10. Mapping and Characterization of the rice gene, *CLUB-SHAPED EMBRYO3, CLE3*

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In previous study, we reported the screening and characterization of rice embryonic mutant from NMU-induced or callus culture-induced mutant libraries (Hong et al., 1995, Imamura et al., 2008). Among these, the *club-shaped embryo1* (*cle1*) mutant was characterized by the "*club-shaped*" structure which extends along the apical-basal axis during the embryonic stage (see Fig. 1G-I compared with A-C). The *cle1* embryo fails to develop most organs and tissues (*i.e.* SAM and RAM) except for the epithelium-like and the vascular-like tissues. It should be noted that a rice homeobox gene *OSH1*, which is specifically expressed in the shoot apical region before the morphological development in WT is misexpressed in *cle1* (Imamura et al., 2008). This expression pattern differs from that of other organless-mutants, (*i.e. organless1*) whose expression pattern is similar to that of the WT embryo (Kamiya et al., 2001). Here, we report a newly identified *club-shaped embryo*, *cle3*, which was also screened by the "*club-shaped*" phenotype of the embryo from the callus culture-induced mutant libraries.

We utilized 7000 mutant lines from the callus culture-induced mutant library to screen for the *club-shaped* embryos, and identified four mutant lines, which were crossed with the *cle1* for allelism test. From these, one line was found to be non-allelic to *cle1* and thus, was named *cle3* (Table 1). We studied the segregation of *cle3* mutant, and the resulting frequency of segregation (17%) did not fit 3:1 (Table 2). This was also true for the segregation of the F_2 progenies derived from crossing the *cle3* mutant (japonica) pistil with Kasalath (indica) pollen. Interestingly, when the Kasalath pistil was crossed with the *cle3* pollen, the frequency of segregation (24%) fitted 3:1 (Table 2). These results suggest that the inheritance of *cle3* is not of Mendelian manner. A similar mode of inheritance was observed in *cle1-1* (data not shown)

The histological analysis of the *cle3* mutant during embryogenesis was studied. In the WT embryo, protrusion of the coleoptile as the first organ differentiation is observed at 4 days after pollination (DAP) (Ito et al., 2005 Fig. 1A). The development of the second leaf primordium from SAM, the radicle from RAM, as well as the vascular tisuue, and the epithelium tissue are completed at 7 DAP (Fig. 1B). The embryo then matures until 10 DAP (Fig. 1C). In contrast to the WT embryo, the protrusion of the coleoptile in the *cle3* embryo was not observed at 4 DAP (Fig. 1D, compared with A). At 7 DAP, the *cle3* embryo elongated along the apical-basal axis, and assumed a "*club-shape*" configuration. At this stage, the radicle was developed, however, SAM, vascular-like tissue, and epithelium-like tissue remained unformed (Fig. 1E). At 10 DAP, the *cle3* embryo began to degenerate of tissues in allover the embryo including the radicle and the vascular-like tissue (Fig. 1F). In summary, the different phenotypes of *cle3* compared with *cle1* embryos are: (1) development of radicle was observed, (2) Establishment of epithelium-like tissue was not observed, and (3) the degeneration of tissues was observed 7 DAP (Fig. 1G-I, compared with D-F).

Towards the isolation of the *CLE3* gene, we used the genomic DNAs of F_2 heterozygous plants from a cross between the *cle3* mutant and Kasalath in this study. We used 30 F_2 heterozygous plants, which were identified by the segregation of the mutant phenotype in F_3 embryos for the linkage analysis. The result demonstrated that *CLE3* is located between TG105 and c16130 on chromosome 6 (Fig. 2). This presents a genetic distance of 9.0 cM (Fig. 2) as inferred from the RGP (Rice Genome Project) map (Harushima et al., 1998). We are currently narrowing down this region containing the *CLE3* gene.

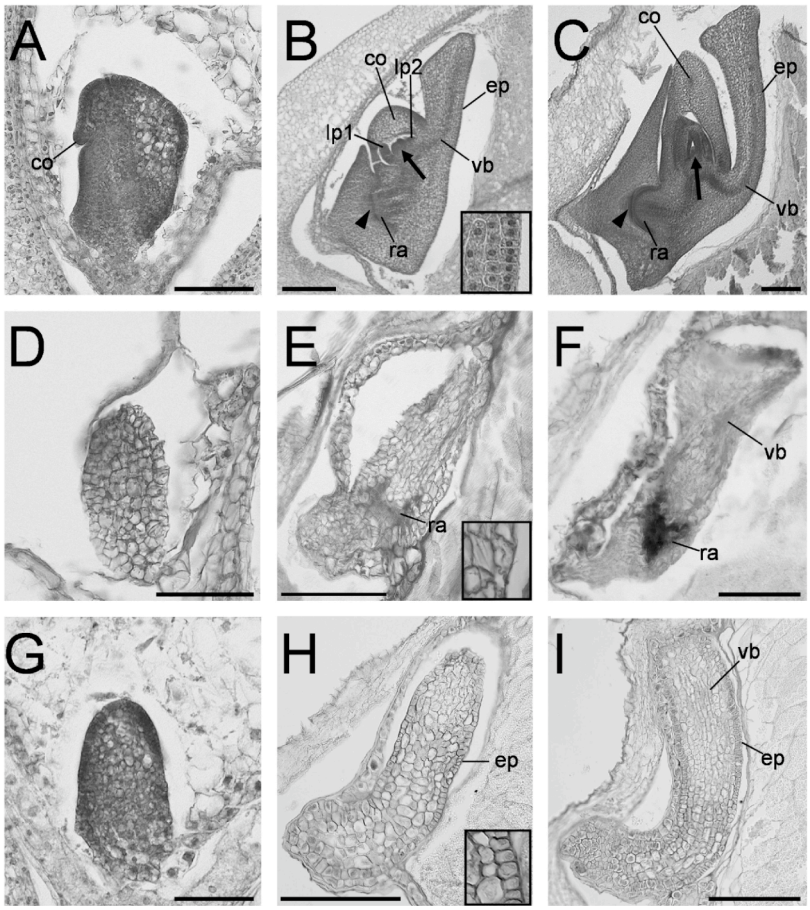

Fig. 1 Embryonic phenotypes of WT, *cle1*, and *cle3*.
Embryonic structures of WT (A-C), *cle3* (D-F), and *cle1-1* (G-I) are shown. A, D, and G were taken at 4 days after pollination (DAP); (B, E, and H) 7 DAP; (C, F, and I) 10 DAP. Insets show enlarged views of the L1 layer of the dorsal side. The palisade-like tissue was not formed in *cle3* (E compared with B and H). Arrow, SAM; arrowhead, RAM; ra, radicle; co, coleoptile; ep, epithelium; lp1, the first leaf primordium; lp2, the second leaf primordium; vb, vasucular bundle. Bars = 200 μ m

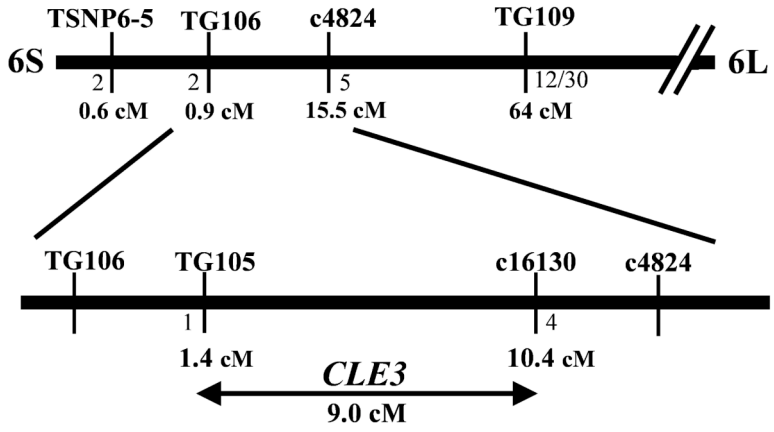

Fig. 2 A linkage map of *CLE3* with DNA makers on chromosome 6.
The horizontal bars and the vertical bars show chromosome 6 and DNA markers, respectively. The numbers under the linkage map indicate recombinants among 30 F_2 plants.

Table 1 Allelism test of *cle3* to *cle1*

Combination[a]	Frequency of embryos (F_1)		
	WT	*cle*	χ^2(3:1)
CLE1-2/cle1-2 × *CLE3/cle3*	22	0	7.33***

[a]Because both mutants are embryonic lethal, the heterozygous plants were used in this study.
***Significantly deviated from a 3:1 ratio at 0.1% level.

Table 2 Segregations of *cle3*

Strain	Frequency of plants (F_1)			Frequency of embryos (F_2)[a]		
	WT	heterozygote (%)	χ^2	WT	*cle* (%)	χ^2(3:1)
CLE3/cle3	14	25 (64)	0.12 (1:2)	338	69 (17)	38.7***
CLE3/cle3 × K	12	18 (62)	1.20 (1:1)	1978	473 (19)	42.5***
K × *CLE3/cle3*	4	6 (52)	0.67 (1:1)	587	186 (24)	0.36

[a]Progenies of the F_1 heterozygous plants were studied.
***Significantly deviated from a 3:1 ratio at 0.1% level. K; Kasalath (*O. Sativa* spp. Indica).

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