

Linkage of AFLP markers to *lhd 1*, a recessive heterochronic gene in Italian ryegrass

Zhen-Sheng Gao, Shinichi Sugita, Seishi Ikeda, Hong-Wei Cai, Tohru Sasaki, and George H. Liang

Abstract: A mutant, *leafy head 1* (*lhd 1*), was discovered in Japan from the progeny of Italian ryegrass (*Lolium multiflorum* Lam.) 'Nioudachi' and local line 'Aichikei #3'. Compared with normal plants, the mutant plant is a dwarf with a larger number of internodes per stem, shorter internodes, and smaller leaves. The plants also head later in the season. Aerial roots are usually produced from the stem nodes during rainy seasons. In characterizing *lhd 1*, it was found to have many branches with small leaves and many small panicles on the upper part of the plant. Panicle development was severely disturbed in *lhd 1* mutants, and the number of leaves produced in the vegetative phase was nearly twice that produced in the wild-type counterpart. The *lhd 1* mutant appears to be a heterochronic mutation that is able to extend the vegetative period during development. The frequency of mutants in segregating populations indicated that *lhd 1* is a recessive allele. To determine the linkage relationship between the *lhd 1* gene and AFLP markers, 768 primer combinations were screened for polymorphisms using bulked segregant analyses in two populations with 316 and 30 plants, respectively. Five AFLP markers were linked to the *lhd 1* locus. E3/M41-1 and E16/M14-2 cosegregated with *lhd 1*. E16/M14-1 and E30/M10-1 flanked the gene at a distance of 0.3 cM and E30/M14-2 was linked to *lhd 1* at a distance of 0.6 cM.

Key words: *Lolium multiflorum*, leafy head mutant, mapping.

Résumé : Un mutant, nommé « *leafy head 1* » (*lhd 1*), a été trouvé au Japon parmi la progéniture du cultivar 'Nioudachi' et d'une lignée locale, 'Aichikei #3', de l'ivraie multiflore (*Lolium multiflorum* Lam.). Par rapport à des plantes normales, le mutant est nain, présente un plus grand nombre d'entre-noeuds par tige, les entre-noeuds sont plus courts et des feuilles plus petites. Les plants du mutant fleurissent aussi plus tardivement. Des racines aériennes sont habituellement produites à partir des nœuds de la tige au cours des saisons de pluie. Au cours de la caractérisation de *lhd 1*, il a été noté qu'il présentait plusieurs branches avec de petites feuilles et plusieurs petites panicules produites dans la partie supérieure de la plante. Le développement de la panicule était très anormal chez *lhd 1* et le nombre de feuilles produites au cours de la phase végétative était près du double du nombre produit chez le type sauvage. *lhd 1* semble être un mutant hétérochronique, chez lequel la phase végétative du développement est prolongée. La fréquence de mutants au sein de populations en ségrégation indique que l'allèle *lhd 1* est récessif. Afin de déterminer la liaison entre *lhd 1* et des marqueurs AFLP, 768 combinaisons d'amorces ont été examinées pour du polymorphisme par analyse de ségrégants en mélange (« bulked segregant analysis ») au sein de deux populations comptant respectivement 316 et 30 plantes. Cinq marqueurs AFLP liés au locus *lhd 1* ont été identifiés. Les marqueurs E3/M41-1 et E16/M14-2 ont montré une parfaite co-ségrégation avec *lhd 1*. Les marqueurs E16/M14-1 et E30/M10-1 bordaient le gène à 0,3 cM tandis que le marqueur E30/M14-2 était situé à 0,6 cM de *lhd 1*.

Mots clés: *Lolium multiflorum*, mutant « *leafy head* », cartographie.

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Introduction

Heterochronic mutations have been identified that affect vegetative development and markedly alter shoot architecture in maize. These include the dominant mutations *Teopod1* (*Tp1*), *Tp2*, *Tp3*, and *Corngrass*, all of which prolong the juvenile phase and change plant organization (Galinat 1966; Poethig 1988; Dudley and Poethig 1991). In *Tp* mutants, juvenile leaves are ectopically expressed during the adult plant phase. Furthermore, several mutations in maize modifying phyllotaxy and the plastochron, both of which also affect the shoot architecture, have been reported. The maize *abphyl* mutant shows variable phyllotaxy, including helical, decussate, and bijugate arrangements associated with an enlarged apical meristem (Greyson and Walden 1972; Greyson et al. 1978). Likewise, the rice *shoot organization 1* (*sho1*) mutant has been reported; it exhibits random phyllotaxis, a short plastochron, and aberrant leaves (Tamura et al. 1992). A modified plastochron may cause heterochrony, as well as the alteration of vegetative shoot architecture. For example, a recessive mutation, *plastochron 1*, shortened the plastochron, but elongated the vegetative phase in rice (Itoh et al. 1998). The phenotype of *plastochron 1* plants was very similar to the *leafy head* rice mutant reported by Hu (1961). These mutants were induced either by γ -ray irradiation or by the chemical mutagen *N*-methyl-*N*-nitrosourea (MNU).

We discovered the new heterochronic mutation in the Italian ryegrass (*Lolium multiflorum* Lam.) 'Nioudachi' and the local line 'Aichikei #3' in Japan. Morphological and organogenic traits of the new mutant were similar to those observed in rice *leafy head* and the *plastochron 1* mutations. Because it is productive and tolerant to local environmental conditions, the grass is an important source for livestock feed. Discovery of the mutant in the grass raises fundamental questions concerning the regulation and mechanism of transition for developmental phases, and provides amenable material for genetic analysis because of its short life cycle.

In this paper, we report the phenotypic characteristics of *leafy head 1* (*lhd 1*) in ryegrass and its genetic mapping in relation to five AFLP marker loci.

Materials and methods

Plant materials and measurement

Two *leafy head* mutants from Italian ryegrass were identified: one appeared from the population of a cross between randomly selected 'Nioudachi' plants (designated plants A $\times$ B and C $\times$ D) (Table 1) and the other was from a local line, 'Aichikei #3', in Japan. There were a total of 316 plants from AB group and 231 from CD group, plus the original A and B plants for a total of 549.

The *leafy head* mutants, their original parents (by vegetative propagation), and the segregating progeny (S_1 , S_2 and F_1 , F_2) of the mutants were planted in a field at a density of 75 $\times$ 40 cm, as well as in pots in a greenhouse. The plants in the greenhouse were grown under a 16 h light (28°C) : 8 h dark (22°C) photoperiod using sodium lights. The genotypes of the S_1 and F_1 families were identified from phenotypes of S_2 and F_2 lines (over 20 plants per line) in the greenhouse. Because Italian ryegrass is a cross-pollinated forage crop

and different plants may have different genetic backgrounds, we used the wild-type siblings of each mutant as controls for characterizing mutant phenotypes. When a new leaf blade had emerged completely from the sheath of the previous leaf, the mutant plants and the wild-type plant were counted for number of leaves in each plant. Other agronomic traits such as leaf width and length, stem diameter, plant height, tiller number, and length of internodes were also investigated.

Preparation of genomic DNA and AFLP reactions

Approximately 0.15 g of young leaves from both mutant and normal plants were harvested and ground in liquid nitrogen to obtain a fine powder. The cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1985) was used to isolate genomic DNA. DNAs were taken from two populations (Table 1) for a total sample of 549 plants.

The AFLP method established by Vos et al. (1995) was slightly modified for analyzing plants with large genomes. Template preparation was performed according to the protocol provided with the AFLP Core Reagent Kit from Life Technologies (Bethesda, Md.). Total genomic DNA (100 ng) was digested with 2.5 U each of *Eco*RI and *Mse*I for 12 h at 25°C. DNA fragments and *Eco*RI and *Mse*I adapters were ligated with T_4 DNA ligase for 2 h at 20°C. The resulting DNA was used as primary template DNA in AFLP analysis. A primer pair based on the sequences of the *Eco*RI and *Mse*I adapters was used for preamplification. The selective amplification was carried out using primers with three additional selective nucleotides; the primer sequences (5'→3') were as follows: E3, IRD800-GACTGCGTACCAATTCAAG; E16, IRD700-GACTGCGTACCAATTCACC; E30, IRD700-GACTGCGTACCAATTCTCT; M10, GATGAGTCCTGAGTAACT; M14, GATGAGTCCTGAGTAACT; and M41, GATGAGTCCTGAGTAACTGA. In each case, the *Eco*RI primer was labeled at the 5' end with fluorescence dye, either IRD700 or 800. The selective PCR was performed in a total volume of 10 μ L, which comprised 30 ng *Mse*I primer, 15 ng IRD700-labeled *Eco*RI primer and 5'-labeled *Eco*RI primer (IRD700:IRD800 = 40:60 v/v), 0.2 mM dNTP mix (MBI Fermentas, Hanover, Md.), and 0.8 U *Taq* DNA polymerase (Qiagen, Valencia, Calif.) and its corresponding reaction buffer. Conditions for running the PCR were set according to Vos et al. (1995) with minor modifications. An initial denaturation step was run at 94°C for 2 min, followed by 94°C for 20 s; 65°C for 30 s; and 72°C for 120 s; 11 cycles in which the annealing temperature was decreased by 0.7°C per cycle; 22 cycles at 94°C for 20 s and 56°C for 30 s; 72°C for 2 min; and finally an ultimate extension at 60°C for 30 min. All PCRs were carried out in a Gene AMP PCR System 9700 (Perkin Elmer, Foster City, Calif.). An equal volume of formamide loading buffer (95% formamide v/v, 10 mM EDTA (pH 8), 0.1% basic fuchsin, and 0.01% bromophenol blue) was added and the samples were denatured at 94°C for 5 min. One microlitre of each sample were then loaded onto a 25-cm denaturing polyacrylamide gel (8%) (Long Ranger, FMC Biozym, Hess. Oldendorf, Germany), which had been preheated for 30 min. Electrophoresis was conducted in the 1.0 Long Run TBE buffer at 1500 V, 40 W, 40 mA, and 48°C. Banding patterns were examined using the LI-COR DNA Sequencer 4200 (LI-COR, Lincoln,

Table 1. The segregation of normal and *leafy head* in different cross combinations.

Cross type or combinations	Normal		<i>lhd</i>		χ^2 (3:1 or 1:1*)	P value
	Homo.	Hete.	Homo.	Total		
A × A (Self)	15	23	13	51	0.0065	0.95–0.90
B × B (Self)	5	7	4	16	0.0000	>0.95
A × B	66	122	61	249	0.0335	0.90–0.80
C × C (Self)	7	15	8	30	0.0444	0.90–0.80
D × D (Self)	0	0	8	8	0.0000	>0.95
C × D	14	42	33	89	5.9438*	0.10–0.02
D × C	0	47	57	104	0.9615*	0.30–0.20
C×D and D×C	14	89	90	193	0.8756*	0.30–0.20

Note: The original parents, A, B, and C, were normal type, and D was *leafy head* mutant; Homo., homozygous genotype; Hete., heterozygous genotype.

Nebr.) with standard protocol and subsequently photographed.

The bulked DNA segregant analysis was used to establish four bulked DNA samples of 20 plants from each of two F₂ populations, A × B and C × D. Genomic DNA isolated from 20 *lhd 1* plants and 20 wild-type plants (homozygous) were mixed in equal proportions to constitute the mutant- and normal-type bulks for bulk segregant analysis.

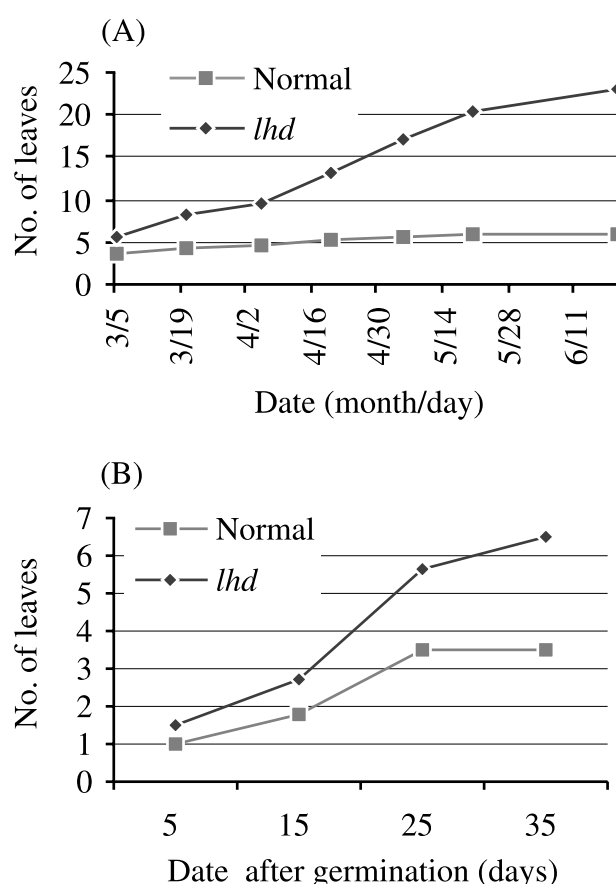
Genetic linkage analysis

AFLP markers that displayed differences among bulks were analyzed for homozygous, heterozygous, and leafy mutant genotypes in the F₁ populations. Linkage distances between dominant AFLP markers and the *lhd 1* locus were calculated, the order of loci determined, and the inter-locus map distances computed. To identify linkage groups, pairwise comparisons and grouping of markers were carried out using the MAPMAKER Macintosh version 2.0 program (Lander et al. 1987) (minimum LOD score = 3.0, maximum theta = 0.45). The arrangements of markers in linkage groups were determined using the “order” and “try” commands.

Results and discussion

Characteristics of *leafy head* mutant

One week after germination, the mutant plants showed obvious increases in leaf number (Fig. 1). During the vegetative growth phase, the mutant had more than twice as many leaves as the normal plants (Tables 2 and 3). Also, the leaf blades on the mutant plants were shorter and narrower than the normal ones. There was a reduction of the internodal length averaging 3 cm and a clear increase in the number of internodes (Fig. 2). After flowering, the average number of internodes was about three times as many as in normal plants (Fig. 2). In addition to tillering from the crown, upper nodes branched out similar to tillering. Later in the vegetative phase, and at the beginning of the heading stage, stems of the mutants were more slender and plants were shorter than normal (Tables 2 and 3). Many lateral or aerial roots developed from nodes on the stem, especially during the rainy season, making asexual reproduction using stem cuttings much easier. In contrast to the normal plants, the vegetative phase of these *lhd 1* mutants was extended by 1–2 weeks before entering the reproductive phase (Table 3).

Fig. 1. Number of leaves in *leafy head* mutant and normal plants.

Development of panicles was impaired and some degenerated panicles appeared in mutant plants (Fig. 4). Some plants had normal heading but panicles were two-thirds smaller than those of the normal plants. In some cases, *lhd 1* plants had primordia on the primary rachis converted into vegetative shoots, where the ectopic shoots continued to grow and repeated the process of initiating panicle development, which formed new rachis/branches again. Thus, formation of vegetative and reproductive phases occurred nearly at the same time. Some *lhd 1* plants did not produce panicles; instead, clumps of leaves corresponding to the panicles were formed.

Table 2. The agronomic traits of *leafy head* mutant and normal plants during vegetation period.

Line name	Pheno-type	Leaf no.	Leaf width (mm)	Leaf length (mm)	Stem diameter (mm)*	Plant height (cm)	Tiller no.
ABG-51	Normal	6.0	11.95	31.09	5.06	75.8	127.0
abg-51	<i>lhd</i>	13.2	5.27	21.81	3.75	56.8	151.1
3D-56	Normal	5.1	11.08	30.15	4.14	59.5	18.0
3d-33	<i>lhd</i>	12.0	5.84	20.53	3.13	47.3	25.2
4D-47	Normal	5.4	11.22	30.62	4.17	73.9	24.6
4d-57	<i>lhd</i>	12.0	5.55	23.26	3.22	64.4	39.1

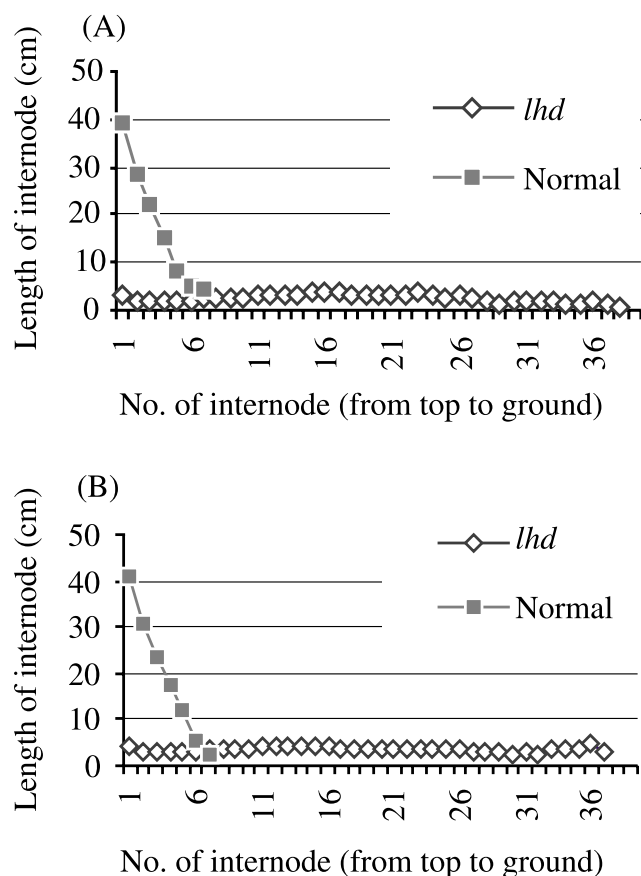
Note: Vegetation period, 20 April 2000.

*Including blade sheath.

Table 3. The agronomic traits of *leafy head* mutant and normal plants at maturation.

Line name	Phenotype	Leaf no.	Leaf width (mm)	Leaf length (mm)	Stem diameter (mm)	Plant height (cm)	Tiller no.	Heading date (month/day)
1D-22	Normal	5.3	7.24	29.78	3.96	101.1	26.0	5/06–5/10
1d-8	<i>lhd</i>	14.1	3.54	16.64	2.53	52.1	43.3	5/11–5/19
3D-56	Normal	5.7	7.71	30.91	4.01	121.0	26.9	5/01–5/13
3d-33	<i>lhd</i>	22.5	3.40	16.97	2.40	75.6	46.4	5/05–5/30
4D-47	Normal	6.1	6.67	29.85	3.98	168.8	29.2	5/01–5/08
4d-57	<i>lhd</i>	23.8	3.13	18.50	2.40	93.1	64.2	5/05–5/13

Note: Maturation date, 20 June 2000.

Fig. 2. Length of internodes in *leafy head* mutant and normal plants. (A) Field-grown plants. (B) Greenhouse-grown plants.

Inheritance analysis of *leafy head* mutant

The *lhd 1* mutant naturally occurred at frequencies of 3.5–5.0% and 1.3–5.0%, respectively, in ‘Nioudachi’, and ‘Aichikei #3’. Among 249 randomly grown F_1 plants of the cross A $\times$ B from ‘Nioudachi’, there were 188 normal plants and 61 *lhd 1* mutants (Fig. 3). Among the S_1 progeny from plant A there were 38 normal plants and 13 mutants. Similarly, among 16 S_1 plants from plant B there were 12 normal and 4 mutant plants (Table 1). The segregation ratios for normal to mutant after pooling the data were very close to the expected 3:1 ($\chi^2 = 0.0335$, $P = 0.80$ – 0.90 , $df = 1$). Both homozygous and heterozygous genotypes were identified from the S_2 and F_2 populations derived by selfing the S_1 and F_1 plants, respectively. Results showed that the ratio among normal homozygotes, normal heterozygotes, and mutant homozygotes was very close to the expected 1:2:1 assuming a one-gene model ($\chi^2 = 0.86$, $P = 0.50$ – 0.75 , $df = 2$). Thus, the *lhd 1* mutation is under the control of a single recessive allele. Crossing different plants from ‘Nioudachi’ also showed similar results. For example, S_1 progeny from normal plant C showed 22 normal : 8 mutant plants and progeny from the cross between mutant plant D and C was composed of 47 normal plants and 57 mutants, or 1:1 ($\chi^2 = 0.96$, $P = 0.2$ – 0.3 , $df = 1$), which was equivalent to the backcross data between a normal heterozygous plant and a recessive mutant and further indicates that *lhd 1* is a recessive mutant under the control of a single allele.

Linkage analysis

Linkage relationships between the *lhd 1* allele and the AFLP markers were determined using bulked segregant analysis from two populations with 316 plants from F_1 (A $\times$ B), S_1 (A), and S_1 (B) and 30 plants from S_1 (C), respectively. There were 768 primer combinations screened from both populations. Among 40 identified polymorphic

Fig. 3. Phenotypes of normal and *lhd 1* (arrowhead) plants in field.



Fig. 4. Phenotypes of panicles in normal and *lhd 1* plants. (A) Clump of leaves corresponds to a panicle. (B) A truncated panicle is formed with one vegetative shoot and enlarged bracts. (C) Normal panicles.



fragments, linkages between the *lhd 1* allele and fragments from four primer combinations, E3/M41-1, E16/M14-1 (E16/M14-2), E30/M10-1, and E30/M1-2 were detected (Fig. 6). Linkage analyses indicated that E3/M41-1 and E16/M14-2 cosegregated with the *lhd 1* allele, E16/M14-1 and E30/M10-1 flanked the locus at a distance of 0.3 cM, and E30/M14-2 was linked to *lhd 1* at a distance of 0.6 cM. The size of AFLP fragment, measured in base pairs, is shown in Fig. 5. A portion of an AFLP gel illustrates the size of E30/M10-1 and E3/M41-1. Other markers were measured in

a similar manner (E30/M14-2, E16/M14-1, and E16/M14-2 fragments were 425 bp, 240 bp, and 114 bp, respectively).

An F_1 population consisting of 103 normal plants and 90 mutants was examined with the linked AFLP markers. Eighty-nine normal heterozygous plants and 90 mutants displayed markers E30/M14-2, E30/M10-1, and E16/M14-2; 14 normal, homozygous plants lacked these bands. All but one of 103 normal plants had E3/M41-1, whereas all 90 mutant plants lacked the bands associated with E3/M41-1 and E16/M14-1. These results further demonstrate that certain

Fig. 5. Portion of an AFLP gel showing the inheritance of AFLP fragments. DNA from the parents, the bulks, and the segregating F₂ individuals scored for the AFLP marker E3/M41-1 (375 bp) and E30/M10-1 (595 bp). M, molecular weight standard; P₁, normal type parent; P₂, *lhd 1* mutant; d20, mutant F₂ bulk (20 plants); D20, normal F₂ bulk (20 homozygous normal-type plants); and lanes abg, ABG, d, and D, segregating F₂ individuals. At the top of each AFLP lane, the genotype of the corresponding F₂ plants are indicated as verified in the F₃ families: DD, homozygous normal type; dd, *lhd 1* mutant; Dd, heterozygous normal-type plant.

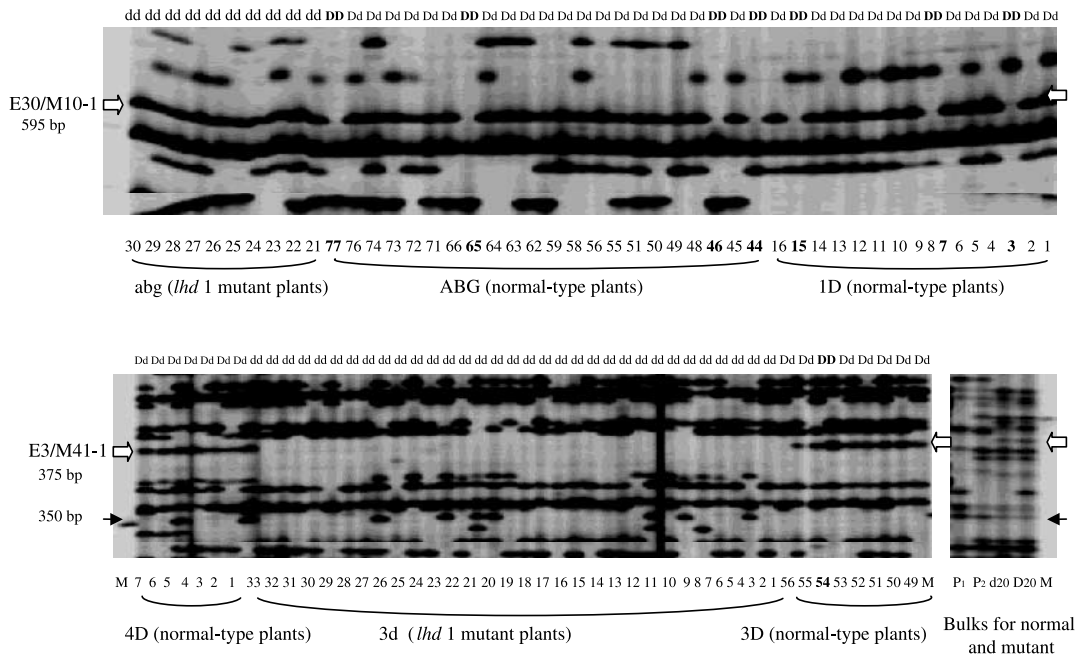
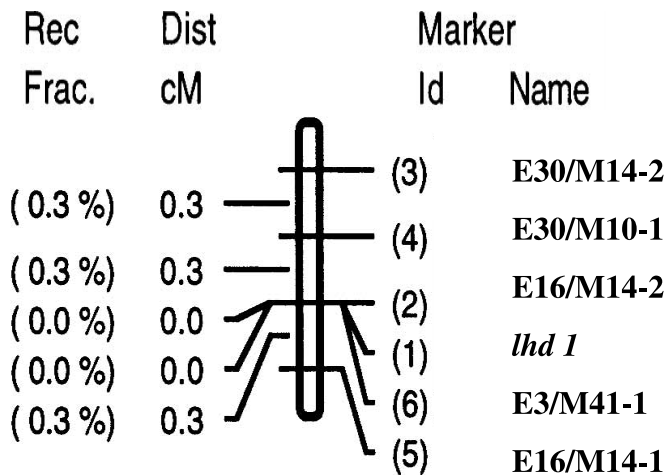


Fig. 6. Linkage map of the *lhd 1* genomic region. The map was constructed with the program MAPMAKER Macintosh version



AFLP markers and the mutant allele, *lhd 1*, were tightly linked. E3/M41-1 and E16/M14-1 markers can be used to distinguish normal, homozygous plants from mutant plants or from the heterozygous normal plants, and E30/M14-2, E30/M10-1, and E16/M14-2 markers can be used to distinguish mutant plants from normal plants.

The genus *Lolium* (2n = 14) includes eight species. Two of the species are major fodder crops that have been cultivated for grassland in temperate regions: perennial ryegrass (*L. perenne*) for grazing and turf, and Italian ryegrass (*L. multiflorum*) for hay and silage. A linkage map from an

interspecific cross (*L. perenne* × *L. multiflorum*) has been constructed that included 106 markers covering a total map distance of 692 cM (Hayward et al. 1998). More recently a genetic map was constructed based on 463 AFLP markers using 17 primer pairs, 3 isozymes, and 5 EST markers (Bert et al. 1999). The map from Bert et al. (1999) covers 930 cM in seven linkage groups.

From 80 accessions of Italian ryegrass, we found the *lhd 1* mutants only from ‘Nioudachi’ and ‘Aichike #3’, with a frequency of 3.5–5.0% and 1.3–5.0%, respectively. *lhd 1* is a recessive mutation under the control of a single allele. To develop markers for leafy head mutant, we demonstrated that five AFLP markers were tightly linked to the *lhd 1* locus. The same five AFLP markers also were used to identify the *lhd 1* gene in another population, indicating that all five markers were consistently tightly linked to the *lhd 1* locus. However, it is not known to which linkage group the *lhd 1* mutant belongs.

During post-embryonic development, the shoot apex of higher plants passes through a juvenile vegetative phase, an adult vegetative phase, and a reproductive phase. In some plants, reproduction is the last phase in the life of the shoot; in others, the growth of the shoot is perpetuated by a lateral vegetative meristem after the terminal meristem becomes reproductive, whereas in some species the primary meristem remains permanently vegetative and only lateral shoots form reproductive structures (Poethig 1990). The phenotypic similarity among *Tp* mutants in maize, *leafy head* mutant in rice, and *lhd 1* in ryegrass suggests that they are under the control of orthologous genes, i.e., homologous genes that have become differentiated in different species that are derived from

a common ancestral species. Identification, sequencing, and comparison of those loci should provide more evidence as to their origin, function, and structure.

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