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Short Communication

RsLFY, a *LEAFY* homologue gene in radish (*Raphanus sativus*), is continuously expressed in vegetative, reproductive and seed development

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Abstract The floral meristem identity gene *LEAFY* (*LFY*) in *Arabidopsis* plays a key role in flower development. We isolated two *LFY*-like genes from radish, designated *RsLFY1* and *RsLFY2*. Comparison of genomic and cDNA sequences revealed that the number and positions of introns are precisely conserved in *RsLFY1*, *RsLFY2* and *LFY*. Using *RsLFY1* full-length cDNA as a probe, genomic DNA blot hybridization analysis detected two hybridizing fragments under high stringency, suggesting that there are two *RsLFY* loci within radish genome. Both genes were expressed during vegetative growth, reproductive growth, flower development and seed development. *RsLFY1* was expressed slightly in leaf primordia and strongly in early floral meristem. It was expressed successively in primordia of sepals, petals, stamens, gynoecium, ovule integument and mature seeds. These results suggest that *RsLFY1* plays a similar role to *LFY* in floral initiation and the developmental stages of floral organs, and that it also may regulate radish seed development, unlike *LFY*.

Key words: Floral meristem identity gene, *LEAFY*, ovule, Radish, seed development.

Radish (*Raphanus sativus* L.) has been long grown as an important crop worldwide, and its cultivars consequently show extreme variation. Radish is believed to have been already in cultivation in the eastern Mediterranean area in 2200 BCE (Kitamura et al. 1958), and subsequently spread to East Asia and Europe. Small-rooted radishes are generally grown in Europe, but the large-rooted form is one of the most important vegetables in Japan, Korea and China. Other forms, such as oilseed radish and rat's tail radish, are grown in South-East Asia (Banga 1976). Cruciferous plants such as radish show considerable variations in the temperature and photoperiod requirements for flowering. But little is known about the molecular function of radish floral development. In *Arabidopsis*, it is known well that *LFY* is a floral meristem identity gene that controls the production of floral meristem primordia and activates floral organ identity genes (Schultz and Haughn 1991; Weigel et al. 1992; Weigel and Nilsson 1995).

We isolated and characterized two *LFY* homologues from radish as a first step to investigate the molecular process of flowering and designated *RsLFY1* and *RsLFY2*. The nucleotide sequence has been submitted to GenBank under accession numbers AB449248 (*RsLFY1*) and AB449249 (*RsLFY2*). In this study, the rat's tail radish cultivar Pakki-hood was used (Nomura et al. 1996). Total RNA extracted from radish shoot apices was used to isolate two *LFY*-like genes by RT-PCR. The longest *RsLFY1* cDNA clone was 1475 bp and encoded a putative protein of 420 amino acids. On the other hand, the longest *RsLFY2* cDNA clone was 1467 bp and encoded a putative protein of 415 amino acids. Comparison of genomic and cDNA sequences revealed that the number and positions of introns were precisely conserved in *RsLFY1*, *RsLFY2* and *LFY*. *RsLFY* genomic DNAs had three exons and two introns. Two introns in each gene contained the canonical GT-AG dinucleotide splice site junctions. Both are 89% identical to LFY (Figure 1), and RsLFY1 is 88% identical to RsLFY2. RsLFY1 and RsLFY2 conserve four typical motifs characteristic of transcription factors: a proline-rich region in the N-terminus, a short leucine zipper consisting of Leu residues, a basic region consisting mainly of Arg and Lys residues, and an acidic region consisting mainly of Asp and Glu residues (Figure 1) (Weigel et al. 1992; Frohlich and Meyerowitz 1997). Using *RsLFY1* full-length cDNA as a probe, genomic DNA blot hybridization analysis detected two hybridizing fragments under high stringency. These results show that *RsLFY1* and *RsLFY2* exist as two-copy genes in radish (Figure 2). There is generally only a single copy of *LFY* in angiosperms (Maizel et al. 2005).

Abbreviations: BCE, Before Common Era; LFY, LEAFY.
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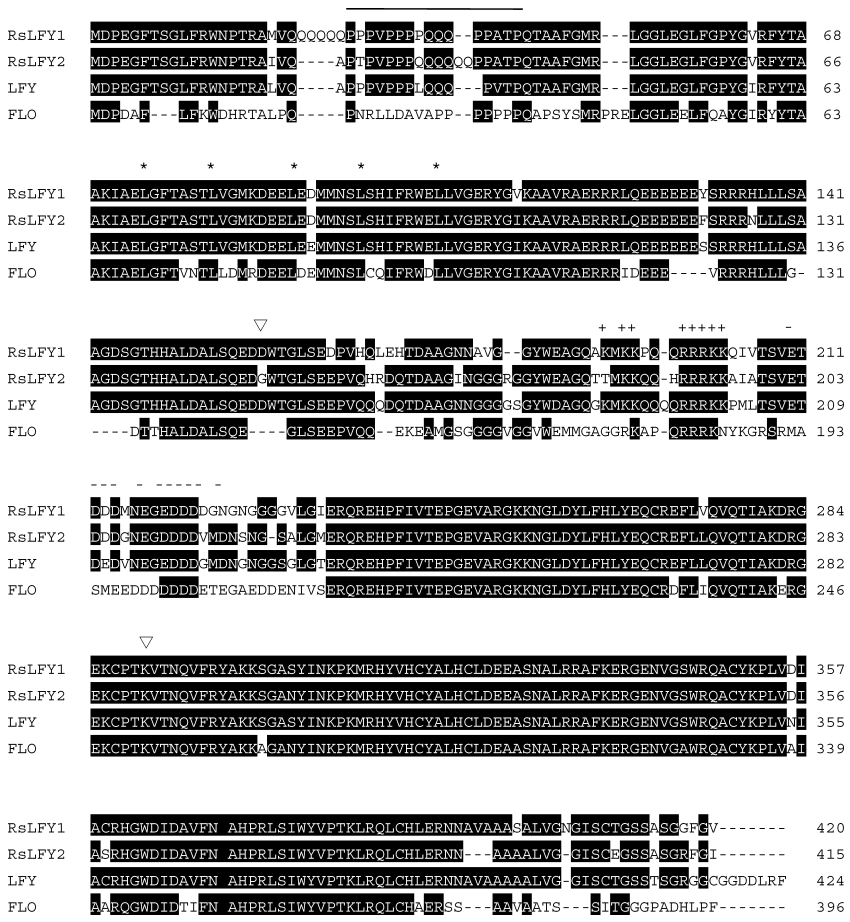

Figure 1. Amino acid sequence alignment of radish RsLFY with *Arabidopsis* LFY (GenBank accession no. M91208) and *Antirrhinum majus* FLO (GenBank accession no. M55525) by CLUSTALW. White letters on black background indicate residues identical to those in RsLFY. Dashes indicate gaps introduced to maximize alignment. A proline-rich domain near the amino terminus is marked with a bar. Asterisks indicate conserved leucine repeats with periodic spacing. +, Basic regions; −, acidic regions. The conserved sites of introns are indicated by triangles.

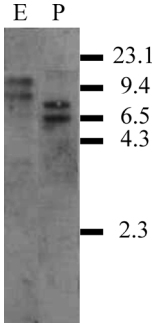

Figure 2. Radish genomic DNA blot analysis. Genomic DNA was digested with *Eco*RI (E) or *Pst*I (P). Numbers on the right indicate molecular size (kb). Full-length *RsLFY1* cDNA was labelled with digoxygenin and used as a probe. Hybridization was carried out as described in the manufacturer's protocol (Roche).

On the other hand, gymnosperm species generally have two *LFY* homologs (Frohlich and Parker 2000). Frohlich and Parker (2000) further proposed that angiosperm lineage might lose one of the *LFY* homologs from ancestral plants. But, there are a couple of active *LFY* homologs in apple and maize genomes (Wada et al. 2002; Bomblies et al. 2003). In this study, it was shown that radish also had retained two copies of active *LFY*-like genes during evolution.

We determined the expression patterns of *RsLFY1* and *RsLFY2* in various radish tissues by RT-PCR. Each PCR primer set amplified a single 381-bp product from total RNA. RT-PCR analyses detected *RsLFY1* and *RsLFY2* expression in vegetative apices, reproductive apices, buds and flowers. Transcripts of both genes were detected in seeds, leaves and stems at low abundance (Figure 3A). We investigated the expression patterns of *RsLFY1* and *RsLFY2* in developing seeds. Both were expressed in 20-, 40- and 60-day seeds after flowering (Figure 3B). This revealed that both were expressed throughout the life cycle of radish. Because both genes show the same expression pattern, they may be redundant.

We investigated the *RsLFY1* expression pattern in detail by *in situ* hybridization. An *RsLFY1* gene-specific cDNA fragment was amplified by PCR. The PCR

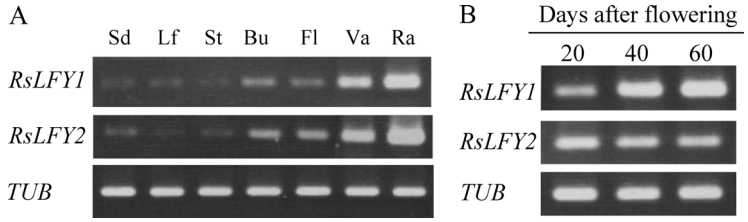

Figure 3. RT-PCR analysis of *RsLFY* expression in radish. Total RNA was extracted from various tissues with an RNeasy Mini Kit (Qiagen). The first-strand cDNA was generated with a SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). The specific primers used for mRNA detection of *RsLFY1* and *RsLFY2* were as follows: *RsLFY1* (5′-TGCCCCACCAAGGTGACGAACCAA-3′ and 5′-GAATAC-TTGGTTCGTCACCTTGGTG-3′); *RsLFY2* (5′-TGCCCTACCAAGGTGACGAACCAG-3′ and 5′-AAACACCTGGTTCGTCACCTTGGTA-3′). The thermocycler program was 2 min/94°C; 30 cycles of 30 s/94°C, 30 s/60°C and 50 s/72°C; and a final extension step of 5 min/72°C. Primer set of *RsLFY1* and *RsLFY2* could amplify specific PCR products, respectively. The same experiments were done more than three times. A fragment of *TUB* (*β-tubulin* gene) was amplified from the same cDNA as a standard control to normalize the amount of cDNA in RT-PCR (Marks et al. 1987). (A) Expression pattern in different tissues. Sd, seed; Lf, leaf; St, stem; Bu, bud; Fl, flower; Va, vegetative shoot apex; Ra, reproductive shoot apex. (B) Expression of *RsLFY* in developing radish seeds. To harvest developing seeds, individual flowers were tagged on the day of flowering. Every 20 d during seed development (20, 40 and 60 d), developing seeds were picked out to extract total RNA.

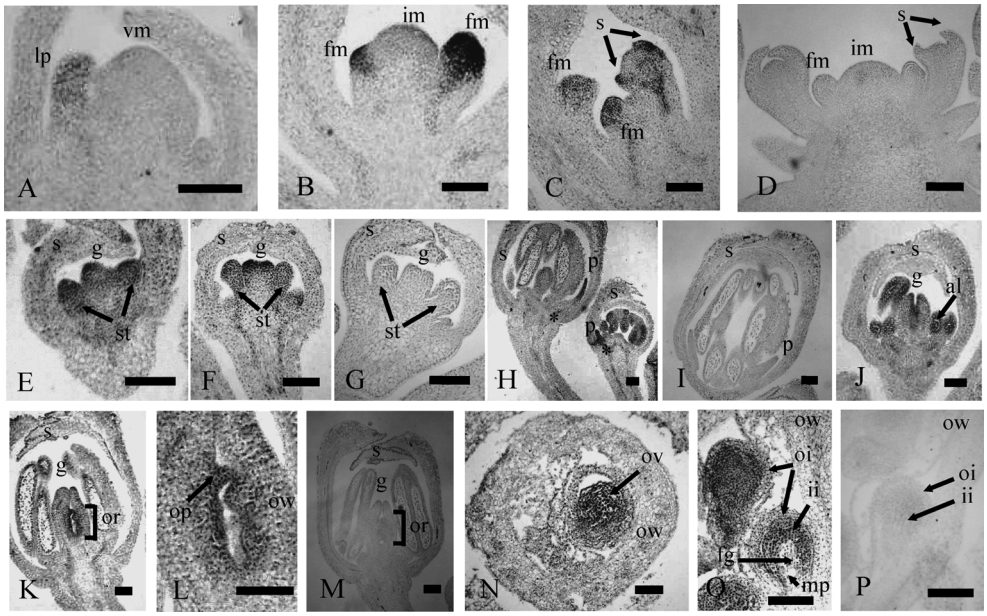

Figure 4. RNA *in situ* hybridization analysis of *RsLFY1* in radish. Each image shows longitudinal sections, except (N). (A) Weak expression in a young leaf primordium (lp) during vegetative growth. (B) Strong expression in floral primordia. (C) Expression in sepal primordia (s) but not between sepals. (E) Emergence of stamens and gynoecium from the central dome of cells. (F) Elongation of gynoecium and stamens. (H) Expression during petal expansion. An asterisk shows the approximate position of petal primordia emergence. (J) Invagination of gynoecium and anther locules. (K) Cells inside closed gynoecium become ovary wall. (L) Ovule primordium arises from the inside of the ovary wall. (N) Transverse section shows expression in the ovule. (O) Expression is detected in the inner integument of the ovule, but not in the outer integument or female gametophyte. D, G, I, M and P are sections hybridized with sense probes. Abbreviations: vm, vegetative apical meristem; lp, leaf primordium; im, inflorescence meristem; fm, floral meristem; s, sepal; st, stamen; g, gynoecium; p, petal; al, anther locule; or, ovary; ow, ovary wall; op, ovule primordium; ov, ovule; ii, inner integument; oi, outer integument; fg, female gametophyte; mp, micropyle. Bars, 100 μm.

product of 240-bp was cloned into the pGEM-T easy vector (Promega) and used as template to produce digoxygenin-labelled antisense and sense RNA probes. This fragment was mainly consisted of 3′ untranslated region of *RsLFY1*. The nucleotide sequence was not identical with high similarity to the corresponding region to *RsLFY2*. Plant tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 8 μm and affixed to slides at 42°C overnight. Hybridization, washing under high stringency and detection were carried out as described in the manufacturer's protocol (Roche). Before the first floral primordia appeared, weak expression was detected in young leaf primordia but no signal was detected in vegetative apical meristem (Figure 4A). After the transition to reproductive growth, *RsLFY1* was strongly expressed throughout early floral primordia (Figure 4B). This suggests that *RsLFY1* controls the transition from vegetative phase to reproductive phase and the induction of floral primordia in the manner of a floral meristem identity gene such as *LFY* (Weigel et al. 1992; Blázquez et al. 1997). Expression was maintained in regions

flanking the growing floral meristem before sepal emergence, but with sepal emergence, it was expressed in the sepal primordia but not between them (Figure 4C). mRNA was present in both dome-shaped staminal primordia and in the gynoecium, which arose from the central cells (Figure 4E). *RsLFY1* was expressed in developing stamens and gynoecium, but the expression was reduced in sepals (Figure 4F). It was expressed in petal primordia but retained its expression only in the petal tip after the petal primordia started to grow towards the top (Figure 4H). Expression was detected in anther locules after the development of stamens (Figure 4J). Because *RsLFY1* is expressed during the initiation of floral organ development, it is suggested that *RsLFY* controls the homoeotic genes of radish flowers in the same way as *LFY* (Weigel et al. 1992; Parcy et al. 1998). As the expression pattern of *RsLFY1* was similar to that of *LFY*, the basic mechanisms involved in flower initiation and development in radish might also be similar to those in *Arabidopsis*.

In the developing gynoecium, *RsLFY1* expression was restricted to the top of gynoecium (Figure 4F). After invagination of the gynoecium, mRNA was present along the inside (Figure 4J). After closure of the gynoecium to form a small space for development of the ovule, *RsLFY1* was weakly expressed in the cells inside the ovary wall (Figure 4K) and the emerging ovule primordia (Figure 4L). mRNA was present also in the ovule which developed in the ovary (Figure 4N). *RsLFY1* was strongly expressed in the inner integument, but no signal was visible in the outer integument or female gametophyte (Figure 4O). Recently, it was reported that angiosperm *LFY* genes fully complement leafy mutant, but gymnosperm *LFY* genes provide only partial rescue, and that *LFY* function has diverged during angiosperm evolution (Maizel et al. 2005). *GmLFY*, a soybean *LFY* homologue, was expressed in the developing seeds. This suggested that *GmLFY* might play an essential regulatory role in seed development (Meng et al. 2007). The expression of *RsLFY1* in the inner surface of the gynoecium and the ovule primordia suggests that *RsLFY1* may regulate the initiation of ovule primordium development, unlike *LFY* in *Arabidopsis* (Figure 4J, K, L). The pronounced expression of *RsLFY1* in the inner integument may play a role during radish seed maturation to regulate seed development or certain seed characteristics dislike *Arabidopsis* (Figure 4O).

Acknowledgements

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