Sex Determination in Ceratopteris richardii Is Accompanied by Transcriptome Changes That Drive Epigenetic Reprogramming of the Young Gametophyte

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ABSTRACT The fern Ceratopteris richardii is an important model for studies of sex determination and gamete differentiation in homosporous plants. Here we use RNA-seq to de novo assemble a transcriptome and identify genes differentially expressed in young gametophytes as their sex is determined by the presence or absence of the male-inducing pheromone called antheridiogen. Of the 1,163 consensus differentially expressed genes identified, the vast majority (1,030) are up-regulated in gametophytes treated with antheridiogen. GO term enrichment analyses of these DEGs reveals that a large number of genes involved in epigenetic reprogramming of the gametophyte genome are up-regulated by the pheromone. Additional hormone response and development genes are also up-regulated by the pheromone. This C. richardii gametophyte transcriptome and gene expression dataset will prove useful for studies focusing on sex determination and differentiation in plants.

KEYWORDS sex determination RNA-seq Ceratopteris gametophyte epigenetics gibberellin antheridiogen transcriptome Genetics of Sex

Ceratopteris richardii is a homosporous fern that produces a single type of haploid spore, with each spore having the potential to develop as a free-living male or hermaphroditic gametophyte. In this and many other fern species, the sex of the gametophyte is determined by a male-inducing pheromone called antheridiogen (Warne and Hickok 1991; Banks 1999). In the absence of $A_{CE}$ (for Antheridiogen Ceratopteris), a spore develops as a hermaphrodite, which begins to secrete biologically detectable amounts of $A_{CE}$ after it loses the competence to respond to its male-inducing effects. In the presence of $A_{CE}$, a spore develops as a male gametophyte. Thus, in a population, spores that germinate first in the absence of $A_{CE}$ develop as hermaphrodites that secrete $A_{CE}$, while spores that germinate later and in the presence of $A_{CE}$ develop as males (Banks et al. 1993; Warne and Hickok 1991). Given that the self-fertilization of a hermaphroditic gametophyte results in a completely homozygous sporophyte (similar to a double haploid), this mechanism of sex determination is presumed to promote outcrossing by increasing the proportion of males in a population of gametophytes (Haufler 2002).

Although small (<3mm), male and hermaphroditic gametophytes are dimorphic and easy to distinguish by size and shape. Each hermaphroditic forms a multicellular, lateral meristem that contributes to its heart-shaped appearance, with multiple archegonia developing after the lateral meristem forms (Figure 1g). The development of this lateral meristem coincides with the loss of competence to respond to $A_{CE}$ as well as the production of $A_{CE}$ in the hermaphrodite (Banks et al. 1993).
Male gametophytes never develop a lateral meristem and are much smaller than hermaphrodites (Figure 1d), with nearly all cells of the male gametophyte terminally differentiating as antheridia. If removed from media containing \( A_{CE} \), undifferentiated cells of a male can form a new hermaphroditic prothallus. Based upon these observations, \( A_{CE} \) has many functions in gametophyte development: it prevents the establishment of the lateral meristem; it promotes the rapid differentiation of antheridia; it prevents its own synthesis or secretion in the male; and is necessary to maintain the male program of expression.

To date, all fern antheridiogens characterized are gibberellins (GAs) (Yamane et al. 1987; Yamane 1998; Takeno et al. 1989; Furber et al. 1989). Although the structure of \( A_{CE} \) is unknown, the GA biosynthetic inhibitors ancyamidol, AMO-1618, and uniconazole-P reduce the proportion of males in a population of Ceratopteris gametophytes suggesting that \( A_{CE} \) and GA share a common biosynthetic pathway (Warne and Hickok 1989). That ABA completely blocks the \( A_{CE} \) response in Ceratopteris is also consistent with \( A_{CE} \) being a GA (Hickok 1983; McAdam et al. 2016).

To understand how \( A_{CE} \) determines the sex of the Ceratopteris gametophyte, mutations affecting sexual phenotype have been characterized and used to develop a genetic model of the sex-determining pathway (Strain et al. 2001; Banks 1994, 1997; Eberle and Banks 1996). Cloning these genes is challenging because of the large genome size of \( C. richardii \), ca. 11Gb (Li et al. 2015), and the lack of a reference genome sequence for this or any other homosporous fern species. An alternative approach to identifying sex-determining genes involves de novo transcriptome assembly using RNA-seq, which provides a means to perform sensitive gene expression studies in organisms that do not have a reference genome (Grabherr et al. 2011; Robertson et al. 2010; Schulz et al. 2012). Here we describe the de novo assembly of the transcriptome of young Ceratopteris gametophytes and identify genes whose expression differs as their sex is being determined by the absence or presence of \( A_{CE} \), thus providing a snapshot of the transcriptional changes that occur as the sex of the spore becomes determined and prior to the differentiation of male or female traits in the developing gametophyte.

MATERIALS AND METHODS

Plants and Growth Conditions

The origins of \( Hn-n \), an isogenic, wild-type strain of Ceratopteris richardii used in this study, is described in (Hickok et al. 1987). The conditions for spore sterilization and gametophyte culture are as previously described (Banks 1994). Medium used to culture gametophytes in the absence of exogenous \( A_{CE} \) is as described in (Banks et al. 1993), and is referred to as fern medium, or FM. \( A_{CE} \) was obtained as a crude aqueous filtrate from media previously supporting gametophyte growth in FM as described in (Banks et al. 1993) and is referred to as conditioned FM (CFM). Scanning electron micrographs (SEMs) were performed on a FEI NOVA nanoSEM on samples prepared as previously described (Banks 1994).

For both RNA-seq and qRT-PCR, spores were grown asexptically in liquid FM at 28° in a growth chamber, shaken at 100 rpm, and at a density of 1g spores/L. Three days after spore inoculation, gametophytes were filtered from media; 1/6 of the spores were added to each of three flasks containing 200 mL sterile FM, which is the \(-A_{CE}\) treatment, and 1/6 were added to each of three flasks containing 200 mL sterile CFM, which is the \(+A_{CE}\) treatment. After 36 hr, gametophytes were vacuum filtered from media and frozen in \( N_2(l) \). Tissue was subsequently stored at -80°. All samples were randomized throughout incubators and during sample preparation and harvesting protocols.

Library Preparation and Sequencing

Frozen tissue was ground under liquid nitrogen for 20 min and total RNA extracted using the RNasy Plant Mini Kit (Qiagen, CA). The TruSeq kit (Illumina, CA) was used to select poly-adenylated mRNA and prepare libraries for sequencing. Libraries were sequenced on an Illumina HiSeq2000 platform using paired-end technology.

Transcriptome Assembly and Annotation

DeconSeq v.0.4.1 was run on each of the FASTQ read files to remove reads aligning to bacterial, viral, rRNA, mitochondrial RNA, and chloroplast DNA (Schmieder et al. 2011; Schmieder and Edwards 2011). After removing adapter sequences and trimming reads based on quality score with Trimomatic v.0.22 (Lohse et al. 2012), reads were assembled using the de novo transcriptome assembler Trinity (Grabherr et al. 2011), with a minimum contig length cutoff of 150 and a fixed k-mer size of 25. An assembly with unique genes was generated by selecting the longest component from each Trinity de Bruijn graph. These were used in subsequent differential expression analyses in order to avoid biasing analyses toward genes that were more difficult to assembly and thus had many more contigs (subcomponents). The program Assembly Stats in the iPlant Discovery environment was utilized to obtain basic assembly statistics (Goff et al. 2011; Earl et al. 2011). Protein-encoding, differentially expressed genes were annotated using the Trinotate workflow (Ashburner et al. 2000;
Differential Expression Analysis

Paired reads were aligned to the assembled transcriptome using RSEM v.1.0.1 (Li and Dewey 2011; Grabherr et al. 2011; Li et al. 2015). Only the transcripts with at least one read aligned in at least one of six samples were used. edgeR v.3.0.8 (Robinson et al. 2010), DESeq v.1.10.1 (Anders and Huber 2010), and EBSeq v.1.1.4 (Leng et al. 2013) were used to identify differentially expressed genes at a Benjamini-Hochberg (Benjamini et al. 2001) corrected FDR of q = 0.01. In edgeR, dispersion was estimated as tagwise dispersion. To retain as much rigor in our methods as possible, genes that were identified as statistically significantly differentially expressed in all three packages and displayed at least a twofold expression difference between conditions were identified as “consensus DEGs” and used in all downstream analyses.

GO Enrichment and Assembly Validation

Because there is no reference genome sequence for C. richardii, GO enrichment was performed by annotating the C. richardii transcriptome and the differentially expressed genes against the Arabidopsis proteome (Araport11) (Hanlon et al. 2015), the non-redundant database, and the Selaginella moellendorfii proteome v. 1.0 (Banks et al. 2011), using BLASTx and an e-value threshold of $10^{-8}$. Gene Ontology (GO) terms were then assigned from the Arabidopsis accession identifier from the best hit associated with the differentially expressed transcripts and the reference C. richardii transcriptome. ClueGO (version 2.3.4), a Cytoscape (version 3.5.1) plug-in (Cline et al. 2007; Smoot et al. 2010; Saito et al. 2012; Shannon et al. 2003), and GO Term Fusion were used to distill and visualize the GO term enrichments within the biological processes category using default parameters, with the following exceptions: the minimum number of genes/cluster was set to 5, the Benjamini-Hochberg method was used to correct the p-values for multiple testing, with a significance threshold of $P < 0.05$ and a custom background model supplied. The GO terms mapping to the entire non-redundant Ceratopteris richardii transcriptome was used as the background when assessing the enrichment of GO terms. To assess the quality of the C. richardii Trinity assembly, the 5133 C. richardii Sanger-generated ESTs available in GenBank were used to blast the entire Ceratopteris richardii transcriptome assembly using BLASTn and a BUSCO (Benchmarking Universal Single-Copy Orthologs) analysis was performed using BUSCO v.2.0 to assess completeness of the assembled transcriptome using the ‘eukaryotic’ dataset, which consists of 303 highly conserved genes (Simão et al. 2015; Waterhouse et al. 2018).

Expression Analysis Validation

Total RNA was isolated from six gametophyte populations cultured and harvested in the same manner as that used to generate the RNA-seq data. RNA was reverse transcribed using the Tetro cDNA Synthesis Kit (BioLine, MA); qRT-PCR was performed using the SYBR green PCR Master Mix (Applied Biosystems), 3ng cDNA template and the StepOne Real-Time PCR System (Applied Biosystems, NY). PCR conditions were: one cycle of 20 min at 95°C, 40 cycles of 3 sec at 95°C and 30 sec at 60°C. Melt curves were analyzed and only those reactions producing a single Tm peak were used. Three technical replicates were performed for each sample. Measurements were normalized to the amount of CrEF1α (GenBank accession number BE642078) transcript in the samples. Reactions without template added served as the negative control. The ΔCt method was used in calculating relative fold changes (Livak and Schmittgen 2001). The primer sequences used are listed in Table S1.

Data Availability

Strains are available upon request. Table S1 contains primers used in qRT-PCR and supplemental figures. Table S3 contains a list of all 1,163 differentially expressed genes found by all three statistics packages with annotation and statistical support included. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GBGN00000000. The version with 82,870 genes used in the differential expression analysis is the second version, GBGN02000000. RSEM results and statistical support for all Trinity predicted transcripts are available upon request. Supplemental material available at Figshare: https://doi.org/10.25387/g3.6100139.

RESULTS AND DISCUSSION

Gametophyte Morphology and Selection of Tissue Samples

The early development of Ceratopteris gametophytes can be divided into distinct stages (Banks et al. 1993). During the first stage (0-3d after spore inoculation), the spore swells but remains intact (Figure 1a). During stage 2 (3-4d), the spore coat cracks along its trilete markings. The first rhizoid emerges from the spore during stage 3 and the two-dimensional protonema (Figure 1b and c) emerges during stage 4 (4-6d). The male and hermaphrodite gametophytes become morphologically distinct at stage 5 (6-7d) (Figure 1c and d) at which time hermaphrodites begin to secrete $A_{CE}$. For a gametophyte to develop as a male, it must continuously be exposed to $A_{CE}$ during stages 2 and 3 (Banks et al. 1993). Because we are interested in identifying genes that are differentially expressed by $A_{CE}$ treatment during the period of time that the sex of the gametophyte is determined, three populations of gametophytes were grown without $A_{CE}$ for three days; at day three (end of stage 1), each population was divided into two and either media without $A_{CE}$ or media with $A_{CE}$ was added to the split samples. All gametophytes were harvested and processed 36hr later (stage 3; (Figure 1b and c)) when the sex of the gametophyte was determined but male and hermaphrodites were morphologically indistinguishable.

Transcriptome Annotation and Assembly

The Ceratopteris transcriptome was assembled from 188 million Illumina paired end reads generated from the six gametophyte samples (see Table S2 for a summary of run metrics, analysis and assembly of the transcriptome). Three biological replicate samples were sequenced and analyzed for each treatment condition. A Trinity (Grabherr et al. 2011) de novo assembly resulted in 82,820 genes with read support of which 24% could be annotated with the Arabidopsis proteome, and 23% could be annotated by the Selaginella proteome. A large number of genes (1,064) could be annotated using the Selaginella proteome but did not have hits in the Arabidopsis proteome. Most of the top hits of these sequences are from the Selaginella moellendorfii genome (302), however many are also from Physcomitrella patens (175), and Marchantia polymorpha (131). Of these, the majority (738 sequences) had hit descriptions of predicted/hypothetical, uncharacterized, or unknown proteins. That most of these sequences do not have known gene functions is not surprising given that Arabidopsis is generally used in annotation of plant datasets. Of the remaining sequences which were annotated, many are sperm-related. Motile sperm are a characteristic of early divergent land plants such as Selaginella and Ceratopteris (reviewed in (Hodges et al. 2012)), and thus it is not surprising that such proteins would be present in these assemblies but notably absent from Arabidopsis. A total of 44 sequences have blast hits to dynin related proteins and 18 have hits to flagellar associated proteins. Additional sequences are also present which are sperm-related,
including radial spoke protein 9 and sporangia induced deflagellation-inducible protein. Of the annotated sequences, 43 are annotated with the cellular component GO term cilium, and 13 are annotated with cilium or flagellum-dependent cell motility; these are likely sperm-related proteins as flagellum are solely found in sperm cells in seedless vascular land plants (Raven et al. 2005).

Following the assembly and annotation of the Ceratopters is gametophyte transcriptome, the quality of the assembly was assessed. First, the quality of the Trinity assembly was assessed by comparing 5,133 Ceratopters Sanger EST sequences available in GenBank to transcript sequences generated by Trinity using BLASTn. 87% of the Sanger ESTs, generated either from C. richardii sporophyte and gametophyte tissues were identical or almost identical (E-value of 0.0) to transcripts in the transcriptome assembly, indicating that Trinity accurately assembled transcript sequences from the short Illumina reads. The expression of the Sanger ESTs not represented in the transcriptome assembly may be age or tissue specific and thus not captured in the transcriptome assembly described here. A BUSCO analysis (Waterhouse et al. 2018; Simão et al. 2015) was also performed to assess the completeness of the transcriptome. BUSCO identifies highly conserved genes as complete, complete and single-copy, fragmented, or missing in the transcriptome. Of the 303 total BUSCO groups searched, 290 were complete (95.7%), 181 were complete and single-copy (59.7%), 10 were fragmented (3.3%), and only 3 were missing (1%). This suggests that the assembled Ceratopters gametophyte transcriptome is quite complete.

Identification and Validation of Differentially Expressed Genes by Antheridiogen Treatment

Three programs, edgeR (Robinson et al. 2010), DESeq (Anders and Huber 2010), and EBSeq (Leng et al. 2013), were used to identify genes that differ in their expression by A_C_E treatment (See Table S2 for number of differentially expressed genes found by each package). A scatterplot (Figure 2) that assesses the overall expression pattern across all transcripts shows that the expression of most transcripts is similar regardless of treatment, as expected. The majority (88%) of differentially expressed genes were more highly expressed in A_C_E treated gametophytes (Figure 2). Of the 1,183 DEGs identified using DESeq, 1,163 were also identified by EBSeq and edgeR; these 1,163 DEGS were used in subsequent analyses. A list of the 1,163 DEGs, their annotation and supporting statistics is provided in Table S3. Of the 133 DEGS more abundant in the non-A_C_E-treated gametophytes, 55% were annotated as protein-encoding genes, while 71% of the 1,030 DEGs more abundant in the A_C_E treated samples could be annotated.

To test the validity of the DEG analysis, the expression of 10 genes, including genes more abundant in A_C_E-treated samples, genes more abundant in the non-A_C_E-treated samples and genes showing a less than twofold difference in abundance between treatments were assessed by qRT-PCR. As shown in Figure S1, the qRT-PCR expression data are consistent with the RNA-seq expression data in the direction of the fold change.

GO-Enrichment of Differentially Expressed Genes

The enrichment of Gene Ontology (GO) Biological Process terms associated with the genes that are up-regulated by A_C_E (Figures 3 and Figure S2) reveals four major networks of enriched GO terms. One cluster includes genes related to various aspects of development, including meristem, shoot and tissue development. Another cluster includes genes involved in hormone (ABA, auxin, ethylene and GA) signaling or responses. A third cluster includes genes that affect chromatin structure and epigenetic regulation of gene expression. The fourth cluster includes genes broadly involved in regulating gene expression; genes within this cluster are included in the “chromatin” cluster. Only a single GO term (response to light stimulus) was enriched for genes that are up-regulated in the non-A_C_E treated samples.

Hormone and Development Genes Responsive to A_C_E

Given that all characterized fern antheridiogens are gibberellins (Yamane 1998), genes involved in GA hormone biosynthesis, signaling and responses are likely to be involved in sex determination in Ceratopters. Of the differentially expressed genes, COPALYL DIPHOSPHATE SYNTHASE/KAURENE SYNTASE (CPS/KS), which encodes a key enzyme in GA biosynthesis (Sun and Kamiya 1994; Hedden and Thomas 2012), is more abundant in gametophytes that will become A_C_E secreting hermaphrodites (Table 1). No other known GA biosynthetic genes, including kaurene oxidase and GA20 oxidase are differentially expressed in C. richardii, indicating that sex-specific A_C_E biosynthesis may be regulated or limited by the expression of the CPS/KS gene in Ceratopters, and that its expression is down-regulated by A_C_E (males do not secrete A_C_E). All major ABA and GA signaling genes (Yamauchi et al. 2004; Chan 2012; Sun 2008) are present in the Ceratopters transcriptome and are listed in Table S4. Ceratopters seems to have all the components seen in Arabidopsis, though instead of the 7 DELLA proteins, responsible for repressing GA responses, Ceratopters has only 2 and two F-box protein encoding genes (SNE and SLY1) involved in GA response in Arabidopsis are not present in the assembled Ceratopters gametophyte transcriptome. Similarities in the sex determining pathway and the GA (Atallah and Banks 2015) and ABA (McAdam et al. 2016; Sussmilch et al. 2017) signaling pathways...
have been described. Furthermore, three independent alleles of *Ceratopteris GAMETOPHYTE INSENSITIVE TO ABA 1* (GAIA1) were shown to be mutations of a gene homologous to *OST1/SLAC1* of *Arabidopsis* (McAdam *et al.* 2016). Wild type gametophytes are hermaphroditic in the presence of *ACE* and ABA, *gaia1* mutants are male in the presence of *ACE* and ABA. However, while the GA receptor (*GID*) and GA signaling (the DELLA *GAI/RGA* and *GID2*) genes are present in the transcriptome, they are not differentially expressed by antheridiogen in *C. richardii* as they are in gametophytes of the fern *Lygodium japonicum* (Tanaka *et al.* 2014). Three *MYB* genes are up-regulated (or de-repressed) by *ACE* treatment in *Ceratopteris* (Table 1). These genes are well-characterized regulators of GA-induced responses during angiosperm seed germination (Gubler *et al.* 2002) and GA-dependent anther development (Aya *et al.* 2011) and may serve similar functions by promoting the development of antheridia and/or suppressing archegonia development in *Ceratopteris* gametophytes exposed to *ACE*.

Although ferns never evolved seeds, there are interesting physiological parallels between seed germination in *Arabidopsis* and sex determination in *Ceratopteris* in that both processes are regulated by two antagonistic hormones, ABA and GA. In germinating *Arabidopsis* seeds, the expression of *Mother of FT and TFL* (*MFT*) gene is modulated by GA and ABA (Xi *et al.* 2010). *MFT* is up-regulated by ABA treatment via the ABA-INSENSITIVE3 (*ABI3*) and ABI5 transcription factors, as well as by DELLA proteins in the GA signaling pathway. Because MFT represses *ABI5*, MFT is central to a negative feedback loop that regulates seed germination by GA and ABA in *Arabidopsis*. Given that the *Ceratopteris MFT* and *ABI3* genes are upregulated by *ACE* and ABA antagonizes the *ACE* response (Hickok 1983; McAdam *et al.* 2016; Sussmilch *et al.* 2017), *ACE* may promote male development by ultimately repressing ABA signaling in the gametophyte through a pathway that involves *MFT* and *ABI3*.

Several additional genes involved in ABA, ethylene, auxin and cytokinin perception, signaling or response are up-regulated by *ACE*

**Figure 3** Functionally grouped Biological Process GO terms specific for *ACE*-up regulated DEGs. The size of each node represents the term enrichment significance. Node labels are shown in the bar graph in Figure S2.
### Table 1  Differentially Expressed Genes Discussed in Text

| Ceratopteris Gene | Annotation | Arabidopsis Accession | Blast E-value | AdjPval  | FoldChange |
|-------------------|------------|------------------------|---------------|----------|------------|
| comp112296        | copalyl diphosphate synthase | AT4G02780.1 | 3.00E-159 | 0.001459703 | 2.2 |
| comp103387        | ABA 8’-hydroxylase | AT4G19230.1 | 0 | 0.003233766 | 2.4 |
| comp112296        | copalyl diphosphate synthase | AT4G02780.1 | 3.00E-159 | 0.001459703 | 2.2 |
| comp112296        | copalyl diphosphate synthase | AT4G02780.1 | 3.00E-159 | 0.001459703 | 2.2 |
| comp108125        | ARR9 | AT2G41310.1 | 3.00E-42 | 1.18E-08 | 5.3 |
| comp82535         | ARR9 | AT2G41310.1 | 2.00E-48 | 1.30E-08 | 4.2 |
| comp119738        | KAR-UP F-box 1 | AT1G31350.1 | 4.00E-32 | 9.17E-05 | 2.3 |
| comp116986        | SCARECROW-like (SCL) | AT5G46770.1 | 1.00E-87 | 0.006147273 | 2.4 |
| comp82755         | GRAS family transcription factor | AT1G53100.1 | 1.00E-92 | 0.000454384 | 2.7 |
| comp103126        | LOST MERISTEMS (LOM) | AT3G4630.1 | 5.00E-49 | 2.53E-05 | 2.9 |
| comp81241         | Latera root primordium (LRP) | AT3G51060.1 | 2.00E-30 | 4.22E-06 | 3.6 |
| comp42166         | MOTHER of FT and TF 1 (MFT) | AT1G18100.1 | 5.00E-62 | 0.000371991 | 2.5 |
| comp111805        | AHK4; cytokinin receptor CRE1a | AT2G01830.1 | 0 | 0.00253886 | 2.2 |
| comp100079        | CKI1 | AT2G47430.1 | 4.00E-108 | 0.000725748 | 2.6 |
| comp115365        | cytosine methyltransferase (MET1) | AT5G49160.1 | 0 | 1.45E-06 | 3.3 |
| comp82159         | chromomethylase (CMT3) | AT1G69770.1 | 1.00E-155 | 0.006306721 | 2.3 |
| comp112176        | DEMETER-like protein 1 (ROS1) | AT2G36490.1 | 8.00E-83 | 0.001457903 | 2.7 |
| comp101920        | NO VEIN (NOV) | AT4G13750.1 | 0 | 0.00253886 | 2.7 |
| comp106375        | PIN-FORMED 4 (PIN4) | AT2G01420.1 | 4.00E-166 | 2.68E-08 | 4.6 |
| comp105872        | PIN-FORMED 3 (PIN3) | AT1G70940.1 | 5.00E-156 | 0.009233133 | 2.2 |
| comp98976         | BIG auxin transport protein | AT3G02260.1 | 0 | 7.20E-09 | 4.2 |
| comp109704        | ABC transporter | AT3G28860.1 | 0 | 7.48E-12 | 4.7 |
| comp97116         | SART-1 family protein DOT2 | AT5G16780.1 | 3.00E-132 | 0.008328934 | 2.5 |
| comp114948        | SAR1 | AT1G33410.2 | 0 | 4.83E-05 | 3 |
| comp105798        | auxin response factor (ARF) | AT1G19220.1 | 5.00E-53 | 0.000175818 | 6.5 |
| comp111805        | AHK4; cytokinin receptor CRE1a | AT2G01830.1 | 0 | 0.00132766 | 3.2 |
| comp100079        | CKI1 | AT2G47430.1 | 4.00E-108 | 0.000725748 | 2.6 |
| comp115365        | cytosine methyltransferase (MET1) | AT5G49160.1 | 0 | 1.45E-06 | 3.3 |
| comp82159         | chromomethylase (CMT3) | AT1G69770.1 | 1.00E-155 | 0.006306721 | 2.3 |
| comp112176        | DEMETER-like protein 1 (ROS1) | AT2G36490.1 | 8.00E-83 | 0.001457903 | 2.7 |
| comp101924        | NERD | AT2G16485.1 | 7.00E-96 | 4.01E-05 | 3 |
| comp109662        | CHR11; chromatin-remodeling 11 | AT3G06400.2 | 0 | 0.007217854 | 2.2 |
| comp83245         | CHR5; chromatin remodeling 5 | AT2G13370.1 | 0 | 2.28E-08 | 3.9 |
| comp302330        | PICKLE (PKL) | AT2G25170.1 | 0.00E+00 | 6.59E-09 | 4.1 |
| comp302330        | PICKLE (PKL) | AT2G25170.1 | 5.00E-124 | 6.59E-05 | 2.8 |
| comp39118         | BRAHMA (BRM) | AT2G46020.2 | 0 | 5.18E-12 | 5 |
| comp43532         | CHR21/INO80 | 1.75E-05 | 3 |
| comp81987         | MBD09; methyl-CpG-binding domain | AT3G01460.1 | 5.00E-103 | 4.26E-09 | 4.1 |
| comp99654         | SUVH4/KYP | AT5G13960.1 | 0 | 0.001159543 | 2.5 |

(continued)
treatment (Table 1). While ABA is known to affect sex determination by blocking the \( A_{CE} \) response, these results point to roles for additional hormones in the sex-determining process. Studies of the effects of exogenous auxin (Gregorich and Fisher 2006; Hickok and Kiriluk 1984), ethylene (Kazmiernczak 2010) and cytokinin (Menéndez et al. 2009) on fern gametophyte development have shown that these hormones can affect the overall size and organization of the gametophyte as well as the number of sex organs in a gametophyte. However, neither auxin, ethylene or cytokinin substitute for or completely block the male-inducing effects of antheridiogen, indicating that \( A_{CE} \) may influence these hormones, or the crossstalk among these hormones, in modulating cell division and expansion in young gametophytes that will become important as they differentiate.

This DEG analysis suggests that \( A_{CE} \) affects the sex of the gametophyte by not only activating genes associated with development, but also by epigenetically reprogramming the nucleus that will divide and ultimately give rise to a male gametophyte. The relatively few genes that are up-regulated in gametophytes not treated with \( A_{CE} \) likely represent genes that are normally expressed in the gametophyte destined to become hermaphrodite but are repressed by \( A_{CE} \).

An Epigenetic Response to \( A_{CE} \)

A striking number of DEGs up-regulated by \( A_{CE} \) encode factors involved in epigenetic regulation of gene expression or epigenetic reprogramming of the genome. These genes were sorted into five groups (Table 1) following the classification of Pikaard and Scheid (Pikaard and Scheid 2014): DNA modification, histone modification, Polycomb-group proteins and interacting components, chromatin formation/ chromatin remodeling and RNA silencing.

The first group includes DNA modification genes that affect cytosine methylation. The DEGs assigned to this group encode DNA Methyltransferase 1 (MET1), which maintains CpG methylation (Saze et al. 2003; Jullien et al. 2012), Chromomethylase 3 (CMT3), which maintains CpHpG methylation (Law and Jacobsen 2010) and Repressor of Silencing 1 (ROS1), a cytosine demethylase (Gong et al. 2002). Differences in global DNA methylation patterns between gametes and adjacent cells of both male and female gametophytes of Arabidopsis have been observed (Pillot et al. 2010; Calarco et al. 2012; Ibarra et al. 2012; Jullien et al. 2012) and are thought to silence transposable elements and reset silenced imprinted genes in sperm cells (Kawahima and Berger 2014; Martinez et al. 2016). While sex determination in a homosporous fern, which occurs during the gametophyte generation, differs from sex determination in the heterosporous angiosperms, which occurs during the sporophyte generation (Tanurdzic and Banks 2004), the up-regulation of these genes during sex determination in Ceratopteris adds another stage of plant development where DNA methylation may play an important role in stabilizing or destabilizing transposable elements and contributes to epigenetic reprogramming of the male gametophyte. Whether the observed differential expression of these DNA methylation genes alters DNA methylation patterns in the genomes of young Ceratopteris gametophytes, and whether additional changes in DNA methylation occur as their gametes differentiate, remain to be tested.

A number of \( A_{CE} \)-up-regulated DEGs encode proteins belonging to the second group, histone-modifying enzymes known to affect gene expression (Table 1). Among them are the histone acetyltransferases HAC1, HAC12 and ROS4, a histone deacetylase (HDA14), the histone methyltransferases TRITHORAX-LIKE PROTEIN 2 and 3 (ATX2 and 3), the SU(VAR)3-9 related proteins SUVH4/KYP and SUVH6, and EARLY FLOWERING IN SHORT DAYS (EFS/SDG8). These proteins are involved in either maintaining transcriptionally active states or transcriptionally inactive states (reviewed in (Liu et al. 2010; Bannister and Kouzarides 2010; Grossniklaus and Paro 2014; Pikaard and Scheid 2014; Xiao et al. 2016)) and can contribute to the maintenance of DNA methylation at silenced loci. ATXR3 is notable in that it is essential for male and female gametophyte development (Berr et al. 2012) and is classified as encoding proteins from the third group of chromatin modifiers: Polycomb proteins. Polycomb proteins and interacting partners are often involved in determining cell proliferation and identify through methylation and chromatin compaction (Grossniklaus and Paro 2014; Kingston and Tamkun 2014). The fourth group of genes, those involved in
chromatic formation/remodeling, are also represented among the genes up-regulated in response to \textit{ACER}, \textit{PICKLE (PKL)}, the gene encoding for a chromatin remodeling factor which is necessary for gibberellin modulated development in \textit{Arabidopsis}, (Park et al. 2017) and INOSITOL-REQUIRING 80 (INO80), are both members of remodeling complexes and are required for normal development (Zhang et al. 2015).

The fifth group of genes involving epigenetic regulation is those relating to RNA-mediated gene silencing pathways (Table 1). Argonaute (AGO) 1, a core member of the RNA-induced silencing complex (RISC) which is involved post transcriptional gene silencing (PTGS) through cleavage or transcriptional inhibition (reviewed in (Czech and Hannon 2011; Martienssen and Moazed 2015)) is significantly up-regulated by \textit{ACER}. Also up regulated are genes encoding two Dicer endonucleases: DCL1 which generates miRNAs of mostly 21nt and DCL4, which generates siRNAs that are 21nt (Pouch-Pélissier et al. 2008). Additional genes involved in RNA-mediated PTGS are \textit{XRN4}, which encodes a nuclease involved in small RNA processing (Cao et al. 2014), and \textit{SUO}, which encodes a component of the miRNA pathway (Yang et al. 2012). \textit{NRPD2}, encoding the catalytic subunit of RNA polymerase IV and V in plants (Ream et al. 2009) is also up-regulated by \textit{ACER}. Pol IV and V are both required for intercellular RNA interference and are involved in PTGS maintenance (Onodera et al. 2005; Pontier et al. 2005; Kanno et al. 2005). Also modulated by \textit{ACER} is a component of the THO/TREX complex, which has a putative role in siRNA biosynthesis (Furumizu et al. 2010). Interestingly, the THO complex represses female germline specification in \textit{Arabidopsis} (Su et al. 2017). Together, these results show that small RNA-mediated PTGS is involved in the suppression of female characteristics in \textit{C. richardii} gametophytes.

All of the epigenetic mechanisms known to occur in plants are represented among the genes up regulated by \textit{ACER}. The importance of epigenetic regulation for sex determination in \textit{C. richardii} should perhaps not come as a surprise. Gametophytes which are removed from \textit{ACER} containing media will over time develop into hermaphrodite gametophytes, thus the promotion of male/suppression of female traits must be reversible. Epigenetic regulation of sex determination would allow for such plasticity in development.

\textbf{Conclusions}

This work reports the first transcriptome of \textit{Ceratopteris richardii}, along with a survey of significant differential gene expression changes between male and hermaphrodite gametophytes as sex is being determined. A high-quality reference gametophyte transcriptome was assembled and used in the identification of genes which may be involved in sex determination. The majority of differentially expressed genes were more highly expressed in the male gametophyte. Many of these up regulated genes are known to be involved in development and in response to hormones. A significant number of differentially expressed genes are involved in chromatin remodeling and epigenetic regulation. Outcomes of this research shed light on the molecular mechanisms involved in sex determination of \textit{C. richardii} as well as provide a resource for other plant science researchers. Future work will probe and functionally classify these differentially expressed genes and as well as survey how these changes persist as the gametophyte moves from sex determination to differentiation.

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\textbf{Author Contributions:} NA performed tissue preparation and treatment as well as harvesting, performed bioinformatics analysis, performed qRT-PCR, interpreted data, and wrote part of the manuscript. FG provided bioinformatics guidance and support. OV participated in designing the experiment as well as provided statistical guidance and support. JB and MT designed the experiment, conceived of experimental design and setup, interpreted data, and wrote manuscript. All authors reviewed and approved the final manuscript.

\textbf{LITERATURE CITED}

Anders, S., and W. Huber, 2010 Differential expression analysis for sequence count data. Genome Biol. 11: R106. https://doi.org/10.1186/gb-2010-11-10-r106

Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler et al., 2000 Gene ontology: tool for the unification of biology. Nat. Genet. 25: 25–29. https://doi.org/10.1038/75556

Atallah, N. M., and J. A. Banks, 2015 Reproduction and the phenotypical regulation of sex type in fern gametophytes. Front. Plant Sci. 6: 100. https://doi.org/10.3389/fpls.2015.00100

Aya, K., Y. Hiwatashi, M. Kojima, H. Sakakibara, M. Ueguchi-Tanaka et al., 2011 The gibberellin perception system evolved to regulate a pre-existing gamyb-mediated system during land plant evolution. Nat. Commun. 2: 544. https://doi.org/10.1038/ncomms1552

Banks, J. A., 1994 Sex-determining genes in the homosporous fern \textit{Ceratopteris}. Development 120: 1949–1958.

Banks, J. A., 1997 The transformer genes of the fern Ceratopteris simultaneously promote meristem and archegonia development and repress antheridia development in the developing gametophyte. Genetics 147: 1885–1897.

Banks, J. A., 1999 Gametophyte development in ferns. Annu. Rev. Plant Biol. 50: 163–186. https://doi.org/10.1146/annurev.arplant.50.1.163

Banks, J. A., L. Hickok, and M. A. Webb, 1993 The programming of sexual phenotype in the homosporous fern \textit{Ceratopteris} richardii. Int. J. Plant Sci. 154: 522–534. https://doi.org/10.1086/297135

Banks, J. A., T. Nishiyama, M. Hasebe, J. L. Bowman, M. Gribskov et al., 2011 The Selaginella genome identifies genetic changes associated with the evolution of vascular plants. Science 332: 960–963. https://doi.org/10.1126/science.1203810

Bannister, A. J., and T. Kouzarides, 2011 Regulation of chromatin by histone modifications. Cell Res. 21: 381–395. https://doi.org/10.1038/cr.2011.22

Benjamini, Y., D. Drai, G. Elmer, N. Kafkafi, and I. Golani, 2001 Controlling the false discovery rate in behavior genetics research. Behav. Brain Res. 125: 279–284. https://doi.org/10.1016/S0166-4328(01)00297-2

Berr, A., E. J. McCallum, R. Ménard, D. Meyer, J. Fuchs et al., 2010 Arabidopsis SET DOMAIN GROUP2 is required for h3k4 trimethylation and is crucial for both sporophyte and gametophyte development. Plant Cell 22: 3232–3248. https://doi.org/10.1105/tpc.110.079962

Calarco, J. P., F. Borges, M. T. Donoghue, F. Van Ex, P. E. Jullien et al., 2012 Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA. Cell 151: 194–205. https://doi.org/10.1016/j.cell.2012.09.001

Cao, M., P. Du, X. Wang, Y.-Q. Yu, Y.-H. Qiu et al., 2014 Virus infection triggers widespread silencing of host genes by a distinct class of endogenous sirnas in \textit{arabidopsis}. Proc. Natl. Acad. Sci. USA 111: 14613–14618. https://doi.org/10.1073/pnas.1407131111
Hau, Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, Gong, Z., T. Morales-Ruiz, R. R. Ariza, T. Roldán-Arjona, L. David, Gregorich, M., and R. Fisher, 2006 Auxin regulates lateral meristem activation in developing gametophytes of Ceratopteris richardii. Botany 84: 973–985.

Finn, R. D., J. Clements, and S. R. Eddy, 2011 Hmmer web server: interactive sequence similarity searching. Nucleic Acids Res. 39: W29–W37. https://doi.org/10.1093/nar/gkr367

Furber, M., L. N. Mander, J. E. Nester, N. Takahashi, and H. Yamane, 2002 Gibberellin signaling in barley aleurone cells. control of SLN1 and pp.010918.

Goff, S. A., M. Vaughn, S. McKay, E. Lyons, A. E. Stapleton, Earl, D., K. Bradnam, J. St John, A. Darling, D. Lin, Eberle, J. R., and J. A. Banks, 1996 Genetic interactions among sex-determining genes in the fern Ceratopteris richardii. Genetics 142: 1081–1093.

Hanlon, M. R., T. Morales-Ruiz, R. R. Ariza, T. Roldán-Arjona, L. David, et al., 2002 ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. Cell 111: 803–814.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, et al., 2011 Full-length transcriptome assembly from RNA-seq data without a reference genome. Nat. Biotechnol. 29: 644–652. https://doi.org/10.1038/nbt.1883

Gregorich, M., and R. Fisher, 2006 Auxin activates lateral meristem initiation in developing gametophytes of Ceratopteris richardii. Botany 84: 1520–1530.

Grossniklaus, U., and R. Paro, 2014 Transcriptional silencing by polycomb-group proteins. Cold Spring Harb. Perspect. Biol. 6: a019331. https://doi.org/10.1101/cshperspect.a019331

Gubler, F., P. M. Chandler, R. G. White, D. J. Llewellyn, and J. V. Jacobsen, 2002 Gibberellin signaling in barley aleurone cells. control of SLN1 and GAMYB expression. Plant Physiol. 129: 191–200. https://doi.org/10.1104/pp.1010981

Hanlon, M. R., M. Vaughn, S. Mock, R. Dooley, W. Moreira et al., 2015 Apartment: an application platform for data discovery. Concurr. Comput. 27: 4412–4422. https://doi.org/10.1002/cpe.3542

Hauter, C. H., 2002 Homospory 2002: An odyssey of progress in pteridophyte genetics and evolutionary biology: Ferns and other homosporous vascular plants have highly polyplid chromosome numbers, but they express traits following diplid models and, although capable of extreme inbreeding, are predominantly outcrossing. A.I.B.S. Bull. 52: 1081–1093.

Hedden, P., and S. G. Thomas, 2012 Gibberellins biosynthesis and its regulation. Biochem. J. 444: 11–25. https://doi.org/10.1042/Bj020245

Hickok, L. G., 1983 Abscisic acid blocks antheridiogen-induced antheridium formation in gametophytes of the fern Ceratopteris. Can. J. Bot. 61: 888–892. https://doi.org/10.1139/b83-098

Hickok, L. G., and R. M. Kiriluk, 1984 Effects of auxins on gametophyte development and sexual differentiation in the fern Ceratopteris thalictroides (L.) brongn. Bot. Gaz. 145: 37–42. https://doi.org/10.1086/337423

Hickok, L. G., T. R. Warne, and M. K. Slocum, 1987 Ceratopteris richardii: applications for experimental plant biology. Am. J. Bot. 74: 1304–1316. https://doi.org/10.1002/j.1537-2197.1987.tb08743.x

Hodges, M. E., B. Wickstead, K. Gull, and J. A. Langdale, 2012 The evolution of land plant cilia. New Phytol. 195: 526–540. https://doi.org/10.1111/j.1469-8137.2012.04197.x

Ibarra, C. A., X. Feng, V. K. Schoff, T.-F. Hsieh, R. Uzawa et al., 2012 Active DNA demethylation in plant companion cells reinforces transposon methylation in gametes. Science 337: 1360–1364. https://doi.org/10.1126/science.1224839

Jullien, P. E., D. Susaki, R. Yelagandula, T. Higashiyama, and F. Berger, 2012 DNA methylation dynamics during sexual reproduction in Arabidopsis thaliana. Curr. Biol. 22: 1825–1830. https://doi.org/10.1016/j.cub.2012.07.061

Kaneshia, M., S. Goto, Y. Sato, M. Furumichi, and M. Tanabe, 2011 Kegg for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 40: D109–D114. https://doi.org/10.1093/nar/gkr988

Kanno, T., B. Huettel, M. F. Mette, W. Aufsatz, E. Jaligot et al., 2005 Atypical RNA polymerase subunits required for RNA-directed DNA methylation. Nat. Genet. 37: 761–765. https://doi.org/10.1038/ng1580

Kawashima, T., and F. Berger, 2014 Epigenetic reprogramming in plant sexual reproduction. Nat. Rev. Genet. 15: 613–624. https://doi.org/10.1038/nrg3685

Kazmierczak, A., 2010 Gibberellic acid and ethylene control male sex determination and development of Anemia phyllitidis gametophytes, pp. 49–65 in Working with Ferns, Springer, New York. https://doi.org/10.1007/978-1-4419-7162-3_5

Kingston, R. E., and J. W. Tamkun, 2014 Transcriptional regulation by trithorax-group proteins. Cold Spring Harb. Perspect. Biol. 6: a019349. https://doi.org/10.1101/cshperspect.a019349

Law, J. A., and S. E. Jacobsen, 2010 Establishing, maintaining and modifi- ding DNA methylation patterns in plants and animals. Nat. Rev. Genet. 11: 204–220. https://doi.org/10.1038/nrg2719

Leng, N. J., A. Dawson, J. A. Thomson, V. Ruotti, A. J. Rissman et al., 2013 EBSeq: an empirical bayes hierarchical model for inference in RNA-seq experiments. Bioinformatics 29: 1035–1043. https://doi.org/10.1093/bioinformatics/btt877

Li, B., and C. N. Dewey, 2011 RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. BMC Bioinformatics 12: 323. https://doi.org/10.1186/1471-2105-12-323

Li, Z., A. E. Baniaga, E. B. Sessa, M. Scascitelli, S. W. Graham et al., 2015 Early genome duplications in conifers and other seed plants. Annu. Rev. Plant Biol. 61: 395–420. https://doi.org/10.1146/annurev.arplant.043008.091939

Lívak, K. J., and T. D. Schmittgen, 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402–408. https://doi.org/10.1016/s1046-8005(00)80235-8

Lohse, M., A. M. Bolger, A. Nagel, A. R. Fernie, J. E. Lunn et al., 2012 RobRNA: a user-friendly, integrated software solution for RNA-seq-based transcriptomics. Nucleic Acids Res. 40: W622–W627. https://doi.org/10.1093/nar/gks540

Martienssen, R., and D. Moazed, 2015 RNAi and heterochromatin assembly. Cold Spring Harb. Perspect. Biol. 7: a019323. https://doi.org/10.1101/cshperspect.a019323

Martínez, G., K. Panda, C. Köhler, and R. K. Slotkin, 2016 Silencing in sperm cells is directed by RNA movement from the surrounding nurse cell. Nat. Plants 2: 16030. https://doi.org/10.1038/nplants.2016.30

McAdam, S. A., T. J. Brodribb, J. A. Banks, R. Hedrich, N. M. Atallah et al., 2016 Abscisic acid controlled sex before transpiration in vascular plants. Proc. Natl. Acad. Sci. USA 113: 12862–12867. https://doi.org/10.1073/pnas.160661113

Menéndez, V., M. Revilla, M. Fal, and H. Fernández, 2009 The effect of cytokinins on growth and sexual organ development in the gametophyte of Blechnum spicant I. Plant Cell Tissue Organ Cult. 96: 245–250 (PCTOC). https://doi.org/10.1007/s11240-008-9481-y
Saito, R., M. E. Smoot, K. Ono, J. Ruscheinski, P.-L. Wang

2014 Epigenetic regulation in plants. Cold Spring Harb. Perspect. Biol. 6: a019315. https://doi.org/10.1101/cshperspect.a019315

Pilott, M., C. Baroux, M. A. Vazquez, D. Autran, O. Leblanc

Pikaard, C. S., and O. M. Scheid, 2014 Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametophytes. Genetics 184: 1271–1281. https://doi.org/10.1093/gene/tzw198

Strain, E., B. Hass, and J. A. Banks, 2001 Characterization of mutations that feminize gametophytes of the fern Ceratopteris. Genetics 159: 1271–1281.

Su, Z., L. Zhao, Y. Zhao, S. Li, S. Won et al., 2017 The tho complex non-cell-autonomously represses female germline specification through the tas3-arf3 module. Curr. Biol. 27: 1597–1609.e2. https://doi.org/10.1016/j.cub.2017.05.021

Sun, T., and Y. Kamiya, 1994 The Arabidopsis GA1 locus encodes the cyclase ent-kaurene synthetase a of gibberellin biosynthesis. Plant Cell 6: 1509–1518. https://doi.org/10.1105/tpc.6.10.1509

Sun, T.-P., 2008 Gibberellin metabolism, perception and signaling pathways in Arabidopsis. The Arabidopsis Book 6: e0103. https://doi.org/10.1199/tab.0103

Sussmilch, F. C., N. M. Atallah, T. J. Brodribb, J. A. Banks, and S. A. McAdam, 2017 Abscisic acid (aba) and key proteins in its perception and signaling pathways are ancient, but their roles have changed through time. Plant Signal. Behav. 12: e1365210. https://doi.org/10.4161/psb.28175

Takeno, K., H. Yamane, T. Yamauchi, N. Takahashi, M. Furber et al., 1989 Biological activities of the methyl ester of gibberellin a73, a novel and principal antheridiogen in Lygodium japonicum. Plant Cell Physiol. 30: 201–205. https://doi.org/10.1093/oxfordjournals.pcp.a077730

Tanaka, J., K. Yano, K. Aya, K. Hirano, S. Takehara et al., 2014 Antheridiogen determines sex in ferns via a spatiotemporally split gibberellin synthesis pathway. Science 346: 469–473. https://doi.org/10.1126/science.1259923

Tanurdzic, M., and J. A. Banks, 2004 Sex-determining mechanisms in land plants. Plant Cell 16: S61–S71. https://doi.org/10.1105/tpc.010667

Warne, T. R., and L. G. Hickok, 1989 Evidence for a gibberellin biosynthetic origin of Ceratopteris antheridiogen. Plant Physiol. 95: 535–538. https://doi.org/10.1104/pp.89.2.535

Warne, T. R., and L. G. Hickok, 1991 Control of sexual development in gametophytes of Ceratopteris richardii: Antheridiogen and abscisic acid. Bot. Gaz. 152: 148–153. https://doi.org/10.1086/337874

Waterhouse, R. M., M. Seppey, F. A. Simão, M. Manni, P. Ioannidis et al., 2018 Busco applications from quality assessments to gene prediction and phylogenomics. Mol. Biol. Evol. 35: 543–548. https://doi.org/10.1093/molbev/msx319

Xi, W., C. Liu, X. Hou, and H. Yu, 2010 Mother of ft and tfl1 regulates seed germination through a negative feedback loop modulating aba signaling in arabidopsis. Plant Cell 22: 1733–1748. https://doi.org/10.1105/tpc.109.073072

Xiao, J., U.-S. Lee, and D. Wagner, 2016 Tug of war: adding and removing histone lysine methylation in Arabidopsis. Curr. Opin. Plant Biol. 34: 41–53. https://doi.org/10.1016/j.pbi.2016.08.002

Yamane, H., 1998 Fern antheridiogens. Int. Rev. Cytol. 184: 1–54. https://doi.org/10.1016/S0074-7696(08)62177-4

Yamane, H., K. Nohara, N. Takahashi, and H. Schraudolf, 1987 Identification of antheridiogen as an antheridiogen in anemia rotundifolia and anemia flexuosa. Plant Cell Physiol. 28: 1203–1207.

Yamauchi, Y., M. Ogawa, A. Kuwahara, A. Hanada, Y. Kamiya et al., 2004 Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of arabidopsis thaliana seeds. Plant Cell 16: 367–378. https://doi.org/10.1105/tpc.018143

Yang, L., G. Wu, and R. S. Poethig, 2012 Mutations in the GW-repeat protein SUO reveal a developmental function for microrna-mediated translesional repression in Arabidopsis. Proc. Natl. Acad. Sci. USA 109: 315–320. https://doi.org/10.1073/pnas.1114673109

Zhang, C., L. Cao, L. Rong, Z. An, W. Zhou et al., 2015 The chromatin-remodeling factor ATIN080 plays crucial roles in genome stability maintenance and in plant development. Plant J. 82: 655–668. https://doi.org/10.1111/tpj.12840

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