Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1979–1982, March 1998
Colloquium Paper

This paper was presented at a colloquium entitled "Protecting Our Food Supply: The Value of Plant Genome Initiatives," organized by Michael Freeling and Ronald L. Phillips, held June 2–5, 1997, sponsored by the National Academy of Sciences at the Arnold and Mabel Beckman Center in Irvine, CA.

Down-regulation of *RFL*, the *FLO/LFY* homolog of rice, accompanied with panicle branch initiation

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ABSTRACT ***FLORICAULA* (*FLO*) of *Antirrhinum* and *LEAFY* (*FLY*) of *Arabidopsis* regulate the formation of floral meristems. To examine whether same mechanisms control floral development in distantly related species such as grasses, we isolated *RFL, FLO-LFY* homolog of rice, and examined its expression and function. Northern analysis showed that *RFL* is expressed predominantly in very young panicle but not in mature florets, mature leaves, or roots. *In situ* hybridization revealed that *RFL* RNA was expressed in epidermal cells in young leaves at vegetative growth stage. After the transition to reproductive stage, *RFL* RNA was detected in all layers of very young panicle including the apical meristem, but absent in the incipient primary branches. As development of branches proceeds, *RFL* RNA accumulation localized in the developing branches except for the apical meristems of the branches and secondary branch primordia. Expression pattern of *RFL* raised a possibility that, unlike *FLO* and *LFY, RFL* might be involved in panicle branching. Transgenic *Arabidopsis* plants constitutively expressing *RFL* from the cauliflower mosaic virus 35S promoter were produced to test whether 35S-*RFL* would cause similar phenotype as observed in 35S-*LFY* plants. In 35S-*RFL* plants, transformation of inflorescence meristem to floral meristem was rarely observed. Instead, development of cotyledons, rosette leaves, petals, and stamens was severely affected, demonstrating that *RFL* function is distinct from that of *LFY*. Our results suggest that mechanisms controlling floral development in rice might be diverged from that of *Arabidopsis* and *Antirrhinum*.**

Gramineae is a large and variable family. Many features of flower development and mature architecture of grass flowers and inflorescences are distinct from those of dicots. Although our knowledge on the genetic network governing initiation and morphogenesis of flowers increased significantly in the last several years, little is known about molecular mechanisms controlling floral development in grass species. Understanding grass flower development may offer general insights into the genetic and developmental bases of morphological evolution among the plant species. Rice (*Oryza sativa* L.) has several advantages that make it a good candidate as a model species to study molecular basis of grass flower development (1). Rice is a diploid species with a small genome (430 Mb/haploid), and analyses of the rice genome and cDNAs have rapidly progressed (2, 3). Moreover, transgenic rice plants can be relatively easily produced by *Agrobacterium*-mediated transformation (4).

Genetic and molecular studies with two dicot plants, *Antirrhinum* and *Arabidopsis*, have shown that the genetic network controlling flower development is conserved at least in the two dicot species (5–7). After the transition from vegetative to reproductive development, floral meristems are initiated by the action of a set of genes called floral meristem identity genes. Among them, *FLORICAULA* (*FLO*) of *Antirrhinum* and its *Arabidopsis* counterpart *LEAFY* (*LFY*) seem to play the most important role for the establishment of floral fate. In strong *flo* and *lfy* mutant plants, flowers are transformed into inflorescence shoots (8, 9). *FLO/LFY* encode putative transcription factors that do not show significant homology to any known genes (8, 9).

To understand molecular mechanisms controlling floral meristem initiation and inflorescence structure of rice we have isolated *RFL*, the *FLO/LFY* homolog of rice, and analyzed its expression and function. We found that the function of *RFL* is distinct from that of *LFY*. Our analysis on *RFL* expression showed that it is unlikely that *RFL* is absolutely required for floral initiation in rice. Instead, the expression pattern of *RFL* suggests its possible involvement in panicle branching. Our results show that the findings obtained from *Arabidopsis* and *Antirrhinum* may not be simply applied as a general model to other distantly related species such as grasses.

MATERIALS AND METHODS

Construction and Screening of cDNA Library. To construct a cDNA library, total RNA was isolated from young panicles of rice (*Oryza sativa* L., cv. Toride 1). Poly(A)$^+$ mRNA was purified from the total RNA by using Magnesphere streptavidin paramagnetic particles (Promega). Double-stranded cDNA was synthesized according to the protocol of supplier (Pharmacia) and cloned into ZAPII vector (Stratagene).

Primers used to amplify rice *FLO/LFY*-like genes by the PCR were designed based on highly conserved sequences found in *FLO/LFY*. The 5′ primer was 5′-TAC/TATA/CAAC/TAAA/GCCA/G/C/TAAA/GATG-3′ and the 3′ primer was 5′-AGCC/TTG/TGTG/TGGG/C/AACA/GTACCA-3′. Genomic DNA of rice cv. Toride 1 was used as templates for the PCR. The PCR product of 235 bp was cloned into pGEM-T (Promega) and used as a probe for screening a cDNA library and Southern blot analysis.

Approximately 10^6 plaques were screened with a gel-purified radiolabeled probe. Hybridization and washes were carried out by standard protocol. The plasmids containing positive cDNA were rescued *in vivo* from phages according to the manufacturer's protocol (Stratagene). Both strands of the

Data deposition: The sequence reported in this paper was deposited in the GenBank database (accession no. AB005620).

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cDNA were sequenced by an automated sequencer (Applied Biosystems 373A) by using dideoxy-cycle sequencing protocol.

DNA Isolation and Southern Blot Analysis. DNA was isolated from young leaves of rice cv. Toride 1 according to the method described previously (10). Two micrograms of the genomic DNA was digested with restriction enzymes and electrophoresed. A 235-bp fragment amplified by PCR was labeled with digoxygenin and used as a probe. Hybridization and washing were carried out as described in the protocol (Boehringer Mannheim).

RNA Isolation and Northern Blot Analysis. RNA was isolated from various tissues by a published method (11). Twenty micrograms of total RNA was separated by electrophoresis. The gel was blotted to nylon membrane (Hybond N+) and hybridized with radiolabeled probe.

***In Situ* Hybridization.** Full-length *RFL* cDNA cloned into pBS(SK+) was linearized and used as a template to produce digoxygenin-labeled antisense RNA probe. Hybridization, washing, and detection were carried out according to Kouchi *et al.* (12).

***Arabidopsis* Transformation.** A 1.5-kb *RFL* cDNA in pBS-(SK+) was digested by *Xba*I and *Eco*RI and subsequently cloned into pIG121-Hm (13) to produce 35S-*RFL* (accession no. AB005620). The 35S-*RFL* construct was introduced to *Arabidopsis*, ecotype Columbia, by vacuum infiltration (14). T_1 generation seeds were sown on MS medium containing 2% sucrose, 0.8% agar, and 30 mg/liter kanamycin. Kanamycin-resistant plants were transferred to soil and grown further in a glass house at 24°C under long day conditions (14 h light/10 h dark).

RESULTS

Isolation of *RFL*. The *FLO/LFY* homolog was obtained by screening a cDNA library prepared from young rice panicles. The longest clone was named as *RFL* and used for further analysis. *RFL* cDNA is 1.5 kb and encodes an ORF of 379 aa (Fig. 1). Deduced amino acid sequence of RFL has a higher homology in the C-terminal half than in the N-terminal half. Overall identity of RFL to FLO and LFY were 48% and 44%, respectively. Southern blot analysis revealed that *RFL* exists as a single-copy gene in rice (data not shown). Comparison between *RFL* cDNA and genomic sequence revealed that the number and position of introns are precisely conserved in *RFL*, *FLO*, and *LFY* (data not shown).

Expression Pattern of *RFL* During Panicle Development. Northern analysis showed that *RFL* is expressed predominantly in young panicles but not in mature florets, leaves, or roots (Fig. 2*A*). Temporal and spatial expression patterns of *RFL* RNA were further examined by *in situ* hybridization.

A vegetative shoot apex produces leaves distichously. After the transition to reproductive growth, a young panicle apex produces several bracts of the panicle. Primary branches grow out from the axil of each bract. Secondary branches are produced from the primary branches. These branches terminate in a single-flowered spikelet (15).

Before transition to reproductive growth, *RFL* RNA was detected in epidermal cells at the marginal region in young leaves but not in the vegetative shoot apical meristem or stem tissue (Fig. 2*B*). After the transition, *RFL* RNA expression was observed in all layers of the very young panicle producing primary branch primordia but was absent in the primary branch differentiation sites (Fig. 2 *C* and *D*). As development of branches proceeded, *RFL* RNA accumulation was localized in the developing branches except for the apical meristem of the branches (Fig. 2 *E* and *F*). The apical meristem of the panicle axis loses its activity and degenerates after the production of several primary branch primordia, leaving a scar on the main rachis (15). The *RFL* RNA expression was observed in the vegetative apical meristem at a very early stage of panicle development (Fig. 2 *B* and *C*), then it started to diminish in the corpus of the panicle axis at the middle stage of primary branch differentiation (Fig. 2*D*). After all the primordia of primary branches had initiated, *RFL* RNA dis-

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RFL  MDPND-AFSA AHPFRWDLGP PAPAPVPPPP PPPPPPPPAN V-PREL---E ELVAGYGVRM    55
LFY  ***EGFTSGL FRWNPTRALV Q**P*****L QQQ*VT*QTA AFGMR*GGL* G*FGP**I*F    60
FLO  ***DAFLFKW D*RTALPQPN RLLDA*A*** ****QA*SYS MR****GGL* **FQA**I*Y    60

RFL  STVARISELG FTASTLLAMT ERELDDMMAA LAGLFRWDLL LGERFGLRAA LRAERGRLMS   115
LFY  Y*A*K*A*** ******VG*K DE**EE**NS *SHI***E** V***Y*IK** V****R**QE   120
FLO  Y*A*K*A*** **VN***DMR DE***E**NS *CQIF***** V***Y*IK** V****R*IDE   120

RFL  LGG-----RH HGHQSGSTVD GASQ--EVLS DEHDMAGSGG MGDDDNGRRM VTGKKQAKKG   168
LFY  EEEEESSR*R *LLL*AAGDS *THHALDA** Q*D*WT*LSE EPVQQQDQTD AA*NNGGGGS   180
FLO  EE----VR*R *LLL---GD- -TTHALDA** QE----*LSE EPVQQE--KE AM*SGGGGV*   165

RFL  S----AARKG KKARRKKVDD LRLDMQEDEM DCCDEDGGGG SESTESSAGG GGGERQREHP   224
LFY  GYWDAGQGKM **QQQQRRRK KPMLTSVETD EDVNEGEDDD GMDNGNGGS* L*T*******   240
FLO  GVWEMMGAG* R**PQRRRKN YKGRSRMASM EE-DDDDDDD ETEGAEDDEN IVS*******   225

RFL  FVVTEPGEVA RAKKNGLDYL FHLYEQCRLF LLQVQSMAKL HGHKSPTKVT NQVFRYAKKV   284
LFY  *I******** *G******** ********D* *****TI**D R*E*C***** *********S   300
FLO  *I******** *G******** ********D* *I***TI**E R*E*C***** *********A   285

RFL  GASYINKPKM RHYVHCYALH CLDEEASDAL RRAYKARGEN VGAWRQACYA PLVDISARHG   344
LFY  ********** ********** *******N** ***F*E**** **S******K ***N*AC***   360
FLO  **N******* ********** ****A**N** ***F*E**** *********K ***A*A**Q*   345

RFL  FDIDAVFAAH PRLAIWYVPT RLRQLCHQAR SSHAAAAAAL PPPLF----- ---------- ---- 389
LFY  W******N** ***S****** K******LE* NNAV****** VGGISCTGSS TSGRGGCGGD DLRF 424
FLO  W***TI*N** ***S****** K******AE* **A*V**TSS ITG------- ----GG-PAD HLPF 396
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FIG. 1. Comparison of amino acid sequences encoded by *RFL, FLO*, and *LFY*. Conserved amino acids are shown by asterisks.

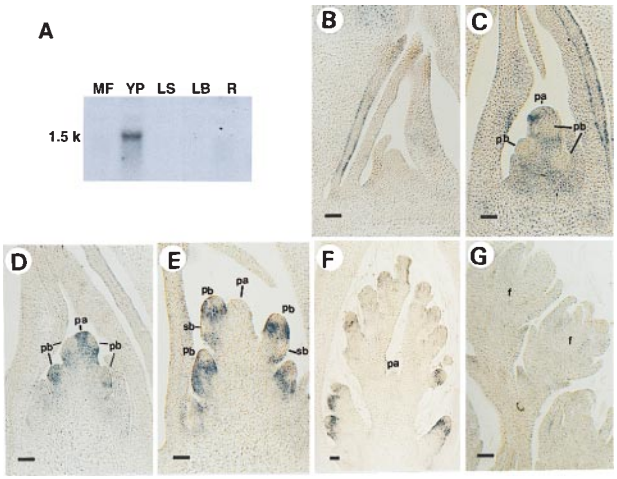

FIG. 2. Distribution of *RFL* RNA in rice plants. (*A*) Northern blot analysis. MF, mature florets; YP, young panicles; LS, leaf sheaths; LB, leaf blades; R, roots. (*B–G*) *RFL* expression analyzed by *in situ* hybridization. (*B*) A vegetative shoot apex. (*C* and *D*) A young panicle at primary branch primordia differentiation stage. (*E*) A young panicle at secondary branch primordia differentiation stage. (*F*) A developing panicle. Four primary branches at various developmental stages in a panicle are shown. The oldest primary branch (arrow) is in the floret differentiation stage. (*G*) Developing florets. All floral organis have developed by this stage. pb, primary branch; sb, secondary branch; pa, panicle apex; f, floret. (Bar = 50 μm.)

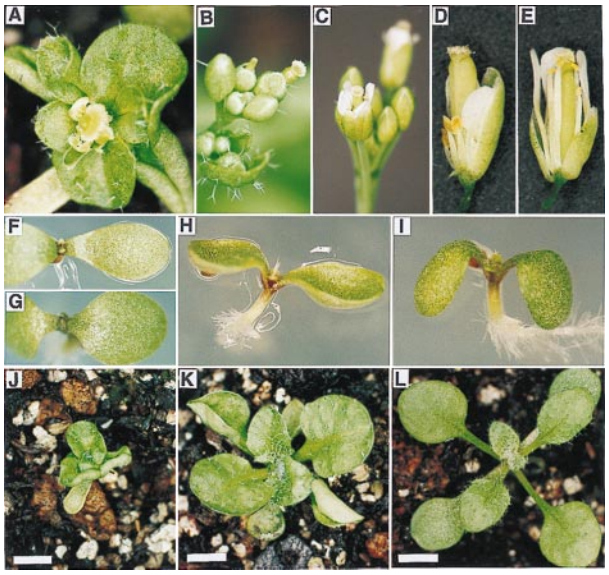

FIG. 3. Transgenic *Arabidopsis* plants transformed with 35S-*RFL*. (*A*) A 35S-*RFL* plant with an abnormal terminal flower. (*B–D*) Flowers with short petals and short stamens in 35S-*RFL* plants (*B* and *D*) and wild-type flowers (*C* and *E*). A sepal and a petal were removed from a flower in *D* and *E*. (*F–H*) Seedlings of 35S-*RFL* (*F* and *H*) and wild-type (*G* and *I*) plants. (*J–L*) Rosette leaves in 35S-*RFL* plants (*J* and *K*) and a wild-type plant at 19 days (*L*). (Bar = 5 mm.)

appeared entirely from the main axis of the panicle (Fig. 2 *E* and *F*). Down-regulation of the *RFL* expression was observed again in incipient or developing secondary branches (Fig. 2*E*). No detectable level of *RFL* RNA expression was found in developing panicles after the branch formation stage (Fig. 2 *F* and *G*). Control sections hybridized with the sense *RFL* RNA probe gave no signal above background (data not shown).

Ectopic Expression of *RFL* in *Arabidopsis*. Ectopic expression of *Arabidopsis LFY* by cauliflower mosaic virus (CaMV) 35S promoter caused transformation of lateral and main inflorescence shoots into floral meristems (16). To examine whether expression of *RFL* gene product in *Arabidopsis* would result in the phenotype similar to that of 35S-*LFY* plants, we made transgenic *Arabidopsis* plants containing 35S-*RFL*.

T_2 plants of 10 independent transgenic lines were grown in a glass house to examine phenotypic alterations. Generally, the phenotype observed in 35S-*RFL* was different from that reported in the 35S-*LFY* plants. We found transformation of inflorescence meristems into flower meristem only in 2 cases out of 100 plants derived from the 10 lines, and the observed terminal flower was extremely abnormal, as shown in Fig. 3*A*. This suggests that *RFL* possesses similar function with *LFY* to a very limited extent. We observed defects in both vegetative and reproductive growth in nine lines, which was not found in 35S-*LFY* transgenic plants. Six lines showed highly abnormal morphology, and three were less severely affected. One line did not show any morphological difference from wild-type plants, and this line was not analyzed further. Cotyledons of 35S-*RFL* plants of all the nine lines were cup-shaped and narrower than those of wild-type plants (Fig. 3 *F–I*). Cosegregation of this cotyledon phenotype and kanamycin resistance was genetically confirmed in all nine lines (data not shown). Rosette leaves were also affected in all nine lines (Fig. 3 *J* and *K*). Curling of rosette leaves was commonly observed. The 35S-*RFL* transgenic plants with severe phenotypes did not have distinct petioles, and plant size was significantly reduced (Fig. 3*J*). The severity of phenotypes in cotyledons correlated well with the curled and wrinkled rosette leaves and shorter petioles. Flowers of 35S-*RFL* plants were also affected. Petals and stamens in 35S-*RFL* plants were shorter than those in wild-type plants (Fig. 3 *B–E*).

DISCUSSION

We found significant differences in *RFL* expression pattern from that of *FLO* and *LFY* (8, 9). *FLO* and *LFY* are expressed in the floral meristem at very early stages of development, consistent with their roles in the initiation of floral meristems. In contrast, *RFL* RNA accumulation was observed in young panicles and developing branches much earlier than the initiation of floral meristem. It should be noted that *RFL* expression has been excluded from the apical meristem of the branches since the initiation of the branch primordia. These meristems are to be converted into floral meristems to form a terminal flower at the top of each primary branch. These demonstrate that initiation of floral meristems takes place without detectable levels of *RFL* RNA accumulation. From this it appears unlikely that *RFL* is required for initiation of floral meristems in rice.

Interestingly, *RFL* RNA accumulation is down-regulated in the cells determined to initiate inflorescence branch primordia. Similar patterns of down-regulation of RNA expression has been reported for *KNOTTED 1* (*KN 1*) gene of maize (17) and *SHOOTMERISTEMLESS* (*STM*) (18) gene of *Arabidopsis. KN 1* and *STM* RNAs are expressed in vegetative shoot meristems and are down-regulated in the incipient and developing leaves. *Arabidopsis* seedlings homozygous for recessive mutations of *STM* exhibit failure to develop and maintain shoot apical meristem. Although the role of the wild-type *KN 1* gene product is unknown, gain-of-function mutations of the *KN 1* cause extra cell division in leaf blade resulting in outgrowth or knot formation. Transgenic plants constitutively expressing *KN 1* developed ectopic meristems (19). From these studies, it has been proposed that the *KN 1* and *KN 1* STM gene products are involved in the maintenance of the indeterminate state of meristems, and that down-regulation of their expression leads to the initiation of determinant lateral organs. Based on the analogy of *KN 1* and *STM* expression, we speculate that *RFL* may play a role in pattern formation of inflorescence

architecture by maintaining an undifferentiated state of cells in the meristems and/or repressing differentiation. An alternative interpretation is that the *RFL* expression is a prerequisite for the initiation of the branch primordium. Identification of loss-of-function phenotypes should help elucidate the exact role of *RFL* during panicle development.

We showed that the coding sequence from the *RFL* gene has a very limited ability to act as a developmental switch to initiate floral meristems compared with *LFY*. The main shoot and all lateral shoots were converted to solitary flowers in transgenic *Arabidopsis* expressing *LFY* under the control of CaMV 35S promoter (16). In contrast, transformation of main or lateral inflorescence shoots to floral meristems was rarely observed in *Arabidopsis* containing 35S-*RFL*. Furthermore, plants containing 35S-*RFL* showed a variety of morphological abnormalities in vegetative organs in contrast to the absence of abnormal phenotypes in the *LFY* mutant plants or 35S-*LFY Arabidopsis* plants (9). Lee *et al.* (20) reported lobed leaves in transgenic *Arabidopsis* carrying 35S-*UFO*, and this phenotype required the presence of functional *LFY*. They also reported that a low level of *LFY* RNA expression is present in young leaf primordia of *Arabidopsis*. Their results and ours suggest that *LFY* plays an unknown role in the development of leaves. Expression of the *RFL* but not *LFY* may disturb the function of endogenous *LFY* function in leaves. It will be interesting to see whether *FLO*/*LFY* homologs from other grass species cause similar phenotypes as 35S-*RFL* when ectopically expressed in *Arabidopsis*. Although conservation of sequences indicates that *RFL* and *FLO*/*LFY* arose from the common ancestral gene, diversification of their functions as well as regulation of their expression have occurred during evolution.

We thank Koji Goto of Kyoto University for providing *Arabidopsis* seeds and help in *Arabidopsis* transformation.

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