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Supplementary information

Dynamic genome evolution in a model fern

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Dynamic genome evolution in a model fern

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY DISCUSSION

Abiotic stress response gene repertoire in ferns

Ferns are known for their tolerance of heavy metals and metalloids in both soil and freshwater[1–4]. Another *Ceratopteris* species, *C. pteridoides*, accumulates cadmium and is a viable candidate for phytoextraction and remediation of cadmium-contaminated ecosystems[5]. Although the genes directly controlling cadmium accumulation have not been identified, we discovered the expansion and functional diversification of the Natural Resistance-Associated Macrophage Protein (NRAMP) heavy metal transporter gene family, which has been shown to control cadmium uptake in *Arabidopsis* (Supplementary Fig. 7)[6]. Further analyses of heavy metal and metalloid tolerance in various fern species alongside genome-informed experimental investigations of gene function in *Ceratopteris* will expand understanding of molecular processes contributing to heavy metal hyperaccumulation in plants. With their abundant natural defenses and phytoremediation potential, ferns provide an important resource for genetic engineering of crop resistance and environmental restoration.

Evolution of the APETALA2 *gene lineage*

The *APETALA2/ETHYLENE RESPONSIVE FACTOR* (*AP2/ERF*) family is essential to plant development across green plants and encompasses genes known to control flower, seed, and fruit development in angiosperms[7]. To identify the *APETALA2* and *AINTEGUMENTA* homologs in *Ceratopteris richardii*, we performed a BLAST search using homologs previously reported across land plants[8,9]. A total of 199 sequences was compiled and used to predict the evolutionary history of this gene lineage. Full nucleotide sequences were aligned using the online version of MAFFT[10] with a gap open penalty of 3.0, offset value of 0.8, and default parameters. To find the nucleotide substitution model that best fits our data, using the Akaike Information Criterion[11], we used jModelTest 2[12], which identified the GTRGAMMA model. RAxML v.8.0.0 was used to estimate phylogenetic relationships under a maximum likelihood (ML) framework[13]. The GTRGAMMA model was assigned, and a full ML search was implemented, using the autoMRE bootstrapping criterion to assess nodal support (-f a -# autoMRE option). Closely related genes from *Arabidopsis* - *ARF3* (*At2g33860*); *RAV1* (*At1g13260*); *RAV2* (*At1g25560*); *DREB1A* (*At4g25480*); *DRE1B* (*At4g25490*) - were used as the outgroup.

The *AP2/ERF* family is split into two major subclades, *euAP2* and *ANT* (*AINTEGUMENTA*), in which three and 11 *Ceratopteris* genes were placed, respectively (Supplementary Fig. 2). *CerAP2* was previously found to be expressed in the inner sporangium wall, young spores, and leaf vasculature during sporogenesis, based on *in situ* hybridization analyses[7]. We found *CerAP2* evenly expressed throughout the ten tissues, while the *Ceratopteris euANT* genes were more highly expressed in stem, root, and young sporophytes and the *Ceratopteris basalANT* genes were also evenly expressed throughout the different tissues (Supplementary Fig. 8).

Co-evolution of LEU- and SEU-like gene families

Publicly available (ncbi.nlm.nih.gov, fernbase.org, congenie.org) *LEUNIG*- (*LUG*) and *SEUSS*- (*SEU*) like genes of *Amborella trichopoda, Arabidopsis thaliana, Azolla filiculoides Chara braunii, Glycine max, Gnetum montanum, Klebsormidium nitens, Marchantia polymorpha, Nicotiana tabacum, Oryza sativa, Physcomitrium patens, Populus trichocarpa, Picea abies, Pinus pinaster, Pseudotsuga menziesii, Salvinia cucullata, Selaginella moellendorfii, Taxus baccata, Vitis vinifera, Volvox carteri f. niagaiensis* and *Zea mays* were identified using BLAST[14]. Phylogenetic trees were constructed using amino acid sequences aligned with MAFFT using MEGA X, maximum likelihood, and automatic trimming (60% site coverage)[15]. Expression patterns of *Ceratopteris LUG*-like and *SEU*-like genes were generated using Heatmapper[16].

LUG- and *SEU*-like genes encode for transcriptional co-regulators that interact and co-evolve even though they belong to different gene families. In flowering plants, these genes are essential for proper flower development, specifically in controlling petal shape and polarity. *LUG*-like genes of seed plants fall into two subclades, the *LEUNIG-HOMOLOG* (*LUH*) clade and the *LUG* clade, but all non-seed land plant sequences, including a single gene, *CrLUG,* from *Ceratopteris*, are members of the *LUG* clade only (Supplementary Fig. 9A). *SEU*-like genes of seed plants also form two clades, the *SEU* and *SEUSS*-like clades. Of the three *SEU*-like *Ceratopteris* genes, two clustered with the seed plant and bryophyte *SEU* genes, and one, *SEUSS*-like 3 (*CrSEU3*), appeared in a clade consisting only of fern sequences (Supplementary Fig. 9B). Neither the *LUG* nor the *SEU* gene families expanded or collapsed in *Ceratopteris* but both kept a moderate number of members during land plant evolution, suggesting that even after gene copies originating from a WGD are purged from the genome, the loss of one gene family member cannot be tolerated. *SEU*- and *LUG*-like *Ceratopteris* genes were expressed in sporophytic and gametophytic tissue alike, with lowest expression in sporangia and developing leaves, and highest expression in the stem, root, expanding leaf, and young sporophyte, suggesting pleiotropic functions for the members of both gene families.

Evolution and selection of cell cycle gene families

Homosporous plants have on average considerably higher chromosome numbers than do heterosporous plants[17,18]. Presumably, this is related either to mechanisms of genome doubling or of genome downsizing, and we hypothesize there could be differences in genes associated with the cell cycle in homosporous and heterosporous plants. Thus, we analyzed genes associated with cyclin-dependent kinases and cyclins, chromatin remodeling, and spindle formation and mapped significant gene copy expansions and contractions on a phylogenetic tree of land plants for which genome sequences are available (Supplementary Fig. 10).

We ran Orthofinder 2.4.0[19] on 21 plant species representing all major clades of land plants, for which whole genomes are currently available (July 2021). We selected genes associated with cyclin-dependent kinases and cyclins[20] and genes associated with spindle fiber formation and chromatin remodeling directly from The *Arabidopsis* Information Resource (TAIR), using the native search function in TAIR and the search terms “spindle” and “chromatin.” We selected 143 orthogroups from the output of Orthofinder and examined the evolution of gene copy number. For

this we used Computational Analysis of gene Family Evolution (CAFE) v5.0[21], which uses a birth and death model to simulate gene family evolution. We then mapped nodes and tips that CAFE indicated had significant expansions or retractions in copy number.

We aligned orthogroups with MUSCLE[22] and synchronized the headers of the peptide orthogroup files to match the corresponding CDS files (https://github.com/carol-rowe666/ortho_group_refile). We then used the peptide alignments to guide the CDS alignment with pal2nal (http://www.bork.embl.de/pal2nal/). Gene models in *Pinus taeda* were often mismatched between CDS and peptide sequences, so this species was removed from the analysis. The pal2nal-aligned CDS files were then used to generate gene trees using IQtree2 (https://github.com/iqtree/iqtree2). Three of the 129 orthogroups included fewer than four sequences and could not be bootstrapped for gene tree estimation. We paired the Newick outputs generated by IQtree2 with the pal2nal-generated aligned CDS orthogroups to test for selection using the absREL model built into Hyphy (http://hyphy.org).

We found no distinct patterns of changes in gene copy number in the three heterosporous lineages. However, in the lineage leading to *Ceratopteris*, we observed five gene expansion events: one associated with a cyclin and four associated with chromatin remodeling. Using HyPhy's implementation of the absREL model, one orthogroup with the largest number of *Ceratopteris* genes under selection included six genes undergoing positive selection. This orthogroup included *Arabidopsis* genes known to be involved in regulation of DNA repair, DNA binding, RNA binding, and endonuclease activity. Otherwise, most other orthogroups had no *Ceratopteris* genes with signs of positive selection and very few orthogroups with either one or two genes undergoing positive selection.

Cytochrome P450 gene family diversity across green plants

Cytochrome P450s (CYP450s) can be found across the major lineages of life but are most diverse in green plants. These enzymes are critical to plant metabolism and have diversified to become one of the largest metabolism-related enzyme families found in Viridiplantae[23]. We identified CYP450 genes from 40 green plant species with multiple representatives from each major lineage, plus three red and brown algae (Rhodophyta and Phaeophyceae, respectively) as outgroups, and classified them into previously identified CYP450 subfamilies (Supplementary Table 12). In total, we identified 11,463 CYP450 genes across these 43 species with the highest CYP450 diversity in hexaploid wheat, *Triticum aestivum*. On average, angiosperms contained 401 CYP450 genes, although this sampling does include recent polyploids such as *T. aestivum*. The gymnosperms had a mean count of 344 CYP450s, ferns had 181, the single lycophyte sample *Selaginella moellendorffii* had 308, bryophytes had 151, charophyte algae had 31, and chlorophyte algae had 17; within ferns, the two water ferns, *Azolla* and *Salvinia*, had 155 and 80 total CYP450 genes, respectively, and *Ceratopteris* had 309. The largest difference in CYP450 subfamily diversity between *Ceratopteris* and the water ferns was in CYP704B (58 in *Ceratopteris*, 7 in *Azolla*, 11 in *Salvinia*), which encode omega fatty acid hydroxylases, enzymes essential for spore

wall polymers[24]. Interestingly, this subfamily has even lower diversity in seed plants, with a mean count of 1.9 CYP704B genes.

Independent of the CYP450 subfamilies, which were largely identified from angiosperm reference genes, we constructed a CYP450 phylogeny from 14 of these taxa, with representatives from each major green plant lineage (Supplementary Figure 11). While the early-diverging CYP450 clades have representative genes from diverse green plant taxa, it is clear that lineage-specific subfamilies are prevalent especially in more recently diverged clades of the CYP450 genes.

Revitalizing the C-Fern Curriculum

Ceratopteris is an exemplary model for teaching plant development and genetics because it has both independent gametophytic and sporophytic life stages, clearly demonstrating the alternation of generations[25,26] typical of all land plants. Students can easily mutagenize the haploid spores using EMS or X-rays and see the resulting mutant phenotypes in the haploid gametophytes[27]. The ability of ferns to undergo intragametophytic selfing (egg fertilized by sperm from a single gametophyte) allows students to produce completely homozygous sporophytes in which any mutant phenotype will also be apparent in the diploid life cycle stage. In addition, true-breeding mutant inbred lines can be maintained long term or can easily be crossed with other genotypes. With the ease and low costs of sequencing, undergraduate students can identify mutant phenotypes in *Ceratopteris,* isolate RNA, prepare libraries, send the libraries off for sequencing, and ultimately receive training in how to map RNA-seq data onto a reference genome within a semester. *Ceratopteris* is also capable of undergoing apogamy (a haploid sporophyte forms from gametophytic tissue without fertilization) and apospory (diploid gametophytes form from sporophytic tissue), readily producing polyploid individuals and essentially permitting virtually every means of plant reproduction with this single species[28,29]. The genome assembly and the sequenced doubled haploid F_2 mapping population of *Ceratopteris* provide the resources and techniques to ensure that *Ceratopteris* and the C-Fern Curriculum will be an important model system for teaching plant development, genetics, genomics, breeding, physiology, and bioinformatics for years to come.

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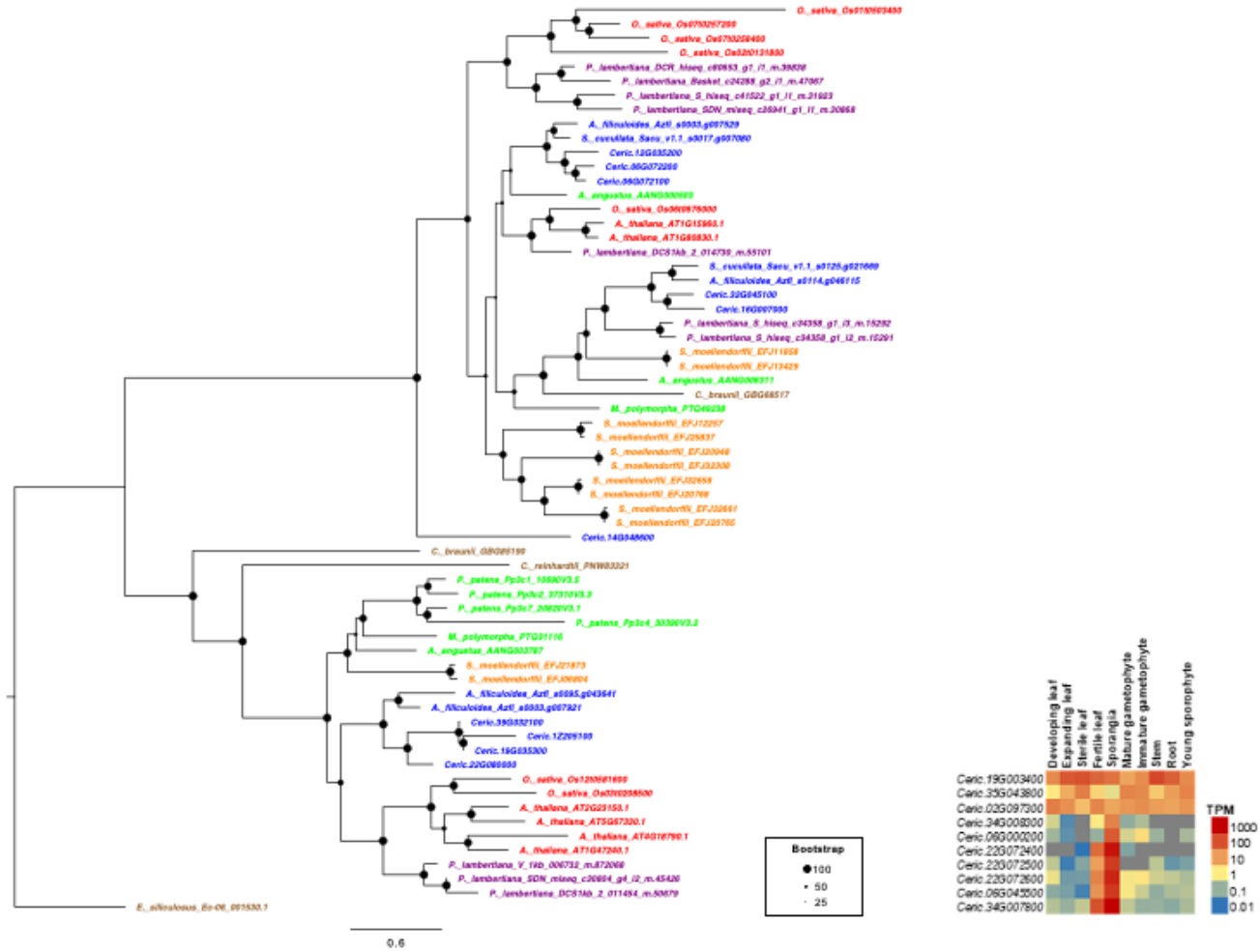
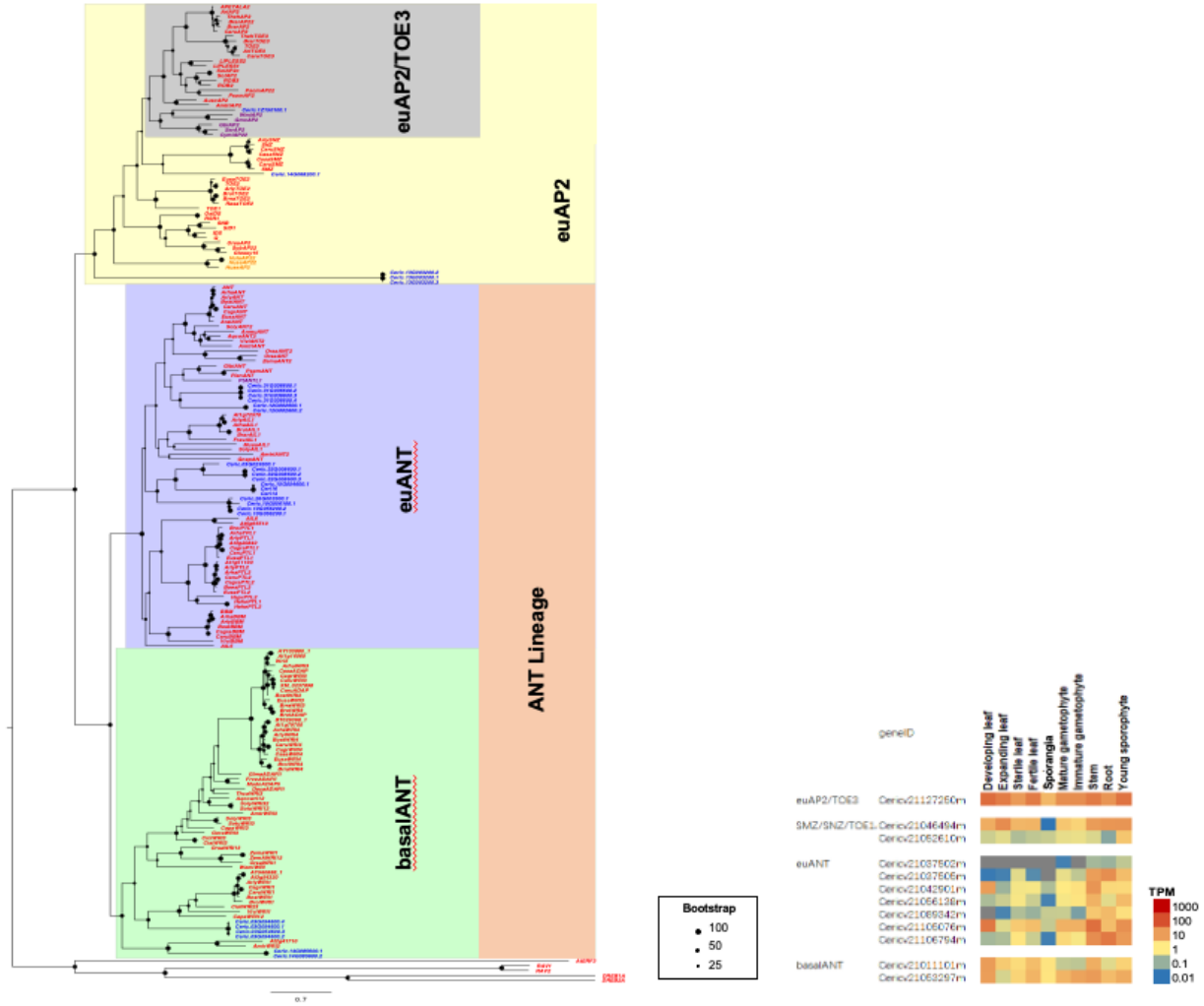
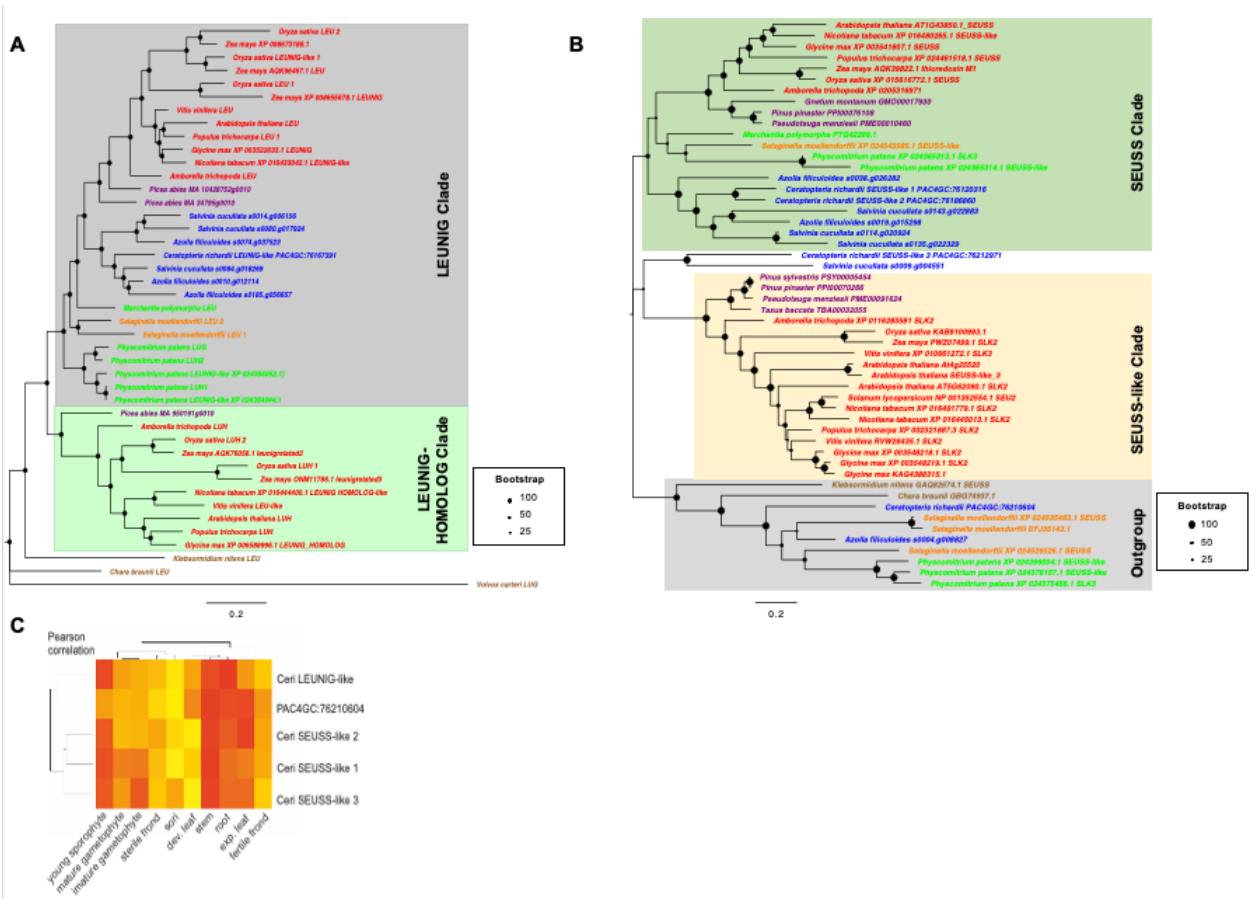
SUPPLEMENTARY FIGURES



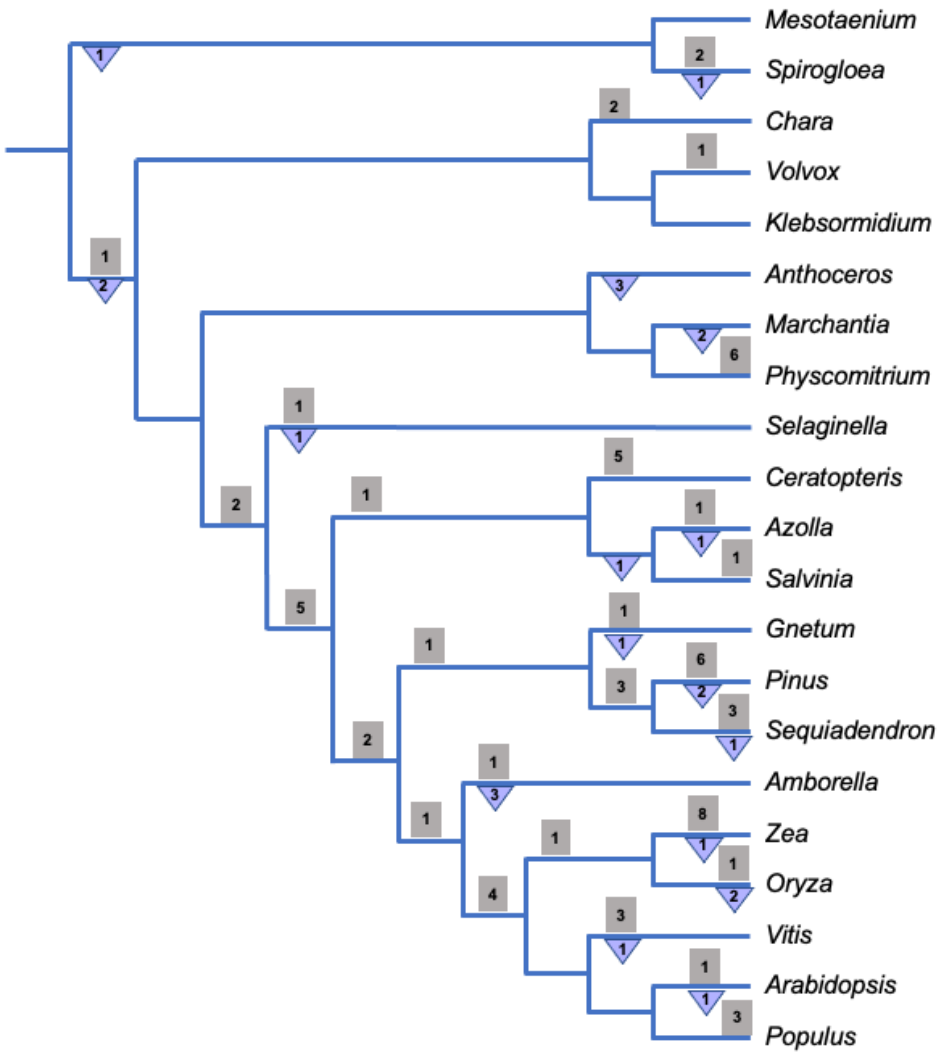
Supplementary Fig. 1 Phylogeny of *NRAMP* metal-ion transporter gene family and *Ceratopteris NRAMP* gene expression. Angiosperm genes are red, gymnosperm genes are purple, fern genes are blue, lycophyte genes are orange, bryophyte genes are green, and algal genes are brown.



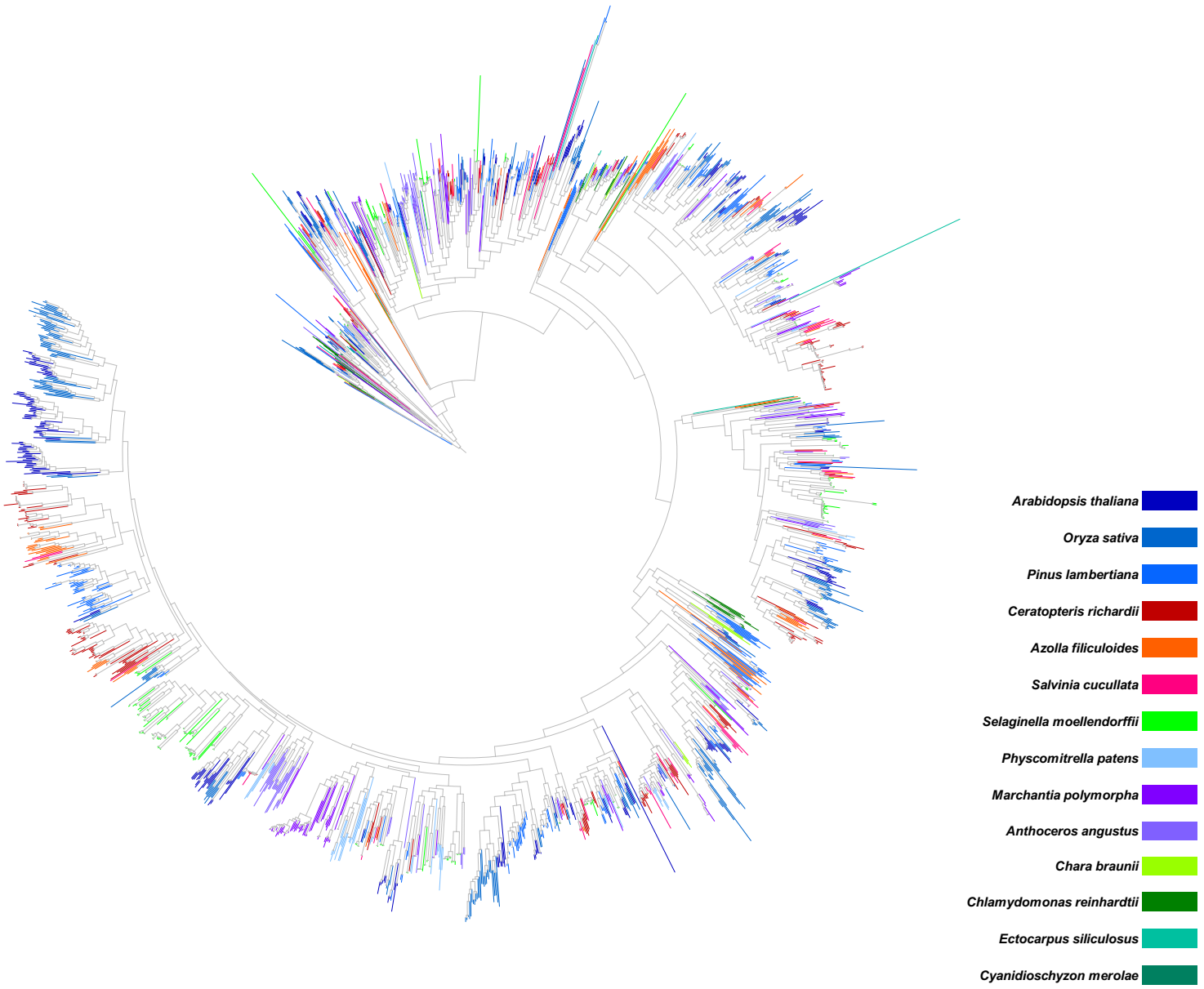
Supplementary Fig. 2 Phylogeny of the *APETALA2/ETHYLENE RESPONSIVE FACTOR* (*AP2/ERF*) across land plants and expression in *Ceratopteris*. Angiosperm genes are red, gymnosperm genes are purple, fern genes are blue, and lycophyte genes are orange.



Supplementary Fig. 3 Co-evolution of transcriptional regulators. (A) Phylogeny of *LEUNIG*-like gene family. (B) Phylogeny of *SEUSS*-like gene family. Angiosperm genes are red, gymnosperm genes are purple, fern genes are blue, lycophyte genes are orange, bryophyte genes are green, and algal genes are brown. (C) Gene expression patterns of *LEU*- and *SEU*-like genes in tissues/life stages of *Ceratopteris*.



Supplementary Fig. 4 Expansion and contraction in copy number of cell cycle-related genes across green plants. Light purple triangles indicate contractions; grey squares show expansions.



332 **Supplementary Fig. 5 CYP450 gene diversity and evolution across green plants.** The color-
333 coding of the 14 green plant taxa spaning 2,193 genes is found in the figure legend.
334