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Genetic dissection of top three leaf traits in rice using progenies from a *japonica* × *indica* cross

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Research Article

Abstract The size of the top three leaves of rice plants is strongly associated with yield; thus, it is important to consider quantitative traits representing leaf size (e.g., length and width) when breeding novel rice varieties. It is challenging to measure such traits on a large scale in the field, and little is known about the genetic factors that determine the size of the top three leaves. In the present study, a population of recombinant inbred lines (RILs) and reciprocal single chromosomal segment substitution lines (SSSLs) derived from the progeny of a *japonica* Asominori × *indica* IR24 cross were grown under four diverse environmental conditions. Six morphological traits associated with leaf size were measured, namely length and width of the flag, second and third leaves. In the RIL population, 49 QTLs were identified that clustered in 30 genomic region. Twenty-three of these QTLs were confirmed in the SSSL population. A comparison with previously reported genes/QTLs revealed eight novel genomic regions that contained uncharacterized ORFs associated with leaf size. The QTLs identified in this study can be used for marker-assisted breeding and for fine mapping of novel genetic elements controlling leaf size in rice.

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INTRODUCTION

Rice (*Oryza sativa* L.) is a primary food crop that serves as a major carbohydrate source for approximately 50% of the world's population. Although numerous factors affect rice yield, photosynthetic efficiency is the most influential (Murchie et al. 1999). Leaves are a principle organ in crop plants, and are involved in numerous fundamental physiological functions, such as photosynthesis and transpiration. Leaf size characteristics, such as length and width, are major determinants of plant architecture. In rice, the size of the top three leaves, i.e., the flag leaf, second leaf and third leaf, is considered to be a key determinant of plant photosynthetic efficiency and grain yield (Peng et al. 2008; Jiang et al. 2010). The larger the size of the top three leaves, the greater the increase in photosynthetic potential and the accumulation of assimilates (Horton 2000; Wang et al. 2011; Wang et al. 2012a).

Leaf size is a typical complex trait that is quantitatively inherited and is sensitive to environmental conditions during plant growth (Gravois and McNew 1993; Yan et al. 1999; Kobayashi et al. 2003; Tsukaya 2005). To date, several quantitative trait loci (QTLs) associated with leaf size have been identified in rice using various genetic populations, such as F_2, doubled haploids, and recombinant inbred lines (RILs) (Zhang et al. 2008; Zhang et al. 2015; Jia et al. 2016). Furthermore, a number of genes controlling leaf length and width have been identified and cloned in rice.

Functional analysis showed that a mutant of *COW1/NAL7*, which is located on the short arm of chromosome 3, had narrower leaves than the wild type, but maintained normal leaf length, and had a 5- to 10-fold higher transpiration rate under low-intensity light and high relative humidity (Woo et al. 2007; Fujino et al. 2008). Loss-of-function of *NAL9/VYL*, also located on chromosome 3, resulted in reduced length and width of the flag and second leaves, and decreased contents of chlorophyll *a* and *b* and carotenoids, thus demonstrating the involvement of *NAL9/VYL* in chloroplast development and photosynthesis in rice (Dong et al. 2013; Li et al. 2013). *NAL1/qFLW4/LSCHL4/qTSN4*, located on chromosome 4, was shown to play an important role in regulating leaf width, whereas it had a limited effect on leaf length, and was also shown to influence photosynthetic rate and carbon source-sink relationships (Qi et al. 2008; Chen et al. 2012; Cho et al. 2014; Zhang et al. 2014; Fabre et al. 2016). *NAL2* and *NAL3*, located on chromosomes 11 and 12, respectively, were found to encode an identical OsWOX3A protein involved in organ (including leaf) development, and *NAL2/NAL3* loss-of-function resulted in plants with reduced leaf width (Cho et al. 2013; Cho et al. 2016). *NRL1/OsCSLD4/DNL1*, located on chromosome 12 of rice, encodes the cellulose synthase-like protein D4 (OsCslD4) and plays a major role in determining plant height and leaf width. Mutation of *NRL1/OsCSLD4/DNL1* caused a reduction in plant height and leaf width, and also altered stomata formation, thereby affecting photosynthesis and transpiration (Hu et al. 2010; Yoshikawa et al. 2013; Ding et al. 2015).

The genetic studies described mainly focused on loss-of-function rice mutants and have allowed the functional characterization of a number of major genes influencing leaf size. However, marker-trait association analyses of bi-parental populations containing high levels of genetic variation are needed to decipher the genetic control of leaf size. Genes/QTLs influencing the size of top three leaves of rice may be used in breeding programs. To this end, Tsunematsu et al. (1996) and Kubo et al. (2002) used two parental lines, namely the *japonica* cultivar Asominori and the *indica* cultivar IR24, to develop a RIL population that contained 71 lines, and reciprocal chromosomal segment substitution lines (CSSLs). These genetic materials have been used in preliminary QTL mapping studies for several rice traits, including grain quality, aluminum tolerance, size of the flag and second leaves, and heading date (Wan et al. 2005; Xue et al. 2007; Zhang et al. 2008; Wei et al. 2010a). The RIL and CSSLs have also been used for map-based cloning of *GW5*, *DTH8*, and *DTH2* (Weng et al. 2008; Wei et al 2010b; Wu et al. 2013), a heterosis study (Wang et al. 2012c), and marker-assisted breeding (Wang et al. 2007). However, the RIL population developed by Tsunematsu et al. (1996) is limited in size, and most of the CSSLs developed by Kubo et al. (2002) contain multiple donor chromosomal segments. Furthermore, these lines were genotyped by restriction fragment length polymorphism (RFLP) analysis, which is no longer commonly used for rice genotyping.

In this study, we further investigated the genetic elements associated with rice leaf size. For this, we generated a novel RIL population by crossing Asominori with IR24, and a single chromosomal segment substitution lines (SSSLs) population via backcrosses between existing CSSLs and their corresponding background parents. Morphological traits for size of the top three leaves were measured in the resulting RIL and SSSL plants. We used these data to: (i) evaluate the correlations between the length and width of the top three leaves of rice grown across four diverse environmental conditions; (ii) identify QTLs associated with length and width of these leaves; and (iii) identify potentially novel QTLs for leaf size, which may be used in future genetic studies and in rice breeding.

RESULTS

Phenotypic distribution, correlation between leaf traits, and ANOVA in the RIL population

For each RIL, we measured six morphological traits associated with the size of the top three leaves, namely flag leaf length (FLL), flag leaf width (FLW), second leaf length (SLL), second leaf width (SLW), third leaf length (TLL), and third leaf width (TLW). Phenotypic frequency distributions of the six traits within the RIL population across four environmental conditions are shown in Figure 1. This analysis demonstrated that trait variation between the two parents clearly differed with varying environmental conditions. IR24 had consistently greater values for each trait in all environmental conditions, indicating the high-level stability of these traits. In the RIL population, transgressive segregation in both directions was observed, but at varying magnitudes for each trait (Figure 1).

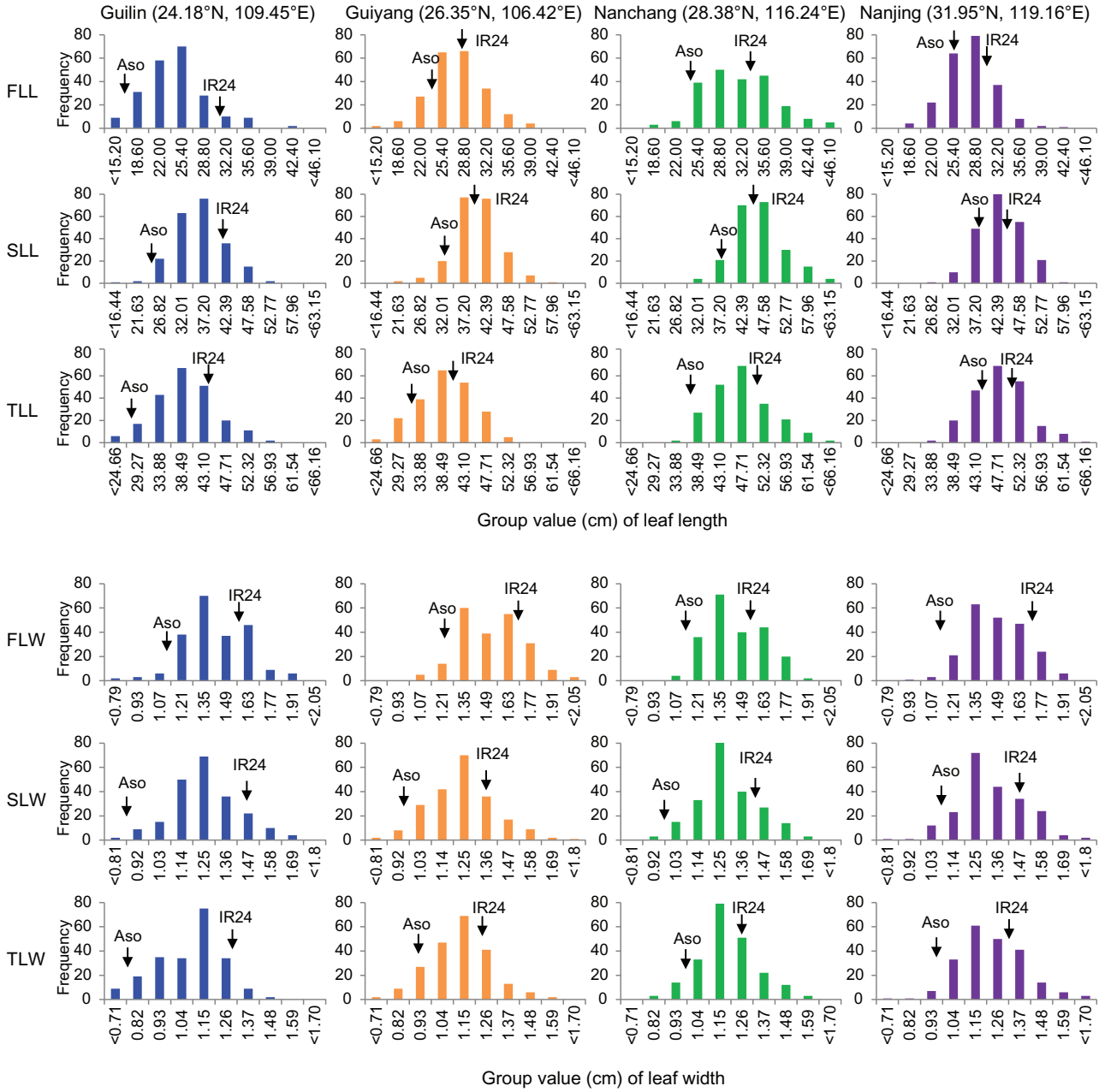

Figure 1. Frequency distribution of the six morphological traits in the RIL population

In each histogram, the two arrows labelled Aso and IR24 indicate the positioning of traits for *Oryza sativa* ssp. *japonica* cv. Asominori and *Oryza sativa* ssp. *indica* cv. IR24, respectively. Analyses are shown for each of the four environmental conditions, namely Guilin, Guiyang, Nanchang and Nanjing. FLL, flag leaf length; FLW, flag leaf width; SLL, second leaf length; SLW, second leaf width; TLL, third leaf length; TLW, third leaf width.

Correlation coefficients calculated from the phenotypic mean of each RIL across the four environmental conditions and two replicates are shown in Table 1. A significance test indicated that the six traits were positively correlated with each other. For the top three leaves, the correlation range was 0.568–0.886 for length, and 0.797–0.923 for width. Correlations between length and width combined were 0.243–0.407, which was much lower than that for length and width alone. For length, the second leaf displayed higher correlation with the flag leaf and third leaf than the correlation between flag leaf and third leaf. Similar results were observed for width (Table 1), which indicates that the second

Table 1. Correlation analysis of the six morphological traits across four environmental conditions determined using the RIL population

Traits	FLL	SLL	TLL	FLW	SLW	TLW
FLL	1.000					
SLL	0.669**	1.000				
TLL	0.568**	0.886**	1.000			
FLW	0.336**	0.390**	0.389**	1.000		
SLW	0.329**	0.335**	0.407**	0.917**	1.000	
TLW	0.343**	0.243**	0.358**	0.797**	0.923**	1.000

Significant differences are indicated at $^{**}P = 0.01$. FLL, flag leaf length; FLW, flag leaf width; SLL, second leaf length; SLW, second leaf width; TLL, third leaf length; TLW, third leaf width.

leaf is potentially representative of the top three leaves.

Genotypic effects, environmental effects, and their interacting effects considered in the ANOVA model were all significant for the six traits. Variance components were estimated by expected mean square methods, based on which two levels of heritability were estimated (Table 2). Genotypic variance was shown to be the most influential component for all traits. Though significant, genotype by environment (GE) interaction had a smaller effect on the traits than the other two components. Heritability in a broad sense was estimated at the plot level as 0.4737–0.6802 for the six traits. However, heritability values were 0.8962–0.9527 when estimated using means across environmental conditions and replicates (Table 2). This level of heritability is considerably high for typical quantitative traits, indicating that the six top leaf traits are suitable for QTL mapping and other genetic studies.

QTLs identified in the RIL population

The LOD threshold used for defining a QTL was 2.65, which was estimated by a total of 6,000 permutation tests on the six traits. Peaks with a LOD score higher than this threshold value in one environmental condition were considered QTLs. Using these parameters, five QTLs were identified for FLL, which were distributed across chromosomes 1, 2, 3, 9, and 10 (qFLL1, qFLL2, qFLL3, qFLL9, and qFLL10, respectively; Table 3). No FLL QTL was found to be significant across the four environmental conditions. However, qFLL2 was significant in three environmental conditions, and qFLL1, qFLL3, qFLL9, and qFLL10 were significant in one environmental condition. Additive effects of the five QTLs on FLL were positive in all environmental conditions, indicating the IR24 alleles were associated with increased FLL. Six QTLs were identified for SLL, with one located on each of chromosomes 1, 3, and 4, and three located on chromosome 2 (Table 3). Of these, qSLL2.1 and qSLL3 were significant in two environmental conditions, and the other four were significant in one environmental condition. In contrast to the other SLL QTLs, qSLL2.3 had a negative effect on SLL. Thus, IR24 alleles increased SLL at five of the six SLL QTLs identified. Seven QTLs were identified for TLL, with one located on each of chromosomes 1, 3, 4, 7, and 12,

Table 2. Variance components and heritability of the six morphological traits determined using the RIL population

	Variance components				Heritability	
Traits	Environment	Genotype	GE interaction	Random error	Plot level	Genotypic mean
FLL	8.8212	12.3227	3.5515	10.1405	0.4737	0.8962
SLL	19.2662	20.9827	4.0994	13.8389	0.5391	0.9194
TLL	24.4894	26.1740	3.6083	11.5797	0.6328	0.9414
FLW	0.0019	0.0260	0.0029	0.0097	0.6731	0.9513
SLW	0.0016	0.0212	0.0021	0.0079	0.6802	0.9527
TLW	0.0046	0.0190	0.0027	0.0083	0.6335	0.9437

FLL, flag leaf length; FLW, flag leaf width; SLL, second leaf length; SLW, second leaf width; TLL, third leaf length; TLW, third leaf width.

Table 3. QTLs identified in the RIL population for length of the top three leaves

Traits	QTL[a]	Ch.	Left marker	Right marker	Guilin (24.18°N, 109.45°E)				Guiyang (26.35°N, 106.42°E)				Nanchang (28.38°N, 116.24°E)				Nanjing (31.95°N, 119.16°E)			
					Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]
FLL	qFLL1	1	RM5496	RM572	1	0.61	1.03	0.51	1	**5.50**	12.54	1.55	4	0.78	1.73	0.72	1	0.92	1.87	0.53
	qFLL2	2	RM1313	RM424	69	**4.37**	7.20	1.29	67	2.02	4.07	0.85	68	**4.60**	8.67	1.58	68	**2.85**	5.75	0.90
	qFLL3	3	RM8269	RM448	163	0.95	1.50	0.59	163	1.12	2.26	0.64	163	**3.14**	5.79	1.30	163	0.54	1.04	0.38
	qFLL9	9	RM8219	RM5688	1	2.34	4.06	0.98	1	1.89	3.72	0.82	1	1.02	1.81	0.73	1	**3.57**	7.18	1.01
	qFLL10	10	RM1146	RM228	37	**3.55**	7.47	1.32	25	1.07	2.05	0.62	25	0.69	1.21	0.61	42	0.00	0.01	0.03
SLL	qSLL1	1	RM488	RM212	84	2.04	4.20	1.18	75	1.25	4.51	1.21	79	**4.35**	11.44	2.04	79	1.55	4.38	1.11
	qSLL2.1	2	RM1313	RM424	66	1.21	2.39	0.88	67	**3.34**	6.92	1.49	65	**5.00**	9.40	1.83	63	0.03	0.06	0.12
	qSLL2.2	2	RM324	RM29	72	1.19	2.52	0.90	73	0.06	0.10	0.18	73	0.13	0.22	0.28	73	**3.04**	5.98	1.29
	qSLL2.3	2	RM6	RM425	132	1.26	2.48	−0.90	132	2.20	3.99	−1.13	132	2.45	4.25	−1.23	135	**3.62**	8.65	−1.55
	qSLL3	3	RM251	RM5748	57	**3.88**	9.69	1.97	54	**3.44**	8.16	1.80	56	1.37	2.79	1.11	56	1.67	3.70	1.13
	qSLL4	4	RM5473	RM131	126	**2.76**	7.29	1.53	125	1.57	3.64	1.08	129	0.96	2.00	0.85	127	1.10	2.68	0.86
TLL	qTLL1	1	RM212	RM1003	86	2.35	4.44	1.30	74	1.94	7.68	1.62	88	**3.17**	5.65	1.47	80	**2.73**	6.84	1.56
	qTLL2.1	2	RM5390	RM1313	65	0.75	1.46	0.74	65	1.22	2.50	0.92	65	**3.60**	6.62	1.56	63	1.06	1.88	0.81
	qTLL2.2	2	RM262	RM6	131	1.79	3.53	−1.15	122	2.19	8.10	−1.65	121	2.16	7.03	−1.61	123	**3.93**	13.66	−2.18
	qTLL3	3	RM251	RM5748	53	**3.65**	7.71	1.87	54	**3.85**	9.43	1.98	53	**2.86**	5.28	1.54	54	2.27	4.62	1.41
	qTLL4	4	RM5473	RM131	125	**3.27**	8.52	1.78	128	0.69	1.70	0.76	132	0.20	0.34	0.35	123	0.61	1.41	0.70
	qTLL7	7	RM180	RM214	25	0.49	0.99	0.61	25	0.36	0.78	0.51	25	0.04	0.08	0.17	25	**2.70**	5.39	1.37
	qTLL12	12	RM277	RM1246	52	0.80	1.49	−0.75	52	0.42	0.84	−0.53	51	**3.65**	6.30	−1.53	51	1.50	2.65	−0.96

[a]A peak was considered to be a QTL if its LOD profile peak was higher than the LOD threshold value in at least one environmental condition, [b]Chromosomal position (cM) of the LOD profile peak, [c]Numbers in bold indicate those LOD profile peak values higher than the threshold value, [d]Percentage of the phenotypic variation explained by the locus at the peak position on the LOD profile, [e]Additive effect of the identified QTL. Positive value indicates the IR24 allele increases the trait; negative value indicates the Asominori allele increases the trait.

and two located on chromosome 2 (Table 3). Of these, qTLL3 was significant in three environmental conditions, qTLL1 was significant in two environmental conditions, and the other five were significant in one environmental condition. Negative effects on traits were observed for qTLL2.2 and qTLL12 across the four environmental conditions. Therefore, IR24 alleles increased TLL at five of the seven TLL QTLs.

Nine QTLs were identified for FLW: one each on chromosomes 3, 4, 6, 8, 11, and 12, and three on chromosome 1 (Table 4). Of these, qFLW3 and qFLW4 were significant in four environmental conditions, qFLW8 was significant in three environmental conditions, qFLW1.3 and qFLW11 were significant in two environmental conditions, and the remaining four were significant in one environment. Both qFLW4 and qFLW12 had negative effects in all environmental conditions, meaning that IR24 alleles increased FLL at seven of the nine FLL QTLs. Nine QTLs were identified for SLL: one each on chromosomes 3, 4, 5, 8, 11, and 12, and three on chromosome 1 (Table 4). Of these, qSLW1.2 and qSLW11 were significant in three environmental conditions, qSLW4 was significant in two environmental conditions, and the other six were significant in one environment. Both qSLW4 and qSLW12 had negative effects in four environmental conditions, indicating that IR24 alleles increased SLL at seven of the nine SLL QTLs. Thirteen QTLs were identified for TLL: one each on chromosomes 4, 5, and 11, two each on chromosomes 3, 8, and 12, and four on chromosome 1 (Table 4). Of these, qTLW1.2 and qTLW12.2 were significant in three environmental conditions, qTLW11 was significant in two environmental conditions, and the other 10 were significant in one environment. The three QTLs qTLW4, qTLW8.1, and qTLW12.2 displayed negative effects. Alleles from IR24 increased TLL for 10 of the 13 TLL QTLs identified.

Confirmation of identified QTLs in SSSLs

Following *t*-test analysis between each SSSL and its corresponding background parent, we selected 15 SSSLs for use in this study to confirm the top three leaf trait QTLs identified in the RIL population. The same morphological traits associated with top three leaf size were measured in these SSSLs (i.e., FLL, FLW, SLL, SLW, TLL, and TLW). Each of the 15 SSSLs showed a significant difference in at least one leaf size-related trait. Significant differences between SSSLs and their respective parents regarding specific traits are shown in Table 5, along with the QTLs contained in the corresponding chromosomal segments. Compared to their parental lines, the four SSSLs ASL2, ISL1, ISL3, and ISL9 possessed significant differences in three leaf size-related traits and the four SSSLs ASL1, ASL5, ISL2, and ISL5 possessed significant differences in two traits. The remaining seven SSSLs displayed significant differences in one trait. Specifically, the chromosomal segments in ASL1, ASL2, and ASL5 had significant effects on two leaf length traits, and those in in ISL1, ISL2, and ISL3 had significant effects on two leaf width traits. Furthermore, the SSSL ISL9 showed significant differences in the three leaf width traits simultaneously compared to that in its corresponding parental line.

Here, an overlap between a QTL genomic region and the donor segment in a SSSL demonstrated was considered as QTL validation. In this study, 22 of the QTLs identified in the RIL population (approximately 45%) were confirmed by the 15 SSSLs (Table 5). For example, two out of five FLL QTLs identified in the RIL population were confirmed. Specifically, qFLL1 was confirmed in ASL1 and ISL1, and qFLL10 was confirmed in ISL8. Furthermore, three out of the six SLL QTLs and three out of the seven TLL QTLs were confirmed in SSSLs. Regarding leaf width traits, six out of the nine FLW QTLs, four out of the nine SLW QTLs, and four out of the thirteen TLW QTLs were confirmed in SSSLs (Table 5).

For comparison, QTL effect estimated using SSSLs and the average QTL effects in SSSLs across the four environmental conditions are also presented in Table 5. There were obvious differences observed between QTL effects estimated using either the RIL or SSSL; however, the tendency of each QTL effect was maintained in both instances, even for those QTLs with relatively small effects.

Identification of novel QTLs affecting leaf size

Locations of the 49 QTLs detailed in Tables 3 and 4 are depicted in a linkage map shown in Figure 2. The 22 QTLs confirmed in SSSLs are underlined. The 49 QTLs were located across 30 marker-delimited genomic regions in the 12 rice chromosomes. The top section of Table 6 details 10 such genomic regions that were found to contain two or more QTLs. Of these, the regions RM1313–RM424 and RM5473–RM131 contained QTLs for leaf length and the regions RM7187–RM317 and RM6948-RM281 contained QTLs for leaf width. The

Table 4. QTLs identified in the RIL population for width of top three leaves

Traits	QTL[a]	Ch.	Left marker	Right marker	Guilin (24.18°N, 109.45°E)				Guiyang (26.35°N, 106.42°E)				Nanchang (28.38°N, 116.24°E)				Nanjing (31.95°N, 119.16°E)			
					Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]
FLW	qFLW1.1	1	RM5496	RM572	8	0.87	2.05	0.03	4	**4.23**	7.89	0.06	5	2.11	4.45	0.04	6	1.43	2.47	0.03
	qFLW1.2	1	RM493	RM488	48	1.66	4.06	0.04	49	0.78	1.71	0.03	43	1.82	5.38	0.04	50	**3.94**	7.47	0.05
	qFLW1.3	1	RM1003	RM486	93	1.52	3.23	0.04	96	**3.07**	4.77	0.04	91	**3.08**	5.47	0.04	92	0.37	0.56	0.01
	qFLW3	3	RM251	RM5748	60	**5.56**	10.31	0.07	60	**5.74**	8.75	0.07	59	**4.99**	9.26	0.06	59	**4.38**	6.71	0.05
	qFLW4	4	RM7187	RM317	92	**5.74**	12.34	−0.07	97	**8.99**	14.45	−0.07	96	**7.35**	13.05	−0.06	94	**7.66**	11.09	−0.06
	qFLW6	6	RM340	RM439	110	0.94	1.72	0.03	110	1.80	2.56	0.03	107	1.40	2.14	0.03	110	**3.80**	5.31	0.04
	qFLW8	8	RM6948	RM281	96	**3.79**	7.23	0.05	94	**4.70**	7.03	0.05	93	2.47	4.04	0.03	97	**3.75**	5.65	0.04
	qFLW11	11	RM286	RM4	1	1.86	3.33	0.04	1	2.21	3.17	0.04	1	**3.68**	6.42	0.05	1	**4.81**	6.96	0.05
	qFLW12	12	RM3331	RM270	85	1.72	2.93	−0.03	82	**5.18**	8.90	−0.06	85	1.00	1.56	−0.02	79	1.22	1.91	−0.02
SLW	qSLW1.1	1	RM5496	RM572	1	1.11	2.39	0.03	3	**5.10**	8.73	0.05	3	1.68	3.59	0.03	1	1.74	3.27	0.03
	qSLW1.2	1	RM493	RM488	62	**3.31**	9.84	0.05	55	0.63	0.86	0.02	44	**2.71**	9.22	0.05	64	**4.59**	13.32	0.06
	qSLW1.3	1	RM1003	RM486	96	1.03	2.23	0.03	95	**3.77**	5.87	0.04	97	2.10	3.57	0.03	97	1.85	3.18	0.03
	qSLW3	3	RM251	RM5748	60	1.61	3.29	0.04	59	**6.36**	9.99	0.06	60	2.20	3.97	0.03	60	2.14	3.89	0.04
	qSLW4	4	RM241	RM7187	91	1.98	4.75	−0.04	92	**7.64**	12.15	−0.06	93	**3.84**	7.83	−0.04	93	2.14	4.10	−0.03
	qSLW5	5	RM430	RM163	50	1.18	2.46	0.03	53	0.91	1.18	0.02	49	**2.70**	4.90	0.03	49	0.70	1.23	0.02
	qSLW8	8	RM531	RM502	90	0.72	1.44	0.02	90	**3.89**	5.29	0.04	90	1.14	1.97	0.02	90	1.40	2.44	0.03
	qSLW11	11	RM286	RM4	2	1.96	4.41	0.04	5	**2.89**	4.58	0.04	1	**4.17**	8.15	0.05	1	**5.55**	11.12	0.06
	qSLW12	12	RM3331	RM270	85	2.31	4.63	−0.04	83	**8.33**	14.07	−0.06	85	2.14	3.75	−0.03	83	1.42	2.97	−0.03
TLW	qTLW1.1	1	RM5496	RM572	1	1.03	1.41	0.02	1	**4.32**	6.75	0.04	1	1.88	3.47	0.03	1	1.18	1.95	0.02
	qTLW1.2	1	RM493	RM488	49	1.16	2.20	0.03	53	**3.62**	6.40	0.04	45	**4.28**	13.37	0.05	63	**5.22**	13.40	0.06
	qTLW1.3	1	RM1003	RM486	96	**6.40**	9.95	0.05	97	1.41	1.94	0.02	97	2.17	3.68	0.03	97	1.73	2.61	0.03
	qTLW1.4	1	RM14	RM5410	151	0.24	0.31	0.01	151	**3.67**	5.41	0.04	151	0.18	0.33	0.01	150	0.14	0.21	0.01
	qTLW3.1	3	RM3467	RM7	34	0.20	0.27	0.01	35	**3.83**	6.45	0.04	34	1.28	2.35	0.02	34	0.99	1.64	0.02
	qTLW3.2	3	RM251	RM5748	60	**6.90**	10.05	0.06	60	1.34	1.93	0.02	60	0.30	0.54	0.01	60	2.56	4.23	0.04
	qTLW4	4	RM7187	RM317	95	1.11	1.42	−0.02	95	**4.21**	6.15	−0.04	95	1.90	3.24	−0.03	97	2.47	4.30	−0.03
	qTLW5	5	RM5558	RM6313	91	**3.40**	8.22	0.05	81	0.27	0.92	0.02	77	1.28	4.45	0.03	80	1.17	4.35	0.03
	qTLW8.1	8	RM72	RM331	45	0.64	0.89	−0.02	38	0.30	0.43	−0.01	38	0.52	0.93	−0.01	40	**6.05**	11.43	−0.05
	qTLW8.2	8	RM6948	RM281	97	**3.85**	6.24	0.04	98	1.31	2.01	0.02	98	1.25	2.44	0.02	96	0.99	1.61	0.02

(Continued)

Table 4. Continued

Traits	QTL[a]	Ch.	Left marker	Right marker	Guilin (24.18°N, 109.45°E)				Guiyang (26.35°N, 106.42°E)				Nanchang (28.38°N, 116.24°E)				Nanjing (31.95°N, 119.16°E)			
					Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]	Pos[b]	LOD[c]	PVE (%)[d]	Add[e]
	qTLW11	11	RM286	RM4	8	1.06	1.57	0.02	2	1.95	3.05	0.03	1	**2.65**	5.11	0.03	1	**4.71**	8.21	0.05
	qTLW12.1	12	RM6296	RM7102	17	**2.77**	3.69	0.03	43	0.37	0.55	0.01	37	0.67	1.80	0.02	43	0.99	1.63	0.02
	qTLW12.2	12	RM3331	RM270	85	**12.34**	18.76	−0.08	83	**6.81**	12.04	−0.06	85	**4.53**	8.55	−0.04	83	0.66	1.21	−0.02

[a]A peak was considered to be a QTL if its LOD profile peak was higher than the LOD threshold value in at least one environmental condition, [b]Chromosomal position (cM) of the LOD profile peak, [c]Numbers in bold indicate those LOD profile peak values higher than the threshold value, [d]Percentage of the phenotypic variation explained by the locus at the peak position on the LOD profile, [e]Additive effect of the identified QTL. Positive value indicates the IR24 allele increases the trait; negative value indicates the Asominori allele increases the trait.

four regions RM493–RM488, RM1003–RM486, RM286–RM4, and RM3331–RM270 contained QTLs for width of each of the top three leaves. The remaining two regions contained QTLs for both leaf length and width. These QTL mapping results were consistent with the data presented in Table 1, in that there is a high correlation between leaf lengths and between leaf widths, and a relatively low correlation between leaf length and width.

Using 71 RILs derived from a comparable cross between Asominori (*japonica*) and IR24 (*indica*) (Tsunematsu et al. 1996), Zhang et al. (2008) identified 19 QTLs for the length and width of the flag and second leaves. Furthermore, these QTLs were investigated in two environmental conditions, with one experimental replication performed in Hainan, China, and two further replications performed in Miyazaki, Japan. Following the association of these QTLs with the physical positions of markers, the three previously reported QTLs qFLL-1a, qFLW-1a, and qSLW-1a were localized to a genomic region delimited by markers RM5496 and RM572, which further harbored qFLL1, qFLW1.1, qSLW1.1, and qTLW1.1 identified in this study. Furthermore, the previously described QTL qSLL-4 was localized to the RM5473–RM131 region, which also harbored qSLL4 and qTLL4 identified here. Finally, the previously described QTLs qFLL-1b and qSLL-1 were localized between RM488 and RM212, corresponding to a genomic region that also contained qSLL1 characterized above (Table 6).

In previous studies, several genes for leaf length and width in rice have been cloned. Four such reported genes were localized within four marker-delimited genomic regions following their association with the physical positions of simple sequence repeat (SSR) markers (Table 6). *NAL9/VYL* (Os03g0411500) was localized alongside the QTLs qSLL3, qTLL3, qFLW3, qSLW3, and qTLW3.2 identified in this study in the RM251–RM5748 region on chromosome 3. *NAL1/qFLW4/LSCHL4/qTSN4* (Os04g0615000) was localized alongside the QTLs qSLL4 and qTLL4 in the RM5473–RM131 region on chromosome 4. *NAL3* (Os12g0101600) was localized alongside the QTL qTLW12 in the RM6296–RM7102 region on chromosome 12. *NRL1/OsCSLD4/DNL1* (Os12g0555600) was localized alongside the QTLs qFLW12, qSLW12, and qTLW12 in the RM3331–RM270 region on chromosomal 12. No reported leaf size-associated genes were identified in the other 14 genomic regions containing QTLs identified here (Table 6).

Table 5. Significance test between SSSLs and the background parent, and details of the QTLs identified in the RIL population located on donor segments in SSSLs

SSSL	Chr.	Donor segment[a]	Traits and effect in SSSL[b]	QTL identified in RIL[c]
ASL1	1	RM5496-RM488	FLL (1.48**), SLL (1.76*),	qFLL1 (0.83), qSLL1 (1.39)
ASL2	1	RM212-RM1003	SLL (1.23**), TLL (1.41**), TLW (0.02**)	qSLL1 (1.39), qTLL1 (1.49), qTLW1.3 (0.03)
ASL3	2	RM6-RM425	SLL (−1.60*)	qSLL2.3 (-1.20)
ASL4	3	RM3131-RM7	TLW (0.35*),	qTLW3.1 (0.02)
ASL5	3	RM7-RM411	SLL (2.14*), TLL (1.30**)	qSLL3 (1.50), qTLL3 (1.70)
ASL6	12	RM19-RM270	FLW (−0.05*)	qFLW12 (-0.03)
ISL1	1	RM5496-RM312	FLL (0.05*), SLW (0.45**), TLW (0.35*)	qFLL1 (0.83), qSLW1.1 (0.04), qTLW1.1 (0.03)
ISL2	1	RM493-RM488	FLW (0.05**), SLW (0.45**)	qFLW1.1 (0.04), qSLW1.2 (0.05)
ISL3	1	RM212-RM5410	TLL (1.92**), FLW (0.06*), SLW (0.03**)	qTLL1 (1.49), qFLW1.2 (0.04), qSLW1.3 (0.03)
ISL4	2	RM262-RM425	TLL (−2.31*)	qTLL2.2 (-1.65)
ISL5	3	RM523-RM251	TLL (2.16**), TLW (0.02**)	qTLL3 (1.70), qTLW3.1 (0.02)
ISL6	4	RM252-RM317	FLW (−0.75**)	qFLW4 (-0.07)
ISL7	6	RM340-RM345	FLW (0.03**)	qFLW6 (0.03)
ISL8	10	RM228-RM6824	FLL (1.10**)	qFLL10 (0.65)
ISL9	11	RM286-RM6288	FLW (0.07*), SLW (0.06*), TLW (0.55*)	qFLW11 (0.05), qSLW11 (0.05), qTLW11 (0.03)

Significant differences from *t*-test are indicated at $^{*}P = 0.05$ and $^{**}P = 0.01$. [a]Donor segments of ASL01–ASL06 were from IR24, and donor segments of ISL01–ISL09 were from Asominori, [b]Values in parentheses are the additive effects in SSSLs. For ISL1–ISL9, the effect is equal to half the difference between SSSL and IR24. For ASL1–ASL6, the effect is equal to half the difference between SSSL and Asominori. A positive value indicates the IR24 allele increases the trait; a negative value indicates the Asominori allele increases the trait. [c]Value in parentheses is the average QTL effect across the four environmental conditions. A positive value indicates the IR24 allele increases the trait; a negative value indicates the Asominori allele increases the trait. FLL, flag leaf length; FLW, flag leaf width; SLL, second leaf length; SLW, second leaf width; TLL, third leaf length; TLW, third leaf width.

Using sequencing data corresponding to genomic sequences of Os03g0411500, Os04g0615000, Os12g0101600, and Os12g0555600, we found that no nonsynonymous substitutions were present in Os03g0411500, Os04g0615000, and Os12g0101600 in the Asominori and IR24 parents. Therefore, *NAL9/VYL* (Os03g0411500), *NAL1/qFLW4/LSCHL4/qTSN4* (Os04g0615000), and *NAL3* (Os12g0101600) were not considered to be causal genes affecting leaf size. In the Os12g0555600 genomic sequence, sequence comparisons between Asominori and IR24 revealed a T-to-C substitution that resembled the *nrl1-1* mutation previously reported by Hu et al. (2010). Thus, *NRL1/OsCSLD4/DNL1* (Os12g0555600) is a likely candidate for the causal gene in the RM3331–RM270 region on chromosome 12 that affects top three leaf width.

Seventeen genomic regions either had at least two QTLs (Table 6, top section) or a single QTL (Table 6, bottom section) that was confirmed in SSSLs. Thus, these QTLs were considered as chromosomal regions that may harbor novel genes that control leaf length or width, and eight of these (indicated with a star symbol in Table 6) merit further investigation. Furthermore, six of these chromosomal regions had two or more QTLs that were confirmed in SSSLs, and two contained QTLs that were both described in previous studies and confirmed here in SSSLs.

DISCUSSION

The top three leaves of rice plants are regarded as the major source of photosynthetic activity, and are thus associated with yield-related traits. Large-scale measurement of leaf size-related traits in the field is time

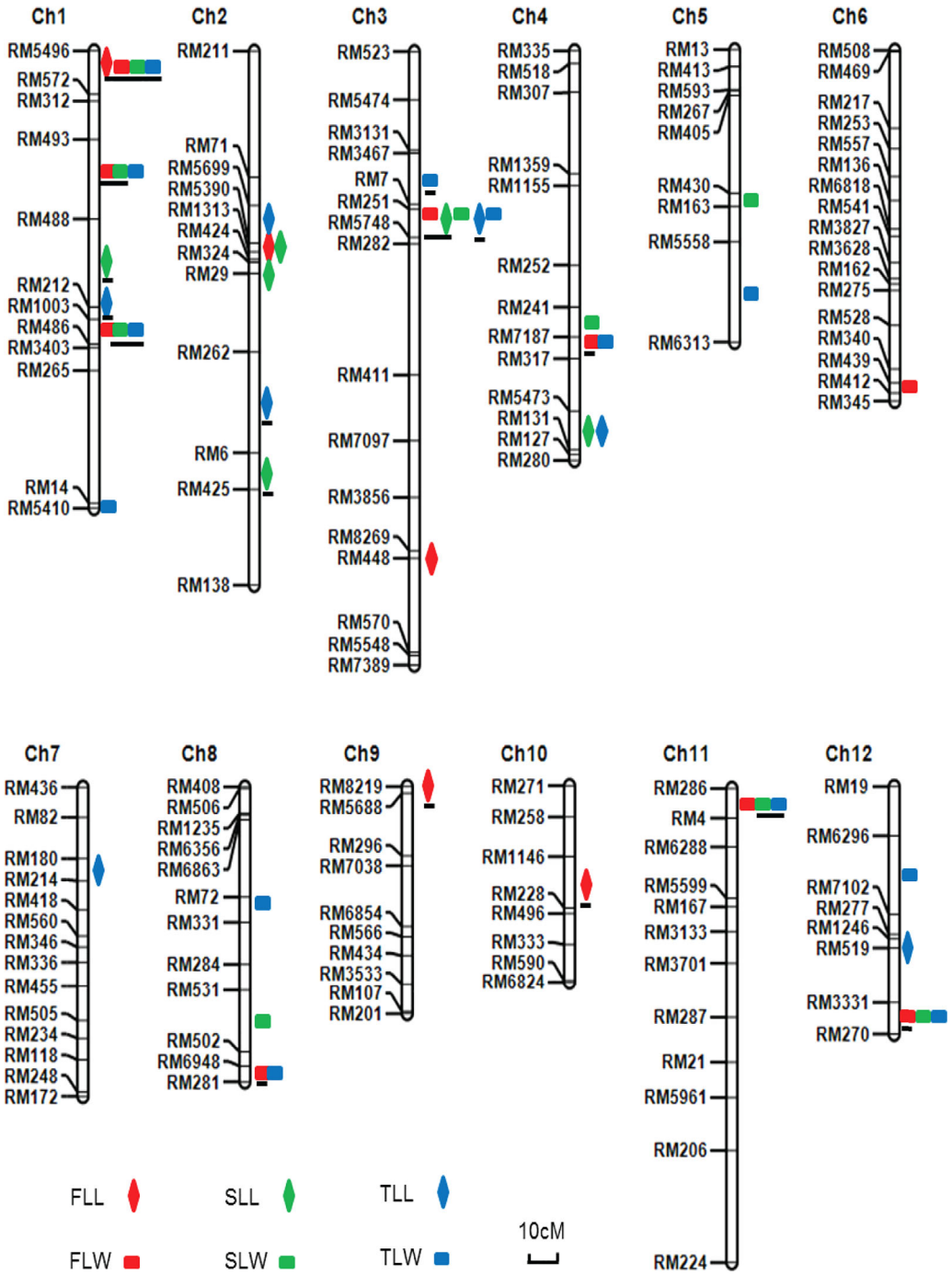

Figure 2. Linkage map locations of the QTLs identified in the RIL population for the six morphological traits
QTLs that were confirmed in SSSLs are underlined. FLL, flag leaf length; FLW, flag leaf width; SLL, second leaf length; SLW, second leaf width; TLL, third leaf length; TLW, third leaf width.

consuming and labor intensive. Photogrammetry and image processing techniques have been developed as a way to measure leaf-related traits. However, this technology remains expensive and may not be suitable for large-scale plant phenotyping in the field (An et al. 2016). In this study, we performed extensive manual length and width measurements of the top three leaves of rice plants grown across four environmental conditions, with two experimental replicates. Leaf area, which can be calculated using leaf length and width

Table 6. QTLs and reported genes/QTLs within a number of genomic regions delimited by select markers

Marker interval	Chromosome	Reported gene/QTL	QTL identified in this study				
RM5496-RM572★	1	qFLL-1a, qFLW-1a, qSLW-1a	**qFLL1**	**qFLW1.1**	**qSLW1.1**	**qTLW1.1**	
RM493-RM488★	1		**qFLW1.2**	**qSLW1.2**	qTLW1.2		
RM1003-RM486★	1		qFLW1.3	**qSLW1.3**	**qTLW1.3**		
RM1313-RM424	2		qFLL2	qSLL2.1			
RM251-RM5748★	3	*NAL9/VYL*	**qSLL3**	**qTLL3**	qFLW3	qSLW3	qTLW3.2
RM5473-RM131★	4	*NAL1/qFLW4/LSCHL4/* qTSN4, qSLL-4	qSLL4	qTLL4			
RM7187-RM317★	4		**qFLW4**	qTLW4			
RM6948-RM281	8		qFLW8	qTLW8.2			
RM286-RM4★	11		**qFLW11**	**qSLW11**	**qTLW11**		
RM3331-RM270	12	***NRL1/OsCSLD4/DNL1***	**qFLW12**	qSLW12	qTLW12.2		
RM488-RM212★	1	qFLL-1b, qSLL-1	**qSLL1**				
RM212-RM1003	1		**qTLL1**				
RM6-RM425	2		**qSLL2.3**				
RM262-RM6	2		**qTLL2.2**				
RM3467-RM7	3		**qTLW3.1**				
RM340-RM439	6		**qFLW6**				
RM1146-RM228	10		**qFLL10**				
RM6296-RM7102	12	*NAL3*	qTLW12				

The top section details genomic regions harboring two or more of the QTLs identified in this study and the bottom section details those with one. In column 3, reported genes are indicated in italics, and causal genes are indicated in bold; QTLs are those described by Zhang et al. (2008). In column 4, QTLs that were confirmed in SSSLs are indicated in bold.

measurements, is a further indicator of leaf size. However, although a number of past studies have used leaf area in genetic analyses (Zhang et al. 2008; Jia et al. 2016), it was shown that the use of mathematically derived traits increased gene number and false discovery rate, caused higher-order of gene interaction, and reduced QTL detection power (Wang et al. 2012b). Therefore, in this study, rather than leaf area, we opted to use direct measurements of leaf length and width as indicators of top three leaf size.

For the six measured top three leaf traits, a total of 49 QTLs were identified in the RIL population derived from a cross between a *japonica* variety and an *indica* variety. The phenotypic variation explained (PVE) by each QTL did not exceed 20%. The PVE for 13 QTLs was higher than 10%, and these QTLs displayed various stabilities across the different environmental conditions. Two common QTLs were detected in each of the four environmental conditions, whereas seven common QTLs were detected with the combination of either two or three environmental conditions, and 33 QTLs were detected when considering a single environmental condition. It should be noted that QTLs not detected across the four environmental conditions displayed non-significant peaks in each environmental condition, and the additive effects displayed the same tendency as those with significant peaks. This illustrates the importance of including multiple environmental conditions in QTL studies, and may also explain the high genotypic variances and low GE interaction observed here.

Of the 30 marker-delimited genomic regions that contained the QTLs identified in the RIL population, two regions contained QTLs affecting both leaf length and width, three regions contained QTLs affecting the lengths of two or three leaves, and eight regions contained QTLs affecting the widths of two or three leaves. In genetics, correlation between traits can be

caused either by the pleiotropic effect of one QTL or by two closely linked QTLs. In this study, we believe correlation between the six traits is more likely caused by pleiotropic effects than by linkage effects. QTLs observed in the same genomic region are likely to be the same QTL. Therefore, lengths of the three top leaves may be controlled by three common QTLs, and widths of the top three leaves may be controlled by eight common QTLs. By contrast, only two common QTLs may control both leaf length and leaf width. This hypothesis is consistent with the observed high correlation among top three leaf lengths and among top three leaf widths, and the relatively low correlation between leaf length and leaf width.

Few common QTLs and a low correlation between leaf length and width have also been reported in maize (*Zea mays*; Tian et al. 2011), wheat (*Triticum aestivum*; Jia et al. 2013; Wu et al. 2016), and barley (*Hordeum vulgare*; Xue et al. 2008). Leaf length and width tend to have considerably different genetic regulation. Leaf development consists of three aspects: thickness and lateral and longitudinal expansion (Sanders and Wyatt 2009). Final leaf size is mainly determined by the extent of lateral and longitudinal expansion. A previous study in *Arabidopsis* indicated that cell expansion along the axis (leaf length) and perpendicular to the axis (leaf width) were independent processes (Czesnick and Lenhard 2015). Therefore, in the RIL population used in this study, we propose that the length of the top three leaves are controlled by distinct genes, but the width of the top three leaves is likely influenced by a number of common genes.

In this study, the fidelity of most of the QTLs is supported by the large mapping population size, the high-level precision phenotyping of the six traits, the high detection power of the mapping method, and the consistent tendency of additive effects across the four environmental conditions. Following QTL confirmation in SSSLs and comparisons between previously reported QTLs and the parental sequences, we identified eight novel marker-delimited genomic regions associated with the size of the top three leaves. These genomic regions contain uncharacterized ORFs that have not been previously associated with leaf size. Therefore, the QTLs and their linked markers described in this study can be confidently used for marker-assisted selection to accelerate the breeding of rice plants with improved grain yield. Furthermore, the eight novel genomic regions can be used for fine mapping and cloning of novel genes controlling leaf size.

MATERIALS AND METHODS

Genetic materials

Between 2007 and 2012, we created a population of 215 F_{12} RILs for use in this study, developed by the single-seed descent method following a cross between *Oryza sativa* ssp. *japonica* cv. Asominori and *Oryza sativa* ssp. *indica* cv. IR24. Genotypic information of the RIL population has been reported in Yin et al. (2015). For convenience, key information is summarized here. The 215 RILs were genotyped using 143 polymorphic SSR markers that had been used in QTL mapping of grain shape-related traits (Yin et al. 2015). No heterozygous segments were found. The Asominori genomic contribution to each RIL was approximately 50%, but with large variation from 10%–90%. Average frequency of Asominori alleles was estimated at 0.4722. The genetic linkage map constructed using the RIL population had a total length of 1,474.31 cm, and the 143 markers were almost evenly distributed among the 12 rice chromosomes.

In additions to the RIL population, backcrosses were performed between existing CSSLs and their corresponding background parents, and donor segments were selected using marker information in segregating generations. This resulted in the isolation of 64 SSSLs in the Asominori background and 57 SSSLs in the IR24 background. SSSLs were genotyped by the same set of SSR markers as described above. As in the RIL population, no heterozygous segments were found. Based on the difference in leaf size between each SSSL and its background parent, 15 SSSLs were selected and used to confirm the QTLs identified in the RIL population. Genotypic information for the 15 SSSLs is shown in Figure 3. ASL01–ASL06 have donor segments from IR24 on chromosomes 1, 2, 3, and 12. ISL01–ISL09 have donor segments from Asominori on chromosomes 1, 2, 3, 4, 6, 10, and 11. ASL01–ASL06 have 94.9%–98.88% genomic contributions from Asominori. ISL01–ISL09 have 94.49%–98.77% genomic contributions from IR24.

Field experiments and trait measurement

The 215 RILs, reciprocal SSSLs, and the two parental lines were grown from May to November in 2013 across

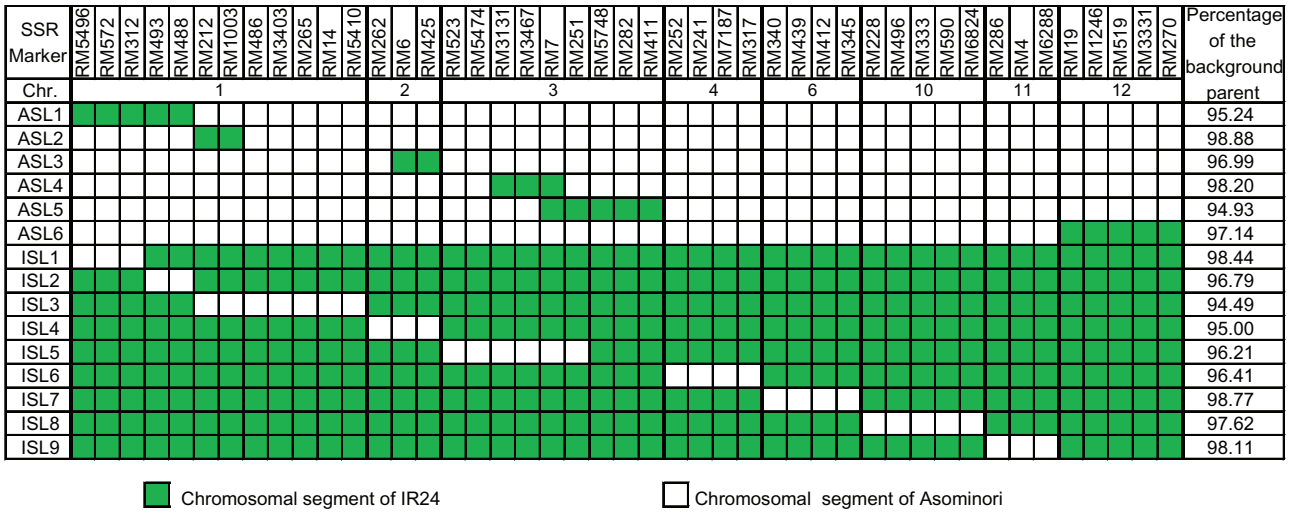

Figure 3. Graphical representation of the genotypes of the 15 SSSLs used to confirm QTLs identified in the RIL population

Six SSSLs in the Asominori background were denoted ASL01–ASL06, and nine SSSLs in the IR24 background were denoted ISL1–ISL9. Donor chromosomal segments in ASL1–ASL6 originated from IR24, and donor chromosomal segments in ISL1–ISL9 originated from Asominori. White and grey colors represent chromosomal segments of Asominori and IR24, respectively. Markers that were conserved among the 15 SSSLs are not shown.

four geologically and ecologically diverse environmental regions in China, namely Guilin (24.18°N, 109.45°E), Guiyang (26.35°N, 106.42°E), Nanchang (28.38°N, 116.24°E), and Nanjing (31.95°N, 119.16°E). Rice is the major cultivated and consumed crop in all of these regions. Checks were inserted every 10 lines. Checks for RILs were the two parental lines, whereas checks for SSSLs were the background parent. A randomized complete block design was applied with two experimental replicates performed in each location. Each entry plot consisted of four rows and each row was cultivated with ten individual plants. Field management during the growing season was according to normal agricultural practices. At full heading stage, three representative individual plants were selected from the central part in each plot. Manual trait measurements were performed on the top three leaves of the main stem.

Phenotypic data analysis and QTL mapping

ANOVA combining the four environmental conditions and heritability estimation were conducted as previously described (Yin et al. 2015). For QTL detection in the RIL population, we used inclusive composite interval mapping, known as ICIM (Li et al. 2007; Wang 2009). The LOD threshold value was obtained by a total of 6,000 permutation tests on the six traits and a genome-wide type I error rate of 0.05. These analyses were completed using the integrated QTL IciMapping software Version 4.1 (Meng et al. 2015). For QTL confirmation in SSSLs, *t*-tests performed in MS Excel were used to estimate the significance probability. SSSL validation of a QTL was considered when the significance probability was below 0.05.

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AUTHOR CONTRIBUTIONS

C.Y. developed the genetic population, conducted the genotyping and field experiments, and drafted the manuscript; C.Y. and H.L. analyzed the data; Z.W., Z.Z., S.L., L.C., X.L., Y.T., J.M., L.X., S.Z., D.Z., and D.L. conducted the field experiments and collected the phenotypic data; J.Wan supervised the lab and field experiments; J.Wang designed the research and finalized the manuscript.

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