Reconstructing the evolution of gene function for a master regulator of flowering: *LEAFY* in the fern *Ceratopteris richardii*

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Abstract

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All land plants have a life cycle with alternation of generations that includes both a haploid, gamete-producing gametophyte and a diploid spore-producing sporophyte. Over evolutionary time, land plants have gone from having independent, longer-living gametophytes with ephemeral, dependent sporophytes, as in the bryophytes, to large, independent sporophytes which support small, dependent, short-lived gametophytes in the seed plants. A critical angiosperm innovation is the production of flowers, which enclose unisexual gametophytes within the sporophyte. The evolution of the flower has been hypothesized to have occurred through a combination of cooption of genes present in the seed plant common ancestor, and expansion of gene families involved in flower development.

A common approach to learning about the evolution of flowering genes is to investigate their homologs in representatives of earlier diverging lineages of non-flowering plants. For instance, ferns represent a lineage in a key phylogenetic placement representative of early-diverging vascular plants and sister to all seed plants, with independent and photosynthetic gametophytes and sporophytes. In more derived lineages, where gametophytes are small and dependent, studying the dependent stage is more challenging and less informative, leaving open questions about the role of genes of interest across both life stages.

In this dissertation, I focus on the homosporous fern *Ceratopteris richardii* (*C. richardii*), to investigate gene function across the plant life cycle. This research aims to broadly inform understanding of the functional evolution of key reproductive genes in land plants. The goal of Chapters 1 and 2 is to investigate the role of the floral meristem identity gene *LEAFY* (*LFY*) in *C. richardii* gametophytes and sporophytes. My work uncovered new roles for the two *C. richardii LFY* paralogs, *CrLFY1/2,* in gametophyte reproduction, particularly in sperm cells and in the

lateral meristem that generates gametangia. In the sporophyte, I found a conserved function in early embryogenesis, in the first division of the fern zygote, a role previously described for a moss species but not in any vascular plant. Together, these findings highlight a novel haploid reproductive function for *LFY* homologs, suggesting that this may have been an ancestral role that was coopted by sporophytes as they became the dominant stage of the plant life cycle.

In Chapter 3, I investigate the developmental genetics of the fern gametophyte. *C. richardii* gametophytes develop as either hermaphrodites, with a multicellular meristem that produces both egg (in archegonia) and sperm (in antheridia), or as ameristic males (developing from a transient apical cell), which produce only sperm. The first spores to germinate develop into hermaphrodites, which secrete the hormone antheridiogen, inducing surrounding gametophytes to develop as males. By comparing transcriptomes of mature male and hermaphrodite gametophytes via RNA-seq, I find a total of 22,719 expressed genes, over half of which (12,424) are differentially expressed between the two sexes, 47.8% upregulated in males and 52.2% upregulated in hermaphrodites. Gene ontology analysis indicated that microtubule genes have high mutual correlation to *CrLFY1*, suggesting a potential novel role of *CrLFY1* in regulating aspects of sperm development.

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Dedication

There are people so dear to me who have not been able to stay in this world. I may not believe that we will meet again, but I do feel the love we shared still, just as much as I did when each of you could walk beside me.

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I cannot thank my parents, my brother, my grandmother and my extended family enough for always encouraging me and forever being enthusiastic about the work that I have done, for always cheering and reassuring me, and for always giving me the space to come home to a perfect meal.

I have been lucky enough to have had many deep, long-standing and incredibly kind and supportive friend groups, all of whom have been instrumental in encouraging my growth. The Cascadia Independence Movement – Audrey, Catie, Lauren and Madeline. It's been quite a journey and I'm so glad to still have you all in my life. The 500 Hearts – Caroline, Genevieve, Janyl and Katie – I can say with a fair amount of certainty since I'm very nearly graduated: Reed was harder than grad school. Y'all are a love that always nurtures me. The Friendz 4ever/Tech Crew – Candice, Killian, Danica, Anna, Sarah, Jeanette – I am constantly inspired seeing the papers published, defenses celebrated, talks given… and the phage pinata.

To those who I've met since my grad journey started, I unfortunately don't have a nice succinct group chat name. Bushra – Hannibal memes and KBBQ forever. Erika – the only other fan of ridiculously astringent tea. Melissa, let Blue run your home. Nick, with excellent movie vibes and good food. Elizabeth and the best musical movie nights. Kyra – anytime I see a cool dead thing, and I'm always reminded of you. Jason, I don't know what will come next, but thank you for the friendship that we had, and for sharing in my questionable music taste. Steven, there's something unspeakingly powerful about your ability to sleep through the loudest musicals.

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Dakota, you have been unwavering in your support and kindness, and in making sure I don't bury myself in C-fern media. I cannot thank you enough. But I will try to bake enough treats to make up for the lack of words

If I have ever said hi to any of your pets, please tell them that I love them and they have also been essential to the completion of this dissertation.

The evolution and development of *LEAFY* function

Authors: Hannah McConnell and Verónica S. Di Stilio

Abstract:

Even though the canonical role of the transcription factor *LEAFY* (*LFY*) is in establishing the identity of angiosperm floral meristems, homologs of this gene are found across all land plants, predating the evolution of flowers. It has been hypothesized that *LFY*'s ancestral role was more generally meristematic, in the regulation of cell division, having more recently acquired its reproductive function. Here, we review mounting evidence from *LFY* orthologs in non-flowering plants that support the hypothesis that *LFY's* reproductive role arose earlier in the land plant lineage than previously thought, in the gametophyte of haploid-dominant plants, and that it was coopted to the sporophyte as land plants evolved towards diploid-dominant life cycles. Additionally, we discuss the impact of novel insights into *LFY*'s mechanism of action on the reconstruction of its functional evolution, including its recent inclusion in the elite category of plant pioneer transcription factors.

Introduction:

*LEAFY'*s roles in angiosperms are well described, first and foremost in its reproductive function as a floral meristem identify gene (Blázquez et al., 1997; Carpenter and Coen, 1990; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1998; Weigel et al., 1992), as well as in its vegetative function in the development of compound leaves of certain legumes (Champagne et al., 2007; Hofer et al., 1997; Jiao et al., 2019; Moriyama et al., 2024; Wang et al., 2013), axillary meristems (Busch and Gleissberg, 2003; He et al., 2020; Kelly et al., 1995; Shu et al., 2000; Souer et al., 1998; Wang et al., 2008; Zhao et al., 2017) and rice panicle branching (Kyozuka et al., 1998; Rao et al., 2008). An evolutionary integration of this body of knowledge beyond angiosperms suggests that *LEAFY's* floral function actually predated angiosperm divergence, while its vegetative function is deeply conserved (Moyroud et al., 2010). "*LEAFY* Blossoms" reviewed *LFYs* functional evolution across land plants and was ahead of its time when proposing that *LFY* homologs play an ancestral role regulating cell division, subsequently acquiring the ability to activate reproductive genetic networks in seed plants (Moyroud et al., 2010). This hypothesis stemmed from findings that, in the moss *Physcomitrium patens*, *LFY* homologs expressed in gametophyte tissue and are necessary for the first division of the zygote (Fig. 1A,B) (Tanahashi et al., 2005), and that expression of *LFY* and the gymnosperm-specific omolog *NEEDLY*, is found in both vegetative meristems and reproductive cones of gymnosperms (Ewa J Mellerowicz et al., 1998; Mouradov et al., 1998; Shindo et al., 2001). From these findings, Moyroud (2010) proposed that *LFY* first played a role in regulating cell

division, as seen in *Physcomitrium*, and then acquired reproductive functions, supported by expression of *LFY* in both vegetative and reproductive tissues of seed plants.

In the years since, functional and expression data in additional land plant clades, in particular lycophytes and ferns, continue to support aspects of the "*LFY* as a cell division gene" hypothesis, while also suggesting that its reproductive role may have arisen earlier, prior to seed plants, in the vascular plant ancestor. Significant novel findings on *LFY's* molecular mode of action provide an opportunity to generate new hypotheses on the evolution of its function and underlying mechanisms. Moreover, the recent incorporation of functional studies in ferns enables a deeper dive into gametophytes, a previously neglected life-stage. Unlike seed plants, the gametophytes of lycophytes and ferns are macroscopic and often independent, with distinct features that enrich the phenotypic characterization of mutants, such as gametangia, rhizoids and single cell or multicellular meristems. Thus, newly described *LFY* functions provide motivation to re-examine previous hypotheses in a new light, prompting us to synthesize the literature that has been published in the interim.

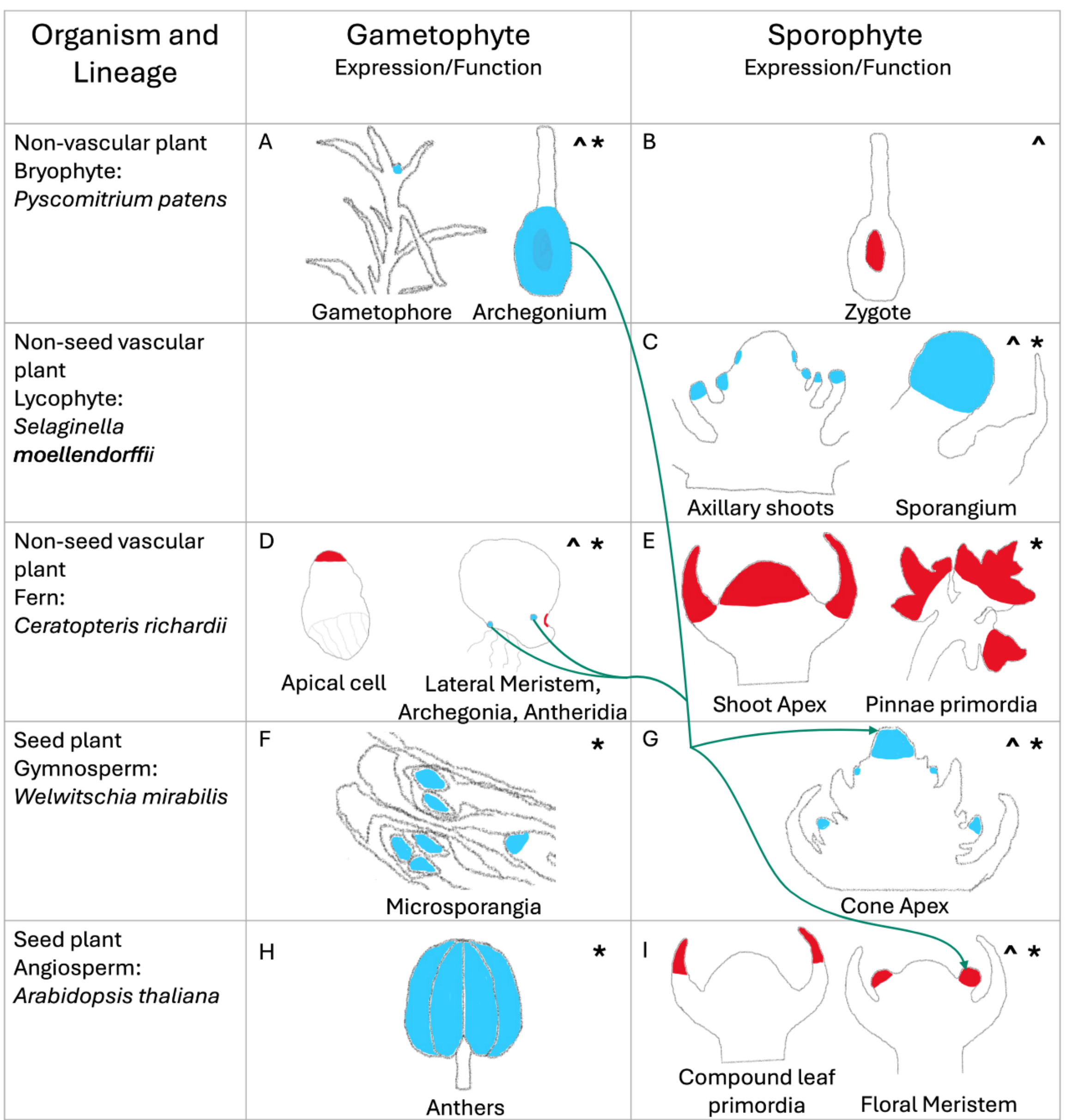

Figure 1: The different roles of *LFY* homologs in land plant gametophytes and sporophytes. Diagrams of gametophytes and sporophytes of representative members of major plant lineages. *LFY* homolog spatial expression is denoted in blue and demonstrated function in red. The teal arrow represents the hypothesis of evolutionary cooption of *LFY* genes from a reproductive role in gametophytes to one in sporophytes. ^ = vegetative function, * = reproductive function. (A-B) *PpLFY* in the moss *Physcomitrium patens* (A) gametophore (the leafy stage of moss gametophytes) and archegonium (the egg-containing gametangium), and (B) zygote within a fertilized archegonium (modified from Tanahashi et al., 2005). (C) *SmLFY* expression in axillary shoots and sporangium of the lycophyte *Selaginella moellendorffii*

(modified from Rodríguez-Pelayo et al., 2022). (D-E) *CrLFY* in the fern *Ceratopteris richardii* (D) (left) the apical cell of the just-germinated gametophyte still attached to the spore coat and (right) the lateral meristem, archegonia and antheridia of the mature gametophyte, and (E) (left) the shoot apex and leaf primordia of the sporophyte and (right) pinnae primordia of developing fronds (modified from McConnell et al., 2025; Plackett, Conway et al., 2018). (F-G) *WmLFY* found in (F) the microsporangia of male cones and (G) the reproductive cone apex of *Welwitschia* (modified from Moyroud et al., 2017). (H-I) *LFY* in (H) the *Arabidopsis* anther and (I) (left) the compound leaf primordia of a pea plant, and (right) floral meristem of *Arabidopsis* (modified from Hofer et al., 1997; Nakabayashi et al., 2005; Schmid et al., 2005a).

***LEAFY* is found in sporophyte vegetative and reproductive meristems across land plants**

Over the past fifteen years, investigations into *LFY* in lycophytes and ferns have provided key evidence that fills a gap for the reconstruction of its ancestral function in land plants (Rodríguez-Pelayo et al., 2022). In the lycophyte *Selaginella moellendorfii*, the *LFY* homolog is expressed in both the apical shoot meristem and in reproductive tissues (Fig. 1 C) (Rodríguez-Pelayo et al., 2022). Two homologs are found in another lycophyte, *Isoetes sinensis*, also localizing to both vegetative and reproductive tissues (Yang et al., 2017). In the horsetail *Equisetum giganteum*, a fern relative, two of three paralogs are expressed highly in reproductive tissue and at a lower level in vegetative tissue (Rodríguez-Pelayo et al., 2022). In the model fern *C. richardii*, a *CrLFY1* paralog was found in the embryonic frond, the shoot apical meristem and the developing compound fronds via promoter-driven β-glucuronidase (*GUS)* reporter (Fig.1 D-E) (Plackett, Conway et al., 2018). In that study, RNAi knockdown of either or both *CrLFY* paralogs resulted in plants that were significantly stunted in their growth, which precluded further investigations into a potential reproductive role (Plackett, Conway et al., 2018). However, two *LFY* paralogs found in the maidenhair fern *Adiantum raddianum* show expression in both vegetative and reproductive tissue, like that found in lycophytes (Rodríguez-Pelayo et al., 2022). Thus, *LFY* homolog expression in both vegetative meristems and reproductive tissue across ferns and lycophytes suggests that the hypothesized ancestral role of *LFY* regulating cell division is likely deeply conserved in vascular plants.

LFY homolog expression is seen in vegetative tissue of several gymnosperms (Fig. 1G) (Mellerowicz et al., 1998; Mouradov et al., 1998; Shindo et al., 2001) and *LFY* regulates cell division in compound leaves of certain angiosperms (Fig. 1I) (Champagne et al., 2007; Hofer et al., 1997). Interestingly, evidence of *LFY* expression in reproductive tissues of both ferns and lycophytes suggests that this function likely arose in the last common ancestor of the vascular plants, as opposed to the last common ancestor of the seed plants and is therefore a considerably older function than previously estimated. One role of *LFY* previously described in *Arabidopsis* but rarely discussed is in indirectly repressing genes involved in the inflorescence meristem, ultimately aiding in floral and fruit determinacy (Lenhard et al., 2001; Lohmann et al., 2001).

This role has recently received new attention following an investigation in *Solanum* that provided evidence that the *LFY* homolog *FALSIFLORA* (*FA)* is also responsible for floral and fruit determinacy (Quevedo-Colmena et al., 2025) a reminder that, even in angiosperms, *LFY* functions are yet to be discovered. An additional unexpected role for LFY has been found in the shoot apical meristem of cucumber; non-flowering roles for LFY in angiosperms had not been previously described in the shoot apex (Zhao et al., 2017).

A role for *LEAFY* in the gametophyte

While transgenic tools allowing for functional studies in non-angiosperm model plants are scarce, a combination of expression data and a limited number of functional studies introduce key insights into *LFY* function in the haploid gametophyte across land plants. In flowering plants such as *Arabidopsis*, the gametophytes are contained within the flower and consist of embryo sacs within ovules, and pollen grains within anthers. The ephemeral nature and small size of this haploid stage complicate functional assays, especially in regard to *LFY*, whose flower meristem identity role is upstream of embryo sac and pollen development. Nevertheless, since *Arabidopsis* is the most studied model plant, its gene atlas shows that *LFY* expression is mostly absent from the mature flower, except for a low amount of expression in stamen (Fig. 1H) (Nakabayashi et al., 2005; Schmid et al., 2005b), suggesting it could arise from pollen grains.

In gymnosperms, *LFY* orthologs and the gymnosperm-specific duplicate *NEEDLY* (*NLY*) are expressed in both male and female reproductive cones. However, gametophyte-specific expression has only been observed in microsporangia, where *NLY* is expressed, while neither *LFY* nor *NLY* is expressed in the female gametophyte (Fig. 1F) (Carlsbecker et al., 2013a; Ewa J Mellerowicz et al., 1998; Mouradov et al., 1998; Moyroud et al., 2017; Shindo et al., 2001; Silva et al., 2016). Further evidence for *LFY* function in the gametophyte comes from representatives of earlier diverging lineages, like bryophytes and ferns, where gametophytes are longer-lived and independent. In the moss *P. patens*, *PpLFY1/2* are necessary for the first cell division of the zygote (the first cell of the sporophyte stage), with *LFY* loss-of-function mutants failing to develop multi-cellular embryos (Fig. 1B) (Tanahashi et al., 2005). No mutant phenotypes were noted in the gametophyte stage, but *PpLFY* promoter-driven *GUS* staining indicates expression in their archegonia (the female gametangia) and the egg cells within them, as well as in the shoot apex of the sporophyte (Fig. 1A) (Tanahashi et al., 2005). The lack of phenotype in mutant moss gametophytes may be indicative of a lack of function despite expression there, or of redundancy. In another bryophyte, the liverwort *Marchantia polymorpha* (*M. polymorpha*)*, MpLFY* is expressed in antheridia (the male gametangia) (Arnoux-Courseaux and Coudert, 2023; Kawamura et al., 2022). In the fern *Ceratopteris richardii* (*C. richardii*), functional studies point to a role of *CrLFY1/2* in the apical cell (a unicellular meristem, or stem cell) of the young gametophyte (Fig. 1D) (Plackett et al., 2018) and the multicellular lateral meristem of the mature hermaphrodite gametophyte (Fig. 1D) (McConnell et al., 2025). *CrLFY1* is also expressed during

the initiation of archegonia, and in the developing and mature sperm cells, based on promoter-*GUS* experiments (Fig. 1D) (McConnell et al., 2025).

Altogether, current evidence suggests that *LFY* regulation of cell division and maintenance of vegetative meristems is conserved in the gametophytes of bryophytes and early vascular plants and additionally points to a conserved reproductive gametophyte role in regulating gametangia development. While *PpLFY* is expressed during moss egg development (Tanahashi et al., 2005), and *MpLFY* in liverwort antheridia (Arnoux-Courseaux and Coudert;Kawamura et al., 2022), both are expressed in gametangia. Taken together, the expression of *LFY* homologs in the gametangia of bryophytes and a fern, as well in the notch meristem that gives rise to gametangia in *C. richardii* (McConnell et al., 2025), suggests that this reproductive role during the haploid stage is ancestral. Moreover, *LFY's* expression in gymnosperm pollen and *Arabidopsis* stamens suggests that this reproductive role in the haploid stage is conserved between spore- to seed-producing plants.

Cooption-from-the-gametophyte hypothesis: from gametangia to sporangia

Comprehensive studies across land plants are further supporting *LFY's* function in the gametophyte. While functional data has yet to elucidate exactly what role *LFY* orthologs play in the development of gametangia in plants with free-living gametophytes, expression data supports a role for *LFY* in the development of gametangia, as described for the bryophytes *P. patens* and *M. polymorpha* (Arnoux-Courseaux and Coudert, 2023; Tanahashi et al., 2005) and for the vascular plant *C. richardii* (McConnell et al., 2025), in addition to roles in cell division. As gametophytes became smaller, shorter-lived and dependent on the sporophyte over evolutionary time in the seed plants, *LFY* appears to have shifted to maintaining sporophytic reproductive meristems in cones and flowers. This sporophyte reproductive role is supported by expression data in gymnosperms, as described above, and by the functional analyses that established *LFYs* canonical role in conferring the identity of the floral meristem (Fig. 1I) (Blázquez et al., 1997; Carpenter and Coen, 1990; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1998; Weigel et al., 1992).Thus, we propose that *LFY's* reproductive role has been coopted over evolutionary time from a yet undefined role in gametangia/gamete development in bryophytes and early vascular plants to one in conferring reproductive meristem identity in the sporophytes of seed plants.

Novel insights into *LEAFY*'s molecular mechanism

Heterologous experiments in an *Arabidopsis lfy* loss-of-function mutant demonstrate that, despite high sequence conservation across land plants, *LFY* orthologs from representatives of early-diverging land plant lineages are unable to rescue flower development (Maizel et al., 2005). While *LFY* orthologs from vascular plants conferred partial rescue (some floral organs were

recovered), only those from closely related angiosperms resulted in normal flowers. A lack of rescue despite high sequence conservation may seem unexpected, but *LFY*'s binding sites vary greatly across land plants (Sayou et al., 2014). Green algae and hornworts (a bryophyte) share the same type of *LFY* binding motif, while mosses recognize a different one. The remaining land plants, liverworts and vascular plants, recognize a third motif (Sayou et al., 2014). While liverwort *LFY* has not been tested, *LFY* homologs from vascular plants are more capable of rescuing the *lfy Arabidopsis* loss-of-function mutant than the those from the other bryophytes (Maizel et al., 2005), suggesting that conserved binding site recognition is critical in the evolution of *LFY* function.

The LFY protein consists of a C-terminal DNA-binding domain (Hamès et al., 2008; et ., 2015; Moyroud et al., 2011; Winter et al., 2011a) and an N-terminal sterile alpha domain that does not directly contact DNA, yet is critical for floral function (Sayou et al., 2016). This N-terminal region also behaves as an oligomerization domain that enables LFY's access to low-affinity binding sites and regions of closed chromatin, and this was the first evidence suggesting that it may be a pioneer transcription factor (Sayou et al., 2016).

LEAFY as a pioneer transcription factor

Pioneer transcription factors belong in a special category of genes capable of accessing target sequences inside nucleosomes, where linker histones are repressing transcription of downstream genes (Iwafuchi-Doi, 2019; Iwafuchi-Doi and Zaret, 2016, 2014; Soufi et al., 2015). While many pioneer transcription factors have been described in animals, only a few are known in plants: *LFY*, *AP1*, *SEP3*, and *LEAFY COTYLEDON1* (*LEC1*) (Lai et al., 2018a). Like other pioneer transcription factors, LFY is able to access these regions of heterochromatin and activate the expression of repressed genes as a homodimer (Jin et al., 2021; Lai et al., 2021; Sayou et al., 2016). Another characteristic of pioneer transcription factors is their ability to recruit chromatin-remodeling factors to open up regions of the genome that otherwise would be inaccessible, allowing other transcription factors to further regulate the genes within those regions (Iwafuchi-Doi, 2019). For example, *LFY* expression is sufficient to induce accessibility to formerly highly repressed regions to direct *LFY* targets such as the floral MADS box genes *AP1* and *AG* (Jin et al., 2021; Lai et al., 2021).

LFY's pioneer transcription factor status has only been described in *Arabidopsis*, and it is therefore unknown if this role is conserved in other plants. Interestingly, highly regulated expression of *LFY* is necessary for the first division of the zygote to proceed in mosses and ferns (McConnell et al., 2025; Tanahashi et al., 2005). While the exact mechanism by which *LFY* regulates this critical first cell division during plant embryogenesis is unknown, it is interesting to note that animal pioneer transcription factors are involved in zygotic genome activation – the initiation of transcription from the new zygote's genome (Fu et al., 2024; Kobayashi and

Tachibana, 2021). Since only a handful of pioneer transcription factors have been identified in plants (Lai et al., 2018a), their developmental roles are still unclear. It is therefore too soon to determine whether *LFY* acts a pioneer transcription in other plants, and whether it also exhibits zygotic genome activation, but this tantalizing explanation for LFY's role in the first cell division of the zygotic seems worth pursuing.

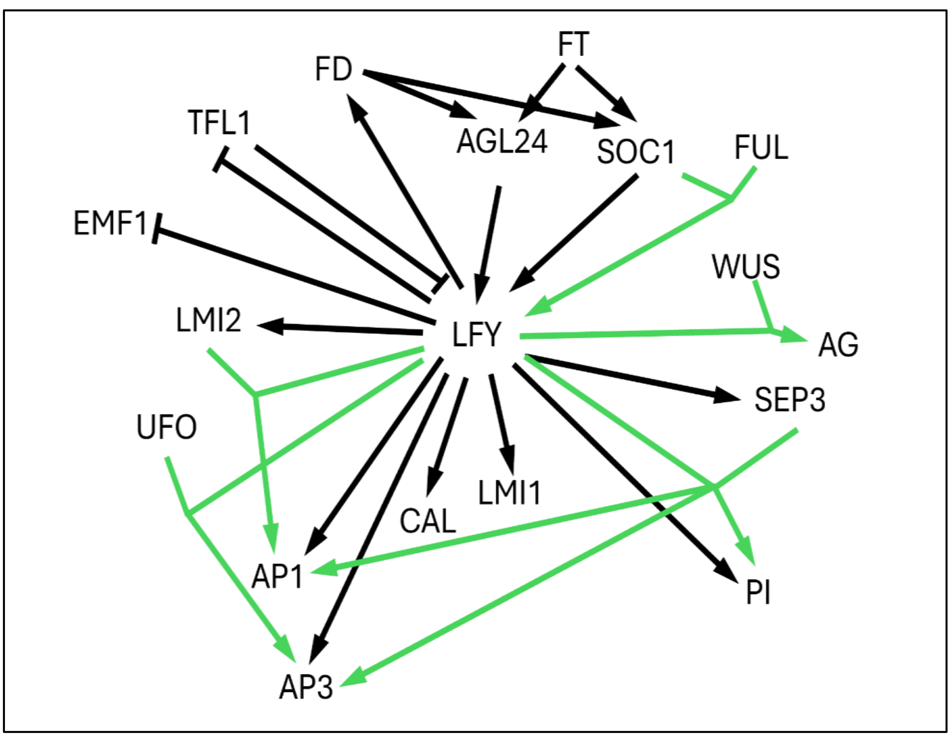

Figure 2: Putative LFY genetic regulatory network for flower development in *Arabidopsis*. Black arrows represent direct inductions and black-capped lines direct repression, and converging green arrows indicate a dimer needed for induction. Regulators of LFY: Balanzà et al., 2014; Zhu et al., 2020). LFY response to regulators: Jaeger et al., 2013; Leal Valentim et al., 2015). Direct initiation: (Chou et al., 2001; Jaeger et al., 2013; Lamb et al., 2002; Parcy et al., 1998; William et al., 2004; Winter et al., 2011a). Heterodimers and complexes: (Hamès et al., 2008; Liu et al., 2009; Pastore et al., 2011; Rieu et al., 2023b, 2023a; Wu et al., 2012).

Evolution of LFY's interactome

While LFY regulates most genes as a homodimer (Jaeger et al., 2013; Lamb et al., 2002; Parcy et al., 1998; William et al., 2004; Winter et al., 2011a), it can also form heterodimers and higher order complexes. These include heterodimers with UNUSUAL FLORAL ORGANS (UFO), which was long hypothesized to signal for LFY degradation; instead, the dimer appears to activate gene expression and redirect LFY to novel cis elements not recognized by the homodimer (Rieu et al., 2023a, 2023b). LFY and WUSCHEL may compete for binding sites to activate expression of flower organ identity genes like *AGAMOUS* (*AG*), although more likely the two proteins also interact as a heterodimer (Hamès et al., 2008). Additionally, LFY forms a complex with LATE MERISTEM IDENTITY2 (LMI2) to activate expression of the A-class gene *APETALA1* (*AP1*) via different binding motifs than the LFY homodimer (Pastore et al., 2011). In addition to upregulating *SEPALATA3* (*SEP3*) (Winter et al., 2011a), LFY also interacts with SEP3 to further upregulate *AP1* and the B-class genes *APETALA3* (*AP3*), and *PISTILATA* (*PI*) (Liu et al., 2009). This LFY-SEP3 complex recruits chromatin remodeling factors SPLAYED and BRAHMA, increasing expression of *AP3* and *AG* by overcoming polycomb repression (Wu et al., 2012).

While in flowering plants LFY forms stable dimers, the key amino acid residues that contribute to this stability are highly variable in non-flowering plants, suggesting weaker interactions (Hamès et al., 2008). LFY's ability to activate downstream genes in angiosperms may be reliant on the stability of these dimers, which would create longer periods of activation. However, this hypothesis cannot be tested without investigations into the LFY interactome of non-flowering plants. It is, therefore, unclear whether the observed variability in key residues for dimer stability leads to less stable dimers that are active for shorter periods of time, or to the functional diversification of LFY orthologs.

The evolution of LFY's GRN

The evolution of LFY's gene regulatory network is central to understanding its functional evolution. In *Arabidopsis*, LFY interacts with a large number of other genes and proteins involved in flowering. It is regulated by a PEBP protein, TFL1, which represses expression (Zhu et al., 2020), as well as another PEBP, FT, which with the bZIP protein FD, indirectly induces expression, by upregulating the MADS-box genes AGL24 and SOC1. These in turn directly upregulate LFY. *FRUITFUL* is another MADS-box gene that forms a heterodimer with SOC1 to bind to the LFY promoter and activate expression of the gene (Balanzà et al., 2014). LFY also then induces further expression of FD and represses TFL1 (Jaeger et al., 2013; Valentim et al., 2015). LFY is directly responsible for initiating transcription of numerous downstream flowering genes, including the MADS box flower organ identity genes *AP1* (Jaeger et al., 2013; Parcy et al., 1998) and *AP3* (Lamb et al., 2002), *CAULIFLOWER (CAL)*, *PI and SEP3* (Winter et al.,

2011a), and *LATE MERISTEM IDENTITY1/2* (William et al., 2004). LFY is additionally responsible for repressing *EMBRYONIC FLOWER1*, a polycomb group protein involved in repressing *AP3*, *AP1* and *AG* (Chou et al., 2001; Winter et al., 2011a). As described above, LFY forms complexes and heterodimers in addition to homodimers, including UFO, LMI2 and possibly WUS and SEP3, which further activate expression of downstream genes like the C-class gene *AG*. Ultimately, LFY is regulated by three gene types, PEBPs, bZIPs and other non-ABC model MADs-Box genes, and in turn regulates all MADs-box flowering classes.

Limited studies have been conducted into the genetic network of LFY outside of *Arabidopsis*, with the majority occurring in other angiosperms. In gymnosperms, there is an overlapping expression between *LFY* paralogs and the C class gene *DAL2* (Sundström and Engström, 2002). Most functional studies involve testing the ability of non-*Arabidopsis LFY* homologs to rescue *lfy* mutants. These heterologous rescues, described above, highlight that the ability of these other homologs to regulate the same genes is quite limited; only *LFY* homologs from other angiosperms were able to activate enough of the downstream MADS-box genes to produce normal flowers (Maizel et al., 2005). What the heterologous assays are unable to determine is what genes *LFY* homologs are regulating endogenously in non-flowering plants. While many of the MADS-box genes involved in flowering do not have homologs in non-flowering plants, B and C genes are found in gymnosperms, and other MADS-box genes are found throughout other non-flowering land plants (Cronk et al., 2002; Qiu et al., 2024; Thangavel and Nayar, 2018; Wang et al., 2010).

The sole study characterizing the genes endogenously regulated by LFY in a non-flowering plant focused on the gymnosperm *Welwitschia mirabilis* (Gnetales) and found that WelLFY can bind to upstream regions of the *Welwitschia* homologs of the B-class genes (Moyroud et al., 2017). While the ability of WelLFY to bind upstream of the C-class gene homolog was not investigated, *WelLFY* expression patterns occurred in the same tissues as *WelAG* expression, but at a slightly earlier stage, prompting the hypothesis that WelLFY may also be involved in regulating a C-class MADS-Box gene. Additionally, while binding success was not tested in other species, conserved binding sites were found upstream of B-class genes in four other gymnosperm species . These experiments provide strong support for the hypothesis that LFY regulation of MADS-box gene predates angiosperms and was coopted into its flowering role.

Nevertheless, the vast majority of evidence supporting the hypothesis that LFY homologs regulate MADS-box genes in other species comes from spatio-temporal comparative expression in gymnosperms. MADS-box genes are found in all land plants and are involved in a wide range of developmental processes (Qiu et al., 2024). Whether or not LFY homologs are able to regulate MADS-box genes in non-seed plants remains an important question that can be hard to address due to unclear orthologous relationships – for instance, are similar binding sites to those found in *Welwitschia* found in any of the bryophyte MADS-box genes and, if so, would a bryophyte LFY

be able to bind to those sites? Ultimately, the investigation of LFY's gene regulatory network in non-flowering plants will help address the degree of conservation of the relationships between this central transcription factor and the orthologs of other flowering genes in land plant evolution.

What's next for *LEAFY*? New frontiers in LFY research

While significant advances have been made in understanding the evolution and development of *LFY* function across the land plants, many unresolved questions remain. Further studies in emerging model plants representing additional lineages are vital, especially as technical advances enable a more thorough exploration of the molecular mechanisms driving *LFY's* mode of action. Whether *LFY* orthologs in other species also behave as pioneer transcription factors is a question worth pursuing to advance understanding of the evolution of this special category of transcription factors in plants.

The evolution of LFY's gene regulatory network is another key question; as described above, much remains unknown about interactions between LFY homologs and MADS box genes in non-seed plants. If LFY were unable to regulate the MADS-box genes of non-seed plants, this would suggest that there were changes to binding sites for these genes in the last common ancestor of the seed plants. Importantly, the regulatory role of LFY could have been coopted from an entirely different developmental process in non-seed plants, in which case there would be a different set of genes regulated by LFY in those species.

A fruitful avenue of investigation to explore the evolution of *LFY* function are domain-swap experiments. LEAFY's C-terminal DNA binding domain is more highly conserved than its N-terminal domain, suggesting that they are under different evolutionary constraints. Domain swap experiments have been performed where both the N- and C-terminal *Arabidopsis* LFY domains were replaced with *C. richardii* LFY domains and vice versa, suggesting that these domains are more critical for LFY function than the other regions of the protein (Maizel et al., 2005), but individual domain replacement remains untested.

Further experiments into *LFY* function in the gametophyte of *Arabidopsis* and of representatives of other plant lineages would enable the investigation of the degree of conservation of its cell division role in the haploid stage, as seen in the apical cell and lateral meristem of the fern gametophyte (McConnell et al., 2025; Plackett, Conway et ., 2018). Functional differences between the haploid and the diploid stage, as well as further evidence of co-option between life stages, would advance understanding of the evolution of this critical transcription factor, while adding insight into potential mechanisms and trajectories for other important transcription factors in plant development.

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LEAFY demonstrates ancestral reproductive functions in the gametophyte and not the sporophyte of the fern *Ceratopteris richardii*

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Abstract:
Flowers are a key reproductive innovation of the angiosperms. They evolved as a modification of the ancestral plant life cycle, whereby the haploid gamete-producing generation (gametophyte) became enclosed within the diploid, spore-producing generation (sporophyte). The transcription factor *LEAFY* (*LFY*) initiates angiosperm floral development, yet its lineage predates flowers and is found across all land plants. *LFY* function outside angiosperms is known from the moss *Physcomitrium patens*, where it controls the first cell division of the sporophyte, and from the model fern *Ceratopteris richardii*, a vascular plant without seeds or flowers where *CrLFY1* and *CrLFY2* maintain vegetative meristem activity. However, how *LFY'*s floral role evolved remains unclear. Using over-expression, we uncover new roles for *CrLFY1/2* in fern gametophyte reproduction, in sperm cells and in the gametophyte's multicellular notch meristem. While no sporophytic reproductive function was detected in terms of time to sporing, over-expression supports a role in frond compounding and a role in the zygote's first cell division. Our findings highlight an ancestral *LFY* function in fern haploid-stage reproduction, presumably co-opted into the sporophyte during the origin of the flower.

Introduction:
Flowers are a key innovation of angiosperms, the most recently diverging clade of land plants, that are largely credited with enabling one of the largest evolutionary radiations of all time (Berendse and Scheffer, 2009). The primary role of the transcription factor *LEAFY* (*LFY*) is as a flower meristem identity gene, with loss-of-function mutants producing leaflike structures instead of flowers (Blázquez et al., 1997; Carpenter and Coen, 1990; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1998; Weigel et al., 1992), and constitutive expression resulting in early flowering (Weigel and Nilsson, 1995). *LEAFY* also falls into a special gene class known as pioneer transcription factors, a few of which are known in plants, with roles in developmental reprogramming (Lai et al., 2018; Yamaguchi, 2021). In its pioneer transcription factor role, *LFY* can bind DNA as either a monomer or dimer in heterochromatic regions, activating the expression of downstream genes by directly binding to promoters in heterochromatic regions and through the recruitment of chromatin-remodeling genes (Jin et al., 2021; Winter et al., 2011).

Even though flower meristem identity is considered *LFY's* canonical role, its homologs are found across land plants, including those without flowers (Moyroud et al., 2010; Sayou et al., 2014),

suggesting that its floral function evolved from a pre-existing ancestral role. *LEAFY* can also be active in vegetative shoot apical meristems of certain angiosperms (Kelly et al., 1995); (Wang et al., 2008); (Zhao et al., 2017); (Shu et al., 2000); (Moriyama et al., 2024); (Souer et al., 1998) and gymnosperms (Mellerowicz et al., 1998; Mouradov et al., 1998; Shindo et al., 2001), the angiosperm sister group that produces seeds without flowers. Other functions for *LFY* homologs include regulating axillary meristems in rice (Rao et al., 2008), and compound leaf development in several angiosperms (Busch and Gleissberg, 2003; Champagne et al., 2007; He et al., 2020; Hofer et al., 1997; Jiao et al., 2019; Wang et al., . In the model fern *Ceratopteris richardii* Brongn. (*C. richardii*)*,* expression of a *LFY* paralog was found in developing fronds, and RNAi-mediated knockdown of its two *LFY* paralogs, *CrLFY1/2*, identified roles in maintaining the vegetative meristem of the diploid sporophyte, in embryo development, and the apical cell (stem cell) of early-stage haploid gametophytes. Those results suggest that *LFY*'s vegetative meristem function was present in the most recent common ancestor (MRCA) of ferns and seed plants (Plackett, Conway et al., 2018). In the moss *Physcomitrium patens*, a bryophyte, expression of two *PpLFY* paralogs is found in the apical cell of the gametophyte, and their disruption results in the zygote failing to divide into a multicellular embryo, indicating that one or both paralogs are necessary for early sporophyte development (Tanahashi et al., 2005) *LFY*'s involvement in regulating vegetative cell divisions across land plants supports the hypothesis that this function is ancestral, whereas its role in floral meristem identity is derived. However, there is also evidence in support of the role of *LFY* homologs in the reproduction of non-flowering plants. In seedless vascular plants, *LFY* expression has been detected in both vegetative and reproductive organs of the sporophyte (Himi et al., 2001; Rodríguez- et al., 2022; Yang et al., 2017), and expression was also seen in reproductive structures (cones) of four conifer genera (Carlsbecker et al., 2013, 2004; Mellerowicz et al., 1998; Mouradov et al., 1998; Vázquez-Lobo et al., 2007). Whether *LFY* also has reproductive functions in *C. richardii* remains unclear, because transgenic knockdown of expression resulted in early termination phenotypes during vegetative development (Plackett, Conway et al., 2018). When heterologously expressed in an *Arabidopsis lfy* loss-of-function mutant, a gymnosperm *LFY* ortholog, and the *C. richardii* paralog *CrLFY2* conferred a partial rescue of flower development, where some but not all floral whorls developed, while a moss version did not (Maizel et al., 2005). Although these experiments raise the hypothesis that *LFY*-dependent floral gene networks could have been co-opted from an ancestral *LFY*-dependent network still present in extant ferns, this remains to be tested since the native binding targets of CrLFY2 are not known.

The evolutionary history of *LFY*'s dual vegetative and reproductive meristematic roles thus presents a compelling question, particularly regarding the emergence of its highly specialized function in floral meristem development, motivating further research as the ability to perform functional studies continues to expand beyond seed plants (Di Stilio and Sinha, 2024). However, few *in planta* functional studies have investigated the potential reproductive role of *LFY* homologs outside of the angiosperms. Ferns are representative of seedless vascular plants, sister

to seed plants, and thus an excellent bridge group, with the homosporous fern *C. richardii* representing an effective model (Hickok et al., 1995, 1987; Hickok and Warne, 1998; Plackett et al., 2015; Renzaglia and Warne, 1995). In *Ceratopteris*, reproductive processes ontogenically equivalent to those of angiosperms occur in both the diploid shoot (sporophyte) and the haploid thallus (gametophyte). In the sporophyte the shoot apex transitions from producing vegetative fronds to sporangium-bearing fronds (sporophylls), and these organs subsequently generate the haploid generation by meiosis, in the form of single-celled spores. Sexual reproduction occurs in the free-living multicellular haploid organism germinating from these spores, the thalloid gametophyte generating gametes (eggs and free-swimming sperm) in specialized organs (archegonia and antheridia) (Conway and Di Stilio, 2020). These gametangia arise from the activity of a 'notch' meristem - archegonia exclusively so (Banks, 1999). Typically described as a vegetative meristem due to its role in generating prothallus tissue, it has recently been shown that archegonia are specified behind the meristem in a position-dependent, cell lineage-independent manner (Geng, 2022), arguing that this meristem has reproductive functions analogous to the angiosperm inflorescence meristem. Unlike angiosperms, with their determinate microscopic, short-lived gametophytes, or bryophytes like *P. patens,* where the diploid sporophyte is short-lived, ferns exhibit free-living macroscopic and indeterminate haploid and diploid phases, making investigations into gene functions in both life stages feasible.

To test whether *LFY* exhibits reproductive activity outside of the seed plants, i.e. in the development of gametangia and sporangia in either the haploid or diploid stage, we characterized transgenic plants constitutively expressing *CrLFY1* and *CrLFY2* during the development of the fern *C. richardii*, bypassing the issue of developmental arrest at the early gametophyte stage caused by RNAi-mediated gene silencing (Plackett, Conway et al., 2018). Single and double over-expressors allowed us to investigate the possibility of sub- or neo-functionalization between the paralogs. On the one hand, our findings do not support the hypothesis of *CrLFY* playing a direct role in reproduction in the sporophyte phase (sporing), as expected from its known angiosperm role. On the other hand, we show new evidence of a reproductive role for *CrLFY* in the gametophyte phase, via its effect on the notch meristem that produces gametangia, and a function in sperm release from antheridia. We also generated new functional evidence supporting a conserved role in the first division of the zygote, as previously described in moss (Tanahashi et al., 2005), with our data further suggesting that *CrLFY*'s expression must be spatio-temporally constrained to enable progression through the zygotic stage. Finally, our study further supports *CrLFY*'s role in the regulation of compound leaf development. Taken together, our findings provide the first evidence of a reproductive role for *LFY* in the gametophyte of the MRCA of ferns and angiosperms, but not in the sporophyte, supporting the hypothesis of evolutionary co-option of gene networks from this haploid phase to floral meristem identity in sporophytes as the latter became the dominant phase in angiosperms.

Results:

Fern *LEAFY* misexpression affects frond development but not the sporing transition in sporophytes

Given that spore production in ferns can be considered analogous to flowering in angiosperms, and that *LFY* overexpression is known to advance flowering, we investigated the hypothesis that *CrLFY* overexpression advances sporing in *C. richardii*. To that end, we generated *35S::CrLFY1*, *35S::CrLFY2* and *35S::CrLFY1+2* transgenic lines and confirmed the presence of the constructs (Fig. S1A-B) and overexpression in the leaves (from here on 'fronds') of transgenic ferns just before the emergence of sporophylls, the site of meiosis and sporogenesis (Fig. S1C-H). Compared to wild type, *35S::CrLFY1* sporophytes had 4.3- to 4.9-fold more expression of *CrLFY1 (*Fig S1C), gametophytes had 2.6-3.7-fold more expression (Fig. S1D), *35S::CrLFY2* sporophytes had 3.5- to 31-fold more expression of *CrLFY2* (Fig. S1E)*,* gametophytes had 1.8-4.1 fold more expression (Fig. S1F) and the double had approximately a five-fold expression of both paralogs (Fig. S1G-H). Although there was high variation in expression amongst transgenic plants, this variation did not directly correlate with phenotype. The number of inserts per transgenic line ranged from 2-12 (Fig. S2).

The time to reproductive transition did not vary between wild-type and transgenic genotypes, i.e., *CrLFY* over-expression did not accelerate sporing in either number of vegetative fronds produced prior to sporing or days post fertilization to sporing (Fig. 1A-B, n=20, p=0.72 or p=0.58 two-way ANOVA). Transgenic plants occasionally produced an unusual number of fronds, either very few or many early in development (Fig. 1A), but on average have the same number of vegetative fronds prior to sporophyll production. During sporophyll development the number of sporangia produced per square cm was not different between wild-type and transgenic fronds (Fig. S3A, n=5-10, p=0.44, two-way ANOVA), nor was the length of sporangia-producing pinnae (Fig. S3B, n=10, p=0.99, two-way ANOVA). The germination rate of transgenic spores was lower at first, presumably due to the effect of antibiotics in the selective media, but caught up with wild type by 180-200 h (Fig. S3C, n=3 (replicate plates, 50 spores each), p=0.46, two-way ANOVA). Thus, there was no advancement in the sporing transition of ferns over-expressing *CrLFY*, contrary to predictions extrapolated from *LFY's* angiosperm role.

Like many ferns, wild-type *C. richardii* develops compound fronds that become increasingly dissected from simple, lobed, pinnate, bipinnate, to tripinnate (Fig. 1C, frond silhouettes). Reproductive fronds (sporophylls) are bi- or tri-pinnate and produce sporangia, the site of meiosis and sporogenesis (Conway and Di Stilio, 2020), Fig. 1C). *LFY* orthologs have been shown to play a role in *C. richardii* frond development (Plackett, Conway et al., 2018) and leaf compounding in legumes (Hofer et al., 1997). In our experiment, the sporophylls of *35S::CrLFY1* and *35S::CrLFY2* plants ranged from simple to tri-pinnate (Fig. 1C, E-F), with no

statistically significant difference from wild-type ratios (Fig. 1C, D, n=20-50, p=0.58, chi-square). In contrast, sporophytes overexpressing both *CrLFY* paralogs had sporophylls that were pinnate or bi-pinnate, but never tri-pinnate, and this was statistically significantly different from wild-type expectations (Fig. 1C, G, n=30, p<0.001, chi-square). Thus, fronds from double over-expressors displayed abnormal development, rather than the expected increased level of dissection from the described role of *LFY* orthologs in compound leaves of certain angiosperms.

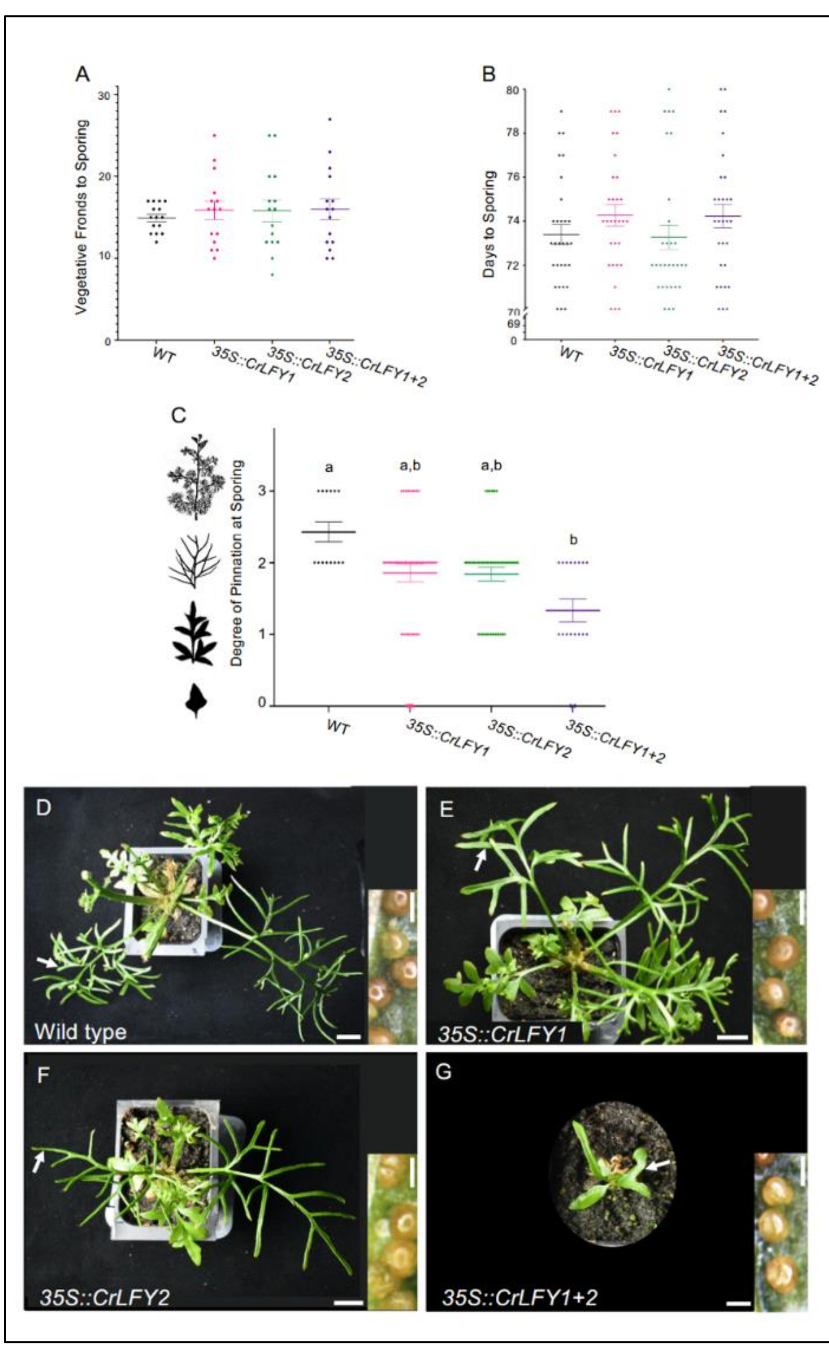

Figure 1: *LFY* orthologs affect frond development but not reproductive transition in *Ceratopteris richardii* sporophytes. Overexpression of *CrLFY1/2* does not change the time to sporing in sporophytes, resulting instead in abnormal development and simpler, less dissected sporophylls (spore-producing fronds) than wild-type at maturity. (A) Number of vegetative fronds produced before sporing. (B) Days to sporing for wild-type (WT) and transgenic plants (n=30, p=0.72, two-way ANOVA). (C) The degree of pinnation at sporing for WT and transgenic fronds: 0=simple or lobed, 1=pinnate, 2=bi-pinnate, and 3=tri-pinnate, with representative frond silhouettes shown on the side (not to scale). Different letters denote a statistically significant difference (n=30, p<0.001, chi-square test). Mean ± standard error of the mean (SEM) shown. (D-G) Sporophyll pinnation 72 days after fertilization (DAF). (D) WT plant producing sporangia on the abaxial side of bi-and tri-pinnate sporophylls. (E) *35S::CrLFY1* and (F) *35S::CrLFY2* plants producing sporangia on pinnate and bi-pinnate fronds. (G) *35S::CrLFY1+2* plant producing sporangia on pinnate fronds. White arrows mark the sites where sporangia were photographed. Scale bars=1 cm in main panels, or 500 µm in insets.

Misexpression of a fern *LEAFY* ortholog alters meristem size in the haploid gametophyte.

Given the established role of *CrLFY* in the apical cell (a unicellular meristem) of the fern haploid stage (gametophyte), we tested the hypothesis that it may also be involved in regulating the multicellular "notch" meristem of hermaphroditic gametophytes (whereas male gametophytes are lacking multicellular meristems (Banks, 1997)). At 13 days post-sowing (dps) notch meristems of *35S::CrLFY2* and *35S::CrLFY1+2* sexually mature gametophytes both contained, on average, 5 additional meristematic cells compared to wild-type (Fig. 2A-B, D-F, n=10, p<0.001). In contrast, notch meristems of *35S::CrLFY1* gametophytes were not significantly different from wild-type (Fig. 2A-C, F, n=10, p=0.51), and native *CrLFY1* expression was not reported in the notch meristem of the previously-published $CrLFY1_{pro}$*::GUS* transgenic reporter line (Fig. S4; (Plackett, Conway et al., 2018)). Additionally, *35S::CrLFY1+2* gametophytes had on average 50.7% larger thalli compared to wild-type, or gametophytes of the same age overexpressing either one of the *CrLFY1/2* paralogs (Fig. 2G, n=10, p<0.001). To test for native *CrLFY* function in the notch, meristem cell numbers were also quantified in *CrLFY*-RNAi lines at a comparable developmental stage, previously shown to knock down both paralogs (Plackett, Conway et al., 2018). One out of seven independent transgenic lines analyzed showed a statistically significant decrease in meristem cell number (Fig. S5, n=5, p<0.01); while there were highly variable expression levels between independent transgenic lines, this particular line was consistently knocked down.

Because archegonia (egg-bearing organs) arise from the notch meristem, we quantified these and antheridia (sperm-bearing organs, which arise from mother cells at the periphery of the notch meristem (Banks, 1999) in *35S::CrLFY* hermaphrodites at 15 dps and 20 dps respectively, when notch-dependent processes could be expected to be even more affected. The average number of

archegonia did not vary between genotypes (Fig. 2H, *n*=30, *p*=0.13, two-way ANOVA) but interestingly, a small proportion of *35S::CrLFY2* gametophytes (3/40 and 1/40, from two independent transgenic lines) failed to develop any archegonia, which was never observed in WT (0/80). Wild-type and transgenic plants did not differ in the number of antheridia either, and all gametophytes produced more antheridia in the absence of fertilization, regardless of genotype (Fig. 2I, *n*=20, *p*=0.56, one-way ANOVA). Together, these results represent the first evidence for a role of *CrLFY* in the multicellular notch meristem of *C. richardii* gametophytes, while also suggesting functional differentiation between the two paralogs.

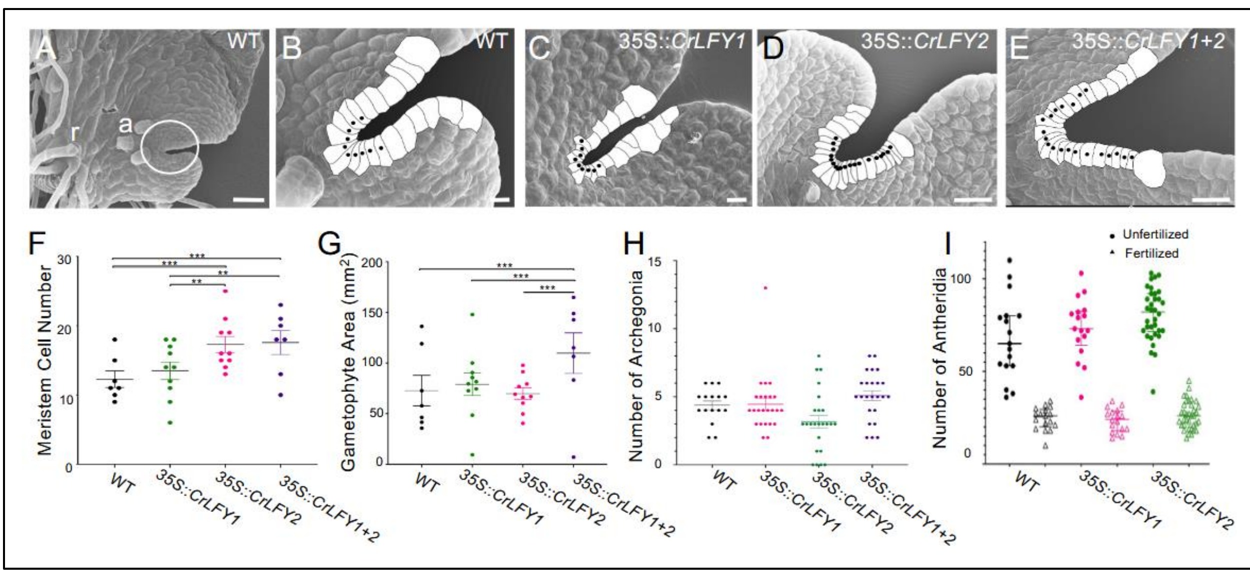

Figure 2: Misexpression of a LEAFY ortholog alters the multicellular meristem of the fern haploid stage. *Ceratopteris richardii* gametophytes overexpressing *CrLFY2,* or both paralogs, produce more meristematic cells and are bigger, but with normal amounts of gametangia (antheridia and archegonia). (A-E) Scanning electron microscopy images before maturity (stage Gh6, (Conway and Di Stilio, 2020) with notch area cells traced and filled in white, and black dots marking the presumed meristematic cells (with a length-to-width aspect ratio greater than 2:1) (A) WT gametophyte, whole thallus (body) showing the notch area, circled and magnified in (B), three archegonia (a), and rhizoids (r). Notch meristem of representative transgenic gametophytes: (C) 35S::*CrLFY1*; (D) 35S::*CrLFY2*; and (E) 35S::*CrLFY1+2*. Scale bars = 20 µm (A-C), 50 µm (D-E). (F) The number of meristematic cells in WT and transgenic gametophytes at 13 days post-sowing (dps, *n*=10, *** = *p*<0.001, two-way ANOVA). (G) Gametophyte surface area in square mm (*n*=10, *** = *p*<0.001, two-way ANOVA). (H) Number of archegonia in mature (15 dps) gametophytes (*n*=30, *p*=0.32, two-way ANOVA). (I) Number of antheridia at 20 dps in gametophytes that were either flooded at 15 dps or kept from fertilizing (*n*=20, *p*=0.90, one-way ANOVA). Mean ± s.e.m. shown.

CrLFYs are expressed in fern sperm cells.

CrLFY1/2 expression had been previously detected in pooled gametophytes without distinction between the sexes (Plackett, Conway et al., 2018). Here, we assessed the sex-specific expression of *CrLFY* in male and hermaphroditic gametophytes before and after sexual maturity. Both paralogs were significantly upregulated in sexually mature males at 14 dps (Fig. 3E), compared to immature males starting to undergo spermatogenesis at 8 dps (Fig. 3C), by 3-fold for *CrLFY1* and by 7-fold for *CrFLY2* (Fig. 3A-B, n=3, p<0.001, two-way ANOVA). Mature males were also significantly upregulated for *CrLFY* compared to immature hermaphrodites (Fig. 3D), by 6.8-fold for *CrLFY1* and by 7-fold for *CrLFY2*, and to mature hermaphrodites (Fig. 3F), by 12-fold for *CrLFY1* and by 27-fold for *CrLFY2* (Fig. 3A-B, n=3, p<0.001, two-way ANOVA). Hermaphrodites produce both archegonia and antheridia while males produce only antheridia, in high numbers. Thus, increased expression coincides with the increased number of antheridia containing fully developed sperm in mature male gametophytes. For *CrLFY1*, this result was supported by GUS localization specifically inside the sperm cells of the $CrLFY1_{pro}$*::GUS* transgenic reporter line (Plackett, Conway et al., 2018) in both hermaphrodite (Fig. 3G) and male gametophytes (Fig. 3H). GUS expression was also detected in the apical cell of germinating spores carrying this reporter gene (Fig. S4), consistent with previously published *in situ* localization data (Plackett, Conway et al. 2018). Compared to wild-type controls (Fig. 3I-K), GUS staining was found in antheridia undergoing spermatogenesis (Fig. 3L), and in mature sperm (Fig. 3M), not in antheridia after sperm release (Fig 3N). Compared to wild-type controls (Fig. 3O, two archegonia shown), GUS activity localized briefly to initiating archegonia (Fig. 3P, archegonium 1) and was not detectable throughout subsequent stages of archegonium development (Fig. 3P, archegonia 2 and 3).

Fern _LFY_ influences sperm release from antheridia

Because *CrLFY* is expressed in *C. richardii* sperm, we evaluated its putative role in sperm development and/or function. We investigated sperm performance parameters in *35S::CrLFY* transgenic gametophytes compared to wild type. There was no difference in the total number of sperm cells produced per gametophyte (Fig. 3Q, n=3, p=0.64, two-way ANOVA), in sperm viability (Fig. 3R, n=3, p=0.11, two-way ANOVA), or sperm swimming speed (Fig. 3S, n=25, p=0.41, two-way ANOVA) between any transgenic line and wild-type. Thus, *CrLFY* overexpression does not appear to influence sperm development or function.

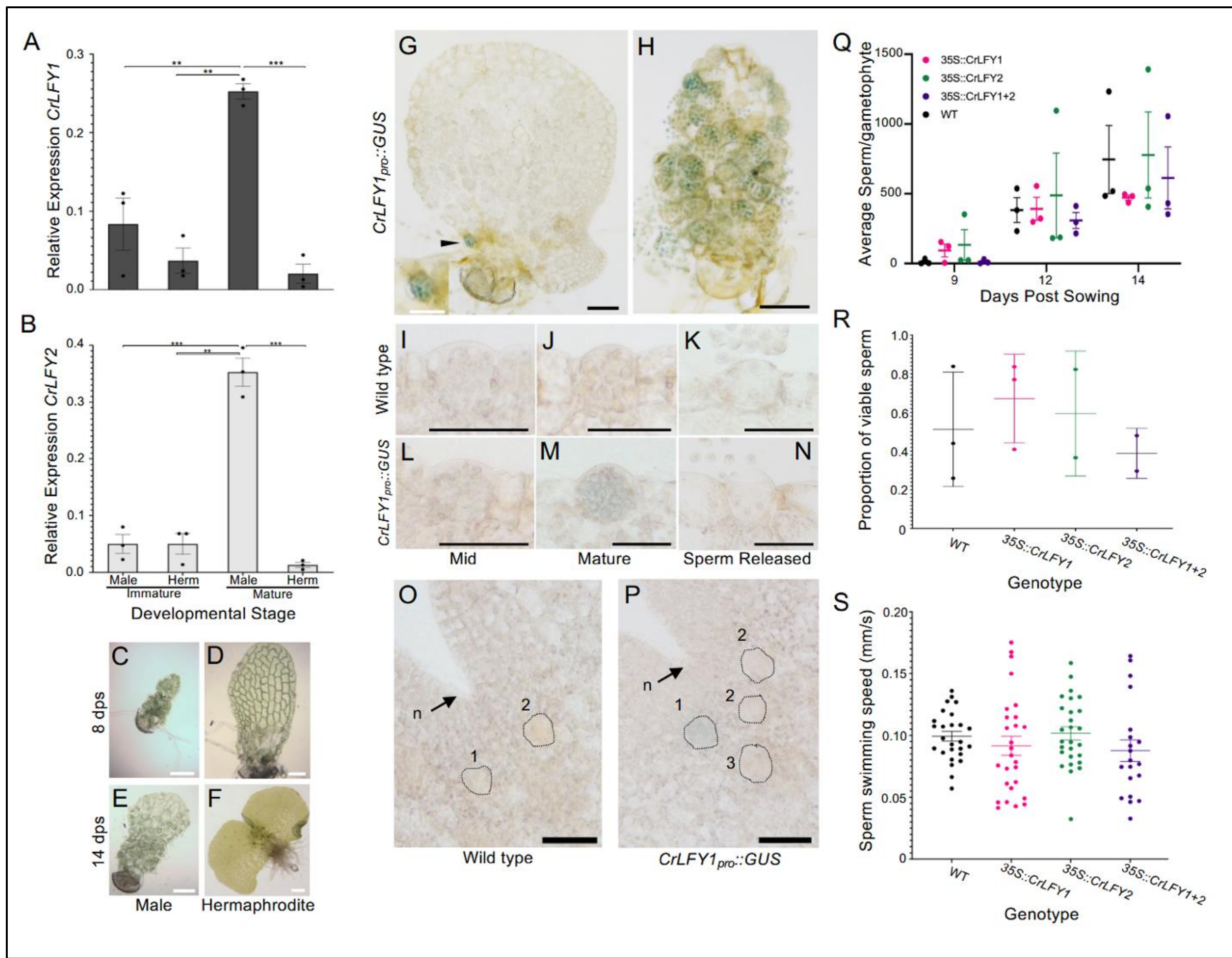

Figure 3: Fern *LFY* paralogs show high expression in mature sperm without a noticeable effect on sperm function when overexpressed. (A-B) Expression of two *C. richardii LFY* paralogs by qPCR (relative to the housekeeping genes *CrACT1* and *CrTBPb*) in gametophytes by sex (male and hermaphrodite, herm) and developmental stage (sexually immature and just starting to undergo spermatogenesis at 8 dps, mature at 15 dps). (A) *CrLFY1* and (B) *CrLFY2* (n=3, **=p<0.01, *** = p<0.001, two-way ANOVA). Representative gametophyte images for the developmental stages used in qPCR: (C) Immature wild-type (WT) male gametophyte at 8 dps, with few immature antheridia. (D) Immature WT hermaphrodite gametophyte at 8 dps. (E) Mature WT male gametophyte at 14 dps, covered in mature antheridia. (F) Mature WT hermaphrodite gametophyte at 14 dps, with antheridia and archegonia. Scale bar=100 µm. (G, H) *CrLFY1$_{pro}$::GUS* expression (blue) in (G) 9-day-old hermaphrodite and (H) male gametophytes. Scale bar=100 µm, or 50 µm (inset). (I-K) Developing antheridia in WT and (L-N) *CrLFY1$_{pro}$::GUS* transgenic gametophytes undergoing spermatogenesis (I, L), with mature sperm (J, M), and after sperm release (K, N). Scale bar=20 µm (O) Archegonia developmental series from the notch meristem (n) in WT and (P) *CrLFY1$_{pro}$::GUS* gametophytes:(1) initiation, (2) development, and (3) fully mature. Scale bar=50 µm. (Q) The average number of sperm cells

released from pools of 100 gametophytes at 9, 12, and 14 dps for WT and transgenic gametophytes (*n*=3, *p*=0.6432, two-way ANOVA). (R) The proportion of viable sperm (with propidium iodide as a viability stain) in WT and transgenic gametophytes *(n*=3 reps, 470 to 11140 sperm analyzed per sample, *p*=0.51, mixed-effects binomial). (S) The swimming speed of sperm (in mm/s) for WT and transgenic gametophytes (*n*=25, *p*=0.4126, two-way ANOVA). Mean ± standard error of the mean (SEM) shown.

In order to investigate a potential role of *CrLFY* in sperm development, we tested whether reduced *CrLFY* expression in *CrLFY*-RNAi (Z*mUbipro::CrLFY1/2-i3)* transgenic lines (Plackett, Conway et al., 2018) caused a sperm phenotype. We identified a significant decrease in the number of sperm released per gametophyte in *CrLFY*-RNAi gametophytes compared to wild-type plants at three different developmental timepoints (Fig. 4A, n=3, *p*<0.0001, two-way ANOVA). *CrLFY*-RNAi gametophytes still contained sperm within antheridia one day post flooding (dpf) in both hermaphrodites (Fig. 4B) and males (Fig. 4C), while wild-type sperm had been released from both sexes (Fig. 4D-E). These results are consistent with *CrLFY* being required either for maturation of the sperm themselves or the surrounding antheridium.

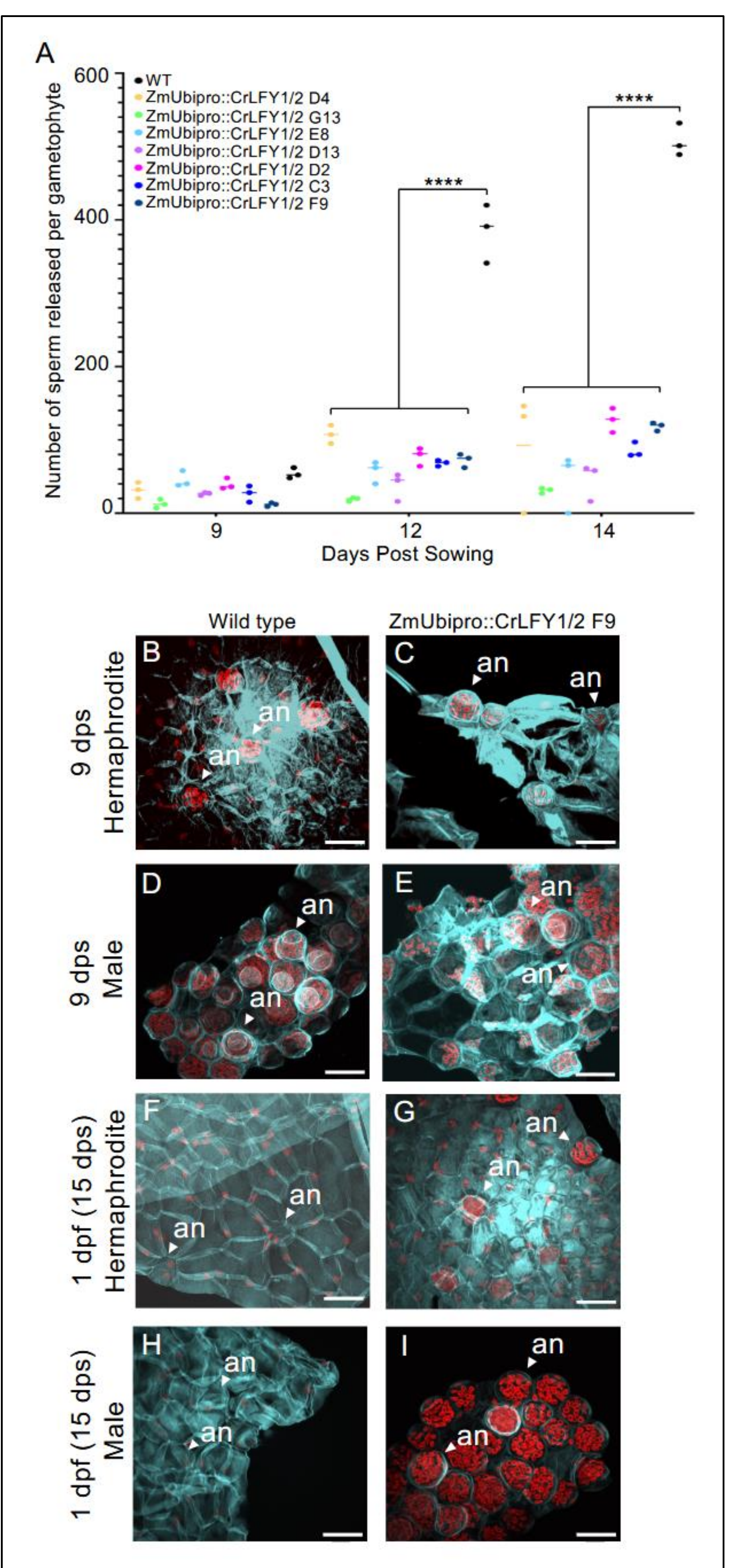
A
Number of sperm released per gametophyte
600
400
200
0
WT
ZmUbipro::CrLFY1/2 D4
ZmUbipro::CrLFY1/2 G13
ZmUbipro::CrLFY1/2 E8
ZmUbipro::CrLFY1/2 D13
ZmUbipro::CrLFY1/2 D2
ZmUbipro::CrLFY1/2 C3
ZmUbipro::CrLFY1/2 F9
****
****
9
12
14
Days Post Sowing
Wild type
ZmUbipro::CrLFY1/2 F9
9 dps Hermaphrodite
9 dps Male
1 dpf (15 dps) Hermaphrodite
1 dpf (15 dps) Male
B
C
D
E
F
G
H
I
an

Figure 4: Gametophytes with reduced *CrLFY* expression fail to release sperm from antheridia. (A) The average number of sperm cells released from pools of 100 gametophytes at 9 (immature), 12 and 14 (mature) day post sowing (dps) for wild-type (WT) and seven independent *CrLFY*-RNAi transgenic lines (transgenic line naming as in Plackett, Conway et al, 2018) (n=3, ****= p<0.0001, two-way ANOVA). (B-I) Partial Z-stacks from confocal images of representative WT and *CrLFY*-RNAi gametophytes at 9 dps; WT (B) and RNAi (C) hermaphrodite, WT (D) and RNAi (E) male. (F-I) Gametophytes one day post flooding (dpf); WT (F) and RNAi (G) hermaphrodite, WT (H) and RNAi (I) male. All samples stained with SR220 (light blue, marking the cell wall) and propidium iodide (red, marking the nuclei), showing multiple antheridia (an). Scale bars = 50 μm.

Misexpression of *C. richardii LEAFY* orthologs disrupts embryo development at the zygote stage.

To further investigate the potential role of *CrLFY* in sperm development, we set up controlled fertilization assays in wild-type and *35S::CrLFY1*, *35S::CrLFY2,* and *35S::CrLFY1+2* transgenic lines and recorded fertilization success as the emergence of visible embryos. Hermaphroditic gametophytes were isolated and flooded with sperm of the same genotype and followed for 14 dpf; by 10 dpf, 80% of wild-type gametophytes contained visible embryos, and by 14 dpf all showed visible signs of embryo development (Fig. 5A). Transgenic gametophytes for all three constructs had significantly fewer embryos from 10 dpf onwards compared to wild-type, with 36% of *35S::CrLFY1*, 31% for *35S::CrLFY2* and 30% of *35S::CrLFY1+2* gametophytes containing visible embryos 10 dpf (Fig. 5A and Table S2, n=36, p<0.01, two-way ANOVA). This result suggests that *CrLFY* overexpression disrupts either fertilization or post-fertilization processes.

To further determine whether the decrease in visible embryos was due to a maternal or a paternal effect, isolated individual wild-type hermaphroditic gametophytes were flooded with transgenic sperm from each of the three genotypes, and the reciprocal experiment consisted of flooding transgenic gametophytes with wild-type sperm. In crosses where wild-type hermaphrodites were flooded with transgenic sperm, there was a significant reduction in the proportion of gametophytes with embryos after 10 dpf compared to the wild-type x wild-type control (Fig. 5B and Table S2, n=36, p<0.01, two-way ANOVA), with 46% of *35S::CrLFY1*, 48% of *35S::CrLFY2* and 44% of *35S::CrLFY1+2* hermaphrodite gametophytes containing visible embryos 10 dpf. In contrast, when transgenic gametophytes were flooded with wild-type sperm, there was a significant reduction in the proportion of gametophytes with embryos between 10-13 dpf (n=36, p<0.01, two-way ANOVA), but by day 14 they experienced a rescue, whereby the proportion of visible embryos was no longer significantly different from wild-type (Fig. 5C and Table S2, n=36, p=0.06, two-way ANOVA). Additionally, the proportion of transgenic gametophytes fertilized with wild-type sperm containing visible embryos was significantly

higher between 10-13 dpf compared to those fertilized with transgenic sperm, whether they were wild-type or transgenic (Supplemental Table 2), with 56% for *35S::CrLFY1* sperm, 57% for *35S::CrLFY2* sperm and 56% for *35S::CrLFY1+2* sperm (n=36, p<0.05, two-way ANOVA). Thus, the reduced proportion of embryos developing from transgenic fertilizations, along with the partial rescue by wild-type sperm, suggest that *CrLFY* misexpression affects early sporophyte development through paternal effects.

To investigate the mechanism underlying the decrease in visible embryos under *CrFLY* misexpression, we examined early embryo development in the archegonia of wild-type and transgenic gametophytes after flooding. Wild-type mature gametophytes produced archegonia with neck cells and a venter containing a ventral canal cell and an egg cell (Fig. 5D-E, Video 1), as expected from prior descriptions (Lopez-Smith and Renzaglia, 2008), and *35S::CrLFY1+2* gametophytes showed similar morphology to these controls (Fig. 5H-I). After flooding, fertilized wild-type gametophytes produce a zygote inside the venter, morphologically distinguishable from the egg cell by the increased cell size, taking up the whole ventral canal space (Lopez-Smith and Renzaglia, 2008). Based on these guidelines, we observed that sperm successfully entered the cytoplasm of both wild-type and transgenic eggs by 2 h post flooding (Fig. 5F, J, Video 2). Within 16 hours, 88% of the wild-type gametophytes (22 out of 25) contained a multicellular embryo (Table 1, Fig. 5G, Video 3) and the rest contained an unfertilized egg cell (Video 4), while only 60% of double transgenic gametophytes (*35S::CrLFY1+2*) contained embryos (15 out of 25) and the rest of the transgenic gametophytes exhibited a single cell with an elongated, more diffuse nucleus that we interpret as a zygote arrested before the first cell division and/or not having undergone proper syngamy (Table 1, Fig. 5J-K, Table 1, Video 5). Occasionally, a wild-type gametophyte with an embryo also contained an arrested zygote from a second fertilization event in another archegonium (Fig. S6), and these had a single condensed nucleus. In gametophytes where *CrLFY1 and -2* were downregulated by RNAi, 84% contained a multicellular embryo 16 hours after fertilization, 8% had not been fertilized and still contained an egg cell and 8% had been fertilized but their zygotes appeared to be arrested (Table 1), and their nuclei were also spherical as in wild type (Fig. S7), rather than the elongated nuclei of zygotes over expressing *CrLFY*.

CrLFY1$_{pro}$::GUS expression was previously reported in the *Ceratopteris* embryo as early as the octant stage (Plackett, Conway et al., 2018) but earlier stages were not reported. Hence, we investigated the earliest timepoint after fertilization where native *CrLFY1* expression is present in this reporter line. We could not detect *CrLFY1$_{pro}$::GUS* staining in the zygote; GUS staining was first visible in the 2-4 cell embryo (Fig. S8). These expression results during early embryo development, together with the decrease in embryos when over expressing *CrLFY* (Fig. 5A-C), suggest that *CrLFY*'s spatio-temporal expression requires precise regulation during zygote development. Misexpression of either *CrLFY* paralog in the zygote may prevent progression to

the first cell division (by an unknown mechanism), causing developmental arrest of the young sporophyte.

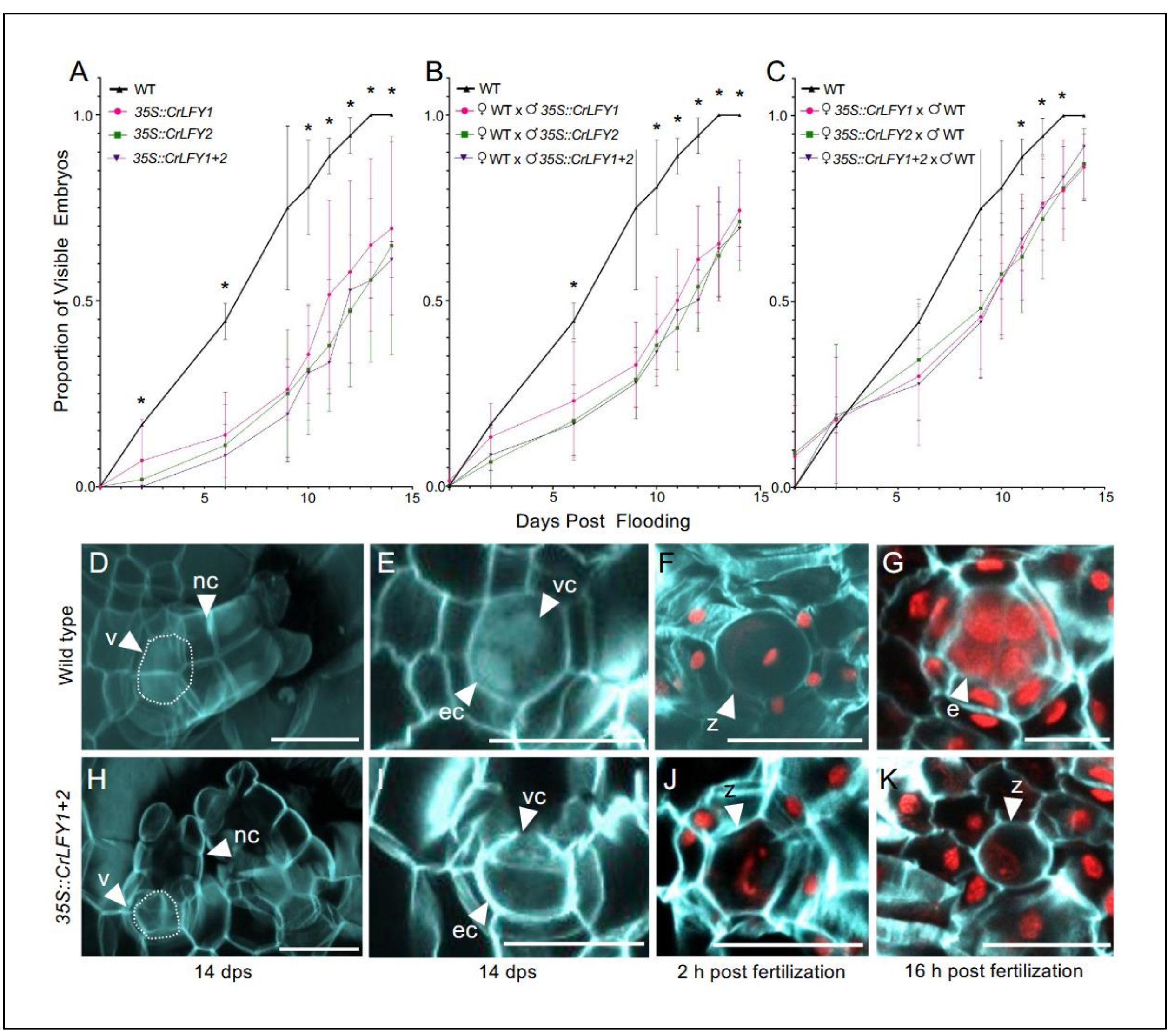

Figure 5: Misexpression of fern LEAFY homologs disrupts early development of the *C. richardii* embryo. In controlled fertilization assays, overexpression of fern *LEAFY* orthologs *CrLFY1/2* prevents zygotes from undergoing cell division and developing into multicellular embryos. Time series of the proportion of gametophytes producing visible sporophytes after flooding (up to 14 dpf) with water containing sperm from the same genotype (A) or outcrossed (B-C). Mean ± s.e.m. shown. (n=36, *=p<0.05 or less, see Supplemental Table 2 for individual p-values). Partial Z-stacks from confocal images comprising the surface of an archegonium in WT (D) and *35S::CrLFY1+2* gametophytes before fertilization (H), stained with SR220 (light blue, cell wall) and Hoechst (light blue, nucleus), denoting the neck canal (nc) and venter (v) cells. White tracing indicates the location of the egg and ventral canal walls, not visible in the

partial stack. A single slice taken from the same Z-stack, further zoomed in, denoting the ventral canal (vc) and egg cell (ec, white arrowheads) at the center of the venter, for WT (E) and transgenic gametophytes (I). A zygote (z) is shown at the center of the archegonial venter for WT (F) and transgenic gametophytes (J-K) one day after flooding, stained with SR2200 and Propidium iodide (red nuclei). A WT multicellular embryo (e) developing inside the archegonial venter one day after flooding (G). Scale bar=20 µm.

Genotype	Fertilization Outcomes		
	Eggs	Zygotes	Multicellular Embryos
WT	0.12	0	0.88
35S::CrLFY1+2	0	0.4	0.6
ZmUbipro::CrLFY1-i3	0.08	0.08	0.84

Table 1: Fertilization outcomes in wild type (WT) and transgenic plants misexpressing *CrLFY*. The proportion of eggs, zygotes and multicellular embryos in gametophytes 16 h post fertilization (n=25) is shown for wild type and transgenic plants over expressing the two fern *CrLFY* paralogs (*35S::CrLFY1+2*) or downregulated for both via RNAi targeted gene silencing (*ZmUbipro::CrLFY1/2-i3*). Note that arrested zygotes are found in over expressing and RNAi transgenic lines, not in wild type.

Discussion:

As the closest living relatives of seed plants, ferns provide a critical link for understanding *LFY* function. Investigating *C. richardii* bridges the phylogenetic gap between angiosperms and bryophytes, offering a framework to trace the evolutionary trajectory of this master regulator of plant development. Here, we asked whether *LEAFY's* sporophytic reproductive function in the angiosperms specifying floral meristem identity (Carpenter and Coen, 1990; Schultz and Haughn, 1991; Weigel et al., 1992) arose from an ancestral vegetative shoot meristem role, or an ancestral reproductive function (in gametogenesis or sporogenesis) predating the angiosperms.

Consistent with prior research indicating that at least one of the *CrLFYs* is necessary to maintain apical meristems in the fern sporophyte (Plackett, Conway et al., 2018), our overexpression

analysis shows that both *CrLFYs* together regulate frond dissection in the sporophyll. Transgenic plants overexpressing both *CrLFYs* developed less dissected fronds, with fewer pinnae and pinnules (the smallest subdivided segment of a frond), which arise from pinnae initial cells (Hill, 2001), suggesting that *CrLFY* are involved in regulating these apical (stem) cells and that their misexpression inhibits pinna initial identity or activity. Given that *LFY* is found mostly as a single copy gene, and that it can function as both a monomer and a dimer in *Arabidopsis* (Winter et al., 2011), it is unclear at this point whether the compound leaf phenotype is due to an additive effect of increased total *CrLFY* expression or to a regulatory interaction between the two paralogs that would suggest sub- or neo-functionalization. Interestingly, decreased leaf compounding in plants overexpressing *CrLFY* matches that of plants where *CrLFY* was downregulated by RNAi (Plackett, Conway et al., 2018), contrary to the predicted increase in dissection based on *CrLFY's* role in promoting leaf apical cell divisions. This unexpected result suggests that the overall spatial and temporal *CrLFY* expression pattern is more critical than the total expression level in determining pinnae and pinnule outgrowth. Moreover, proper maintenance of pinnae initial identity would require the absence of *CrLFY* expression in surrounding cells for leaf development to proceed normally, consistent with GUS expression patterns reported previously for *CrLFY1* (Plackett, Conway et al., 2018). Alternatively, it is also possible that *CrLFY* affects leaf development in a way other than by regulating cell division at pinnae initials. Several angiosperm *LFY* homologs promote compound leaf development, e.g., in legumes (Champagne et al., 2007; He et al., 2020; Hofer et al., 1997; Jiao et al., 2019; Wang et al., 2025); and this role in marginal meristems leading to compound leaves may have arisen separately in ferns and legumes, given the growing consensus that fronds have evolved independently from seed plant leaves (Tsuda, 2024). However, there is also evidence of *LFY* homologs regulating compound leaf development in California poppy, an early-diverging eudicot, (Busch and Gleissberg, 2003), which together with our findings, suggests the alternative hypothesis that this function could represent a deep homology present in the ancestor of ferns and seed plants.

Given that overexpression of *LFY* accelerates flowering in *Arabidopsis* (Weigel et al., 1992), the lack of acceleration of the sporing transition in our transgenic plants was unexpected. However, an increase in *CrLFY* expression from vegetative fronds to sporophylls is not naturally found in *C. richardii* (Plackett, Conway et al., 2018) and, consistent with this evidence, *CrLFY* overexpression did not alter the development of reproductive structures (sporangia and spores) in sporophylls. While early termination of the *Ceratopteris* vegetative shoot in loss-of-function experiments (Plackett, Conway et al, 2018) prevented addressing this question, the evidence shown here suggests that *LFY's* floral function did not arise from an ancestral role in promoting a reproductive transition in the fern sporophyte shoot. Other fern and lycophyte *LFY* orthologs show increased expression in sporophylls compared to vegetative fronds (Rodríguez-Pelayo et al., 2022), however, fern expression data did not distinguish between sporangia and sporophyll

tissue and the only available expression data specifically in sporangia are from heterosporous lycophytes (Rodríguez-Pelayo et al., 2022; Yang et al., 2017). Considering that *C. richardii* is a homosporous fern, a sporing transition role for *LFY* could either have been lost in the *Ceratopteris* lineage or gained in connection with the evolution of heterospory.

Our findings also support the capacity of *CrLFY2* to regulate the multicellular 'notch' meristem of hermaphrodite gametophytes. In addition to its floral meristem identity role, *LFY* orthologs are involved in the maintenance of shoot apical meristems in several dicot and monocot angiosperms (Kelly et al., 1995; Moriyama et al., 2024; Shu et al., 2000; Souer et al., 1998; Wang et al., 2008; Zhao et al., 2017), of axillary meristems in rice (Rao et al., 2008), and of marginal meristems in compound leaf development (already described). In gymnosperms, *LFY* paralogs are expressed in the vegetative shoot of *Gnetum* and *Pinus radiata* (Mellerowicz et al., 1998; Shindo et al., 2001), and either one or both *CrLFYs* are necessary to maintain the apical cell in the early fern gametophyte (Plackett, Conway et al., 2018). However, this meristem function had not been previously shown in multicellular meristems of haploid gametophytes. Our overexpression experiments suggest that *CrLFY2* (but not *CrLFY1*) contributes to notch meristem proliferation, and we see a decrease in the number of notch meristem cells in strong RNAi knockdown plants, suggesting a native function in notch meristem maintenance. *$CrLFY1_{pro}$::GUS* expression is not reported in the notch meristem, supporting a paralog-specific role for *CrLFY2*. That only one of the two paralogs shows an effect when overexpressed suggests the possibility of sub- or neo-functionalization in regulating this meristem. Although the notch meristem is typically considered a vegetative meristem, it is specifically associated with the initiation of egg-producing gametangia (archegonia) at its periphery (Banks, 1999; Conway and Di Stilio, 2020; Geng et al., 2022; Hickok et al., 1987). Archegonium initiation has recently been demonstrated to be dependent on positional cues in relation to the notch meristem rather than cell lineage (Geng, 2022). This supports reclassification of the notch as a reproductive meristem, with functions analogous to the angiosperm inflorescence or floral meristem, and therefore *CrLFY2* may play a reproductive function in the fern gametophyte. This hypothesis is supported by the fact that *CrLFY2* has been shown to partially complement an *Arabidopsis lfy* loss-of-function mutant (Maizel et al., 2005), further suggesting that *LFY's* floral function may have been recruited from an ancestral reproductive role in early, macroscopic gametophytes with multicellular meristems. In additional support of this hypothesis, we detected roles for *LFY* in fern gametangia. While we did not find differences in the average number of antheridia and archegonia between wild-type and transgenic plants, we observed a small number of *35S::CrLFY2* transgenic plants without archegonia, and we also detected *CrLFY1pro::GUS* signal in early archegonium development.

Our findings also identified the novel expression of *CrLFY* in fern sperm. We determined that in the gametophyte generation, *CrLFYs* are most highly expressed in mature male gametophytes, and that in both sexes *CrLFY1* expression is localized to developing sperm within antheridia.

Moreover, sperm is withheld in the antheridia of RNAi knockdown plants, rather than being released in response to flooding from otherwise mature gametophytes, indicating a role of *CrLFY* in sperm maturation and/or release. *LFY* homolog expression has been reported in *Arabidopsis* stamens (containing pollen, the sperm-producing male gametophytes) (Nakabayashi et al., 2005; Schmid et al., 2005), and in antheridia of the bryophyte *Marchantia polymorpha* (Arnoux-Courseaux and Coudert, 2024; Kawamura et al., 2022), although not of moss (Tanahashi et al., 2005). It is therefore possible that this sperm function is ancestral.

Stalled zygote development in a small proportion of *CrLFY* knockdown gametophytes suggests that decreased expression of *CrLFY* might prevent cell division in the zygote. This role was previously hypothesized based on *CrLFY1pro::GUS* expression in the early embryo (Plackett, Conway et al., 2018), and the evidence presented here represents the first functional support for *CrLFY'*s role in promoting the transition of the zygote into a multicellular embryo. The presence of arrested zygotes in *PpLFY* disruptant mutants of the moss suggested that this is an ancestral role of *LFY* in the early development of the sporophyte (Tanahashi et al., 2005), and evidence presented here suggests that this function was retained in vascular plants and subsequently lost in angiosperms (or in seed plants).The first zygotic cell division in *Arabidopsis*, *Ceratopteris* and *Physcomitrium* is asymmetric (Gooh et al., 2015; Johnson and Renzaglia, 2008; Mansfield and Briarty, 1991; Tanahashi et al., 2005; Ueda et al., ; Wang et al., 2020), and analogous to animal embryology, there is evidence of morphometric gradients behind this asymmetry in *Arabidopsis* (Lukowitz et al., 2004; Wang et al., 2006). The molecular mechanisms underlying zygote polarity and the first asymmetric division in land plants remain unknown (Matsumoto and Ueda, 2024). *LFY* is not expressed or known to function in the *Arabidopsis* zygote and embryo, and hence this role has presumably been lost in angiosperms, although this aspect deserves further exploration in other angiosperms (Blázquez et al., 1997; Klepikova et al., 2016; Weigel et al., 1992). Native *CrLFY* expression is asymmetric in early multicellular fern embryos (Plackett, Conway et al., 2018), but GUS staining is insufficiently sensitive to detect possible intracellular gradients in late zygote development. Alternatively, *CrLFY* may influence or establish another native intracellular gradient necessary for the first division of the zygote. Auxin has long been suggested as a zygotic gradient (Zhang and Laux, 2011), and in *Arabidopsis* floral tissues *LFY* is both regulated by and regulates auxin biosynthesis (Li et al., 2013), providing a potential mechanism to explain the developmental arrest observed.

Intriguingly, we also found that overexpression of *CrLFY* causes zygotic arrest in *C. richardii*. In gametophytes flooded with sperm overexpressing *CrLFY*, regardless of the genotype of the egg, more than half of the zygotes failed to form embryos. The fact that zygotic arrest occurred in similar proportions across *CrLFY1* and *CrLFY2* overexpressing genotypes, suggests that both *CrLFYs* are capable of interfering with zygote development. We determined that zygote arrest through *CrLFY* overexpression occurred when it was introduced through the sperm and was associated with an abnormally elongated and uncondensed nucleus not seen in stalled *CrLFY*-

RNAi zygotes, suggesting that development was affected at a different stage. Given that we detected no native zygotic expression of *CrLFY1* between 4- and 24-hours post flooding, we speculate that *LFY* must first be repressed during fertilization to allow proper development of the zygote. It is unclear whether a similar repression of *LFY* homologs is necessary for zygote development in *Physcomitrium* or other bryophytes. The nuclear morphology observed could potentially represent incomplete syngamy. Zygotic genome activation (ZGA) is the transition of the zygote genome from silent to transcriptionally active (Fu et al., 2024), and while most evidence suggests a higher role for the maternal genome in this process, there is also evidence for a paternal contribution (Gehring et al., 2004; Kermicle, 1970; Pignatta et al., 2018). In mammals, pioneer transcription factors are heavily involved in ZGA (Fu et al., 2024; Kobayashi and Tachibana, 2021), and LFY has been described as a pioneer transcription factor in *Arabidopsis* (Jin et al., 2021; Yamaguchi, 2021). Further investigations will be necessary to determine whether *CrLFYs* also behave as pioneer transcription factors that contribute to ZGA, in which case, their overexpression could advance the timing of ZGA and affect proper nuclear fusion. Our results thus raise the testable hypothesis that *CrLFY* expression is tightly controlled for zygote development to progress through to the first cell division and that any disruptions in this expression pattern, whether via gene silencing or over expression, will result in developmental arrest. The ultimate mechanism for *CrLFY's* involvement in the proper formation and division of the first cell in the sporophytic phase will certainly require further investigation.

In conclusion, while we do not find evidence for a reproductive role of *CrLFY* in the sporophyte of the fern *C. richardii* via advanced sporing, we show that *LFY* homologs are involved in the regulation of reproductive functions in the haploid gametophyte, both in its multicellular reproductive meristem and in a novel sperm development role. Hence, taking an evolutionary lens that considers available data across land plants, we hypothesize that *LFY* homologs may have regulated multicellular meristems in both the haploid gametophyte and diploid sporophyte stages of the MRCA of non-seed vascular plants and seed plants, this function becoming exclusive to the sporophyte in seed plants, where gametophytes became determinate and hence ameristic. Our findings further support the hypothesis that *LFY's* derived reproductive meristem role in flowering plants originated in the last common ancestor of ferns and seed plants and was potentially co-opted from the gametophyte to the sporophyte phase as vascular plants became increasingly sporophyte-dominant in their evolutionary history. Moreover, *LFY's* regulation of zygote development appears to be conserved through at least the non-seed vascular plants and may be more nuanced than previously thought.

Methods:

Plant materials and growth conditions:

All experiments were conducted in wild-type *Ceratopteris richardii* Hn-n accession background (Hickok et al., 1995). Spores were surface-sterilized by a 10-minute treatment of 10% Hypochlorite and 0.1% Tween (Sigma-Aldrich, St. Louis, MO) at room temperature, rinsed four times and then imbibed in sterile MilliQ water for 2-6 days in the dark to synchronize germination ("Dark Start", (Hickok and Warne, 1998)1998). Spores were then sown onto C-fern media at pH 6 in 1% Difco Bacto agar (Carolina Biologicals, Burlington, NC) with 20 µg/mL of Hygromycin B (Millipore Sigma, Burlington, MA) as the selective agent for transgenic plants, and grown in a Percival chamber at 28ºC, 16 h light/ 8 hr dark, 80 µmol/m^2/s fluorescent light under humidity domes. Once gametophytes were sexually mature (Gh7 (Conway and Di Stilio, 2020), the plates were flooded with 1 mL of sterile water to induce fertilization and incubated until the resulting sporophytes had 5-6 leaves. Young sporophytes were transplanted to soil (Sunshine #4, Sun Gro® Horticulture, Agawam, MA) in 24-well plug trays kept in standing water in a Conviron Chamber (28ºC, 70% humidity, 16 h light/8 h dark) under 80 µmol/m^2/s fluorescent lights. After approximately 6 weeks, plants were transferred to 10 x 10 cm pots and moved to the greenhouse, kept in standing water, and fertilized every two weeks (Plant Marvel Nutriculture, 20-10-20+, 9.6 g/10 gal). Mature sporophylls were cut and placed in glassine bags to mature for at least three months, after which spores were collected into microcentrifuge tubes and stored at room temperature in the dark.

Transgenic line preparation:

Generation of Constructs: The coding sequences (CDS) of *CrLFY1* and *CrLFY2* have previously been validated by cloning (Himi et al., 2001; Plackett et al., 2018). No other *LFY* homologs were detectable by amino acid sequence homology within the *C. richardi*i genome v2.1 (Marchant et al., 2022a). Each CDS was amplified from wild-type Hn-n *C. richardii* cDNA by RT-PCR and cloned separately into the *35S::ocs* expression cassette of the pART7 cloning vector (Gleave, 1992) as an *Eco*RI-*Bam*HI restriction fragment. The *35S::CrLFY1::ocs* and *35S::CrLFY2::ocs* constitutive expression cassettes were each cloned separately into the 'pBOMBER' *Ceratopteris* transformation vector, carrying a hygromycin resistance cassette driven by the Gateway *nos* promoter (Plackett et al., 2015), as a *Not*I-*Not*I restriction fragment.

Generation of transgenic lines: Transformation of 35S::*CrLFY1* and 35S::*CrLFY2* constructs into Hn-n *C. richardii* callus was performed by microparticle bombardment as previously described (Plackett et al., 2015). Each construct was transformed separately. T_0 sporophyte shoots were regenerated from transformed tissue under 40 μg/ml hygromycin selection. Spores were collected from regenerated plants, and germinated to yield T_1 gametophytes, which were

self-fertilized to produce T_2 sporophytes after growing on hygromycin and genotyping to confirm construct presence. Double over-expressing transgenic lines were obtained by crossing the two validated T_2 single over-expressing lines BA14 and BD5, flooding single isolated hermaphrodites overexpressing one paralog with sperm overexpressing the other paralog. The resulting sporophytes were grown on hygromycin plates until transferred to soil and genotyped to confirm the presence of both constructs.

Validation of transgenic plants:

Sporophyte leaves from selfed T_1 plants were collected at the simple leaf stage (S2, (Conway and Di Stilio, 2020), flash frozen and ground, and genomic DNA was extracted from 10 mg of tissue using a modified CTAB method (Doyle and Doyle, 1987). To validate the presence of the transgene, PCR was performed with construct-specific primers and confirmed T_2 sporophytes were assessed for construct insert number by digital droplet PCR (ddPCR), with primers and probes designed on the same exon region as the qPCR primers for both genes (Supplemental Table S1). Thermocycling conditions were determined following the BioRad ddPCR Application guide (BioRad, Hercules, CA, USA). After droplet generation and thermocycling, samples were transferred to a QX200 droplet reader (Bio-Rad, Hercules, CA). Droplet counts were analyzed with QuantaSoftTM version 1.7 (BioRad, Hercules, CA) with default or manually adjusted settings for threshold determination to distinguish positive and negative droplets. *CrLFY1* copy number was calculated using *CrLFY2* as the internal reference in each sample, and the reverse was done for *CrLFY2* copy number calculation, with the wild-type reference copy number set to 1 in both cases.

Phenotyping of transgenic plants:

Phenotyping of transgenic plants was carried out in the T2 generation. Isogenic lines were produced by isolating hermaphrodite gametophytes at the G4 developmental stage when hermaphrodites and males can be distinguished (Conway and Di Stilio, 2020), in a 24-well plate and flooding them at stage G7 when they had developed mature gametangia. All transgenic lines were grown alongside WT Hn-n. Phenotype characterization included: spore germination success, quantification of gametangia, gametophyte notch meristem size and cell number throughout development, quantification of archegonia with zygotes/embryos after fertilization, number of pinnae at sporing, pinnae length, days to production of sporangia, and number of sporangia in sporophytes. Statistical analyses included ANOVA, for multiple comparisons, and χ^2 goodness of fit test for the number of pinnae per sporophyll.

Gene expression analysis:

RNA was extracted from flash-frozen whole single leaves (50-100 mg) or entire young sporophytes using the Spectrum Total Plant RNA kit (Sigma-Aldrich, St. Louis, MO). Primers

for *CrLFY1*, *CrLFY2*, and the housekeeping genes *Actin* and *TBP* were as previously designed (Plackett et al., 2018). Primer amplification efficiency was determined with a plasmid serial dilution using the slope of the linear regression line. Primer specificity was tested via melting curve analysis. qRT-PCR of three biological replicates and three technical replicates each were performed in a Bio-Rad CFX Connect with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). *CrLFY* expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) normalized against the geometric mean of housekeeping gene expression (Hellemans et al., 2007). The standard deviation of the Ct values of each gene was calculated to ensure minimal variation in gene expression. Error bars represent the standard error of the mean for the $2^{-\Delta\Delta Ct}$ values. Relative Expression values of *CrLFY1/2* were compared amongst genotypes by one-way ANOVA followed by Tukey's comparisons.

GUS staining:

The $CrLFY1_{pro}$::GUS transgenic reporter lines used here were previously established (Plackett, Conway et al., 2018). Whole gametophytes were stained for GUS as described in (Plackett et al., 2015, 2014), using 1 mg/mL X-GlcA and 20 μM potassium ferricyanide to increase staining strength and specificity. GUS solution was infiltrated into tissue without pre-fixation using a gentle vacuum over two minutes and incubated for 16-24 hours at 37ºC in darkness. GUS-stained tissues were cleared by incubating in 70% ethanol solution at room temperature.

Microscopy:

Live gametophytes were photographed on agar using a Q-imaging MicroPublisher 3.3 RTV camera mounted on an Amscope dissecting microscope, or a Zeiss Axio Zoom. For fluorescent microscopy, tissue was fixed in FAA (4% formaldehyde, 5% acetic acid, 50% ethanol) at 4ºC overnight. ClearSee (Kurihara et al., 2015) was used for clearing for 7-14 days after fixation. Plants were stained with SCRI Renaissance 2200, SR2200 (Renaissance Chemicals, Selby, North Yorkshire, UK), and either Propidium Iodide or Hoechst 33342 (Thermo Fisher, Waltham, MA). Images were obtained with a Nikon A1R HD25 confocal microscope. Frond and whole-plant photos were taken using a Nikon D3400 hand-held camera with a macro lens attachment. Sporangia quantification was performed by dissecting 2 cm regions of mature sporophylls to reveal sporangia. Images were minimally processed for brightness and contrast using ImageJ (Schindelin et al., 2012). GUS-stained tissue was imaged under Kohler illumination using a Leica DM500 microscope (Leica Microsystems (UK) Ltd., Milton Keynes, UK) mounted with a GXCAM-U3PRO-6.3 digital camera (GT Vision Ltd, Newmarket, UK). GUS staining images were minimally processed for brightness and contrast in Photoshop 2022 (Adobe Inc., San Jose, California, USA).

Fertilization Assays:

WT and *35S::CrLFY* spores were sown on C-fern media as described above. Once gametophytes had developed enough for males and hermaphrodites to be differentiated (stage Gh4, Conway and Di Stilio, 2020), individual hermaphrodites were isolated into 24-well plates, 36 plants per each WT or transgenic line, and the rest of the population was allowed to continue to grow. Once gametophytes were sexually mature (Gh7, (Conway and Di Stilio, 2020), the original plates they had been collected from were flooded with 2 mL water, and 1 mL of post-flood water (containing sperm released from the gametophytes) was collected with a pipette and used to flood the isolated plants. For the first assay, all plants were flooded with water containing sperm of the same genotype. In the second assay, transgenic sperm was used to flood WT gametophytes, and in the third, WT sperm was used to flood transgenic gametophytes. Plants were checked under a dissection microscope for evidence of embryos at 2, 6, and 9 days after flooding, then daily for 14 days. Differences in the proportion of visible embryos were tested by two-way ANOVA followed by Tukey's comparisons.

Meristem Quantification:

WT and 35S::*CrLFY* gametophytes were fixed in FAA overnight just before sexual maturity (Gh6 (Conway and Di Stilio, 2020)). After overnight fixation in FAA, gametophytes were dehydrated to 100% EtOH through an alcohol series, critical point dried, and sputter coated with gold particles. Samples were imaged on a JEOL JSM-6010 Plus scanning electron microscope and images were analyzed in FIJI v2.10 (NIH, USA) to determine the length and width of cells in the notch meristem area. Hermaphrodite gametophytes transition from dividing from a single apical cell to a multicellular lateral notch meristem as they develop, and because cells that have recently divided periclinally in the notch meristem are narrow and long, we used a 2:1 length-to-width ratio to define meristematic cells. Meristematic cells were counted, and the total surface area of the gametophytes was determined in ImageJ. Differences in the number of meristematic cells, the size of gametophytes and the number of gametangia (antheridia and archegonia) were compared by two-way ANOVA followed by Tukey's comparisons.

Sperm performance assays:

Sperm number and viability: Sperm number was determined from samples acquired by a BD FACSymphony A3 Cell Analyzer (Becton, Dickinson and Company, Franklin Lakes, NJ) using FACSDiva v. 8.0. Sperm viability, defined as the proportion of live sperm, was determined from samples acquired by a BD Accuri C6 Plus (Becton, Dickinson and Company, Franklin Lakes, NJ). Spermatocytes were stained with propidium iodide and detected with a blue laser of 488 nm. All analyses were conducted in FlowJo v10.9.0 Software (BD Life Sciences) and gated

initially on singlet cells. To determine viability, cells were gated to split two distinct propidium iodide staining intensities, where intense staining indicates a non-viable cell.

Sperm Swimming Speed: 200 male gametophytes were collected and placed into distilled water. The suspension was pipetted off and placed on a microscope slide with a cover slip. Video of moving sperm was recorded on a Samsung Galaxy A54, mounted on a Leica DM1000 LED compound scope at 10X magnification. Raw videos were processed by DVR-Scan 1.6 software to make a black-and-white mask of the sperm for tracking; these masks were processed by TrackR v0.1.2. Scaled coordinates and frame number (30 frames per second) were used to calculate the speed from frame to frame of the first 10 sperm in view 5 minutes after the initial release of the sperm. These frame-to-frame speeds were then averaged for each of the sperm. Speed differences were compared by two-way ANOVA followed by Tukey's comparisons.

Sperm Count: 100 male gametophytes were collected at differing degrees of maturity – immature (Gm4, Conway and Di Stilio, 2020), mature (Gm7, Conway and Di Stilio, 2020) and two days after reaching sexual maturity, placed in water and allowed to release sperm for one minute. The suspension was then pipetted off and placed in phosphate-buffered saline. These samples were stored at 4°C for at least 2 weeks. Propidium iodide at a final concentration of 0.4 mg/mL was added 45 min before analysis on the BD FACSymphony (Becton, Dickinson and Company, Franklin Lakes, NJ). Using a HTS plate reader (Bruker, Billerica, MA), samples were analyzed at a flow rate of 1 µl/second. The number of sperm cells was compared by two-way ANOVA followed by Tukey's comparisons.

Sperm Viability: Sperm viability assay was designed following Zhang et al. (1992). 100 male gametophytes were collected at sexual maturity (Gm7, Conway and Di Stilio, 2020), placed in distilled water, and allowed to release sperm for 60 seconds. The suspension was then pipetted off and placed in MilliQ water. This suspension was filtered through 30 µm nylon mesh (MTC Bio, Sayreville, NJ). Propidium iodide at a final concentration of 0.17 mg/ml was added to the suspension. Spherotech Accucount Blank particles (Biocompare, San Francisco, CA) with a concentration of 10^6 were added to a final concentration of 77,000. After 6 minutes from gametophytes first being placed in water, samples were analyzed using a BD Accuri C6 Plus (Becton, Dickinson and Company, Franklin Lakes, NJ) at a flow rate of 35 µL per second and a

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Supplementary Materials for McConnell et al.

LEAFY demonstrates ancestral reproductive functions in the gametophyte and not the sporophyte of the fern *Ceratopteris richardii.*

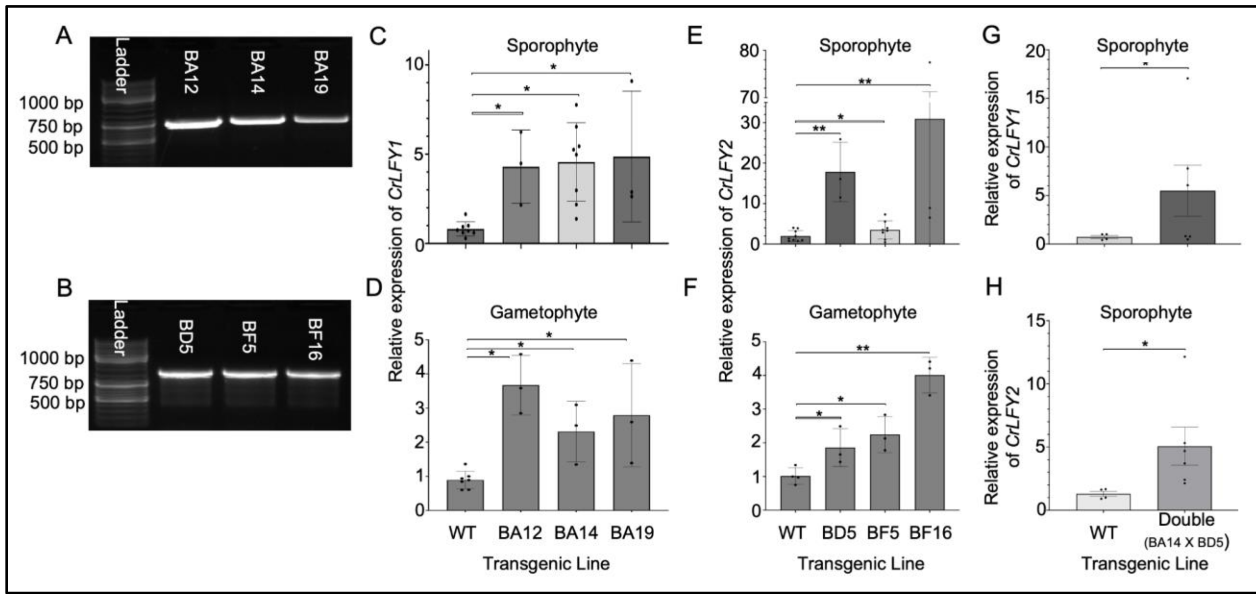

Figure S1: **Molecular and expression validation of transgenic plants in this study.** (A) gDNA amplification of three independent transgenic lines per construct characterized here; primers specific for the *35S::CrLFY1* cassette showing bands of the expected size (753 bp). (B) Same for *35S::CrLFY2* transgenic lines (824 bp). (C-D) Relative expression of *CrLFY1* to the housekeeping genes *CrACT1* and *CrTBPb* comparing wild-type (WT) to three independent *35S::CrLFY1* transgenic lines characterized in this study, for (C) sporophytes or (D) gametophytes. (E) Relative expression of *CrLFY2* in WT and *35S::CrLFY2* for (E) sporophytes and (F) gametophytes. (G-H) *CrLFY1* and *CrLFY2* expression in the double transgenic line. Expression of *CrLFY1/2* was significantly higher in transgenic plants compared to wild-type controls (n=3-8, * = p<0.05, **=p<0.01, one-way ANOVA). Mean ± standard error of the mean (SEM) shown. See Table S1 for primer sequences.

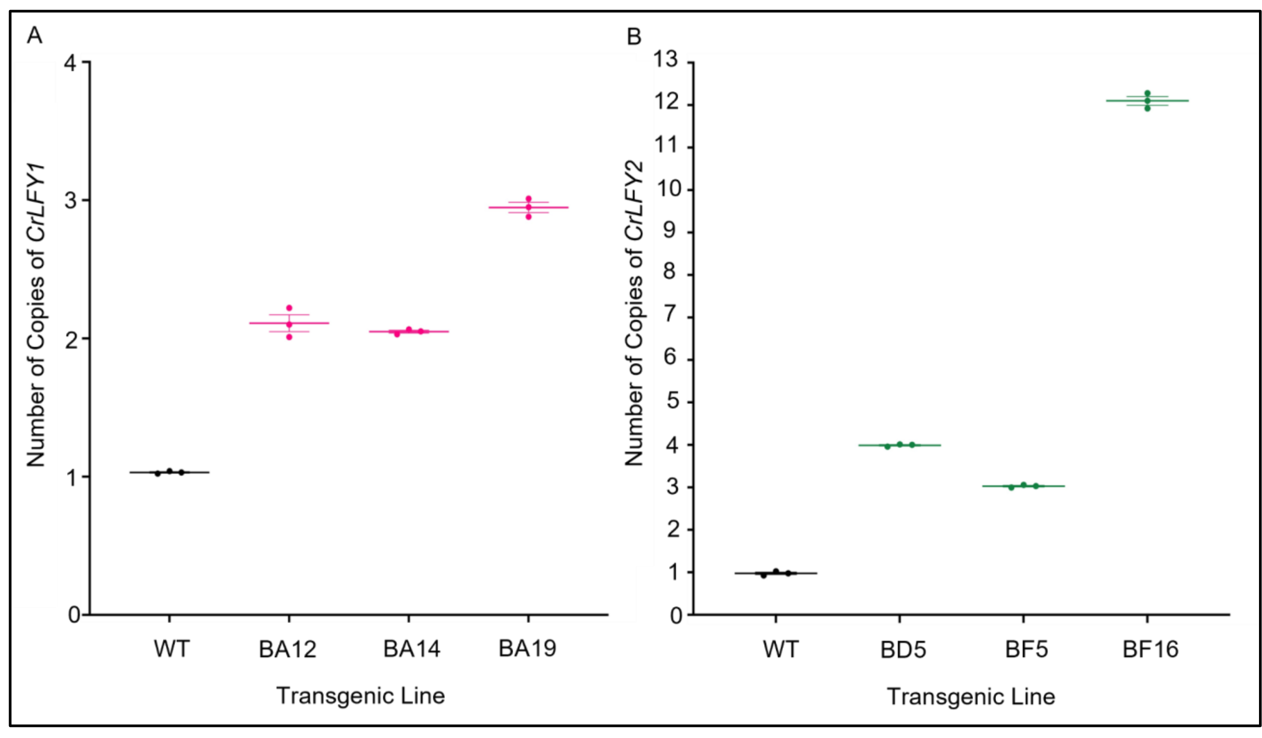

Figure S2: Insert copy number estimation by digital droplet PCR for the transgenic plant lines characterized in this study.

(A) Number of *35S::CrLFY1* construct insertions in the three transgenic lines used in this study, compared to wild-type (WT). Two transgenic lines, BA12 and BA14, contain 2 total copies, or one endogenous and one inserted copy of *CrLFY1*, while BA19 contains 3 total copies (i.e., 2 insertions). (B) Number of copies of *35S::CrLFY2* construct insertions in the three transgenic lines used. transgenic line BD5 contains 4 total copies of *CrLFY2*, or one endogenous and 3 inserted, while BF5 contains 3 total copies, and BF16 contains 12. Mean ± standard error of the mean (SEM) shown.

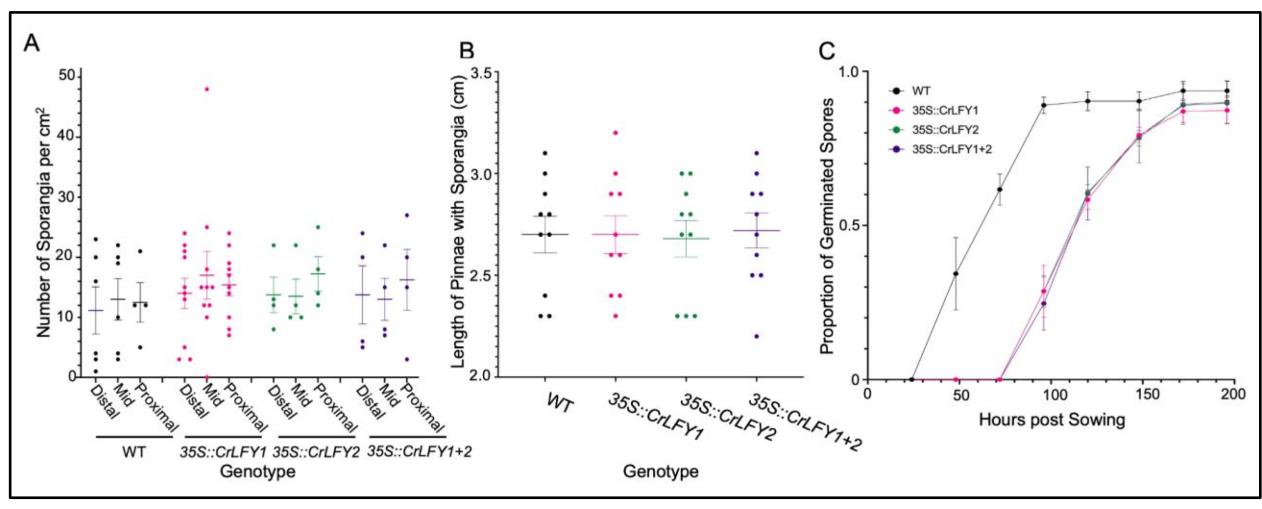

Figure S3: Number of sporangia, length of sporophyll pinnae, and spore germination success in wild-type (WT) and transgenic *C. richardii*. (A) The average number of sporangia across the tip, middle, and bottom regions of a frond (*n*=5-10, *p*=0.44, two-way ANOVA); (B) Length of individual pinnae from sporophylls bearing sporangia (*n*=10, *p*=0.99, two-way ANOVA); and (C) Proportion of WT and transgenic spores that germinate 200 hrs after sowing, out of 50, from 3 independent plates (*n*=3, *p*=0.46, two-way ANOVA). Mean values ± standard error of the mean (SEM) shown.

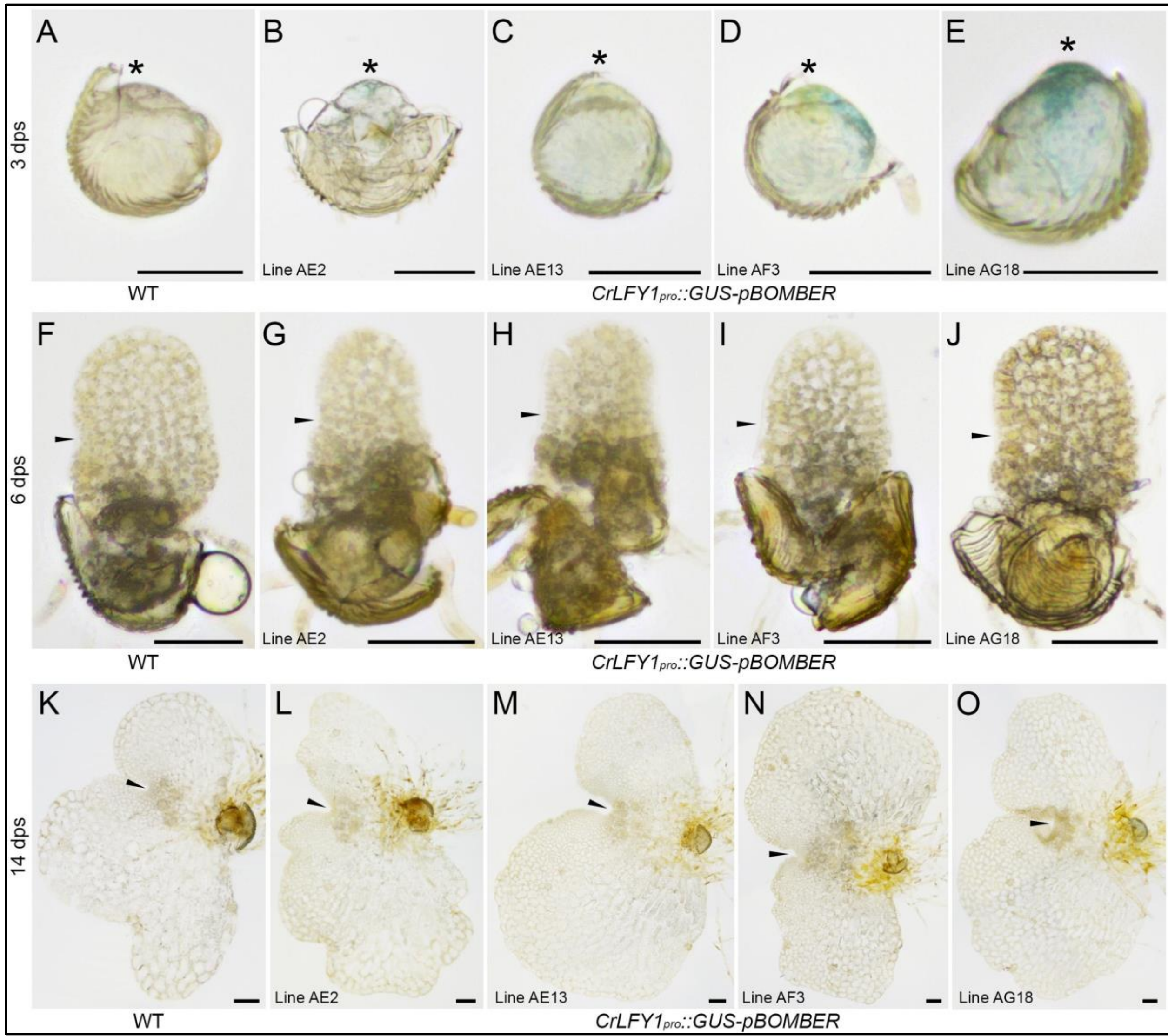

Figure S4: Additional CrLFY1pro::GUS gametophyte expression. GUS-stained whole gametophytes at 3dps during spore germination (A-E), 6 dps during notch meristem initiation (F-G) and 14 dps at sexual maturity with a fully active notch meristem (K-O), comparing WT (A, F, K) to four independent transgenic lines carrying CrLFY1pro::GUS (B-E, G-J, L-O), as previously described (Plackett, Conway et al. 2018). GUS staining is visible in the apical cell at spore germination, consistent with previous in situ localization results. GUS staining was not detected in the notch meristem. Scale bars = 100 μm, asterisks = apical cell, arrowheads = notch meristem.

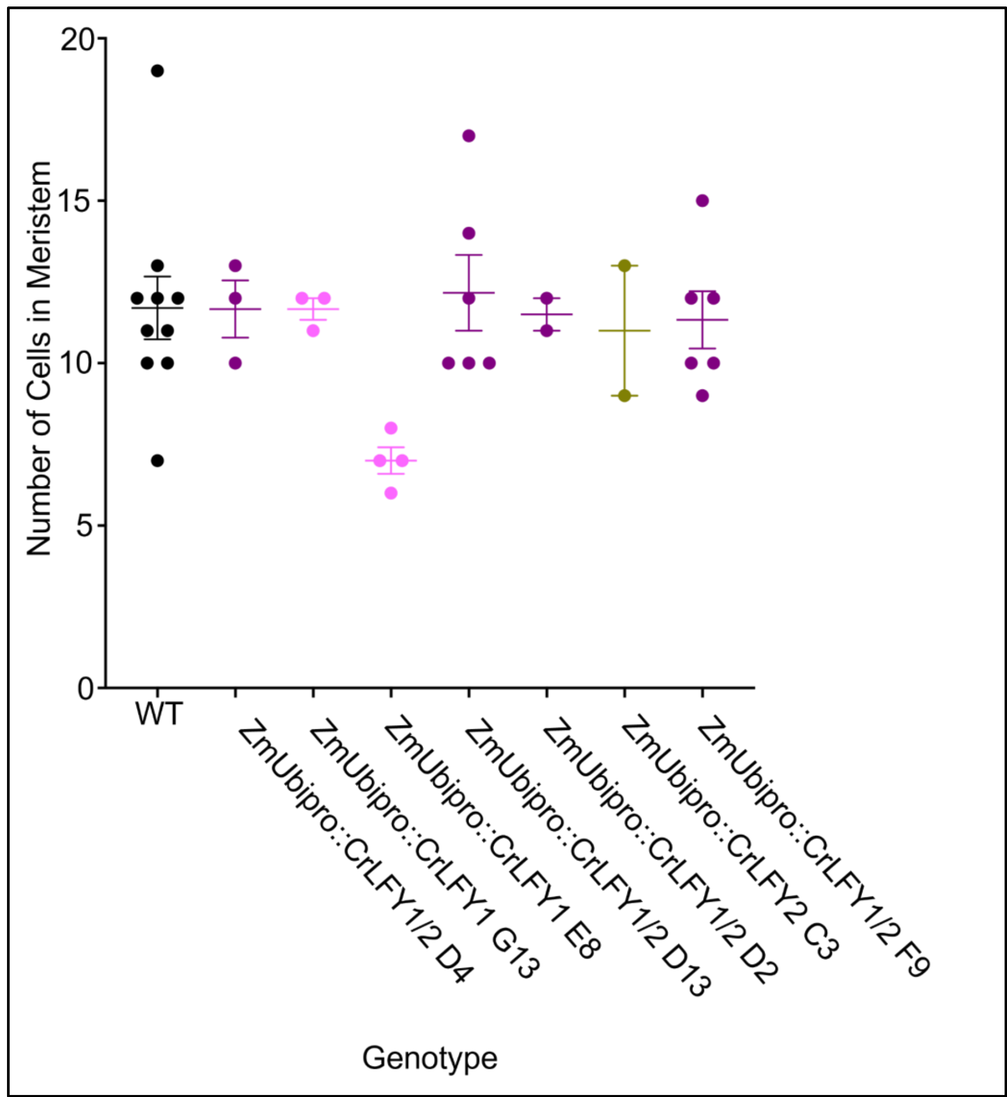

Figure S5: Quantification of notch meristematic cells in CrLFY-RNAi transgenic lines. The number of meristematic cells in WT and transgenic gametophytes at 13 days post-sowing. Letter and number combinations indicate independent transgenic lines.

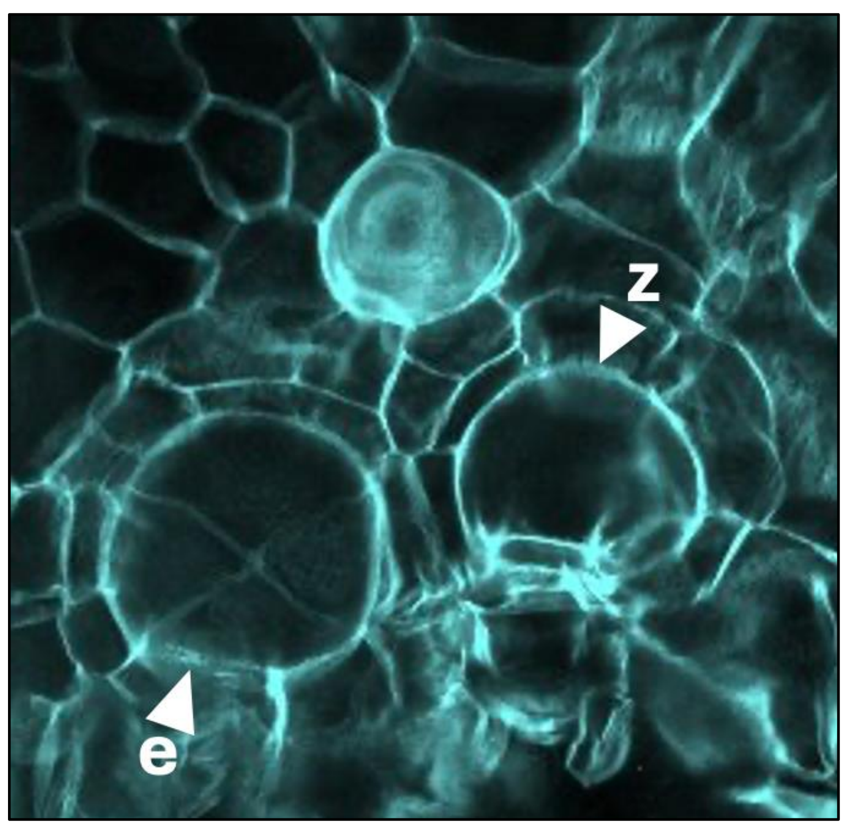

Figure S6: Example of secondary fertilization in wild-type gametophytes, with multicellular embryo and an arrested zygote. Wild-type gametophytes produce a multicellular embryo (e) within 16 h of fertilization. Representative photo from low-frequency secondary fertilization, with a multicellular embryo (4 cells visible, but likely at 8-cell stage), and a single cell zygote (z) that will not develop into a sporophyte beside it.

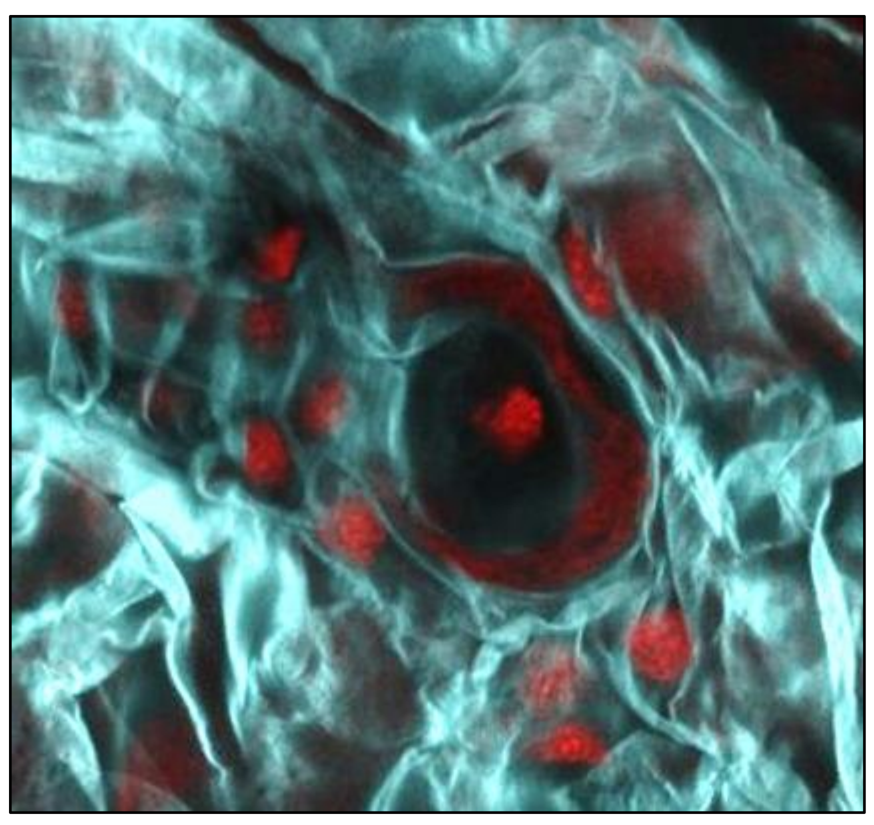

Figure S7: Decrease in Fern *LFY* expression results in failure of zygote progression. Partial Z-stacks from confocal images of an archegonium in a RNAi-*CrLFY* (*ZmUbipro::CrLFY1/2-i)* gametophyte one day after flooding stained with SR220 (light blue, cell wall) and Propidium Iodide (red, nuclei). The zygote has not progressed beyond the one-cell stage and is representative of all stalled zygotes found in the RNAi-*CrLFY* plants, whereas multicellular embryos are found in wild type controls at this stage.

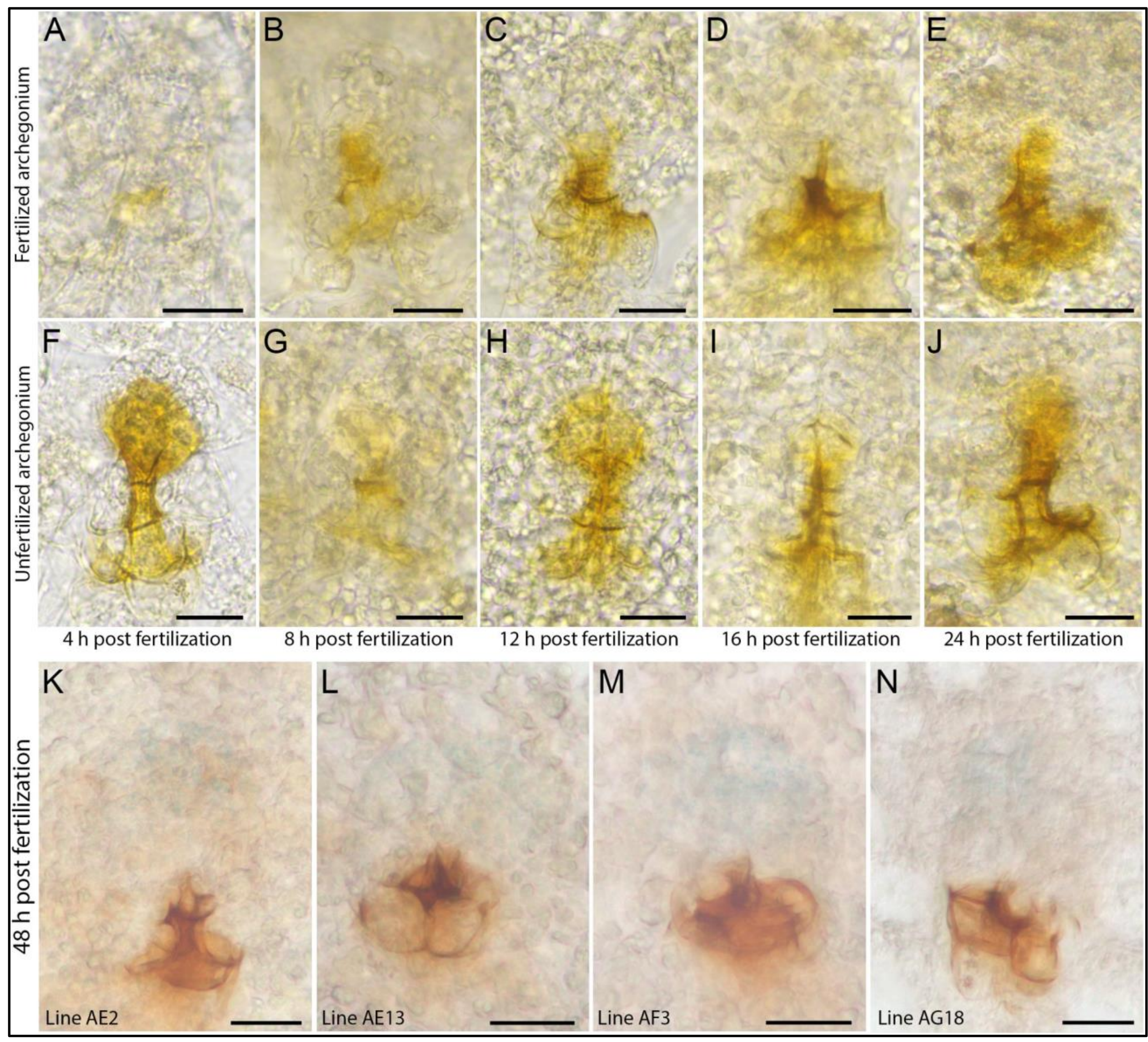

Figure S8: CrLFY1pro::GUS expression during zygote and early embryo development. (A-J) *CrLFY1*$_{pro}$*::GUS* archegonia (line AG18) GUS-stained 4 (A,F), 8 (B,G), 12 (C,H), 16 (D,I) and 24 hours (E,J) after fertilization, showing fertilized archegonia (A-E) compared to unfertilized archegonia (F-J) from the same gametophyte per timepoint. (K-N) The earliest developmental stage post fertilization where GUS staining became visible, comparing four four independent transgenic lines carrying *CrLFY1*$_{pro}$*::GUS* as previously described (Plackett, Conway et al. 2018). Scale bars = 25 μm.

Primer name	Sequence 5'→ 3'	Purpose
qCrLFY1F	ACA AGC ATG CTA TTA TCC ATT GGT	qPCR gene expression
qCrLFY1R	TCA CTG TCC TTG CTC TTC TCT AAA	qPCR gene expression
qCrLFY2F	GAC TCC TTG CTC TAC CTG AAC CTA	qPCR gene expression
qCrLFY2R	CTT CAC CAG GCT CTG TCA CTA TAA	qPCR gene expression
qCrACT1_F	GAG AGA GGC TAC TCT TTC ACA ACC	qPCR reference gene
qCrACT1_R	AGG AAG TTC GTA ACT CTT CTC CAA	qPCR reference gene
qCrTBPb_F	ATG AGC CAG AGC TTT TCC CC	qPCR reference gene
qCrTBPb_R	TTC GTC TCT GAC CTT TGC CC	qPCR reference gene
HygF2	CTTCTACACAGCCATCGGTC	Transgenic validation
HygR	CCGATGGTTTCTACAAAGATCG	Transgenic validation
CrLFY1_F	AAATAGGGCCACCTGGACTC	Transgenic validation

CrLFY1_R	CATTCTTCTTTCCCCTTGCC	Transgenic validation
CrLFY2_F	TGTAGAAGGCACCAGGGAAC	Transgenic validation
CrLFY2_R	TCCCCGTCCTCACCAGGCTC	Transgenic validation
35S_end	AAACCTCCTCGGATTCCATT	Transgenic validation
OCSend	TTAGAATGAACCGAAACCGG	Transgenic validation
CrLFY1_4,707 F	ACCTGGACTCCTGGCTCTAC	ddPCR insert number
CrLFY1_4,874 R	TTTCCCCTTGCCACTTCACC	ddPCR insert number
CrLFY2_1,868 F	ACTGCTGCTCAGAATGGTCCC	ddPCR insert number
CrLFY2_2,070 R	TCTCTGGTCCTGTCATCCCC	ddPCR insert number

Table S1: Primers used in this study (qPCR primers from Plackett, Conway et al., 2018).

Sperm Contribution	Construct	Day 2	Day 6	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Same genotype	35S::*CrLFY1*	**<0.05**	**<0.01**	0.10	**<0.05**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	35S::*CrLFY2*	**<0.01**	**<0.01**	0.07	**<0.01**	**<0.001**	**<0.01**	**<0.01**	**<0.05**
	35S::*CrLFY1 +2*	**<0.001**	**<0.05**	0.06	**<0.05**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
Transgenic flooding WT	35S::*CrLFY1*	0.39	**<0.01**	0.13	**<0.05**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	35S::*CrLFY2*	**<0.05**	**<0.01**	0.11	**<0.05**	**<0.001**	**<0.001**	**<0.001**	**<0.01**
	35S::*CrLFY1 +2*	0.37	**<0.05**	0.09	**<0.05**	**<0.01**	**<0.01**	**<0.05**	**<0.05**
WT flooding transgenic	35S::*CrLFY1*	0.98	0.070	0.24	0.09	**<0.05**	**<0.05**	**<0.01**	0.07
	35S::*CrLFY2*	0.98	0.25	0.27	0.11	**<0.05**	**<0.05**	**<0.01**	0.07
	35S::*CrLFY1 +2*	0.65	0.14	0.23	0.11	**<0.05**	**<0.05**	**<0.05**	0.08
WT flooding transgenic vs Same genotype	35S::*CrLFY1*	0.43	0.17	**<0.05**	**<0.05**	0.57	**<0.05**	**<0.05**	**<0.05**
	35S::*CrLFY2*	0.24	0.03	0.12	**<0.05**	0.06	**<0.05**	**<0.05**	**<0.05**
	35S::*CrLFY1+2*	0.07	0.27	0.33	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**

Table S2: Statistics (*p*-values) for the proportion of multicellular embryos observed up to two weeks after fertilization (flooding) from two-way ANOVA. The proportion of multicellular embryos for each genotype was compared to the proportion of multicellular embryos found in wildtype on each day, *n*=36, bold indicates a statistically significant difference.

Sex-specific gene expression in mature gametophytes of *Ceratopteris richardii* and their correlation to expression of one of two *LEAFY* orthologs

Authors: Hannah McConnell, Julin Maloof, Verónica S. Di Stilio

Abstract: The homosporous fern *Ceratopteris richardii* produces two different types of haploid gamete-producing organisms (gametophytes). Gametophytes develop from spores, and differentiate into either ameristematic males which produce sperm, or hermaphrodites which grow via a multicellular notch meristem and produce both sperm and egg. Gametophyte sex determination is based on sensitivity to the hormone antheridiogen. This differential hormone exposure causes a cascade of transcriptional events leading to vastly different gene expression between sexes. Here, we compare transcriptomes of mature male and hermaphrodite gametophytes to identify differentially expressed genes (DEGs) by RNA-seq, finding 22,719 genes expressed in total, out of 36,857 known protein-coding genes in the genome, with 12,424 differentially regulated between the sexes. Of these DEGs, about half were upregulated in each sample type. Critically, almost half of the most highly expressed genes in males have no gene annotations, revealing what may be an entire program of gene regulation and function not available for investigation or modeling in other land plants. Gene ontology analysis revealed that most of the genes upregulated in hermaphrodites involve processes necessary for continued growth and metabolism, while in males it includes genes involved in microtubule-based movement, likely associated with flagella in motile sperm. There was also ontogenetic variation in gene expression for both sexes. Genes involved in microtubule development were also highly correlated with the *C. richardii LEAFY* ortholog *CrLFY1*, suggesting a putative novel role for this critical meristem identity gene in flagella development in gametophytes. These results continue to provide insight into the development of the fern gametophyte and contribute to the investigation of sex determination in the haploid stage of land plants.

Introduction:

Ceratopteris richardii (*C. richardii*) is a homosporous fern, and sex-determination in gametophytes of this species is based on early exposure to antheridiogen, a hormone hypothesized to act as a gibberellin or brassinosteroid (Banks et al., 1993; Burow et al., 2024; Conway and Di Stilio, 2020). The first gametophytes to germinate from spores develop as hermaphrodites (producing both male and female gametangia) that secrete antheridiogen, which induces later-germinating spores to develop as males. Regardless of antheridiogen uptake, the earliest developmental stages of the gametophytes look the same, but over time they differentiate, with hermaphrodite gametophytes growing into a larger heart-shaped thallus (Fig.

1A), producing gametangia for both eggs and sperm, and males remaining smaller and spatulate (Fig. 1B), producing dozens of sperm-containing gametangia (Banks et al., 1993; Conway and Di Stilio, 2020).

Previous work has investigated the differences between gene expression in young males and hermaphrodites, detailing the transcriptional changes that occur early in gametophyte development, as sex is being determined (Atallah et al., 2018). However, while the initial changes that occur as sex determination is happening are essential to the overall development of the two sexes of the fern gametophyte, the entire developmental trajectory between males and hermaphrodites is quite different, and while some of the genes responsible for those differences are likely expressed early in development, many may not become expressed until later, and would not be found in the transcriptome of the early gametophytes. Importantly, hermaphrodite gametophytes undergo significantly more cell division and will continue to grow until an egg is fertilized, unlike males, which contain a transient apical cell and stop growing at maturity (Conway and Di Stilio, 2020). Additionally, genes necessary for the final aspects of gamete development, such as those responsible for sperm and egg maturation, are less likely to be expressed early in gametophyte development. Therefore, an investigation into differential gene expression in mature gametophytes can offer insight into what genes are responsible for later stages of gametophyte development.

Additionally, given that we found marked expression of the *CrLEAFY1* (*CrLFY1*) promoter visualized by GUS (Chapter 1) in mature sperm within antheridia and that *CrLEAFY2* (*CrLFY2*) had an effect on the notch meristem of hermaphrodite gametophytes (Chapter 1), we asked whether differential gene expression in gametophytes might be regulated by either of these paralogs.

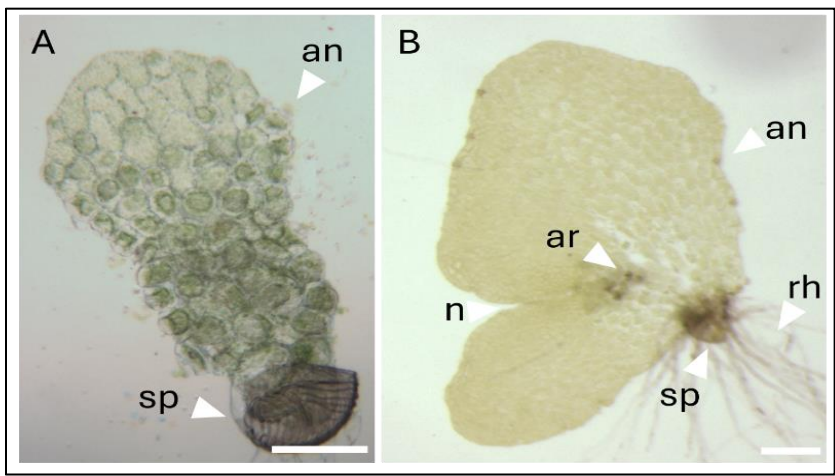

Figure 1: ***C richardii* mature male and hermaphrodite gametophytes.** (A) Mature male gametophyte 14 days post sowing (Gm7, Conway and Di Stilio, 2020) with prominent antheridia (sperm-producing organs). (B) Mature hermaphrodite gametophyte 14 days post sowing (Gh7, Conway and Di Stilio, 2020) with archegonia (egg-producing organs) and a few antheridia. an = antheridia, ar = archegonia sp = spore coat, rh = rhizoids, n = notch meristem. Scale bar=100 µm

Results and Discussion: Differential gene expression between hermaphrodite and male gametophytes:

We found 22,719 total genes expressed in *C. richardii* gametophytes, with 10,295 genes expressed at similar levels in both sexes, 5,944 significantly upregulated in mature males (Fig. 1A), and 6,480 upregulated in mature hermaphrodites (Fig.1B, 2A). In males, the top 50 upregulated genes exhibit 11- to 16-fold higher expression than in hermaphrodites (Fig. 2B), while in hermaphrodites, the top 50 upregulated genes are expressed 16- to 20-fold higher than in males. The total number of expressed genes in our study is lower than the number of expressed genes previously reported between the haploid gametophyte and diploid sporophyte stage, with 33,026 genes expressed in pooled gametophytes and sporophytes and 273 genes expressed specifically in pooled gametophytes (Marchant et al., 2022b). This is likely due to a larger total number of reads in the Marchant samples as well as our application of a filter requiring more than 5 counts per million in 3 or more samples for a gene to be considered expressed. Removing the filter from our data set reveals 28,614 genes with at least one read.

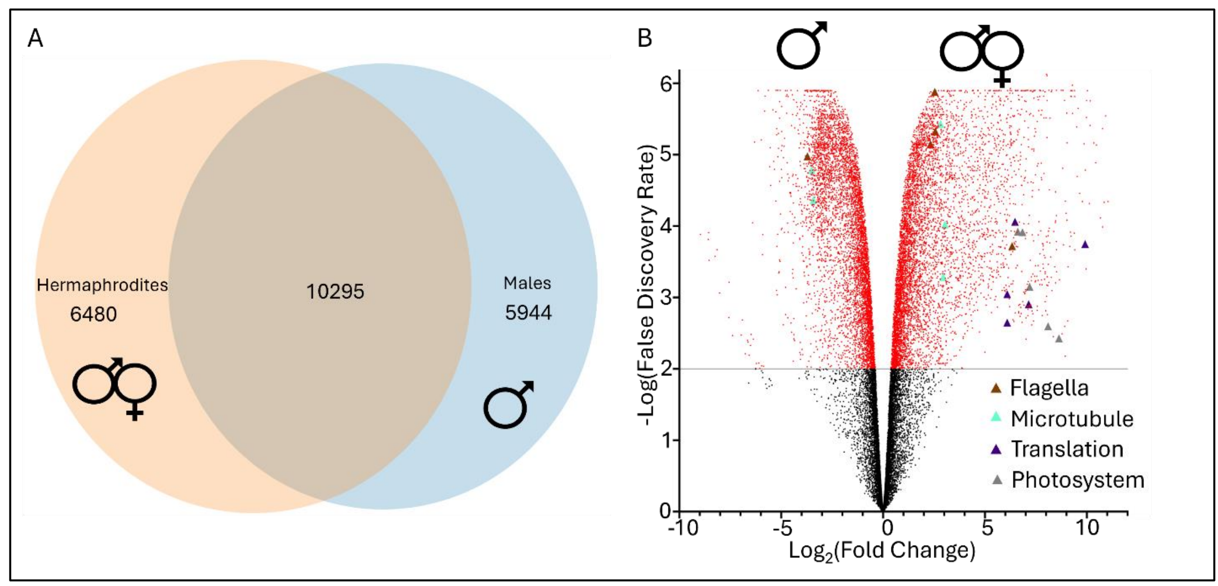

Figure 2: Differential gene expression in male and hermaphrodite gametophytes of the fern *C. richardii.* (A) Venn diagram of numbers of transcripts expressed in male and hermaphrodite gametophytes of *C. richardii*-with shared expressed genes at the center. (B) Volcano plot (false discovery rate vs log fold change in expression) of pooled male (n=3) and hermaphrodite (n=3) gametophyte samples. The black line at -Log(2) marks a p-value <0.01 threshold, with red dots representing differentially expressed genes. Transcripts more highly expressed in male gametophytes are shown to the left, and those more highly expressed in hermaphrodites to the right. Colored triangles represent differentially regulated genes of interest. Figure caption notes GO terms associated with coordinating-colored genes.

Top Gene Ontology sex-specific categories

The top Gene Ontology (GO) terms found among genes upregulated in the hermaphrodite gametophytes include metabolic processes, translation, and photosynthesis (Fig. 3). This is indicative of the hermaphrodites undergoing significantly more continued growth and cell division, energetically supported by performing more photosynthesis than the males. Hermaphrodite gametophytes can continue to grow for an extended period of time if they are not fertilized and will continue to undergo cell division and produce archegonia (each with an egg) and antheridia up until fertilization. Because males have a transient apical cell, as they mature, they stop dividing and photosynthesizing and instead undergo cell differentiation to produce large numbers of gametangia, or antheridia. As such, they are not expected to continue to express genes relating to cell division or photosynthesis, and they die off after releasing sperm.

The only gene ontology (GO) term upregulated in males compared to hermaphrodites is for microtubule-based movement. Given that we sampled mature gametophytes prior to sperm release, this GO term is likely due to the high number of unreleased sperm in males. Even though hermaphrodites also produce some antheridia with sperm (24-25 on average, see Chapter 1), males are completely covered in antheridia and thus are expected to have significantly more sperm-related DEGs. Representatives of early diverging vascular plant lineages, such as ferns, produce motile sperm that is released upon contact with water and swim towards the egg-containing archegonia following chemical cues (Banks et al., 1993; Conway and Di Stilio, 2020). In *C. richardii*, sperm motility is achieved by 70 flagella per sperm cell (Lopez-Smith and Renzaglia, 2008), each driven by dyneins, microtubule-based motors, explaining the DEG GO term for males.

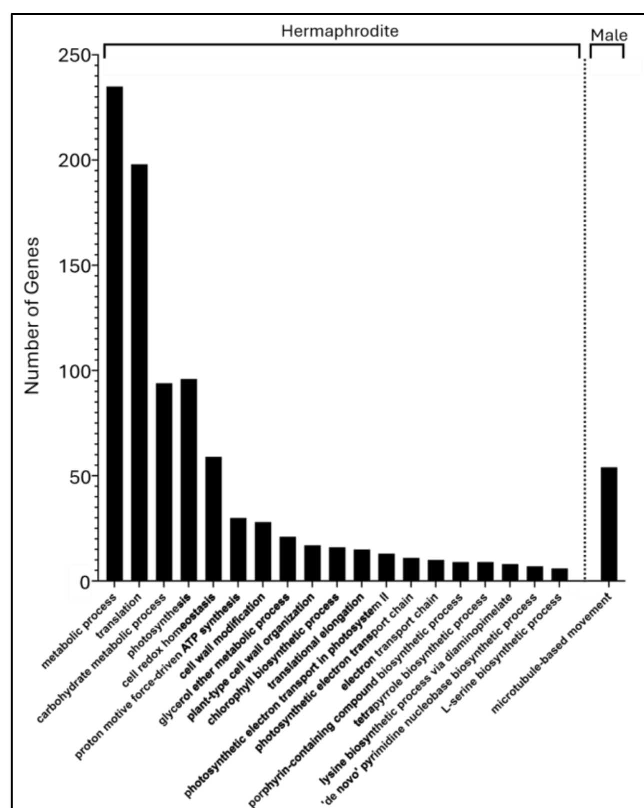

Figure 3: Top Gene Ontology terms for differentially expressed genes between hermaphrodite and male gametophytes of *Ceratopteris richardii*. Number of genes expressed in the top GO categories for hermaphrodites and males ($p<0.05$).

Top sex-specific genes:

Among the top 50 differentially expressed genes in hermaphrodites (Fig. 4), 16 lack annotations in the *C. richardii* genome and do not have a BLAST (REF) match to annotated genes in any of the other available genomes on Phytozome. Among the remaining genes, most do not cluster into any given role, although several belong to gene families involved in cell division, metabolic processes, and photosynthesis, in line with the top GO terms found for hermaphrodite-upregulated genes. In males (Fig. 4), 23 of the top 50 differentially expressed genes lack annotations in the *C. richardii* genome and do not blast to annotated genes in any of the other available genomes on Phytozome. Among the remaining genes, there are several coding for cell membrane and cell wall proteins often involved in removing excess cytoplasm from developing sperm (Myles, 1978).

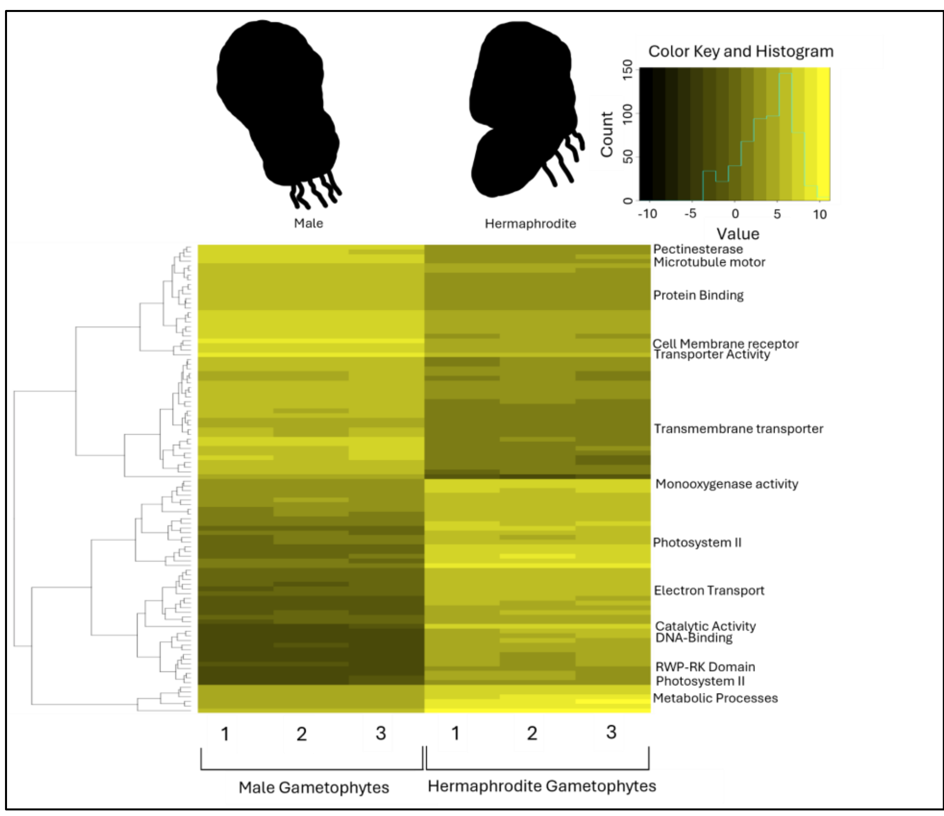

Figure 4. Differentially expressed genes (DEGs) between mature male and hermaphrodite gametophytes. Heatmap characterizing expression of the top fifty up-regulated sex-specific differentially expressed genes. Colors indicate mean-centered $\log_2$ (counts per million reads). Cells closer to black indicate downregulation, while those closer to yellow indicate upregulation.

Gametophyte Sex	**Gene Name/Family**	**Gene Function**	**Number of hits in BLAST**
Male	Flagellar Associated Proteins	Flagella	102
	Dynein Light Chain Type 1 Family, Dynein assembly factor	Dynein	36
	Cell Division Cycle Proteins	Cell Division	8
	RWP-RK family	Nitrogen Uptake/Gametogenesis/ Immune Response	3
Hermaphrodite	Flagellar Associated Proteins	Flagella	37
	Dynein Light Chain Type 1 Family, Dynein assembly factor	Dynein	11
	RWP-RK family	Nitrogen Uptake/Gametogenesis/ Immune Response	3

Table 1: Highly expressed candidate gene families of interest for male and hermaphrodite gametophytes of *Ceratopteris richardii.*

Candidate genes upregulated in male gametophytes:

Since male gametophytes produce significantly larger quantities of sperm than hermaphrodites, we searched specifically for differential expression of genes known to be involved in the production of motile sperm. We found 36 genes involved in dynein production and 102 genes related to flagella function upregulated in males compared to hermaphrodite gametophytes (Table 1). Interestingly, 37 flagella-related genes were upregulated in hermaphrodites, despite the apparent absence of other flagella (outside of sperm cells) in the fern gametophyte (Table 1). We speculate that these genes are also involved in sperm development. There were also 11 dynein-related genes upregulated in hermaphrodites. Since dyneins have also been found in seed plants, which do not produce motile sperm (Gibbons, 1996), they are likely performing other microtubule-based roles in the hermaphrodites in connection with cell division and/or expansion (Table 1).

Unexpectedly, seven genes involved in cell division were upregulated in males, even though cell division is not expected in ameristic mature males (Table 1) (Conway and Di Stilio, 2020). Spermatids divide quite early in the process of spermiogenesis, well before the male gametophyte is fully mature (Lopez and Renzaglia, 2014); it is possible that these upregulated genes are specific to spermatid division and simply are delayed in their degradation. It is also possible that this is evidence of endoreduplication in the male gametophyte; this feature of genome replication without cell division has been described in other male fern gametophytes (Kaźmierczak, 2010) but has not been investigated in *C. richardii*.

An additional interesting finding was the high expression of *RWP-RK* family genes in the male gametophytes. In flowering plants, *RWP-RK* genes are generally involved in floral development and are also found in the female gametophyte of *Arabidopsis* (Chardin et al., 2014; Tedeschi et al., 2017). However, in certain algae, such as *Chlamydomonas reinhardtii*, *RWP-RK* genes are involved in sex determination and production of gametes (Ferris and Goodenough, 1997; Geng et al., 2023, 2014). We found three *RWP* genes more highly expressed in hermaphrodites, one in fact among the top differentially expressed genes, and three others more highly expressed in males (Table 1), suggesting that the specialization found in algae could have been conserved through the early vascular plants, and that *RWP*'s role in sperm development could have been lost in the more recently diverged angiosperm lineage.

Differentially expressed Gene Families:

The *AUXIN RESPONSE FACTOR (ARF)* gene family is found in all land plants in three classes, as well as in their green algal relatives (Finet et al., 2013; Flores-Sandoval et al., 2018; Kato et al., 2020; Mutte et al., 2018; Rienstra et al., 2023). In *C. richardii*, *ARF* genes are involved in developmental processes, including the initiation of sporophytic growth. (Woudenberg et al.,

2024). However, the role of ARF genes in gametophyte development is understudied, especially regarding sex-specific differences. We found five *ARFs* upregulated in mature hermaphrodites, and five upregulated in mature males. Because several of these *ARFs* lack annotations, it is challenging to determine their roles, especially in light of the multiple aspects of development that *ARF*s affect (Fig. 5). Nevertheless, one of the *ARFs* upregulated in hermaphrodites is a homolog of *Arabidopsis ARF1*, which is involved in the initiation of flowering and in stamen development (Ellis et al., 2005). Upregulation in *C. richardii* mature hermaphrodite gametophytes may be due to a similar reproductive role, although a lack of *ARF1* in the male gametophytes suggests that this is not a conserved role. Other *ARF* genes do appear to have some conservation between ferns and angiosperms. In mature male gametophytes, one of the upregulated *ARFs* is a homolog of *ARF8*, a gene necessary for pollen viability (the male gametophyte) in *Arabidopsis*. Similarly to how the *ARFs* that are involved in reproductive roles in angiosperms show specialization, there also appears to be sex-specific specialization of the *ARFs* in *C. richardii.*

Arabinogalactan proteins are found throughout sperm development in *C. richardii* and other plants (Çiftçi, 2012; Lopez and Renzaglia, 2014) and are also involved in egg development in other plant species (Tucker and Koltunow, 2014). In addition to roles in gamete production, some arabinogalactan proteins are involved in cell elongation, expansion, differentiation, and embryogenesis (Hervé et al., 2016). We found 13 arabinogalactan genes upregulated in hermaphrodites, and four upregulated in males (Fig. 5), suggesting that arabinogalactan proteins could be involved in both egg and sperm development in *C. richardii*, with sex-specific expression of different members of the gene family.

In petunia and *Medicago truncatula*, *NO APICAL MERISTEM (NAM)* mutants fail to develop shoot apical meristems (Cheng et al., 2012; Souer et al., 1996) and develop abnormal flowers, with deformed anthers and carpels. In *Arabidopsis*, the related genes, *CUP-SHAPED COTYLEDON-1-3, (CUC1-3)* (together with *NAM* genes, the *NAC* family) have overlapping meristem roles (Hibara et al., 2006). Unlike in the flowering plants, where multiple copies of these genes are somewhat redundant, in *C. richardii* gametophytes, a single copy of a *NAM* homolog is highly expressed in mature hermaphrodites, and two different copies are highly expressed in the mature males (Fig. 5). Unlike in flowering plants, where it appears that the same *NAM* homologs are involved in both male (anther) and female (carpel) development, sex-specific expression in the fern gametophytes suggests sub functionalization.

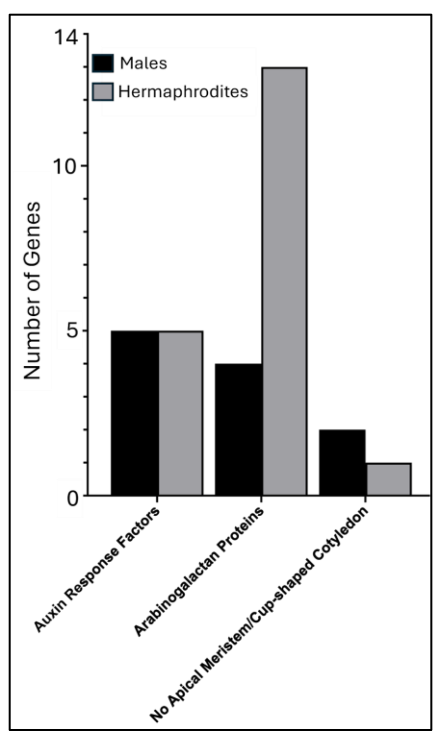

Figure 5: **Gene Families with sex-specific expression in the gametophytes of *Ceratopteris richardii*.**

Ontogenetic differences in gene expression:

While early gene expression differences between male and hermaphrodite gametophytes have already been investigated in *C. richardii* (Atallah et al., 2018), genes responsible for differences in mature gametophytes may not be expressed until much further in development, including those necessary for the maturation of gametes. Therefore, comparison between immature and mature gametophyte gene expression can provide key insights into ontogenetic changes in gene expression between the sexes. Three transcripts coding for copalyl diphosphate synthase genes are highly expressed in immature hermaphrodite gametophytes and are not found later in development (Table 2). Copalyl diphosphate synthase genes catalyze a key intermediate for diterpenes, including gibberellins (Ivamoto-Suzuki et al., 2023). While the chemical makeup of *C. richardii* antheridiogen is yet unknown, it has been hypothesized to act as a gibberellin (Warne and Hickok, 1989), as for all known antheridiogens in other fern species (Atallah and Banks, 2015), or potentially a brassinosteroid (Burow et al., 2024). In some fern species, antheridiogen is produced until the fertilization of the egg (Atallah and Banks, 2015), however, the lack of gibberellin-associated genes in mature hermaphrodite gametophytes of *C. richardii* suggests that its production may be halted earlier in this species.

Sex	Stage	Gene/Gene Family
Hermaphrodite	Immature	Copalyl diphosphate synthase
		ARABIDOPSIS RESPONSE REGULATOR 9
	Mature	*ARABIDOPSIS RESPONSE REGULATOR 8*
Males	Immature	*LOST MERISTEMS*
		KEEP ON GOING
		NO VEIN
		PIN-FORMED 3
		PIN-FORMED 4
		SART-1 Family
		DOT2
		SAR1

Table 2: Genes and gene families upregulated during immature gametophyte development (Atallah et al., 2018) that were investigated as candidates in this study.

Two homologs of *ARABIDOPSIS RESPONSE REGULATORS* (*ARR*) known to respond to the hormone cytokinin (To et al., 2004) are expressed in hermaphrodite gametophytes of *C. richardii*, one with closest homology to *ARR8*, and the other to *ARR9*. The *ARR9* homolog is found in immature hermaphrodite gametophytes, while the *ARR8* homolog is found in mature hermaphrodites (Table 2). *ARR8* and *ARR9* are in the same functional group, the Type-A Response Regulators, they share similar expression patterns, and double mutants have heightened sensitivity to cytokinin (To et al., 2004). Their temporal separation in the hermaphrodite gametophyte suggests sub functionalization.

LOST MERISTEMS (*LOM*), a gene necessary for the regulation of vegetative meristems in *Arabidopsis* (Schulze et al., 2010) is upregulated in young male gametophytes of *C. richardii*. At this early stage, male gametophytes are undergoing cell division both at the transient apical cell as well as in the early developing gametangia (antheridia), where spermatid precursor cells are starting to divide (Conway and Di Stilio, 2020; Lopez and Renzaglia, 2014). *LOM* homologs are no longer expressed in mature males, likely because they are no longer undergoing cell division, either at the apical cell or for sperm production within the antheridia.

KEEP ON GOING (KEG) is involved in intracellular trafficking processes (Gu and Innes, 2012) and in abscisic acid signaling (Liu and Stone, 2010; Stone et al., 2006) in *Arabidopsis*. The *C. richardii* homolog is highly expressed in immature males and not found in mature males (Table 3). Since intracellular trafficking is often a precursor to excretion from the cell itself, *KEG* expression in *C. richardii* may result from the need to move cytoplasm out of the sperm cells prior to or during fertilization (Lopez-Smith and Renzaglia, 2008; Myles, 1978). Alternatively, abscisic acid signaling is involved in several aspects of plant development (Chen et al., 2020), and may be involved in early spermiogenesis, but stops being expressed as the sperm reach maturity.

Auxin plays a critical role in numerous developmental processes in land plants, and in *C. richardii* gametophyte this has primarily been described in the rhizoids (Woudenberg et al., 2024) and the lateral "notch" meristem of hermaphroditic gametophytes (Withers et al., 2023). Auxin has also been shown to play a role in antheridia production in mosses (Ohishi et al., 2021). Several auxin-related genes, including *NO VEIN, PIN-FORMED3, PIN-FORMED4, SART-1 FAMILY PROTEIN DOT2*, and *SAR1*, are expressed highly early in male gametophyte development only (Table 2), suggesting that these genes may be involved in the differentiation of antheridia.

Male gametophytes have highly expressed genes correlated with *CrLFY1*:

There are two paralogs of *LEAFY* (*LFY*) in *C. richardii*; both *CrLFY1* and *CrLFY2* are expressed more highly in males than hermaphrodites, with *CrLFY1* expressed 4.4 (FDR= 0.000004) times and *CrLFY2* expressed 1.25 (FDR=0.021) times more highly in males. Expression of *CrLFY1* has been shown via promoter-driven β-glucuronidase (*GUS)* reporter in sperm (McConnell et al., 2025); expression patterns for *CrLFY2* are currently unknown. *LFY* homologs in flowering plants are responsible for maintaining the identity of the floral meristem (Blázquez et al., 1997; Carpenter and Coen, 1990; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1996; Weigel et al., 1992); in certain legumes and many non-flowering plants, they are also responsible for regulating vegetative meristems, in both the sporophyte and gametophyte phase (Busch and Gleissberg, 2003; Champagne et al., 2007; He et al., 2020; Hofer et al., 1997; Jiao et al., 2019; Kelly et al., 1995; McConnell et al., 2025; Ewa J Mellerowicz et al., 1998; Moriyama et al., 2024; Mouradov et al., 1998; Plackett et al., 2018; Rao et al., 2008; Shindo et al., 2001; Shu et al., 2000; Souer et al., 1998; Wang et al., 2008, 2013; Zhao et al., 2017). While *CrLFY1* is expressed in sperm, its function there is unknown, and as no other sperm role has been found for a *LFY* homolog, it is difficult to speculate the role of a meristem identity gene in an ameristematic gametophyte. To investigate whether *CrLFY1* or *CrLFY2* might be involved in male gametophyte differentiation, we created mutual rank (MR) co-expression networks (Obayashi and Kinoshita, 2009) for each *CrLFY* paralog using 59 gametophyte RNAseq samples that were independent of the other male and hermaphrodite samples described in this chapter. Genes in the *CrLFY1* MR network (Fig. 6 A-D) but not those in the *CrLFY2* MR network (Fig. 6 E-F) were enriched for genes upregulated in males. A group of 1180 genes was identified within this highly correlated cluster using density-based clustering (Fig. 6 C-D). The top four GO terms associated with these clustered genes are involved with microtubule creation or function (Table 3).

	Gene Ontology ID	Term	p value
1	GO:0007018	Microtubule-based movement	$5.9e^{-13}$
2	GO:0007017	Microtubule-based process	$1.92e^{-5}$
3	GO:0031122	Cytoplasmic microtubule organization	$2.7e^{-5}$
4	GO:0007029	Microtubule nucleation	$3.7e^{-5}$

Table 3. Gene Ontology (GO) terms associated with genes highly correlated with expression of the *C. richardii LEAFY* ortholog *CrLFY1*.

As described above, microtubules are critical to the proper function of motile sperm, which consists of 70 flagella (Lopez-Smith and Renzaglia, 2008). The correlation seen between *CrLFY1* and the genes associated with these microtubule-related GO terms suggests that *CrLFY1* may be involved in regulating these genes.

While no *LFY* homolog has been described as having a role in any aspect of sperm development, in addition to *CrLFY1* expression found in sperm, expression of *LFY* has been found in the antheridia of *Marchantia* (Arnoux-Courseaux and Coudert, 2024; Kawamura et al., 2022), and in anthers of *Arabidopsis* (Nakabayashi et al., 2005; Schmid et al., 2005b). This suggests the possibility that a sperm-related role for LFY could be ancestral in land plants, and later lost with the loss of sperm motility in most seed plants and all angiosperms. Any vestigial *LFY* function in flowering plant sperm development would therefore not be related to flagella function and remains undescribed.

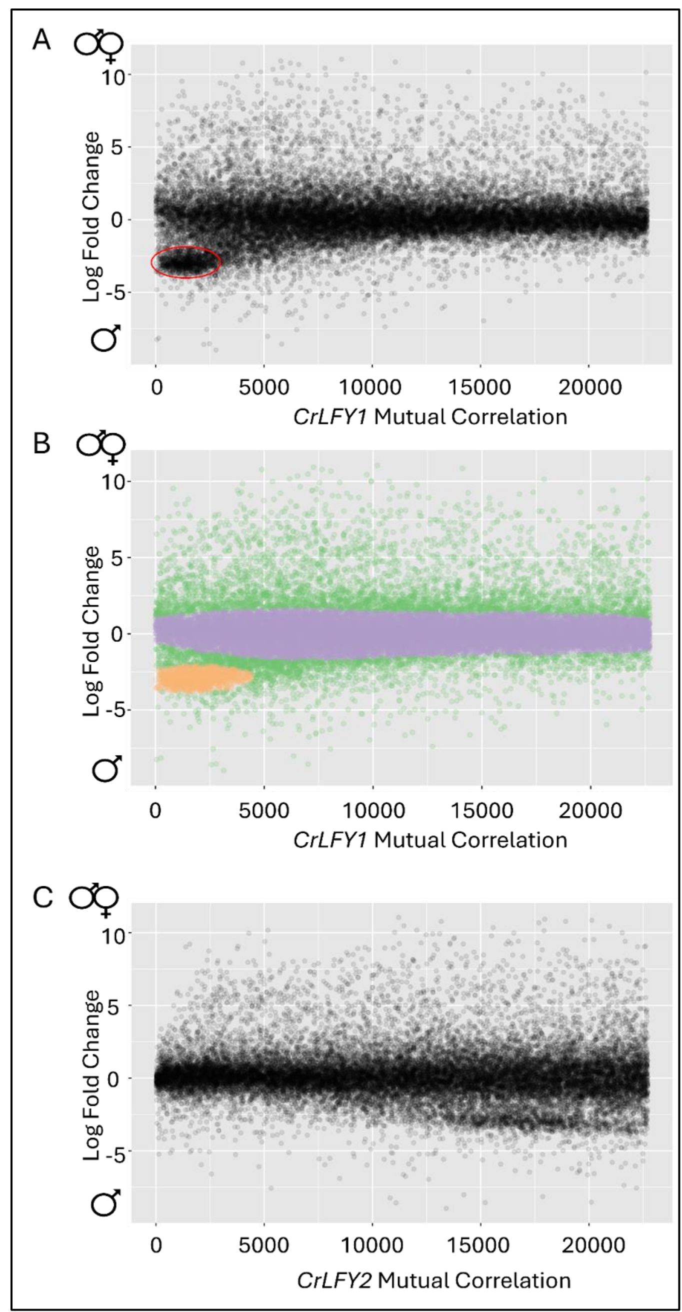

Figure 6. Genes mutually correlated with *CrLFY1* are upregulated in male gametophytes. (A) Mutual Rank Correlation between Differentially Expressed genes (DEGs) and CrLFY1. More positive Log Fold Change values indicate high expression in hermaphrodites. More negative Log Fold Change values indicate high expression in males A cluster of correlated genes upregulated in male gametophytes is circled in red. (B) The same plot as in B, color-coded for groups built from density-based clustering. (C) Mutual Rank Correlation between DEGs and CrLFY2. None of the correlated genes appear upregulated by gametophyte sex in C.

Trends and future directions:

We see expected differences between the sexes in which GO terms are upregulated, with those relating to cell division and metabolism upregulated in hermaphrodites, signaling their continued proliferation, and one upregulated in males relating to the development of flagella. Relatedly, one hundred and two flagella-related and thirty-six dynein genes are upregulated in males compared to hermaphrodites. Unexpectedly, we see three RWP-RK family genes upregulated in both sexes; while these genes have only been shown to have a female gametophyte role in other plants (Chardin et al., 2014; Tedeschi et al., 2017), in certain algal relatives they are involved in the development of both male and female gametangia (Ferris and Goodenough, 1997; Geng et al., 2023, 2014), suggesting that this role has been conserved in the early-diverging vascular plants, but lost in the flowering plants.

Between the sexes, there is evidence of differential gene expression between genes belonging to the same family, including members of the *Auxin Response Factor* Family, Arabinogalactan proteins, and the *NAC* genes, suggesting sex-specific specialization. Across development, we see several genes that are highly expressed in young gametophytes (Atallah et al., 2018) have decreased in expression in the mature gametophyte while also seeing that in some cases, other members of the same gene family have increased in expression, suggesting that specialization across development may have occurred.

While *LFY* homologs have well-described functions in maintaining floral meristem identity (Blázquez et al., 1997; Carpenter and Coen, 1990; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1996; Weigel et al., 1992), and have also been described as being involved in other vegetative and reproductive meristems in both sporophyte and gametophyte life stages (Busch and Gleissberg, 2003; Champagne et al., 2007; He et al., 2020; Hofer et al., 1997; Jiao et al., 2019; Kelly et al., 1995; McConnell et al., 2025; Mellerowicz et al., 1998; Moriyama et al., 2024; Mouradov et al., 1998; Plackett et al., 2018; Rao et al., 2008; Shindo et al., 2001; Shu et al., 2000; Souer et al., 1998; Wang et al., 2008, 2013; Zhao et al., 2017), *LFY* function in sperm development has only been observed so far in *C. richardii* (McConnell et al., 2025). Here, we provide evidence that *CrLFY1* is highly correlated to genes involved in microtubule development in sperm and may therefore regulate the expression of these genes. However, further investigation will be necessary to determine whether the observed correlation is truly indicative of regulation in *C. richardii*, and whether other *LFY* homologs play a similar role in additional land plants.

When investigating the top differentially expressed genes from our dataset, 32% of the genes most highly expressed in hermaphrodites and 46% of the genes most highly expressed in males

had no annotations in Phytozome or NCBI, highlighting the need for additional studies in emerging model organisms such as *C. richardii*. Not only will elucidating the identity and function of these genes clarify the role they play in the developmental processes of fern gametophytes, but it will also provide critical evidence for how these gene families, and their developmental roles, have evolved across the land plants.

Methods:

Plant Growth Conditions:

All experiments were conducted in wild-type *Ceratopteris richardii* Hn-n accession background (Hickok et al., 1995). Spores were surface-sterilized by a 10-minute treatment of 10% Hypochlorite and 0.1% Tween (Sigma-Aldrich, St. Louis, MO) at room temperature, rinsed four times and then imbibed in sterile MilliQ water for 2 days in the dark to synchronize germination ("Dark Start", (Hickok and Warne, 1998)1998). Spores were then sown onto C-fern media at pH 6 in 1% Difco Bacto agar (Carolina Biologicals, Burlington, NC) and grown in a Percival chamber at 28ºC, 16 h light/ 8 hr dark, 80 µmol/m^2/s fluorescent light under humidity domes. Once gametophytes were sexually mature, males and hermaphrodites were collected by visual inspection under a dissecting microscope with forceps and flash frozen in liquid nitrogen.

Library Preparation and Sequencing

RNA was extracted from flash-frozen pooled sex-segregated gametophytes using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) was used to extract total RNA.

RNAseq libraries were prepared and sequenced by Azenta Life Sciences (South Plainsfield, New Jersey). Total RNA samples were quantified using Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina using manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were initially enriched with Oligod(T) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNA were subsequently synthesized. cDNA fragments were end-repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by PCR with limited cycles. The sequencing library was validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA) and quantified by using Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA). The sequencing libraries were multiplexed and clustered onto a flowcell on the Illumina NovaSeq instrument according to the manufacturer's instructions. The samples were sequenced using a 2x150bp Paired End (PE) configuration. Image analysis and base calling were conducted by the NovaSeq Control Software (NCS). Raw sequence data (.bcl files) generated from Illumina NovaSeq were converted into fastq files and de-multiplexed using Illumina bcl2fastq 2.20 software. One mismatch was allowed for index sequence identification.

RNA-seq analysis

Reads were sorted by barcode, quality checked by FastQC ("Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data," n.d.) and multiQC (Ewels et al., 2016), and trimmed using Trimmomatic v 0.39 (Bolger et al., 2014) settings "ILLUMINACLIP:TruSeq3-PE.fa:2:25:10 LEADING:3 SLIDINGWINDOW:4:20 MINLEN:50". Reads were mapped to *Ceratopteris richardii* genomic reference sequence v2.1 (Marchant et al., 2022b), downloaded from Phytozome (Goodstein et al., 2012) using STAR v2.7.10b (Dobin et al., 2013), and bam files were sorted and indexed with samtools (Li et al., 2009). The mapping rate was 81.4%, resulting in an average of 21.1 million mapped reads per sample. Genes were filtered to retain those expressed with more than 5 counts in 3 or more samples (22,719 genes were retained). The remaining analyses were performed in R (R Core Team, 2025). The trimmed mean of M-values (TMM) method from the edgeR package was used to normalize libraries (Robinson et al., 2010; Robinson and Oshlack, 2010). Differentially expressed genes were identified using a negative binomial generalized linear model with quasi-dispersion in edgeR (Chen et al., 2025; Lun et al., 2016; Lund et al., 2012). GeneOntology enrichment analysis was performed using GoSeq (Young et al., 2010) using the GO terms included with the *C. richardii* genome version 2.1 annotation.

Investigation of Differentially Expressed Genes

Investigation of differentially expressed genes was performed in both a targeted and non-targeted manner. Non-targeted analysis was conducted by investigating the top 100 most differentially expressed genes by the lowest p-value. Targeted expression analysis was performed by investigating the expression differences of specific genes and gene families of interest. These included the genes shown to be most differentially expressed in young gametophytes (Atallah and Banks, 2015), and orthologs of genes involved in flowering, arabinogalactan genes, genes involved in motile sperm development (flagella, dynein, cilia, radial spoke proteins), and gibberellins.

CrLFY MR network analysis

We created a mutual rank (MR) co-expression network (Obayashi and Kinoshita, 2009) for gametophyte expressed genes. The network was constructed from 59 RNAseq samples, including 47 samples downloaded from the NCBI Short Read Archive and 12 samples from our *CrLFY* overexpression and RNAi lines (Table S1). Importantly, the 6 samples used for differential expression analysis in this manuscript were not included in the network. Reads were mapped to the *C. richardii* reference genome, normalized as described above, and transformed to log2(counts per million). Pairwise correlations between all genes were calculated, the (absolute) correlation values were transformed to ranks for each gene, and the mutual rank was calculated

as the geometric mean of ranks for each pair of genes, using R. Lower mutual ranks indicate more similar expression patterns across the samples.

The relationship between male/hermaphrodite differentially expressed genes and location in the *CrLFY* networks was determined by plotting log2 fold change expression differences (in male vs hermaphrodite) against the *CrLFY* mutual rank for each gene (Figure 6). To identify the distinct cluster of genes that are highly differentially expressed in males and have low MR with *CrLFY1*, we used density-based clustering as implemented in the dbscan R package (Hahsler et al., 2019), with parameters eps=0.25, minPts=500, borderPoints=TRUE).

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Supplementary information:

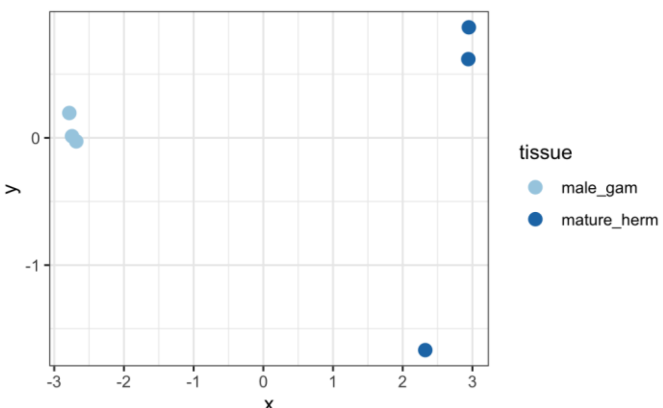

Figure S1: NMDS plot of male and hermaphrodite gametophyte RNA-seq samples in this study. 84% of the variation is found on the X axis, and 7% is found on the y axis.

Sample	Group	Description	Rep
ERR3436537	PRJEB33372	mature gametophyte	1
SRR29324701	PRJNA1121398	5 DAG WT hermaphrodite	1
SRR29324700	PRJNA1121398	5 DAG WT hermaphrodite	2
SRR29324688	PRJNA1121398	5 DAG WT hermaphrodite	3
SRR29324689	PRJNA1121398	5 DAG WT hermaphrodite	4
SRR29324682	PRJNA1121398	5 DAG CrHAM KF hermaphrodite	1
SRR29324681	PRJNA1121398	5 DAG CrHAM KF hermaphrodite	2
SRR29324680	PRJNA1121398	5 DAG CrHAM KF hermaphrodite	3
SRR29324679	PRJNA1121398	5 DAG CrHAM KF hermaphrodite	4
SRR29324686	PRJNA1121398	5 DAG WT male	1
SRR29324678	PRJNA1121398	5 DAG WT male	2
SRR29324699	PRJNA1121398	5 DAG WT male	3
SRR29324698	PRJNA1121398	5 DAG WT male	4
SRR29324696	PRJNA1121398	5 DAG CrHAM KF male	1
SRR29324695	PRJNA1121398	5 DAG CrHAM KF male	2
SRR29324694	PRJNA1121398	5 DAG CrHAM KF male	3
SRR29324697	PRJNA1121398	5 DAG CrHAM KF male	4
SRR29324693	PRJNA1121398	8 DAG WT hermaphrodite	1
SRR29324692	PRJNA1121398	8 DAG WT hermaphrodite	2
SRR29324691	PRJNA1121398	8 DAG WT hermaphrodite	3
SRR29324690	PRJNA1121398	8 DAG WT hermaphrodite	4
SRR29324687	PRJNA1121398	8 DAG CrHAM KF hermaphrodite	1
SRR29324685	PRJNA1121398	8 DAG CrHAM KF hermaphrodite	2
SRR29324684	PRJNA1121398	8 DAG CrHAM KF hermaphrodite	3
SRR29324683	PRJNA1121398	8 DAG CrHAM KF hermaphrodite	4
SRR30294755	PRJNA1149654	whole gametophyte DMSO	1
SRR30294754	PRJNA1149654	whole gametophyte DMSO	2
SRR30294747	PRJNA1149654	whole gametophyte DMSO	3
SRR30294746	PRJNA1149654	whole gametophyte DMSO	4
SRR30294745	PRJNA1149654	whole gametophyte IAA	1
SRR30294744	PRJNA1149654	whole gametophyte IAA	2
SRR30294743	PRJNA1149654	whole gametophyte IAA	3
SRR30294742	PRJNA1149654	whole gametophyte IAA	4
SRR12605697	PRJNA651769	mature gametophyte	2
SRR12605696	PRJNA651770	immature gametophyte	1
SRR13179615	PRJNA681601	Cr_Male	1
SRR13179616	PRJNA681601	Cr_Male	2
SRR13179617	PRJNA681601	Cr_Male	3
SRR13179613	PRJNA681601	Cr_Herm	1
SRR13179614	PRJNA681601	Cr_Herm	2
SRR13179621	PRJNA681601	Cr_Herm	3
SRR13179622	PRJNA681601	Cr_Herm	4
SRR13179623	PRJNA681601	Cr_Herm	5
SRR13179624	PRJNA681601	Cr_Herm	6
SRR13179625	PRJNA681601	Cr_Herm	7
SRR13179626	PRJNA681601	Cr_Herm	8
SRR13179627	PRJNA681601	Cr_Herm	9
BA12-gam	CrLFY1-OX-gam	CrLFY1-OX-gam	1
BA14-gam	CrLFY1-OX-gam	CrLFY1-OX-gam	2
BA19-gam	CrLFY1-OX-gam	CrLFY1-OX-gam	3
BD5-gam	CrLFY2-OX-gam	CrLFY2-OX-gam	1
BF16-gam	CrLFY2-OX-gam	CrLFY2-OX-gam	2
BF5-gam	CrLFY2-OX-gam	CrLFY2-OX-gam	3
Cross-gam-1	Cross-gam	Cross-gam	1
Cross-gam-2	Cross-gam	Cross-gam	2
Cross-gam-3	Cross-gam	Cross-gam	3
RNAi-gam-1	RNAi-gam	RNAi-gam	1
RNAi-gam-2	RNAi-gam	RNAi-gam	2
RNAi-gam-3	RNAi-gam	RNAi-gam	3

Table S1: Samples used for the Mutual Rank (MR) co-expression network. Columns 'Sample' and 'Group' give the NCBI biosample and bioproject accession numbers where appropriate.