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Evolution of MADS-Box Gene Induction by *FLO/LFY* Genes

Saiko Himi,[1] Ryosuke Sano,[2,3] Tomoaki Nishiyama,[2] Takako Tanahashi,[4] Masahiro Kato,[4] Kunihiko Ueda,[1] Mitsuyasu Hasebe[2,5,6]

[1] Department of Biology, Faculty of Science, Kanazawa University, Kanazawa 920-1192, Japan
[2] National Institute for Basic Biology, 38 Nishigonaka, Myodaiji-cho, Okazaki 444-8585, Japan
[3] Department of Biology, Faculty of Science, Chiba University, Chiba 263-0022, Japan
[4] Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan
[5] Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki 444-8585, Japan
[6] PRESTO, Japan Science and Technology Corporation

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Abstract. Some MADS-box genes function as floral homeotic genes. The *Arabidopsis LFY* gene is a positive regulator of floral homeotic genes, and homologs of the *FLO/LFY* gene family in other angiosperms and gymnosperms are likely to have a similar function. To investigate the origin of the floral homeotic gene regulatory cascade involving the *FLO/LFY* gene, *FLO/LFY* homologs were cloned from a leptosporangiate fern (*Ceratopteris richardii*), two eusporangiate ferns (*Angiopteris lygodiifolia* and *Botrychium multifidum* var. *robustum*), three fern allies (*Psilotum nudum*, *Equisetum arvense*, and *Isoetes asiatica*), and a moss (*Physcomitrella patens*). The *FLO/LFY* gene phylogenetic tree indicates that both duplication and loss of *FLO/LFY* homologs occurred during the course of vascular plant evolution. The expression patterns of the *Ceratopteris LFY* genes (*CrLFY1* and *2*) were assessed. *CrLFY1* expression was prominent in tissues including shoot tips and circinate reproductive leaves, but very weak in other tissues examined. Expression of *CrLFY2* was also prominent in tissues, including shoot tips and circinate reproductive leaves. These patterns of expression are dissimilar to that of any *Ceratopteris* MADS-box gene previously reported, suggesting that the induction of MADS-box genes by *FLO/LFY* is not established at the stage of ferns.

Key words: *Leafy* — *FLORICAULA* — *FLO/LFY* — MADS-box — Pteridophyte — Fern — Floral homeotic gene — Reproductive organ — Flower

Introduction

The diploid reproductive structure of flowering plants is the flower, which is composed of four whorls of floral organs: sepals, petals, stamens, and a gynoecium. The development of these floral organs is governed by floral homeotic genes, most of which are members of the MADS-box gene family (reviewed in Wolpert et al. 1998). The floral homeotic genes are specifically expressed in the floral primordia where each floral homeotic gene plays its own role. In angiosperms, the expression of most floral homeotic genes in the floral primordia is induced by the *FLORICAULA/LEAFY* (*FLO/LFY*) gene (Coen et al. 1990; Weigel 1992). The interaction between floral homeotic genes and co-regulation of the *FLO/LFY* gene and some other genes results in distinct patterns of floral homeotic gene expression (Parcy et al. 1998; Busch et al. 1999). The MADS-box gene family is composed of about 10 phylogenetically distinct groups, and floral homeotic genes belong to several of these groups (Theissen et al. 2000). The expression patterns and functions of MADS-box genes other than the floral

Correspondence to: Dr. Mitsuyasu Hasebe; *present address:* National Institute for Basic Biology, 38 Nishigonaka, Myodaiji-cho, Okazaki 444-8585, Japan; *email:* mhasebe@nibb.ac.jp

Table 1. List of taxa used in this study

Species	Locality	Gene name	Accession no.
Leptosporangiate ferns			
Ceratopteris richardii Brongn.	Strain Hnn*	*CrLFY1*	AB049974
		CrLFY2	AB049975
Eusporangiate ferns			
Angiopteris lygodiifolia Rosenst.	Cultivated**	*AlLFY1*	AB050090
		AlLFY2	AB050091
		AlLFY3	AB050092
Botrychium multifidum var. robustum C. Chr.	Japan (Kanazawa)	*BaLFY1*	AB050086
Fern allies			
Psilotum nudum L.	Cultivated***	*PnLFY1*	AB050085
Equisetum arvense L.	Japan (Kanazawa)	*EaLFY1*	AB050088
Isoetes asiatica (Makino) Makino	Japan (Hokkaido)	*IaLFY1*	AB050087
Moss			
Physcomitrella patens subsp. *patens* Tan	Cultivated****	*PpLFY1*	AB052251
		PpLFY2	AB052252

* Scott and Hickok (1987); **The Botanic Gardens of Toyama; ***Botanical Gardens, Faculty of Science, University of Tokyo, ****Ashton and Cove (1977)

homeotic genes are diverse (reviewed in Theissen et al. 2000).

MADS-box genes have been identified in some gymnosperms, and some of these genes are orthologous to the floral homeotic genes of angiosperms. This means that floral homeotic genes had already diverged in the common ancestor of angiosperms and gymnosperms (reviewed in Hasebe 1999; Theissen et al. 2000). The expression patterns of several gymnosperm floral homeotic gene orthologs have been studied in conifers (Tandre et al. 1998; Rutledge et al. 1998; Mouradov et al. 1999; Sundström et al. 1999) and *Gnetum* (Winter et al. 1999; Shindo et al. 1999), and all orthologs are specifically expressed in their respective reproductive organs, but not in vegetative organs. This is similar to angiosperm floral homeotic genes, which are specifically expressed in reproductive organs. Gymnosperm *FLO*/*LFY* genes likely induce these patterns of reproductive organ-specific expression in a manner similar to that of the angiosperm *FLO*/*LFY* gene, which causes the floral organ-specific expression of the floral homeotic genes. This inference is based on the observations that *in situ* expression patterns of *NEEDLY* (*NLY*), the conifer *Pinus radiata FLO*/*LFY* gene, are similar to those of the conifer floral homeotic gene orthologs, and that *NLY* can complement *LFY* function in *Arabidopsis* when it is introduced in the *Arabidopsis lfy* mutant (Mouradov et al. 1998). Therefore, the regulatory system of floral homeotic gene orthologs involving the *FLO*/*LFY* gene was already established in the common ancestor of angiosperms and gymnosperms.

Three groups of several fern MADS-box genes have been reported, although the phylogenetic relationship of these groups to seed plant MADS-box genes have not been established with statistical confidence (Münster et al. 1997; Hasebe et al. 1998). Like seed plants, ferns form several sterile vegetative leaves at the shoot meristem after embryogenesis. In time, the shoot meristem undergoes a phase change to form reproductive leaves bearing spores. All fern MADS-box genes previously studied are expressed in both reproductive and vegetative organs, and no MADS-box genes are specifically expressed in a reproductive organ as an floral homeotic gene. We hypothesized that the specification of the expression of generally expressed MADS-box genes to reproductive organs was important for the evolution of the flower (Hasebe et al. 1998; Hasebe 1999; Hasebe and Ito 1999). Furthermore, since the *FLO*/*LFY* gene induces floral homeotic genes in seed plants, establishing the induction of MADS-box genes by a *FLO*/*LFY* gene was likely to be a key event in the evolution of floral homeotic genes from generally expressed MADS-box genes. This hypothesis can be tested by analyzing the *FLO*/*LFY* genes in non-seed plants, such as pteridophyte, including ferns and fern allies. In this study, we have cloned *FLO*/*LFY* genes from three ferns, three fern allies, and a moss, and inferred the phylogenetic relationships among all *FLO*/*LFY* genes. The patterns of *FLO*/*LFY* gene expression were assessed in the fern *Ceratopteris richardii*. The evolution of the induction of MADS-box genes by the *FLO*/*LFY* gene is discussed.

Materials and Methods

Cloning of FLO/LFY *Homologs. FLO*/*LFY* homologs were cloned from the species listed in Table 1. Voucher specimens are deposited in the herbarium of Kanazawa University (KAN). All collected materials were immediately frozen in liquid nitrogen for RNA extraction. Total RNA was extracted according to Shindo et al. (1999). The extracted RNA was further purified using ISOGEN LS (Nippon Gene, Tokyo, Japan). Complementary DNA was synthesized from the total RNA using Superscript II reverse transcriptase and the adapter primer with poly-T sequences (5′-CUACUACUACUAAGGCCACGCGTCGAC-TAGTACT$_{16}$-3′) supplied by Life Technologies (Rockville, MD). PCR was performed using the cDNA as template, the adapter primer, and a *FLO*/*LFY*-specific primer encoding the amino acids MRHYVHCYA. The sequence of the degenerate primer is 5′-CAUCAUCAUCAUAT-

GCGICAYTAYGTICAYTGTTAYGC-3′, where nucleotides are represented by the one letter IUPAC code. U and I represent a deoxy uracil and a deoxy inosine, respectively. This primer is based on the primer DW1089 kindly provided by D. Weigel (Salk Institute, CA). The CAU and CUA stretches at the 5′ end of each primer are designed for the CLONEAMP System (Life Technologies). Using this system, PCR products with the same primers at both ends are not cloned. The PCR procedures followed those of Hasebe et al. (1998). The PCR products were separated on 1% agarose gels. Approximately 0.5–1 kb fragments were purified with a Gene Clean II Kit (Bio 101, Vista, CA), and cloned into the pAMP1 plasmid (Life Technologies). The cloned PCR products were sequenced with an Auto Read Sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ALF autosequencer (Amersham Pharmacia Biotech), or an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems). The 5′ region of the amplified fragment was obtained using a 5′ RACE system (Life Technologies), and sequenced. The sequences of gene-specific primers used in the 5′ RACE are deposited in the DNA database with the corresponding *FLO*/*LFY* homologs. To exclude PCR errors, at least two clones obtained from independent PCR reactions were sequenced for each gene. When discrepancies were found, the majority from at least three independently amplified PCR products was determined to be the correct sequence.

Phylogenetic Analysis. *FLO*/*LFY* genes were sought in the nr dataset at NCBI using the programs BLAST X (version 2.0.10) (Altschul et al. 1997). The amino acid sequences of 31 *FLO*/*LFY* genes obtained from the DNA database and 11 *FLO*/*LFY* homologs sequenced in this study were aligned using CLUSTAL W version 1.8 (Thompson et al. 1994). Two hundred and forty amino acid sites without indels were used for the phylogenetic analysis.

To search for the maximum likelihood (ML) tree, we used most parsimonious (MP) trees as starting trees for local rearrangement search (Adachi and Hasegawa 1996). The MP trees were heuristically searched using the tree bisection and reconnection option of PAUP*4.0 b4a (Swofford 1998). To obtain the ML tree, the MP trees were subject to a local rearrangement search to evaluate log likelihoods under the JTT model using ProtML in the MOLPHY, version 2.3b3 package (Adachi and Hasegawa 1996). Using the tentative ML tree, a further exhaustive search under a constraint was performed to obtain the final ML tree. The local bootstrap probability of each branch was estimated by the resampling-of-estimated-log-likelihood (RELL) method (Kishino et al. 1990; Hasegawa and Kishino 1994).

Northern Hybridization. Northern hybridization followed Shindo et al. (1999). Total RNA (20 μg) was electrophoresed on 1% agarose denaturing gels, and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) using 10 × SSC. As both *CrLFY* genes are more strongly expressed in the tissues, including shoot apices and circinate leaves, than other tissues in preliminary experiments, 6 μg of total RNA was loaded for these tissues. Hybridization and washing were performed at 65°C with a buffer (Church and Gilbert 1984). The *CrLFY1*- and *2*-specific probes were 790 bp *Sca*I-*Nhe*I and 870 bp *Nru*I-*Nco*I fragments, respectively. Each gene-specific fragment was labeled with [^{32}P] dCTP (Amersham Pharmacia Biotech) using a Random Primer DNA Labeling Kit, version 2.0 (TaKaRa, Kyoto, Japan).

Results and Discussion

Isolation of FLO/LFY *Genes from Ferns and Fern Allies*

FLO/*LFY* homologs were cloned from a leptosporangiate fern (*Ceratopteris richardii*), two eusporangiate ferns (*Angiopteris lygodiifolia* and *Botrychium multifidum* var. *robustum*), three fern allies (*Psilotum nudum, Equisetum arvense*, and *Isoetes asiatica*), and a moss (*Physcomitrella patens* subsp. *patens*). Two *FLO*/*LFY* homologs were obtained from *Ceratopteris,* three from *Angiopteris,* and two from *Physcomitrella.* A single *FLO*/*LFY* homolog was isolated from each of the remaining species (for names of these homologs, see Table 1). These 11 *FLO*/*LFY* genes and 31 vascular plant *FLO*/*LFY* genes obtained from the DNA database were aligned. The proline-rich region and the acidic region observed in angiosperm *FLO*/*LFY* genes (Coen et al. 1990) were not found in fern and fern allies *FLO*/*LFY* genes, nor are they found in gymnosperms (Mouradov et al. 1998; Frohlich and Meyerowitz 1997; Frohlich and Parker 2000), indicating that they evolved in the angiosperm lineage.

Phylogeny of FLO/LFY *Genes*

Forty-two *FLO*/*LFY* homologs were aligned (EMBL accession number: ds42823), and 240 amino acids sites were used for the phylogenetic analysis. As *JaLEAFY1* and *JaLEAFY2* have identical amino acid residues at all 240 sites, they were treated as a single operational taxonomic unit. Of the 240 sites, 120 were parsimony-informative. MP search found 6 MP trees of 815 steps. In the ML tree found by the local rearrangement search of these MP trees, a monocot clade (*Juncus* and *Oryza*) and a Brassicaceae clade (*Arabidopsis, Jonopsidium,* and *Brassica*) formed a sister group (data not shown). This result is incongruent with the species phylogeny inferred by previous studies (Mathews and Donoghue 1999; Soltis et al. 1999; Qiu et al. 1999).

As the branch length of the monocot clade was much longer than the lengths of other branches, we tested whether the evolutionary rate of *FLO*/*LFY* homolog in the monocot lineage is accelerated using the relative rate test (Sarich and Wilson 1973). We used *NymodLFY* as a reference, since it locates at the base of angiosperm *FLO*/*LFY* genes. The basal position of *Nymphaea* is concordant with the species phylogeny of angiosperms (Mathews and Donoghue 1999; Soltis et al. 1999; Qiu et al. 1999). The differences in the distances between a monocot *FLO*/*LFY* gene-the *NymodLFY* gene and any other dicots *FLO*/*LFY* gene-the *NymodLFY* gene was calculated. The standard error was estimated by a bootstrap method; resampling amino acid sites based on pseudorandom numbers generated by the minimal standard random number generator (Park and Miller 1988), and calculating a difference for each set. The calculated differences were significantly positive (data not shown) except for one set: *RFL* (a rice *FLO*/*LFY* homolog)-*NymodLFY* and *PepspLFY-NymodLFY,* for which the difference was 0.057 ± 0.053, and 92.9% in 10,000 bootstrap replicates were positive. These results indicate that the evolutionary rates of monocots *FLO*/*LFY* homologs are higher than those of other angiosperms.

The rice *FLO*/*LFY* gene, *RFL,* is suggested to have functions different from those of other angiosperm *FLO*/

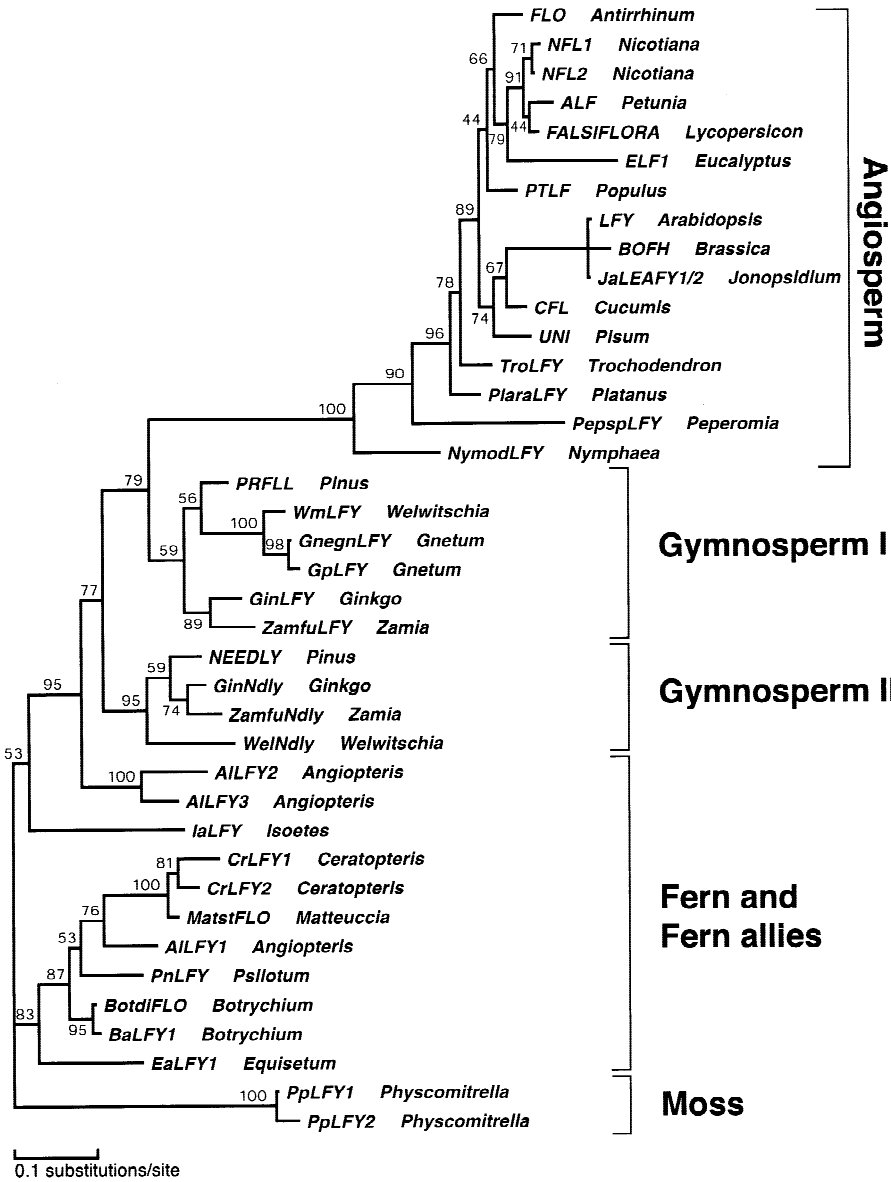

Fig. 1. The maximum likelihood tree of the *FLO*/*LFY* genes. Local bootstrap probabilities are shown on branches where available. The horizontal branch length is proportional to the estimated evolutionary distance. The genus name is indicated after the name of each *FLO*/*LFY* homolog.

LFY genes (Kyozuka et al. 1998). Unlike other angiosperm *FLO*/*LFY* genes, *RFL* expression is not detected in floral primordia, and the ectopic expression of *RFL* in *Arabidopsis* does not enhance the transition of shoot meristem into floral meristem, as the *Arabidopsis LFY* and other angiosperm *FLO*/*LFY* genes do when ectopically expressed in *Arabidopsis* (Weigel and Nilsson 1995). The acceleration of amino acid substitution and subsequent divergence of amino acid sequences between *FLO*/*LFY* genes in monocots and other dicots *FLO*/*LFY* genes may be related to their functional differences.

We preferred a phylogenetic analysis without monocot *FLO*/*LFY* genes because of their unusual clustering in the preliminary ML tree. In a heuristic search using tree bisection and reconnection 54 trees of 722 steps were found. In a local rearrangement search starting from these trees, a tree with log likelihood of −4788.21 was found. We prepared 105 constraint trees in which six clades recognized in the tentative ML tree (an angiosperm clade, two gymnosperm clades, two fern clades, and a moss clade) are fixed. In a local rearrangement search starting from the 105 trees, a tree with a better log likelihood of −4783.19 ± 276.46 was found (Fig. 1). We tried to make some of other constraint trees, but no tree with a better log likelihood than the ML tree in Fig. 1 was obtained. The topology of this tree is mostly similar to that of the ML tree, including monocot *FLO*/*LFY* genes.

In the ML tree (Fig. 1), angiosperm *FLO*/*LFY* genes formed a clade, and the phylogenetic relationship is mostly concordant with the species tree (Mathews and Donoghue 1999, Soltis et al. 1999, Qiu et al. 1999). Gymnosperm *FLO*/*LFY* homologs are separated into two clades (Gymnosperm I and II clades in Fig. 1), which is consistent with previous studies (Frohlich and Meyerowitz 1997, Mouradov et al. 1998, Mellerowicz et al.

1998, Frohlich and Parker 2000). The Gymnosperm I clade forms a sister group with an angiosperm *FLO*/*LFY* gene clade. Angiosperm *FLO*/*LFY* genes clustering with the Gymnosperm II clade have not been found in searches of the DNA database, even in the entire *Arabidopsis* genome. In each gymnosperm clade, *Ginkgo* and *Zamia* formed a sister group with relatively high bootstrap values, but their relationships to other gymnosperm groups were ambiguous. A close relationship between confers and gnetales has been inferred in some molecular analyses using combined data for mitochondria, chloroplast, and nuclear genes (Bowe et al. 2000, Chaw et al. 2000), but the inference was not strongly supported in the *FLO*/*LFY* tree. The sister relationship between *Ginkgo* and cycads was also not supported in the three genome analyses, and further study using more data is necessary to confirm the phylogenetic relationship of gymnosperms.

The fern and fern allies *FLO*/*LFY* homologs were unexpectedly separated into three lineages: the first consisted of two *Angiopteris* genes (*AlLFY2* and *3*), the second contained only *IaLFY*, and the third included all other *FLO*/*LFY* homologs of ferns and fern allies. The position of *IaLFY* is ambiguous with a low bootstrap value. The *AlLFY2* and *3* clade is sister to seed plant *FLO*/*LFY* genes with high local bootstrap values (95%). Other fern *FLO*/*LFY* genes formed a clade with the *Equisetum EaLFY1* gene and *Psilotum PnLFY* gene. *Ceratopteris CrLFY1* and *2* are sister genes, indicating that these genes were duplicated after *Ceratopteris* diverged from *Matteuccia.* Two leptosporangiate ferns (*Ceratopteris* and *Matteuccia*) and two eusporangiate ferns (*Angiopteris* and *Botrychium*) formed a clade with *Psilotum* gene with 87% of local bootstrap value. The close relationship of *Psilotum* to ferns instead of fern allies is concordant with the previous phylogenetic inferences using chloroplast 16S rDNA (Manhart 1995), *atpB* gene (Wolf 1997), a data set combining *rbcL, atpB,* 18S rDNA and morphological data (Wolf et al. 1998), and a data set combining *rbcL, atpB, rps4,* and 18S rDNA (Pryer et al. 2001). The latter two analyses also support a monophyletic relationship of ferns and *Equisetum.* The *FLO*/*LFY* tree and these previous studies support the monophyly of leptosporangiate ferns, eusporangiate ferns, *Psilotum,* and *Equisetum.*

Assuming that the *FLO*/*LFY* tree is reliably rooted by the two moss *Physcomitrella* genes and that our *FLO*/*LFY* tree well represents the whole diversity of *FLO*/*LFY* genes, there should be at least two *FLO*/*LFY* genes in the common ancestor of vascular plants. One was then duplicated in the common ancestor of seed plants and the other, corresponding to the Gymnosperm II clade, was lost in the angiosperm lineage.

Some gene families involved in plant development, such as MADS-box genes and homeobox genes, increased in number by gene duplications during the course of evolution (Hasebe and Ito 1999). It is speculated that the increase in gene number is related to the functional divergence of gene family, resulting in morphological diversity (Hasebe and Ito 1999, Sakakibara et al. 2001). This is not the case for *FLO*/*LFY* genes, as the number of genes in a genome decreased in angiosperms compared with gymnosperms and ferns (Fig. 2). Further studies on the function of *FLO*/*LFY* genes in different clades of the gene tree in Fig. 1 will give insights into the evolution of *FLO*/*LFY* genes.

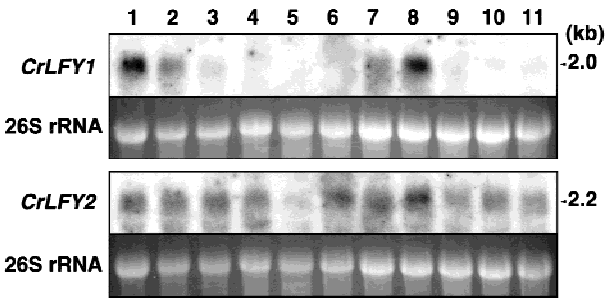

Fig. 2. RNA gel blot analyses using *CrLFY1* and *2* gene-specific probes. Each of lanes 1–3 contained 6 μg of total RNA, and each of other lanes contained 20 μg of total RNA. The RNA was from reproductive shoot tips including shoot apices, adventitious roots, and young leaf primordia less than 5 mm long (lane 1); circinate young reproductive leaves 0.5–2 cm long without petioles (lane 2); circinate young reproductive leaves 2–10 cm long without petioles (lane 3); expanded reproductive leaves before spore maturation, without petioles (lane 4); expanded vegetative leaves without petioles (lane 5); roots (lane 6); vegetative shoots with 2 mature vegetative leaves (lane 7); vegetative shoot tips with 4 mature vegetative leaves (lane 8); mixture of male and hermaphrodite gametophyte tissue cultured 5 (lane 9), 11 (lane 10), or 18 (lane 11) days after inoculation. 26S rRNA was used as a loading control.

Expression of CrLFY1 *and* 2 *mRNA*

Some MADS-box genes involved in floral development are directly induced by *FLO*/*LFY* gene in *Arabidopsis* (Parcy et al. 1998; Busch et al. 1999). To assess the origin and evolution of the MADS-box gene induction by *FLO*/*LFY* gene, expression patterns of *FLO*/*LFY* genes and previously reported MADS-box genes (Münster et al. 1997; Hasebe et al. 1998) in the fern *Ceratopteris richardii* were compared. Expression patterns of MADS-box genes have not been reported in other ferns and fern allies. A study of this fern is important because of its tractable genetics, its relatively rapid life cycle, and the large body of information known about its development (Banks 1999).

Ceratopteris richardii germinates from its spore 5 days after inoculation (dai), and develops into male and hermaphroditic gametophytes. Male gametophytes are induced by the pheromone antheridiogen secreted by hermaphroditic gametophytes (Banks 1999). Male gametophytes have a spatula-like shape and form only antheridia around 11 dai. Hermaphroditic gametophytes are

heart-shaped and form both antheridia and archegonia around 11 dai. An egg in an archegonium is fertilized by one of the sperms released from the antheridia. In our culture conditions, the approximate ratio of males to hermaphrodites is 4 to 1. At 18 dai, some eggs are fertilized, and sporophytes start to develop. Sporophytes form several vegetative leaves during early stages of development, then switch to form reproductive leaves with sporangia in which mitosis occurs to form spores. The expression of *CrLFY1* and *2* mRNA was assessed by northern hybridization using a specific probe for each gene (Fig. 2). *CrLFY1* and *2* transcripts were detected in both vegetative and reproductive shoot tips and circinate reproductive leaves. Three times less RNA was loaded in lane 1 and 2 than in lane 7 and 8, suggesting that signals in reproductive shoot tips (lane 1) are stronger than those of vegetative shoot tips (lanes 7 and 8 in Fig. 2). A weak signal was also detected in other tissues, including vegetative shoots, roots, and gametophytes. The signals at the circinate reproductive leaves were weaker than the fertile shoot tips. *CrLFY2* was more ubiquitously expressed than *CrLFY1*, but its expression in the shoot tips was stronger than any other tissues examined.

Several MADS-box genes have been cloned in *Ceratopteris*, and the expression of *CMADS1, 2, 3, 4* and *CRM3*/*CMADS6* genes has been examined in detail (Münster et al. 1997; Hasebe et al. 1998). *CMADS1, 2,* and *3* genes are expressed in the shoot apex, sterile leaves, fertile leaves, and roots. Expression at the shoot apex is weaker than in other tissues. *CMADS4* is strongly expressed in roots, and weakly expressed in other sporophytic tissues. *CRM3*/*CMADS6* is predominantly expressed in gametophytes, but expression is detectable in sporophytic tissue. These patterns of expression are different from those of *CrLFY1* and *2*, which are predominantly expressed at the fertile shoot tip (Fig. 2). This indicates that neither of the *CrLFY* genes directly induces these *Ceratopteris* MADS-box genes, although we cannot rule out the possibility that *CrLFY* indirectly induces these genes. When a gene targeting or a transformation technique that is feasible in the lower land plant *Physcomitrella patens* (Nishiyama et al. 2000) becomes available in *Ceratopteris*, a more detailed analysis of the regulation of *Ceratopteris* MADS-box genes will be possible. In *Arabidopsis*, floral homeotic MADS-box genes are directly induced by the *LFY* gene (Parcy et al. 1998; Busch et al. 1999). Other angiosperm *FLO*/*LFY* orthologs are likely to have similar functions based on expression patterns which are similar to the *LFY* gene (Coen et al. 1990; Kelly et al. 1995; Hofer et al. 1997; Southerton et al. 1998; Souer et al. 1998; Shu 1999; Rottmann et al. 2000). Gymnosperm *FLO*/*LFY* orthologs are also likely to induce the gymnosperm orthologs of floral homeotic genes in conifers (Tandre et al. 1998; Rutledge et al. 1998; Mouradov et al. 1999; Sundström et al. 1999) and *Gnetum* (Winter et al. 1999), as the expression patterns of *FLO*/*LFY* orthologs and floral homeotic gene orthologs are similar (Mouradov et al. 1998). Together, our results indicate that the direct induction of floral homeotic gene homologs by the *FLO*/*LFY* gene was established after the divergence of ferns from the seed plant lineage (ca. 400 million years ago: Stewart and Rothwell 1993), and before the divergence of gymnosperms and angiosperms (ca. 300 million years ago: Stewart and Rothwell 1993). According to the *FLO*/*LFY* gene tree (Fig. 1), we have to be careful that *CrLFY1* and *2* are not orthologous to seed plant *FLO*/*LFY* genes, and we cannot rule out the possibility that pteridophyte *FLO*/*LFY* orthologs (*AlLFY2, 3,* and *IaLFY1*) may regulate their MADS-box genes. Since no MADS-box genes have been analyzed in either *Angiopteris* or *Isoetes*, further study of these species will be necessary to examine the origin of the *FLO*/*LFY* gene—MADS-box genes cascade. More detailed analyses of the expression patterns of *CrLFY* genes will also give further insights into their function in *Ceratopteris.*

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