The Evolution of *euAPETALA2* Genes in Vascular Plants: From Plesiomorphic Roles in Sporangia to Acquired Functions in Ovules and Fruits

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Abstract

The field of evolutionary developmental biology can help address how morphological novelties evolve, a key question in evolutionary biology. In *Arabidopsis thaliana*, *APETALA2* (*AP2*) plays a role in the development of key plant innovations including seeds, flowers, and fruits. *AP2* belongs to the *AP2/ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR* family which has members in all viridiplantae, making it one of the oldest and most diverse gene lineages. One key subclade, present across vascular plants is the *euAPETALA2* (*euAP2*) clade, whose founding member is *AP2*. We reconstructed the evolution of the *euAP2* gene lineage in vascular plants to better understand its impact on the morphological evolution of plants, identifying seven major duplication events. We also performed spatiotemporal expression analyses of *euAP2/TOE3* genes focusing on less explored vascular plant lineages, including ferns, gymnosperms, early diverging angiosperms and early diverging eudicots. Altogether, our data suggest that *euAP2* genes originally contributed to spore and sporangium development, and were subsequently recruited to ovule, fruit and floral organ development. Finally, euAP2 protein sequences are highly conserved; therefore, changes in the role of *euAP2* homologs during development are most likely due to changes in regulatory regions.

Key words: **AP2/TOE3, cooption, evo–devo, gene lineage evolution, morphological innovations, sporangium development.**

Introduction

One of the key questions in biology is how new structures evolve. Evolutionary developmental biology (evo–devo) which focusses on how changes in the genetic networks underlying development influence the evolution of taxa can help address this question. In particular, changes in *cis-* and *trans-* regulatory regions and variation in the protein sequences, or their interactors, have allowed the identification of preexisting genes or networks coopted for the development of novel traits (Jacob1977; Carroll 2000; Sanetra et al. 2005; Monteiro and Podlaha 2009). One powerful approach to assess how evolutionary novelties arise is the combined assessment of gene lineage evolution in parallel to the study of expression and function in a comparative manner across distantly related taxa. In plants, several morphological innovations, such as the seed, the flower, and the fruit, have evolved at different timepoints and correlate with the diversification and ecological dominance of specific plant lineages (Burleigh and Mathews 2004; Soltis and Soltis 2004; Zanne et al. 2014). Moreover, these structures are critical for plant reproduction as they provide protection and nourishment to the spores and sometimes to the zygote. To investigate how morphological innovations may evolve, we focus on the *euAPETALA2* (*euAP2*) gene lineage known to be a key player in the development of seeds, flowers, and fruits in *Arabidopsis thaliana* (Arabidopsis) (Bowman et al. 1989; Jofuku et al. 1994; Yant et al. 2010; Ripoll et al. 2011).

The *APETALA2/ETHYLENE RESPONSIVE FACTOR* (*AP2/ERF*) family is one of the most important gene lineages in plant development, with over 4,000 members present across Viridiplantae (Bowman et al. 1989; Jofuku et al. 1994; Ohme-Takagi and Shinshi 1995; Elliott et al. 1996; Moose and Sisco 1996; Kim et al. 2006; http://planttfdb.gao-lab.org/family.php?fam=AP2; last accessed May 14, 2020). According to the number of AP2 domains, the lineage has been divided into two classes; the AP2-like class encode proteins with two AP2 domains, namely AP2-R1 and AP2-R2, while the ERF-like class proteins only have the AP2-R1 domain (Jofuku et al. 1994; Elliott et al. 1996; Nole-Wilson et al. 2005; Yant et al. 2010; Li et al. 2016). These domains consist of three $_{\beta}$ sheets and one

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amphipathic $_{\alpha}$ helix, which are important for DNA binding (Jofuku et al. 1994; Allen et al. 1998). Within seed plants, there are two sister clades: *euAP2* and *AINTEGUMENTA* (*ANT*) (Kim et al. 2006). For the purpose of this paper, we will focus on the *euAP2* lineage (*sensu* Kim et al. 2006 followed by Zumajo-Cardona and Pabón-Mora 2016; called the AP2 group in Wang et al. 2016). This gene clade includes the canonical *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*), *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3*, and *AP2*. Gene evolution topologies from this clade recover two groups (Kim et al. 2006; Wang et al. 2016), one with *TOE1/2*, *SMZ*, and *SNZ* (TOE type *sensu* Wang et al. 2016) and one with *AP2* and *TOE3* (*AP2* type *sensu* Wang et al. 2016). The latter subclade *euAP2/TOE3* includes the two Arabidopsis paralogues *AP2* and *TOE3* as the result of a duplication event specific to Brassicaceae (Kim et al. 2006; Wang et al. 2016; Zumajo-Cardona and Pabón-Mora 2016). Two other major duplication events occurred in the *euAP2/TOE3* lineage, one in monocots coinciding with the radiation of commelinids and another duplication previous to the diversification of basal eudicots (Zumajo-Cardona and Pabón-Mora 2016).

In Arabidopsis, *AP2* is involved in the floral transition and contributes to sepal and petal identity (Koornneef et al. 1983; Jofuku et al. 1994; Yant et al. 2010). Mutant *ap2* plants exhibit homeotic conversions of sepals and petals into leaf-like organs with stigmatic surfaces carrying ovules, and into stamens respectively (Pruitt et al. 1987; Komaki et al. 1988; Bowman et al. 1989; Kunst et al. 1989; Drews et al. 1991). Thereafter, *AP2* was included in the ABC model for flower development, as an A-class gene (Bowman et al. 1991; Coen and Meyerowitz 1991). Originally A and C-class genes were thought to be mutually antagonistic in order to mark boundaries between the perianth and the stamens/carpels (Gustafson-Brown et al. 1994; Mandel and Yanofsky 1995). This was supported by the observations of *AGAMOUS* ectopic expression in the *ap2* mutant (Bowman et al. 1993). In wild-type Arabidopsis, downregulation of *AP2* in stamens and carpels primarily occurs via microRNA *miR172* activity (Zhao et al. 2007; Wollmann et al. 2010). It is likely that a *miR172-AP2* regulatory module is present throughout euphyllophytes, as *miR172* has been identified in angiosperms, gymnosperms, and ferns (Axtell and Bartel 2005; Zhang et al. 2006). *AP2* also functions in integument development and seed coat cell patterning as well as embryo development by controlling cell size and number (Jofuku et al. 1994; Ohto et al. 2005). In addition, *AP2* also controls the reproductive transition via induction of *AGAMOUS-LIKE 15* expression and by repression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (Yant et al. 2010). Finally, *AP2* is part of the Arabidopsis fruit development network, controlling replum growth and valve margin formation by a direct negative regulation of *SHATTERPROOF*, *BREVIPEDICELLUS* and *REPLUMLESS* (Ripoll et al. 2011).

Previous studies have identified homologues of *euAP2/TOE3* across seed plants for which the protein sequences appear to be more similar to AP2 than to the TOE3 protein (Kim et al. 2006; Zumajo-Cardona and Pabón-Mora 2016; Najafi et al. 2018; Sharma et al. 2018; Kerstens et al. 2020), and have highlighted the pleiotropic roles of *AP2* homologues in eudicots and monocots. In snapdragon (*Antirrhinum majus*, Plantaginaceae), the *AP2* orthologues *LIPLESS1* and *2* are expressed in bracts, sepals, the distal portions of the petals, and weakly in the carpels. The double *lipless1/2* mutant shows homeotic conversion from sepals to bract-like organs and smaller petals (Keck et al. 2003). In petunia, *PhAP2A* (also known as *REPRESSOR OF B-FUNCTION1*, *ROB1*), is expressed in leaves and in all floral organ primordia, but *phap2a* mutants do not show any abnormal phenotype compared with wild-type plants (Maes et al. 2001; Morel et al. 2017). Additionally, *PhAP2A* expression is detected in the integuments during ovule development and the endosperm during seed development (Maes et al. 2001). Two additional paralogues have been identified in petunia, *ROB2* and *3*. Like *PhAP2A*, they too are broadly expressed in all floral whorls (Morel et al. 2017). Genetic analyses indicate redundancy among the three paralogues and only the triple mutant, *rob1,2,3*, shows defects in sepal, petal, and carpel development (Morel et al. 2017). In tomato, there are three paralogues *SlAP2a*,*SlAP2b*,and *SlAP2c*. RNAinterference-mediated repression targeting the three paralogs, show mutants with longer fused sepals and exhibit yellow-orange pigmentation linked to an increased $_{\beta}$-carotene to lycopene ratio during fruit ripening. In addition, defects in the seed coat are also observed (Chung et al. 2010; Karlova et al. 2011). *SlAP2a* specific CRISPR-Cas9 knockouts confirm its function in fruit ripening and show that the sepal identity function is not dependent on *AP2a* alone (Wang et al. 2019). Finally, in kiwi, a natural mutant with a *miR172* insensitive version of *euAP2* results in aberrant flowers with multiple perianth whorls (Varkonyi-Gasic et al. 2012).

The *AP2* homologues in monocots influence inflorescence architecture and the transition from the spikelet meristem to the floral meristem (Chuck et al. 2008; Lee and An 2012; Bommert and Whipple 2018). *euAP2/TOE3* homologues include *INDETERMINATE SPIKELET 1* (*IDS1*) and *SISTER OF INDETERMINATE SPIKELET 1* (*SID1*) in maize (Chuck et al. 1998), *SUPERNUMERARY BRACT* (*SNB*), *OsIDS1* (Lee et al., 2007; Lee and An, 2012) and *RICE STARCH REGULATOR 1* (*RSR1*) in rice (Fu and Xue 2010), and *Q* (*AP2L5*) and *AP2L2* in wheat (Faris et al. 2003; Debernardi et al., 2020). *IDS1* in maize plays a role in inflorescence architecture by controlling the number of floral meristems produced (Chuck et al. 1998,2007,2008). On the other hand, single *sid1* mutants do not have different phenotypes from the wild type plants, but both *IDS1* and *SID1* are needed for inflorescence meristem branching, spikelet determinacy, and floral meristem identity (Chuck et al. 2008). In rice, the *snb* mutant has a delayed floral transition and produces additional glumes (interpreted as bracts) before the formation of the floral meristem. The *snb osids1* double mutant also has chimeric floral organs, with organ defects in paleas (sepal homologues) and lodicules (petal homologues) as well as homeotic conversion of lodicules into carpels (Lee et al. 2007; Lee and An 2012). Additionally, the rice *RSR1* negatively regulates starch synthesis genes during the development of the endosperm (Fu and Xue 2010). The wheat *Q* locus controls many domestication-

related traits that facilitate its harvest, such as the free-threshing character (Simons et al. 2006; Gil-Humanes et al. 2009), rachis fragility (Jantasuriyarat et al. 2004), spike length, plant height (Kato et al. 1998,2000) and flowering time (Kato et al. 1998). More recently, *Q* (*AP2L5*) and *AP2L2* were shown to control the specification of axillary floral meristems, lemmas (bracts), paleas (sepals), and lodicules (petals), with conversion of lodicules into carpels (Debernardi et al. 2020).

Functional characterization of *euAP2/TOE3* homologues is lacking in early diverging angiosperms, gymnosperms, and ferns. Nevertheless, real-time (RT)-PCR has been used to assess the expression of *AP2* homologues in *Amborella trichopoda*, where it is found in all floral organs and leaves (Kim et al. 2006). In the gymnosperms,*Larix marschlinsii* and *Picea thunbergii* (with two paralogues each: *LmAP1L1*, *LmAP2L2* and *PtAP2L1*, *PtAP2L2*, respectively), different paralogues are differentially expressed during somatic embryogenesis and the first stages of embryo development (Vahala et al. 2001; Shigyo and Ito 2004; Guillaumot et al. 2008). *PtAP2L1* and *PtAP2L2* expression has been also detected in the nucellus and the integument primordia (Shigyo and Ito 2004). Finally, in *Ginkgo biloba*, the expression of an *AP2* homologue was detected in ovules and leaves (Zumajo-Cardona and Pabón-Mora 2016). In addition, ectopic expression of *Picea abies* homologues, *PaAP2L1*, *PaAP2L2*, and *PaAP2L3*, in an *ap2* mutant background in Arabidopsis, was able to promote petal identity suggesting functional conservation at the molecular level across seed plants (Nilsson et al. 2007).

With the goal of assessing how *euAP2/TOE3* homologues have acquired these functions over evolutionary time, we wanted to investigate their putative plesiomorphic roles outside core eudicots and monocots. Because functional characterization techniques are currently unavailable for many of the targeted taxa, we present detailed spatiotemporal expression analyses for *AP2* homologues in the basal eudicot *Bocconia frutescens* (Papaveraceae), the early divergent angiosperm *Aristolochia fimbriata* (Aristolochiaceae), two gymnosperms, *Ginkgo biloba* (Ginkgoaceae) and *Gnetum gnemon* (Gnetaceae), and one fern, *Ceratopteris richardii* (Pteridaceae). These comprehensive expression analyses spanning major vascular plant lineages have enabled us to show that plesiomorphic roles for *AP2-like* homologues include sporangium and ovule development. Only more recently, in angiosperms, have these homologues been recruited into flowering transition, floral organ identity, and fruit development. Across vascular plants, there are few changes in the protein sequences of *euAP2/TOE3* homologues, therefore the cooption of *euAP2/TOE3* homologues in the development of morphological novelties are most likely due to independent evolution of *cis-* or *trans*-regulatory regions.

Results

euAP2/TOE3 Gene Evolution in Vascular Plants

Here, we present an extended phylogenetic hypothesis of *euAP2/TOE3* genes in tracheophytes. We isolated 84 genes from eudicots, 59 from monocots, 27 from early diverging angiosperms, 30 from gymnosperms, 25 from ferns, and 7 from lycophytes. The aligned matrix contained 750,384 characters. The gene tree obtained by ML analysis shows that eu*AP2/TOE3* homologues have remained as single copy in ferns, gymnosperms, early diverging angiosperms, and most core eudicots (fig. 1). Gene lineage duplications identified here include two major angiosperm duplication events previously described (Zumajo-Cardona and Pabón-Mora 2016), one in basal eudicots and another duplication event prior to the diversification of Brassicaceae (fig. 1). With extended sampling within Solanaceae, it was possible to identify two duplications specific to this family (fig. 1; supplementary fig. S3). Furthermore, here we present two additional duplication events within Poaceae (Bootstrap (BS) values= 89 and 79) and one corresponding to the diversification of Zingiberales (BS= 99). For the most part, the gene tree is consistent with known phylogenetic relationships among seed plants. However, the tree topology shows gymnosperm sequences forming a clade (AP2L clade *sensu* Wang et al. 2016). In addition, we recover a clade with fern and lycophyte *AP2* sequences as sister to gymnosperm homologues. Because the most recent hypothesis for vascular plants suggests the relations (lycophytes [ferns {gymnosperms + angiosperms}]), our topology may still require additional sampling to fully sort gene lineage evolution in vascular plants (fig. 1). Despite the sampling effort, no eu*AP2/TOE3* homologues were recovered from non-vascular plants.

The euAP2/TOE3 proteins are highly conserved across vascular plants, with the AP2-R1 and AP2-R2 binding domains present in all sequences analyzed (supplementary fig. S4; Kim et al. 2006). The *miR172* binding site (AAASSFG[S/P]) was detected in ferns and seed plant homologues but not in lycophytes. When found, the miR172 motif is located at the C-terminus, except in two fern proteins, EqboAP2 and EqboAP2-1, from *Equisetum bogotense*, which have the miR172 motif in the N-terminus of the protein, before the AP2-R1 domain. In addition, the start motif (MW[D/N]LNxxP) is conserved and corresponds to an EAR repressor motif (DLNxxP; Ohta et al. 2001; Hiratsu et al. 2003; Kim et al. 2006; Guillaumot et al. 2008). Only the maize GLOSSY15 and a few gymnosperm, fern, and lycophyte proteins lack this EAR repressor motif. Toward the C-terminus of the AP2-R2 domain is another ERF-associated Amphiphilic Repression motif (EAR: LxLxLX; Ohta et al. 2001; Hiratsu et al. 2003) which is conserved across seed plants (Zumajo-Cardona and Pabón-Mora 2016) but is absent in GLOSSY15, most *Equisetum giganteum* homologues and in all lycophyte proteins (supplementary fig. S4). In general, all angiosperm protein sequences are shorter, with around 400–560 amino acids and have more conserved motifs, compared with gymnosperm, fern, and lycophyte homologues with around 550–770 amino acids and more variable regions.

In order to assess the possible roles of *AP2* in plant development across tracheophytes, we carried out spatiotemporal expression analyses in selected species belonging to poorly explored plant lineages. Here, we present our results from six *AP2* homologues across major vascular plant lineages namely, early diverging eudicots, early diverging angiosperms, gymnosperms, and ferns.

FIG. 1. ML tree of *euAP2/TOE3* gene lineage across vascular plants. Yellow stars indicate large-scale duplication events. These duplications are (from top to bottom): 1) before the diversification of Brassicaceae; 2) two duplications specific to Solanaceae; 3) with the radiation of Papaveraceae; 4) two duplications before the radiation of Poales; 5) one within Zingiberales. BS values > 60% were placed at the nodes. Colors in the tree follow the top left conventions for each major group of seed plants. *Black arrows* point to the *AP2* sequences where expression was analyzed by In Situ hybridization in each species.

Expression of *euAP2/TOE3* Homologues in the Basal Eudicots *Eschscholzia californica* and *Bocconia frutescens* (Papaveraceae)

Two paralogues were identified in both *Eschscholzia californica* and *Bocconia frutescens* (Papaveraceae) as a result of the duplication event described above for basal eudicots (fig. 1). *Eschscholzia californica* flowers have two nonpersistent sepals, four petals, multiple stamens, and two carpels. In *E. californica*, the expression of the two paralogues named *EscaAP2-1* and *EscaAP2-2* was evaluated with RT-PCR and

quantitative real-time (qRT)-PCR. The results show that *EscaAP2-1* is more broadly expressed across different stages of flower development as well as in leaves; however, this paralogue is highly expressed in young floral buds, carpel in anthesis, and the mature fruit. Conversely, *EscaAP2-2* has a more specific expression in the petals and the carpels during anthesis and the mature fruit (supplementary fig. S5).

Bocconia frutescens flowers are also dimerous, with two nonpersistent sepals, apetalous, with two whorls of numerous stamens, and a bicarpellate gynoecium with a gynophore and a prominent bifurcated stigma (fig. 2A; Arango-Ocampo et al. 2016). The fruit has two valves, which at maturity fall apart leaving the single seed attached to a commissural ring-like structure (fig. 2A). *Bocconia frutescens* flower and fruit developmental stages have been previously described in detail (Zumajo-Cardona et al. 2017), the same stages are used here to describe the expression results obtained for the two *AP2* paralogues isolated, named *BofrAP2-1* and *BofrAP2-2* (fig. 1). Our results show different expression patterns for the two copies. *BofrAP2-1* is strongly expressed in the vegetative shoot apical meristem (SAM) and the young developing leaves (fig. 2B). *BofrAP2-1* is slightly expressed in the floral primordia (fig. 2C). During early stages of flower development, *BofrAP2-1* is not found to be expressed in sepals, stamens, or carpel primordia (fig. 2D and E). The expression of *BofrAP2-1* is detected later in the sepals, stamens, carpel, and ovule as these start to elongate (fig. 2F) and it is maintained in the carpel tips as they overtop and close above the single ovule (fig. 2G). Later in flower development, as the style and stigma differentiate, the expression of *BofrAP2-1* in the sepals is restricted to their growing tips and is maintained more homogenously in the other floral organs including stamens and carpels, as well as the single ovule (fig. 2H). In the anthetic flower, *BofrAP2-1* expression becomes restricted to the epidermal layers of the carpel walls, to the growing tips of the stigma, the adaxial flanks of the massive stigmas, the transmitting tract, and the growing tips of the two integuments in the developing ovule (fig. 2Iand J). The expression of *BofrAP2-1* persists in the young fruit, especifically in the medial inner region of the commissural ring, where the commissural ring meets the valve margins (fig. 2Kand L). *BofrAP2-1* expression does not extend to the dehiscence zone through which the two valves split apart.

Conversely, the paralogue, *BofrAP2-2*, is expressed in the SAM at lower levels compared with *BofrAP2-1*, and it cannot be detected in the young leaves (fig. 2M). In general, the expression of *BofrAP2-2* during flower development is lower than the one identified for *BofrAP2-1*. *BofrAP2-2* is not expressed in the floral primordia (fig. 2N), or when the stamen and carpel primordia start to develop (fig. 2O). However, *BofrAP2-2* can be detected at the base and at the tip of the sepals, as well as at the growing tips of carpels as they overtop the single ovule (fig. 2P to R). The expression at the base of the sepals is maintained throughout flower development and corresponds to the sepal abscission zone active at anthesis (fig. 2P to R). When the style and stigma begin to differentiate, the expression of *BofrAP2-2* marks the meeting point between the two stigmas on their adaxial flanks (fig. 2Q). This expression continues as the flower develops during stigmatic fusion (fig. 2R) and the formation of a massive style and during the differentiation of the transmitting tract (fig. 2S). Later in fruit development, *BofrAP2-2* is broadly expressed in the valves, more strongly in the exocarp and the endocarp than in the mesocarp (fig. 2T). Finally, *BofrAP2-2* is also found throughout the commissural ring, especially in the medial inner region, and like its paralogue it is lacking from the dehiscence zone (fig. 2T and U).

Expression of euAP2/TOE3 Homologues in the Early Divergent Angiosperm *Aristolochia fimbriata* (Aristolochiaceae)

The *A. fimbriata* flower grows solitary, axillary to each leaf. The flower consists of a perianth formed by three petaloid sepals fused to form a tubular structure divided in three regions: the proximal utricle, the medial tube, and the distal limb; petals are lacking (fig. 3A). Stamens and stigmas fuse forming a gynostemium crowning the inferior ovary (González and Stevenson 2000; Pabón-Mora et al. 2015). The fruit is an acropetally dehiscent septicidal capsule, leaving "baskets" full of seeds (fig. 3A). A single *AP2* homologue was identified from the selected species *A. fimbriata* (fig. 1). We describe the expression results for *AfimAP2*, based on detailed descriptions of the developmental stages of flowers and fruits previously identified (Pabón-Mora et al. 2015). Our expression analyses show that *AfimAP2* is not expressed in the SAM or in the floral bud prior to and during sepal initiation (fig. 3B). However, *AfimAP2* is expressed in young leaves (fig 3B). *AfimAP2* expression is detected in the sepals, in the stamen primordia, and in the ovary later in development (fig. 3C). Similar expression patterns are maintained throughout flower development, as the perianth curves and the thecae differentiate (fig. 3D and E), and later on when the utricle, tube and limb can be distinguished in the perianth (fig. 3E and F). When ovule differentiation begins, the expression of *AfimAP2* can be seen in the ovary wall, in the nucellus, and in the two integuments (fig. 3G and H). In a preanthetic floral bud, *AfimAP2* expression is no longer detected in the gynostemium, neither the stamens nor the stigmas, but it is maintained in the utricle and in the ovary (fig. 3I). Interestingly, *AfimAP2* expression in the ovary is stronger around the main vascular trace and in the inner layers of the wall, and lacking from the septum (fig. 3H and J).

Expression of euAP2/TOE3 Homologues in the Gymnosperms: *Ginkgo biloba* and*Gnetum gnemon*

In *Ginkgo biloba*, the funiculus, ovule-bearing stalk, is initiated axillary to the leaf and it usually dichotomizes producing two ovules, which are characterized by having a single integument covering the nucellus. To perform the expression analyses, ovules of *G.biloba* at four different stages were collected. At the first stage, the ovules are immature with a thickened collar, a vegetative structure unique to *Ginkgo*, that develops from the hypodermal layer proximal to the ovule (Douglas et al. 2007; fig. 4A). At the second stage, when the ovules are ready to be fertilized, the integument forms a more

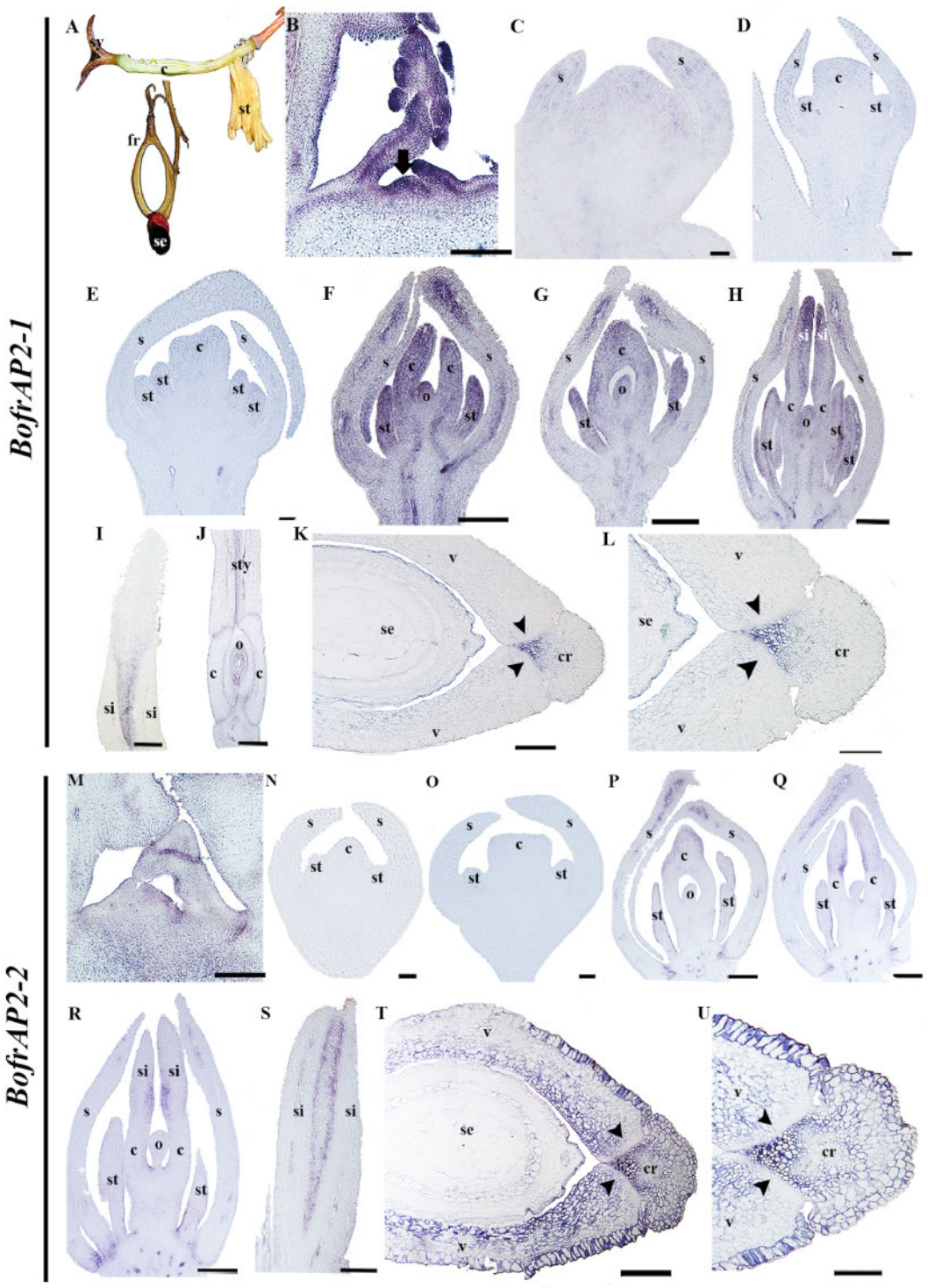

FIG. 2. Expression analyses in *Bocconia frutescens* (*A*) of the two *AP2* homologues: *BofrAP2-1* and (*B* to *L*) and *BofrAP2-2* (*M*to *U*). (*A*:*B*) *frutescens* flower in anthesis and mature fruit.(B)Expression in the shoot apical meristem.(*C* to *H*)Floral stages3–9. (***I***) Stigmas of a preanthetic flower. (*J*) Ovary and stigma of a preanthetic flower. (*K* and *L*) Cross section of a young fruit. Note the expression is restricted to the commissural ring.(*M*)Leaf primordia. (*N* to *R*) Floral stages3–9. (*S*) Longitudinal section through the stigmas. (*T* and *U*) Cross section of a young fruit. Note the expression is restricted to the valves and commissural ring, no expression is detected in the dehiscence zone. *Black arrowheads* pointing to the dehiscence zone of the fruit; *black arrow* pointing to the shoot apical meristem; c, carpel; cr, commissural ring; fr, fruit; o, ovule; s, sepal; se, seed; st, stamen; si, stigma; sty, style; v, valve. *Scales*: 50 μm (*C* to *E*, *N* and *O*), 75 μm (*P* and *Q*), 100 μm (*I*,*J*, and *S*), 125 μm (Band *M*), 150 μm (*F* to *H*, *R*), 0.1 mm (*K*, *T*, and *U*).

conspicuous micropyle which releases the pollination drop (fig. 4*B*). The next stage is characterized by fleshy growth in the integument, largely linked to the formation of multiple mucilage canals; the collar remains as a small structure at the base of the ovules (fig. 4*C*). In the seed, the integument, now the fleshy testa turns yellow and ripens (fig. 4*D* and *E*). An

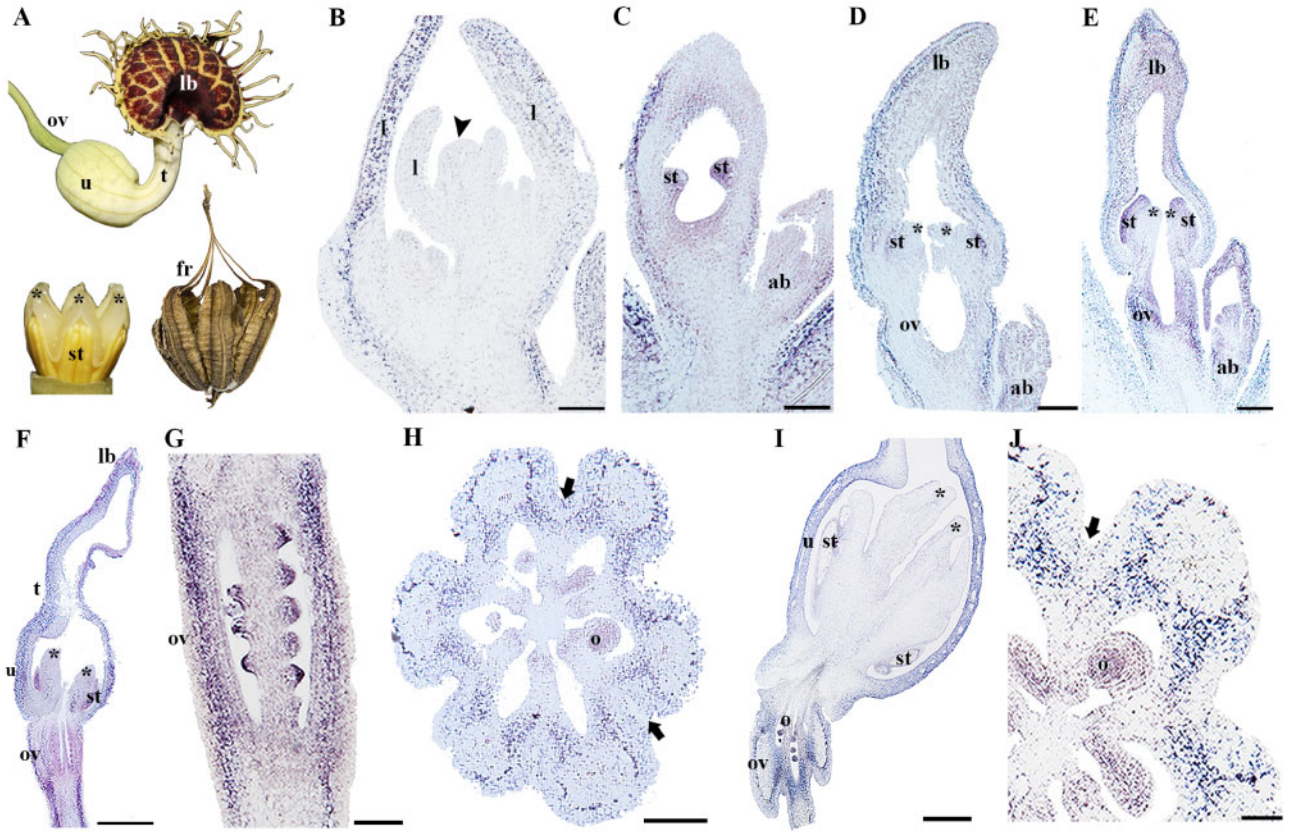

Fig. 3. Expression of *AfimAP2*. (*A*) *Aristolochia fimbriata* flower in anthesis and mature fruit. (*B*) Inflorescence apex with floral buds in stage 1–2, note no expression floral and sepal primordia.(*C*) Flowers stage 3, expression in the adaxial side of the sepal, stamen primordia. (*D* and *F*) Floral buds at stages 4–7, note the expression in the ovary and ovules. (*G*) Ovary of a flower at stage 8.(*H*) Cross section through the ovary, *AfimAP2* is expressed in the nucellus and integuments. (*I*) Utricle and ovary of a flower in preanthesis. (*J*) Cross section of the ovary of a flower in preanthesis. Note there is no expression in the septum. *Asterisk*s indicate stigmas; *black arrowhead* pointing to the inflorescence meristem; *black arrow* pointing to the septum; ab, accessory bud; fr, fruit; l, leaf; lb, limb; o, ovule; ov, ovary; st, stamen; t, tube; u, utricle. *Scales*: 50 μm (*H* and *I*), 60 μm (*J*), 80 μm (*G*), 100 μm (*B* to *F*).

immature pollen cone was included in the expression analysis as well (fig. 4*F*). One *AP2* homologue was identified in *G. biloba* and was named *GibiAP2* (fig. 1). The expression of *GibiAP2* was evaluated in ovulate and pollen cones of *G. biloba*. One feature that is different in gymnosperms, compared with angiosperms, is that the tissue processing during the insitu hybridization experiment, results in brownish mucilage canal walls and tannin oxidation (fig. 4*G* to *L*; see also Lovisetto et al. 2013). Thus, brown color represents background and only the purple colorimetric reaction represents gene expression. *GibiAP2* is expressed in the adaxial leaf surface (fig. 4*G*). Expression in the young developing ovule was detected in the vegetative tissues such as the stalk and collar, as well as the proximal portion of the ovule, the nucellus and slightly less in the integument (fig. 4*H*). As the ovule reaches the fertilization stage with a well-developed megagametophyte, two different integumentary layers are visible, the outer most layer is characterized by the presence of tannins and mucilage canals. At this point,*GibiAP2* expression is detected in the integument, the nucellus, and the developing megagametophyte (fig. 4*I*). As the integument thickens, the expression *of GibiAP2* is found in the integument and in the nucellus (fig. 4*J*). The same expression can be seen until the fertilized ovule transitions to a ripening seed (fig. 4*K* and *L*). In the pollen cone, weak expression of *GibiAP2* is detected in the endothecium and the tapetum of the microsporangium and the microspores (fig. 4*M*).

In the other gymnosperm species evaluated, *Gnetum gnemon*, the strobili develops axillary to the leaf and it has multiple nodes with rings of ovules (fig. 5*A*). The ovules are characterized by having an integument and two additional envelopes, for a total of three protective layers covering the megasporangium and later the megagametophyte. Thus, from the inside to the outside, the first layer corresponds to the integument, the second layer is the middle envelope, and the third layer is the outer envelope. The outer envelope becomes fleshy and colored, usually red (Endress1996; Rydin et al. 2006). Unlike, *G. biloba*, *G. gnemon* has bisexual cones, in each node of the strobili, an upper ring of ovules is formed and several rings of microsporangia develop basipetally, in the bisexual strobilus the ovules are usually abortive (fig. 5*A*; Pearson 2010). Ovules at seven different stages of development were collected, based on size and color. The first stage available was the ovule

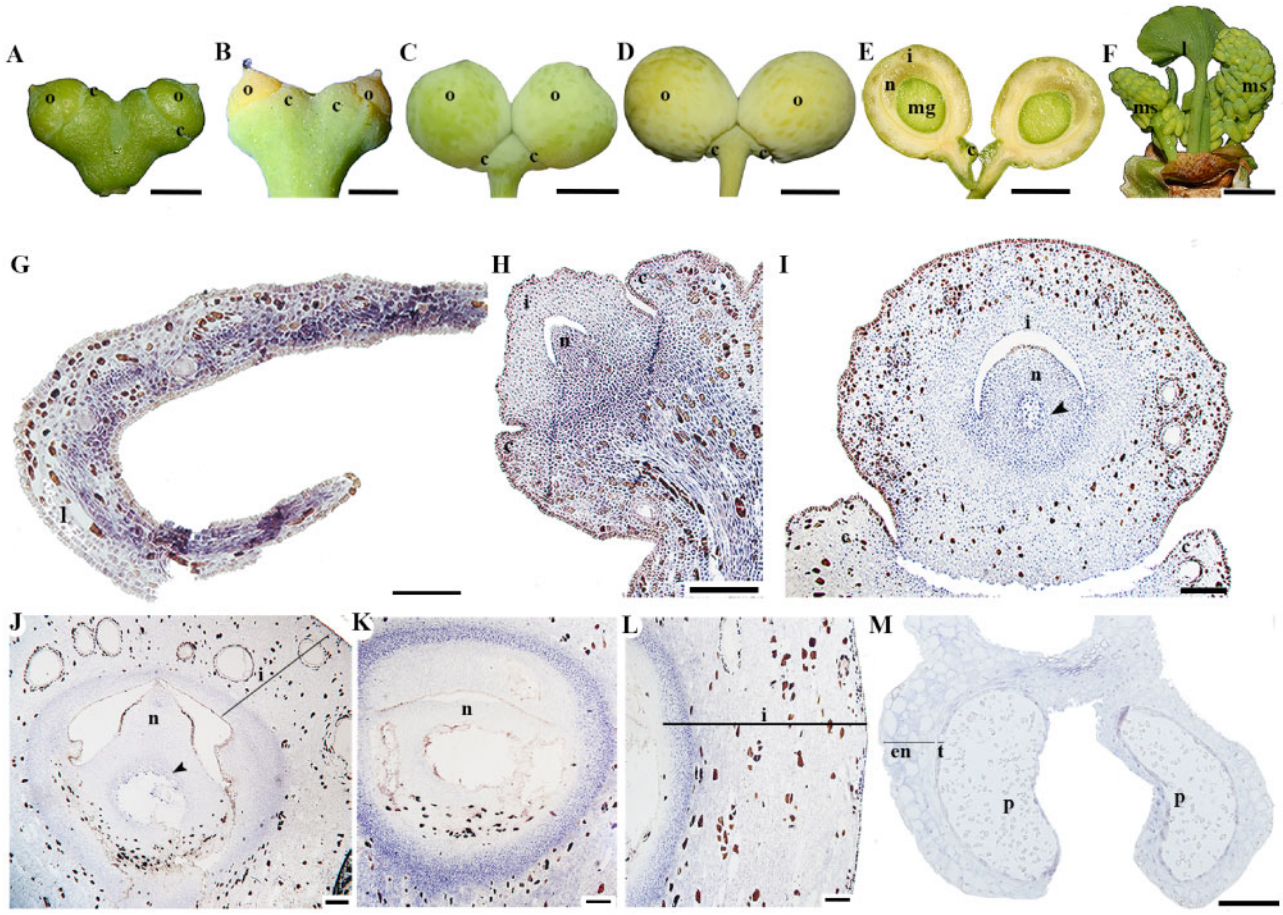

Fig. 4. Expression of *GibiAP2* in *Ginkgo biloba*.(*A* to *D*). Ovules at different developmental stages. (*E*) Hand section of a mature ovule as shown in (*D*). (*F*) Young pollen cones in the axils of leaves. (*G* to *M*)*GibiAP2* expression patterns. (*G*) Cross section of a leaf. (*H* to *L*)Ovule at different stages, note the expression in the integuments and nucellus is maintained throughout ovule development. (*L*) Close-up of theintegument of a fully mature ovule. (*M*) Pollen cone. *Black arrowhead* pointing to the megagametophyte; c, collar; en, endothecium; i, integument; l, leaf; mg, megagametophyte; ms, microsporangium; n, nucellus; o, ovule; p, pollen; st, sporogenous tissue; t, tapetum. *Scales*: 50 μm (*J* to *L*), 100 μm (*G* to *I*, *M*), 5 mm (*A*,*B*, *F*), 1 cm (*C* to *E*).

collected from a bisexual cone where the micropyle was not yet visible (fig. 5*A*). For the next two stages, the ovules have a green and coriaceous outer envelope (fig. 5*B*). The ovules at the next stage are still green but they have continued to grow, becoming fleshier with a clearly distinguishable micropyle (fig. 5*C*). At the following stage, the ovules turn yellow indicating they are competent for fertilization (fig. 5*D*). As the ovule grows, the micropyle closes (fig. 5*E*). Finally, the ovule turns into a red fleshy seed (fig. 5*F*). Like *G. biloba*, a single *AP2* homologue was identified in *G. gnemon* and was named *GneAP2* (fig. 1). In the earliest developmental stage of the bisexual cones sampled, the expression of *GneAP2* was detected in the nucellus and the middle envelope of a young ovule, as well as in the sporogenous tissue of the microsporangia (fig. 5*G*). The expression of *GneAP2* in the nucellus is maintained during early stages of ovule development (fig. 5*H*). As all three protective layers differentiate, the expression of *GneAP2* is reduced in the nucellus but remains strong in the integument and in the proximal portion of the middle envelope (fig. 5*I*). *GneAP2* is also expressed in the megagametophyte and in the pollen chamber, the region of the nucellus that receives the pollen (fig. 5*J*). These expression patterns are maintained as the ovule continues to develop, the expression is particularly strong in the integument including the region forming the micropyle (fig. 5*K*). Later on, *GneAP2* expression is found in the proximal portion of the integument and in the archegonia of the megagametophyte (fig. 5*L*). The expression of *GneAP2* in the mature seed is not detected (fig. 5*M*).

Expression of *euAP2/TOE3* Homologues in the Fern *Ceratopteris richardii*

Ceratopteris richardii is a homosporous fern, meaning that there is no differentiation of female and male sporangia and spores, thus, all the spores are the same size and identity. The sporangia develop on the abaxial side of the leaf and as the plant matures, the leaf margins curve to cover the sporangia (Conway and Di Stilio 2020; fig. 6*A* and *B*). The development of the sporangium begins with the transverse division of a single superficial initial cell, with a series of subsequent cell divisions the lower cells will form the stalk and the apical cells will form the spore containing capsule or "head" of the sporangium (Foster and Gifford 1974). *CerAP2* expression is detected at early stages of sporangium

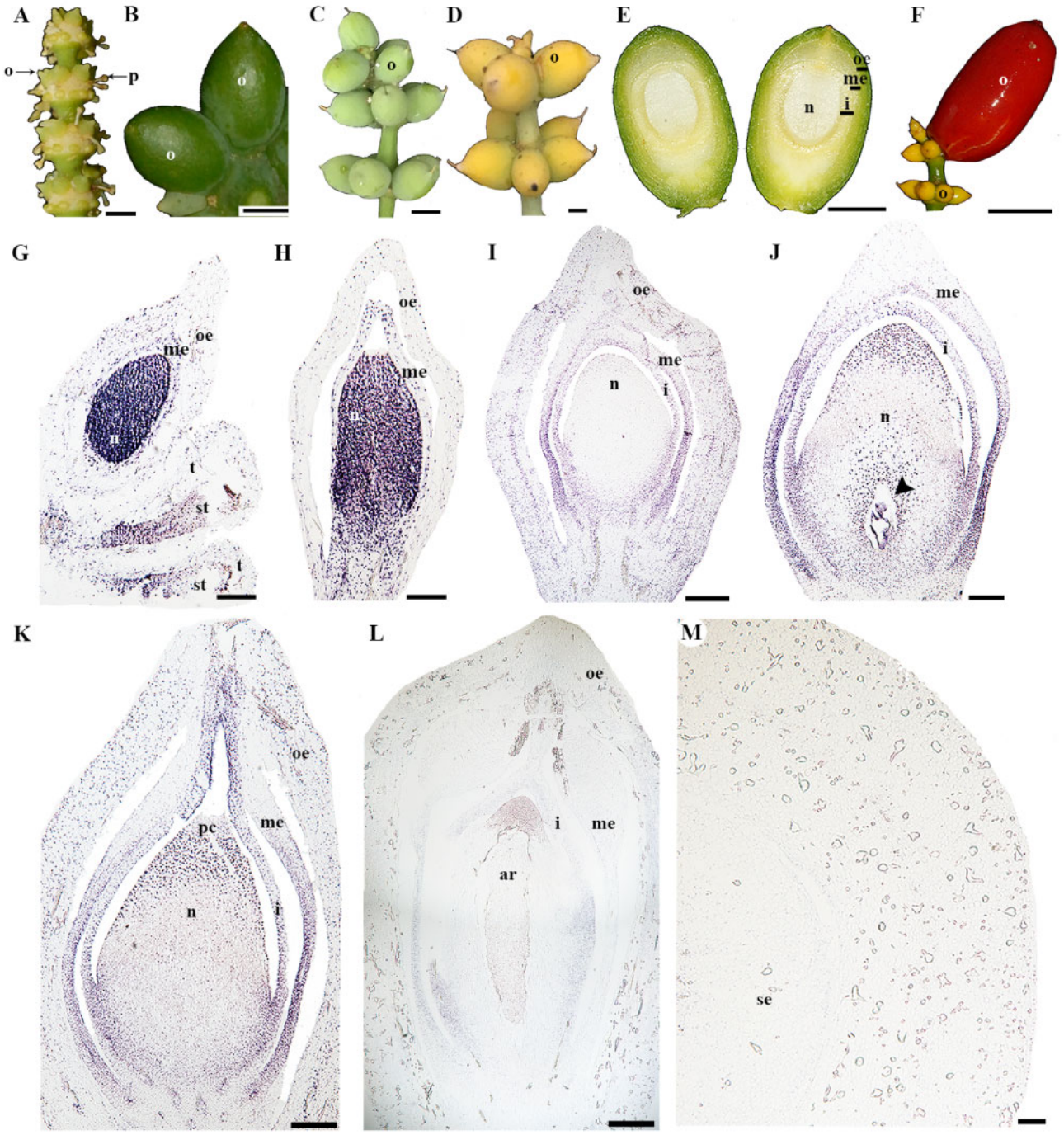

Fig. 5. Expression analyses in *Gnetum gnemon*.(*A*) Bisexual cone with a ring of ovules and rings of pollen cones developing basipetally at each node. (*B* to *F*) Ovules at different stages of development. (*E*) Inside of the mature ovule showing three different layers protecting the nucellus. (*F*) Fully mature seed. (*G*) Pollen cone with ovule at the top. (*H* to *L*) Developing ovules, note the expression of *GneAP2* is restricted to the nucellus, integument, the middle envelop and later in the archegonia and pollen chamber. (*M*) Cross section of a young seed. *Black arrowhead* pointing to the megagametophyte; ar, archegonia; i, integument; me, middle envelop; ms, microsporangium; n, nucellus; o, ovule; oe, outer envelope; p, pollen; pc, pollen chamber; se, seed; t, tapetum. *Scales:* 50 μm (*J* to*M*), 100 μm (*G* to *I*), 0.5 cm (*A* to *D*), 2 cm (*E* and *F*).

development (fig. 6*C*). As the inner cell develops, *CerAP2* is expressed in the sporogenous tissue where the spores will develop and in the few cells forming the stalk (fig. 6*D*). Expression of *CerAP2* in the sporangium wall and sporogenous tissue is maintained as the sporangia develops (fig. 6 *D* and *E*) but is no longer detected in the stalk (fig. 6*F* and *G*). Following the maturation of the sporangium, *CerAP2* expression is maintained in the young spores, the inner wall of the sporangium (tapetum), and in the vasculature of the leaf (fig. 6*H* and *I*).

Discussion

APETALA2 was first recognized as a perianth identity gene in Arabidopsis, and molecular genetic analyses placed it in the

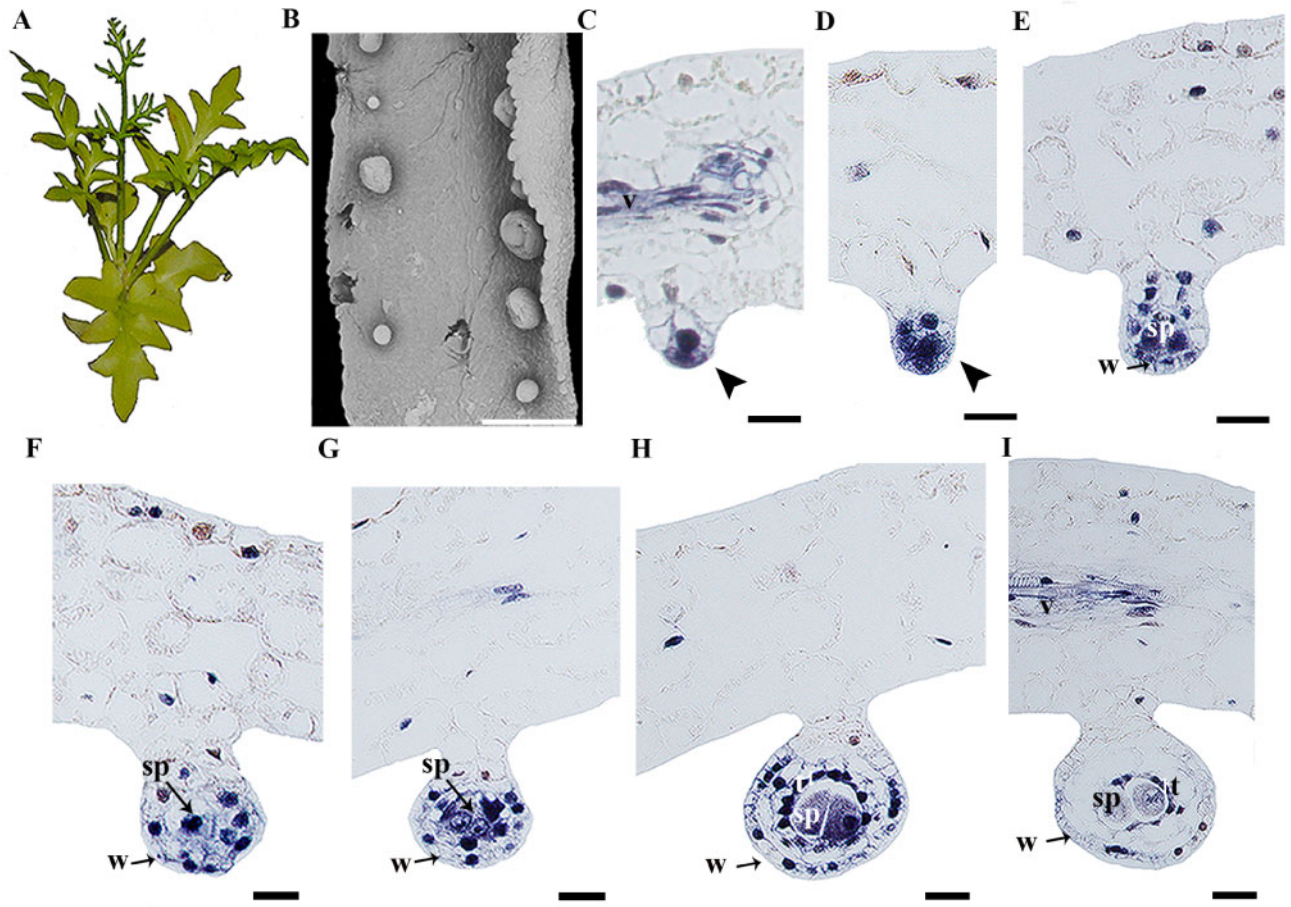

FIG. 6. Expression analyses in *Ceratopteris richardii*.(*A*) Mature plant (note sterile leaves with wider laminae) (*B*) Scanning Electron Microscope Photograph of a fertile leaf (narrow lamina from A). (*C* to *I*) *CerAP2* expression at different stages of sporangium development. Note the expression restricted to the sporangium, no expression is detected in the leaf. *Black arrowhead* pointing to the sporangium primordia; sp, sporogenous cells; t, tapetum; v, vasculature; w, sporangium wall. *Scales*: 500 μm (*B*); 10 μm (*C* to *I*).

ABC model of floral development as an A-class gene (Bowman et al. 1989; Kunst et al. 1989; Bowman et al. 1991; Coen and Meyerowitz 1991; Bowman et al. 1993). *AP2* homologues have been shown to play roles in fruit and seed development in Arabidopsis as well as in other core eudicots (Jofuku et al. 1994; Chung et al. 2010; Karlova et al. 2011; Ripoll et al. 2011), and have also been implicated in inflorescence patterning and yield in monocots (Chuck et al. 2008; Lee and An 2012; Bommert and Whipple 2018). Moreover, *euAP2* genes are present in vascular plants but have remained little explored outside flowering plants. Importantly, the *euAP2* genes are sister to the *AINTEGUMENTA* genes a lineage dating back to bryophytes as suggested by the presence of *ANT* homologues in *Physcomitrella* (Kim et al. 2006). This points to an ancient gene lineage with likely pleiotropic developmental roles in vascular plants. However, the data available so far does not explain why *AP2* homologues perform so many different roles across angiosperms, nor does it clarify the ancestral roles of the gene lineage early in angiosperm evolution or in gymnosperms or ferns.

In order to infer the functional evolution of this gene lineage, we have: 1**)** updated the gene phylogeny with more inclusive gene sampling from vascular plants identifying seven major duplication events in the *euAP2/TOE3* angiosperm subclade (Zumajo-Cardona and Pabón-Mora 2016), and homologues from gymnosperms, ferns, and lycophytes. These results differ from previous analyses in the finding of fern and lycophyte *euAP2/TOE3* homologues (Kim et al. 2006; Dinh et al. 2012; Wang et al. 2016); 2**)** performed detailed spatiotemporal expression analyses in representative species from major vascular plant groups. Our results point to plesiomorphic roles of the gene lineage in the sporangia development in ferns and later recruitment of *euAP2/TOE3* homologues in ovule and seed development in gymnosperms, with putative new roles in fruit development in angiosperms and their recruitment in perianth development in some angiosperms (fig. 7). Below, we discuss the implications of these findings in a gene lineage with pleiotropic developmental roles.

Early Recruitment of *euAP2/TOE3* Homologues: From Sporangium Development in Ferns to Ovule Development in Seed Plants

Our expression analyses allow us to unequivocally place the ancestral role of *euAP2/TOE3* homologues at the core of vascular plant reproduction. In the homosporous fern *C.richardii*, *CerAP2* is expressed throughout sporangia development,

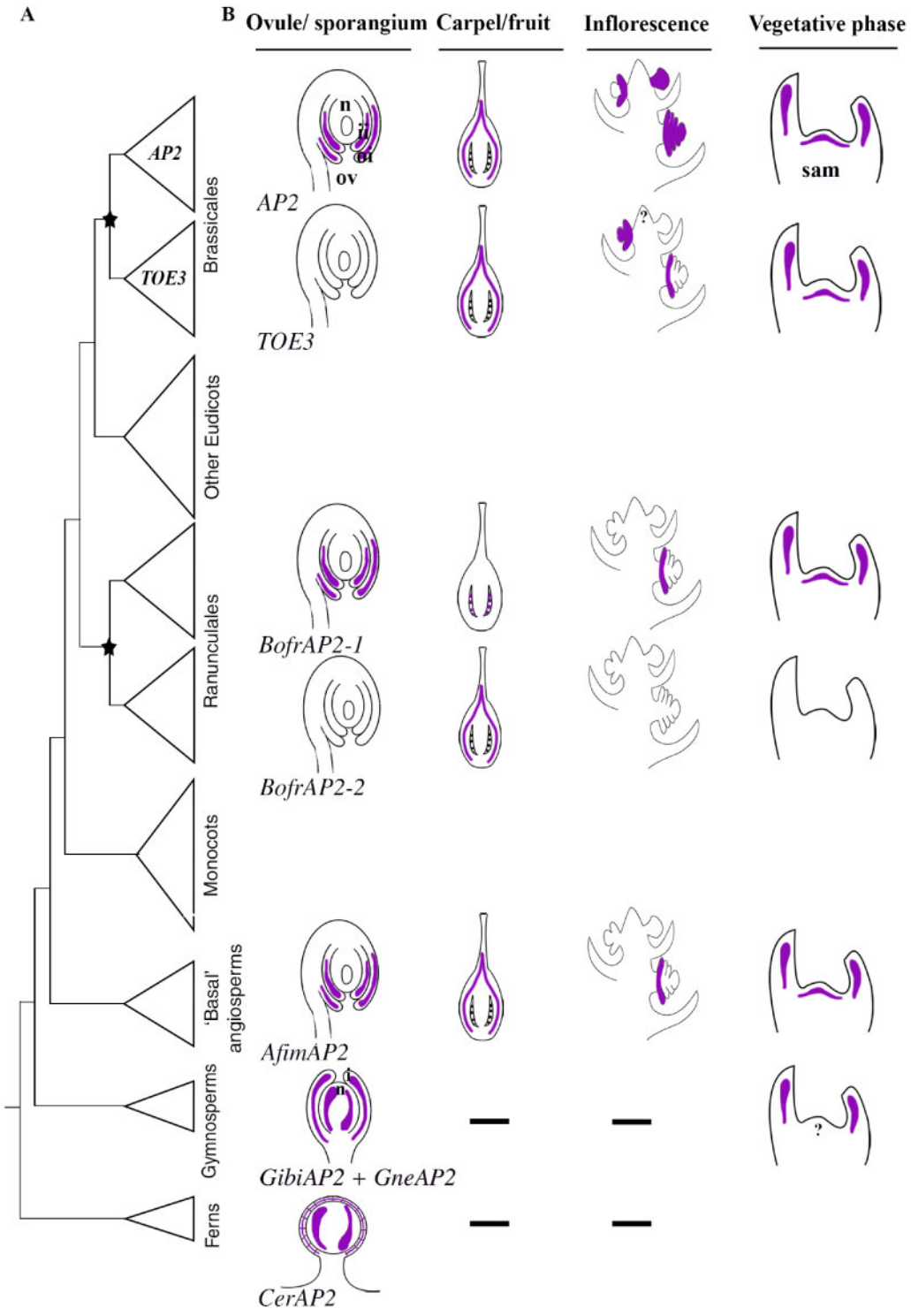

Fig. 7. Summary of (*A*) duplication events of the *euAP2/TOE3* gene lineage across vascular plantsand (*B*) Spatiotemporal expression of *euAP2/TOE3* homologues during reproductive and vegetative phases, the purple color indicates the areas where expression has been reported for that clade. *AP2* and *TOE3* expression patterns in Arabidopsis as reported by previous studies (Würschum et al. 2006; Wollmann et al. 2010; Ripoll et al. 2011; Jung et al. 2014). Question mark denotes tissues for which expression information is not available . Black bars denote the absence of the tissue in that plant lineage; i, integument; ii, inner integument; n, nucellus; oi, outer integument; ov, ovule; sam, shoot apical meristem.

from early sporangium development until the differentiation of the wall and the spores (fig. 6). Expression of *euAP2/TOE3* homologues in the two gymnosperms *G. biloba* and *G. gnemon* indicate that they were retained over evolution as key factors in the development of the mega- and the microsporangia (figs. 4and 5). Perhaps more interesting is the fact that from early seed plant diversification, *euAP2/TOE3* genes expand their expression domains to the protective integuments, forming around the megasporangia (figs. 4and 5), which are major innovations occurring in the

seed plants (Brenner and Stevenson 2006). Another important observation is that in ferns and gymnosperms the expression of *euAP2/TOE3* homologues in sporangia development is broader at first and at maturity becomes more restricted. Specifically expression of *euAP2/TOE3* homologues remains closer to the tapetum and the spores in ferns, or in the nucellus and integument in gymnosperms. Because our data cannot be used to assess the function of the *euAP2/TOE3* homologues, we can only hypothesize that they are associated with the nourishing and protective tissues of the meiotic products, the gametophytes, and perhaps even the young embryo(s). This is consistent with data in other gymnosperms like *L. marschlinsii* and *P. abies*, where *euAP2/TOE3* homologues are expressed during the first stages of embryo development (Vahala et al. 2001; Shigyo and Ito 2004; Guillaumot et al. 2008).

The expression of *AP2-like* homologues in ovules and the two integuments was retained in angiosperms and it is clearly one of the plesiomorphic roles of the gene lineage present during flowering plant evolution. Evidence for this includes: 1**)** the expression of *AfimAP2* in *A.fimbriata* (fig. 3*F* to *L*) and *BofrAP2-1* in *B. frutescens* (fig. 2*C* to *G*) in the integuments and the nucellus; and 2**)** the recruitment of *AP2* in Arabidopsis during integument development, as the *ap2* mutant has in the first whorl carpel-like structures with placental tissue and ovules with defective or lacking integuments (Jofuku et al. 1994). Additionally, when studied with other ovule development genes, *BELL1* and *AG*, the ovules developed in all whorls seem to be only enlarged cells that resemble a nucellus without actual integuments (Western and Haughn 1999). It is important to highlight that bryophytes (mosses, liverworts, and hornworts) also have sporangia but, no *euAP2* homologues have been identified in this plant lineage so far (fig. 1; Kim et al. 2006). This could be because meristematic tissues vary between liverworts, mosses, hornworts, and vascular plants (Campbell1913; Ligrone et al. 2012). Liverworts have a sporangium or capsule, from which the spores develop, which grows by cell divisions in the absence of any localized area recognizable as a meristem (Gunning et al. 1978; Thomas1980). In mosses, an unifacial meristematic area is formed producing the sporangium primordium acropetally, this is a transient basal meristem (Renzaglia1978). On the other hand, hornworts also have a basal meristem but it remains active for the life of the sporophyte, a characteristic more similar to vascular plants (Campbell1913; Ligrone et al. 2012).

New Roles of *euAP2/TOE3* Homologues in Flowering Plants Include Fruit Development

Our expression analyses of *euAP2/TOE3* homologues in the early divergent angiosperm *A. fimbriata* and the basal eudicot *B. frutescens* point to their involvement in carpel and fruit development (figs. 2and 3). Carpels and fruits are major innovations in angiosperms which provided additional prezygotic barriers for egg cell fertilization in the ovules, as well as an efficient vessel for seed dispersal aiding in plant fitness. *euAP2/TOE3* genes had already been identified as important players in fruit patterning of model core eudicot species (Alkio et al. 2012; Mühlhausen et al. 2013). In Arabidopsis, *AP2* acts as an upstream regulator by directly repressing valve margin genes such as *SHATTERPROOF* (*SHP*) and replum identity genes such as *BREVIPEDICELLUS* and *REPLUMLESS* (Ripoll et al. 2011). In tomato, one of the *AP2* homologues is involved in fruit ripening (Chung et al. 2010). It is remarkable that *AP2-like* copies in both *A. fimbriata* and *B. frutescens* are broadly expressed in the fruit walls (or the commissural ring), but always remain absent from the dehiscence zones, which are the regions through which the pericarp will break to release the seeds. Previous studies of *RPL* homologues in *B. frutescens* show mutually exclusive expression patterns with *BofrAP2* copies, as *BofrRPL* is restricted to the dehiscence zone (Zumajo-Cardona et al. 2018). Taken together, the data suggest that the repression AP2–RPL may be a largely conserved module across angiosperms. Interestingly, *SPATULA* genes in *B. frutescens* share overlapping expression patterns with *RPL* homologues in the deshicence zone, suggesting that they too can be directly or indirectly repressed by *AP2-like* homologues. On the other hand, *SHP* is the result of a core-eudicot duplication, but, it has been suggested that its function in fruit development may be maintained by the *paleoAGAMOUS*, preduplication genes (Pabón-Mora et al. 2014). However, there is no evidence that *paleoAG* and *AP2-like* genes interact in any way so far. Thus, to confirm this hypothesis, more studies such as spatiotemporal expression analyses for *paleoAGAMOUS* are still required. As for early diverging angiosperms, it is less clear what are the putative genes controlling fruit histogenesis. However, in *A. fimbriata*, both *AfimSPT* (a *SPATULA* homologue) and *AfimSTK* (a *SEEDSTICK* homologue) have restricted expression to the septum and future dehiscence zones (Suárez-Baron et al. 2017; Peréz-Mesa et al. 2020). Altogether, the data does suggest that *AP2-like* homologues control aspects of fruit wall development and may repress other fruit development transcription factors to the deshisence zones in dry dehiscent fruits in noncore eudicots (figs 2and 3). Thus, *AP2* function in fruit ripening seems to be present in dry dehiscent fruits and fleshy fruits, although further studies are required to determine if the function is conserved in dry indehiscent fruits (Ripoll et al. 2011; Wang et al. 2019).

Roles of *euAP2/TOE3* Genes in Flower Development Are Broad, but Its Canonical A Function Appears Restricted to the Brassicaceae and Poaceae

In Arabidopsis, *AP2* was classified as an A function gene based on its role in perianth development and the mutual antagonism of C function genes within the ABCE model of floral organ development (Bowman et al. 1991; Drews et al. 1991; Bowman et al. 1993; Tissier et al. 1999; Pelaz et al. 2000). Functional studies demonstrated that these dual roles of *AP2* could be separated genetically (Bowman et al. 1991; Coen and Meyerowitz 1991). *euAP2/TOE3* homologues have been unequivocally identified as A function genes by functional analyses in Arabidopsis and Brassica (Drews et al. 1991; Zhang et al. 2018). Canonical A function appears conserved in Poaceae, as molecular and genetic analyses in rice

and wheat, show that *AP2* orthologues are integral for perianth development and antagonism of C function; *ap2* knockouts have homeotic conversions in the grass perianth (Lee et al, 2007; Lee and An 2012; Debernardi et al. 2020). *euAP2/TOE3* gene expression has been detected in the floral meristem, sepals, petals, the ovary wall, and ovules in Arabidopsis, maize, petunia, rice, snapdragon, and wheat suggesting putative roles in all these organs (Bowman et al. 1989,1991,1993; Maes et al. 2001; Keck et al. 2003; Lee et al. 2007; Chuck et al. 2008; Lee and An 2012). Knockouts of *euAP2/TOE3* in petunia and snapdragon affect perianth development, however, no homeotic conversion or ectopic expression of C class genes were detected in these mutants, indicating that the antagonism between A function, as specified by euAP2/TOE3, and C functions is not conserved in these species (Keck et al. 2003; Causier et al. 2010; Morel et al. 2017). The conservation of the ABC model of floral organ identity and, particularly, how the perianth whorl is specified across angiosperms is still unknown (Causier et al. 2010).

A conservation in the ABC model of floral organ development would suggest that the A and E function orthologues of *APETALA1/FRUITFULL* (*AP1/FUL*), *euAP2/TOE3* (A class genes) and *SEPALLATA* (*SEP*, E class genes), would specify the perianth whorl (Pelaz et al. 2001; Causier et al. 2010). Surprisingly, we did not detect expression of *euAP2/TOE3* orthologues in the perianth primordia of *B. frutescens* or *A. fimbriata* suggesting that, although they are expressed late in sepal development, these orthologues do not play a role in perianth specification or antagonism of C class genes early in floral development of these species (figs. 2 and 3). *Bocconia frutescens* has two caducous sepals and no petals. *AP1/FUL* and *SEP* orthologues are expressed in the sepals of *B. frutescens* which suggest these genes may specify this perianth whorl (Arango-Ocampo et al. 2016). It was hypothesized that the lack of petals in *B. frutescens* was due to a lack of a B function orthologue and expanded expression of the C function orthologue of *AGAMOUS* (*AG*; Arango-Ocampo et al. 2016). *Aristolochia fimbriata* has three petaloid sepals and also lacks petals. Expression analyses of *AfimFUL*, *AfimSEP*, and *Afim AGAMOUS-Like 6* (*AGL6*) orthologues suggests that they are important for the specification of sepals in *A. fimbriata* (Pabón-Mora et al. 2015). Furthermore, the interaction of AfimSEP2 and AfimAGL6 proteins by yeast-2-hybrid indicate that these two protein orthologues are key players in perianth specification in *A. fimbriata* (Perez-Mesa et al. 2019) In turn, available data strongly suggest that *AfimAP2* contribution may not be needed for perianth initiation. Knockouts of ABC floral organ identity orthologues are needed to better understand how the perianth is specified in these species.

Putative Functions of *euAP2/TOE3* Homologs in Stem Cell Niche Maintenance in Flowering Plants

One of the key roles of *euAP2/TOE3* genes is in reproductive transition and control of maintenance of the SAM as it has recently been included in the genetic pathway controlling meristem maintenance (Würschum et al. 2006; Balanzà et al. 2018). The mechanism of action of *AP2* is the result of direct repression of *FRUITFULL* (*FUL*) thus, controlling global proliferative arrest (GPA), and in turn *AP2* (and other *euAP2* genes), directly or indirectly or controlling *WUSCHEL* maintaining the SAM (Würschum et al. 2006; Balanzà et al. 2018). Although this study does not include vegetative meristem versus reproductive meristem in all taxa, we detected expression of *BofrAP2-1* in the SAM suggesting that it is conserved in basal eudicots and that its role in meristem development is putatively maintained (fig. 2*B*). In grasses, the spikelet is the basic unit of the inflorescence and *AP2* homologues have been extensively reported to be involved in reproductive transition, from the spikelet meristem to the floral meristem (Chuck et al. 1998,2008; Lee and An 2012; Bommert and Whipple 2018; Debernardi et al. 2020). In maize *IDS1* and *SID1* are required to initiate the floral meristems and to control spikelet determinacy (Chuck et al. 1998; Chuck et al. 2008). A similar scenario is observed in wheat, where *Q* is involved in the initiation of the floral meristem (Debernardi et al. 2020). Altogether, it seems that the function of the *AP2* homologues in SAM and reproductive transition is conserved across angiosperms, and it could be the ancestral role of the gene lineage like *euAP2* genes (*SMZ*, *SNZ*, *TOE1*, *2*, and *3*) are also involved in flowering time (Schmid et al. 2003; Würschum et al. 2006; Huijser and Schmid 2011). Further studies in ferns and lycophytes are still required to predict if the transition to reproduction is conserved across vascular plants.

euAP2/TOE3 Gene Expression Domains Coincide with Major Seed Plant Innovations

Our results together with the data available on the *euAP2/TOE3* genes allow us to provide different scenarios regarding functional evolution of the pleiotropic *euAP2/TOE3* genes (fig. 7).

(1) Our expression analyses suggest that the putative ancestral role of the *euAP2/TOE3* gene lineage may be associated with sporangia development, as indicated by the expression of these genes in fern, gymnosperm, and angiosperm sporangia. Moreover, *euAP2/TOE3* genes have been recruited in the formation of the integuments, a synapomorphy for seed plants, and in carpels and fruits, two major apomorphies for flowering plants (fig. 7). These results place the *euAP2/TOE3* genes at the core of plant reproduction, linked to the occurrence of novel features across euphyllophytes. The underlying mechanisms controlling the shifting expression patterns of *euAP2/TOE3* genes are still unknown, but we hypothesize that *cis*-regulatory elements and noncoding gene regions have likely diverged during the course of vascular plant evolution (Sharma et al. 2018).

(2) Comparative sequence analyses show a conservation in the repression domains (RD, EAR domains) in AP2/TOE3 proteins and a conserved miR172 binding site in *AP2/TOE3* genes across most euphyllophytes (Kim et al. 2006; Guillaumot et al. 2008). In turn, although the study did not include vegetative versus reproductive meristems for all taxa, we cannot rule out that the repression from

vegetative to reproductive transition is part of the plesiomorphic roles of the *euAP2/TOE3* gene lineage (fig. 7; Aukerman and Sakai 2003; Schmid et al. 2003; Chuck et al. 2007; Jung et al. 2007; Huijser and Schmid 2011; Balanzà et al. 2018; Debernardi et al. 2020). Even more, when TOE3 repression of AG is critical for floral patterning in Arabidopsis (Jung et al. 2014). It is well known that the RD functions through the recruitment of TOPLESS (TPL) and TPL-related corepressors, are also conserved across land plants (Ohta et al. 2001; Causier et al. 2012). Thus, it is possible that the repression of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) or their homologues in euphyllophytes are conserved (Yant et al. 2010; Klintenäs et al. 2012).

(3) The canonical A function of *euAP2/TOE3* genes is characteristic of a large number of representatives in eudicots and Poaceae (fig. 7). The fact that most members of the *euAP2/TOE3* gene clade have the *miR172* binding sites suggest that this post-transcriptional regulation is conserved across euphyllophytes, and that the *miR172* regulation is not the only mechanism associated with the floral organ identity function (supplementary fig. S4; Park et al. 2002; Griffiths-Jones 2004; Axtell and Bartel 2005; Sunkar and Jagadeeswaran 2008; Luo et al. 2013; Zumajo-Cardona and Pabón-Mora 2016). In turn, the coupling of *euAP2/TOE3* genes to floral organ identity roles may be linked to the acquisitions of specific and new protein–protein interactions in some angiosperms lineages.

Thus, it is clear that *euAP2/TOE3* genes are part of the developmental toolkit that has allowed major innovations in seed plants: seeds, flowers, and fruits. We stress the importance of sequencing more genomes and the standardization of functional tools across land plants in their native context. Future studies need to focus on understanding shifting *cis*-regulatory elements that can help explain the expansion from sporangia expression into new domains in integuments, the carpels, the fruits and the perianth, as well as the changes in the genetic regulatory network integrating euAP2/TOE3 proteins across euphyllophytes.

Materials and Methods

Isolation of Homologues and Phylogenetic Analyses of the *euAP2*/TARGET of EAT 3 Gene Lineage

To reconstruct the evolution of the *euAP2/TOE3* gene lineage across vascular plants we have included sequences from our own generated transcriptomes across flowering plants as follows: for several eudicots including *Brugmansia suaveolens* (Solanaceae,Hernández-Ciro and Pabón-Mora 2020), *Coffea arabica*, *Condaminea corymbosa*, *Palicourea angustifolia* (Rubiaceae, Salazar-Duque, Alzate, Urrea-Trujillo, Ferrandiz and Pabón-Mora *In preparation*) and *Pilostylesboyacensis* (Apodantaceae; González et al. 2020); for some monocots including *Masdevallia coccinea*, *Masdevallia wendlandiana*, *Maxillaria aurea*, *Miltonia roezlii*, *Oncidium sp*, *Stelis pusilla*, *Tolumnia sp.*, (Orchidaceae, Madrigal et al. 2019); and for the ferns,*Adiantum capillus-veneris* (Pteridiaceae) *Anemia villosa* (Anemiaceae), *E. bogotense*, and *Equisetum cf. giganteum* (Equisetaceae, Rodr¡guez-Pelayo, Vasco, Ambrose and Pabón-Mora *In preparation*).Other fern and lycophyte homologues were retrieved from the OneKP transcriptome database (https://db.cngb.org/onekp/; last accessed January 9, 2021).

These sequences were recovered by a BLAST search using the *euAP2/TOE3* sequences previously reported (Zumajo-Cardona and Pabón-Mora 2016), as well as the paralogues of Arabidopsis (*AP2*: AT4G36920 and *TOE3*: AT5G67180; supplementary table 1). The search was thoroughly performed in *Physcomitrella patens* (a moss), in the transcriptome available in cosmoss.org, as well as in other bryophytes and algae transcriptomes available in the OneKP database and in the genomes available through Phytozome. However, no *euAP2/TOE3* homologues were retrieved outside vascular plants.

A total of 209 sequences were compiled and edited manually to keep only the open reading frame using AliView (Larsson2014) and then aligned using the online version of MAFFT (Katoh and Standley 2014) with a gap open penalty of 3.0, offset value of 0.8 and all the other default parameters. To find the nucleotide substitution model that best fits our data, according to Akaike Information Criterion (Akaike1974), we used the jModelTest 2 (Darriba et al. 2012), which identified the GTRGAMMA model as the best-fit model for our dataset. RAxML v.8.0.0 was used to estimate phylogenetic relationships under a maximum likelihood (ML) framework (Stamatakis et al. 2008; Stamatakis2014) using the complete nucleotide alignment of all homologues. The GTRGAMMA model was assigned and a full ML search was implemented, using the autoMRE bootstopping criterion to assess nodal support (-f a -# autoMRE option). Closely related genes from Arabidopsis: *SMZ* (AT3g54990), *SNZ* (AT2g39250), and *TOE2* (AT5g60120), were used as the outgroup. The 44 newly isolated sequences were deposited in GenBank (GenBank numbers MW375853-MW375896).

To identify reported domains, new motifs, and obtain a comprehensive overview of protein sequence evolution in the lineage, a total of 103 sequences of euAP2/TOE3 were selected representing major vascular plant lineages (77 angiosperms; 22 gymnosperms; 11 ferns; 5 lycophytes). Complete sequences were permanently translated and uploaded as amino acids to the online Multiple Em for Motif Elicitation server (http://meme.nbcr.net; last accessed May 14, 2020) (Bailey et al. 2006), and run with all the default options.

Expression Analyses of Selected *euAP2/TOE3* Homologues from Vascular Plants

To perform qRT-PCR, floral buds and flowers in anthesis from *E. californica* were collected from the grounds of the New York Botanical Garden (NYBG) and immediately frozen in liquid nitrogen. Total RNA extraction was carried out using TRIzol reagent (Invitrogen) from all dissected organs: floral bud, sepal, petal stamens, and carpels in preanthesis as well as in anthesis, immature fruit, mature fruit, and leaf. Genomic

DNA contamination was removed using DNaseI (RNase-free, Austin, TX, USA) following the manufacturer's instructions. First-strand cDNA was synthesized from 3ug of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) with oligodT_{20} primers, following manufacturer's instructions. The resulting cDNA was diluted 1:4 and the target fragment was amplified using locus-specific primers for each paralogue with the help of online tools available at https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool (last accessed May 14, 2020) (supplementary table 2).

For in situ hybridization analyses, inflorescences, floral buds, and fruits of *B. frutescens* were collected in the field (voucher: Colombia, Antioquia, Medellín, Km. 17 750, V$_i$a El Escobero Las Palmas, sobre la via principal, January 2019, *C. Zumajo-Cardona, F. Gonzalez* and *N. Pabón-Mora* 14) and immediately fixed in formaldehyde–acetic acid–ethanol (FAA; 3.7% formaldehyde: 5% glacial acetic acid: 50% ethanol). Plants of *A. fimbriata* were grown in the laboratory with 15 h light. Developing SAMs in reproductive stages, floral buds and carpels were collected and fixed FAA. *Ginkgo biloba* young ovules and pollen cones were collected from the grounds of NYBG (Accession number: 1353/97) and immediately fixed in FAA. *Gnetumgnemon* young ovules and pollen cones were collected from the Nolen green houses at NYBG (Accession number: 2153/2002*C). The fern *C.richardii* strain RN3 was grown in a growth chamber under controlled conditions, 16-hour light at 26 °C (voucher: USA, New York, Pfizer lab, NYBG, November 2019, *C. Zumajo-Cardona, T. Smalls* and *B. Ambrose* 25 NY).

All samples were incubated in Formaldehide, Glacial Acetic Acid, and Ethanol (FAA) for 2–3 hours. Samples were then dehydrated in a standard ethanol series, embedded in paraffin (Paraplast-Xtra, Fisher Healthcare, Houston, TX, USA) and stored at 4 °C until use. The samples were sectioned at 10 μm with a Microm HM355 rotary microtome (Fisher Scientific, Pittsburgh, PA, USA). DNA templates for the synthesis of RNA probes were obtained by PCR amplification of 210–360 bp fragments with primers specific to each *AP2* homologue (supplementary fig. S1; supplementary table 2). The fragments were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Digoxigenin labeled RNA probes were prepared using T7 RNA polymerase (Roche, Switzerland), a murine RNAse inhibitor (New England Biolabs, Ipswich, MA, USA), and RNA labeling mix (Roche, Switzerland) according to each manufacturer's protocol. In situ hybridization of RNA was carried out according to Ambrose et al. (2000) and Ferrandiz et al. (2000), and hybridized overnight at 55 °C. The probes were diluted 1:50 for all the experiments. Slides were permanently mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Additionally, in situ hybridization experiments were performed with *sense* probes, to assess any nonspecific background signal (supplementary fig. S2). Sections were viewed on a Zeiss optical microscope and digitally photographed with a Nikon DXM1200C digital camera and ACT-1 software.

All flower and fruit developmental stages described here are based on previous descriptions for *Bocconia frutescens* (Zumajo-Cardona et al. 2017; Zumajo-Cardona et al. 2018) and *A. fimbriata* (Pabón-Mora et al. 2015).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Data Availability

The data underlying this article are available in the GenBank Nucleotide Database with accession numbers provided in the methods and supplemental material. The alignment data underlying this article is available upon request to the corresponding author.

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