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# Spatial localization of conserved developmental regulators during apogamic sporophyte initiation in the fern *Dryopteris affinis*

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## Abstract

Apogamy represents an alternative reproductive strategy in ferns in which sporophyte development occurs without fertilization, although its molecular regulation remains poorly understood. Here, we investigated conserved regulatory pathways potentially associated with apogamic development in gametophytes of the obligately apogamous fern *Dryopteris affinis*. A transcriptome-wide, homology-guided analysis combined with conserved domain annotation and manual curation identified 17 candidate genes, including components of small RNA silencing, chromatin and Polycomb-mediated regulation, and embryogenic AP2/AIL transcription factors. Protein–protein interaction analysis identified two major predicted regulatory modules associated with small RNA-mediated gene silencing and Polycomb-mediated chromatin regulation. Differential expression analysis showed significant upregulation of embryogenesis-related AP2/AIL/PLT regulators, particularly BBM, AIL1, and PLT2. PCR amplification and sequencing confirmed the identity of 12 candidate transcripts, including BBM, SERK1, MEE29, SE, AIL1, PLT2, AGO1, FIS1, FIE2, WOX13, DCL1, and MSI1. RNA fluorescence in situ hybridization (RNA-FISH) revealed spatially restricted signals for BBM, AIL1, MEE29, and SE in the apogamic centre of heart-shaped gametophytes, whereas no signal was detected at the earlier filamentous or spatulate stages. Together, these results reveal stage- and region-specific expression of conserved developmental regulators in apogamous gametophytes, linking their expression to the developmental transition associated with apogamic sporophyte initiation.


## 1. Introduction

Apogamy is an asexual reproductive mode in ferns in which the sporophyte develops directly from somatic cells of the gametophyte without fertilization (Liu et al. 2012; Grusz et al. 2021). Unlike seed plants, where apomixis is restricted to relatively few evolutionary lineages (Koltunow and Grossniklaus 2003; Hand and Koltunow 2014), apogamy occurs naturally in numerous fern lineages and represents one of the most widespread modes of asexual reproduction in this group (Raghavan 1989; Sheffield and Bell 2002). Because ferns possess two independent free-living generations, the gametophyte and the sporophyte, they provide a unique experimental system for investigating the developmental and molecular mechanisms that initiate sporophyte formation in the absence of fertilization (Atallah and Banks 2015). Studying apogamy not only improves our understanding of the origin and regulation of this reproductive strategy in ferns but also provides a valuable evolutionary framework for investigating fertilization-independent reproduction across land plants (Grossniklaus 2007; Goeckeritz et al. 2024). Furthermore, understanding the molecular basis of apogamy may ultimately contribute to the development of novel strategies for engineering clonal reproduction in crop species, a long-standing objective in plant breeding (Koltunow and Grossniklaus 2003; Qu et al. 2024).

During the past two decades, considerable progress has been made in elucidating the molecular basis of apomixis in flowering plants (Hand and Koltunow 2014; Goeckeritz et al. 2024). However, this progress has been uneven across the different developmental components of the process. Functional evidence has been obtained primarily for parthenogenesis, where genes such as *PsASGR-BBML* in aposporous grasses (Conner et al. 2015) and *ToPAR* in diposporous *Taraxacum* (Underwood et al. 2022) have been shown to initiate embryo development from the unfertilized egg cell. By contrast, the molecular mechanisms underlying apomeiosis, including both apospory and diplospory, as well as autonomous endosperm development and sporophytic apomixis, remain largely unresolved, with no universally conserved regulatory genes identified to date (Goeckeritz et al. 2024). In

ferns, however, our understanding of the molecular regulation of natural apogamy remains extremely limited, and no gene has yet been functionally demonstrated to regulate this developmental process.

*Dryopteris affinis* (Lowe) Fraser-Jenk. ssp. *affinis* is an obligately apogamous fern, due to the absence of female gametangia, which prevents sexual reproduction (Fraser-Jenkins, 1980). This species has served as a model system in a series of studies by our group addressing its transcriptome, proteome and molecular features associated with apogamous development (Grossmann et al. 2017; Wyder et al. 2020; Fernández et al. 2021; Ojosnegros et al. 2022, 2023, 2024; Fernández et al. 2026). Previous analysis of the *D. affinis* gametophyte transcriptome identified homologs of several *Arabidopsis thaliana* regulators associated with embryogenesis, RNA silencing, epigenetic control, meristem organization and reproductive development (Ojosnegros et al. 2024). These findings suggested that conserved regulatory pathways involved in reproductive and developmental transitions in seed plants may also be associated with gametophyte development and apogamic sporophyte initiation in *D. affinis*.

Although transcriptomic analyses can identify candidate genes potentially involved in developmental reprogramming, they do not reveal the tissues, cell types or developmental stages in which these genes are expressed. Because spatially restricted gene expression plays a fundamental role in cell fate specification and developmental patterning in plants, characterization of transcript localization is essential for linking candidate gene expression to specific developmental contexts. RNA in situ hybridization approaches, including fluorescence in situ hybridization, enable the visualization of specific transcripts within intact tissues, thereby establishing spatial associations between gene expression patterns and developmental processes (Jackson 1992; Houben et al. 2006; Wang et al. 2008; Yang et al. 2016).

Despite their widespread application in flowering plants, these approaches have only rarely been applied to ferns and, to our knowledge, have not been used to investigate the spatial expression of candidate genes potentially involved in apogamy in the gametophytes of obligately apogamous ferns.

In the present study, we systematically explored the *D. affinis* gametophyte transcriptome to identify regulatory genes potentially associated with reproductive and developmental reprogramming. Candidate identification combined sequence homology, protein-domain information, and manual functional curation, revealing putative regulators associated with embryogenic competence, RNA-mediated silencing, epigenetic regulation, meristematic activity, and developmental signaling. A subset of 17 candidates was selected for further characterization through differential expression analysis and, for selected genes, amplification and sequencing to confirm their identity. Spatial and temporal expression patterns of four genes were further investigated by RNA fluorescence in situ hybridization (RNA-FISH) coupled with confocal microscopy. Together, these analyses were used to test the hypothesis that conserved developmental regulators show stage- and region-specific expression associated with apogamic sporophyte initiation in fern gametophytes.

## 2. Material and methods

# 2.1. Computational protein annotation

The protein annotation was performed using the assembled transcriptome of *D. affinis* obtained previously (Wyder et al. 2020) (Fig. 1). The original transcriptome assembly was generated from RNA-seq libraries prepared using the TruSeq RNA Sample Prep Kit v. 2 and sequenced on an Illumina HiSeq 2000 platform. The

Transcriptome Shotgun Assembly project is available in the European Nucleotide Archive (ENA) under accession number PRJEB18522, while the assembled transcript sequences in FASTA format and the corresponding annotation are available in Zenodo (https://doi.org/10.5281/zenodo.1040330).

Open reading frames were predicted using TransDecoder v5.7.1, and the resulting peptide sequences were subjected to homology searches against the curated SwissProt database using DIAMOND v2.1.9. Two complementary levels of homology analysis were used: best-hit (top1) assignments provided a non-redundant functional overview, whereas the five best hits per query (top5) were retained to increase sensitivity in large and paralog-rich gene families. Protein domain annotation was independently performed using HMMER v3.4 against the Pfam-A database, and top5 homology results were integrated with Pfam domain annotations for candidate discovery and structural validation.

A targeted screening was subsequently performed using a predefined list of reproduction-related keywords and gene-family identifiers, including AP2, AIL, BABY BOOM, PLETHORA, ARGONAUTE, DICER, RDR, WOX, Polycomb, FIE, MSI, meiosis, gametophyte, and embryogenesis. Because no genes uniquely responsible for apogamy have been identified to date, candidate discovery was guided by regulatory systems repeatedly implicated in plant reproductive reprogramming, including small RNA-mediated silencing pathways, DNA methylation machinery, chromatin modifiers, Polycomb-associated regulators, and embryogenic transcription factors.

The resulting set of putative reproduction-related genes was manually curated based on E-value, bit score, sequence identity, concordance of functional annotation among the five best SwissProt hits, and the presence of diagnostic Pfam domains when applicable. Particular weight was given to consistency of functional assignment among the top five matches to reduce the impact of potentially spurious best hits, yielding a refined set of high-confidence candidate proteins.

## 2.2. Protein-protein interactions and domain analyses

Potential functional associations among the selected candidate proteins were explored using STRING v12.0 (Szklarczyk et al. 2023). Because *D. affinis* is not represented in STRING, the corresponding *Arabidopsis thaliana* homologs identified by sequence homology were used to construct the interaction network, applying a minimum required interaction score of 0.700 (high confidence). STRING associations derived from the available evidence channels, including experimental data, curated databases, text mining, co-expression, genomic context, and homology, were considered. Accordingly, the resulting network was interpreted as a reference-based prediction of conserved functional associations rather than as evidence of direct protein–protein interactions in *D. affinis*.

The sequences of the selected *D. affinis* proteins were analyzed using SMART v10.0 (Letunic and Bork 2026) to identify conserved protein domains. Domain architectures were compared with those of the corresponding *A. thaliana* homologs analyzed using the same procedure. *A. thaliana* protein sequences were retrieved from the TAIR database.

## 2.3. Transcript quantification and differential expression analysis

The de novo transcriptome assembly of *D. affinis* gametophytes generated by Wyder et al. (2020) was used as reference for transcript quantification. RNA-seq reads were quantified against the reference transcriptome using Salmon. Transcript abundance estimates were imported into R (R Core Team 2025) using the tximport package

and analyzed using DESeq2. Differential expression was assessed between one-dimensional (1D) and two-dimensional (2D) gametophytes, with three biological replicates per developmental stage. Differential expression analysis was performed using the negative binomial generalized linear model implemented in DESeq2. Normalization factors were estimated using the median-of-ratios method, and dispersion parameters were estimated from biological replicates. Differentially expressed transcripts were identified based on $\log_2$ fold change ($\log_2$FC) values and Benjamini–Hochberg adjusted p-values (padj). Transcripts with padj < 0.05 were considered significantly differentially expressed. Positive $\log_2$FC values indicate higher transcript abundance in 2D relative to 1D gametophytes.

## 2.4. Phylogenetic analysis of selected candidate genes

To provide additional support for the annotation of selected candidate genes belonging to multigene families, phylogenetic analyses were performed. Reference protein sequences and corresponding alignments for the WOX and euANT (AP2/AIL) gene families were obtained from the phylogenetic studies of Wu et al. (2019) and Dipp-Álvarez and Cruz-Ramírez (2019), respectively. The euANT phylogeny comprised 128 sequences (124 reference sequences plus 4 *D. affinis* candidates), whereas the WOX phylogeny included 7 sequences (26 reference sequences plus the *D. affinis* WOX13 candidate). The predicted *D. affinis* protein sequences were incorporated into the corresponding reference alignments using MAFFT v.7 (Katoh and Standley 2013). Maximum-likelihood phylogenetic trees were inferred with IQ-TREE 2 (Minh et al. 2020) using the substitution model employed in each reference study. Branch support was assessed with 1,000 SH-aLRT replicates and 1,000 ultrafast bootstrap replicates. The resulting phylogenies were used to provide additional support for the annotation of the selected candidate genes.

## 2.5. In vitro spore culture, collection and freezing of gametophytes

Fronds from sporophytes of *D. affinis*, collected in Turón forest (Asturias, Spain; 43°12'10" N – 5°43'43" W; 477 m a.s.l.), were placed between paper sheets to release spores from sporangia. The collected spores were stored at 4 ºC until use. Before *in vitro* culture, spores were surface-sterilized. To this end, 5 mg of spores were immersed in distilled water at room temperature overnight for hydration, then centrifuged at 2,500 rpm for 5 min to remove residual sporangial material and treated with a 5 g/L NaClO solution for 10 min. After sterilization, spores were rinsed three times with distilled water, with centrifugation at 2,500 rpm for 4 min between each wash. Sterilized spores were then cultured in Erlenmeyer flasks containing 50 mL of liquid Murashige and Skoog medium (Murashige and Skoog 1962), supplemented with 2% (w/v) sucrose, with the pH adjusted to 5.7. Cultures were maintained on a rotary shaker at 75 rpm in a growth chamber at 25°C, under a photosynthetically active radiation intensity of 70 µmol/$m^2$·s and a 16 h light photoperiod. Approximately three weeks after culture initiation, spores began to germinate and were transferred to culture vessels containing solid Murashige and Skoog medium, supplemented with 0.7% (w/v) agar as a solidifying agent. Cultures were maintained under the same conditions described above (25°C, light intensity of 70 µmol/$m^2$·s and a 16 h photoperiod). Approximately one month after transferring to solid medium, gametophytes at the cordate stage were collected into Eppendorf tubes. Each tube contained 100 mg of gametophytes. They were immediately frozen in liquid nitrogen (– 196°C) and stored at – 80°C until use.

## 2.6. Gametophyte homogenization, RNA extraction and quantification

Two tubes containing cordate stage gametophytes stored in the freezer were homogenized by grinding in liquid nitrogen using a pre-chilled mortar until a fine powder was obtained. RNA was extracted using the GeneMATRIX Universal RNA Purification Kit (EURX Molecular Biology Products), following the manufacturer's instructions for plant tissue. RNA quantification was performed using a Nabi Nano UV-Vis spectrophotometer (MicroDigital Co., South Korea). The two tubes with RNA samples were stored at − 80°C.

## 2.7. cDNA synthesis

cDNA was synthesized from 0.5 µg of total RNA using the NG dART RT-PCR Kit (EURX Molecular Biology Products), according to the manufacturer's protocol (version 3.0). Each 10 µL reaction contained 2 µL of 5× NG cDNA Buffer, 0.25 µL of Oligo(dT)20 primer, 0.25 µL of random hexamer primer, 0.5 µL of NG dART RT Mix, RNA template, and RNase-free water. RNA input was normalized to ensure equal amounts of total RNA in each reaction. Reverse transcription was performed at 25°C for 10 min, 50°C for 1 h, and 85°C for 5 min, followed by holding at 4°C. Synthesized cDNA was stored at − 20°C until use.

## 2.8. Selection of candidate genes for experimental validation and primer design

Based on the integrative computational analyses described above, a representative and experimentally manageable subset of 17 candidate genes was selected for experimental validation. Candidate selection aimed to encompass the major regulatory pathways potentially associated with reproductive and developmental reprogramming identified in the computational analysis, including RNA silencing: *ARGONAUTE 1 (AGO1), ARGONAUTE 4 (AGO4), ARGONAUTE 9 (AGO9)* and *DICER-LIKE 1 (DCL1)*; embryogenesis and stem-cell identity: *BABY BOOM (BBM)* and *PLETHORA 2 (PLT2)*; receptor-mediated signaling: *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1)*; Polycomb-associated epigenetic regulation: *FERTILIZATION-INDEPENDENT ENDOSPERM (FIE), FERTILIZATION INDEPENDENT SEED 1 (FIS1), FERTILIZATION-INDEPENDENT ENDOSPERM 2 (FIE2)* and *MULTICOPY SUPPRESSOR OF IRA 1 (MSI1);* meristem organization: *FASCIATED STEM 4 (FAS4);* RNA processing: *SERRATE (SE);* embryo development: *MATERNAL EFFECT EMBRYO ARREST 29 (MEE29)*; and transcriptional regulation associated with developmental processes: *WUSCHEL RELATED HOMEOBOX 13 (WOX13)* and the AP2-like ethylene-responsive transcription factors AIL1 and AIL7.

## 2.9. Candidate genes amplification and sequencing

Lyophilized primers were resuspended in ultrapure water to obtain stock solutions at equal concentrations according to the amount supplied by the manufacturer and subsequently diluted 1:10 for use. Primer stock solutions were stored at − 20°C. Synthesized cDNA was diluted 1:10 with ultrapure water before PCR amplification. The expected amplicon length for each candidate gene was considered when determining extension times, using an approximation of 1 min per kilobase.

PCR reactions were performed in a final volume of 50 µL using the NG dART RT-PCR Kit (EURX Molecular Biology Products) according to the manufacturer's instructions. Amplification conditions consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing at 52–56°C for 30 s (optimized for each gene), and extension at 72°C for 3 min, followed by a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gels in 1× sodium borate buffer and visualized using GeneSys software. Products corresponding to each candidate gene were purified using the GeneMATRIX Basic DNA Purification Kit (EURX Molecular Biology Products), and DNA concentration was

determined using a Nabi Nano UV-Vis spectrophotometer (MicroDigital Co., South Korea). Purified products were subjected to Sanger sequencing at the Scientific-Technical Services of the University of Oviedo (Oviedo, Spain), which provided the corresponding sequence data for each amplified product.

## 2.10. Microscopic observation, collection, and fixation of gametophytes at different developmental stages

To obtain gametophyte samples for subsequent fluorescence in situ hybridization, spores were germinated in vitro in liquid MS medium and the resulting gametophytes were transferred to solid medium as previously described. Development was monitored weekly by light microscopy. Ten days after transfer, filamentous gametophytes were collected and fixed in FAA (3.9% formaldehyde, 50% ethanol, 5% glacial acetic acid) for 2 h, following 2 min of vacuum infiltration and gentle mixing every 30 min. Samples were then washed five times with PBS (pH 7.4) and stored in 70% ethanol at 4°C until further processing. Gametophytes at the spatulate and heart-shaped stages were collected at successive 10-day intervals and fixed following the same procedure.

## 2.11. RNA fluorescence in situ hybridization (RNA-FISH)

Four genes were selected from the previously sequenced candidates: BABY BOOM (BBM), an AP2-like ethylene-responsive transcription factor (AIL1), SERRATE (SE), and MATERNAL EFFECT EMBRYO ARREST 29 (MEE29). Gene-specific probes were designed to hybridize to a specific region of each target mRNA and were labelled with Cyanine 5 (Cy5) at the 5' end.

Fluorescence in situ hybridization was performed on fixed gametophytes at the filamentous, spatulate, and heart-shaped stages using Cy5-labelled probes targeting the selected candidate genes. Samples were rehydrated through an ethanol series, washed in PBS, and treated with 2% cellulase for 1 h at 37°C, followed by permeabilization with 0.2 N HCl for 15 min and equilibration in 2× SSC. Pre-hybridization was performed in HB50 buffer (50% formamide, 2× SSC) for 1 h at 37°C. Samples were denatured at 78°C for 5 min and hybridized overnight at 55°C in buffer containing 50% formamide, 2× SSC, 10% dextran sulfate, and Cy5-labelled probes. Negative controls were processed in parallel without probes. After hybridization, samples were sequentially washed in 2×, 1×, and 0.5× SSC, counterstained with DAPI, and mounted in Mowiol for microscopic observation.

Gametophytes at the three developmental stages subjected to FISH were imaged using a Leica TCS SP8 confocal microscope at the Scientific-Technical Services, University of Oviedo (Oviedo, Spain). Cy5 fluorescence was detected using excitation at 630–650 nm and emission at 660–700 nm. A total of 786 gametophytes were subjected to FISH, comprising 298 filamentous, 373 spatulate, and 115 heart-shaped gametophytes. Approximately 35% of the FISH-processed gametophytes from each developmental stage were subsequently examined by confocal microscopy.

# 3. Results

## 3.1. Homology-guided identification

Analysis of the *D. affinis* gametophyte transcriptome identified a diverse set of homologues of genes associated with reproductive and developmental regulation (Fig. 2), including embryogenic transcription factors, epigenetic and chromatin regulators, small RNA pathway components, hormone-related developmental regulators, and genes associated with gametophyte development and meiosis. Most of the identified genes belonged to the

category of chromosome dynamics / cohesion / cell-cycle control, followed by post-transcriptional / RNA processing with reproductive relevance, and auxin / hormone-dependent reproductive patterning.

A representative subset of 17 candidates selected for subsequent analyses is summarized in Table 1, together with the differential expression and experimental evidence obtained in the present study.

Table 1

Summary of candidate genes potentially associated with apogamy and related developmental processes identified in the *Dryopteris affinis* gametophyte transcriptome. Differential expression and supporting evidence obtained in the present study are also indicated.

| Gene | Transcript identifier | Annotation | $log_2$ FC | padj | Supporting evidence | Function associated with the homologous gene |
|---|---|---|---|---|---|---|
| *BBM* | 369145-36 | BBM/AP2-like | 5.746 | $1.29 \cdot 10^{-10}$ | Domain comparison + PCR + sequencing + FISH | Embryogenic competence / cellular reprogramming |
| *SERK1* | 394361-25 | SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 | -0.234 | 0.167 | Domain comparison + PCR + sequencing | Development-associated signaling |
| *FIE* | 78705-284 | FIE/FIE2-like Polycomb group protein | 0.027 | 0.965 | Domain comparison + Transcriptome annotation | PRC2-mediated epigenetic regulation |
| *FIE2* | 15170-627 | FIE2-like Polycomb group protein | -0.201 | 0.526 | Domain comparison + PCR + sequencing | PRC2-mediated epigenetic regulation |
| *FAS4* | 214887-125 | FAS4/DEAH13-like | 0.048 | 0.905 | Domain comparison + PCR | Regulation of development / proliferation |
| *MSI1* | 765–1800 | MSI1-like | -0.138 | 0.63 | Domain comparison + PCR + sequencing | PRC2 / chromatin complex |
| *MEE29* | 77-3381 | MEE29/DEAH6-like | -0.067 | 0.873 | Domain comparison + PCR + sequencing + FISH | Embryogenesis-associated processes |
| *SE* | 284827-77 | SERRATE/SRRT-like | 0.068 | 0.841 | Domain comparison + PCR + sequencing + FISH | Small RNA processing |
| *AIL1* | 113636-233 | AIL1/AP2-like | 2.405 | $1.08 \cdot 10^{-29}$ | Domain comparison + PCR + sequencing + FISH | Embryogenic competence |
| *AIL7* | 196074-141 | AP2/AIL family candidate | 0.602 | $5.32 \cdot 10^{-5}$ | Domain comparison + PCR | Embryonic development regulation |
| *PLT2* | 45126-379 | PLT2/AP2-like | 4.142 | $7.45 \cdot 10^{-14}$ | Domain comparison + | Embryonic patterning and development |

| Gene | Transcript identifier | Annotation | $log_2$ FC | padj | Supporting evidence | Function associated with the homologous gene |
|---|---|---|---|---|---|---|
| | | | | | PCR + sequencing | |
| *AGO1* | 3555 – 1139 | AGO1-like | 0.533 | $3.39 \cdot 10^{-5}$ | Domain comparison + PCR + sequencing | Small RNA-mediated gene silencing |
| *FIS1* | 10125-750 | FIS1-related | -0.132 | 0.656 | Domain comparison + PCR + sequencing | Epigenetic regulation/development |
| *WOX13* | 20354-552 | WOX13-like | -0.557 | $1.73 \cdot 10^{-3}$ | Domain comparison + PCR + sequencing | Developmental reprogramming |
| *DCL1* | 212798-127 | DCL1-like | -0.095 | 0.811 | Domain comparison + PCR + sequencing | miRNA biogenesis |
| *AGO4* | 283466-78 | AGO4-like | 0.67 | $9.8 \cdot 10^{-3}$ | Domain comparison + Transcriptome annotation | RNA-directed gene silencing / epigenetic regulation |
| *AGO9* | 353942-42 | AGO9-like | -0.035 | 0.975 | Domain comparison + Transcriptome annotation | Reproductive gene silencing |

*$log_2$FC, $log_2$ fold change; padj, Benjamini–Hochberg-adjusted p-value.

# 3.2. Protein-protein interactions and domain analyses

Protein-protein interactions among the genes identified in the *D. affinis* gametophyte transcriptome that are potentially related to apomixis were analyzed using STRING (Fig. 3). The resulting network consisted of 17 nodes, of which 12 were interconnected by 13 edges, while the remaining five nodes showed no interactions, with a PPI enrichment p-value of $8.01 \cdot 10^{-7}$, indicating that the proteins exhibited significantly more interactions than would be expected for a random set of proteins of similar size and degree distribution. The average node degree was 1.53, the average local clustering coefficient was 0.49, and the expected number of edges was 2. The proteins with the highest number of interactions were ARGONAUTE 1 (AGO1), DICER-LIKE 1 (DCL1) and SERRATE (SE), each with 4 interactions. Regarding the type and strength of the predicted interactions, the strongest co-occurrence evidence was identified between FASCIATED STEM 4 (FAS4) and MATERNAL EFFECT EMBRYO ARREST 29 (MEE29). The strongest homology was observed between ARGONAUTE 1 (AGO1) and 9 (AGO9). The strongest co-expression evidence was observed between MATERNAL EFFECT EMBRYO ARREST 29 (MEE29) and SERRATE (SE). The strongest association based on experimental evidence was identified between FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) and FERTILIZATION-INDEPENDENT SEED 1 (FIS1), whereas the strongest databases- and text mining-supported associations were found between FERTILIZATION-

INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1). In contrast, no evidence of associations based on neighborhood or gene fusion was observed.

A comparison of the amino acid domains of the selected proteins and their corresponding homologues in *A. thaliana* was also performed using SMART (Fig. 4 and Supplementary Fig. 1). Domain comparison of the predicted proteins showed that several candidates displayed complete conservation in both the number and composition of functional domains relative to their corresponding *A. thaliana* homologs, such as BABY BOOM (BBM), the two AP2-like ethylene-responsive transcription factors (AIL1 and AIL7), PLETHORA 2 (PLT2), WUSCHEL-RELATED HOMEOBOX 13 (WOX13), and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE). Two candidates exhibited differences in domain architecture. For example, MSI1 differed in both domain composition and number between *D. affinis* and *A. thaliana*. MSI1 from *D. affinis* contained six WD40 domains, whereas its *A. thaliana* homologue contained the same six WD40 domains plus an N-terminal CAF1C_H4-bd domain.

FERTILIZATION-INDEPENDENT ENDOSPERM (FIE):

MULTICOPY SUPPRESSOR OF IRA 1 (MSI1):

# 3.3. Identification and expression patterns of candidate genes associated with apogamy

Differential expression analysis of the 17 selected candidate genes revealed distinct expression patterns between one-dimensional and two-dimensional gametophytes (Table 1). The identified transcripts included key regulators belonging to the AP2/AIL/PLT family, such as *BABY BOOM (BBM)*, *AINTEGUMENTA-LIKE 1 (AIL1)* and *PLETHORA 2 (PLT2)*. They showed marked differential abundance in the analyzed dataset, with positive $\log_2$ fold changes of 5.75, 2.41 and 4.14, respectively, and highly significant adjusted p-values (padj = $1.29 \cdot 10^{-10}$, $1.08 \cdot 10^{-29}$ and $7.45 \cdot 10^{-14}$). An additional AP2/AIL family transcript, corresponding to an *AIL7*-like candidate, was also detected and showed increased abundance ($\log_2$ FC = 0.6; padj = $5.32 \cdot 10^{-5}$).

Several genes associated with small RNA-mediated regulations were also identified. The validated transcripts included *ARGONAUTE 1 (AGO1)*, *4 (AGO4)*, and *9 (AGO9)*, *DICER-LIKE 1 (DCL1)*, and *SERRATE (SE)*. Among these, *AGO1* and *AGO4* showed significant changes in transcript abundance (*AGO1*: $\log_2$ FC = 0.53, padj = $3.39 \cdot 10^{-5}$; *AGO4*: $\log_2$ FC = 0.67, padj = $9.8 \cdot 10^{-3}$), whereas *AGO9*, *DCL1* and *SE* showed no significant differential accumulation under the analyzed conditions.

Candidates related to chromatin regulation and Polycomb-associated pathways were also recovered, including *FERTILIZATION-INDEPENDENT ENDOSPERM (FIE)*, *FERTILIZATION-INDEPENDENT ENDOSPERM 2 (FIE2)*, and *MULTICOPY SUPPRESSOR OF IRA 1 (MSI1)*. These transcripts were detected in the gametophyte transcriptome but did not show significant differential abundance (*FIE*: $\log_2$ FC = 0.03, padj = 0.965; *FIE2*: $\log_2$ FC = − 0.2, padj = 0.526; *MSI1*: $\log_2$ FC = − 0.14, padj = 0.63).

Additional developmental and signaling candidates included *WUSCHEL RELATED HOMEOBOX 13 (WOX13) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1). WOX13* showed significantly lower transcript abundance in two-dimensional gametophytes ($\log_2$FC = − 0.56; padj = $1.73 \times 10^{-3}$), whereas SERK1 did not show significant differential abundance ($\log_2$FC = − 0.23; padj = 0.167). The embryogenesis-associated candidates

*MATERNAL EFFECT EMBRYO ARREST 29 (MEE29)* and *FASCIATED STEM 4 (FAS4)* were also detected but showed no significant differential accumulation (*MEE29*: $\log_2$FC = − 0.07, padj = 0.873; *FAS4*: $\log_2$FC = 0.05, padj = 0.905).

## 3.4. Phylogenetic analyses

Phylogenetic analyses were performed to provide additional support for the annotation of selected candidate genes belonging to the WOX and euANT (AP2/AIL) gene families. The *D. affinis* WOX candidate was placed within the WOX13 lineage (Fig. 5). Within the euANT family, the AIL candidates were placed within the AIL lineage, whereas *DaBBM* and *DaPLT2* were positioned within the BBM and PLT lineages, respectively (Fig. 6). Overall, the phylogenetic results were consistent with the annotations obtained from sequence similarity searches and conserved-domain analyses, providing additional support for the assignment of these *D. affinis* candidate genes.

## 3.5. Sequence validation

Gene-specific primers were designed for amplification of the 17 selected candidate genes. The corresponding transcript sequences are provided in Supplementary Table 1, and the forward and reverse primer sequences are shown in Supplementary Table 2.

Specific PCR amplification was obtained for 14 of the 17 candidate genes. Single bands of the expected size were obtained for *BABY BOOM (BBM), SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), FASCIATED STEM 4 (FAS4), MATERNAL EFFECT EMBRYO ARREST 29 (MEE29), SERRATE (SE)*, the two AP2-like ethylene-responsive transcription factors AIL1 and AIL7, *PLETHORA 2 (PLT2), ARGONAUTE 1 (AGO1), FERTILIZATION INDEPENDENT SEED 1 (FIS1), FERTILIZATION-INDEPENDENT ENDOSPERM 2 (FIE2), WUSCHEL RELATED HOMEOBOX 13 (WOX13), DICER-LIKE 1 (DCL1*), and *MULTICOPY SUPPRESSOR OF IRA 1 (MSI1). SERK1, FAS4, MEE29, AGO1, FIS1, FIE2, WOX13, DCL1*, and *MSI1* were successfully amplified at an annealing temperature of 56°C; *BBM, SE*, and *PLT2* at 54°C; and *AIL1* and *AIL7* at 52°C. In contrast, *ARGONAUTE 4 (AGO4)* and *ARGONAUTE 9 (AGO9)* produced multiple bands, whereas no amplification product was detected for *FERTILIZATION-INDEPENDENT ENDOSPERM (FIE).* Thus, specific amplification was not obtained for these three candidates.

Following purification and Sanger sequencing of the PCR products, the obtained sequences were aligned with their corresponding transcripts from the *D. affinis* transcriptome. Sequence comparison confirmed the identity of 12 candidate transcripts: *BBM, SERK1, MEE29, SE, AIL1, PLT2, AGO1, FIS1, FIE2, WOX13, DCL1*, and *MSI1*.

## 3.6. In situ localization

Following sequence validation of the selected candidates, the spatial and temporal expression patterns of four genes, *BABY BOOM (BBM)*, the AP2-like ethylene-responsive transcription factor AIL1, *MATERNAL EFFECT EMBRYO ARREST 29 (MEE29)*, and *SERRATE (SE)*, were investigated in *D. affinis* gametophytes using fluorescence in situ hybridization (FISH). Confocal microscopy revealed hybridization signals for all four transcripts in heart-shaped gametophytes, with the signals spatially localized in the apogamic center (Fig. 7). In contrast, no hybridization signal was detected for any of the four transcripts at the earlier filamentous or spatulate stages. Thus, detectable FISH signals for these four candidate transcripts were restricted to the heart-shaped developmental stage and localized within the apogamic center.

# 4. Discussion

Apogamy is a genetically determined reproductive strategy in ferns whose molecular regulation remains poorly understood. Although genetic factors are known to contribute to apomictic reproduction, increasing evidence from flowering plants indicates that epigenetic mechanisms, including DNA methylation and gene silencing, can also participate in the regulation of fertilization-independent reproductive development (Nogler, 1984; Grimanelli, 2012; Hernández-Lagana et al. 2016). Together with transcriptional and post-transcriptional regulatory pathways, these mechanisms may contribute to the developmental reprogramming underlying asexual reproduction (Barcaccia and Albertini, 2013; Xu et al. 2022). In this context, the identification and characterization of conserved developmental and epigenetic regulators in *D. affinis* provide a molecular framework for investigating the mechanisms potentially associated with apogamy in ferns.

# 4.1. Identification and functional characterization of candidate regulatory genes

The identification of homologues belonging to several conserved regulatory pathways indicates that *D. affinis* possesses components of multiple molecular systems involved in reproductive development. Although their functional involvement in fern apogamy remains to be demonstrated, these genes constitute a coherent set of candidates that could plausibly contribute to this process, based on their established functions in seed plants.

The interactome of the set of proteins potentially related to apomixis was analyzed. AGO1, DCL1 and SE exhibited the highest number of interactions, consistent with their central roles in microRNA biogenesis and RNA-mediated gene silencing. Together, these proteins regulate post-transcriptional gene expression and contribute to plant development and epigenetic regulation through small RNA-mediated pathways in *A. thaliana* (Rogers and Chen 2013; Bologna and Voinnet 2014). Their connectivity in the predicted interaction network is consistent with the potential involvement of small RNA-mediated regulatory mechanisms in gametophyte development and apogamy in *D. affinis*.

The different evidence channels supporting the predicted interactions further reinforced the biological coherence of the network. The co-occurrence of FAS4 and MEE29 suggests that these genes have been evolutionarily conserved together across species, which may reflect their participation in related biological processes or shared functional pathways. The highest homology was observed between AGO1 and AGO9, consistent with their membership in the ARGONAUTE protein family. Both proteins share the conserved PAZ and PIWI domains, reflecting their common evolutionary origin and their central roles in small RNA-mediated gene silencing in *A. thaliana* (Vaucheret 2008; Bologna and Voinnet 2014). The strongest co-expression relationship was detected between MEE29 and SE, suggesting coordinated gene expression and a potential functional association during plant development. Experimental evidence most strongly supported the interaction between FIE and FIS1, consistent with their well-established association as core components of the Fertilization-Independent Seed Polycomb Repressive Complex 2 (FIS-PRC2). FIS-PRC2 is a specialized PRC2 complex involved in the regulation of seed and endosperm development. PRC2 plays a central role in epigenetic gene silencing through H3K27 trimethylation in *A. thaliana* (Köhler and Villar 2008; Hennig and Derkacheva 2009). Likewise, the strongest databases- and text mining-supported associations were identified between FIE and MSI1, another core component of FIS-PRC2. The extensive experimental and literature support for these interactions reflects the well-established role of the PRC2 complex in chromatin remodeling and epigenetic regulation. Given the identification of homologous proteins in *D. affinis*, these findings suggest that Polycomb-mediated regulatory mechanisms may also contribute to the control of reproductive development and represent potential molecular

components involved in apomixis. Overall, the interaction network revealed associations corresponding to two major conserved regulatory modules, namely small RNA-mediated gene silencing and Polycomb-dependent chromatin regulation. The identification of homologues belonging to both pathways provides further support for the hypothesis that conserved epigenetic regulatory mechanisms may contribute to reproductive development and apogamy in ferns.

Domain comparison showed that most candidate proteins displayed identical domain composition and organization to their corresponding *A. thaliana* homologues. In contrast, MSI1 differed in both the number and composition of conserved domains between the two species. The predicted *D. affinis* protein contained six WD40 domains, whereas the *A. thaliana* homologue additionally contained a CAF1C_H4-bd domain. WD40 domains mediate protein–protein interactions and function as scaffolds for the assembly of multiprotein complexes, including PRC2 and CAF-1, where it plays essential roles in chromatin assembly and epigenetic regulation (Hennig et al. 2003; Köhler et al. 2003). The CAF1C_H4-bd domain is a Pfam-defined histone H4-binding domain identified in members of the CAF-1 protein family (Mistry et al. 2021). The conservation of the six WD40 domains suggests that the core scaffolding function of MSI1 is likely retained in *D. affinis*. However, the apparent absence of a detectable CAF1C_H4-bd domain could reflect structural divergence affecting histone H4 binding or other chromatin-associated interactions. Alternatively, this difference may result from an incomplete protein prediction rather than genuine evolutionary divergence. Additional genomic and functional analyses will be required to determine this, and whether it has functional consequences for epigenetic regulation in *D. affinis*.

## 4.2. Molecular validation of candidate genes

Previous transcriptomic studies of *D. affinis* identified numerous candidate genes potentially associated with reproductive development and apogamy through homology searches against *A. thaliana* (Wyder et al. 2020; Ojosnegros et al. 2024). In the present study, the successful amplification and sequencing of twelve selected candidates provided experimental support for these transcriptome-based predictions. The validated genes encompass several major regulatory modules, including transcription factors (*BBM, AIL1, PLT2* and *WOX13*), components of epigenetic and chromatin-mediated regulation (*FIS1, FIE2* and *MSI1*), RNA-mediated silencing pathways (*AGO1* and *DCL1*), and signaling proteins associated with developmental reprogramming (*SERK1*). The recovery of representatives from these distinct regulatory pathways suggests that multiple conserved developmental mechanisms may contribute to the regulation of apogamy in *D. affinis*.

Importantly, the integration of these experimentally validated candidates with the transcriptomic differential expression dataset provided additional evidence supporting their potential involvement in apogamic development. Several key regulators associated with embryogenic competence showed marked changes in transcript abundance. In particular, the AP2/AIL/PLT module displayed a strong transcriptional activation pattern, with *BBM*, *AIL1* and *PLT2* homologues showing increased abundance in the analyzed dataset ($\log_2$ FC = 5.75, 2.41 and 4.14, respectively). Similarly, the *AIL7*-like candidate also showed increased transcript abundance. These results show that the transition to two-dimensional gametophyte development is accompanied by increased transcript abundance of several conserved developmental regulators associated with embryogenic reprogramming.

Phylogenetic analyses of selected candidates from the *WOX* and *euANT* (AP2/AIL) gene families provided additional support for their annotation. The *D. affinis* sequences clustered within the expected gene lineages, in agreement with the annotations inferred from sequence similarity and conserved domain analyses. Together,

these complementary approaches strengthen the annotation in the identity of the selected candidate genes, particularly those belonging to complex multigene families.

Among the validated candidates, the identification of a *BABY BOOM (BBM)* homologue in *D. affinis* is particularly noteworthy. In ferns, heterologous expression of *BBM* from *Brassica napus* has been shown to induce spontaneous apogamous sporophyte formation, while overexpression of the *Ceratopteris richardii AINTEGUMENTA (ANT)* homolog produces similar effects (Boutilier et al. 2002; Bui et al. 2017). These findings suggest that regulatory pathways controlling embryogenesis and developmental reprogramming in seed plants may also operate during apogamy in ferns. Consistent with this hypothesis, homologs of other AP2/ERF family transcription factors, including *AIL1* and *PLT2*, were also validated in *D. affinis*. Members of this family are known to regulate stem-cell maintenance, organ initiation and developmental competence in *A. thaliana* (Aida et al. 2004; Horstman et al. 2014), making them plausible candidates for the regulation of apogamic sporophyte initiation. In addition, the presence of the homolog *WUSCHEL-RELATED HOMEOBOX 13 (WOX13)* was confirmed. *WOX* genes are central regulators of plant development and cell fate determination, and *WOX* homologs have been implicated in both gametophyte and sporophyte development in the fern *C. richardii* (Youngstrom et al. 2019). Although the direct involvement of *WOX13* in apogamy remains unknown, its developmental functions suggest that it may contribute to the regulatory network associated with sporophyte initiation in *D. affinis*.

Gene silencing represents another potential mechanism underlying apomixis in ferns (Barcaccia and Albertini, 2013). The presence of the homologous gene *SERRATE (SE)*, whose protein regulates gene silencing and shoot apical meristem activity in *A. thaliana* (Dong et al. 2008), was detected. Additional validated candidates included *FERTILIZATION-INDEPENDENT ENDOSPERM 2 (FIE2), FERTILIZATION INDEPENDENT SEED 1 (FIS1)*, and *MULTICOPY SUPPRESSOR OF IRA 1 (MSI1)*. Their proteins are components of the Polycomb Repressive Complex 2 (PRC2), which mediates gene silencing through chromatin modification in *A. thaliana* (Köhler et al. 2003). These proteins repress in this species the development of unfertilized embryos and endosperm, and loss of their function can lead to phenotypes resembling parthenogenesis (Ohad et al. 1999; Köhler et al. 2003; Guitton and Berger 2005; Weinhofer et al. 2010). The homologues *ARGONAUTE 1 (AGO1)* and *DICER-LIKE 1 (DCL1)* were also validated in the transcriptome of *D. affinis*. In *A. thaliana*, both genes are involved in RNA-mediated post-transcriptional gene silencing (Qu et al. 2008; Cuperus et al. 2010; Bologna and Voinnet 2014). Together, these genes support the hypothesis that epigenetic and RNA-mediated regulatory mechanisms may contribute to the developmental reprogramming associated with apogamy in *D. affinis*.

RNA helicases may also contribute to apomixis-related processes. Among the validated candidates, *MATERNAL EFFECT EMBRYO ARREST 29 (MEE29)* is of particular interest. Its homolog in *A. thaliana* and related proteins in other plant species are involved in RNA processing and embryogenesis (Barcaccia and Albertini 2013). Although its specific role in fern apogamy remains unknown, the presence of *MEE29* in *D. affinis* further supports the potential importance of post-transcriptional regulation during reproductive reprogramming.

Additional validated candidates included a *SERK1* receptor kinase. Members of the SERK family participate in developmental signaling pathways involved in somatic embryogenesis, cell fate determination and reproductive development in several plant species (Hecht et al. 2001; Albertini et al. 2005; Wang et al. 2015). Although the precise function of the *D. affinis* homologue remains unknown, its identification further expands the repertoire of candidate regulators potentially involved in apogamic development.

Although *AGO4, AGO9, FIE, FAS4*, and *AIL7* were not successfully sequenced in the present study, they remain candidates of interest because of their reported involvement in reproductive development or apomixis in other plant species. Future studies will be required to clarify their possible roles in *D. affinis*.

## 4.3. Spatial and temporal expression analysis of candidate genes

To our knowledge, this represents the first application of RNA-FISH to investigate candidate genes associated with apogamy in *D. affinis*. To investigate the spatial and temporal expression patterns of candidate apogamy-related genes, *BBM, AIL1, MEE29* and *SE* were analyzed by RNA fluorescence *in situ* hybridization (RNA-FISH).

*In situ* hybridization (ISH) has been widely used to investigate gene expression patterns in plants; however, its application in ferns remains comparatively limited. Previous studies have employed ISH approaches to investigate developmental regulators in ferns, including meristem-associated genes and WOX transcription factors (Ambrose and Vasco 2016; Youngstrom et al. 2019). ISH has also been used to study phytochrome expression in *Adiantum capillus-veneris*, revealing tissue-specific expression patterns in developing fronds (Okamoto et al. 1997), and more recently in *C. richardii* to analyze transcription factor expression during organogenesis (Xiao and Li 2024). Fluorescence *in situ* hybridization (FISH) offers several advantages over conventional ISH, including higher spatial resolution and increased sensitivity, making it particularly suitable for the analysis of small and structurally simple tissues such as fern gametophytes.

In the present study, confocal microscopy revealed hybridization signals for *BBM*, *AIL1, MEE29* and *SE* in heart-shaped gametophytes of *D. affinis*, localized in the apogamic centre. In contrast, no signal was detected in filamentous or spatulate gametophytes. These observations are consistent with both previous transcriptomic analyses (Wyder et al. 2020) and the differential expression analysis performed in the present study, which identified increased transcript abundance of several developmental regulators associated with apogamy. The coincidence of temporal expression and spatial localization provides additional evidence of an association between the expression of these genes and the developmental transition leading to sporophyte initiation. Particularly noteworthy is the localization of the signals in the apogamic centre, the region from which the sporophyte originates, suggesting that these genes may participate in the cellular reprogramming events associated with apogamy. Although functional validation will be required to establish causal relationships, the results are consistent with the hypothesis that conserved developmental regulators associated with embryogenic reprogramming have been recruited during the evolution of apogamy in ferns.

The findings presented here further establish the gametophyte of *D. affinis* as a valuable model system for investigating plant reproduction, particularly apogamy. These results build upon a growing body of previously conducted studies on this species (Grossmann et al. 2017; Wyder et al. 2020; Fernández et al. 2021; Ojosnegros et al. 2022, 2023, 2024; Fernández et al. 2026).

In conclusion, by combining homology searches, protein domain analyses and manual curation, several candidate genes potentially involved in apogamy were identified in the gametophyte transcriptome of *D. affinis*. Interaction network and domain analyses indicated that these genes are associated with conserved regulatory pathways involved in small RNA-mediated gene silencing and Polycomb-dependent chromatin regulation, consistent with a potential contribution of epigenetic mechanisms to reproductive development in this fern. PCR amplification and sequencing experimentally validated 12 candidate transcripts, including *BBM, MEE29, SE, AIL1,*

*PLT2, AGO1, FIS1, FIE2, WOX13, DCL1, SERK1*, and *MSI1*. In addition, RNA fluorescence in situ hybridization revealed the spatial and temporal expression patterns of BBM, AIL1, MEE29, and SE, with hybridization signals detected specifically in heart-shaped gametophytes in the apogamic center but absent from filamentous and spatulate stages. Together, these findings reveal a stage- and region-specific expression pattern of conserved developmental and regulatory genes during the transition from gametophyte to sporophyte and are consistent with the hypothesis that conserved developmental pathways have been recruited during the evolution of apogamic reproduction in ferns.

## Declarations

*Main Conclusion*. Conserved developmental regulators show spatially restricted expression in the apogamic centre, supporting their association with apogamous sporophyte initiation in *Dryopteris affinis*.

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# Author contribution:

Conceptualization: HF, JMA; Methodology: SO, ASA, JMA; Investigation: SO, ASA; Formal analysis: HF, SO, ASA; Software: HF, SO, ASA, JMA; Supervision: UG; Project administration: HF; Writing – original draft: HF; Writing – review and editing: SO, ASA, JMA, AEV, UG.

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# Data availability:

The RNA-seq data analysed in this study are publicly available in the European Nucleotide Archive (ENA) under accession number PRJEB18522. The assembled transcript sequences and corresponding annotations are available in Zenodo (doi:10.5281/zenodo.1040330). Nucleotide sequences of the candidate transcripts analysed in this study are provided in the Supporting Information (Supplementary Table 1).

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# Figures

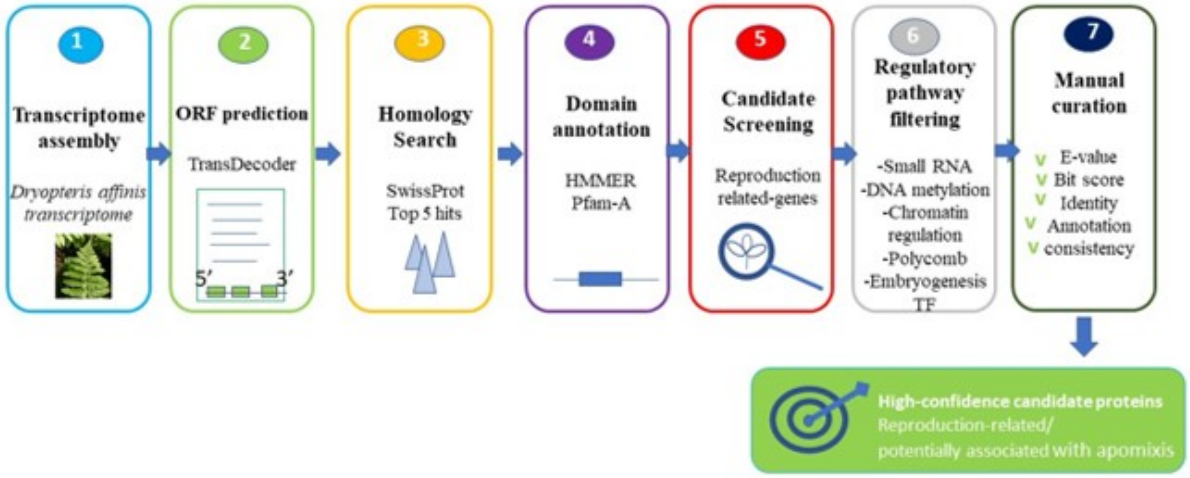



**Figure 1**

Schematic workflow of the computational analysis performed on the gametophyte transcriptome of the fern *Dryopteris affinis* to identify candidate genes potentially associated with apogamy. Created by the authors.

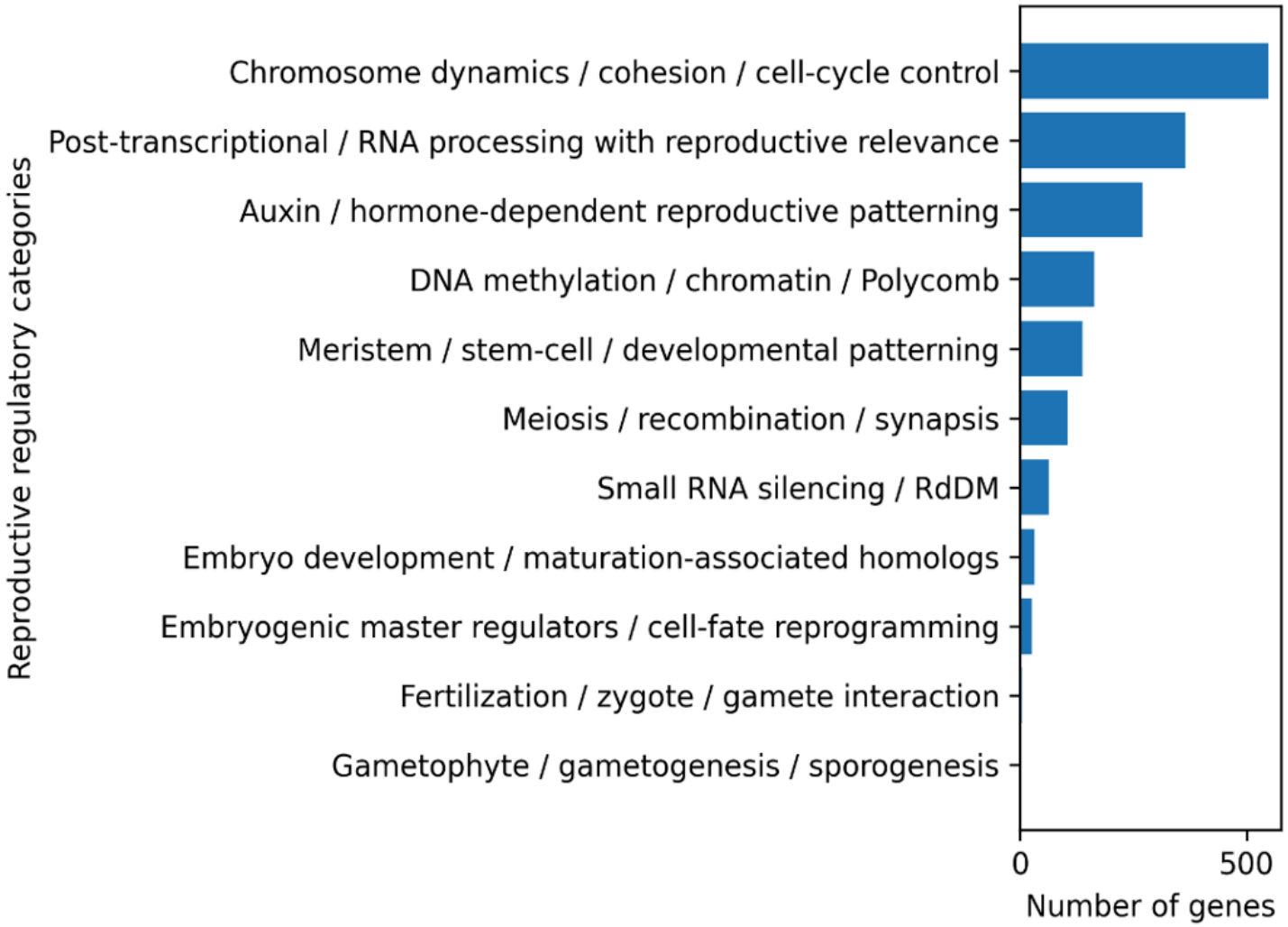



**Figure 2**

Functional classification of homologues of genes associated with reproductive and developmental regulation identified in the gametophyte transcriptome of *Dryopteris affinis*.

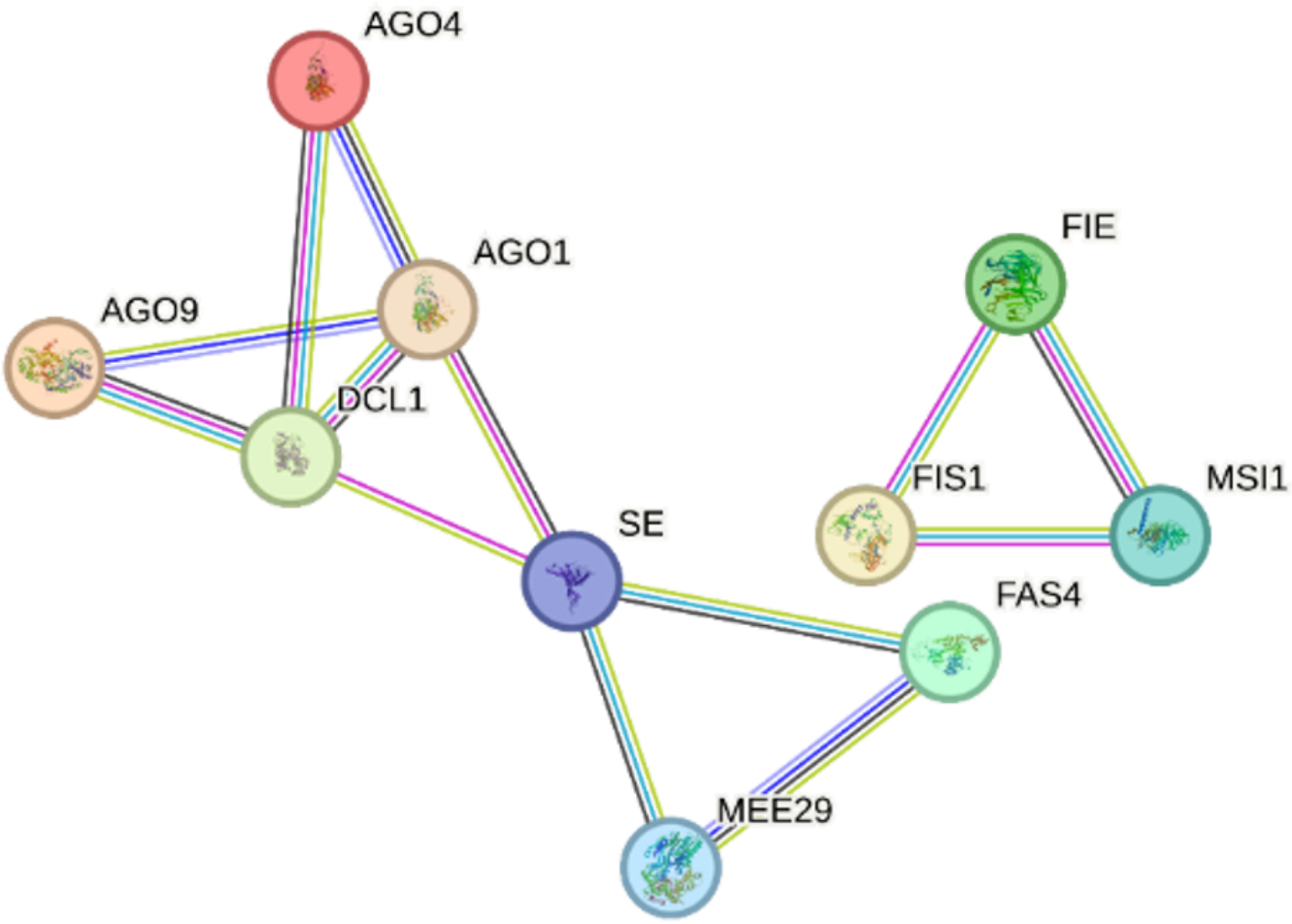



**Figure 3**

Protein-protein interactions network of the genes identified in the *Dryopteris affinis*gametophyte transcriptome that are potentially related to apomixis, generated using STRING. Edge colors indicate the type of evidence supporting each predicted interaction: pink, experimental evidence; yellow, text mining; black, co-expression; light blue, databases; dark blue, co-occurrence; and purple, homology.

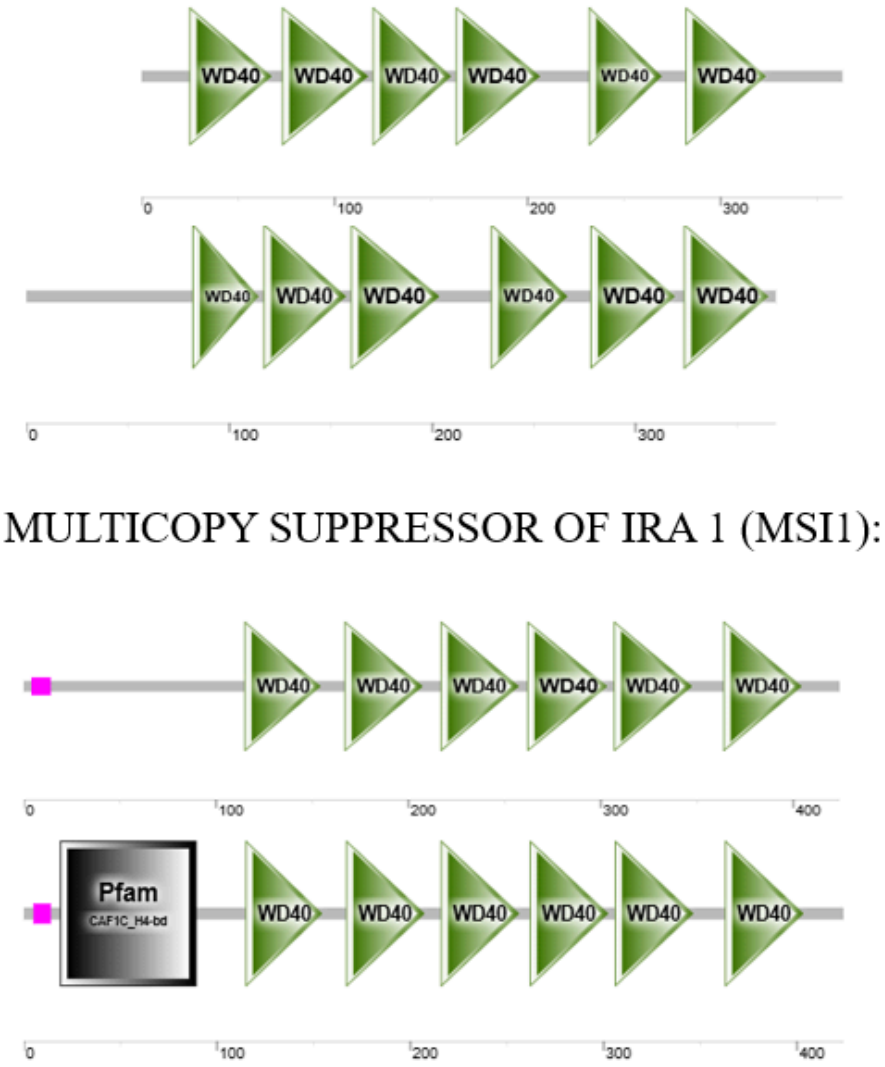



**Figure 4**

Amino acid domains of proteins from *Dryopteris affinis* gametophytes potentially involved in apomixis (top) and their corresponding homologues from *Arabidopsis thaliana*(bottom), as identified using SMART.

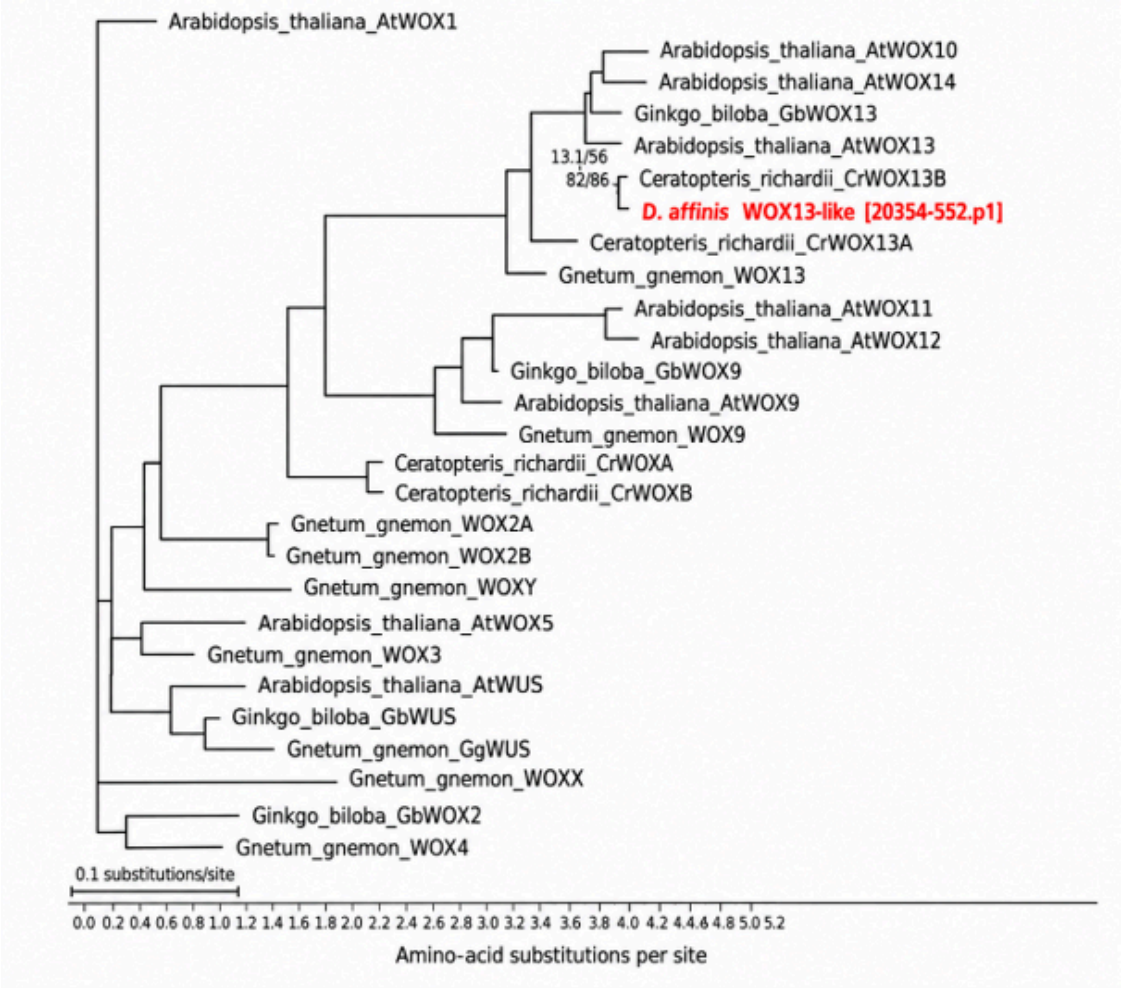



**Figure 5**

Phylogenetic placement of the WOX13-like candidate identified in *Dryopteris affinis* ssp. *affinis*. Maximum-likelihood phylogeny based on 27 WOX protein sequences representing selected major land-plant lineages. The *D. affinis*candidate 20354-552.p1 is highlighted in red and was recovered as sister to *Ceratopteris richardii* CrWOX13B (SH-aLRT/UFBoot = 82/86), supporting its annotation as a WOX13-like sequence. Branch lengths represent amino-acid substitutions per site. Support values are shown for nodes relevant to the phylogenetic placement of the *D. affinis* candidate.

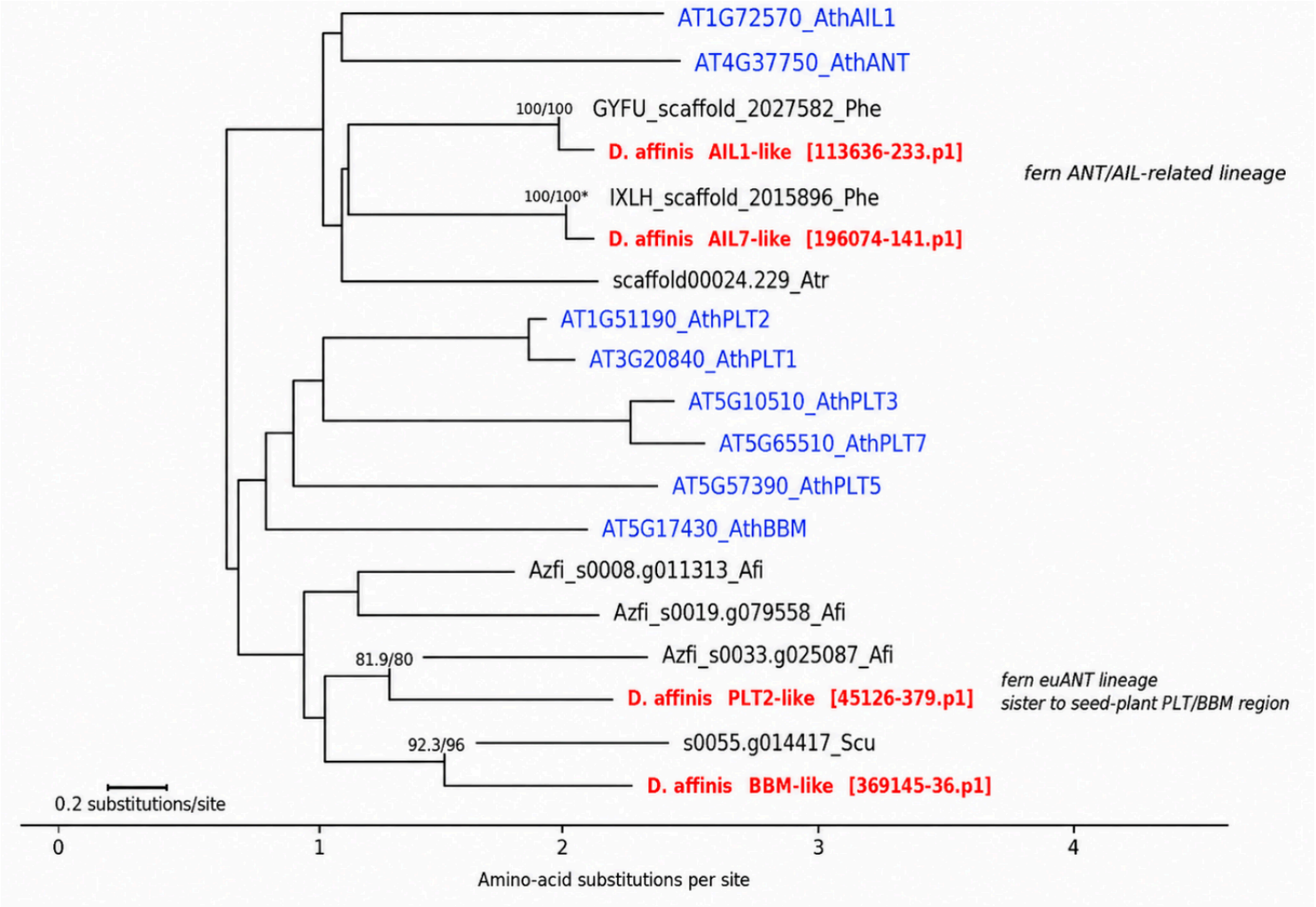



**Figure 6**

Phylogenetic placement of euANT/AP2-AIL candidates identified in *Dryopteris affinis* ssp. *affinis*. Focused representation of the maximum-likelihood phylogeny inferred from the 128-sequence euANT/AP2-AIL dataset. The four *D. affinis*candidates are highlighted in red, whereas selected *Arabidopsis thaliana*reference proteins are shown in blue. The *D. affinis* sequences are recovered within two fern euANT lineages associated with the ANT/AIL-related and PLT/BBM-related regions of the phylogeny. SH-aLRT/UFBoot support values are shown for the immediate placements of the *D. affinis* candidates. Branch lengths represent amino-acid substitutions per site. For clarity, only a subset of reference sequences is displayed; the topology was not recalculated after pruning.

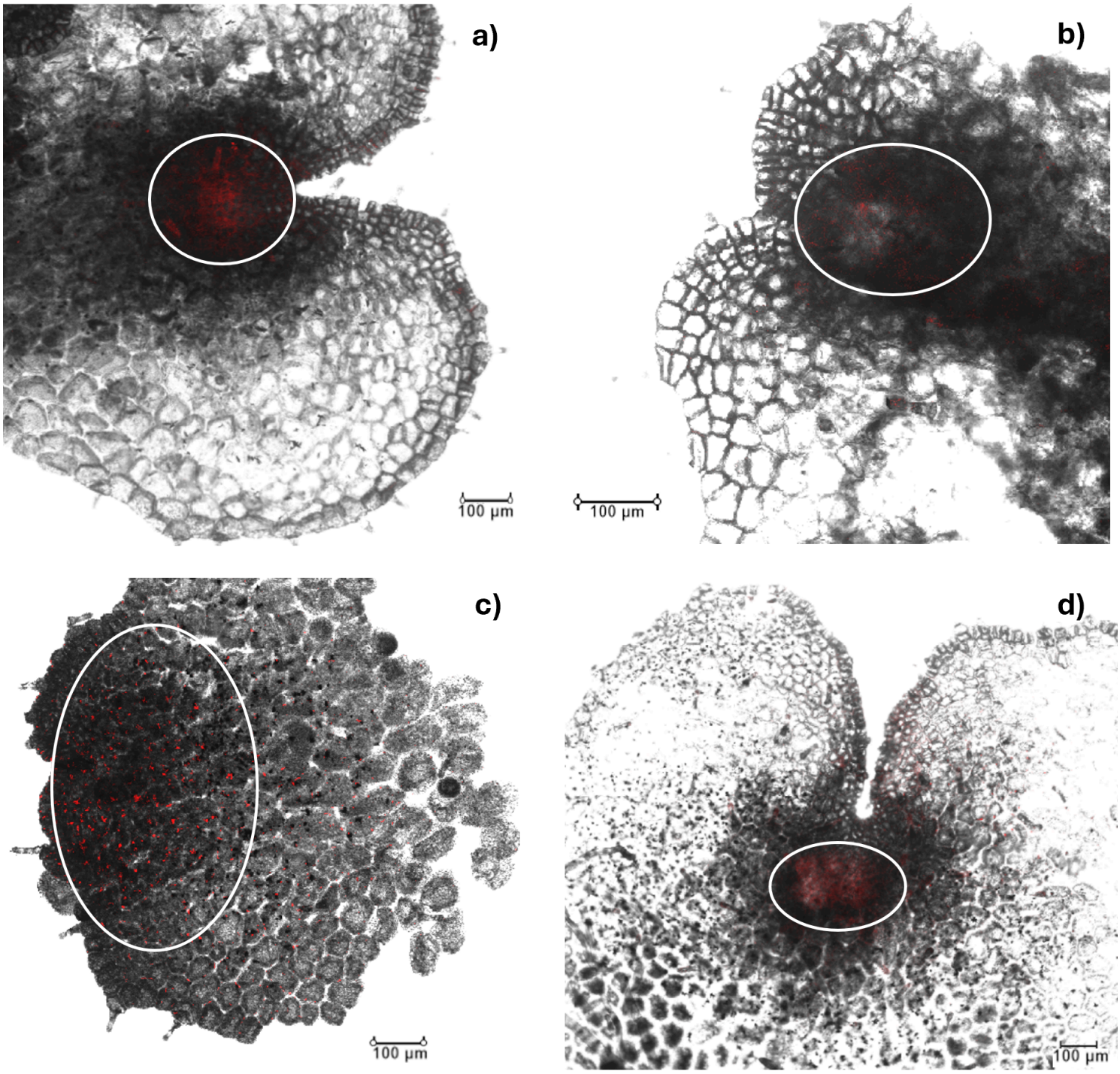



**Figure 7**

Images of heart-shaped gametophytes of the fern *Dryopteris affinis* subjected to fluorescent *in situ* hybridization (FISH) for the genes: (a) *BABY BOOM (BBM),* (b) an AP2-like ethylene-responsive transcription factor (*AIL1*), (c) *MATERNAL EFFECT EMBRYO ARREST 29 (MEE29),* and (d) *SERRATE (SE)*. The Cy5 signal is shown in red. White circles indicate regions with stronger Cy5 fluorescence. All probes were labelled with Cyanine 5 (Cy5). All images were obtained with a Leica TCS SP8 confocal microscope.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.docx
- SupplementaryTable1.docx
- SupplementaryTable2.docx