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Molecular mapping of a gene '*ld*(t)' controlling cleistogamy in rice

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Abstract Cleistogamy is the self-pollination within closed spikelets and is expected to be a useful genetic tool for prevention of possible gene transfer in transgenic crops, for maintenance of genetic purity in autogamous crops, and for increased tolerance to biotic and abiotic stresses. Mapping of the gene *ld*(t), which is responsible for lack of lodicules inside spikelets and causes cleistogamy, was carried out using F_2 and F_3 populations derived from a cleistogamous (CL) mutant CL-SNU × Milyang 23 cross. A number of STS markers along chromosomes were developed and bulked segregant analysis was adopted for preliminary mapping. The results showed that the *ld*(t) was located at the end region of chromosome 1L, flanked by *S01178b* (an STS marker developed for the locus at 178 cM based on the rice genetic map reported by Japanese Rice Genome Project) at 0.8 cM and co-segregated with *S01181a* and *S01181b* (an STS marker developed for the locus at 181 cM).

Introduction

Cleistogamy is the self-pollination and self-fertilization without opening of the floret and has been reported in 287 species from 56 families (Lord 1981), and in 19 of the 60 grass tribes (Connor 1979). Lord (1981) suggested four groups of CL plants: (1) pre-anthesis cleistogamy, in which bud pollination occurs followed by anthesis; (2) pseudo-cleistogamy, in which no morphological differences occur between cleistogamous (CL) and chasmogamous (CH) flowers other than a lack of expansion and anthesis in CL flowers and which could be caused by environmental factors; (3) complete cleistogamy, in which some species produce only CL flowers; and (4) "true" cleistogamy, in which various floral dimorphisms result from divergent developmental pathways in a single species or individual. Cleistogamy has been reported in several cereal crops including genus *Avena* (Connor 1979), genus *Sorghum* (Merwine et al. 1981), barley (Kurauchi et al. 1993; Reid 1985), and wheat (Sethi and Chhabra 1990). In rice, Nagao and Takahashi (1963) identified a CL mutant with compact panicles, small spikelets, and relatively short height. The mutant had abnormal glumes in which the lower parts could not be differentiated, and the lemma and palea were united. As a result, the spikelets were tightly held in at flowering time while having normally swelled lodicules. The gamadiness was reported to cause the cleistogamy in rice due to incomplete exertion of panicles from the flag leaf-sheath and subsequent CL selfing without opening of spikelets within the flag leaf-sheath (Heu and Shretha 1983; Maekawa 1983). Li et al. (2002) reported that a CL line, CL-01, had less-developed lodicules and thus the florets did not open during pollination and fertilization. Won et al. (1998) identified a CL mutant among rice collections and reported that the lack of lodicules inside the spikelets was the direct cause of cleistogamy of the mutant and the CL phenotype was stable under different air temperatures, day lengths and fertilizer levels. During anthesis in rice, spikelets opened by splitting the joint between lemma and palea as lodicules swell (Hoshikawa 1993). However, spikelets of the lodiculeless mutant could not open and remain closed during anthesis.

Recently, cleistogamy has been focused on as a useful genetic tool for prevention of possible gene transfer in transgenic crops (Daniel 2002), for maintaining genetic purity of autogamous crops across generations (Saxena et al. 1993), and for other advantages such as tolerance to biotic and abiotic stresses (Hughes 1996; Li et al. 2002).

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Genetic control of cleistogamy has been studied in several species. Single recessive genes for the cleistogamy have been reported in pigeon pea (Saxena et al. 1993), in Japanese two-rowed barley cultivars (Kurauchi et al. 1993), in durum wheat (Chhabra and Sethi 1991). Chhabra and Sethi (1991) further noted that cleistogamy in durum wheat was associated with poorly developed lodicules and a stiff perianth, and that flower phenotypes were unaffected by environmental conditions. Two independent genes were reported to be involved in the cleistogamy in sorghum (Merwine et al. 1981) and in early maturing cultivars of Japanese soybean (Takahashi et al. 2001). Three independent recessive genes were also reported for the cleistogamy in wheat (Fujita et al. 2005). Turuspekov et al. (2004, 2005) reported that the expression of cleistogamy in barley was controlled by two tightly linked genes or different alleles of the same gene, *cly*1 and *Cly*2, which were located on the same region of chromosome 2HL, and they postulated a hypothetical model of the regulation for the type of flowering in barley. In rice, Nagao and Takahashi (1963) reported that a single recessive gene *d*7 determined the CL mutant phenotype and that the gene was possibly linked to the gene *d*6 on chromosome 4. Won et al. (1998) reported that lack of lodicules of the CL mutant was under the control of a single recessive gene, which was designated as *lodiculeless spikelet*(t), *ld*(t). In this study, the gene *ld*(t) which is responsible for the cleistogamy was located on the molecular map. This is the first report on the mapping of a cleistogamy gene in rice.

Fig. 1 Panicles and dehulled spikelets of the wild type (**a**) and a cleistogamous (**b**) line. *Arrows* indicate the lodicule which is present only in the wild-type spikelet. Spikelets of wild-type panicle are opened during flowering, while spikelets of cleistogamous panicle stay unopened

Materials and methods

Plant materials and phenotype evaluation

The cleistogamy mutant line CL-SNU (Won et al. 1998), which was identified as a natural mutant from *japonica* rice germplasms introduced from worldwide and genetically fixed, was crossed with Milyang23, a genetically divergent *tongil*-type rice [a high yielding plant type derived from an *indica* × *japonica* cross in Korea (Chung and Heu 1991) and similar to *indica* in its genetic make-up], and though the F_1 plants were semi-sterile, an F_2 population was produced and used for mapping. Parents and their F_2 progeny of 396 plants were planted in one plant per hill in the paddy field of the Experimental Farm, Seoul National University, Suwon. Leaves were collected from each plant at the maximum tillering stage and used for DNA extraction. During anthesis at heading stage, each plant was identified as CL or CH type by observing panicles and spikelets (Fig. 1); plants which had spikelets opened were classified as CH type, whereas plants which had spikelets unopened were regarded as CL type, and subsequently for CL type plants, five spikelets from each plant were further examined for the absence of lodicules inside the spikelet. The genotype (homozygous or heterozygous) of each of CH type F_2 plants was determined by progeny testing using F_3 plants which were raised from the seeds harvested from each of CH type F_2 plants.

DNA marker analysis and mapping procedure

DNA was extracted from leaves of each F_2 plant and the parents according to Causse et al. (1994). Equal amounts of DNA from each of six CL- and CH-homozygous plants were used to construct bulks from F_2 segregants. Bulked segregant analysis (BSA) (Michelmore et al. 1991) was adopted for preliminary mapping. A number of STS markers for the entire genome were developed by designing primers based on the differences in DNA sequences between *indica* and *japonica* rices, which are available at http://www.ncbi.nlm.nih.gov/ (for *indica*) and at http://www.rgp.dna.affrc.go.jp/ (for *japonica*), respectively. After BSA, location of the *ld*(t) gene was determined, and consequently, fine mapping was conducted with neighboring STS markers. Primer pairs of STS markers used for fine mapping were S01164F (5′-GGGAC-CAAGACCTTTCCAAT-3′) and S01164R (5′-CG

GAAGCAGGAGAATCGTAG-3′), S01167F (5′-CTAGGCATTCGAAGGCAATC-3′) and S01167R (5′-TCAAAAAGCTGTGCCTCTCA-3′), S01178aF (5′-CCCGATCGAAAGCATTGTAA-3′) and S01178aR (5′-AGACACGGTTGTGAGCCTCT-3′), S01178bF (5′-TTTCTAGCATTGCCCATATTCA-3′) and S01178bR (5′-TTCTTTACGCTGCCATGACG-3′), S01181aF (5′-CCTTTGTCACGCAGGTAGGT-3′) and S01181aR (5′-CCAACCACTGATCCATTCCT-3′), and S01181bF (5′-AGATCGATCGAGCAACGAAC-3′) and S01181bR (5′-CCCAACCTGCATCGATTATT-3′). PCR was performed in a reaction volume of 20 μl containing 40 ng of template DNA, 0.2 μM of each primer, 200 μM of each dNTP, 10 mM Tris–Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.5 U of *Taq* DNA polymerase. Amplification was carried out in a PTC100 96U thermocycler (MJ Research, USA) as follows: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and 5 min at 72°C for final extension. PCR products were separated in 3.0% agrose gels. Linkage analysis was conducted using a computer software "MAPMAKER version 3.0" (Lander et al. 1987). Map distances were estimated by the Kosambi function (Kosambi 1944).

Results

Phenotype of parents and genetic segregation

Spikelets of the CL-SNU remained closed during flowering, while spikelets of wild-type variety opened and anthers protruded outside the spikelets, as shown in Fig. 1. The mutant line CL-SNU and all of the CL type plants in F_2 completely lacked lodicules which are typically located just below the base of the stamens on the lemma side and push the lemma outward leading to the opening of the spikelet during anthesis in CH type plants. Thereby, CL or CH type plants were easily distinguished in the F_2 population of CL-SNU/Milyang23 cross through observation of physical appearance of panicles and spikelets. The segregation ratio in F_2 was fitted to 1 CH (homozygous) plants:2 CH (heterozygous) plants:1 CL (homozygous) plants (Table 1). Here, CH homozygous or CH heterozygous genotypes of F_2 plants were determined by progeny testing in F_3 plants using the seeds harvested from each of the CH plants in F_2. All of the STS markers surrounding the target locus were also segregated into 1:2:1 ratio, as expected as in Table 1.

Genetic mapping of the gene *ld*(t)

Four to five STS markers belonging to each of the 12 rice chromosomes were used for BSA (Michelmore et al. 1991) to identify the chromosomal locus of the *ld*(t) gene. The results revealed that the *ld*(t) gene was located at the end region of chromosome 1L. Six of STS markers originally designed for the target region containing *ld*(t) were mapped (Fig. 2) using an F_2 population of 396 plants derived from CL-SNU/Milyang23 cross. The order of markers in this region of chromosome 1 is the same as in the frame map reported by the Japanese Rice Genome Project (JRGP) (http://www.rgp.dna.affrc.go.jp/). However, total distance from the locus *S01164* to the locus *S01181* in this study was much shorter (9.3 cM) than that in the map developed by JRGP in which the distance between the two loci was 17 cM. The marker loci closest to the *ld*(t) gene were *S01178b* and *S01181a*/*S01181b* at a distance of 0.8 cM and 0 cM, respectively. The *S01181a* and *S01181b* co-segregated with *ld*(t) without recombinant between them. In addition, no recombinant was found between *S01181a* and *S01181b* though there was a 169 kb distance between two markers. Figure 3 shows an example of co-segregation of the *ld*(t) gene and an STS marker *S01181a* in the F_2 population of CL-SNU/M.23 cross.

Table 1 Segregation of CL genotype and flanking markers surrounding the cleistogamy locus '*ld*(t)' at the bottom-end region of chromosome 1L in F_2 of CL-SNU/Milyang23 cross

Marker or cleistogamy	Number of F_2 plants in each genotypic class				X^2 (1:2:1)	*P*
	M23[b]	Het	Clei	Total		
S01164[a]	85		107	393	2.668	0.50–0.25
S01168	81	205	101	387	3.434	0.25–0.10
*S01178*a	81	205	110	396	4.742	0.10–0.05
*S01178*b	81	207	108	396	4.500	0.25–0.10
ld(t)—cleistogamy	85	205	106	396	2.722	0.50–0.25
*S01181*a	82	204	105	391	3.446	0.25–0.10
*S01181*b	83	204	105	392	3.122	0.25–0.10

[a]Nomenclature of STS markers: the first two digits after S indicate the chromosome number, and the latter three digits denote cM based on the map constructed by the Japanese Rice Genome Project. When more than two markers originated in the same cM region, though physically distant, an alphabet was affixed to the marker name

[b]The genotypes, normal (CH) homozygotes and heterozygotes, were determined by the progeny test in F_3. *M23* normal (CH) homozygotes, *Het* heterozygotes, *Clei* cleistogamy homozygotes

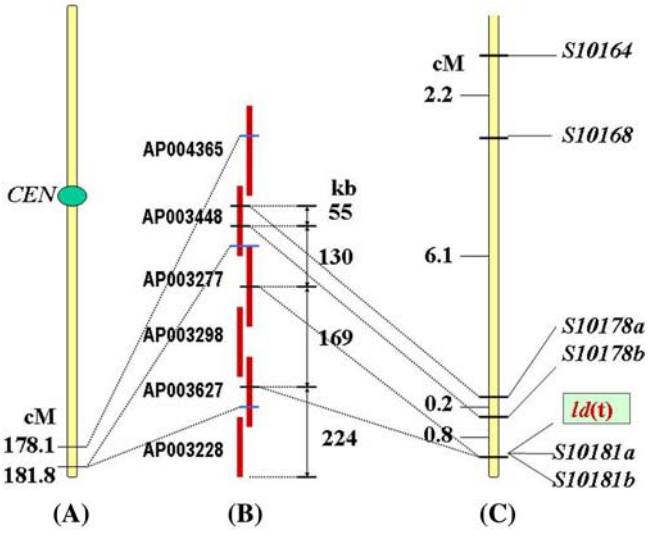

Fig. 2 Linkage map of the end region of chromosome 1L of rice showing the location of the '*ld*(t)' and linked markers. **a** Chromosome 1 reported by JRGP, *CEN* centromere. **b** Contig map of PAC clones corresponding to 178.1–181.8 cM of JRGP map. Physical length of the target region containing *ld*(t) locus from *S10178b* to the end of chromosome 1L is approximately 520 kb. **c** Linkage map surrounding the *ld*(t) locus constructed using 396 F_2 plants

Discussion

Cleistogamy in rice and its utilization

Cleistogamy is a powerful genetic tool for prevention of possible gene transfer in autogamous transgenic plants through blocking of pollen transfer to neighboring plants or to sympatric species capable of inter-mating (Daniel 2002). Also, cleistogamy will be useful to maintain the genetic purity of cultivars of autogamous crops across generations (Saxena et al. 1993) and to increase the seed setting under unfavorable environments during anthesis (Li et al. 2002). Several mechanisms causing cleistogamy have been reported in rice: sheathed panicle (Heu and Shretha 1983; Maekawa 1983), less-developed lodicules (Li et al. 2002), floral deformity (Nagao and Takahashi 1963), and lack of lodicules (Won et al. 1998). Among them, the cleistogamy caused by lack of lodicules (Won et al. 1998) is more likely to be applicable to breeding programs as well as transgenic plants, because it was controlled by a single recessive gene *ld*(t) and might have no deleterious effect on agronomic traits such as days to heading, culm length, panicle length, number of panicles and grain fertility as reported by Won et al. (1998), in which they observed and compared the traits between cleistogamous and non-cleistogamous plants in F_2 populations derived from two crosses between the cleistogamy line and two regular cultivars. Cleistogamy directly caused by lack of lodicules has also been reported in grasses such as *Thyridolepis* (Blake 1972, cited in Connor 1979) and *Calyptochloa* (Hubbard 1933, cited in Connor 1979).

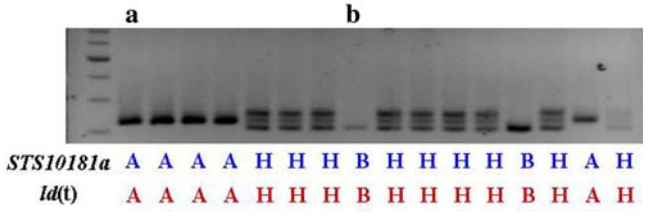

Fig. 3 An example of co-segregation of *ld*(t) gene and its flanking STS marker, *STS10181a*, in the F2 population of a cleistogamous line/M.23 cross. *Lane a* cleistogamous line, *Lane b* M.23; Genotype A: cleistogamy, Genotype B: M.23, Genotype H: heterozygous

Genetic mapping

A gene *ld*(t), which is responsible for the lack of lodicules inside spikelets and thereby causes cleistogamy (Won et al. 1998), was located at the end region below 178.1 cM of chromosome 1L. The physical distance from *S01178b* to the end of chromosome 1 including telomere region was approximately 520 kb, where five PAC clones are connected with overlapped margins (http://www.rgp.dna.affrc.go.jp/) (Fig. 2). The physical length of 520 kb is not short enough to adopt a candidate gene approach for the isolation of *ld*(t) gene. The STS markers developed for this study were basically designed to detect *InDel* polymorphisms between *indica* and *japonica* parents. Therefore, additional markers including SNP should be developed to narrow down the target locus using larger segregating populations or F_3 progenies of the F_2 recombinants in which the breakpoints existed near the target locus.

Total distance from the locus *S01164* to the locus *S01181* in this study was much shorter (9.3 cM) than that in the map developed by JRGP in which the distance between the two loci was 17 cM. One possible explanation for the difference in map distance is that populations used for mapping were different from each other.

This is the first report of mapping cleistogamy gene in rice, while molecular mapping of cleistogamy genes was reported in barley by Turuspekov et al. (2004) in which the expression of cleistogamy in barley was controlled by two tightly linked genes or different alleles of the same gene, *cly*1 and *Cly*2, which were located on the same region of chromosome 2HL. The syntenic relationship between barley and rice chromosomes has not been fully understood in detail so far. However, based on (1) the analysis using a genomics DB "Gramene" (Ware et al. 2002) in which chromosome 2H of barley corresponds to rice chromosome 4, 7, 3, and 11 in a scattered manner and (2) the reports on comparative mapping (Sorrells et al. 2003, Devos 2005) where rice chromosome 1 exhibited the overall synteny to wheat chromosome 3, while wheat chromosome 2 was roughly matched to rice

chromosome 4 and 7, it is inferred that chromosome 2HL of barley is unlikely to have a homology with chromosome 1L of rice where the *ld*(t) locus resides. This implies that the *ld*(t) gene in this study might be different from the genes for cleistogamy reported in barley. Now we are trying to isolate the gene *ld*(t). Once isolated, the developmental pathway of lodicules in relation to the development of floral organs will be elucidated. It may be helpful to uncover the developmental and genetic relationship between lack of lodicules and less-developed lodicules in CL mutants of grass species. Backcross breeding is in progress to develop CL varieties with the genetic background of commercial cultivars.

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