

RESEARCH ARTICLE

Validation and dissection of quantitative trait loci for leaf traits in interval RM4923-RM402 on the short arm of rice chromosome 6

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Abstract

Validation and dissection of a QTL region for leaf traits in rice which has been reported in a number of independent studies were conducted. Three sets of near isogenic lines (NILs) were originated from a residual heterozygous line derived the *indica* cross Zhenshan 97B/Milyang 46. They were overlapping and totally covered a 4.2-Mb heterogenous region extending from RM4923 to RM402 on the short arm of rice chromosome 6. Each NIL set consisted of 10 maternal lines and 10 paternal lines. They were measured for the length, width, perimeter and area of the top three leaves and the number of spikelets per panicle, number of grains per panicle and grain weight per panicle. In NIL sets 6-4 and 6-7, differing in intervals RM4923-RM225 and RM19410-RM6119, respectively, significant variations with the enhancing alleles from the female parent ZS97 were shown for the length, perimeter and area except for the area of the third leaf from top in 6-4, but the effects were lower in 6-4 than in 6-7. No significant effects were detected for the three traits in the remaining NIL set. It was shown that flag leaf length (FLL) is the primary target of the QTLs detected. Two QTLs for FLL linked in repulsion phase were resolved, of which *qFLL6.2* located in the 1.19-Mb interval RM3414-RM6917 had a major effect with the enhancing allele from Zhenshan 97B, and *qFLL6.1* located in the 946.8-kb interval RM19350-RM19410 had a smaller effect with the enhancing allele from Milyang 46. The two QTLs also exerted pleiotropic effects on the yield traits.

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Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops, feeding about half the world's population. In rice plant, the top three leaves on a stem, particularly the flag leaf, are the primary source of carbohydrate production accumulated in the grains (Ghosh *et al.* 1990; Li *et al.* 1998). In rice breeding efforts for high-yielding potential, selection for desirable morphological traits of the flag leaf is often a major task for rice breeders (Yuan 1997; Lu *et al.* 2005; Ma *et al.* 2008). Identification of genetic factors underlying these traits will help to formulate an efficient breeding strategy.

Over the last three decades, quantitative trait locus (QTL) mapping has become a standard procedure for understanding the genetic basis for continuous variation. QTLs under-

lying varietal difference for morphological traits of the flag leaf in rice were first reported by Li *et al.* (1998) using an F_{2:4} population derived from the inter-subspecies cross Lemont/Teqing. This work was followed by using recombinant inbred lines (RILs), and test cross and backcross F₁ populations derived from the same cross (Mei *et al.* 2003, 2005). Similar studies were conducted in populations derived from six other inter-subspecies crosses (Li *et al.* 2000; Kobayashi *et al.* 2003; Yue *et al.* 2006; Cao *et al.* 2007; Fan *et al.* 2007; Xiao *et al.* 2007) and three *indica-indica* crosses (Cui *et al.* 2003; Shen *et al.* 2003; Tong *et al.* 2007). The QTLs identified are distributed on all 12 chromosomes of rice, among which a QTL responsible for the width and length of the flag leaf was located on chromosome 4 using RILs (Kobayashi *et al.* 2003) and validated using heterogenous inbred families (Kobayashi *et al.* 2006).

In an earlier investigation (Shen *et al.* 2003), we have used RILs derived from the *indica* rice cross, Zhenshan 97B/

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Milyang 46, to determine QTLs for the area, length, width, perimeter and length/width ratio of the top three leaves. A total of 24 QTLs located in nine intervals were detected, among which a cluster of QTLs responsible for seven traits was located in the RM197-RZ516 region on the short arm of chromosome 6. Significant effects for morphological traits of the rice leaf have also been detected in similar locations in other populations (Cui *et al.* 2003; Wang *et al.* 2005; Yue *et al.* 2006; Cao *et al.* 2007; Fan *et al.* 2007). In this study, near isogenic lines (NILs) carrying heterogenous segments extending from RM4923 to RM402 on the short arm of rice chromosome 6 were developed and used to analyse QTLs for leaf traits in this region.

Materials and methods

Plant materials

Three sets of NILs with overlapping heterogenous segments in a 4.2-Mb region flanked by simple sequence repeat (SSR) markers, RM4923 and RM402, on the short arm of rice chromosome 6 were developed and used in this study. The NIL sets, named as 6-4, 6-7 and 6-10, carried heterogenous segments RM4923-RM225, RM19410-RM6119 and RM6119-RM402 (figure 1) corresponding to the physical positions of 2.17–3.42, 2.91–4.43 and 4.43–6.40 Mb on rice chromosome 6 (<http://www.gramene.org>), respectively.

The three NIL sets were developed from an F_{2:3} population derived from a residual heterozygous line (RHL) carrying a 7.3-Mb heterozygous segment extending from RM4923 to RM19784 on the short arm of rice chromosome 6. This RHL was identified from an F₇ population of the *indica* cross Zhenshan 97B/Milyang 46 (Du *et al.* 2008), in which Zhenshan 97B (ZS97) and Milyang 46 (MY 46) are the maintainer and restore lines of the major commercial three-line hybrid rice Shanyou 10, respectively. Three F₃ plants carrying heterozygous segments RM4923-RM225, RM19410-RM6119 and RM6119-RM402, respectively, in a homogeneous background were selected. Seeds from the three plants were sown and the resultant populations were assayed with SSR markers shown in figure 1. In each population, 20 non-recombinants including 10 maternal homozygotes and 10

paternal homozygotes were selected. Thus each of the three NIL sets consisted of 10 maternal lines and 10 paternal lines differing at the entire heterozygous region of the immediate RHL from which they were derived.

DNA marker analysis

DNA was extracted following the method of Zheng *et al.* (1995). PCR amplification was performed according to Chen *et al.* (1997), but the products were visualized on 6% nondenaturing polyacrylamide gels using silver staining. All the SSR markers were selected from Gramene (<http://www.gramene.org>).

Phenotypic evaluation and data analysis

Sixty rice lines of the three NIL sets were planted in the summer–autumn seasons at the China National Rice Research Institute, Zhejiang, People's Republic of China, in 2006. They were grown at the spacing of 23 cm between rows and 20 cm between plants in a row in a completely randomized block design with two replicates. Twelve plants per line were planted in each replicate, and the middle 10 plants were sampled. They were measured with CI-203 (CID Bio-Science, Camas, USA) at heading stage for the length, width, perimeter and area of the top three leaves. They were also harvested at maturity and measured for the number of spikelets per panicle (NSP), number of grains per panicle (NGP) and grain weight per panicle (GWP).

Two-way ANOVA was conducted to test phenotypic variation between the two genotypic groups in each NIL set. The analysis was performed with a mixed model using SAS procedure GLM (SAS 1999), as described previously (Dai *et al.* 2008). Given the detection of significant difference ($P < 0.05$), the same model was applied to estimate the additive effect of the QTL and the proportion of phenotypic variance explained by the QTL.

Results

Analyses of the three sets of NILs with overlapping heterogenous segments covering a 4.2-Mb region flanked by RM4923 and RM19784 on the short arm of rice chromosome 6 (figure 1) provided an opportunity to validate and separate QTLs located in the target region. Since the two genotypic groups in each NIL set differ only in a portion of this region, significant phenotypic variation between them would be an indication of the presence of a QTL in the given portion. Correspondingly, nonsignificant phenotypic differences between the genotypic groups would be an indication of the absence of a QTL in the given portion, which may provide additional evidence for the presence of a QTL in a neighbouring region.

Variation of the leaf traits in the three sets of NILs

In each of the three NIL sets, two-way ANOVA was performed to test the differences of the leaf traits between the

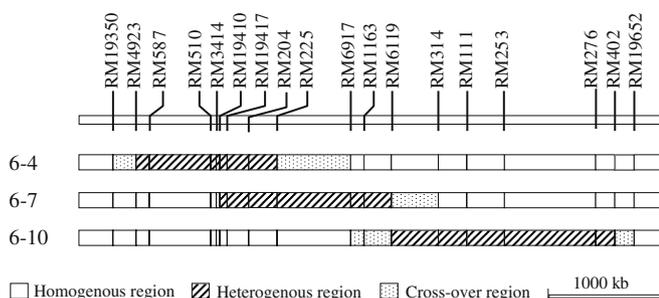


Figure 1. Genotypic composition of NIL sets 6-4, 6-7 and 6-10 in interval RM19350-RM19652 on the short arm of rice chromosome 6.

maternal and paternal lines (table 1). It was shown that NIL set 6-7 exhibited the most significant variation and NIL set 6-10 had the least.

In NIL set 6-4, significant variations were observed for the length, perimeter and area of the top three leaves except for the area of the third leaf from top, whereas no significant variations were observed for leaf width. The contributions of the genotypic effect to the phenotypic variance of the length, perimeter and area of the top two leaves ranged from 18.5% to 21.2%, and the contributions on the length and perimeter of the third leaf from top were 10.3% and 10.2%, respectively. In addition, the enhancing alleles were all derived from the female parent ZS97. These results suggest that QTLs for the length, perimeter and area of the top

three leaves were located in the heterogenous region in NIL set 6-4.

In NIL set 6-7, significant variations were observed for all the 12 traits, and the variation was much more distinct for the length, perimeter and area than for the width, especially at the top two leaves. The contributions of the genotypic effect to the phenotypic variance of the length, perimeter and area were 85.5, 85.7 and 81.3% at the flag leaf, 75.7, 75.7 and 71.1% at the second leaf from top, and 33.8, 33.8 and 22.4% at the third leaf from top, respectively. Again, the enhancing alleles were all derived from the female parent ZS97. Evidently, major QTLs responsible for the length, perimeter and area of the top three leaves were located in the heterogenous region in NIL set 6-7. Regarding the leaf width, the

Table 1. Effects of the QTLs located in interval RM4923-RM402 on the length, width, perimeter and area of the top three leaves in rice estimated from three sets of NILs.

NIL set	Leaf position	Trait	Genotypic mean ^a		F	P	A ^b	R ² (%) ^c	
			Z	M					
6-4	Top 1st	length (cm)	44.92	36.76	5.29	0.0336	-4.08	20.8	
		perimeter (cm)	90.72	74.42	5.29	0.0336	-8.15	20.8	
		area (cm ²)	52.58	42.00	6.42	0.0208	-5.18	21.2	
	Top 2nd	width (cm)	1.55	1.54	0.28	0.6034			
		length (cm)	49.17	43.71	6.18	0.0230	-2.73	21.1	
		perimeter (cm)	99.09	88.20	6.21	0.0227	-5.45	21.2	
	Top 3rd	area (cm ²)	52.34	45.27	6.53	0.0199	-3.53	18.5	
		width (cm)	1.37	1.34	2.46	0.1340			
		length (cm)	44.82	42.33	4.69	0.0441	-1.24	10.3	
	6-7	Top 1st	perimeter (cm)	90.32	85.33	4.71	0.0436	-2.49	10.2
			area (cm ²)	41.93	39.59	1.39	0.2545		
			width (cm)	1.22	1.20	0.47	0.5026		
Top 2nd		length (cm)	48.88	32.23	206.19	<0.0001	-8.33	85.5	
		perimeter (cm)	98.68	65.48	206.72	<0.0001	-16.60	85.7	
		area (cm ²)	55.30	36.13	111.34	<0.0001	-9.59	81.3	
Top 3rd		width (cm)	1.46	1.52	6.84	0.0176	0.03	16.9	
		length (cm)	49.91	39.48	151.57	<0.0001	-5.22	75.7	
		perimeter (cm)	100.55	79.86	150.25	<0.0001	-10.34	75.7	
6-10		Top 1st	area (cm ²)	52.67	40.05	84.89	<0.0001	-6.31	71.1
			width (cm)	1.37	1.29	14.82	0.0012	-0.04	29.9
			length (cm)	44.36	40.82	21.91	0.0002	-1.77	33.8
	Top 2nd	perimeter (cm)	89.41	82.32	21.86	0.0002	-3.54	33.8	
		area (cm ²)	41.38	36.96	13.88	0.0015	-2.21	22.4	
		width (cm)	1.23	1.15	14.06	0.0015	-0.04	20.9	
	6-10	Top 1st	length (cm)	36.25	35.81	0.14	0.7140		
			perimeter (cm)	73.46	72.56	0.15	0.7015		
			area (cm ²)	43.15	40.50	2.96	0.1026		
		Top 2nd	width (cm)	1.59	1.51	12.66	0.0022	-0.04	19.6
			length (cm)	44.64	43.71	0.98	0.3349		
			perimeter (cm)	90.11	88.25	0.98	0.3364		
Top 3rd		area (cm ²)	46.64	44.53	2.67	0.1198			
		width (cm)	1.32	1.30	1.19	0.2894			
		length (cm)	43.51	43.12	0.08	0.7815			
6-10	Top 3rd	perimeter (cm)	87.74	86.97	0.07	0.7920			
		area (cm ²)	40.97	39.32	1.13	0.3019			
		width (cm)	1.21	1.17	3.20	0.0907			

^aZ, Zhenshan 97B homozygote; M, Milyang 46 homozygote. ^bAdditive effect of replacing a maternal allele by a paternal allele. ^cProportion of phenotypic variance explained by the putative QTL.

genotypic effects were smaller and displayed inconsistent allelic directions across the three leaves. The MY46 allele increased the width of the flag leaf but decreased the width of the second and third leaves from top, with the genotypic effects contributing 16.9, 29.9 and 20.9% to the phenotypic variances, respectively.

In NIL set 6-10, no significant variations were observed for the length, perimeter and area of the top three leaves, indicating that QTLs for these traits were not present in the heterogenous region in NIL set 6-10. Significant variation was detected for the leaf width, but at the flag leaf only, explaining 19.6% of the phenotypic variance. Together with the observations that the leaf width exhibited no significant variations in 6-4, and given the inconsistent and relatively small genotypic effect in 6-10, it can be assumed that leaf width is not a major target of QTLs having genotypic difference between ZS97 and MY46 in interval RM4923-RM402.

Dissection of QTLs for flag leaf length (FLL) in the RM4923-RM402 region

As described above, analyses of the phenotypic variances indicated that QTLs responsible for the length, perimeter and area of the top three leaves were located in the heterogenous regions in NIL sets 6-4 and 6-7. It was also shown that the contributions of the genotypic effect to the phenotypic variance were generally similar for the three traits, and in all cases the enhancing alleles were derived from the female parent ZS97. Since leaf length is a primary trait and the largest contribution for the leaf length was observed at the flag leaf, it is reasonable to conclude that FLL is the primary target of the QTLs for leaf morphological traits located in the heterogenous regions of NIL sets 6-4 and 6-7.

Although the alleles for increasing FLL were both derived from ZS97 in NIL sets 6-4 and 6-7, the additive effect and the contribution to the phenotypic variance estimated were much smaller from NIL set 6-4 than from 6-7 (table 1). This suggests that a major QTL for FLL with the enhancing allele from the female parent ZS97 was located in the common heterogenous region presented in NIL sets 6-4 and 6-7; moreover, another QTL having a smaller effect with the

enhancing allele from the male parent MY46 was located in a region which was heterogenous in NIL set 6-4 and homogenous in NIL 6-7. In addition, both QTLs were located in regions which were homogenous in NIL set 6-10 showing no significant variances for FLL.

Since the genotype in the cross-over region of an RHL was undetermined, a cross-over region may be heterogenous or homogenous in the NIL set derived. The undetermined region should be included in the possible region of a QTL. As shown in figure 1, RM19410-RM225 was heterogenous in both the NIL sets 6-4 and 6-7, while RM3414-RM19410 was heterogenous in 6-4 and undetermined in 6-7, and RM225-RM6917 was undetermined in 6-4 and heterogenous in 6-7. Thus a major QTL for FLL was located in the interval flanked by RM3414 and RM6917, which corresponds to a 1.19-Mb region extending from 2,881,884 to 4,067,714 bp on rice chromosome 6. As expected, this region was homogenous in NIL set 6-10 which showed no significant variations on FLL.

Similarly, taking into account the interval RM19350-RM4923 which was undetermined in NIL set 6-4 and RM3414-RM19410 undetermined in 6-7, the region which could be heterogenous in 6-4 and homogenous in 6-7 was flanked by RM19350 and RM19410. Thus the other QTL for FLL was located in interval RM19350-RM19410, which corresponds to a 946.8-kb region extending from 1,967,316 to 2,914,081 bp on rice chromosome 6. As expected, this region was also homogenous in NIL set 6-10 which showed no significant variations for FLL.

Following the order of the two QTL regions in the rice physical map, the minor QTL in RM19350-RM19410 and the major QTL in RM3414-RM6917 were designated as *qFLL6.1* and *qFLL6.2*, respectively.

Effects of *qFLL6.1* and *qFLL6.2* on yield traits

Results of the two-way ANOVA on the three yield traits are presented in table 2. In NIL sets 6-4 and 6-7, variations detected for the yield traits were similar to those observed for the leaf traits (table 1). First, significant variations were detected in both sets, and the enhancing alleles were always

Table 2. Effects of the QTLs located in interval RM4923-RM402 on the number of spikelets per panicle (NSP), number of grains per panicle (NGP) and grain weight per panicle (GWP).

NIL set	Trait	Genotypic mean		<i>F</i>	<i>P</i>	<i>A</i>	<i>R</i> ² (%)
		<i>Z</i>	<i>M</i>				
NIL6-4	NSP	151.55	129.93	9.58	0.0062	-10.81	26.8
	NGP	112.31	99.26	5.41	0.0320	-6.52	19.9
	GWP (g)	2.70	2.41	3.73	0.0694		
NIL6-7	NSP	158.90	114.33	345.29	<0.0001	-22.29	89.0
	NGP	107.97	84.99	41.30	<0.0001	-11.49	49.2
	GWP (g)	2.55	2.09	21.97	0.0002	-0.23	38.3
NIL6-10	NSP	140.82	124.83	77.08	<0.0001	-7.99	57.0
	NGP	110.40	98.66	20.99	0.0002	-5.87	34.0
	GWP (g)	2.72	2.51	9.82	0.0057	-0.10	21.3

derived from ZS97. Second, the additive effect and the contribution to the phenotypic variance estimated were much smaller from NIL set 6-4 than from 6-7. Third, similar proportions of the phenotypic variance explained were observed for FLL and NSP. These suggest that the minor QTL *qFLL6.1* and the major QTL *qFLL6.2* had exerted pleiotropic effects on NSP, NGP and GYP, especially on NSP.

In NIL set 6-10, which showed insignificant variation for the leaf traits, significant variations were detected for all of the three yield traits analysed. This suggests that QTLs which were responsible for yield traits and exhibited little effect on the leaf traits were located in a region which was heterogenous in NIL set 6-10 and homogenous in 6-4 and 6-7.

Discussion

The length, width and area are the three traits determining the shape and size of a leaf, among which the area is attributable to the length and width with higher correlations between length and area than between width and area (Yan and Wang 1990; Li *et al.* 1998, 2000; Lu *et al.* 2005; Peng *et al.* 2007; Xiao *et al.* 2007). The improvement of flag leaf traits through plant breeding had led to a drastic increase in grain yield (Yonezawa 1997), and FLL has long been recognized as one of the key factors in the formulation of new plant ideotype for high-yielding potential in rice (Yuan 1997; Ma *et al.* 2008). In the present study, NILs covering a 4.2-Mb heterogenous region flanked by RM4923 and RM402 on the short arm of rice chromosome 6 were developed and used to test the effects of QTLs for leaf traits and yield traits in this segment. The target region was found to contain two QTLs responsible for the length, area and perimeter of the rice leaves, with FLL as the primary target. One QTL, *qFLL6.2*, was located in a 1.19-Mb region flanked by RM3414 and RM6917 and had a major effect on FLL with the enhancing allele from the female parent ZS97. Another QTL, *qFLL6.1*, was located in a 946.8-kb region flanked by RM19350 and RM19410 and had a smaller effect on FLL with the enhancing allele from the male parent MY46. It was also shown that the two QTLs had exerted pleiotropic effects on the spikelet number, grain number and grain weight measured on a per-panicle basis.

Many QTLs for leaf morphological traits in rice have been recently mapped using DNA markers, among which some were detected in independent studies. The region we report here has been found to affect leaf traits in six of the 10 crosses analysed, including two *indica* crosses between parental lines of major commercial hybrids Shanyou 63 (Cui *et al.* 2003; Wang *et al.* 2005) and Shanyou 10 (Shen *et al.* 2003), respectively, one other *indica* cross (Tong *et al.* 2007) and three inter-subspecies crosses (Cao *et al.* 2007; Fan *et al.* 2007; Yue *et al.* 2006). Mapping results in this study have not only verified the importance of the target region in the genetic control of leaf traits in rice, but also provided good candidates for QTL fine-mapping and map-based cloning.

NILs which have been commonly used in QTL fine-mapping are usually developed by advanced backcrossing in combination with marker-assisted selection (MAS). However, it requires a large population to select enough recombinants for high-resolution mapping (Li *et al.* 2004). Recent uses of RHL and its likeness have provided an alternative choice of developing genetic resources for QTL fine mapping in crop species (Tuinstra *et al.* 1997; Loudet *et al.* 2005; Yamanaka *et al.* 2005). For the development of an RHL-derived NIL set, a single plant selected from an RIL population on the basis of its genotype is generally sufficient. The procedure of repeated backcrossing and MAS are not required. Additionally, the workload for marker analysis and the number of lines for trait evaluation are decreased. The region harbouring a QTL of interest could be further delimited to a smaller interval by using NILs derived from the sub-RHLs (Yamanaka *et al.* 2005; Kobayashi *et al.* 2006; Cheng *et al.* 2007). In the present study, sub-RHLs were selected from a RHL-derived F_{2:3} population previously used in QTL mapping for yield traits of rice. Genotyping was performed using a small number of SSR markers. Not only the effect of the target interval on the morphological traits of rice leaves was verified, but also two QTLs tightly linked in repulsion phase were resolved. Thus, our study provides another example in support of the high efficiency of using RHLs for QTL fine-mapping as demonstrated by Yamanaka *et al.* (2005).

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