Two LEAFY homologs ILFY1 and ILFY2 control reproductive and vegetative developments in Isoetes L.

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LEAFY (LFY) is a plant-specific transcription factor, which is found in algae and all land plants. LFY homologs exert ancestral roles in regulating cell division and obtain novel functions to control floral identity. Isoetes L. is an ancient genus of heterosporous lycophytes. However, characters about LFY homologs in lycophytes remain poorly investigated. In this study, two LFY homologs, ILFY1 and ILFY2, were cloned from five Isoetes species, including I. hypsophila, I. yunguensis, I. sinensis, I. orientalis, and I. taiwanensis. The full length of ILFY1 was 1449–1456 bp with an open reading frame (ORF) of 927–936 bp. The full length of ILFY2 was 1768 bp with ORF of 726 bp. Phylogenetic tree revealed that ILFY1 and ILFY2 were separated into two clades, and I. hypsophila were separated with the others. Expression analysis demonstrated that IsLFY1 and IsLFY2 for I. sinensis did not show functional diversity. The two transcripts were similarly accumulated in both vegetative and reproductive tissues and highly expressed in juvenile tissues. In addition, the IsLFY1 and IsLFY2 transgenic Arabidopsis similarly did not promote precocious flowering, and they were inactive to rescue lfy mutants. The results facilitate general understandings about the characteristics of LFY in Isoetes and evolutionary process.

Flowering is a common and critical event during the life cycle of angiosperm plants. During vegetative phase, apical meristems give rise to leaves and lateral shoots. Then plants switch from vegetative into reproductive developments in Arabidopsis LFY, controls the branching of inflorescence12. The function of LFY is paralogous with NEEDLY in gymnosperms17. But only LFY in gymnosperms is orthologous with LFY in angiosperms. The LFY and NEEDLY paralogs in gymnosperms are specifically expressed in both vegetative and reproductive meristems18, 19. The LFY paralog provides more activity to rescue Arabidopsis lfy mutant phenotypes than NEEDLY1–18.

In addition, LFY homologs are also present in free-sporing land plants, including lycophytes, ferns or their allies and bryophytes1–2, 20. Expressions of CrLFY1 and CrLFY2 in Ceratopteris richardii are prominent in shoot tips and circinate reproductive leaves20. But CrLFY2 only has some ability to rescue severe Arabidopsis lfy mutants, which is less than LFY homologs in gymnosperms1. Two PpLFY paralogs in the moss Physcomitrella patens are
markedly expressed in gametophyte and sporophyte. The PpLFY proteins play critical roles in controlling first zygotic cell division. Whereas they are inactive to complement Arabidopsis lfy mutants. Taken together, these observations imply that translation from the vegetative to reproductive development and directed induction of the floral homeotic MADS-box genes by LFY are established after the divergence of mosses and before the divergence of vascular plant lineage. LFY homologs probably regulate cell division, expansion and arrangement in free-sporing land plants, and they control both floral identity and cell division in seed plants. Additionally, LFY homolog is not specific to land plants and it is also found in algae, indicating ancestral roles predating land plants.

Isoetes L. is an ancient genus of heterosporous lycopsids. Phylogenetic analyses show that Isoetes is one of the earliest basal vascular plants, which can date back to the Devonian. Isoetes has approximately 200 species recognized by a strong reduced plant body. Furthermore, this genus is the only survival of ancient taxa as the closest relatives of the famous tree lycopods. The plants have fleshy corms with a range of spirally arranged microphylls and multiple dichotomizing roots along with median furrows of the corm. Microsporangia and megasporangia are embedded in the bottom of fertile leaves. With the corms growing in girth, the leaves and sporangia are becoming mature from inside to outside. It is prevalent to identify species and analyze phylogenetic relationships using second intron of LFY homologs. Although Isoetes possesses an important position in phylogenetic evolution, characters and functions of LFY homologs for Isoetes (referred as LFY) are still ambiguous.

In this present study, we cloned two LFY paralogs, including ILFY1 and ILFY2. Totally, we identified the two genes in five Isoetes species, including I. hypsophila, I. yunguiensis, I. sinensis, I. orientalis, and I. taiwanensis. Roles of the two LFY paralogs in I. sinensis (IhLFY1 and IhLFY2) were further investigated using the quantitative real-time PCR (qRT-PCR) and in situ hybridization assays. Moreover, we created transgenic Arabidopsis plants, which were constitutively expressed IhLFY1 and IhLFY2 under the control of the cauliflower mosaic virus 35S promoter, respectively. This comprehensive study will facilitate our understandings about the roles of LFY homologs in Isoetes and evolutionary process.

Results
Cloning of ILFY1 and ILFY2 sequences. The full-length of ILFY genes were cloned from five Isoetes species, including I. hypsophila, I. yunguiensis, I. sinensis, I. orientalis, and I. taiwanensis. In total, we isolated two ILFY paralogs, including ILFY1 and ILFY2, based on the 5′ and 3′ rapid amplifications of cDNA ends (RACE) systems. The full length of ILFY1 was 1449–1456 bp with an open reading frame (ORF) of 927–936 bp (Fig. 1). The full length of ILFY2 was 1768 bp with ORF of 726 bp. More detailed information about ILFY1 and ILFY2 was list in Table S1, including the full length cDNA, 5′UTR, open reading fragment (ORF), 3′UTR of the two homologs in the five species. The ORF sequences of ILFY1 and ILFY2 were identical for I. yunguiensis, I. orientalis, and I. taiwanensis. ILFY1 for the four species occupied 94.86% identity with IhLFY1, and ILFY2 shared 98.68% identity with IhLFY2. Furthermore, The ORF fragments for ILFY1 and ILFY2 for I. yunguiensis, I. sinensis, I. orientalis, and I. taiwanensis were shared 63.41% identity. The ORF fragments for IhLFY1 and IhLFY2 displayed 62.57% identity. The corresponding sequences have been submitted into the GenBank database with the accession numbers from KX229755 to KX229764.

We also amplified and sequenced genomic ILFY1 and ILFY2 sequences in the five Isoetes species. Comparison analysis revealed that both ILFY1 and ILFY2 in the five Isoetes species contained three exons and two introns (Fig. 1). The length of first intron for ILFY1 was 319–325 bp and second intron was 957–992 bp in length. The first intron for ILFY2 was 200 bp in length and second intron was 914–926 bp in length.

Protein comparison and phylogenetic tree. The predicted ILFY1 and ILFY2 amino acid sequences were aligned with a diverse of LFY homologs downloaded from the NCBI database. The comparison analysis revealed that the N-terminal regions were partially conserved and C-terminal regions were highly conserved in evolutionary process (Fig. 2). Moreover, the clearly conserved C-terminal domains were comprised of two β-sheet and seven α-helix structures. Identical levels of amino acid sequences were shown in Table S2 through comparing deduced proteins of IhLFY1, IhLFY2, IsLFY1, and IsLFY2 with LFY in A. thaliana, PRLFY1 and NEEDLY in
Pinus radiata, CRLFY2 in Ceratopteris richardii, SmLFY1 in Selaginella moellendorffii, and PpILFY1 in *P. patens*, respectively. A conserved histidine residue (His) is substituted by Asp in the C regions for PpILFY1 and PpILFY2 in *P. patens*. Nonetheless, the ILFY1 and ILFY2 proteins in the C regions consistently contained the conserved His residue, which was similar to that in numerous land plants. To determine the phylogenetic relationships of ILFY1 and ILFY2, we constructed a phylogenetic tree of LFY amino acid sequences using the maximum likelihood method (Fig. 3). The phylogenetic tree showed that topology of LFY homologs was concordant with the species topology. The ILFY1 and ILFY2 paralogs in the five *Isoetes* species were separated into two clades. Moreover, the two paralogs in *I. hypsophila* were separated with the other four species. In addition, we also used nucleotide sequences of the conserved N-terminal and C-terminal regions to reveal the phylogenetic relationships of ILFY1 and ILFY2. The phylogenetic tree was similar with the phylogenetic tree based on the amino acid sequences (Figure S1). The ILFY1 and ILFY2 paralogs in the five *Isoetes* species were also separated into two clades and the two paralogs in *I. hypsophila* were separated with the other four species.

**qRT-PCR.** The expression levels of *IsLFY1* and *IsLFY2* were investigated using qRT-PCR approach, respectively. The materials included roots, corms, and each whorl of leaves, megasporangia, and microsporangia. Averagely, there were about 12 whorls for each plant, including the former 11 whorls of sporophylls and last whorl of immature leaves. We collected all whorls of sporophylls or immature leaves and sporangia, including the former 7 whorls of megasporangia and latter 4 whorls of microsporangia. Expression analysis revealed that the *IsLFY1* transcripts were slightly expressed in the former 4 whorls of megasporophylls and megasporangia, and expression levels were increasing from the 5th to 7th whorls (Fig. 4). Moreover, the expression levels in the 7th whorl of megasporangia were the highest in all collected samples. Generally, the expression levels were higher in the 8th to 11th whorls of microsporophylls and microsporangia than that from the 1th to 5th whorls, respectively. Furthermore, expression level was higher in the 11th whorl of microsporangia than the other whorls of sporangia. For the 12th whorl for immature leaves, the expression levels were relatively higher than the other whorls of sporophylls. In addition, the expression levels at roots were prominently strong. For the *IsLFY2* transcripts, expression levels in all tissues were similar with that of the *IsLFY1* transcripts (Fig. 4). But *IsLFY2* expressions were significantly higher than that of *IsLFY1* at the corms. Similarly, the expression level of *IsLFY2* was the highest in the 7th whorl of megasporangia, followed by the roots and 11th whorl of microsporangia.

**In situ hybridization.** Expression patterns of the *IsLFY1* and *IsLFY2* transcripts were further characterized using in situ hybridization assays. A range of tissues were selected, containing roots, corms, and the 6th whorl of leaves and megasporangia, and 8th whorl of microsporangia. The *IsLFY1* and *IsLFY2* transcripts showed similar expression patterns in all examined tissues. The transcripts in roots were accumulated in all parts of roots except periderm (Figs 5a and 6a). Moreover, the two transcripts were significantly detected in endodermis, connective...
parts, and vascular bundles. Leaves of \( I. \) \( sinensis \) are quadrangular in outline and the two transcripts were detected all parts of the leaves, including parenchyma cells and vascular bundles (Figs 5b and 6b). Corm of \( I. \) \( sinensis \) is a short tuberous body35. In situ hybridization for longitudinal sections of the corms showed that the two transcripts were dramatically expressed at the zone of parenchyma cells which surrounded vascular bundles (Figs 5d and 6d). The expressions were slight in the cortical cells from transverse sections of the corm (Figs 5c and 6c). In addition, megasporangia and microsporangia are in the bottom of the sporophylls (Figure S2), and the spores are separated by trabeculae29. In general, a large number of microspores are located in microsporangia and limited megaspores are in megasporangia. \( IsLFY1 \) was strongly expressed in trabeculae or intine of the microsporangia, and partial microspores (Fig. 5e). \( IsLFY2 \) was also strongly expressed in trabeculae or intine of the microsporangia, whereas it is faintly detected in microspores (Fig. 6e). In addition, the two transcripts were expressed at high levels in trabeculae or intine of the megasporangia and expressed at a low level in megaspores (Figs 5f and 6f).

**Arabidopsis transgenes.** To investigate whether the roles of \( IsLFY1 \) and \( IsLFY2 \) were conserved in evolutionary process, we created transgenic **Arabidopsis** plants, which constitutively expressed \( IsLFY1 \) and \( IsLFY2 \) under the control of the cauliflower mosaic virus 35S promoter, respectively. The T1 seeds were selected using kanamycin resistance and resistance plants were grown under long-day conditions. In total, we isolated 30 \( IsLFY1 \) transgenic **Arabidopsis** lines, and only two accelerated flowering. Totally, 25 \( IsLFY2 \) transgenic lines were identified and only one showed precocious flowering. As shown in Table S3, the remaining transgenic plants did not differ significantly in appearance from the wild-type controls and they did not cause precocious flowering (Fig. 7a–d).

In addition, we also created the transgenic plants that the full-length \( IsLFY1 \) and \( IsLFY2 \) cDNAs were transformed into severe \( ify-1 \) **Arabidopsis** mutants under the control of the cauliflower mosaic virus 35S promoter, respectively. In total, we produced 6 \( IsLFY1 \) transgenic and 5 \( IsLFY2 \) transgenic lines (T1). The phenotypic characters were investigated from the F2 progeny. For \( ify-1 \) mutants, early-arising flowers are replaced by bracts and completely transformed into inflorescence shoots (Fig. 7e). The later-arising flowers are abnormal without petals and stamens5. Whereas wild-type flowers include four sepals, four petals, two carpels, and six stamens (Fig. 7d).
Among the 200 T1 lines examined, 30 were homozygous for lfy-1 mutants. However, only one IsLFY1 transgenic lines partially complement the lfy phenotype. The remaining plants showed inactive to rescue the lfy mutants.

Figure 4. Expression patterns of IsLFY1 and IsLFY2 using quantitative real-time PCR assays. The abbreviations L, Me, Mi, R, Co represent leaves, megasporangia, microsporangia, roots, and corms, respectively. The megasporangia were in the bottom of fertile leaves from 1th to 7th whorls, and microsporangia were from 8th to 11th whorls. The immature leaves were in the 12th whorl. Values represent the means ± SE. Asterisk indicated that the expression levels of IsLFY1 and IsLFY2 were significantly different with the P value less than 0.01.

Figure 5. In situ hybridization of IsLFY1 expressions. (a) Transverse section of roots. (b) Transverse section of leaves. (c) Transverse section of corms. (d) Longitudinal section of corms. (e) Transverse section of microsporangia. (f) Transverse section of megasporangia. Scale bars were 300 μm for megasporangia and microsporangia, 200 μm for root, leaves, and 100 μm for corms.
In addition, among the identified 28 transgenic \( \text{lfy-1} \) mutants, only one \( \text{IsLFY2} \) transgenic lines partially complement the \( \text{lfy} \) phenotype and the remaining plants similarly were inactive to rescue the \( \text{lfy} \) mutants (Fig. 7g). The early flowers were replaced by bracts, which subtended secondary inflorescences (Fig. 7h). The later flowers consisted of sepals and carpels (Fig. 7i,j). Phenotypes of the transgenic plants were indistinguishable from the \( \text{lfy} \) mutants (Table S4).

Discussion

Analysis of the deduced ILFY1 and ILFY2 protein in five \( \text{Isoetes} \) species showed that ILFY1 and ILFY2 amino acid sequences were common in \( \text{I. yunguiensis} \), \( \text{I. sinensis} \), \( \text{I. orientalis} \), and \( \text{I. taiwanensis} \). There was 94.86% identity for ILFY1 and 98.68% identity for ILFY2 in the four species compared with \( \text{IhLFY1} \) and \( \text{IhLFY2} \), respectively. Moreover, the ORF fragments of ILFY1 and ILFY2 shared 63.41% identity for the four species and 62.57% identity for \( \text{I. hypsophila} \). The five \( \text{Isoetes} \) species in China display a space order of the distribution pattern, \( \text{I. hypsophila-I. yunguiensis-I. sinensis-I. orientalis-I. taiwanensis} \), from high altitude to low altitude and from west to east distribution36, 37. Therefore, geographic isolation probably results in a slight diversity of the ILFY homologs in phylogenetic evolution. LFY homologs possess a markedly conserved C-terminal domain and partially conserved N-terminal domain 1, 38. Comparison analysis revealed that ILFY1 and IFLY2 in the C-terminal regions were highly conserved in the evolutionary process. The clearly conserved C-terminal regions include DNA binding domain to regulate downstream genes, whereas the partially conserved N-terminal regions play key roles in forming LFY dimerization and higher complex with other TFs or chromatin remodelers39–41. Moreover, An amino acid substitution from His to Asp in the C-terminal domain causes PpLFY proteins inactive by binding a canonical LFY-binding domain38. Nonetheless, ILFY1 and IFLY2 consistently shared the conserved His, which were resembled that in vascular plants.

Information about the expression patterns of \( \text{IsLFY1} \) and \( \text{IsLFY2} \) suggested that functions for the two genes might be conserved, probably being redundant in both paralogs, despite differences outside the DNA binding domains17, 42. In general, there is a single copy in most angiosperms and more than two copies in nonflowering plants. Nonetheless, major subfunctionalization is not occurred for the additional copies20, 43. \( \text{I. sinensis} \) includes sporophylls and immature leaves, and the leaves are closely spirally arranged on the fleshy corms29. The megasporophylls are located outside of the corm, and microsporophylls are growing inside of the corm. Moreover, the leaves are becoming mature from inside to outside, and the immature leaves are in the center of the corm28. Generally speaking, the \( \text{IsLFY1} \) and \( \text{IsLFY2} \) transcripts were consistently higher in microsporangia than megasporangia, and expression levels were higher in the inside microsporophylls than outside megasporophylls. Expression levels of the two genes were similarly highest in the 7th whorl of megasporophylls, followed by roots, the 11th whorls of microsporangia, and the transcripts were highly expressed in young tissues. Thus, the results demonstrated that the two ILFY paralogs played general roles in both reproductive and vegetative developments.
In situ hybridization analysis further revealed that *IsLFY1* and *IsLFY2* were ubiquitously expressed in all active parts of leaves and roots, trabeculae and intine of the megasporangia or microsporangia, and the zone of parenchyma cells which surrounded vascular bundles of the corms. The cambium appears in parenchyma cells which surrounded vascular bundles of the corms and it is not differentiated. Moreover, the transcripts were significantly detected in endodermis of the roots, which are poorly differentiated. The results probably indicated that *IsLFY1* and *IsLFY2* probably have an ancestral role in controlling cell division or arrangement and developmental process.

Constitutive overexpression of LFY in *Arabidopsis* promotes early flowering and reduces number of adult leaves. The transgenic plants transformed lateral shoots into terminal flowers. LFY is strongly expressed in floral primordia, and faintly expressed in cauline leaf primordia. Moreover, constitutive overexpression of LFY homologs results in precocious flowering in most angiosperms and gymnosperms. However, most of the *IsLFY1* and *IsLFY2* transgenic lines did not show precocious flowering relative to the wild-type plants, suggesting that the two paralogs were not functional orthologs with *LFY*. The early-arising flowers in severe *lfy* mutants are converted into leaves and lateral shoots, and the late-arising flowers are only composed of sepals and carpels. Observation revealed that the 35S::*IsLFY1* and 35S::*IsLFY2* transgenes were similarly inactive to complement *lfy* mutants, which were consistent with *PpLFY1* and *PpLFY2* in *P. patens*. Previous research proposed that complementation ability is gradually increasing from mosses to angiosperms, and a continuum of nonneutral change is
responsible for the functional changes. Moreover, the gradual activity among nonflowering plants implies alteration of the DNA binding specificity of LFY homologs. It is necessary to further investigate the interaction targets of LFY to elucidate the evolutionary process.

In summary, we cloned two LFY paralogs in five Isoetes species, including I. hypsophila, I. yunguiensis, I. sinensis, I. orientalis, and I. taiwanensis. Expression patterns of IsLFY1 and IsLFY2 in I. sinensis demonstrated that the two genes did not have functional divergences. The two transcripts are expressed not only in reproductive tissues but also in vegetative tissues. Moreover, the transcripts were expressed at a high level in juvenile tissues, indicating LFY genes control both vegetative and reproductive developments in Isoetes. In addition, overexpression of IsLFY1 and IsLFY2 in Arabidopsis similarly did not show precocious flowering compared with wild-type plants. Furthermore, the two paralogs were inactive to complement severe ify mutant phenotypes, implying that the two homologs were not functional orthologs with LFY. Overall, the study provides important information for understanding the characters and functions of LFY homologs in Isoetes and evolutionary process.

Methods

Plant materials. Totally, five Isoetes species were collected in China and cultivated in a greenhouse of Wuhan University, including I. hypsophila, I. yunguiensis, I. sinensis, I. orientalis, and I. taiwanensis. The material I. hypsophila was collected from Daoheng in Sichuan Province, China (N29°29′; E100°14′); I. yunguiensis was collected from Longfeng Lake in Sichuan Province, China (N26°29′; E106°58′); I. sinensis was collected from Xianin River in Zhejiang Province, China (29°28′N; 119°14′E); I. orientalis was collected from Songyang in Zhejiang Province, China (N28°47′; E119°12′); and I. taiwanensis was collected from Jinmen in Taiwan Province, China (N24°27′; E118°23′).

RNA isolation and cloning of LFY homologs. Initially, approximately 0.15 g juvenile leaves from the five Isoetes species were immediately sampled and frozen by liquid nitrogen before RNA extraction, respectively. Then total RNA was isolated using RNaioo Plus (Takara, Da Lian, China), according to the manufacturer's protocols. Total RNA (6 μg) was incubated at 37 °C for 15 min to eliminate genomic DNA using 1 μL RNase-free DNase I (Promega, Madison, WI, USA). The treated total RNA was reverse transcribed into single-strand cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and Oligo(dT)18-adaptor primer. Partial LFY segments were amplified using specific primers (5′TTCAAATGGGAGCCCAAGATACG3′ and 5′GACATTGAGGGTGCTGGAT3′), which were designed according to Saiko Himi et al. The obtained cDNA initially was diluted 10-fold, and used as templates for polymerase chain reactions (PCR) amplification. The reaction mixture (20 μL) contained 2 μL of buffer with 2 mM MgCl2, 0.25 pmol forward and reverse primers, 0.1 mM dNTP, 2.0 μL diluted cDNA, 12.6 μL sterile water and 0.5 μL Primer Star HS DNA polymerase (Takara, Dalian, China). The PCR conditions included initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 55 °C for 40 s, and finally 72 °C for 1 min. Then the PCR products were purified (QiQuick PCR cleanup kit), cloned into the pEASY-Blunt vector (TransGen Biotech Company, Beijing, China), and sequenced in both directions on an ABI 3730 DNA Sequencer using BigDye Terminator version 3.1 (Applied Biosystems).

To obtain full-length ORF of LFY sequences in the five Isoetes species, we performed 5′ and 3′ RACE PCR using the 5′ RACE System for Rapid Amplification of cDNA Ends and 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) Kits, respectively. The gene-specific primers for the 5′ RACE PCR were included (5′GATCTTTGGCTTCTCTCACTATCC3′ and 5′CCAAGTCGACCTTTGGATATTTG3′). The gene-specific primers for the 3′ RACE PCR were used (5′GGTTCCAGGGATAGAATGGGAG3′ and 5′TTAGTGACTTCCCTTAA3′). Then the products for the 5′ and 3′ RACE PCR were purified and cloned into pEASY-Blunt Cloning vector, respectively. Eight positive clones were sequenced in both directions for each sample.

DNA isolation, amplification and sequencing. Total genomic DNA of the five Isoetes species was isolated from juvenile leaves using the CTAB method. The two ILFY genes were amplified using specific primers (ILFY1: 5′ATGACTGAGCTGGGTTTCACCG3′ and 5′TTAGCTGCTTTCTTTTCT3′; ILFY2: 5′TCAAATGGGAGCCCAAG3′ and 5′TTAGTGACTTCCCTTAA3′) respectively. The PCR program was an initial denaturation step of 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 57 °C for 50 s, and finally 72 °C for 2 min. The products were further purified and ligated with pEASY-Blunt Cloning vector. Three positive clones for each sample were sequenced in both directions for each sample.

Construction of a phylogenetic tree. LFY homologs were searched using the program BLAST X model in the NCBI database (http://www.ncbi.nlm.nih.gov/). The amino acid and nucleotide sequences were aligned using Clustal X 2.0 software, including 36 LFY homologous sequences and 10 ILFY homologous sequences obtained in this study. The phylogenetic analysis was conducted based on the JTT substitution model and maximum likelihood approach implemented in the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7.0) program. Support for internal nodes was estimated based on 1000 bootstrap replicates, and bootstrap less than 50% was removed above the relevant branch.

qRT-PCR. Given that I. sinensis is relatively widespread in China and easily adapts to the greenhouse environment in Wuhan University, I. sinensis was employed to investigate the expression patterns, including IsLFY1 and IsLFY2. The leaves and sporangia were arranged on corms in whorls, and they are becoming mature from inside to outside. Each whorl of green leaves and sporangia was collected, respectively. All sampled materials were immediately frozen by liquid nitrogen for RNA extraction. In addition, we also sampled the materials of corms and roots for RNA isolation. Total RNA of all samples was isolated using RNaioo Plus (Takara, Da Lian, China). Then the isolated RNA was treated with RNase-free DNase I (Takara, Da Lian, China) for 45 min according to the manufacturer’s protocols. Subsequently, 1 μg treated RNA was reverse-transcribed into single-strand cDNA using DNA isolation, amplification and sequencing.
expression levels were calculated using 2−ΔΔCt method, and normalized to the geometric average of Ct values with Actin as an internal control.

All experiments and analyses were performed in triplicate.

In situ hybridization. *I. sinensis* was further employed to investigate spatial-specific expression patterns of IsLFY1 and IsLFY2 using in situ hybridization assays. Samples were fixed using 4% paraformaldehyde buffers, including roots, corms, and the 6th whorl of sporophylls and megasporangia, and 8th whorl of microsporangia. In order to ensure the specific hybridization patterns of IsLFY1 and IsLFY2, the non-conserved regions of IsLFY1 and IsLFY2 were used to design the specific probes. Partial sequences of IsLFY1 were amplified using IsFY1-specific primers (5′-AATCTATGCTGTGACTAGGGG-3′ and 5′-TTGTAGTGCACTGTTATG-3′), and partial sequences of IsLFY2 were amplified using IsFY2-specific primers (5′-GGACTATGCTGTAGACCCGG-3′ and 5′-CACAGTGCTTGTAATGTGG-3′). The two amplification segments were introduced into the pGEM-T Vector (Promega, Madison, WI, USA). The antisense probes for the two paralogs were synthesized using SacII (Takara, Da Lian, China) and SP6 RNA polymerases (Promega, Madison, WI, USA). Sense strand controls for the IsLFY1 and IsLFY2 probes were synthesized using SacI (Takara, Da Lian, China) and T7 RNA polymerases (Promega, Madison, WI, USA). Both the sense and antisense probes were ligated with the binary vector pBI121 with a cauliflower mosaic virus 35S promoter, respectively. After digestion, the two amplification segments of IsFY1 and IsFY2 were generated into single-stranded digoxigenin-11-UTP-labeled RNA probes, respectively. Tissues materials were fixed, embedded with paraplast, sectioned into 8 μm in thickness, and hybridized using the probes, described by Bechtold et al. All slides were photographed using an OLYMPUS X73 microscope.

Plasmids construction and Arabidopsis transgenes. The coding regions of IsLFY1 and IsLFY2 were ligated with the binary vector pBI121 with a cauliflower mosaic virus 35S promoter, respectively. Initially, BamHI and Smal restriction fragments were added to the upstream of start codon sequences and downstream of stop codon sequences for the two genes, respectively. The introduced restriction fragments of IsLFY1 and IsLFY2 were generated by PCR amplification using a set of primers (IsLFY1: 5′-CGGGATCCATGACAGATTCAGAGATAGAAGAAC-3′ and 5′-TTGAGTCGCACGTCGTAT3′; and partial sequences of IsLFY2 were amplified using IsFY2-specific primers (5′-GGACTATGCTGTAGACCCGG-3′ and 5′-CACAGTGCTTGTAATGTGG-3′). The two amplification segments were introduced into the pGEM-T Vector (Promega, Madison, WI, USA). The antisense probes for the two paralogs were synthesized using SacI (Takara, Da Lian, China) and SP6 RNA polymerases (Promega, Madison, WI, USA). Sense strand controls for the IsLFY1 and IsLFY2 probes were synthesized using SacI (Takara, Da Lian, China) and T7 RNA polymerases (Promega, Madison, WI, USA). Both the sense and antisense probes were ligated with the binary vector pBI121 with a cauliflower mosaic virus 35S promoter, respectively. The introduced restriction fragments of IsFY1 and IsFY2 were generated into single-stranded digoxigenin-11-UTP-labeled RNA probes, respectively. Tissues materials were fixed, embedded with paraplast, sectioned into 8 μm in thickness, and hybridized using the probes, described by Bechtold et al. All slides were photographed using an OLYMPUS X73 microscope.

The identified 35S::IsLFY1 and 35S::IsLFY2 were introduced into *Agrobacterium tumefaciens* strain GV3101, and further crossed into the Arabidopsis plants (Columbia ecotype) using the floral dip method. In addition, the 35S::IsLFY1 and 35S::IsLFY2 were also crossed into *Iyf* heterozygous Arabidopsis plants, respectively. The heterozygous lines were isolated from a bulk of *Iyf*-1 mutant seeds, which were obtained from TAIR (http://www.arabidopsis.org/). All transgenic plants (T1) were selected on MS medium containing 50 mg/mL kanamycin. The 35S::IsLFY1 and 35S::IsLFY2 lines were identified using PCR amplifications through plating 200 F1 seeds, and homozygous lines (T2) were selected for phenotypic analysis. The seeds were kept for 3 days at 4 °C before sowing. All resistant and wild plants were grown at 23 °C under 16 h light/8 h dark.

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**Author Contributions**

T.Y. cultured the plant tissue and isolated RNA, performed the experiments, analyzed the data, and drafted the manuscript. M.D. performed partial experiments, X.L. and Y.G. revised the manuscript. All authors reviewed the final manuscript.
Additional Information

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