BR, Blum A (1997) Breeding for drought resistance in rice: physiology and molecular genetics considerations. Crop Sci 37:1426–1434

Pflieger S, Lefebvre V, Causse M (2001) The candidate gene approach in plant genetics: a review. Mol Breed 7:275–291

Price AH, Courtois B (1999) Mapping QTLs associated with drought resistance in rice: progress, problems and prospects. Plant Growth Regul 29:123–133

Price AH, Tomos AD (1997) Genetic dissection of root growth in rice (*Oryza sativa* L.). II. Mapping quantitative trait loci using molecular markers. Theor Appl Genet 95:143–152

Price AH, Steele KA, Moore BJ, Barraclough PP, Clark LJ (2000) A combined RFLP and AFLP linkage map of upland rice (*Oryza sativa* L.). Theor Appl Genet 100:49–56

Price AH, Cairns JE, Horton P, Jones RGW, Griffiths H (2002a) Linking drought-resistance mechanisms to drought avoidance in upland rice during a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. J Exp Bot 53:989–1004

Price AH, Steele KA, Moore BJ, Jones RGW (2002b) Upland rice grown in soil-filled chambers and exposed to contrasting water-deficit regimes. II. Mapping quantitative trait loci for root morphology and distribution. Field Crop Res 76:25–43

Touzet P, Winkler RG, Helentjaris T (1995) Combined genetic and physiological analysis of a locus contributing to quantitative variation. Theor Appl Genet 91:200–205

Watt RK, Ludden PW (1999) Nickel-binding proteins. Cell Mol Life Sci 56:604–625

Wu J, Machara T, Shimokawa T, Yamamoto S, Harada C, Takazaki Y, Ono N, Mukai Y, Koike K, Yazaki J, Fujii F, Shomura A, Ando T, Kono I, Waki K, Yamamoto K, Yano M, Matsumoto T, Sasaki T (2002) A comprehensive rice transcript map containing 6,591 expressed sequence-tag sites. Plant Cell 14:525–535

Yadav R, Courtois B, Huang N, McLaren G (1997) Mapping genes controlling root morphology and root distribution in a doubled-haploid population of rice. Theor Appl Genet 94:619–632

Yoshida S, Forno DA, Cock JH, Gomez KA (1976) Laboratory manual for physiological studies of rice, 3rd edn. The International Rice Research Institute, Manila, The Philippines

Zhang WP, Shen XY, Wu P, Hu B, Liao CY (2001) QTLs and epistasis for seminal root length under a different water supply in rice (*Oryza Sativa* L.). Theor Appl Genet 103:118–123

Zheng HG, Babu RC, Pathan MS, Ali ML, Huang N, Courtois B, Nguyen HT (2000) Quantitative trait loci for root penetration ability and root thickness in rice: comparison of genetic backgrounds. Genome 43:53–61