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Genetic analysis and gene mapping of *leafy head* (*lhd*), a mutant blocking the differentiation of rachis branches in rice (*Oryza sativa* L.)

DUAN Yuanlin[1], WU Weiren[2,1], LIU Huaqing[3], ZHANG Danfeng[1], ZHOU Yuanchang[1], PAN Runsheng[1], LIN Lihui[1], CHEN Zhiwei[1], GUAN Huazhong[1], MAO Damei[1], LI Weiming[1] & XUE Yongbiao[4]

1. College of Crop Science, Fujian Agriculture & Forestry University, Fuzhou 350002, China;
2. College of Agriculture & Biotechnology, Zhejiang University, Hangzhou 310029, China;
3. Academy of Agricultural Science, Fujian Province, Fuzhou 350003, China;
4. Institute of Genetics & Developmental Biology, Chinese Academy of Sciences, Beijing 100080, China

Correspondence should be addressed to Wu Weiren (e-mail: zjweiren@pub5.fz.fj.cn) or Xue Yongbiao (e-mail: ybxue@genetics.ac.cn)

Abstract **A rice mutant called *leafy head* (*lhd*), in which the differentiation of rachis branches is blocked, was identified in a doubled haploid (DH) population derived through F_1 anther culture from a cross between rice (*Oryza sativa* L.) indica cultivar Gui-630 and japonica cultivar Taiwanjing. The mutant is shorter in plant height, possessing smaller and clumpy leaves, and always stays at the vegetative growth stage. Genetic analysis suggests that *lhd* is controlled by a single recessive gene, which is temporarily named *lhd*(*t*). The phenotype of the mutant suggests that *LHD*(*t*) is a key gene controlling the differentiation of rachis branches. In order to map the gene, two F_2 populations were constructed by crossing the *lhd* heterozygote with varieties Minghui-77 (*indica*) and Jinghua-8 (*japonica*). In the F_2 of *lhd* heterozygote × Jinghua-8, some mutant plants appeared as the "medium type", suggesting that the *lhd* phenotype could be influenced by genetic backgrounds. With the published SSR markers of RM series and additional SSR markers developed by ourselves and using the methods of bulked segregant analysis (BSA) and mutant analysis (with 498 mutant plants in total), *LHD*(*t*) gene was mapped onto the distal region of the long arm of chromosome 10. Markers SSR1, RM269, RM258, RM304 and RM171 were located on one side with distances of 6.4, 16.6, 18.4, 22.2 and 26.3 cM to *LHD*(*t*); whereas markers SSR4 and SSR5 were on the other side with distances of 0.6 and 2.2 cM to *LHD*(*t*). The results will facilitate the positional cloning and functional study of the *LHD*(*t*) gene.**

Keywords: ***Oryza sativa*** **L., *LHD*(*t*), rachis differentiation, SSR marker, gene mapping.**

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Flowers, fruits and seeds are products of plant reproductive development and provide the important sources of foods for humans. Therefore, the molecular genetic mechanisms of floral development have been a hotspot of research of plant developmental biology[1]. Rice is one of the most important staple food crops. The outcome of its reproductive development would determine the yield and quality of grains. Rice is also a model plant of cereals. Hence, the study of rice reproductive development, especially the molecular genetic mechanisms controlling the induction of flowering and the development of floral organs has important implications both in theory and in practice. Mutants have played an important role in the efforts of uncovering the mystery of reproductive development, namely, the functions and interactions of related genes in the process of reproductive development, in plants. Recent advances of researches on the flower development in *Arabidopsis* and *Antirrhinum* have revealed the molecular mechanisms controlling flowering and floral development[2,3]. Nevertheless, the research of flower development in cereals like rice has far lagged behind. A major reason could be attributed to the fact that few floral mutants have been obtained in cereals. To date, most studies on the mechanisms of genetic regulation of flower development in rice have been performed based on gene cloning on homology[4—6].

The initiation of flowering is controlled by both endogenous and environmental signals. With the induction of appropriate environmental conditions, the vegetative meristem in the shoot apex is transformed into the reproductive meristem. This is a critical transition point in the ontogeny of plant, which is marked by a number of changes in the shoot apex at molecular, physiological and morphological levels. Once the transition is initiated, the formation of the inflorescence structure will begin. In rice, flower development can be divided into three distinct stages, namely, rachis branch differentiation, spikelet differentiation and floral organ differentiation, where the rachis branch differentiation is a key stage deciding whether the plant can enter and complete the process of reproductive development. We have found a rice mutant called *lhd* (*leafy head*) from the progeny of a cross between Gui-630 (*indica*) and Taiwanjing (*japanica*) derived by F_1 anther culture. The mutant is shorter in height, has smaller and clumpy leaves that emerge more quickly, and always stays in the vegetative growth state. In other words, the differentiation of rachis branches in the mutant is blocked. Hu[7] and Itoh et al.[8] also found several mutants phenotypically similar to *lhd*, but they only performed morphological and classical genetic studies on the mutants. As we could not test the allelism of our mutant to those reported by Hu and Itoh et al., we temporarily named our mutant gene *lhd*(*t*). In this study, we carried out extensive genetic mapping of gene *LHD*(*t*) as the first step towards its positional cloning and functional study.

1 Materials and methods

The *lhd* mutant was found from a doubled haploid line derived from the F_1 of Gui-630 (*indica*) × Taiwanjing (*japanica*) by anther culture in 1995. Since the mutant is sterile, in order to maintain the mutant gene *lhd*(*t*), all normal plants in the line were harvested individually. Afterwards, in each generation, the progenies were always planted in lines and only the lines showing the segregation of mutant plants (this means that their parents were heterozygotes at the mutant locus) were harvested for the preservation of the mutant gene. The progeny lines were planted in different cropping seasons and the segregation of the mutant trait was investigated. All the data collected in different seasons were jointly analyzed to determine the genetic pattern of the mutant trait.

F_2 populations for gene mapping were constructed from crosses between the *lhd* heterozygote and two varieties, Minghui-77 (*indica*) and Jinghua-8 (*japonica*). Normal plants from the *lhd*-segregating lines were randomly selected and crossed with Minghui-77 and Jinghua-8 as well as selfed for 1 or 2 panicles. In the next season, the F_1's and the corresponding selfed progeny were planted simultaneously to examine whether the parental plants were *lhd* heterozygotes or not. In the F_1 generation of *lhd* heterozygote × Minghui-77 or Jinghua-8, half of the progeny would expect to be heterozygotes *LHD*(*t*)/*lhd*(*t*), from which the F_2 populations required were obtained. The detailed procedure of constructing F_2 populations is similar to that described in ref. [9].

The performance of the target trait in the F_2 population from *lhd* heterozygote × Minghui-77 was investigated. The CTAB method was used to extract genomic DNA from fresh leaves of an individual plant. The method of bulked segregant analysis (BSA)[10] was used to search for SSR markers linked to the target gene. Namely, 20 normal plants and 20 mutant plants were randomly selected from the F_2 population to make two DNA pools, and the DNAs of Minghui-77, Jinghua-8 and the original mutant were used as controls. The SSR markers obtained by BSA were used to assay the mutant plants in the F_2 population. The band-type of the original mutant was recorded as 1, whereas that of Minghui-77 was recorded as 2, and the heterozygous band-type was recorded as 3. Linkage analysis among the target gene and the SSR markers was performed with the software MAPMAKER/EXP3.0, and the chromosome position was determined by referring to the SSR marker linkage map reported by Temnykh et al.[11].

Preliminary mapping of the target gene was conducted using the published RM-series SSR markers. In order to find closer linked markers, several new SSR primers (named with prefix SSR for distinction) were designed based on the published rice sequence of the region containing the target gene[12]. PCR amplification, electrophoresis and silver-stain in the SSR analysis were performed according to Panaud et al.[13] with a slight modification.

2 Results

(ⅰ) Morphological features of the mutant *lhd*. Within 10 d after germination, the mutant had no apparent difference from the wild-type in plant morpha. Beginning from 10—15 d after germination, the mutant exhibited a higher rate of leaf emerging and produced more leaves, but developed shorter internodes (Fig. 1(a)). When the wild-type was reaching the heading stage, the mutant showed a significant dwarfism, with more, smaller and clumpy leaves (Fig. 1(b)). The reproductive development of wild-type began after finishing vegetative growth, but the reproductive development of the mutant was completely blocked; its primordial of the primary rachis branches was all converted into vegetative shoots. Obviously, the mutant gene *lhd*(*t*) starts to express at the early stage of the vegetative growth, but its main effect appears at the stage of transition from vegetative growth to reproductive development.

In addition, the mutant gene also appeared to affect tiller development. The mutant entered the active tillering stage more quickly and reached the tillering peak earlier than the wild-type, but their final tiller numbers were not significantly different (data not shown).

(ⅱ) Genetic basis of the mutant trait. Segregation of the mutant trait in two successive progeny generations of an *lhd* heterozygote was investigated. Table 1 shows that among the 1277 plants observed, 944 plants exhibited normal phenotype and 333 plants exhibited *lhd* phenotype, showing a good fit to the expected ratio of 3∶1. The ratio did not alter in three cropping seasons. In addition, the mutant phenotype was also expressed typically and stably when the mutant was planted in a greenhouse (Fujian Province, China) during winter. These results suggest that *lhd* is controlled by a single recessive gene and can be inherited steadily, not affected by the climate factors (day length and temperature) and cropping conditions. We temporarily named the gene *lhd*(*t*).

Table 1 Inheritance pattern of *lhd* in three cropping seasons

Cropping season	Population size	Normal plants	*lhd* plants	χ^2 (3∶1)
Early	521	379	142	1.41
Middle	327	251	76	0.54
Late	429	314	115	0.75
Total	1277	944	333	0.79

$\chi^2_{0.05,1} = 3.84$.

(ⅲ) Effects of genetic background on the phenotype of the *lhd* mutant. In the two F_2 populations, the ratio between normal plants and *lhd* plants were also 3∶1 (Table 2), verifying that *lhd* was controlled by a single reces-

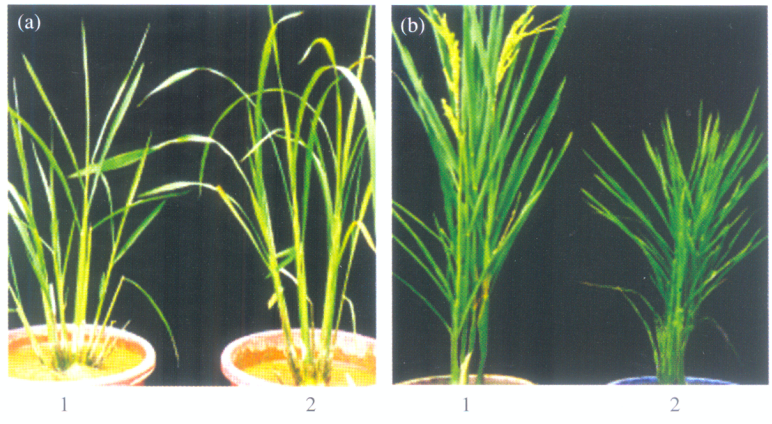

Fig. 1. Morphological appearances of the *lhd* mutant and the wild-type of rice. (a) Plants at 40 d after germination; (b) plants at the heading stage.

Table 2 Segregation and phenotype of *lhd* mutant in the F_2 populations

Combination	Year	Population size	Wild-type plants	*lhd* plants	χ^2 (3 : 1)	No. of medium-type	Medium-type/mutant
Cross with Jinghua-8	1999	80	61	19	0.08	4	0.21
	2000	120	89	31	0.04	5	0.16
Cross with Minghui-77	1999	678	520	158	1.04	0	0
	2000	523	399	124	0.46	0	0

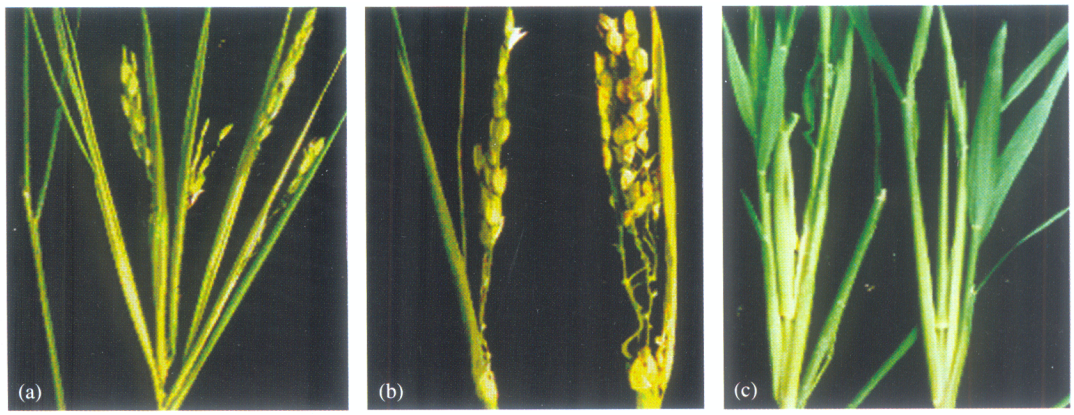

Fig. 2. Medium-type ((a) and (b)) and typical (c) branches and buds of *lhd* plants.

sive gene. In the F_2 of *lhd* heterozygote × Minghui-77, the mutant phenotype was typical (Fig. 1), but in the F_2 of *lhd* heterozygote × Jinghua-8, the mutant phenotype in part of the mutant plants was markedly attenuated into a "medium type" (Fig. 2), of which some top branches could continuously differentiate and produce a few short panicles, but the spikelets developed abnormally and could not produce seeds. These results suggest that genetic background could affect the phenotype of *lhd* mutant. Although the *lhd* mutant came from the progeny of a cross between an *indica* rice variety and a *japanica* rice variety, the wild-type of *lhd* is more like an *indica* rice type according to its plant morpha. Since Minghui-77 is an *indica* rice variety while Jinghua-8 is a *japonica* rice variety, the results imply that the extent of influence of genetic background on the *lhd* phenotype is correlated to the genetic distance between the parents.

(iv) Chromosomal mapping of *LHD*(*t*). Two hundred pairs of SSR primers of RM series evenly distributed in the rice genetic map were selected to screen for polymorphic markers between the *lhd* mutant and Minghui-77, and 84 pairs (46.2%) exhibited polymorphism. The 84 pairs of primers were then used to detect polymorphisms between the normal DNA pool and the mutant DNA pool. Four SSR markers on chromosome 10, RM269, RM258, RM304 and RM171, were found to be linked to *LHD*(*t*). Further analysis with the 4 SSR markers on 498 mutants from the F_2 population showed that the four markers were all located on the same side with distances of 16.9, 18.7, 22.6 and 26.4 cM, respectively, to *LHD*(*t*). It could thus be

deduced that *LHD*(*t*) is located on the distal region of the long arm of chromosome 10[13].

(v) Fine mapping of *LHD*(*t*). Because the published markers nearby the target locus were rare, it was necessary to look for additional new markers. According to the published sequences of BAC clones of rice chromosome 10[12], 9 pairs of new SSR primers (SSR1—SSR9) were designed and synthesized for the fine mapping of *LHD*(*t*). Three pairs, SSR1, SSR4 and SSR5 (Table 3), showed polymorphisms and close linkage to *LHD*(*t*). Among them, SSR1 is on the same side as RM269, RM258, RM304 and RM171, with a distance of 6.4 cM to *LHD*(*t*), while SSR4 and SSR5 are on the other side with distances of 0.6 and 2.2 cM to *LHD*(*t*) (Fig. 3).

3 Discussion

Although the expression of the mutant phenotype was complete in the original *lhd* mutant, it was attenuated in part in the mutant plants from the F_2 population of *lhd* heterozygote × Jinghua-8. This indicates that genetic background could influence the expression of *lhd* mutant phenotype. The influence might come from redundant genes, modifying genes and/or interactive genes. The phenomenon of the mutant phenotype attenuation was also found in the *lhd*-alike mutants reported by Hu[7] and Itoh et al.[8]. The mutant found by Hu could partly tassel under the short day condition, and the mutant found by Itoh et al. could partly tassel even under the normal condition, similar to the "medium-type" found in this study. However, there is a difference in that the panicles generated in their "medium-type" plants developed normally and could produce seeds. This suggests that the genetic basis of the transition from vegetative growth to reproductive development in rice is likely to be more complicated. If the mutant genes found in this study and by Hu and Itoh et al. are not mutually allelic, it is likely that there may be several key genes controlling the initiation of reproductive development in rice. If these mutant genes are allelic, it implies that there may be only one or very few key genes controlling the initiation of reproductive development in rice, but their functions may be modified or compensated by other genes. To answer this question, it is necessary to clone *LHD*(*t*) and study its function.

In gene mapping, in order to acquire polymorphic markers more easily, it is usual to use genetically less related materials as parents. However, the genetic relationship between parents should not be too far, otherwise the fertility of the F_1 generation could be very low, making the construction of genetic population difficult. The phenomemon was particular striking in the present study, probably because of that the target gene is involved in the reproductive development. In the two crosses used, the F_1 of *lhd* heterozygote × Jinghua-8 (*indica* × *japanica*) was severely sterile so that the F_2 seeds obtained were not sufficient for constructing a large population required. Therefore, we had to utilize the combination of *lhd* heterozygote × Minghui-77 (*indica* × *indica*) for the gene mapping. Although the problem of F_1 sterility was solved in this combination, the polymorphism between the parents was low, bringing about the difficulty of screening polymorphic markers. Hence, how to find a balance between genetic polymorphism and F_1 sterility should be critically considered in practical studies.

In this study, the fact that *LHD*(*t*) is near to the region of centromere also adds the difficulty of finding closely linked markers. The published molecular markers in this region are very rare and the degree of polymorphism in this region is also relatively low in general. We utilized the published rice genomic sequence of the region near to *LHD*(*t*) on chromosome 10 and the methods of bioinformatics to search for and develop new SSR markers. Among the 9 SSR loci detected, 3% or 33.3% exhibited polymorphism. Although the proportion of polymorphic markers is 46.2% lower than the average value (this

Table 3 List of the SSR primers designed according to the published genomic sequences

Primer name	Primer sequence	SSR motif	Annealing temperature/℃	Band size (bp)
SSR1	F: 5′-ATGATTTCCCCGTTCTCTTCC-3′ R: 5′-TGAAATGGTTGGTGCGTGAT-3′	$(AT)_{28}$	55	192
SSR4	F: 5′-TGCCTCCTTTTTCTCTCCTT-3′ R: 5′-TTTTGACTTCTTGTAGCCCCC-3′	$(TC)_{19}$	53	160
SSR5	F: 5′-GTGGCTTCCGACATATAGTTAC-3′ R: 5′-GCTGGGTATTCCTAAATCTACAA-3′	$(AT)_{34}$	52	216

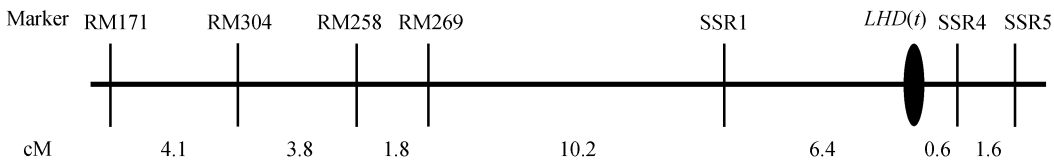

Fig. 3. Linkage map of the region where *LHD*(*t*) is located on chromosome 10.

also reflects that the polymorphism level in this region is really lower), it still demonstrates that the efficiency of our method for searching for polymorphic markers is quite high. Therefore, the method would be useful for other similar gene mapping efforts in rice.

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