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Identification of *qRL7*, a major quantitative trait locus associated with rice root length in hydroponic conditions

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Root system development is an important target for improving yield in rice. Active roots that can take up nutrients more efficiently are essential for improving grain yield. In this study, we performed quantitative trait locus (QTL) analyses using 215 recombinant inbred lines derived from a cross between Xieqingzao B (XB), a maintainer line with short roots and R9308, a restorer line with long roots. Only a QTLs associated with root length were mapped on chromosomes 7. The QTL, named *qRL7*, was located between markers RM3859 and RM214 on chromosome 7 and explained 18.14–18.36% of the total phenotypic variance evaluated across two years. Fine mapping of *qRL7* using eight BC_3F_3 recombinant lines mapped the QTL to between markers InDel11 and InDel17, which delimit a 657.35 kb interval in the reference cultivar Nipponbare. To determine the genotype classes for the target QTL in these BC_3F_3 recombinants, the root lengths of their BC_3F_4 progeny were investigated, and the result showed that *qRL7* plays a crucial role in root length. The results of this study will increase our understanding of the genetic factors controlling root architecture, which will help rice breeders to breed varieties with deep, strong and vigorous root systems.

Key Words: rice (*Oryza sativa* L.), root length, QTL, *qRL7*, hydroponic conditions.

Introduction

The root system performs essential functions in plant development, including anchoring the plant, and the uptake of water and nutrient (Dorlodot *et al.* 2007, Fujii 1961, Gewin 2010, Liao *et al.* 2004). For example, a deep, thick and branched root system results in better survival under adverse conditions, such as water or nutrient deficits (Price *et al.* 2002, Yano *et al.* 2005, Zheng *et al.* 2000). The high adaptive plasticity of roots complicates the genetic dissection of genes controlling variation in root structure, representing a bottleneck for the efficient selection of specific root ideotypes (Kamoshita *et al.* 2002a). The genetic basis of root structural variation has been studied mainly through QTL analysis. Many QTLs for root-releated traits have been identified in rice under normal or abiotic stress conditions (Kamoshita *et al.* 2002a, 2002b, Li *et al.* 2005, Qu *et al.* 2008, Rebouillat *et al.* 2009, Steele *et al.* 2006, 2013). Most researchers were interested in QTLs affecting rice root traits under specific environments. However, for rice production, the climate is usually normal, with few adverse conditions; therefore, mapping QTLs for rice roots under normal conditions becomes more important.

The fibrous rice root system contains many roots from each plant at the mature stage, making them difficult to study; consequently, there have been few studies of rice root traits at the reproductive stage (Courtois *et al.* 2009). Yield is mostly determined during the ripening stage; therefore, studies on rice roots during later stages would be more useful. The super hybrid rice, 'Xieyou 9308', is a 'late-stage vigor' rice variety with vigorous leaf and root growth during the late growth period (Cheng *et al.* 2005). During the late growth stage, its leaves remain green until the mature panicle stage (Cheng *et al.* 2005, 2007a).

In this study, we performed QTL analyses using 215 recombinant inbred lines (RILs) derived from a cross between Xieqingzao B (XB), a maintainer line with short roots and R9308, a restorer line with long roots. A QTL associated with root length was mapped on chromosome 7. The QTL, named *qRL7*, was located between markers RM3859 and RM214 on chromosome 7 and explained 18.14–18.36% of the total phenotypic variance evaluated over 2 years. Furthermore, by using advanced-backcross progeny, we validated the genetic effect of *qRL7* for root length on chromosome 7 and delimited its candidate genomic region. The results of this study will increase our understanding of the genetic

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factors controlling root architecture, which will help rice breeders to breed varieties with deep, strong and vigorous root systems.

Materials and Methods

Experimental materials

For QTL analyses, 215 RIL lines (F_{16}) were developed by the single-seed-descent method. XB is the maintainer line of 'Xieyou 9308' and has short roots. R9308 is the restorer line of 'Xieyou 9308' and has long roots.

To perform fine mapping of the QTL for root length on chromosome 7, we developed six BC_3F_3 lines in which recombination had occurred within the region containing the target QTL. MAS was used to select these lines from advanced backcross progeny derived from a cross between R9308 (the recurrent parent) and one RIL line (S4) in which the target QTL region was homozygous for the XB (donor parent) allele. Six BC_3F_2 recombinant plants (BC_3F_2-1-1, BC_3F_2-7-6, BC_3F_2-4-3, BC_3F_2-5-1, BC_3F_2-8-4 and BC_3F_2-9-2) were obtained and self-pollinated to produce homozygous recombinant BC_3F_3 lines. Two NILs (*qRL7*-NIL1 and *qRL7*-NIL2) were obtained in the BC_3F_3 using a MAS strategy. *qRL7*-NIL1 harbored the XB allele at *qRL7*, while *qRL7*-NIL2 carried the R9308 allele. These eight lines were genotyped with DNA markers distributed across all chromosome regions, as described in "DNA marker screening and QTL analysis". To determine the genotype classes for the target QTL in the six BC_3F_3 recombinants, the root lengths of their BC_3F_4 progeny were investigated.

Hydroponic experiments

The hydroponic experiments were conducted on the experimental base of the China National Rice Research Institute (CNRRI) in 2010 and 2011 in Fuyang city, Zhejiang province, China. Each pool was 12 m × 3 m × 1.5 m (length × width × depth). Each foamed plastic was 1.5 m × 1 m, resulting in 24 foamed plastics in each pool. Forty rice plants were planted in each foamed plastic; therefore, a 25 cm × 15 cm row-column design was used (Fig. 1). Forty holes were drilled in each foamed plastic, one hole for each seedling. In May, germinated seeds were sown in the field. After one month, the seedlings were removed carefully to floated plastics in the pool. Each seedling was bundled with sponges to avoid the seedling falling away from the hole, because the hole was too large for the seedling. The foamed plastics containing the planted seedlings were placed in full water pools. Three days after transplanting, the first fertilizer was added to the plant in the sponge. Ten days after the first fertilizer, the second fertilizer was applied. The third fertilizer was applied 10 days later. The fertilizer was a Nitrogen-Phosphorus-Potassium mixed fertilizer ($N : P_2O_5 : K_2O = 15 : 15 : 15$) (Sinochem Group), microelements and rapeseed cake in a quality ratio of 10 : 1 : 20. Thereafter, no fertilizer should be required during the rest of the growing season. About 6 g fertilizer was applied for each plant and insecticide (Triazophos, Sinochem Group) was sprayed every week until the heading stage. Three trials were performed for each design, and each line was grown in a randomized complete block design, with one plant of each line per hole. For each RIL, five plants

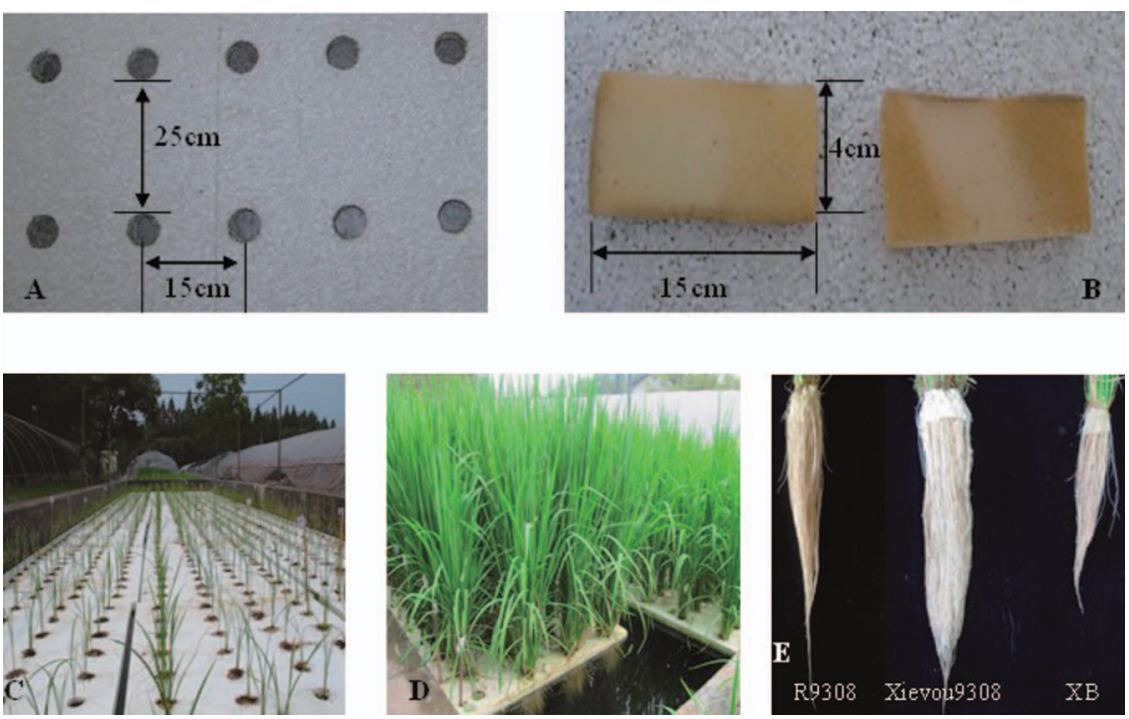

Fig. 1. Hydroponic method and phenotypes of plants. A: Image for foamed plastics, B: Image for sponge. C: Image taken from seedling stage. D: Image of heading stage. E: Image taken after the plants were removed from the foamed plastics.

Table 1. Statistical analysis of the four traits of the RIL population

Years	Item	RL[a] (cm)	PH[b] (cm)	DWR[c] (g plant^{-1})	DWS[d] (g plant^{-1})
2010	Mean ± SD[e]	29.6 ± 5.23	90.73 ± 10.41	4.37 ± 0.39	19.99 ± 3.52
	Range	16.3–68.3	43.1–121.4	0.32–8.35	1.25–61.84
	Kurtosis	0.42	0.49	1.28	0.47
	Skewness	0.35	–0.13	0.91	0.88
2011	Mean ± SD[e]	30.43 ± 5.75	92.03 ± 9.43	4.35 ± 0.28	28.82 ± 3.23
	Range	14.3–47.7	35.9–124.5	0.44–8.28	1.89–60.71
	Kurtosis	0.56	0.43	–0.01	–0.08
	Skewness	–0.73	–0.4	–0.11	0.13
XB	Mean ± SD[e]	25.26 ± 3.59	87.64 ± 5.31	3.31 ± 0.36	18.26 ± 2.89
R9308	Mean ± SD[e]	35.2 ± 5.62	128.7 ± 7.78	4.95 ± 0.56	29.51 ± 3.9
	Significance level	**	**	**	**

RL[a], root length; PH[b], plant height; DWR[c], dry weight of roots.
DWS[d], dry weight of shoot; SD[e], standard deviation.
** significance at P = 0.01 between two parents.

were grown in a randomized complete block design, with one plant of each line per hole.

Phenotype evaluation

Plant height (PH), root length (RL), dry weight of roots (DWR) and dry weight of shoot (DWS) were measured for each plant at the heading stage (Fig. 1). After measuring the root length, the roots were cut off, placed in an envelope and dried in an oven at 80°C. The root traits were measured for three of the five plants (excluding border plants, to avoid edge effects); the average value of the three plants was used as the mean value for each RIL. Six BC_3F_3, BC_3F_4 lines and two NILs were grown on the experimental base of CNRRI in 2012 for fine mapping of *qRL7*.

DNA marker screening and QTL analysis

The linkage map for the RILs, which comprised 198 simple sequence repeat (SSR) markers, spanned 1814.5 cM, with an average spacing of 9.2 cM between adjacent markers. Among them, 165 were from the work of Shen *et al.* (2008); the other 33 SSR markers were added to fill the gaps in the map (Feng *et al.* 2010). Using criteria set for a rice molecular marker linkage map from a previous study (McCouch *et al.* 2002), the molecular map was determined to be suitable for QTL analysis. Putative QTLs were detected using the composite interval mapping (CIM) function of QTL Cartographer 2.5 (Wang *et al.* 2005). The CIM threshold was based on the results of 1000 permutations at a 5% significance level (Churchill and Doerge 1994). The additive effect and the percentage of phenotypic variance explained (PVE) by each QTL were estimated at the maximum logarithm of odds (LOD) score.

For genotyping the BC_3F_2 and BC_3F_3 populations, 121 SSR markers were used. PCR amplifications from the RILs were performed as previously described (Shen *et al.* 2008). To narrow down the candidate region of the *qRL7* on chromosome 7, an additional forty-two insertion-deletion (Indel) markers in the interval between RM3859 and RM214 were selected. The genome resequencing of the two parents, XB and R9308, have been completed (unpublished); therefore, Indel marker design is very effective. Forty-two Indel markers were designed using software Primer premier 3.0 software (Chin *et al.* 2010), which generated 100–300 bp amplicons across the *qRL7* regions. The Indel markers were tested in both parents. Among these 42 markers, nine showed a polymorphism between XB and R9308 when assayed by acrylamide gel electrophoresis, and were named as Indel1, Indel4, Indel7, Indel11, Indel13, Indel16, Indel17, Indel21 and Indel41 (Supplemental Table 1). Gene annotation within the specific genomic regions was carried out using IRGSP1.0 from the Rice Annotation Project (RAP, http://rapdb.dna.affrc.go.jp/) (Pan *et al.* 2013, Rice Annotation Project *et al.* 2008). The genotypes of each line were estimated from the results of Dunnett's test at a significance level of 0.1%.

Results

Phenotypic variation of four traits among RILs

As shown in Table 1, significant phenotypic differences ($P < 0.01$) were found between the parents, XB and R9308. Transgressive segregations for the four traits were observed over 2 years (Fig. 2). Most of the RILs had RLs that fell between the values of the two parental lines and ranged from 25.26 cm to 35.2 cm (Fig. 2). The phenotypic correlation coefficients (PCC) among the four traits in the 215 RILs over 2 years were shown in Table 2. Highly significant correlations were found for each pair of traits (RL, PH, DWR and DWS). Every PCC was greater than or equal to 0.80 and the highest significant correlations were found between DWR and DWS. This indicated that the root length, plant height, the biomass of roots and shoots are probably regulated by same main factors.

QTLs for four traits in the RILs

Seven QTLs for these four traits were detected on chromosomes 6, 7, 8 and 10 in during the 2 years. The LOD thresholds ranged from 4.23 to 8.89, with PVEs of 12.56%

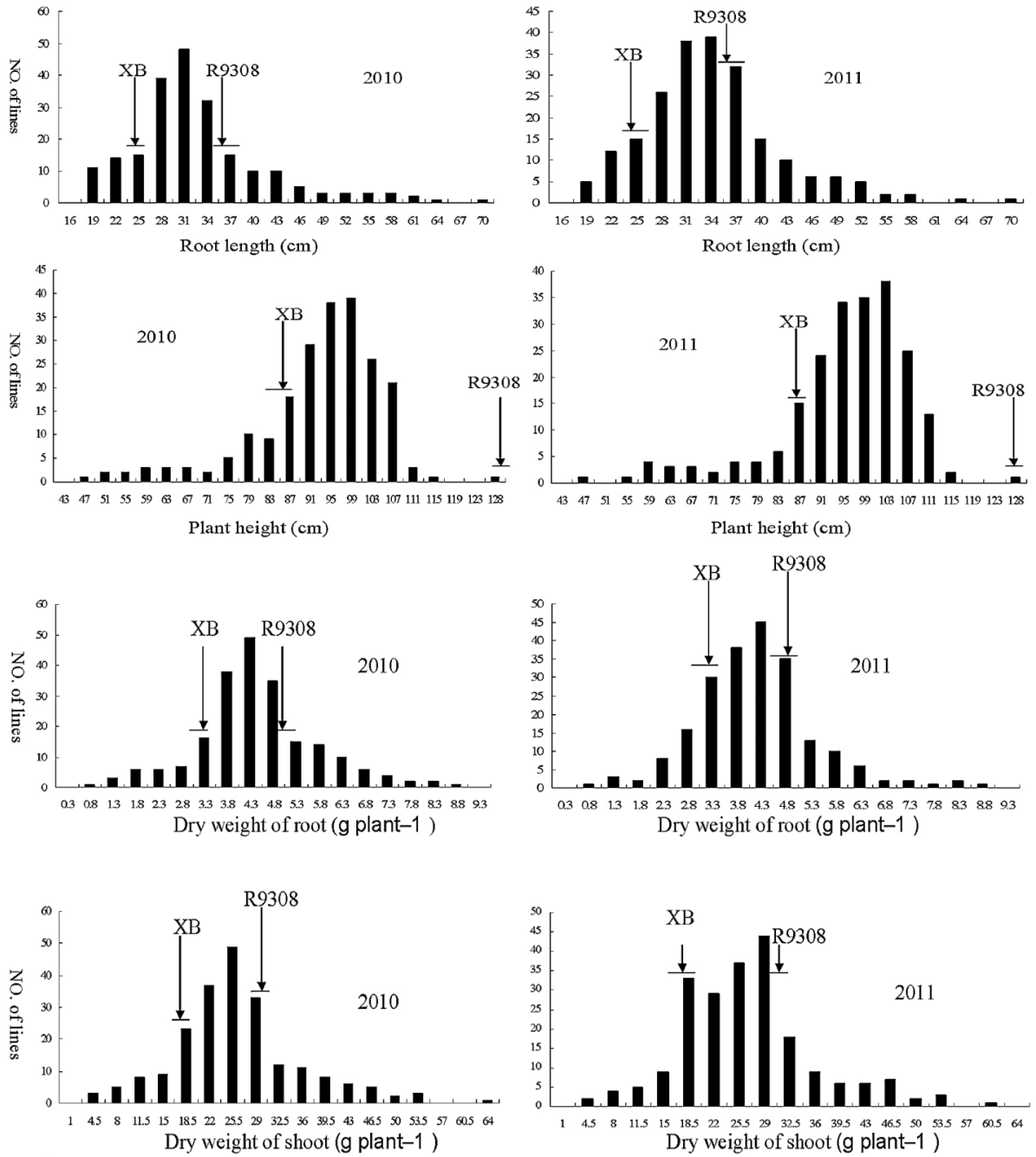

Fig. 2. Frequency distribution of four traits (RL, PH, DWR, DWS) in RILs. Arrowheads and horizontal lines indicate the mean values and standard deviation for the two parents. XB Xieqingzao B.

to 23.68%, respectively. In particular, four QTLs were detected on chromosome 7 (Table 3 and Fig. 3). The QTL, *qRL7*, which affected RL, was detected in the interval RM3859-RM214 with PVEs of 18.14% and 18.36% over 2 years. Three QTLs (*qPH7*, *qPH8* and *qPH10*), one (*qDWR7*) and two QTLs (*qDWS6* and *qDWS7*) affecting PH, DWR and DWS, respectively, were found in the RIL population. Among these, *qPH10* was only detected in 2010. The additive effects of R9308 at all QTLs increased RL, PH, DWR and DWS by 0.29% to 9.43% over the value for XB.

Development of NILs harboring qRL7

qRL7 for RL on chromosome 7 was responsible for high phenotypic variance over 2 years. To verify the genetic effect of this QTL at the heading stage, two NILs differing only at the *qRL7* region were developed using a MAS strategy (Fig. 4). We then investigated the RLs of two NILs (BC_3F_3) lines for the candidate region of chromosome 7 from either XB (*qRL7*-NIL1) or R9308 (*qRL7*-NIL2) (Fig. 4). None of the *qRL7*-NIL1 plants had roots that were as long as those of R9308, although some plants roots were

Table 2. Correlation coefficients among the traits measured in the RIL population

Traits	2010				2011			
	RL	PH	DWR	DWS	RL	PH	DWR	DWS
RL								
PH	0.92**				0.90**			
DWR	0.93**	0.95**			0.92**	0.93**		
DWS	0.82**	0.94**	0.99**		0.85**	0.93**	0.99**	

** $P < 0.01$. For definitions of RL, PH, DWR and DWS, see the footnote to Table 1.

Table 3. Identification of QTLs associated with four traits in the RILs over 2 years

QTL	Chromosome	Interval	2010			2011		
			LOD	PVE[a] (%)	AE[b]	LOD	PVE[a] (%)	AE[b]
qRL7	7	RM3859-RM214	6.73	18.14	4.93	6.85	18.36	5.36
qPH7	7	RM214-RM5875	7.25	20.32	6.37	8.89	23.68	9.43
qPH10	10	RM6100-RM3773	4.23	12.56	1.79	–	–	–
qPH8	8	RM5647-RM8266	7.97	21.49	8.91	7.18	19.75	8.35
qDWR7	7	RM3859-RM214	6.45	17.56	0.38	6.25	16.26	0.29
qDWS7	7	RM214-RM5875	5.89	15.84	2.09	5.18	14.86	1.42
qDWS6	6	RM7434-RM162	7.29	18.87	2.55	7.05	19.58	2.63

[a] Percentage of phenotypic variance explained by each QTL.
[b] Additive effect of each QTL.
'–' means not detected.

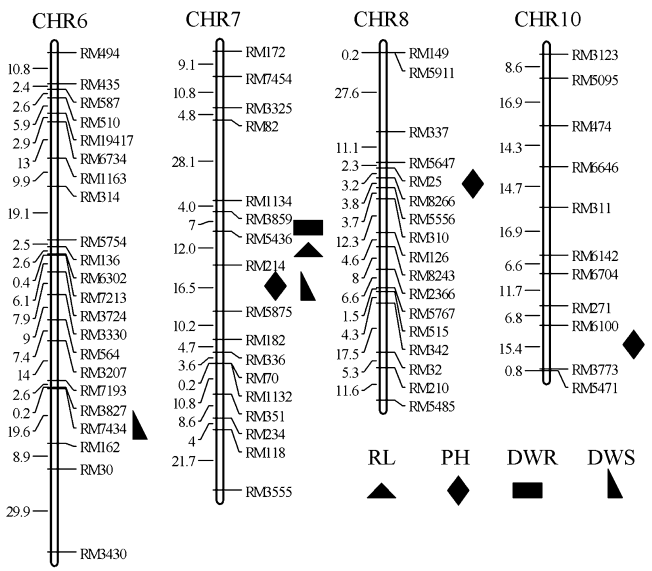

Fig. 3. Chromosome locations of QTLs for four traits (RL, PH, DWR, DWS) in RILs over two years.

slightly elongated. *qRL7*-NIL2 showed a mean root length and standard deviation (SD) of 33.1 ± 2.9 cm, similar to R9308 (35.69 ± 4.3 cm), whereas the mean root length and SD of *qRL7*-NIL1 was 26.3 ± 2.1 cm. These results confirmed that the XB allele of the RL QTL on chromosome 7 conferred short root length at the heading stage.

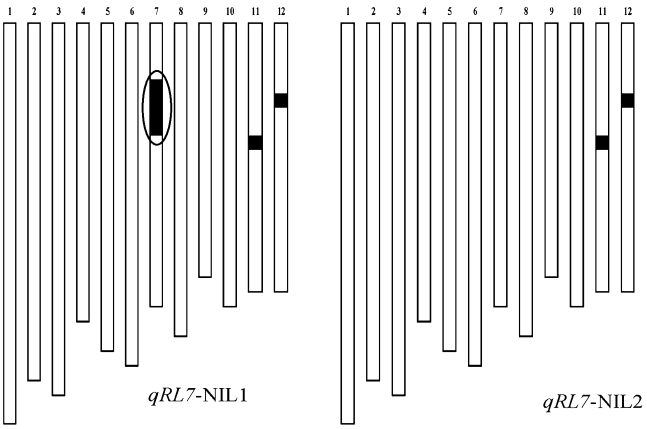

Fig. 4. Graphical representation of the genotypes of two NILs for each of the two *qRL7* alleles. Chromosome numbers are indicated above each linkage map. White and black boxes represent regions that are homozygous for marker alleles from R9308 or homozygous for marker alleles from XB, respectively. The circle was the position of *qRL7*.

Substitution mapping of qRL7

Six recombinant lines were used to map the *qRL7* as a single locus (Table 4). Progeny testing classified the six BC_3F_3 lines into two groups, those with short roots and those with long roots. Three lines (BC_3F_3-1-1, BC_3F_3-7-6 and BC_3F_3-4-3) had relatively short roots, ranging from 26.1 cm to 26.3 cm, whereas the other three lines (BC_3F_3-5-1, BC_3F_3-8-4 and BC_3F_3-9-2) had root lengths of 33.1 cm to 33.2 cm, which were similar to that of *qRL7*-NIL2 (Table 4). These phenotypic groups were predicted to be associated with genotype classes that were homozygous for the XB allele and for the R9308 allele. These results clearly showed that the RL QTL is located between markers InDel11 and InDel17 on chromosome 7 (Fig. 5). The candidate genomic region of *qRL7* between InDel11 and InDel17 spans 657.35 kb in the Nipponbare genome (Fig. 5).

Discussion

The materials and DNA markers

Progress in rice genome sequencing has provided valuable resources and tools for rice molecular genetics (Mergemann *et al.* 2000). In particular, large numbers of genetic markers, such as SSRs, have helped the genetic dissection of complex traits in rice using QTL analysis (Yamamoto *et al.* 2009). The genome of the two parents of XB and R9308 were resequenced in 2010 to design Indel markers. Xieyou9308 is a commercial super hybrid rice released in 1996 in China and is a 'late-stage vigor' rice variety: its leaves remain green until the rice spike matures, and its roots in the reproductive stage remain vigorous and a white color, they do not turn brown (Cheng *et al.* 2005, 2007b). When the gene representing *qRL7* is molecularly identified in Xieyou 9308, which will help us to improve the root lengths of these rice varieties and gain higher yields.

Table 4. Genotypes of nine DNA markers on chromosome 7 in the BC_3F_3 lines and root lengths in the BC_3F_4 progeny

lines	Genotype of marker in BC_3F_3 lines[a]									Root length (cm) in BC_3F_4 lines		
	InDel1	InDel4	InDel7	InDel11	InDel13	InDel16	InDel17	InDel21	InDel41	mean ± SD	P[b]	Predicted genotype of *qRL7*[c]
XB	A	A	A	A	A	A	A	A	A	25.26 ± 3.59	0.95	XB
R9308	B	B	B	B	B	B	B	B	B	35.2 ± 5.62	$<1 \times 10^{-6}$*	R9308
qRL7-NIL1	A	A	A	A	A	A	A	A	A	26.3 ± 2.3	–	*qRL7*-NIL1
qRL7-NIL2	B	B	B	B	B	B	B	B	B	35.1 ± 1.9	$<1 \times 10^{-6}$*	*qRL7*-NIL2
BC_3F_3-5-1	A	A	B	B	B	B	B	B	B	33.1 ± 1.4	$<1 \times 10^{-6}$*	R9308
BC_3F_3-8-4	B	B	B	B	B	B	A	A	A	33.2 ± 1.9	$<1 \times 10^{-6}$*	R9309
BC_3F_3-9-2	B	B	B	B	B	B	A	A	A	33.1 ± 1.5	$<1 \times 10^{-6}$*	R9308
BC_3F_3-1-1	B	B	B	B	A	A	A	A	A	26.1 ± 1.1	0.87	XB
BC_3F_3-7-6	A	A	A	A	A	A	A	B	B	26.3 ± 1.5	0.91	XB
BC_3F_3-4-3	A	A	A	A	A	A	B	B	B	26.2 ± 0.9	0.89	XB

[a] Genotypes of DNA markers are represented by A (white) for XB homozygous and B (black) for R9308 homozygotes.

[b] P, probability of no significant difference between control line (*qRL7*-NIL1) and recombinant BC_3F_3 line in Dunnett's test.

* Indicates significance at the 0.1% level.

[c] Genotypes of *qRL7* were predicted from the results of Dunnett's test using a 0.1% level of significance.

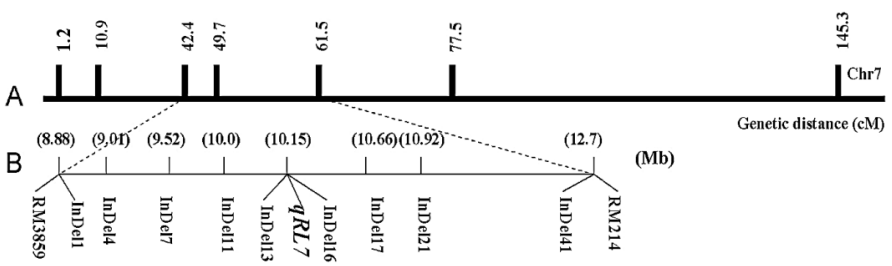

Fig. 5. Location of *qRL7* on rice chromosome 7. A: Linkage map of the RILs derived from XB × R9308. B: Linkage map constructed from the six BC_3F_3 recombinants. Numbers in parentheses beside the DNA markers indicate their physical map position (Mb) on chromosome 7 of Nipponbare.

Advantages and defects of the hydroponic method

Generally, it is difficult to evaluate the whole root system when rice is planted in soil. Using hydroponics, the whole rice root system can be obtained for detailed evaluation. Adverse environments affect genetic mechanisms of root architecture. Some studies found that certain genes controlling rice root traits were expressed in soil culture conditions, but not in hydroponic conditions. For example, the major QTL, Phosphorus uptake 1 (*Pup1*), which confers tolerance to low phosphorus treatment, was expressed in field conditions, but not in hydroponic conditions (Joong *et al.* 2011). Therefore, some scientists doubt the results of studies of rice roots in hydroponic conditions. However, these QTLs or genes were all related to specific environment conditions: the environment stimulated the expression of genes or QTLs controlling rice root traits. Thus, they should be named conditional QTLs or conditional genes, and may not represent the intrinsic genetic program of rice root traits. Therefore, stable environmental control is needed to investigate rice root architecture (Uga *et al.* 2012). The hydroponic method provides homogeneous growth conditions for rice roots and QTLs detected in this environment reflect the intrinsic genetic program of rice root traits.

A major QTL for root length in rice

"Thick with leaves and deep-rooted" is a term used to mean that if you want a rice plant to grow luxuriantly, the roots must be advanced. Rice root traits have been extensively studied, for example, *qSOR1*, a major rice QTL involved in soil-surface rooting in paddy fields was studied (Uga *et al.* 2012) and QTLs for root morphology of a rice population adapted to rainy lowland conditions were analyzed (Kamoshita *et al.* 2002a). Some QTLs associated with root traits that increase yield in upland rice have been investigated (Steele *et al.* 2013). A major QTL, *Dro1*, involved in deep rooting of rice under upland field conditions, was detected on chromosome 9 (Uga *et al.* 2011). A distinct QTL-*qREP-6* involved in root elongation induced by phosphorus deficiency was detected on the long arm of chromosome 6 (Shimizu *et al.* 2008). *Brt4*, a major QTL conferring basal root thickness, was located to chromosome 4 (Liu *et al.* 2008). Other QTLs associated with rice roots have been mapped (Adnan *et al.* 2002, Kondo *et al.* 2001, Zheng *et al.* 2003, 2006). However, there has been no research on rice root traits at the heading stage, because the roots at this stage are difficult to investigate if the rice plants are planted in the field. The present study, using hydroponic, seven QTLs were detected for four traits and a major QTL, *qRL7*, was

identified for root length at the heading stage. Further study using eight BC_3F_3 recombinant lines, allowed *qRL7* to be mapped to a 657.35 kb region between markers InDel11 and InDel17 (Table 4 and Fig. 5). The IRGSP1.0 from the Rice Annotation Project (RAP, http://rapdb.dna.affrc.go.jp/) predicts 96 genes in the candidate region for *qRL7*. To clone the gene(s) represented by *qRL7*, a large population derived from the cross of *qRL7*-NIL1 and *qRL7*-NIL2 would be useful.

Potential applications of QTLs for root length in rice

In rice, water deficiency is one of the dominant abiotic stresses limiting productivity under upland and rainy lowland conditions (Kirk *et al.* 1998). Almost 50% of the soils in rice cultivation areas of the world are currently water deficient (Ismail *et al.* 2007); therefore, enhancement of water acquisition efficiency is a very important breeding target in rice. QTLs for root traits of deep root ratio and deep root mass were detected on chromosomes 2, 3, 4, 9 and 11 (Kamoshita *et al.* 2002b). A major QTL, *qRL6.1*, for root length of rice seedlings grown under a wide range of NH_4^+ concentrations in hydroponic conditions was fine-mapped (Mitsuhiro *et al.* 2010). *qRL6.1* could explain 13.5% to 21.1% of the phenotypic variation and enhance rice yield. However, these studies for architectural features of the root system were not performed at the heading stage. Root traits have a major effect on rice yield from the heading stage to the ripening stage. The major QTL *qRL7* can improve root length significantly at the heading stage and probably has an important effect on yield. Further fine mapping and map-based cloning of *qRL7* will be helpful for understanding the 'late-stage vigor' mechanism of Xieyou9308 and accelerate molecular breeding using *qRL7*.

Acknowledgements

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