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Fine mapping of the quantitative trait locus *qFLL9* controlling flag leaf length in rice

Shukun Jiang · Xijuan Zhang · Jiayu Wang · Wenfu Chen · Zhengjin Xu

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Abstract The morphological traits of leaves, such as size and shape, are major determinants of plant architecture and strongly affect high yield performance. To understand the molecular mechanism governing flag leaf length, we analyzed quantitative trait loci (QTLs) affecting flag leaf length by employing 176 F_2 individuals derived from a cross between two *japonica* rice cultivars: Shennong265 (SN265) and Lijiangxintuanheigu (LTH). We identified *qFLL3*, *qFLL6* and *qFLL9* from this F_2 population. Flag leaf length was increased by SN265 alleles at *qFLL3* and *qFLL6*, but by LTH allele at *qFLL9*. In order to eliminate the influence of *qFLL3* and *qFLL6*, one single residual heterozygous plant for *qFLL9* region, RH-*qFLL9*, was selected based on the genotypes of 114 simple sequence repeat (SSR) markers and used as the parent of a segregating population. Using this segregating population of 889 plants, this region was narrowed down to an interval between RM24423 and RM24434. According to the rice annotation project database, there are 17 predicted genes in the 198-kb target region.

Keywords SSR marker · Rice · Cytochrome P450 · Fine mapping · Flag leaf length

S. Jiang · J. Wang · W. Chen · Z. Xu (✉)
Key Laboratory of Crop Physiology, Ecology, Genetics and Breeding, Ministry of Agriculture, Key Laboratory of Northern Japonica Rice Breeding of Liaoning Province, Shenyang Agricultural University, 110866 Shenyang, China
e-mail: xuzhengjin@126.com.cn

S. Jiang
e-mail: sk.jiang@hotmail.com

X. Zhang
Dandong Academy of Agricultural Sciences, Dandong 118109, China

Introduction

Grain yield of rice is largely determined by the top three leaves (*Oryza sativa* L.). The top three leaves on a stem are the primary source of carbohydrate production. Particularly, the flag leaf produces 40–60% of carbohydrates accumulated into grains, which finally determines grain yield (Gladun and Karpov 1993a; Foyer 1987; Kholupenco et al. 1996; Gladun and Karpov 1993b).

Li et al. (1998) used an $F_{2:4}$ population and 115 well-distributed restriction fragment length polymorphism (RFLP) markers to analyze the genetic basis underlying the relationship between source leaves (the top two leaves) and sink capacity, and discovered that *QLl3a*, *QLl3b*, *QLl7* and *QLl9* controlling leaf length are located on chromosomes 3, 7 and 9, while *QLw3*, *QLw4* and *QLw8* controlling leaf width are located on chromosomes 3, 4 and 8. Kobayashi et al. (2003) analyzed QTLs affecting flag leaf development and

mapped nine QTLs affecting flag leaf length and six QTLs controlling flag leaf width. Cui et al. (2003) mapped seven QTLs affecting leaf area. Rice *constitutively wilted 1 (cow1)* mutant exhibits the narrow and rolling leaf phenotype, and *COW1* gene encodes a flavin-containing monooxygenase (FMO) which belongs to a new member of the YUCCA protein family (Woo et al. 2007). Fujino et al. (2008) identified a spontaneous mutation of narrow leaf, termed *narrow leaf 7* (*nal7*), which displayed significantly decreased leaf blade width. The *NAL7* gene also encodes a FMO and shows sequence similarity to the *YUCCA* gene family which catalyzed tryptamine to create *N*-hydroxytryptamine in auxin biosynthesis. Further analysis indicated that *NAL7* is involved in auxin biosynthesis (Fujino et al. 2008). Qi et al. (2008) characterized a classic rice dwarf mutant named *narrow leaf1 (nal1)* which exhibits a characteristic phenotype of narrow leaves. *nal1* encodes a plant-specific protein with unknown biochemical function, affects polar auxin transport as well as the vascular patterns of rice plants and plays an important role in control of lateral leaf growth. Compared with the greater available information regarding the regulation mechanism for leaf width, the molecular mechanism governing flag leaf length remains obscure.

In this paper, we identified three QTLs affecting flag leaf length and conducted fine mapping of one of the three detected QTLs, *qFLL9*, to a 198-kb interval on chromosome 9. These results provide more information for better understanding of the molecular mechanism governing flag leaf length.

Materials and methods

Plant materials

To identify QTLs more easily, we selected and crossed parental *japonica* varieties that showed highly significant differences in flag leaf length: Lijiangxintuanheigu (LTH) with a very long flag leaf (43.2 ± 3.5 cm) and Shennong265 (SN265) with a short flag leaf (22.2 ± 3.3 cm), to produce an F_2 population of 176 plants. QTL analysis for flag leaf length was carried out based on this F_2 population.

For fine mapping of *qFLL9* as described later, five individuals from the F_2 population were selected according to QTL analysis results. Foreground and background selection were conducted in the 125 F_5 plants. Consequently, one F_5 plant, which was heterozygous at the *qFLL9* region on chromosome 9 and homozygous in other regions (termed RH-*qFLL9*), was selected based on genotyping results of 114 SSR markers. A segregating population of 899 individuals for the region of *qFLL9* was developed. The scheme for constructing the RH-*qFLL9* plant used in this study is summarized in Fig. 1.

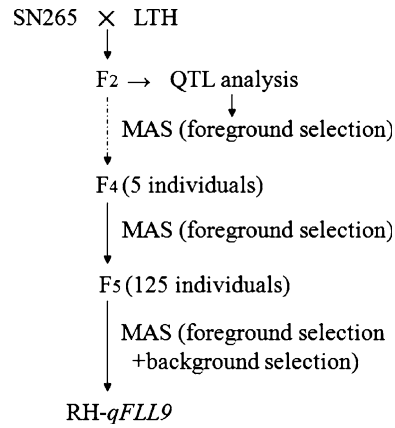

Fig. 1 Breeding scheme for the selection of the RH-*qFLL9* plant in this study

Cultivation and trait measurements

The 176 F_2 plants and 889 segregating plants from RH-*qFLL9* were laid out in a field for phenotypic evaluation in the rice growing seasons of 2006 and 2009, respectively, on the experimental farm of the Rice Research Institute of Shenyang Agricultural University, Shenyang, China. The sowing date was April 12, and 15 plants per line with 2 LTH plants at each end to remove edge effects were transplanted on May 18, with 13.3 cm between plants and 30 cm between rows. Field management essentially followed standard agricultural practice. Fertilizers applied were 60, 90 and 90 kg/ha of N, P_2O_5 and K_2O, respectively.

The parents and the two segregating populations showed a few days variation in heading date, therefore all plants were planted and investigated at the same time. Flag leaf length was defined as length from collar to top of leaf. At 25 days after heading, mean values for flag leaves on three main stems of each plant were used for data collection.

Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from young leaves of a single plant using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). The SSR markers, developed by Temnykh et al. (2001) and Huang and Zhang (2003), were denoted by "RM" and "PM", respectively. DNA amplification was performed using a GeneAmp PCR system 9700 thermocycler (Perkin Elmer Cetus, Norwalk, CT). Each reaction of 15 μl PCR mixture contained 20 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 8.8), 0.1% Triton-X, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 0.2 μM of each primer, 5% (v/v) dimethyl sulphoxide and 0.5 U Taq DNA polymerase (Tiangen Biotech, Beijing, China). Amplification conditions consisted of initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 45 s, 55–60°C for 45 s and 72°C for 1 min, followed by final extension at 72°C for 5 min. To detect polymorphisms of markers, polymerase chain reaction (PCR) products were separated on 6% polyacrylamide denaturing gels, and DNA fragments were detected by silver staining (Panaud et al.1996).

Genetic linkage map construction

The linkage map was constructed using the Mapmaker/Exp 3.0 program (Lander et al. 1987) on a personal computer. The command "GROUP" was used to verify the linkage group (LOD = 3, max distance = 50 cM), and the order of the linkage groups was determined by using the "ORDER" and "RIPPLE" commands. The "TRY" command was used to insert marker. The Kosambi function was used to calculate genetic distances.

QTL analysis and fine mapping of *qFLL9*

F_2 population

Mapping of QTLs and estimation of their effects were carried out for flag leaf length by using the approach of composite interval mapping (CIM) in the computer package Windows QTL Cartographer version 2.5 (Wang et al. 2007). For CIM, a 2-cM window size was used for genome scans. The permutation method was used to obtain the thresholds of the experiment based on 1,000 runs of randomly shuffling the trait values ($P = 0.05$). The peak points of the LOD in the linkage map were taken as the putative positions of the QTLs, and additive effects of these QTLs were taken from the points showing the largest LOD. The relative contribution of a genetic component was calculated as the proportion of phenotypic variation explained by that component (Zeng 1994).

Segregating population from RH-qFLL9

For fine mapping of *qFLL9*, the bulked-extreme and recessive-class approach as described by Zhang et al. (1994) was used to calculate recombination frequencies between *qFLL9* and molecular markers in the 187 homozygous plants with long flag leaf out of 889 segregating plants from RH-*qFLL9*. Thus, the recombination frequency = $(N_1 + N_2/2)/N$, where N is the total number of long-flag-leaf plants, N_1 is the number of long-flag-leaf plants with the marker genotype of the short-flag-leaf parent and N_2 is the number of long-flag-leaf plants with heterozygous marker genotype.

Results

Construction of linkage map and QTL mapping of flag leaf length using 176 F_2 plants

An SSR linkage map of 114 marker loci was constructed (Fig. 2). All marker positions were in good agreement with previous maps (www.gramene.org). The mapped markers covered 12 rice chromosomes in 1397.5 cM of genetic distance with an average of 12.3 cM between two markers. The total genetic distance in the present population was 91.4% of that in the Japanese Rice Genome Research Program (RGP) map according to location information from www.gramene.org.

We mapped three QTLs for flag leaf length in this F_2 population (Table 1). Among them, a major QTL for flag leaf length, *qFLL9*, was located between RM434 and RM242 on chromosome 9, at which the LTH allele contributed to long flag leaf (Fig. 3a). The additive effect of *qFLL9* was –9.23. The amount of phenotypic variation explained by *qFLL9* was 42.9% (Table 1).

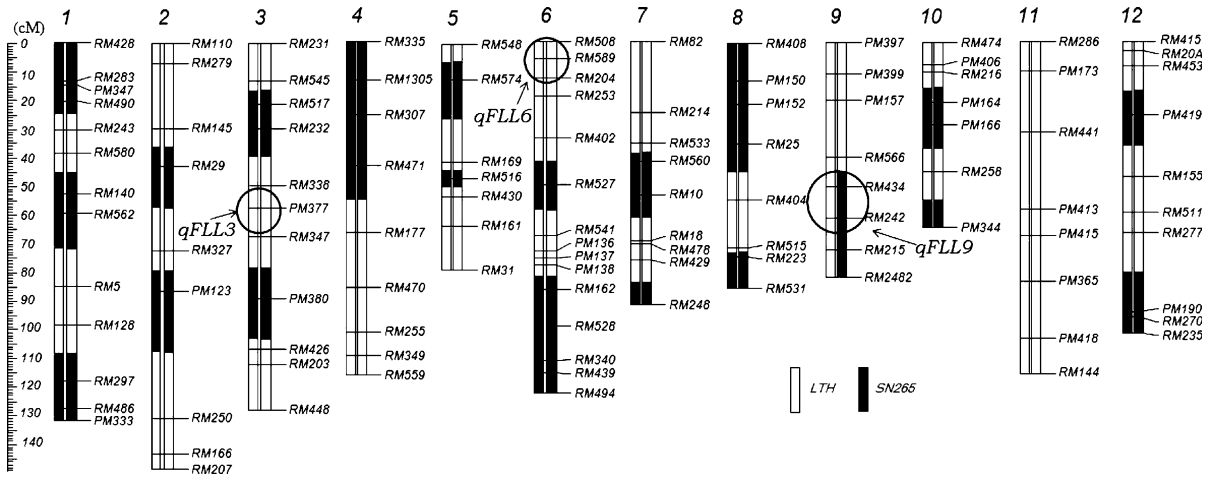

Fig. 2 Graphical genotype of the RH-*qFLL9* plant determined using 114 SSR markers. Solid and open bars represent SN265 segments and LTH segments, respectively. Putative location of each QTL is *circled*, and the size of each *circle* indicates the relative magnitude of their genetic effect

Table 1 Quantitative trait loci affecting flag leaf length in the F_2 population derived from SN265/LTH

QTL	Chromosome	Marker interval	Peak LOD	PEV (%)[a]	Additive effect[b]
qFLL3	3	RM338-RM347	2.49	12.2	2.94
qFLL6	6	RM508-RM204	3.00	14.5	3.04
qFLL9	9	RM434-RM242	10.72	42.9	−9.23

[a] Percentage of explained phenotypic variation (PEV)

[b] Positive and negative values indicate that alleles of SN265 and LTH, respectively, caused flag leaf elongation

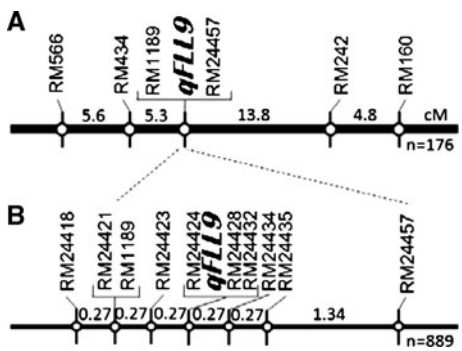

Fig. 3 Genetic map of *qFLL9* controlling flag leaf length. The values between markers are mean recombination frequencies. **a** Location of *qFLL9* on rice chromosome 9 estimated using 176 F_2 plants. **b** High-resolution linkage map of the *qFLL9* region produced based on 889 segregating plants from RH-*qFLL9*

Phenotypic performance of flag leaf length in the segregating population from RH-*qFLL9*

The RH-*qFLL9* plant was homozygous at all SSR loci except for the region of *qFLL9* on the long arm of chromosome 9 (Fig. 2). This enabled us to handle the *qFLL9* locus as a single Mendelian factor in the segregating population. In fact, clear monogenic segregation for flag leaf length was observed in Fig. 4b, in apparent contrast to the results in Fig. 4a. We found that the heterozygous RH-*qFLL9* plant had short flag leaf, and the segregation ratio of plants with short flag leaf and long flag leaf in the segregating population was 658:231 = 2.85:1.00, fitting well to the 3:1 ratio ($\chi^2 = 0.4593$, $P > 0.05$). These results revealed that flag leaf length was controlled by a single gene and that long flag leaf is a recessive trait in this population.

Fine mapping of *qFLL9*

For fine mapping of *qFLL9*, 187 homozygous plants with long flag leaf from the RH-*qFLL9* plant and 18 new molecular markers surrounding *qFLL9* were used to calculate the recombination frequency. Of these markers, three (RM24424, RM24428 and RM24432)

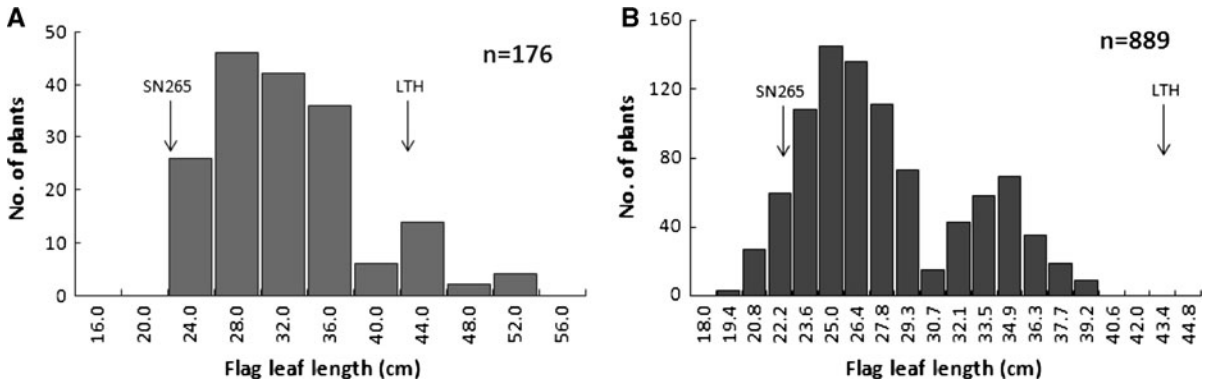

Fig. 4 Frequency distribution of flag leaf length in the F_2 population from SN265/LTH (**a**) and a segregating population derived from RH-*qFLL9* (**b**)

Table 2 Candidate genes in the198-kb target region corresponding to the region of *qFLL9* (referred from UNIPLOT)

Name	Location	Protein	Function
AK063191	16973644–16975242	Hypothetical protein	Unknown
AK073097	16979658–16983622	BRCT domain containing protein	DNA break and repair
AK119560	16983842–16992222	Similar to Aldehyde dehydrogenase family seven member A1 (EC 1.2.1.3) (Antiquitin 1) (Matured fruit 60 kDa protein) (MF-60)	Cellular aldehyde metabolic and oxidation reduction process
AK104963	17000791–17001602	Ctr copper transporter family protein	Copper ion transporting
J033108L14	17010418–17013567	Similar to SBT1 protein (Subtilisin-like protease)	Proteolysis with serine-type endopeptidase activity
AK107584	17015034–17017000	Similar to cytochrome P450 monooxygenase CYP92A1	Brassinosteroids biosynthesis
AK111616	17041551–17044099	Similar to elicitor-inducible cytochrome P450	Brassinosteroids biosynthesis
J065094C22	17057348–17061785	Similar to cytochrome P450	Brassinosteroids biosynthesis
AK101247	17064862–17069562	Whey acidic protein, core region domain containing protein	Reduced length of the inflorescence internode
AK111977	17076001–17078929	Protein kinase-like domain containing protein	ATP binding and protein serine/threonine kinase activity
AK066748	17092853–17097289	Similar to Oryzain γ-chain precursor (EC 3.4.22.–)	Expresses only in seeds and induced by gibberellic acid
AK106970	17098053–17101678	t-snare domain containing protein	Vesicle-mediated transportation
AB095097	17109055–17112026	Similar to RSH2	Guanosine tetraphosphate metabolic process
J013063M05	17115362–17121338	SNF2-related domain containing protein	Zinc-finger, ATP, DNA and protein binding
AK061348	17124697–17128191	Conserved hypothetical protein	Transferring glycosyl groups
AK100287	17130777–17135403	Similar to Axi 1 (auxin-independent growth promoter)-like protein	Expresses only in chloroplast and Golgi apparatus
AK063205	17159191–17162517	Conserved hypothetical protein	Transferring glycosyl groups

were co-segregating completely with *qFLL9* (Fig. 3b). Finally *qFLL9* was mapped between RM24423 (16,965,639 bp) and RM24434 (17,163,735 bp) (http://rapdb.dna.affrc.go.jp/). *qFLL9* was narrowed down to a 198-kb genomic region according to the information of these two markers. According to the rice annotation project database (http://rapdb.dna.affrc.go.jp/), there are 17 predicted genes in the 198-kb

target region (Table 2). The functional information of these predicted genes was obtained from the UNIPROT protein knowledgebase (http://www.uniprot.org/).

Discussion

For fine mapping of QTL, it is necessary to carry out high-resolution linkage analysis using a large number of plants that segregate only around the QTL being investigated. Near-isogenic, introgression and chromosome segment substitution lines (Tian et al. 2006; Yu et al. 2007; Ebitani et al. 2005; Tan et al. 2008) developed by advanced backcrossing with marker-assisted selection have been widely used. Alternatively, a segregating population from a single plant with a heterozygous target QTL region on a homozygous background has increasingly frequently been used in recent years owing to its simple development (Yamanaka et al. 2005; Tuinstra et al. 1997). In this study, we used 889 individuals derived from the RH-*qFLL9* plant to dissect the QTL affecting flag leaf length down to a single Mendelian factor. Because of the similar genetic background of the two parents (both being *japonica* rice varieties with small diversity), it is difficult to develop enough molecular markers to narrow down the target region. There are two approaches to resolve this problem; one is to increase the population size, and the other is to change the parent SN265 to an *indica* rice variety with short flag leaf. We are now taking these two approaches to obtain more recombination in the target region.

The most significant finding in our study was the delimitation of the *qFLL9* gene to a DNA fragment of approximately 198-kb in length. Seventeen predicted genes were detected in the target region, including three genes (AK107584, AK111616 and J065094C22) related to cytochrome P450. Cytochrome P450 proteins are well known to be heme-binding enzymes, with monooxygenase activity such as oxidation, hydroxylation, isomerization and dehydration of various kinds of compounds (Nelson et al. 1996). D2/CYP90D2 and D11/CYP724B1 with shortening of clum, grain length and leaf length of rice have been already cloned encoding cytochrome P450 which play a role in brassinosteroid biosynthesis (Hong et al. 2003; Tanabe et al. 2005). Zhu et al. (2006) isolated an elongated uppermost internode mutation and revealed that the *Eui* gene causing this mutation encodes a previously uncharacterized P450, CYP714D1, as a gibberellins catabolism gene. Therefore, these three genes might be associated with control of flag leaf length. On the other hand, AK101247, whey acidic protein and core region domain containing protein, was identified as *DENSE ELECT PANICLE 1 (DEP1)* of rice, reducing the length of the inflorescence internodes, increasing the number of grains per panicle and grain yield and causing semi-dwarf stature. The parent material SN265 used in this study has the *dep1* gene. Although *dep1* is expressed in root, leaf, culm, inflorescence meristem and young inflorescence, it is expressed strongly in both the inflorescence meristem and the intercalary meristem but not in leaf (Huang et al. 2009). So, we considered that *dep1* is different from *qFLL9*. Moreover, AK119560, similar to aldehyde dehydrogenase family seven member A1 and AK066748, similar to oryzain γ-chain precursor is expressed only in fruits of apple and seeds of soybean (Watanabe et al. 1991; Yamada et al. 1999). AK100287, similar to Axi 1 (auxin-independent growth promoter)-like protein, is expressed in chloroplast or Golgi apparatus (http://www.uniprot.org/). According to their organ specificity of gene expression, the latter four genes (AK101247, AK119560, AK066748 and AK100287) would not be target genes of *qFLL9*. Considering the organ specificity of gene expression and the molecular function information from the protein knowledgebase, AK107584, AK111616 and J065094C22 might be the most likely candidate genes, but we do not have enough data [polymorphisms or expression levels of these genes in leaves by reverse-transcription (RT)-PCR or quantitative real-time RT-PCR] to rule out the other ten candidate genes.

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