J. Plant Biol. (2015) 58:402-410
DOI 10.1007/s12374-015-0402-4

ORIGINAL ARTICLE

Genetic Dissection of Leaf-related Traits using 156 Chromosomal Segment Substitution Lines

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Received: Augut 18, 2015 / Accepted: October 9, 2015

Abstract A two-line super-hybrid rice (*Oryza sativa* L.) variety [Liangyoupei9 (LYP9)] demonstrated superiority over its both parents, viz. elite inbred lines 93-11 and Pei-ai64S (PA64S), as well as other conventional hybrids, and had long been exploited in China. However, the genetic basis of its leaf-related traits, supposed to be an important component for yield potential, remains elusive. Here, initially a set of chromosome segment substitution lines (CSSLs) was constructed, in which the genome of Pei-ai64S has been introgressed into the background of 93-11. This set was developed by marker aided selection, based on 123 polymorphic SSR markers. The introgressed chromosomal segments presented in the 156 CSSLs covered 96.46% of Pei-ai64S genome. Afterwards, the CSSLs were deployed to assess the genetic basis of leaf size (length and width) and chlorophyll content of top three leaves across five different environments. The CSSLs showed transgressive segregation for all of the traits, and significant correlations were detected among most of the traits. A total of 27 quantitative trait loci (QTL) were identified on ten chromosomes, and three QTL cluster affecting related traits were found on chromosome 3, 6, and 8, respectively. Remarkably, two key QTLs, *qALW3-1* and *qALW3-2*, both controlling the antepenultimate leaf width, were identified in all five environments, and their effect were further validated by CSSLs harboring the two QTL alleles. Our results indicate that developing CSSLs is a powerful tool for genetic dissection of quantitative traits. Meanwhile, the QTLs controlling leaf-related traits uncovered here provide useful information for marker-assisted selection in improving the performance of leaf morphology and photosynthetic ability.

Keywords: Chlorophyll content, Chromosome segment substitution line (CSSL), Leaf length, Leaf width, Quantitative trait loci (QTL), Rice (*Oryza sativa* L.)

Introduction

The rapid increasing population has spawned tremendous concerns about increasing crop yield. Historically, dramatic increase in crop yield, known as the Green Revolution, largely resulted from the development of genetically improved high-yielding varieties (Hedden 2003). Among the genetic approaches used to improve yield potential, "ideal-type breeding" was used to modify plant architecture (morphology). So far, plant breeders have harnessed a lot of natural variations in cultivars to modify plant architecture (Hedden 2003).

Rice is a staple food crop in most of Asia and part of Africa. Several studies have indicated that the top three leaves contribute more than 80% of the total assimilates in the grains (Tomoshiro et al. 1983; Gladun and Karpov 1993a, 1993b). Leaf size (length and width) is an essential component for leaf architecture, influencing canopy morphology and sunshine interception ability and, as a result, overall yield (Tian et al. 2011). Previous studies showed leaf size was inherited quantitatively and influenced greatly by the environments (Farooq et al. 2010; Bian et al. 2014; Lim et al. 2014; Yang et al. 2015). With the availability of molecular markers and genetic maps, a number of quantitative trait loci (QTL) for leaf morphology have been detected in rice (Mei et al. 2003; Yue et al. 2006; Xue et al. 2008; Farooq et al.

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2010; Bian et al. 2014; Lim et al. 2014). For instance, one QTL for flag leaf size was mapped to chromosome 4 using a doubled haploid (DH) population derived from a cross between Azucena and IR64 (Yan et al. 1999). With a set of recombinant inbred lines (RILs) from a cross between Lemont and Teqing and two backcross (BC) populations, two QTLs for flag leaf length and one for flag leaf width were detected (Mei et al. 2003). A major QTL on chromosome 1 accounting for a large proportion of the genetic variation in flag leaf size was detected in a BC inbred line (BIL) population derived from Zhenshan 97 and 93-11(Wang et al. 2012). Other two QTL, viz. *qFL1* associated with flag leaf length and *qFLW4* related with flag leaf width, have been fine mapped (Wang et al. 2011; Chen et al. 2012). *Nal1* and *Nal7*, both associated with flag leaf width, have been cloned (Fujino et al. 2008; Qi et al. 2008). Recently, with the aid of a high-throughput leaf scorer, a total of 73 new loci, together with nine known ones, were detected for traits associated with leaf size on a panel of 533 rice accessions (Yang et al. 2015).

Apart from leaf size, chlorophyll content directly affects photosynthetic ability and thus is another important physiological trait affecting yield in rice. A few reports regarding genetic dissection of chlorophyll content in rice leaves were carried out (Dong et al. 2007; Zuo et al. 2007; Yang et al. 2008; Takai et al. 2010; Jiang et al. 2012; Jiang et al. 2014). Dong et al. (2007) identified seven QTL for leaf chlorophyll content at tillering and heading stages. Zuo et al. (2007) detected 10 QTLs responsive for chlorophyll content before heading. Zhang et al (2014) found a major QTL (*qLSCHL4*) related to both flag leaf shape and chlorophyll contents in RILs constructed by 93-11 and Nipponbare. Additionally, Wang et al (2015) demonstrated that *Grain number, plant height, and heading date7* (*Ghd7*) was a major locus for natural variation of chlorophyll content at the heading stage. Although these studies have contributed significantly to our understanding of the genetic control of leaf traits, there may be additional, unknown genes/ QTLs controlling leaf development and photosynthetic capability, especially in elite rice varieties.

Chromosome segment substitution lines (CSSLs), in which each line carries a single or a few defined chromosome segment of donor genome and has a pure genetic background from a recurrent genotype, is an approach to conduct QTL mapping in order to improve the mapping precision (Nadeau and Frankel 2000). A set of CSSLs, where 93-11 was used as background (or called recipient) parent and Pei-Ai 64S (PA64S) as donor parent, was developed in the present study. 93-11 is an elite *indica* restoring line in China and has been whole-genome sequenced in 2002 (Yu et al. 2002). PA64S is a thermo-sensitive genic male sterile line carrying wide compatibility genes, and possesses three quarter of *indica* genetic background and one quarter of gene introgressions from *japonica* and *javanica* accessions. A pioneer two-line rice hybrid, Liangyoupei9 (LYP9), deriving from crossing of PA64S and 93-11, produced 20% to 30% higher yields per hectare than their parents and other hybrid or inbred cultivars. Hence it has long been deployed in rice breeding of China to increase yield since released in 2001 (Fig. 1, Yuan 2003; Cheng et al. 2007). However, genetic mechanisms regulating leaf-related traits in LYP9 remain elusive.

Fig. 1. Plant architecture of 93-11, PA64S and their F1 hybrid variety Liangyoupei9.

The present study was aimed to identify QTLs associated with leaf size and chlorophyll content by means of LYP9-derived CSSLs. Clarifying genetic architecture of the traits will speed molecular manipulation for future breeding material improvement.

Results

Features of the CSSLs

As shown in Supplementary Fig. 1 and Table 2, substituted chromosome segments in the CSSLs covered most of 12 chromosomes except two small gaps at the distal end of the short arm of chromosome 10 (defined by SSR markers RM5095, RM222, RM244 and RM311, respectively) and long arm of chromosome 12 (defined by SSR markers RM7102, RM3331, RM235 and RM12, respectively). The average percentage of background genome segment was 97.61%, with a range from 96.6% for chromosome 9 and 11 to 98.3% for chromosome 2. Hence each CSSL could be deemed as near-isogenic lines (NILs) of 93-11. Totally 124 CSSLs contained less than three introgressed chromosome segments, among which 98 lines were singletons. Based on

Table 1. Characteristics of five environments where the chromosome segment substitute lines were grown

Code	Cropping location	Cropping season	Average daily air temperature (°C)	Photoperiod (sunlight/daytime, hour)	Rainfall (mm/month)
E1	Lingshui, Hainan, China, N 18.2, E 108.9	Dec, 2009-May, 2010	22.6	6.4/12.1	78.9
E2	Nanjing, Jiangsu, China, N 31.2, E 118.4	May-Oct, 2010	23.8	6.6/15.0	129.4
E3	Lingshui, Hainan, China, N 18.2, E 108.9	Dec, 2011-May, 2011	22.5	6.5/12.4	80.9
E4	Nanjing, Jiangsu, China, N 31.2, E 118.4	May-Oct, 2011	24.8	6.6/14.9	125.4
E5	Lingshui, Hainan, China, N 18.2, E 108.9	Dec, 2012-May, 2012	22.4	6.5/12.2	85.6

Table 2. Parameters of the chromosome segment substitution lines

Chr. No	Length of chromosome	Average length of substituted segment	Total length of substituted segment	Coverage length	Percentage of coverage (%)
1	181.80	34.72	347.20	181.80	100.00
2	157.90	30.95	495.20	157.90	100.00
3	166.40	24.11	217.00	166.40	100.00
4	129.60	17.99	179.90	129.60	100.00
5	122.00	28.11	196.80	122.00	100.00
6	124.40	28.09	252.80	115.90	93.17
7	118.60	19.51	175.60	118.60	100.00
8	121.20	20.19	161.50	108.30	91.36
9	93.50	26.45	158.70	93.50	100.00
10	83.80	20.22	101.10	72.80	92.87
11	117.90	18.13	145.00	117.90	100.00
12	109.20	22.21	155.50	96.20	90.13
Overall average	127.19	24.22	215.53	123.41	96.46

the recombinant breakpoint occurred between two adjacent markers, the target chromosomal segments substituted from PA64S in each CSSL ranged from a minimum 1.1 cM to a maximum 53 cM, with an average of 27.1 cM. The percentages of donor segment to whole genome varied from 0.1% to 14.1% in the CSSLs, with 91% of the total introgression lines lower than 5%. The overall donor coverage percentage was 96.46%.

Variation of Phenotypic Traits

Table 3 showed the phenotypic variation of the CSSLs and their parents for nine leaf traits across five environments from 2010 to 2012. Significant differences were observed on all the traits between the parents. For example, 93-11 had higher phenotypic values than PA64S for seven traits including flag leaf length (FLL), flag leaf width (FLW), penultimate leaf length (PLL), penultimate leaf width (PLW), antepenultimate leaf length (ALL), antepenultimate leaf width (ALW) and flag leaf chlorophyll (FLC), but lower for penultimate leaf chlorophyll content (PLC) and antepenultimate leaf chlorophyll content (ALC) in five environments. Meanwhile, the CSSLs showed continuous distribution with transgressive segregation for these traits. The mean and variation of the CSSLs both remained largely consistent for each leaf trait in five environments, except that ALL showed a much greater variation. In addition, the ANOVA indicated that variances among the genotypes (the CSSLs), the five environments, and the Genotype × Environment interactions were significant for all the traits. The environmental variances accounted for over 60% of the total phenotypic variation for nine leaf traits except FLW and PLC (Table 4).

Correlations

All leaf size traits showed significant correlations in the CSSLs (Table 5), with the highest correlation between FLL and PLL ($r = 0.82^{**}$). Similarly, highly significant positive correlations were observed between any two traits about leaf chlorophyll content among FLC, PLC, and ALC, in which the strongest correlation was observed between FLC and ALC ($r = 0.78^{**}$) and the lowest correlation between PLC and ALC ($r = 0.49^{**}$). No or weak significant positive correlations were observed between leaf size and chlorophyll content traits except those between FLC and ALL ($r = 0.35^{*}$), as well as FLC and ALW ($r = 0.34^{*}$).

QTL Analysis

A total of 27 QTLs influencing nine leaf-related traits were identified and mapped on ten chromosomes except 9 and 11,

Table 3. Summary statistics of phenotypic performance of the chromosome segment substitution lines (CSSLs) population and its parents for eight leaf traits in five environments

Traits[a] and environments[b]	Parents		The CSSLs				
	93-11	PA64S	Mean	SE[c]	Min	Max	CV[d]
FLL							
E1	25	23	27.1	4.1	19.0	45.0	16.8
E2	26	24	27.3	4.0	19.0	40.0	16.0
E3	28	26	26.9	4.1	18.0	39.0	16.9
E4	20	18	27.0	4.2	18.0	38.0	17.5
E5	26	24	27.5	4.3	19.0	43.0	18.2
FLW							
E1	1.8	1.4	2.1	0.1	1.6	2.5	0.02
E2	1.8	1.4	2.0	0.1	1.8	2.4	0.02
E3	2	1.6	2.0	0.1	1.7	2.4	0.02
E4	1.8	1.4	2.0	0.1	1.6	2.3	0.02
E5	1.9	1.5	2.1	0.2	1.7	2.5	0.03
PLL							
E1	32	30.5	35.8	5.0	25.0	50.0	25.1
E2	31	29.5	35.8	5.6	24.0	57.0	30.9
E3	42	40.5	35.8	5.2	22.0	51.0	27.4
E4	28	26.5	35.8	5.8	23.0	52.0	33.1
E5	34	32.5	36.0	5.5	22.0	50.0	30.7
PLW							
E1	1.8	1.7	1.9	0.1	1.6	2.4	0.02
E2	1.6	1.5	1.9	0.1	1.6	2.3	0.01
E3	1.8	1.7	1.9	0.1	1.6	2.2	0.02
E4	1.8	1.7	1.9	0.1	1.5	2.2	0.02
E5	1.9	1.8	1.9	0.1	1.6	2.3	0.02
ALL							
E1	42	41.4	48.8	7.1	1.9	63.0	51.0
E2	47	46.4	49.3	7.2	1.9	65.0	52.5
E3	54	53.4	49.0	7.1	1.8	65.0	50.1
E4	42	41.4	49.2	7.5	1.6	64.0	56.2
E5	47	46.4	49.2	7.5	1.8	66.0	56.1
ALW							
E1	1.7	1.5	2.1	4.0	1.3	52.0	16.1
E2	1.5	1.3	2.1	4.4	1.5	57.0	19.4
E3	1.6	1.4	2.1	3.6	0.7	47.0	13.1
E4	1.6	1.4	2.1	4.2	1.4	54.0	17.4
E5	1.7	1.5	2.2	4.6	1.5	59.0	20.9
FLC							
E1	39.9	37.4	40.4	3.4	30.4	71.2	11.3
E2	36.9	34.4	34.7	3.9	25.0	43.6	15.5
E3	37	34.5	33.2	3.3	21.7	40.6	10.7
E4	38	35.5	36.3	2.7	27.5	44.4	7.0
E5	37	34.5	37.8	2.3	31.2	47.9	5.2
PLC							
E1	48	48.6	36.1	3.5	25.2	48.0	12.5
E2	35.2	36.2	34.7	4.0	23.9	44.9	15.7
E3	47	48.2	28.5	3.7	19.6	39.1	13.5
E4	46	47	36.4	2.6	28.2	42.0	6.6
E5	45	46	35.3	2.9	27.9	41.5	8.6
ALC							
E1	35.7	37.6	31.9	3.7	22.4	40.5	13.5
E2	30.6	32.5	31.6	4.1	22.1	44.2	16.5
E3	36	37.9	25.6	3.8	16.8	36.8	14.4
E4	34	36.9	34.4	2.9	26.2	45.7	8.2
E5	30	31.9	29.2	3.9	20.6	37.4	15.3

[a]FLL, Flag leaf length; FLW, Flag leaf width; PLL, Penultimate leaf length; PLW, Penultimate leaf width; ALL, Antepenultimate leaf length; ALW, Antepenultimate leaf width; FLC, Flag leaf chlorophyll content; PLC, Penultimate leaf chlorophyll content; ALC, Antepenultimate leaf chlorophyll content
[b]E1-E5 indicate five environments as described in Table 1.
[c]Standard error
[d]Co-efficient of variation

with LOD values ranging from 3.1 to 14.4 (Fig. 2, Table 6).

Out of nine QTLs affecting six leaf size properties, four QTLs (*qALL3-1, qALL3-2, qALW3-1* and *qALW3-2*) were mapped near the RM448–RM571 region on chromosome 3. In contrast to *qALW3-1* and *qALW3-2* which were simultaneously identified in five environments, *qALL3-1* was repeatedly detected across two environments (E1 and E5) whereas *qALL3-2* only one environment. Additionally, four QTLs (*FLL1*, *qFLW8*, *qPLL8*, and *qPLW6*) were detected on chromosome 1, 6 and 8, respectively, and showed low repeatability (in only 1 environment).

Nine QTLs affecting FLC were detected in which *qFLC3*

Table 4. Analysis of variance of leaf-related traits in the chromosome segment substitute lines across five environments

Source	Degree of freedom	Mean square (MS)									*F*-value								
		FLL[a]	FLW	PLL	PLW	ALL	ALW	FLC	PLC	ALC	FLL	FLW	PLL	PLW	ALL	ALW	FLC	PLC	ALC
Environment	4	121.1	148.9	246.7	319.5	43.1	74.1	165.2	15.1	86.3	24.1**	4.3*	4.1*	4.7**	8.8*	5.4*	119.1**	78.2**	23.4**
Genotype	155	41.2	178.5	123.7	122.3	10.4	56.2	137.4	41.6	33.2	83.4**	62.2**	19.5**	5.6*	9.4**	10.5**	7.8*	9.5*	19.4**
GxE	620	16.9	28.4	78.2	212.4	8.1	8.5	4.0	16.3	37.8	5.5*	3.4*	5.1*	1.6*	1.4**	7.1*	6.9*	8.5*	6.2*
Pooled error	775	14.7	9.1	52.1	9.8	21.8	8.2	11.3	26.2	14.1									

[a]Abbreviations of the traits are same as Table 2.
*, **Significance levels are 0.05 and 0.01, respectively.

Table 5. Phenotypic correlations among leaf size and chlorophyll content from 156 chromosome segment substitute lines with the 93-11 genetic background based on the mean values in five environments

Trait[a]	FLL	FLW	PLL	PLW	ALL	ALW	FLC	PLC
FLW	0.40*							
PLL	0.61**	0.60**						
PLW	0.28*	0.82**	0.44*					
ALL	0.27*	0.48**	0.51**	0.42*				
ALW	0.51**	0.44**	0.43**	0.45**	0.69**			
FLC	NS[b]	NS	-0.16	NS	0.35*	0.34*		
PLC	0.14	NS	NS	NS	-0.20	NS	0.77**	
ALC	0.17	NS	NS	NS	NS	NS	0.78**	0.49**

[a]Abbreviations of the traits are same as Table 2.
[b]Non-significant
*, **indicates significance levels of 0.05 and 0.01, respectively.

Table 6. QTLs affecting rice leaf size and chlorophyll content detected by 156 PA64/93-11 chromosome segment substitute lines across five environments

Trait[a]	Env[b]	Loci	Chr.	Marker	LOD[c]	PVE (%)[d]	Additive effect[e]
FLL	E3	qFLL1	1	RM246	5.2	22.5	7.4
FLW	E2	qFLW8	8	RM223	4.3	16.8	3.5
PLL	E2	qPLL8	8	RM25	3.3	9.3	10.6
PLW	E3	qPLW6	6	RM162	3.3	19.4	-0.1
ALL	E1	qALL3-1	3	RM571	3.3	9.2	-6.8
	E4	qALL3-2	3	RM448	4.3	11.8	-5.8
	E5	qALL3-1	3	RM571	3.3	9.4	-7.2
ALW	E1	qALW1	1	RM495	7.3	19.2	5.0
		qALW3-1	3	RM448	4.1	11.5	3.1
		qALW3-2	3	RM571	9.5	24.4	6.2
	E2	qALW3-1	3	RM448	4.2	11.7	3.4
		qALW3-2	3	RM571	9.5	24.4	6.8
	E3	qALW3-1	3	RM448	4.1	11.5	2.7
		qALW3-2	3	RM571	9.7	24.8	5.6
	E4	qALW3-1	3	RM448	4.2	11.5	3.2
		qALW3-2	3	RM571	9.6	24.5	6.5
	E5	qALW3-1	3	RM448	4.2	11.6	3.5
		qALW3-2	3	RM571	9.6	24.6	7.1
FLC	E1	qFLC1	1	RM243	14.4	35.5	6.6
		qFLC2-1	2	OSR17	3.6	6.8	2.6
		qFLC3	3	RM5548	3.1	15.6	4.9
		qFLC8-1	8	RM25	3.2	7.9	-2.7
		qFLC8-2	8	RM310	3.2	8.5	-5.9
	E2	qFLC2-2	2	RM530	4.3	14.1	-3.5
	E3	qFLC3	3	RM5548	3.8	24.4	5.6
	E4	qFLC3	3	RM5548	6.4	18.5	5.2
	E5	qFLC3	3	RM5548	9.1	22.8	4.9
PLC	E1	qPLC6	6	RM469	3.5	10.5	2.9
		qPLC7	7	RM248	3.4	9.2	-1.9
	E2	qPLC10	10	RM467	3.1	9.9	-1.6
	E4	qPLC5	5	RM3437	5.5	16.6	3.5
	E5	qPLC6	6	RM469	4.5	17.5	2.7
ALC	E1	qALC6	6	RM469	3.5	21.2	3.2
		qALC12	12	RM3331	4.1	23.4	2.3
	E2	qALC3	3	RM5548	3.5	16.6	4.9
		qALC10	10	RM271	4.2	19.5	3.6
	E3	qALC6	6	RM469	3.8	9.9	4.1
	E5	qALC4	4	RM7187	3.7	17.5	4.5

[a]See Table 2 for abbreviations of the traits
[b]see Table 1 for naming of five environments (env)
[c]LOD, logarithm of odds
[d]PVE, phenotypic variance explained
[e]Positive number means the addictive effect coming from 93-11 allele, while negative value from PA64S allele

was repeatedly detected near the marker RM5548 across three environments (E3-E5), while *qFLC1, qFLC2-1, qFLC3, qFLC8-1, qFLC8-2*, and *qFLC2-2* were detected only in 1 or 2 environments. For PLC, four QTLs (*qPLC5, qPLC6, qPLC7,* and *qPLC10*) were identified in 1 or 2 environments, with explained phenotypic variation of 9.2-17.5%. For ALC, five QTLs (*qALC3, qALC4, qALC6, qALC10,* and *qALC12*) were mapped on chromosome 3, 4, 6, 10, and 12, respectively. Except for *qALC6*, the other four QTLs were detected only once.

Most positive effects were contributed by 93-11 alleles (Table 6). Three QTL clusters (three QTLs each) were found on chromosome 3, 6, and 8, respectively.

QTLs with High Stability

Two CSSLs (CSSL66 and CSSL67) having chromosome region substituted by PA64S alleles at *qALW3-1* locus (defined by SSR markers RM5548-RM448) were selected to confirm the repeatability of the QTL. Meanwhile, other two CSSLs (CSSL68 and CSSL69) harboring RM468-RM571 region substituted by PA64S alleles at *qALW3-2* locus were selected as well. Analyses of *t*-tests showed significant difference of phenotypic values between 93-11 and the CSSL carrying any of the target QTL alleles (Fig. 3). The results indicated that the effects of *qALW3-1* and *qALW3-2* were significant and repeatable in five environments. However, the other 25 QTLs were environment-specific as their significant effects were only detected in 1 to 3 environments.

Discussion

Here, a set of CSSLs was constructed and used to dissect genetic components controlling leaf-related traits in an elite hybrid rice variety, LYP9. The CSSLs have several advantages over primary mapping populations such as F_2, F_3, DH, and RIL populations previously used in conducting QTL studies for leaf traits (Dong et al. 2007; Farooq et al. 2010; Jiang et al. 2012; Jiang et al. 2014). Firstly, each CSSL carries a single or fewer PA64S segments in the near-isogenic background of 93-11, and thus genetic interactions between PA64S alleles are minimized. Due to reduced genetic background noise, the location and effect of QTL can be more precisely

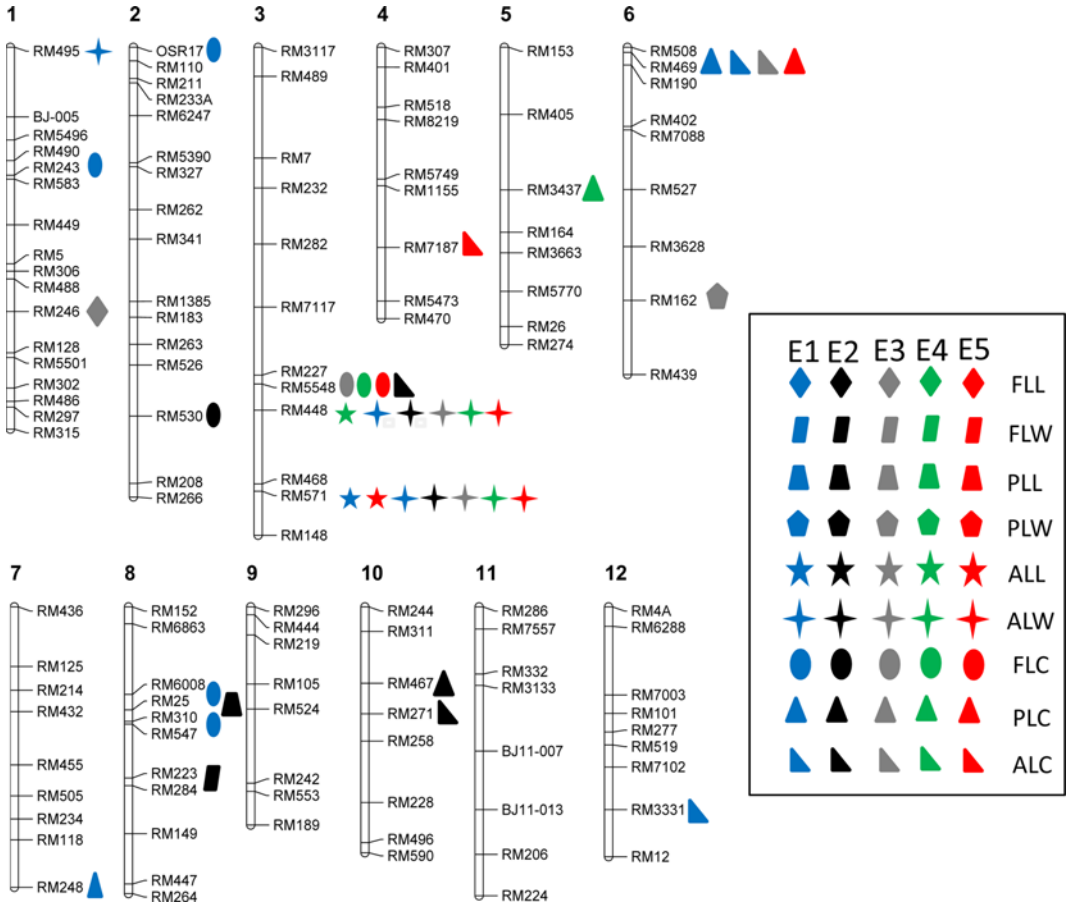

Fig. 2. Chromosomal locations of QTLs for the six leaf size properties and three chlorophyll content characteristics detected in five environments. See Table 1 for naming of five environments (E1-E5), and Table 2 for abbreviations of the traits

estimated, particularly for small effect QTL (Yano 2001). Second, high-resolution mapping of putative QTLs as Mendelian factors and further map-based cloning will be feasible, using secondary F_2 population derived from a cross between a QTL CSSL and the recurrent parent (Eshed and Zamir 1995; Takahashi et al. 2001). In addition, a secondary F_2 population between different target CSSLs can be used to precisely detect and confirm epistasis between QTLs (Lin et al. 2000; Yamamoto et al. 2000). Finally, the CSSLs can be used for simultaneous identification, mapping, and transfer of multiple desirable QTLs for target traits, especially for QTL pyramiding in plant breeding programs (Li 2001). In rice, some CSSLs have been developed so far [Kubo et al. 1999; Bian et al. 2010; Rice Genome Resource Center (RGRC, http://www.rgrc.dna.affrc.go.jp/stock.html)]. Compared with them, our CSSLs have bigger size (156 here vs. 39-108 lines for others) and were developed from a widely grown commercial hybrid variety LYP9. Hence the PA64S/93-11 CSSLs provide a powerful tool for future genetic analysis and breeding in rice. All CSSLs will be provided in timely manner for non-profit use.

As numerous QTLs affecting related traits were mapped to similar genomic regions to form QTL clusters (Fig. 2), classical quantitative genetics assumed that either pleiotropic effects or the tight linkage of genes was the probable genetic basis of the high trait correlations, which in this study ranged from 0.34* between FLC and ALW to 0.78** between FLC and ALC. If pleiotropy is implicated, a coincidence of location and direction of genetic effect is expected for positively related traits. The results obtained here are mostly in agreement with this expectation (Fig. 2; Table 6). However, we also found the chromosomal segments harboring a cluster of QTL on chromosome 3 contained two close loci for ALW, suggesting gene linkage might play a role in this region.

Of 27 QTLs identified in five individual environments, six QTLs (*qFLC1*, *qFLC3*, *qALC4*, *qALC12*, *qPLW8*, and *qFLL1*) are located in the vicinity of QTLs affecting these traits detected in the other mapping populations (Mei et al. 2003; Dong et al. 2007; Farooq et al. 2010; Jiang et al. 2012; Zhang et al. 2014; Bian et al. 2014). These six QTLs each accounted for a significant proportion of the overall phenotypic variation, with an average PVE of 28.5%, 20.4%, 18.5%, 21.4%, 19.2% and 22.6%, respectively across various studies. This is taken to mean that the effects of these six QTLs are less affected by epistasis from varied genetic backgrounds.

Molecular markers tightly linked to QTLs across different environments would be highly useful in marker-assisted selection and early generation selection of desirable recombinants (Moose and Mumm 2008). In this study, intriguingly two QTLs (*qALW3-1* and *qALW3-2*) with relatively stable effect across all five environments are reported here for the first time (Fig. 2) and have been validated in their corresponding CSSLs

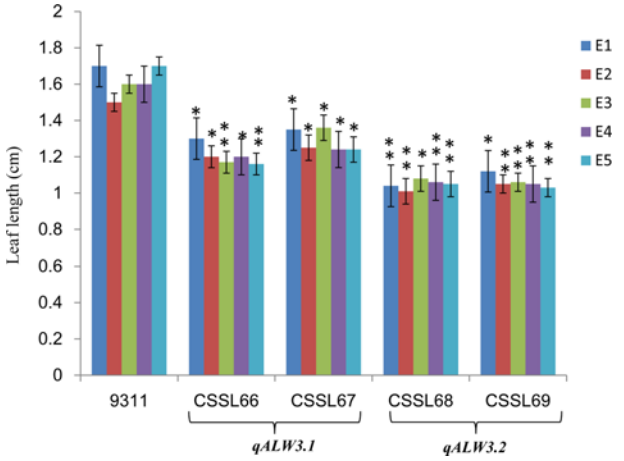

Fig. 3. Confirmation of two stable QTLs (qALW3.1 and qALW3.2) controlling antepenultimate leaf width in five environments. See Table 1 for codes of E1-E5. All values in individual environment were compared to mean of 93-11 using t-test. Significance levels: *P < 0.05, **P < 0.01.

(Fig. 3). Secondary F_2 populations between the target CSSLs and 93-11 are currently being exploited for the fine mapping and positional cloning of the both QTLs.

In summary, we have been able to identify some new QTLs underlying leaf-related traits using a set of CSSLs from two parents of an elite hybrid variety. The markers closely linked to the QTLs (Table 6) could eventually be useful for breeding of rice cultivars with better leaf architecture and photosynthetic capability. Moreover, two QTLs associated with ALW were constantly detected in all five environments and both of them provide useful information for marker-assisted selection in improving leaf traits.

Materials and Methods

Plant Materials and Field Experimental Design

The construction of CSSLs including 156 PA64S introgression lines in 93-11 background was initiated in 1999 by crossing PA64S with 93-11. Then resulting hybrids were backcrossed to 93-11 successively for three generations to produce 405 BC_3F_1 plants in 2000 to 2003. In 2004, self-pollinated progenies from the BC_3F_1 were grown in the field. The leaves of 200 plants, each from one BC_3F_2 line, were collected and their DNAs were extracted for genotyping. Totally 123 polymorphic SSR markers evenly distributed across all 12 rice chromosomes were used for defining genotype of each CSSL. In the first round of marker analysis, 86 CSSLs with relative clean background (viz. containing 1-3 introgressed PA64S segments) were picked out. The remaining 114 individuals were either self-pollinated or backcrossed by 93-11 again. The resultant lines were screened using similar marker-assisted selection (MAS) approach as described above, and other 70 CSSLs were ultimately obtained in 2005 to 2007.

The two parents and 156 CSSLs were planted in five different environments (E1-E5, Table 1) including two locations (Nanjing and Hainan) and three continuous cropping years (2010-2012). Nanjing

represents natural long-day whereas Hainan indicates natural short-day growth environments. A randomized complete block design with two replications was applied in each environment. Each entry plot contained two rows, and each row included ten individual plants. The 20 individual plants of each genotype in one entry plot were planted with spacing of 16.5 × 16.5 cm^2. A wide-row spacing of 23.5 cm was set between the plots. The fertilizer management and control of diseases and insect pests were applied as recommended.

DNA Preparation and PCR for Genotyping Analysis

Fresh leaves from plants were collected and stored under -20°C for use. Genomic DNA was extracted by the CTAB method (Murray and Thompson 1980). For PCR, each 10 μL amplification reaction contained 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM dNTP, 0.2 μM SSR primers, 0.5U Taq polymerase (TaKaRa, Dalian, China) and 20 ng DNA template. The amplification regime consisted of a denaturation step (94°C, 5 min), following by 35 cycles of 94°C/30s, 55°C/30s and 72°C/60s, and a final extension step of 72°C/7 min. The PCR products were electrophoresed through 8% non-denaturing polyacrylamide gels in 0.5×TBE buffer and bands were scored visually.

Phenotypic Evaluation

Six plants in the middle from each CSSL were subjected to investigation for leaf length and width around heading stage. For each CSSL, the fully extended leaves from flag leaf, the second top leaf (also called penultimate leaf) and third top leaf (antepenultimate leaf) were measured separately with a ruler and data were collected in the field. Chlorophyll content was determined on intact flag, penultimate, and antepenultimate leaves at 15-20 d after heading using a handy chlorophyll meter (SPAD-502PLUS, Konica-Minolta, Japan), as described by Zhang et al. (2014) and Wang et al. (2015). Five readings around the middle of each leaf blade were averaged.

Data Analysis

The marker distances, length of chromosomes, introgressed segments and overall genome size were estimated based on a high density molecular linkage map (McCouch et al. 2002) and the International Rice Genome Sequencing Project SSR database (http://www.gramene.org/microsat).The construction of graphical genotypes and calculation of the percentage of the total genome in each CSSL were performed using Graphical GenoTypes (GGT) software (http://www.dpw.wau.nl/pv/pub/ggt/).

According to field data and/or marker genotypes either in individual or multiple environments, analyses of variance (ANOVA) and QTL detection in CSSL population were conducted by software QTL IciMapping (Li et al. 2007; Wang et al. 2007). A RSTEP-LRT-ADD mapping method was adopted and 1,000 permutations were run to determine logarithm of odds (LOD) score for declaring a QTL with significant effect, as suggested by Wang et al. (2006). QTL nomenclature followed the scheme suggested by McCouch et al. (1997).

In addition, a *t*-test was employed to determine the presence of significant differences between the phenotypic values of 93-11and those of CSSLs harboring target QTL alleles.

Acknowledgments

This research was supported by the grants from Jiangsu Science and Technology Development Program (BE2012303); National Transform Science and Technology Program (2013ZX08001004-002); the Chinese National High Technology Research and Development Program ("863" Program, Nos. 2014AA10A604).

Author's Contributions

XL, LL, and YX performed research; SL, YT, LC, ZW, and LJ analyzed data; ZZ and JW designed research and wrote the paper. Each of the authors agreed on the contents of the paper and posted no conflicting interest.

Supporting Information

Fig. S1. Graphical genotypes of the 156 chromosome segment substitution lines with PA64S as donor (black box) and 93-11 as receipt (genetic background, in white).

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