LEAFY maintains apical stem cell activity during shoot development in the fern Ceratopteris richardii

Andrew RG Plackett†‡, Stephanie J Conway‡§, Kristen D Hewett Hazelton‡, Ester H Rabbinowitsch†, Jane A Langdale†*, Verónica S Di Stilio‡*

1Department of Plant Sciences, University of Oxford, Oxford, United Kingdom; 2Department of Biology, University of Washington, Seattle, United States

Abstract

During land plant evolution, determinate spore-bearing axes (retained in extant bryophytes such as mosses) were progressively transformed into indeterminate branching shoots with specialized reproductive axes that form flowers. The LEAFY transcription factor, which is required for the first zygotic cell division in mosses and primarily for floral meristem identity in flowering plants, may have facilitated developmental innovations during these transitions. Mapping the LEAFY evolutionary trajectory has been challenging, however, because there is no functional overlap between mosses and flowering plants, and no functional data from intervening lineages. Here, we report a transgenic analysis in the fern Ceratopteris richardii that reveals a role for LEAFY in maintaining cell divisions in the apical stem cells of both haploid and diploid phases of the lifecycle. These results support an evolutionary trajectory in which an ancestral LEAFY module that promotes cell proliferation was progressively co-opted, adapted and specialized as novel shoot developmental contexts emerged.

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Introduction

Land plants are characterized by the alternation of haploid (gametophyte) and diploid (sporophyte) phases within their lifecycle, both of which are multicellular (Niklas and Kutschera, 2010; Bowman et al., 2016). In the earliest diverging bryophyte lineages (liverworts, mosses and hornworts) the free-living indeterminate gametophyte predominates the lifecycle, producing gametes that fuse to form the sporophyte. The sporophyte embryo develops on the surface of the gametophyte, ultimately forming a simple determinate spore-producing axis (Kato and Akiyama, 2005; Ligrone et al., 2012). By contrast, angiosperm (flowering plant) sporophytes range from small her-baceous to large arborescent forms, all developing from an indeterminate vegetative shoot apex that ultimately transitions to flowering, and gametophytes are few-celled determinate structures produced within flowers (Schmidt et al., 2015). A series of developmental innovations during the course of land plant evolution thus simplified gametophyte form whilst increasing sporophyte complexity, with a prolonged and plastic phase of vegetative development arising in the sporophyte of all vascular plants (lycophytes, ferns, gymnosperms and angiosperms).

Studies aimed at understanding how gene function evolved to facilitate developmental innovations during land plant evolution have thus far largely relied on comparative analyses between bryophytes and angiosperms, lineages that diverged over 450 million years ago. Such comparisons have revealed examples of both sub- and neo-functionalization following gene duplication, and of co-option of existing gene regulatory networks into new developmental contexts. For example, a single bHLH transcription factor in the moss Physcomitrella patens regulates stomatal differentiation, whereas gene duplications have resulted in three homologs with sub-divided stomatal patterning
eLife digest  The first plants colonized land around 500 million years ago. These plants had simple shoots with no branches, similar to the mosses that live today. Later on, some plants evolved more complex structures including branched shoots and flowers (collectively known as the “flowering plants”). Ferns are a group of plants that evolved midway between the mosses and flowering plants and have branched shoots but no flowers.

The gradual transition from simple to more complex plant structures required changes to the way in which cells divide and grow within plant shoots. Whereas animals produce new cells throughout their body, most plant cells divide in areas known as meristems. All plants grow from embryos, which contain meristems that will form the roots and shoots of the mature plant. A gene called LEAFY is required for cells in moss embryos to divide. However, in flowering plants LEAFY does not carry out this role, instead it is only required to make the meristems that produce flowers.

How did LEAFY transition from a general role in embryos to a more specialized role in making flowers? To address this question, Plackett, Conway et al. studied the two LEAFY genes in a fern called Ceratopteris richardii. The experiments showed that at least one of these LEAFY genes was active in the meristems of fern shoots throughout the lifespan of the plant. The shoots of ferns with less active LEAFY genes could not form the leaves seen in normal C. richardii plants. This suggests that as land plants evolved, the role of LEAFY changed from forming embryos to forming complex shoot structures.

Most of our major crops are flowering plants. By understanding how the role of LEAFY has changed over the evolution of land plants, it might be possible to manipulate LEAFY genes in crop plants to alter shoot structures to better suit specific environments.

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roles in the angiosperm Arabidopsis thaliana (hereafter ‘Arabidopsis’) (MacAlister and Bergmann, 2011); class III HD-ZIP transcription factors play a conserved role in the regulation of leaf polarity in P. patens and Arabidopsis but gene family members have acquired regulatory activity in meristems of angiosperms (Yip et al., 2016); and the gene regulatory network that produces rhizoids on the gametophytes of both the moss P. patens and the liverwort Marchantia polymorpha has been co-opted to regulate root hair formation in Arabidopsis sporophytes (Menand et al., 2007; Pires et al., 2013; Proust et al., 2016). In many cases, however, interpreting the evolutionary trajectory of gene function by comparing lineages as disparate as bryophytes and angiosperms has proved challenging, particularly when only a single representative gene remains in most extant taxa – as is the case for the LEAFY (LFY) gene family (Himi et al., 2001; Maizel et al., 2005; Sayou et al., 2014).

The LFY transcription factor, which is present across all extant land plant lineages and related streptophyte algae (Sayou et al., 2014), has distinct functional roles in bryophytes and angiosperms. In P. patens, LFY regulates cell divisions during sporophyte development (including the first division of the zygote) (Tanahashi et al., 2005), whereas in angiosperms the major role is to promote the transition from inflorescence to floral meristem identity (Carpenter and Coen, 1990; Schultz, 1991; Weigel et al., 1992; Blázquez et al., 1997; Souer et al., 1998; Molinero-Rosales et al., 1999). Given that LFY proteins from liverworts and all vascular plant lineages tested to date (ferns, gymnosperms and angiosperms) bind a conserved target DNA motif, whereas hornwort and moss homologs bind to different lineage-specific motifs (Sayou et al., 2014), the divergent roles in mosses and angiosperms may have arisen through the activation of distinct networks of downstream targets. This suggestion is supported by the observation that PpLFY cannot complement loss-of-function lfy mutants in Arabidopsis (Maizel et al., 2005). Similar complementation studies indicate progressive functional changes as vascular plant lineages diverged in that the lfy mutant is not complemented by lycophyte LFY proteins (Yang et al., 2017) but is partially and progressively complemented by fern and gymnosperm homologs (Maizel et al., 2005). Because LFY proteins from ferns, gymnosperms and angiosperms recognize the same DNA motif, this progression likely reflects co-option of an ancestral LFY gene regulatory network into different developmental contexts. As such, the role in floral meristem identity in angiosperms would have been co-opted from an unknown ancestral
Figure 1. *CrLFY1* and *CrLFY2* arose from a recent gene duplication event. Inferred phylogenetic tree from maximum likelihood analysis of 64 LFY amino acid sequences (see Supplementary file 1 for accession numbers) sampled from within the fern lineage plus lycophyte sequences as an outgroup. Bootstrap values are given for each node. The tree shown is extracted from a phylogeny with representative sequences from all land plant lineages (Figure 1—figure supplement 1). The *Ceratopteris richardii* genome contains no more than two copies of LFY (Figure 1—figure supplement 2, indicated by *). Different taxonomic clades within the fern lineage are

Figure 1 continued on next page
context in non-flowering vascular plants, a context that cannot be predicted from existing bryophyte data. The role of LFY in non-flowering vascular plant lineages has thus far been hypothesized on the basis of expression patterns in the lycophyte *Isoetes sinensis* ([Yang et al., 2017](#)), several gymnosperm species ([Mellerowicz et al., 1998](#); [Mouradov et al., 1998](#); [Shindo et al., 2001](#); [Carlsbecker et al., 2004](#); [Vázquez-Lobo et al., 2007](#); [Carlsbecker et al., 2013](#)) and the fern *Ceratopteris richardii* (hereafter ‘Ceratopteris’) ([Himi et al., 2001](#)). These studies reported broad expression in vegetative and reproductive sporophyte tissues of *I. sinensis* and gymnosperms, and in both gametophytes and sporophytes of Ceratopteris. Although gene expression can be indicative of potential roles in each case, the possible evolutionary trajectories and differing ancestral functions proposed for LFY within the vascular plants ([Theissen and Melzer, 2007](#); [Moyroud et al., 2010](#)) cannot be resolved without functional validation. Here we present a functional analysis in Ceratopteris that reveals a stem cell maintenance role for at least one of the two LFY homologs in both gametophyte and sporophyte shoots and discuss how that role informs our mechanistic understanding of developmental innovations during land plant evolution.

**Results**

The *CrLFY1* and *CrLFY2* genes duplicated recently within the fern lineage

The LFY gene family is present as a single gene copy in most land plant genomes ([Sayou et al., 2014](#)). In this regard, the presence of two LFY genes in Ceratopteris ([Himi et al., 2001](#)) is atypical. To determine whether this gene duplication is more broadly represented within the ferns and related species (hereafter ‘ferns’), a previous amino acid alignment of LFY orthologs ([Sayou et al., 2014](#)) was pruned and supplemented with newly-available fern homologs (see Materials and methods) to create a dataset of 120 sequences, ~50% of which were from the fern lineage ([Supplementary file 1](#)–3). The phylogenetic topology inferred within the vascular plants using the entire dataset ([Figure 1—figure supplement 1](#)) was consistent with previous analyses ([Qiu et al., 2006](#); [Wickett et al., 2014](#)). Within the ferns (64 in total), phylogenetic relationships between LFY sequences indicated that the two gene copies identified in *Equisetum arvense*, *Azolla caroliniana* and *Ceratopteris* each resulted from recent independent duplication events ([Figure 1](#)). Gel blot analysis confirmed the presence of no more than two LFY genes in the Ceratopteris genome ([Figure 1—figure supplement 2](#)). Given that the topology of the tree excludes the possibility of a gene duplication prior to diversification of the ferns, *CrLFY1* and *CrLFY2* are equally orthologous to the single copy LFY representatives in other fern species.

*CrLFY1* and *CrLFY2* transcripts accumulate differentially during the Ceratopteris lifecycle

The presence of two LFY genes in the Ceratopteris genome raises the possibility that gene activity was neo- or sub-functionalized following duplication. To test this hypothesis, transcript accumulation patterns of *CrLFY1* and *CrLFY2* were investigated throughout the Ceratopteris lifecycle (shown as a schematic in [Figure 2](#) for reference).
The developmental stages sampled spanned from imbibed spores prior to germination of the haploid gametophyte (Figure 3A), to differentiated male and hermaphrodite gametophytes (Figure 3B–D), through fertilization and formation of the diploid sporophyte embryo (Figure 3E), to development of the increasingly complex sporophyte body plan (Figure 3F–K). Quantitative real-time PCR (qRT-PCR) analysis detected transcripts of both CrLFY1 and CrLFY2 at all stages after spore germination, but only CrLFY2 transcripts were detected in spores prior to germination (Figure 3L). A two-way ANOVA yielded a highly significant interaction ($F(10,22) = 14.21; p<0.0001$) between gene copy and developmental stage that had not been reported in earlier studies (Himi et al., 2001), and is indicative of differential gene expression between CrLFY1 and CrLFY2 that is dependent on developmental stage. Of particular note were significant differences between
Figure 3. CrLFY1 and CrLFY2 are differentially expressed during the Ceratopteris lifecycle. (A-K) Representative images of the developmental stages sampled for expression analysis in (L). Imbibed spores (A); populations of developing gametophytes harvested at 5 (B, C) and 8 (D) days after spore-sowing (DPS), comprising only males (B) or a mixture of hermaphrodites (h) and males (m) (C, D); fertilized gametophyte subtending a developing sporophyte embryo (em) (E); whole sporophyte shoots comprising the shoot apex with 3 (F) or five expanded entire fronds attached (G); individual vegetative fronds demonstrating a heteroblastic progression in which frond complexity increases through successive iterations of lateral outgrowths (pinnae) (H–J); complex fertile frond with...
CrLFY1 and CrLFY2 transcript levels during sporophyte development (Supplementary file 4). Whereas CrLFY2 transcript levels were similar across sporophyte samples, CrLFY1 transcript levels were much higher in samples that contained the shoot apex (Figure 3F,G) than in those that contained just fronds (Figure 3H–K). These data suggest that CrLFY1 and CrLFY2 genes may play divergent roles during sporophyte development, with CrLFY1 acting primarily in the shoot apex and CrLFY2 acting more generally.

Spatial expression patterns of CrLFY1 are consistent with a retained ancestral role to facilitate cell divisions during embryogenesis

Functional characterization in P. patens previously demonstrated that PpLFY promotes cell divisions during early sporophyte development (Tanahashi et al., 2005). To determine whether the spatial domains of CrLFY1 expression are consistent with a similar role in Ceratopteris embryo (early sporophyte) development, transgenic lines were generated that expressed the reporter gene B-glucuronidase (GUS) driven by a 3.9 kb fragment of the CrLFY1 promoter (CrLFY1 pro::GUS). This promoter fragment comprised genomic sequence encoding the entire published 5'UTR (Himi et al., 2001) plus a further 1910 bp upstream of the predicted transcription start site (Figure 1—figure supplement 2A). In the absence of a genome sequence, repeated attempts to isolate an analogous fragment of CrLFY2 sequence were unsuccessful (see Materials and methods for details). Construct maps plus DNA blot and PCR validation of transgenic lines are shown in Figure 4—figure supplements 1–4. GUS activity was monitored in individuals from three independent transgenic lines, sampling both before and up to six days after fertilization (Figure 4A–O), using wild-type individuals as negative controls (Figure 4P–T) and individuals from a transgenic line expressing GUS driven by the constitutive 35S promoter (35S pro::GUS). Notably, no GUS activity was detected in unfertilized archegonia of CrLFY1 pro::GUS gametophytes (Figure 4A,F,K) but by two days after fertilization (DAF) GUS activity was detected in most cells of the early sporophyte embryo (Figure 4B,G,L). At 4 DAF, activity was similarly detected in all visible embryo cells, including the embryonic frond, but not in the surrounding gametophytic tissue (the calyptra) (Figure 4C,H,M). This embryo-wide pattern of GUS activity became restricted in the final stages of development such that by the end of embryogenesis (6 DAF) GUS activity was predominantly localized in the newly-initiated shoot apex (Figure 4D,E,I,J,N,O). Collectively, the GUS activity profiles indicate that CrLFY1 expression is induced following formation of the zygote, sustained in cells of the embryo that are actively dividing, and then restricted to the shoot apex at embryo maturity. This profile is consistent with the suggestion that CrLFY1 has retained the LFY role first identified in P. patens
Figure 4. The CrLFY1 promoter drives reporter gene expression in proliferating tissues of the developing Ceratopteris embryo. (A–Y) GUS activity detected as blue staining in developing embryos of three independent CrLFY1::GUS transgenic reporter lines (A–O), a representative negative wild-type control line (P–T) and a representative positive 35S::GUS control line (U–Y). Tissues are shown prior to fertilization (A, F, K, P, U), or 2 (B, G, L, Q, V), 4 (C, H, M, R, W), and 6 (D, I, N, S, X) days after fertilization (DAF). In CrLFY1::GUS lines, GUS activity first became visible within the first few divisions of embryo development (but not in surrounding gametophyte tissues) at 2 DAF (B, G, L) and was expressed in cells of the embryo frond as it proliferated (C, H, M). GUS activity was visible in the shoot apex and in frond vascular tissue at 6 DAF (D, I, N), with staining in the shoot apical cell (sac), subtending shoot apex tissues and newly-initiated fronds, including the frond apical cell (fac) (E, J, O). No GUS activity was detected in wild-type samples (P–T), whereas the majority of cells in the constitutively expressing 35S::GUS samples stained blue (U–Y). Embryos develop on the surface of the gametophyte thallus when an egg cell (ec) within the archegonium (which comprises a venter (v) and neck cells (nc) to allow sperm...


Kofuji and Hasebe, 2014

Plant Biology

Hou and Hill, 2004

Figure 4 continued

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promoter sequences in T-DNA insertions. embryonic sporophytes, GUS activity was assessed in between pinnae (特点是proliferation in the indeterminate shoot apex. To monitor

tative development (特点是recruited to regulate cell division processes in the shoot when sporophytes evolved from determinate structures.

(Tanahashi et al., 2005), namely to promote the development of a multicellular sporophyte, in part by facilitating the first cell division of the zygote.

**CrLFY1 is expressed in dividing tissues throughout shoot development**

Both mosses and ferns form embryos, but moss sporophyte development is determinate post-embryogenesis (Kato and Akiyama, 2005; Kofuji and Hasebe, 2014) whereas fern sporophytes are elaborated post-embryonically from indeterminate shoot apices (Bierhorst, 1977; White and Turner, 1995). The Ceratopteris shoot apex comprises a single apical cell that generates daughter cells through asymmetric divisions, and individual lateral organs (fronds and root) arise from their own apical cells specified within the grouped descendants of these daughter cells (Hou and Hill, 2002; Hou and Hill, 2004). **CrLFY1** expression in the shoot apex at the end of embryogenesis (Figure 4E,F) and elevated transcript levels in shoot apex-containing sporophyte tissues (Figure 3L) suggested an additional role for **CrLFY1** relative to that seen in mosses, namely to promote proliferation in the indeterminate shoot apex. To monitor **CrLFY1** expression patterns in post-embryonic sporophytes, GUS activity was assessed in **CrLFY1** lines at two stages of vegetative development (Figure 5A–O) and after the transition to reproductive frond formation (Figure 5—figure supplement 1A–L). Wild-type individuals were used as negative controls (Figure 5P–T; Figure 5—figure supplement 1M–P) and **35S****::GUS** individuals as positive controls (Figure 5U–Y; Figure 5—figure supplement 1Q–T). In young sporophytes (20 DAF), GUS activity was primarily localized in shoot apical tissues and newly-emerging frond primordia (Figure 5A,F,K), with very little activity detected in the expanded simple fronds produced at this age (Figure 5B,G,L). In older vegetative sporophytes (60 DAF), which develop complex dissected fronds (Figure 5C,H,M), GUS activity was similarly localized in the shoot apex and young frond primordia in two out of the three fully characterized lines (Figure 5D,I,N) and in a total of 8 out of 11 lines screened (from seven independent rounds of plant transformation). GUS activity was also detected in developing fronds in regions where the lamina was dividing to generate pinnae and pinnules (Figure 5E,J,O). In some individuals GUS activity could be detected in frond tissues almost until maturity (Figure 5C). Notably, patterns of **CrLFY1** expression were the same in the apex and complex fronds of shoots before (60 DAF) (Figure 5C–E,H–J,M–O) and after (~115 DAF) the reproductive transition (Figure 5—figure supplement 1A–L). Consistent with a general role for **CrLFY1** in promoting cell proliferation in the shoot, GUS activity was also detected in shoot apices that initiate de novo at the lamina margin between pinnae (Figure 5Z–AD). Together these data support the hypothesis that LFY function was recruited to regulate cell division processes in the shoot when sporophytes evolved from determinate to indeterminate structures.
Figure 5. The *CrLFY1* promoter drives reporter gene expression in proliferating shoot tissues of the Ceratopteris sporophyte. (A–Y) GUS activity detected as blue staining in post-embryonic sporophytes from three independent *CrLFY1*::GUS transgenic reporter lines (A–O), negative wild-type controls (P–T) and positive 35S::GUS controls (U–Y). Sporophytes were examined at 20 DAF (A, B, F, G, K, L, P, Q, U, V) and 60 DAF (C–E, H–J, M–O, R–T, W–Y). GUS staining patterns are shown for whole sporophytes (A, C, F, H, K, M, P, R, U, W), shoot apices (arrowheads) (B, D, G, I, L, N, Q, S, V, X) and developing fronds (E, J, O, T, Y). In *CrLFY1*::GUS sporophytes at 20 DAF (producing simple, spade-like fronds) GUS activity was restricted to the shoot apex (A, F, K) and newly-initiated frond primordia, with very low activity in expanded fronds (B, G, L). In *CrLFY1*::GUS sporophytes at 60 DAF (producing complex, highly dissected fronds) GUS activity was similarly seen in the apex (C, H, M), but persisted for longer during frond development. Activity was initially detected throughout the frond primordium (D, I, N), before becoming restricted to actively proliferating areas of the lamina (E, J, O). Scale bars = 2 mm (A, F, K, P, U), 500 μm (B, D, G, I, L, N, Q, S, V, X) 10 mm (C, H, M, R, W), 1 mm (E, J, O, T, Y). *GUS staining in maturing frond. GUS staining patterns were the same in leaves formed after the reproductive transition (Figure 5—figure supplement 1). (Z–AD) Fronds can initiate *de novo* shoots (white arrowheads) from marginal tissue between Figure 5 continued on next page
CrLFY regulates activity of the sporophyte shoot apex

To test the functional significance of CrLFY expression patterns, transgenic RNAi lines were generated in which one of four RNAi constructs targeted to CrLFY1, CrLFY2 or both were expressed from the maize ubiquitin promoter (ZmUbi pro). Construct maps plus DNA blot and PCR validation of transgenic lines are shown in Figure 6—figure supplements 1–5. Genotypic screening identified 10 lines.

Table 1. Summary of CrLFY RNAi transgenic lines and their phenotypic characterization.

| RNAi transgene | Line | Transformation replicate | Gametophyte phase | Sporophyte phase | % arrested |
|---------------|------|--------------------------|-------------------|-----------------|------------|
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i1 | B16  | 1 | + | - | - | 99.86 | + | + | - | - | <5% |
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i1 | B19  | 1 | + | - | - | 50.00 | + | + | + | - | <5% |
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i1 | D13  | 2 | + | - | - | 99.80 | + | + | - | - | <5% |
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i1 | D2   | 3 | + | + | + | 0.00 | + | + | - | - | <5% |
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i1 | D4   | 3 | + | + | + | 0.00 | + | + | - | - | <5% |
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i2 | F9   | 4 | + | - | - | 0.00 | + | + | - | - | <5% |
| ZmUBi pro::CrLFY1 pro::CrLFY1-2 i2 | F14  | 5 | - | - | - | 100.00 | - | - | - | - | 0 |
| ZmUBi pro::CrLFY1 pro::CrLFY1-1 i3 | E8   | 6 | + | +/- | +/- | 100.00 | - | - | - | - | 0 |
| ZmUBi pro::CrLFY1 pro::CrLFY1-1 i3 | G13  | 7 | + | + | + | 0.00 | + | + | - | - | <5% |
| ZmUBi pro::CrLFY2 pro::CrLFY2-1 i4 | C3   | 8 | + | + | + | 0.00 | + | + | - | - | <5% |
| NHC-1 (control) | D20  | 9 | + | + | + | 0.00 | + | + | - | - | 0 |
| NHC-2 (control) | F3   | 10 | + | + | + | 0.00 | + | + | - | - | 0 |
| NHC-3 (control) | F4   | 10 | + | + | + | 0.00 | + | + | - | - | 0 |

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Figure 6. Suppression of CrLFY expression causes early termination of the Ceratopteris sporophyte shoot apex. (A–L) Sporophyte phenotype 25 days after fertilization (DAF) in no hairpin control, NHC-1 (A) and transgenic lines carrying RNAi constructs against CrLFY1 (ZmUbi<sub>pro</sub>::CrLFY1-i3) (B, C), CrLFY2 (ZmUbi<sub>pro</sub>::CrLFY1-i4) (D) and both CrLFY1 and CrLFY2 (ZmUbi<sub>pro</sub>::CrLFY1/2-i1 and ZmUbi<sub>pro</sub>::CrLFY1/2-i2) (E–L). In some lines, both aborted and phenotypically normal sporophytes were identified (compare E and I; F and J; G and K; H and L). The presence of the RNAi transgene in phenotypically normal sporophytes was validated by genotyping (Figure 6—figure supplement 5). Scale bars = 1 mm (A–H), 5 mm (I–L). (M–Q) Sporophyte phenotype of two no hairpin control
which contained the complete transgene cassette and three lines that contained a fragment of the transgene which included the antibiotic resistance marker but not the RNAi hairpin (Table 1).

In the no hairpin control (NHC) plants, post-embryonic shoot development initiated with the production of simple, spade-like fronds from the shoot apex (Figure 6A) as in wild type. In eight transgenic lines, sub-populations of sporophytes developed in which this early stage of sporophyte development was perturbed, one line (E8) failing to initiate recognizable embryos (Figure 6B) and the remainder exhibiting premature shoot apex termination, typically after producing several distorted fronds (Figure 6C–H). Sub-populations of phenotypically normal transgenic sporophytes were also identified in some of these lines (Figure 6I–L). The two remaining lines exhibited less severe shoot phenotypes, one undergoing shoot termination after the production of simple (B19a) or lobed (B19b) fronds at the stage when control sporophytes produced complex dissected fronds (Figure 6M–O), and the other (D4b) completing sporophyte development but reduced in size to approximately 50% of controls (Figure 6P,Q). Despite the predicted sequence specificity of CrLFY1-i3 and CrLFY2-i4 (Supplementary file 5), qRT-PCR analysis found that all four RNAi constructs led to suppressed transcript levels of both CrLFY genes (Figure 6R,S). The severity of the shoot phenotype was correlated with the level of endogenous CrLFY transcripts detected across all lines (Figure 6R,S), with relative levels of both CrLFY1 and CrLFY2 significantly reduced compared to controls in all early-terminating sporophytes (E8, G13, C3, D2, D4a, D13, F9) (p<0.01 or less). In phenotypically normal transgenic siblings CrLFY2 transcript levels were not significantly lower than controls (indeed in line D2, levels were higher p<0.01) whereas CrLFY1 levels were significantly reduced (p<0.0001), as in arrested siblings. Together, these data indicate that CrLFY2 can compensate for some loss of CrLFY1, but at least 22% of CrLFY1 activity is required for normal development (line D4a pn, Figure 6J,S). It can thus be concluded that CrLFY1 and CrLFY2 act partially redundantly to maintain indeterminacy of the shoot apex in Ceratopteris, a role not found in the early divergent bryophyte P. patens, nor known to be retained in the majority of later diverging flowering plants.
Figure 7. Suppression of CrLFY expression causes early termination of the Ceratopteris gametophyte apical cell. (A–C) In no hairpin control lines, the gametophyte established a triangular apical cell (ac) shortly after spore (sp) germination (A). Divisions of the apical cell established a photosynthetic thallus in both hermaphrodite and male gametophytes. At 10 days post spore sowing (DPS) both gametophyte sexes were approaching maturity, with the hermaphrodite (B) having formed a chordate shape from divisions at a lateral notch meristem (n) and having produced egg-containing archegonia (ar), sperm-containing antheridia (an), and rhizoids (rh). The male (C) had a more uniform shape with antheridia across the surface. These phenotypes were identical to wild-type. (D–J) When screened at 10–17 DPS, gametophytes from multiple RNAi lines (as indicated) exhibited developmental arrest, mostly associated with a failure of apical cell activity. Arrest occurred at various stages of development from failure to specify an apical cell, resulting in only a rhizoid being produced and no thallus (D) through subsequent thallus proliferation (E–I). Gametophyte development in one line progressed to initiation of the notch meristem but overall thallus size was severely reduced compared to wild-type (J). (K–R) In situ hybridization with antisense probes detected CrLFY transcripts in the apical cell and immediate daughter cells of wild-type gametophytes at 4
CrLFY promotes apical cell divisions in the gametophyte

In six of the RNAi lines that exhibited sporophyte developmental defects, it was notable that 50–99% of gametophytes arrested development prior to the sporophyte phase of the lifecycle (Table 1). This observation suggested that LFY plays a role in Ceratopteris gametophyte development, a function not previously demonstrated in either bryophytes or angiosperms. During wild-type development, the Ceratopteris gametophyte germinates from a single-celled haploid spore, establishing a single apical cell (AC) within the first few cell divisions (Figure 7A). Divisions of the AC go on to form a two-dimensional photosynthetic thallus in both the hermaphrodite, where a notch meristem forms on growth (Figure 7B), and male sexes (Figure 7C) (Banks, 1999). In contrast, the gametophytes from six RNAi lines (carrying either ZmUbi::CrLFY1-i3, ZmUbi::CrLFY1/2-i1 or ZmUbi::CrLFY1/2-i2) exhibited developmental arrest (Figure 7D–J), which in five lines clearly related to a failure of AC activity. The point at which AC arrest occurred varied, in the most severe line occurring prior to or during AC specification (Figure 7D) and in others during AC-driven thallus proliferation (Figure 7E–I). Failure of AC activity was observed in both hermaphrodites (Figure 7E) and males (Figure 7H,I). The phenotypically least-severe line exhibited hermaphrodite developmental arrest only after AC activity had been replaced by the notch meristem (Figure 7J). A role for CrLFY in maintenance of gametophyte AC activity was supported by the detection of CrLFY transcripts in the AC and immediate daughter cells of wild-type gametophytes by in situ hybridization (Figure 7K–N). By contrast CrLFY transcripts were not detected in arrested ZmUbi::CrLFY1/2-i1 lines (Figure 7O–R) in which the presence of the transgene was confirmed by genotyping of individual arrested gametophytes (Figure 7—figure supplement 1). CrLFY1 and CrLFY2 transcripts could not be clearly distinguished in situ due to sequence similarity (see Supplementary file 6), and hence the observed phenotypes could not be ascribed to a specific gene copy. However, these data support a role for at least one CrLFY homolog in AC maintenance during gametophyte development, and thus invoke a role for LFY in the regulation of apical activity in both the sporophyte and gametophyte phases of vascular plant development.

Discussion

The results reported here reveal a role for LFY in the maintenance of apical cell activity throughout gametophyte and sporophyte shoot development in Ceratopteris. During sporophyte development, qRT-PCR and transgenic reporter lines demonstrated that CrLFY1 is preferentially expressed in the shoot apex (whether formed during embryogenesis or de novo on fronds, and both before and after the reproductive transition); in emerging lateral organ (frond) primordia; and in pinnae and pinnules as they form on dissected fronds (Figures 3–5). Notably, active cell division is the main feature in all of these contexts. CrLFY2 transcript levels were more uniform throughout sporophyte shoot development, in both dividing tissues and expanded fronds (Figure 3), and expression has previously been reported in roots (Himi et al., 2001). Simultaneous suppression of CrLFY1 and CrLFY2 activity by RNAi resulted in developmental arrest of both gametophyte and sporophyte shoot apices, with any fronds produced before termination of the sporophyte apex exhibiting abnormal morphologies (Figures 6 and 7). The severity of phenotypic perturbations in sporophytes of transgenic lines correlated with combined CrLFY1 and CrLFY2 transcript levels, with wild-type levels of CrLFY2 able to fully compensate for up to a 70% reduction in CrLFY1 levels (Figure 6). The duplicate CrLFY genes therefore act at least partially redundantly during shoot development in Ceratopteris.
Figure 8. Evolutionary trajectory of LFY function. The phylogeny was reconstructed from selected LFY protein sequences representing all extant embryophyte lineages (as highlighted) and the algal sister-group. Coloured bars at the terminal branches represent different developmental functions of LFY determined from functional analyses in those species (see Supplementary file 8 for references). Coloured numbers indicate the putative points of origin of different functions inferred from available data points across the tree. 1, cell division within the sporophyte zygote; 2, maintenance of indeterminate cell fate in vegetative shoots through proliferation of one or more apical cells (AC); 2a, maintenance of indeterminate cell fate in vegetative lateral/axillary apices; 2b, maintenance of indeterminate cell fate in the margins of developing lateral organs (compound leaves); 3, specification of floral meristem identity (determinate shoot development producing modified lateral organs) and shoot transition to the LEAFY state.

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A function for LFY in gametophyte development has not previously been reported in any land plant species. In the moss *P. patens*, *PpLFY1* and *PpLFY2* are expressed in both the main and lateral apices of gametophytic leafy shoots but double loss-of-function mutants develop normally, indicating that LFY is not necessary for maintenance of apical cell activity in the gametophyte (Tanahashi et al., 2005). By contrast, loss of *CrLFY* expression from the gametophyte shoot apex results in loss of apical cell activity during thallus formation in Ceratopteris (*Figure 7*). The different DNA binding site preferences (and hence downstream target sequences) of *PpLFY* and *CrLFY* (Sayou et al., 2014) may be sufficient to explain the functional distinction in moss and fern gametophytes, but the conserved expression pattern is intriguing given that there should be no pressure to retain that pattern in *P. patens* in the absence of functional necessity. The thalloid gametophytes of the two other extant bryophyte lineages (liverworts and hornworts) resemble the fern gametophyte more closely than mosses (Ligron et al., 2012), but LFY function in these contexts is not yet known. Overall, the data are consistent with the hypothesis that in the last common ancestor of ferns and angiosperms, LFY functioned to promote cell proliferation in the thalloid gametophyte, a role that has been lost in angiosperms where gametophytes have no apical cell and are instead just few-celled determinate structures.

The range of reported roles for LFY in sporophyte development can be rationalized by hypothesizing three sequential changes in gene function during land plant evolution (*Figure 8*). First, the ancestral LFY function to promote early cell divisions in the embryo was retained in vascular plants after they diverged from the bryophytes, leading to conserved roles in *P. patens* (Tanahashi et al., 2005) and *Ceratopteris* (*Figure 4*). Second, within the vascular plants (preceding divergence of the ferns) this proliferative role expanded to maintain post-embryonic apical cell activity, and hence to enable indeterminate shoot growth. This is evidenced by *CrLFY* activity at the tips of shoots, fronds and pinnae (*Figures 4–6*), all of which develop from one or more apical cells (Hill, 2001; Hou and Hill, 2004). Whether fern fronds are homologous to shoots or to leaves in angiosperms is an area of debate (Tomescu, 2009; Vasco et al., 2013; Harrison and Morris, 2018), but there are angiosperm examples of LFY function in the vegetative SAM (Ahearn et al., 2001; Zhao et al., 2018), axillary meristems (Kanrar et al., 2008; Rao et al., 2008; Chatha et al., 2013) and in actively dividing regions of compound leaves (Hofer et al., 1997; Molinero-Rosales et al., 1999; Champagne et al., 2007; Wang et al., 2008; Moniaux et al., 2017) indicating that a proliferative role in vegetative tissues has been retained in at least some angiosperm species. Consistent with the suggestion that the angiosperm floral meristem represents a modified vegetative meristem (Theibon et al., 2016), the third stage of LFY evolution could have been co-option and adaptation of this proliferation-promoting network into floral meristems, with subsequent restriction to just the flowering role in many species. This is consistent with multiple observations of LFY expression in both vegetative and reproductive shoots (developing cones) in gymnosperms (Mellerowicz et al., 1998; Mouradov et al., 1998; Shindo et al., 2001; Carlsbecker et al., 2004; Vázquez-Lobo et al., 2007; Carlsbecker et al., 2013; Moyroud et al., 2017) and suggests that pre-existing LFY-dependent vegetative gene networks might have been co-opted during the origin of specialized sporophyte reproductive axes in ancestral seed plants, prior to the divergence of angiosperms.

The proposed evolutionary trajectory for LFY function bears some resemblance to that seen for KNOX protein function. Class I KNOX genes are key regulators of indeterminacy in the vegetative shoot apical meristem of angiosperms (Gaillochet et al., 2015), and are required for compound leaf formation in both tomato and *Cardamine hirsuta* (Bar and Ori, 2015). In ferns, KNOX gene expression is observed both in the shoot apex and developing fronds (Sano et al., 2005; Ambrose and Vasco, 2016), and in *P. patens* the genes regulate cell division patterns in the determinate sporophyte (Sakakibara et al., 2008). It can thus be speculated that LFY and KNOX had overlapping functions in the sporophyte of the last common ancestor of land plants, but by the divergence of
ancestral angiosperms from gymnosperms, KNOX genes had come to dominate in vegetative meristems whereas LFY became increasingly specialized for floral meristem function. Unlike LFY, however, there is not yet any evidence for KNOX function in the gametophyte of any land plant lineage, and thus if a pathway for regulating stem cell activity was co-opted from the gametophyte into the sporophyte, the LFY pathway is the more likely one.

Materials and methods

Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|----------------------------------|-------------|---------------------|-------------|------------------------|
| **Gene** (Ceratopteris richardii) | CrLFY1 (CrLFY1) | Himi et al. (2001), PMID:11675598, This paper | NCBI:AB049974.2; NCBI:MH841970 | cDNA only; ORF plus contiguous promoter |
| **Gene (C. richardii)**          | CrLFY2 (CrLFY2) | Himi et al. (2001), PMID:11675598, This paper | NCBI:AB049975.2; NCBI:MH841971 | cDNA only; ORF plus contiguous promoter |
| Strain, strain background (C. richardii) | Wild type (Hn-n) | Warne and Hickok, 1987, PMID:16665325 | | |
| Genetic reagent (C. richardii)   | CrLFY1/2-i1 | This paper | | C. richardii transgenic line; RNAi knockdown of CrLFY1 and CrLFY2 expression. |
| Genetic reagent (C. richardii)   | CrLFY1/2-i2 | This paper | | C. richardii transgenic line; RNAi knockdown of CrLFY1 and CrLFY2 expression. |
| Genetic reagent (C. richardii)   | CrLFY1-i3 | This paper | | C. richardii transgenic line; RNAi knockdown of CrLFY1 expression. |
| Genetic reagent (C. richardii)   | CrLFY2-i4 | This paper | | C. richardii transgenic line; RNAi knockdown of CrLFY2 expression. |
| Genetic reagent (C. richardii)   | CrLFY1pro-GUS | This paper | | C. richardii transgenic line; CrLFY1pro-GUS reporter. |
| Genetic reagent (C. richardii)   | 35Spro::GUS | This paper | | C. richardii transgenic line; 35Spro::GUS reporter. |
| Recombinant DNA reagent          | CrLF1pro | This paper | NCBI:MH841970 | CrLFY1 5’ genomic fragment; Figure 1—figure supplement 2 |
| Recombinant DNA reagent          | CrLFY2pro fragment 1 | This paper | NCBI:MH841971 | CrLFY2 5’ genomic fragment; Figure 1—figure supplement 2 |
| Recombinant DNA reagent          | CrLFY2pro fragment 2 | This paper | | CrLFY2 5’ genomic fragment; Figure 1—figure supplement 2, Supplementary file 7 |
| Recombinant DNA reagent          | GUS | Ulmasov, 1997, PMID:9401121 | | B-Glucuronidase (GUS) coding sequence |
| Recombinant DNA reagent          | pANDA (RNAi vector) | Miki and Shimamoto (2004), PMID:15111724 | | |
| Recombinant DNA reagent          | pCR4-TOPO (Cloning vector) | Invitrogen | Thermo Scientific: K457502 | |
| Recombinant DNA reagent          | pDONR207 (Gateway vector) | Invitrogen | | |
| Recombinant DNA reagent          | pBOMBER (Binary vector) | Plackett et al. (2015), PMID:26146510 | NCBI:MH841969 | Modified pART27 (PMID:1463857); Hygromycin resistance antibiotic selection marker |
| Recombinant DNA reagent          | pART7 (Cloning vector) | Gleave 1992, PMID:1463857 | | |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|-------------------|-------------|------------------------|
| Recombinant DNA reagent          | ZmUbi<sub>pro</sub>::CrLFY1/2-i1-pANDA | This paper |  | RNAi construct targeting CrLFY1 and CrLFY2; Figure 6—figure supplement 1; Figure 6—figure supplement 2 |
| Recombinant DNA reagent          | ZmUbi<sub>pro</sub>::CrLFY1/2-i2-pANDA | This paper |  | RNAi construct targeting CrLFY1 and CrLFY2; Figure 6—figure supplement 1; Figure 6—figure supplement 2 |
| Recombinant DNA reagent          | ZmUbi<sub>pro</sub>::CrLFY1-i3-pANDA | This paper |  | RNAi construct targeting CrLFY1; Figure 6—figure supplement 1; Figure 6—figure supplement 2 |
| Recombinant DNA reagent          | ZmUbi<sub>pro</sub>::CrLFY2-i4-pANDA | This paper |  | RNAi construct targeting CrLFY2; Figure 6—figure supplement 1; Figure 6—figure supplement 2 |
| Recombinant DNA reagent          | CrLFY1<sub>pro</sub>::GUS-pBOMBER | This paper |  | GUS reporter construct, CrLFY1; Figure 4—figure supplement 1 |
| Recombinant DNA reagent          | 35Spro::GUS-pBOMBER | This paper |  | GUS reporter construct, 35S control; Figure 4—figure supplement 1 |
| Recombinant DNA reagent          | CrLFY1 in situ probe (antisense) | This paper |  | In situ hybridisation probe; Supplementary file 6 |
| Recombinant DNA reagent          | CrLFY1 in situ probe (sense) | This paper |  | In situ hybridisation probe; Supplementary file 6 |
| Recombinant DNA reagent          | CrLFY2 in situ probe (antisense) | This paper |  | In situ hybridisation probe; Supplementary file 6 |
| Recombinant DNA reagent          | CrLFY2 in situ probe (sense) | This paper |  | In situ hybridisation probe; Supplementary file 6 |
| Recombinant DNA reagent          | 32P-CrLFY1 probe 1 | This paper |  | DNA gel blot probe for CrLFY1; Figure 1—figure supplement 2 |
| Recombinant DNA reagent          | 32P-CrLFY1 probe 2 | This paper |  | DNA gel blot probe for CrLFY1; Figure 1—figure supplement 2 |
| Recombinant DNA reagent          | 32P-CrLFY2 probe 1 | This paper |  | DNA gel blot probe for CrLFY2; Figure 1—figure supplement 2 |
| Recombinant DNA reagent          | 32P-CrLFY2 probe 1 | This paper |  | DNA gel blot probe for CrLFY2; Figure 1—figure supplement 2 |
| Recombinant DNA reagent          | 32P-HygR probe | Plackett et al. (2014), PMID:24623851 |  | DNA gel blot probe, T-DNA specific; Figure 4—figure supplement 1 |
| Recombinant DNA reagent          | 32P-GUS probe | Plackett et al. (2014), PMID:24623851 |  | DNA gel blot probe, T-DNA specific; Figure 4—figure supplement 1 |
| Recombinant DNA reagent          | 32P-GUSlinker probe | This paper |  | DNA gel blot probe, T-DNA specific; Figure 6—figure supplement 2 |
| Sequence-based reagent           | CrLFY1ampF | This paper |  | ORF amplification, CrLFY1: 5'-ATGGATGTCTCTTTATTGCCAC-3' |
| Sequence-based reagent           | CrLFY1ampR | This paper |  | ORF amplification, CrLFY1: 5'-TCACATCATAGATGCAGCTATCAGTC-3' |
| Sequence-based reagent           | CrLFY1ampF | This paper |  | ORF amplification, CrLFY2: 5'-ATGGATGTCTCTTTATTGCCAC-3' |
| Sequence-based reagent           | CrLFY1ampR | This paper |  | ORF amplification, CrLFY2: 5'-TCACATCATAGATGCAGCTATCAGTC-3' |
| Sequence-based reagent           | CrLFY1invF | This paper |  | Inverse PCR, CrLFY1: 5'-CTATGGAGTACGAAGCACCAC-3' |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|------------|------------------------|
| Sequence-based reagent            | CrLFY1invF2 | This paper          |            | Inverse PCR, CrLFY1: 5'-CGATCATTTCTTGTACTGCTCTC-3' |
| Sequence-based reagent            | CrLFY1invF3 | This paper          |            | Inverse PCR, CrLFY1: 5'-CAGTGCAATGACCTCTGATATTG-3' |
| Sequence-based reagent            | CrLFY1invR  | This paper          |            | Inverse PCR, CrLFY1: 5'-CAGTTGTTCGATCTGCAG-3' |
| Sequence-based reagent            | CrLFY1invR2 | This paper          |            | Inverse PCR, CrLFY1: 5'-CTCCGGTTTTTCTATTGGAACG-3' |
| Sequence-based reagent            | CrLFY1invR3 | This paper          |            | Inverse PCR, CrLFY1: 5'-CAAGAACCCGCTGGAGTAAC-3' |
| Sequence-based reagent            | CrLFY2invF  | This paper          |            | Inverse PCR, CrLFY2: 5'-GCTTTTAAATTGTGACGTGTG-3' |
| Sequence-based reagent            | CrLFY2invF2 | This paper          |            | Inverse PCR, CrLFY2: 5'-CAAGAACCCGCTGGAGTAAC-3' |
| Sequence-based reagent            | CrLFY2invF3 | This paper          |            | Inverse PCR, CrLFY2: 5'-CACTGAAAGGTGCTATCAAC-3' |
| Sequence-based reagent            | CrLFY2invF4 | This paper          |            | Inverse PCR, CrLFY2: 5'-CATGGTCGACCTTGAGAAG-3' |
| Sequence-based reagent            | CrLFY2invF5 | This paper          |            | Inverse PCR, CrLFY2: 5'-GCTGAGCATGACTCC-3' |
| Sequence-based reagent            | CrLFY2invF6 | This paper          |            | Inverse PCR, CrLFY2: 5'-CAAGAACCCGCTGGAGTAAC-3' |
| Sequence-based reagent            | CrLFY2invF7 | This paper          |            | Inverse PCR, CrLFY2: 5'-CAATAGTAGATTCTCCCTTCATAC-3' |
| Sequence-based reagent            | CrLFY2invF8 | This paper          |            | Inverse PCR, CrLFY2: 5'-GCTCTTTAATTTGAATCAGTC-3' |
| Sequence-based reagent            | CrLFY2invF9 | This paper          |            | Inverse PCR, CrLFY2: 5'-GCAAATGTTGCACTCGACT-3' |
| Sequence-based reagent            | CrLFY2invF10| This paper          |            | Inverse PCR, CrLFY2: 5'-CATGGTCCGATGGGAAACAG-3' |
| Sequence-based reagent            | CrLFY2invF11| This paper          |            | Inverse PCR, CrLFY2: 5'-GCTTCCTTTAATTTGAATCAGTC-3' |
| Sequence-based reagent            | CrLFY2invF12| This paper          |            | Inverse PCR, CrLFY2: 5'-GTTTCCAGATATCTGCTGTC-3' |
| Sequence-based reagent            | CrLFY2invF13| This paper          |            | Inverse PCR, CrLFY2: 5'-GCTCTTTAATTTGAATCAGTC-3' |
| Sequence-based reagent            | CrLFY2invF14| This paper          |            | Inverse PCR, CrLFY2: 5'-GCTACTATCATCAGTACAC-3' |
| Sequence-based reagent            | CrLFY2invF15| This paper          |            | Inverse PCR, CrLFY2: 5'-GCTACTATCATCAGTACAC-3' |
| Sequence-based reagent            | CrLFY2invF16| This paper          |            | Inverse PCR, CrLFY2: 5'-GCTACTATCATCAGTACAC-3' |
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|-------------------|------------|------------------------|
| Sequence-based reagent            | CrLFY2invR  | This paper        |            | Inverse PCR, CrLFY2: 5'-CAGTTGTCAC AGAGGTAGCGAGC-3' |
| Sequence-based reagent            | CrLFY2invR2 | This paper        |            | Inverse PCR, CrLFY2: 5'-CCCTACGATG TATACCTTGTGTC-3' |
| Sequence-based reagent            | CrLFY2invR3 | This paper        |            | Inverse PCR, CrLFY2: 5'-CAGTGACGA GATGTCATGACAG-3' |
| Sequence-based reagent            | CrLFY2invR4 | This paper        |            | Inverse PCR, CrLFY2: 5'-GAGGAGCT GAAATGCAACTC-3' |
| Sequence-based reagent            | CrLFY2invR5 | This paper        |            | Inverse PCR, CrLFY2: 5'-CCTGCGCTCC TATGAAAACAC-3' |
| Sequence-based reagent            | CrLFY2invR6 | This paper        |            | Inverse PCR, CrLFY2: 5'-CCTGTAAGGG AGGGAGAATCTAC-3' |
| Sequence-based reagent            | CrLFY2invR7 | This paper        |            | Inverse PCR, CrLFY2: 5'-GCACCTCAAC GATGATGACAC-3' |
| Sequence-based reagent            | CrLFY2invR8 | This paper        |            | Inverse PCR, CrLFY2: 5'-GCTGTACTA AGGCAACATC-3' |
| Sequence-based reagent            | CrLFY2invR9 | This paper        |            | Inverse PCR, CrLFY2: 5'-CATCTATGATA GCACAACATC-3' |
| Sequence-based reagent            | CrLFY2invR10| This paper       |            | Inverse PCR, CrLFY2: 5'-CATCTATGATA GCACAACATC-3' |
| Sequence-based reagent            | CrLFY2invR11| This paper       |            | Inverse PCR, CrLFY2: 5'-CTGCCTCTGTA TGAACACAGA-3' |
| Sequence-based reagent            | CrLFY2invR12| This paper       |            | Inverse PCR, CrLFY2: 5'-CTAGCTTCTTGTAGGTGCTACG-3' |
| Sequence-based reagent            | CrLFY2invR13| This paper       |            | Inverse PCR, CrLFY2: 5'-CATGCAAGA AGCATGCAATTC-3' |
| Sequence-based reagent            | CrLFY2invR14| This paper       |            | Inverse PCR, CrLFY2: 5'-GCTGTACTA AGGCAACATC-3' |
| Sequence-based reagent            | CrLFY2invR15| This paper       |            | Inverse PCR, CrLFY2: 5'-CATGAGGCC GAGGTGACCTAC-3' |
| Sequence-based reagent            | CrLFY2invR16| This paper       |            | Inverse PCR, CrLFY2: 5'-CGTAACAGA CGACCTCGATATAATAG-3' |
| Sequence-based reagent            | CrLFY2invR17| This paper       |            | Inverse PCR, CrLFY2: 5'-CTCTTTGCTCA TATAGCTTGAACAGA-3' |
| Sequence-based reagent            | CrLFY1 + 2 (1)-RNAi-F | This paper |            | T-DNA cloning, CrLFY1/2-i1: 5'-ATGGGT TTCACTGATGTC-3' |
| Sequence-based reagent            | CrLFY1 + 2 (1)-RNAi-R | This paper |            | T-DNA cloning, CrLFY1/2-i1: 5'-CTCTCTCT TTGTCTCCTTTG-3' |
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Sequence-based reagent            | CrLFY1 + 2 (2)-RNAi-F | This paper | T-DNA cloning, CrLFY1/2-i2: 5'-ATGGGTTTCACGTAGTAC-3' |
| Sequence-based reagent            | CrLFY1 + 2 (2)-RNAi-R | This paper | T-DNA cloning, CrLFY1/2-i2: 5'-TCTCCTCTTGTATAGTAC-3' |
| Sequence-based reagent            | CrLFY1-RNAi-F | This paper | T-DNA cloning, CrLFY1-i3: 5'-CATGGAATTCCATAGTAC-3' |
| Sequence-based reagent            | CrLFY1-RNAi-R | This paper | T-DNA cloning, CrLFY1-i3: 5'-GACGAGTTCCATAGTAC-3' |
| Sequence-based reagent            | CrLFY2-RNAi-F | This paper | T-DNA cloning, CrLFY2-i4: 5'-GCCATTGCTACTGAGGAG-3' |
| Sequence-based reagent            | CrLFY2-RNAi-R | This paper | T-DNA cloning, CrLFY2-i4: 5'-CATCTAGAGAGGAGTTCC-3' |
| Sequence-based reagent            | pCrLFY1amp-NotF | This paper | T-DNA cloning, pCrLFY1amp-NotF: 5'-CATCTAGACAGGAGTTCC-3' |
| Sequence-based reagent            | pCrLFY1amp-XbaR | This paper | T-DNA cloning, pCrLFY1amp-XbaR: 5'-CCATCTAGACAGGAGTTCC-3' |
| Sequence-based reagent            | GUSamp-XbaF | This paper | T-DNA cloning, GUS CDS: 5'-CATCTAGACAGGAGTTCC-3' |
| Sequence-based reagent            | GUSamp-XbaR | This paper | T-DNA cloning, GUS CDS: 5'-GAGGAGTTCCATAGTAC-3' |
| Sequence-based reagent            | qCrLFY1_F2 | This paper | qRT-PCR, qCrLFY1: 5'-GTCCGCTATTCGTGCAGAGA-3' |
| Sequence-based reagent            | qCrLFY1_R2 | This paper | qRT-PCR, qCrLFY1: 5'-AATTCAAGGGGTCGACGAGACGAAG-3' |
| Sequence-based reagent            | qCrLFY2_F3 | This paper | qRT-PCR, qCrLFY2: 5'-GACGGGAGAAGCTACTGAG-3' |
| Sequence-based reagent            | qCrLFY2_R3 | This paper | qRT-PCR, qCrLFY2: 5'-AGGAATCTGACCACTGCTAC-3' |
| Sequence-based reagent            | qCrTBPb_F | Ganger et al. (2015), DOI: 10.1139/cjb-2014–0202 | qRT-PCR, qCrTBP: 5'-ATGACGAGCAAGCTACTGAG-3' |
| Sequence-based reagent            | qCrTBPb_R | Ganger et al. (2015), DOI: 10.1139/cjb-2014–0202 | qRT-PCR, qCrTBP: 5'-ATGACGAGCAAGCTACTGAG-3' |
| Sequence-based reagent            | qCrACT1_F | Ganger et al. (2015), DOI: 10.1139/cjb-2014–0202 | qRT-PCR, qCrACT1: 5'-GAGAGAGGCTACTTCAACCC-3' |
| Sequence-based reagent            | qCrACT1_R | Ganger et al. (2015), DOI: 10.1139/cjb-2014–0202 | qRT-PCR, qCrACT1: 5'-GAGAGAGGCTACTTCAACCC-3' |
| Sequence-based reagent            | CrLFY1_ISH_F | This paper | In situ probes, CrLFY1: 5'-GAGCAGACACACGAATAGCAGG-3' |
| Sequence-based reagent            | CrLFY1_ISH_R | This paper | In situ probes, CrLFY1: 5'-GAGCAGACACACGAATAGCAGG-3' |

*Continued on next page*
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|-------------------|-------------|------------------------|
| Sequence-based reagent            | CrLFY2_ISH_F | This paper        |             | In situ probes, CrLFY2: 5’-GGCTGGTTGTATTA CGGATAGC-3' |
| Sequence-based reagent            | CrLFY2_ISH_R | This paper        |             | In situ probes, CrLFY2: 5’-TTATTCATAG CTGCGACTGTACCTG-3' |
| Sequence-based reagent            | CrLFY1_Probe1F | This paper |             | Copy number analysis, CrLFY1 probe 1: 5’-CAGG CAAAGGAACAAAG-3' |
| Sequence-based reagent            | CrLFY1_Probe1R | This paper |             | Copy number analysis, CrLFY1 probe 2: 5’-CACCTTGAAGTTAGCT TTATGGTAAAG-3' |
| Sequence-based reagent            | CrLFY2_Probe1F | This paper |             | Copy number analysis, CrLFY2 probe 1: 5’-CATAGC TGCAGCTACCTG-3' |
| Sequence-based reagent            | CrLFY2_Probe1R | This paper |             | Copy number analysis, CrLFY2 probe 2: 5’-CTGTAG AAGGTAAGTACCTG-3' |
| Sequence-based reagent            | CrLFY2_Probe2F | This paper |             | Copy number analysis, CrLFY2 probe 2: 5’-CTGTAG AAGGTAAGTACCTG-3' |
| Sequence-based reagent            | CrLFY2_Probe2R | This paper |             | Copy number analysis, CrLFY2 probe 2: 5’-CTGTAG AAGGTAAGTACCTG-3' |
| Sequence-based reagent            | HygF2        | Plackett et al. (2014), PMID:24623851 | T-DNA gel blot probe, Hyg R: 5’-CTTCTACA CAGCCATCGGTC-3' |
| Sequence-based reagent            | HygR         | Plackett et al. (2014), PMID:24623851 | T-DNA gel blot probe, Hyg R: 5’-CCGATGGTT TCTGACTACAAAGATCG-3' |
| Sequence-based reagent            | GH3seqF3     | Plackett et al. (2014), PMID:24623851 | T-DNA gel blot probe, GUS: 5’-CCTGCGCT GTAGCGGTCTTCG-3' |
| Sequence-based reagent            | GH3seqR4     | Plackett et al. (2014), PMID:24623851 | T-DNA gel blot probe, GUS: 5’-CAGCGGTCTTCTCG-3' |
| Sequence-based reagent            | GUSlinkerseqF | This paper |             | T-DNA gel blot probe, RNAi: 5’-CTGATT AACCACAAACCGTTCTAC-3' |
| Sequence-based reagent            | GUSlinkerseqR | This paper |             | T-DNA gel blot probe, RNAi: 5’-CTGATT AACCACAAACCGTTCTAC-3' |
| Sequence-based reagent            | HPT-F        | Miki and Shimamoto (2004), PMID:15111724 | RNAi genotyping, Hyg R: 5’-GAGCCGTACCTA TTGACTTCGG-3' |
| Sequence-based reagent            | HPT-R        | Miki and Shimamoto (2004), PMID:15111724 | RNAi genotyping, Hyg R: 5’-GAGCCGTACCTA TTGACTTCGG-3' |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|------------------------------------|-------------|--------------------|-------------|------------------------|
| Sequence-based reagent            | pVec8F      | *Miki and Shimamoto (2004)*, PMID:15111724 | RNAi genotyping, RNAi hairpin: 5'-TTTAGC CCTGCCCTCATACG-3' |
| Sequence-based reagent            | pVec8R      | *Miki and Shimamoto (2004)*, PMID:15111724 | RNAi genotyping, RNAi hairpin: 5'-ATTGC CAAATGTTTGAAAGA-3' |
| Sequence-based reagent            | PW64F       | This paper         | RNAi genotyping, RNAi hairpin: 5'-ATCCAC GCGTATTCCG-3' |
| Sequence-based reagent            | PW64R       | This paper         | RNAi genotyping, RNAi hairpin: 5'-ATCCAC GCGTATTCCG-3' |
| Sequence-based reagent            | pCrLFY1genoF1 | This paper         | *CrLFY1*pro::GUS genotyping: 5'-CTTAGA TGCTTGAGATGCTAC-3' |
| Sequence-based reagent            | pCrLFY1genoF2 | This paper         | *CrLFY1*pro::GUS genotyping: 5'-CTCTCT TCTTGCTTGTGTTGTG-3' |
| Sequence-based reagent            | pCrLFY1genoF3 | This paper         | *CrLFY1*pro::GUS genotyping: 5'-CAACCTGCAACAGGTGTGACTTACG-3' |
| Sequence-based reagent            | pCrLFY1genoF4 | This paper         | *CrLFY1*pro::GUS genotyping: 5'-CACTGCTATTCACTGCATTCCG-3' |
| Sequence-based reagent            | pCrLFY1genoR | This paper         | *CrLFY1*pro::GUS genotyping: 5'-AGGAGGCACTTCTTTTACGTG-3' |
| Sequence-based reagent            | GUSgenoR    | This paper         | *CrLFY1*pro::GUS + 35S*pro::GUS genotyping: 5'-CATGTGTACGCCTCCCTCGC-3' |
| Sequence-based reagent            | 3SigenoF    | This paper         | 35S*pro::GUS genotyping: 5'-CTGAGCTTAGACACAGGTTG-3' |
| Sequence-based reagent            | OCS3'genoR  | This paper         | 35S*pro::GUS genotyping: 5'-CATCACTAGTAGCTAGCTTGCGC-3' |
| Commercial assay or kit           | Phusion high-fidelity polymerase | Thermo Scientific | Thermo Scientific: F5305 |
| Commercial assay or kit           | Gateway LR clonase II enzyme mix | Invitrogen | Invitrogen: 11791100 |
| Commercial assay or kit           | QIAGEN Plasmid Maxi Kit | QIAGEN | QIAGEN:12163 |
| Commercial assay or kit           | Whatman Nytran nylon blotting membrane | GE Healthcare | GE Healthcare: 10416294 |
| Commercial assay or kit           | Random Primers DNA Labelling kit | Invitrogen | Invitrogen: 18187013 |
| Commercial assay or kit           | Carestream Kodak autoradiography GBX developer and fixer | Sigma-Aldrich | Sigma-Aldrich: Z354147 |
| Commercial assay or kit           | Carestream Kodak Biomax XAR film | Sigma-Aldrich | Sigma-Aldrich: F5763 |
| Commercial assay or kit           | iTaq universal SYBR Green mastermix | Bio-Rad | Bio-Rad: 1725120 |
| Commercial assay or kit           | DIG-labelling mix | Roche Applied Sciences | Roche: 11277073910 |
| Commercial assay or kit           | T3 RNA polymerase | Roche Applied Sciences | Roche: 11031163001 |
| Commercial assay or kit           | T7 RNA polymerase | Roche Applied Sciences | Roche: 10881767001 |

*Continued on next page*
### Plant materials and growth conditions

All experimental work was conducted using *Ceratopteris richardii* strain Hn-n (*Warne and Hickok, 1987*). Plant growth conditions for Ceratopteris transformation and DNA gel blot analysis of transgenic lines were as previously described (*Plackett et al., 2015*).

### Phylogenetic analysis

A dataset of 99 aligned LFY protein sequences from a broad range of streptophytes was first retrieved from *Sayou et al.* (2014). The dataset was pruned and then supplemented with further sequences (*Supplementary file 1*) to enable trees to be inferred that would (i) provide a more balanced distribution across the major plant groups and (ii) infer fern relationships. Only a subset of available angiosperm sequences was retained (keeping both monocot and dicot representatives) but protein sequences from other angiosperm species where function has been defined through loss-of-function analyses were added from NCBI – *Antirrhinum majus* FLO AAA62574.1 (*Coen et al., 1990*), *Pisum sativum* UNI AAC49782.1 (*Hofer et al., 1997*), *Cucumis sativus* CsLFY XP_004138016.1 (*Zhao et al., 2018*), *Medicago truncatula* SGL1 AY928184 (*Wang et al., 2008*), *Petunia hybrida* ALF AAC49912.1 (*Souer et al., 1998*), *Nicotiana tabacum* NFL1 AAC48985.1 and NFL2 AAC48986.1 (*Kelly, 1995*), *Eschscholzia californica* EcFLO AAO49794.1 (*Busch and Gleissberg, 2003*), *Gerbera hybrida* cv. ‘Terraregina’ GhLFY ANS10152.1 (*Zhao et al., 2016*), *Lotus japonicus* LjLFY AAX13294.1 (*Dong et al., 2005*) and *Populus trichocarpa* PTLF AAB51533.1 (*Rottmann et al., 2000*). To provide
better resolution within and between angiosperm clades, sequences from Spirodea polyrhiza (32G0007500), Zostera marina (27g00160.1), Aquilegia coerulea (5G327800.1) and Solanum tuberosum (PGSC0003DMT400036749) were added from Phytozone v12.1 (https://phytozone.jgi.doe.gov/pz/portal.html). Genome sequence from the early-diverging Eudicot Thalictrum thalictroides was searched by TBLASTX (Altschul et al., 1990) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastx&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab) with nucleotide sequence from the Arabidopsis LFY gene. A gene model was derived from sequence in two contigs (108877 and 116935) using Genewise (Birney et al., 2004) (https://www.ebi.ac.uk/Tools/psa/gene-wise/). Gymnosperm sequences were retrieved from Ginkgo biloba and from a subset of conifers included in Sayou et al. (2014), whilst sequences from conifers where in situ hybridization patterns have been reported were added from NCBI – Pinus radiata PRFLL AAB51587.1 and NLY AAB68601.1 (Mellerowicz et al., 1998; Mouradov et al., 1998) and Picea abies PaLFY AAV49504.1 and PaNLY AAV49503.1 (Carlsbecker et al., 2004). Fern sequences were retained except Angiopteris spp sequences which consistently disrupted the topology of the tree by grouping with gymnosperms. To better resolve relationships within the ferns, additional sequences were identified in both NCBI and 1KP (Matasci et al., 2014) databases. The protein sequence from Matteuccia struthiopteris AAF77608.1 MatsFLO (Himi et al., 2001) was retrieved from NCBI. Further sequences from horsetails (2), plus eusporangiate (1) and leptosporangiate (53) ferns were retrieved from the 1KP database (https://db.cngb.org/blast/) using BLASTP and the MatsFLO sequence as a query. Lycophyte and bryophyte sequences were all retained, but the liverwort Marchantia polymorpha predicted ORF sequence was updated from Phytozone v12.1 (Mpo0113s0034.1.p), the hornwort Nothoceros genome scaffold was replaced with a translated full length cDNA sequence (AHJ90704.1) from NCBI and two additional lycophyte sequences were added from the 1KP dataset (Isoetes tegetiformans scaffold 2013584 and Selaginella kraussiana scaffold 2008343). All of the charophyte scaffold sequences were substituted with Coleochaete scutata (AHJ90705.1) and Klebsormidium subtile (AHJ90707.1) translated full-length cDNAs from NCBI.

The new/replacement sequences were trimmed and amino acids aligned to the existing alignment from Sayou et al. (2014) using CLUSTALW (Li et al., 2015) (Supplementary file 2 and 3). The best-fitting model parameters (JTT + I + G4) were estimated and consensus phylogenetic trees were run using Maximum Likelihood from 1000 bootstrap replicates, using IQTREE (Nguyen et al., 2015). Two trees were inferred. The first contained only a subset of fern and allied sequences to achieve a more balanced distribution across the major plant groups (81 sequences in total) (Figure 8), whereas the second used the entire dataset (120 sequences – 50% of which are fern and allied sequences – Figure 1—figure supplement 1). The data were imported into ITOL (Letunic and Bork, 2016) to generate the pictorial representations. All branches with less than 50% bootstrap support were collapsed. Relationships within the ferns (Figure 1) were represented by pruning the lycophyte and fern sequences (68 in total) from the tree containing all available fern sequences (Figure 1—figure supplement 1).

**CrLFY locus characterization and DNA gel blot analysis**

Because no reference genome has yet been established for Ceratopteris (or any fern), CrLFY copy number was quantified by DNA gel blot analysis. Ceratopteris genomic DNA was hybridized using both the conserved LFY DNA-binding domain diagnostic of the LFY gene family (Maizel et al., 2005) and also gene copy-specific sequences (Figure 1—figure supplement 2). CrLFY1 and CrLFY2 share 85% amino acid similarity, compared to 65% and 44% similarity of each to AtLFY. DNA gel blotting and hybridization was performed as described previously (Plackett et al., 2014). The results supported the presence of only two copies of LFY within the Ceratopteris genome. All primers used in probe preparation are supplied in the Key Resources Table.

Genomic sequences for CrLFY1 and CrLFY2 open reading frames (ORFs) were amplified by PCR from wild-type genomic DNA using primers designed against published transcript sequences (Himi et al., 2001). ORFs of 1551 bp and 2108 bp were obtained, respectively (Figure 1—figure supplement 2). Exon structure was determined by comparison between genomic and transcript sequences. The native promoter region of CrLFY1 was amplified from genomic template by sequential rounds of inverse PCR with initial primer pairs designed against published CrLFY1 5’UTR sequence and additional primers subsequently designed against additional contiguous sequence that was retrieved. A 3.9 kb contiguous promoter fragment was isolated for CrLFY1 containing the
entire published 5'UTR and 1.9 kb of additional upstream sequence (Figure 1—figure supplement 2). Repeated attempts were made to obtain a CrLFY2 promoter fragment but this proved impossible in the absence of a reference genome. Some sequence contiguous with the CrLFY2 ORF was obtained by inverse PCR using primers designed against the previously published 5'UTR sequence of the CrLFY2 transcript (Himi et al., 2001). This sequence was extended to 1016 bp in length using additional primers against the isolated genomic sequence but this fragment did not contain the entire published 5'UTR. Numerous rounds of inverse PCR generated a second 3619 bp genomic fragment containing sequence identical to the remaining 5'UTR (see Figure 1—figure supplement 2, Supplementary file 7) but the presumed connecting sequence between these two fragments could not be amplified despite many attempts. It was eventually concluded that either the intervening promoter fragment was too long to amplify or that it was too GC rich for amplification. All primers used in ORF amplification and inverse PCR are supplied in the Key Resources table. The contiguous sequences obtained for the CrLFY1 and CrLFY2 genomic loci have been submitted to Genbank (accessions MH841970 and MH841971, respectively).

**qRT-PCR analysis of gene expression**

RNA was extracted from Ceratopteris tissues using the Spectrum Total Plant RNA kit (Sigma-Aldrich, St. Louis, MO) and 480 ng were used as template in iScript cDNA synthesis (Bio-Rad). CrLFY1 and CrLFY2 locus-specific qRT-PCR primers were designed spanning intron 1. Amplification specificity of primers was validated via PCR followed by sequencing. qRT-PCR of three biological replicates and three technical replicates each was performed in a Bio-Rad CFX Connect with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer amplification efficiency was checked with a cDNA serial dilution. Efficiency was determined using the slope of the linear regression line as calculated by Bio-Rad CFX Connect software. Primer specificity was tested via melting curve analysis, resulting in a single peak per primer set. CrLFY expression was calculated using the 2^ΔΔCt method (Livak and Schmittgen, 2001) and normalized against the geometric mean of the expression of two endogenous reference genes (Hellemans et al., 2007), CrACTIN1 and CrTATA-BINDING PROTEIN (TBP) (Ganger et al., 2015). The standard deviation of the Ct values of each reference gene was calculated to ensure minimal variation (<3%) in gene expression. Error bars represent ± the standard error of the mean of the 2^ΔΔCt values. All primers used in qRT-PCR are supplied in the Key Resources table.

Relative expression values of CrLFY from qRT-PCR were compared by one or two-way analysis of variance (ANOVA) for developmental stages followed by Tukey’s or Sidak’s multiple comparisons, respectively. To test whether genes were downregulated in transgenic RNAi lines, two-way ANOVA was performed with gene (CrLFY1 or CrLFY2) and transgenic line as factors, with ‘gene’ as a repeated factor when all transgenic lines had the same number of replicates. Where appropriate, expression of each gene in each line was compared to the expression of the respective control by Dunnet comparisons. Control plants had been transformed and were hygromycin-resistant, but did not contain the RNAi hairpin that triggers gene silencing (non-hairpin controls, NHC). For all experiments, NHCs were grown alongside transgenic lines. qRT-PCR of transgenic lines was necessarily conducted across several plates, each including a representative NHC, and statistical comparisons were performed within each plate relative to its respective control. The significance threshold (p) was set at 0.05. All statistical analyses were performed in Prism v. 6.0 (GraphPad Software, Inc., La Jolla, CA).

**Generation of GUS reporter constructs**

The CrLFY1 pro:::GUS reporter construct (Figure 4—figure supplement 1) was created by cloning the CrLFY1 promoter into pART7 as a NotI-XbaI restriction fragment, replacing the existing 35S promoter. A β-glucuronidase (GUS) coding sequence (Ulmasov, 1997) was cloned downstream of pCrLFY1 as an XbaI-XbaI fragment. The same GUS XbaI-XbaI fragment was also cloned into pART7 to create a 35S pro:::GUS positive control (Figure 4—figure supplement 4). The resulting CrLFY1 pro:::GUS::ocs and 35S pro:::GUS::ocs cassettes were each cloned as NotI-NotI fragments into the pART7-based binary transformation vector pBOMBER carrying a hygromycin resistance marker previously optimized for Ceratopteris transformation (Plackett et al., 2015). All primers used in GUS reporter component amplification are supplied in the Key Resources table.
Generation of RNAi constructs

RNAi constructs were designed and constructed using the pANDA RNAi expression system (Miki and Shimamoto, 2004). Four RNAi fragments were designed, two targeting a conserved region of the CrLFY1 and CrLFY2 coding sequence (77% nucleotide identity) using sequences from either CrLFY1 (CrLFY1/2-1) or CrLFY2 (CrLFY1/2-2), and two targeting gene-specific sequence within the 3′UTR of CrLFY1 (CrLFY1-1) or CrLFY2 (CrLFY2-1) (Figure 6—figure supplement 1). Target fragments were amplified from cDNA and cloned into Gateway-compatible entry vector pDONR207 (Invitrogen, Carlsbad, CA). Each sequence was then recombined into the pANDA expression vector via Gateway LR cloning (Invitrogen, Carlsbad, CA). All primers used in RNAi target fragment amplification are supplied in the Key Resources table.

Generation of transgenic lines

Transformation of all transgenes into wild-type Hn-n Ceratopteris callus was performed as previously described (Plackett et al., 2015). T₀ sporophyte shoots were regenerated from transformed callus tissue, with each round of transformation using multiple separate pieces of callus as starting material. Transgenic T₁ spores were harvested from these T₀ shoots, germinated to form T₁ gametophytes and then self-fertilized to produce T₁ sporophytes. T₁ sporophytes were assessed for T-DNA copy number by DNA gel blot analysis (Figure 4—figure supplement 2; Figure 6—figure supplement 3) and the presence of full-length T-DNA insertions was confirmed through genotyping PCR (Figure 4—figure supplements 3 and 4). All primers used in genotyping reactions are supplied in the Key Resources table. For characterization of RNAi lines, T₂ spores were collected from individuals that either contained the full transgene construct or from segregants in which the RNAi hairpin was absent.

GUS staining

GUS activity analysis in CrLFY1pro::GUS transgenic lines was conducted in the T₁ generation. GUS staining was conducted as described previously (Plackett et al., 2014). Optimum staining conditions (1 mg/ml X-GlcA, 5 μM potassium ferricyanide) were determined empirically. Tissue was cleared with sequential incubations in 70% ethanol until no further decolorization occurred. GUS-stained gametophytes were imaged with a Zeiss Axioplan microscope and GUS-stained sporophytes imaged with a dissecting microscope, both mounted with Q-imaging Micro-published 3.3 RTV cameras. Images were minimally processed for brightness and contrast in Photoshop (CS4).

Phenotypic characterization

Phenotypic characterization of RNAi transgenic lines was conducted in the T₂ or T₃ generation. Iso- genetic lines were obtained by isolating hermaphrodite gametophytes in individual wells at approximately 7 DPS (or when the notch became visible, whichever came first) and flooding them once they had developed mature gametangia (at approximately 9 DPS). All transgenic lines were grown alongside both wild-type and no hairpin controls, and phenotypes observed and recorded daily. Gametophytes exhibiting altered phenotypes were imaged at approximately 10 DPS with a Nikon Microphot-FX microscope. Sporophytes with abnormal phenotypes were imaged with a dissecting microscope.

In situ hybridization

Antisense and sense RNA probes for CrLFY1 and CrLFY2 were amplified and cloned into pCR 4-TOPO (Invitrogen) and DIG-labelled according to the manufacturer’s instructions (Roche, Indianapolis, IN). Probes were designed to include the 5′UTR and ORF (CrLFY1 521 bp 5′UTR and 1113 bp ORF; CrLFY2 301 bp 5′UTR and 1185 bp ORF) (Supplementary file 6). All primers used in situ probe amplification are supplied in the Key Resources table. We were unable to identify fragments that distinguished the two genes in whole mount in situ hybridizations. Tissue was fixed in FAA (3.7% formaldehyde, 5% acetic acid; 50% ethanol) for 1–4 hr and then stored in 70% ethanol. Whole mount in situ hybridization was carried out based on Hejástko et al. (2006), with the following modifications: hybridization and wash steps were carried out in 24-well plates with custom-made transfer baskets (0.5 mL microcentrifuge tubes and 30 μm nylon mesh, Small Parts Inc., Logansport, IN). Permeabilization and post-fixation steps were omitted depending on tissue type to avoid damaging...
fragile gametophytes, Acetic Anhydride (Sigma-Aldrich) and 0.5% Blocking Reagent (Roche) washing steps were added to decrease background staining, and tissue was hybridized at 45°C. Photos were taken under bright-field with a Q-imaging Micro-publisher 3.3 RTV camera mounted on a Nikon Microphot-FX microscope. Images were minimally processed for brightness and contrast in Photoshop (CS4).

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|-------------------------------|----------------------------------------|----------------------------|
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Author contributions
Andrew RG Plackett, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Cloned open reading frames and upstream promoter fragments of CrLFY1 and CrLFY2, Made the CrLFY1pro::GUS reporter constructs, Generated and validated transgenic reporter lines, Conducted GUS staining, Performed gel blot analysis of CrLFY copy number; Stephanie J Conway, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Screened, validated and characterized RNAi lines, Carried out in situ hybridizations; Kristen D Hewett Hazelton, Formal analysis, Validation, Investigation, Methodology, Writing—review and editing, Screened, validated and characterized RNAi lines, Performed ontogenetic gene expression analysis; Ester H Rabbinowitsch, Investigation, Generated and maintained T0 transgenic lines; Jane A Langdale, Conceptualization, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Writing—original draft, Carried out the phylogenetic analyses; Verónica S Di Stilio, Conceptualization, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Writing—original draft, Cloned the CrLFY coding sequences and made the RNAi constructs during a sabbatical visit to the University of Oxford, Carried out the statistical analyses.

Author ORCIDs
Andrew RG Plackett https://orcid.org/0000-0002-2321-7849
Jane A Langdale https://orcid.org/0000-0001-7648-3924
Verónica S Di Stilio https://orcid.org/0000-0002-6921-3018
Additional files

**Supplementary files**

- Supplementary file 1. LFY sequences included in phylogenetic analysis (in addition to Sayou et al., 2014) dataset.
  DOI: https://doi.org/10.7554/eLife.39625.027
- Supplementary file 2. Alignment of all 120 LFY amino acid sequences used in phylogenetic analysis.
  DOI: https://doi.org/10.7554/eLife.39625.028
- Supplementary file 3. Alignment of LFY amino acid sequences used in phylogenetic analysis (ferns only).
  DOI: https://doi.org/10.7554/eLife.39625.029
- Supplementary file 4. Statistical comparison of CrLFY transcript levels between different ontogenetic stages.
  DOI: https://doi.org/10.7554/eLife.39625.030
- Supplementary file 5. Specificity of CrLFY RNAi target sequences. Alignment (prepared using Clustal Omega) of the full length transcript sequences for CrLFY1 and CrLFY2, with nucleotide identity between the two gene copies denoted by a subtending asterisk. The CDS for each gene is highlighted in bold. The target sequences for each RNAi construct are highlighted in light blue (CrLFY1/2-i1), light green (CrLFY1/2-i2), dark blue (CrLFY1-i3) or dark green (CrLFY2-i4). The CrLFY1/2-i1 and CrLFY1/2-i2 target sequences each have 77% similarity to the opposing gene transcript (BLAST2n, discontiguous megablast for highly similar sequences). The full-length CrLFY1-i3 and CrLFY2-i4 target sequences do not demonstrate significant sequence similarity to the opposing gene transcript or 3'UTR alone (BLAST2n, blastn for somewhat similar sequences) but short regions of similarity within the target sequence might explain the cross-reactivity observed.
  DOI: https://doi.org/10.7554/eLife.39625.031
- Supplementary file 6. Predicted hybridization and specificity of CrLFY in situ hybridization probes. Alignment (prepared using Clustal Omega) of full length CrLFY1 and CrLFY2 transcript sequences, with nucleotide identity between the two paralogs denoted by a subtending asterisk. The coding sequence (CDS) for each gene copy is highlighted in bold. Predicted sites of hybridization for the two probes are highlighted in blue (CrLFY1) and yellow (CrLFY2) respectively, with PCR primer sites underlined. The in situ probes span the complete CDS and 5'UTR of each gene copy. The CrLFY1 probe sequence shows 79% nucleotide identity to the CrLFY2 transcript (BLAST2n, discontiguous megablast for highly similar sequences). The CrLFY2 probe shows 79% nucleotide identity to the CrLFY1 transcript (BLAST2n, discontiguous megablast for highly similar sequences).
  DOI: https://doi.org/10.7554/eLife.39625.032
- Supplementary file 7. Amplified CrLFY2 genomic fragment (3619 bp), not connected directly to CrLFY2 open reading frame. Sequence highlighted in green corresponds to published CrLFY2 5'UTR (Himi et al., 2001)
  DOI: https://doi.org/10.7554/eLife.39625.033
- Supplementary file 8. Summary of published reports of LFY function in a range of angiosperm species. All citations included in reference list of main article (Bradley et al., 1996; Blázquez et al., 1997; Bradley et al., 1997; Kyozuka et al., 1998; Pnueli et al., 1998; Ratcliffe et al., 1999; Gourlay et al., 2000; Bomblies et al., 2003; Becker et al., 2005; Meng et al., 2007; Souer et al., 2008; Wreath et al., 2013).
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- Transparent reporting form
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Data availability
All data generated or analysed during this study are included in the manuscript and supporting files. Source data files have been provided for Figures 3 and 6. Sequences and alignments for phylogenetic analyses are included in Supplementary files 1-3.

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