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by

Doudou Kong

from Gansu, China

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Reviewer

Prof. Dr. Annette Becker
Institute of Botany
Justus-Liebig-University Gießen

Prof. Dr. Agnieszka Golicz
Department of Agrobioinformatics
Justus-Liebig-University Gießen

Examiner

Prof. Dr. Sigurd J. Braun
Institute of Genetics
Justus-Liebig-University Gießen

Prof. Dr. Nikola-Michael Prpic-Schäper
Institute of Zoology and Developmental Biology
Justus-Liebig-University Gießen

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Dedication

献给那些知道我不完美却依然爱我的人

To those who know I am not perfect but still love me

Acknowledgments

I have never been good at writing, yet I wanted to say goodbye properly. So please forgive me if this turns out a bit long. I wondered many times if I would feel lost when writing this because it marks the end of my student life. It's a strange mix of feelings: exciting and a little sad. What I do know is that I could never have finished this thesis without the support, encouragement, and kindness of so many people around me.

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"你太专注于未来, 却没有意识到, 今天正是你多年前希望的模样。"

"You're so focused on the future that you don't see that today is exactly the life you once wished for."

May these words reach you as they reached me, offering the same courage and kindness along the way.

Summary

Comparative transcriptomics reveals how conserved regulators and flexible gene expression programmes shape the stability and diversity of carpel identity and differentiation. Taking this perspective further, we integrate floral morphogenesis with cross-species transcriptome data across eudicots to test how regulatory change accompanies morphological innovation.An orthogroup (OG) is a set of genes across species that descend from a single gene in their most recent common ancestor (MRCA), encompassing orthologs. On this basis, We mapped OGs to expression profiles and identified conserved and lineage-specific patterns. These patterns are then linked to morphological traits. The findings suggest that a small number of deeply conserved factors are fundamental to carpel development, and that shifts in expression and timing are associated with lineage-specific carpel morphologies.

In the first part of this thesis, floral morphogenesis in eudicots is summarized with an emphasis on the origin and diversity of ring meristems. Ring meristems, which generate multiple whorls of stamens, are widespread in Ranunculales and exhibit multiple patterns of initiation. Subsequently, the floral morphogenesis of *Pteridophyllum racemosum* (Papaveraceae, Ranunculales), a sister lineage to the remaining Papaveraceae, is described for the first time. Its floral organs are relatively simple and lack a ring meristem. *P. racemosum* produces flowers with two sepals, four petals in two whorls, four stamens, and a syncarpous gynoecium of two carpels, a combination rare within Papaveraceae but consistent with reconstructions of the family's ancestral flower.

The second part focuses on transcriptomics of carpel development in eudicots. Transcriptomes of carpels are generated for *Arabidopsis thaliana*, *Eschscholzia californica*, and *Solanum lycopersicum* across four developmental stages. Comparison of OGs revealed that most regulators of carpel development are present in all three species at the genome level, but their expression pattern often differs. Only a few regulators, like *HECATE* (*HEC*) and *FRUITFULL* (*FUL*), follow conserved expression patterns. Detailed mapping from expression of OGs to published regulatory pathways showed that the *NGATHA* (*NGA*) is conserved both in expression and in function, representing a core component of the regulatory network for stigma and style development, while other network, such as those involving polarity establishment, is divergent. These results indicate that carpel development relies on both core regulators and flexible components whose evolutionary role may mediate through expression.

In conclusion, this thesis integrates morphological studies with comparative transcrip-

tomics to investigate the genomics and expression of floral organ evolution in eudicots. The results show that conserved carpel regulators maintained in genome, while flexible expression patterns may be inferred to contribute to differentiation. These results provide valuable gene resources for future functional studies once stable transformation systems are established in non-model systems.

Zusammenfassung

Die vergleichende Transkriptomik zeigt, wie konservierte Regulatoren und flexible Genexpressionsprogramme die Stabilität und Vielfalt der Identität und Differenzierung von Fruchtblättern beeinflussen. Ausgehend von dieser Perspektive integrieren wir die Blütenmorphogenese mit transspeziesübergreifenden Transkriptomdaten von Eudikotyledonen, um zu untersuchen, wie regulatorische Veränderungen mit morphologischen Innovationen einhergehen. Ein OG ist eine Gruppe von Genen verschiedener Spezies, die von einem einzigen Gen in ihrem MRCA abstammen und Orthologe umfassen. Auf dieser Grundlage haben wir OGs auf Expressionsprofile abgebildet und konservierte und stammespezifische Muster identifiziert. Diese Muster werden dann mit morphologischen Merkmalen in Verbindung gebracht. Die Ergebnisse deuten darauf hin, dass eine kleine Anzahl tief konservierter Faktoren für die Entwicklung der Fruchtblätter von grundlegender Bedeutung ist und dass Verschiebungen in der Expression und im Zeitpunkt mit stammespezifischen Fruchtblattmorphologien verbunden sind.

Im ersten Teil dieser Arbeit habe ich die Blütenmorphogenese bei Eudikotyledonen mit Schwerpunkt auf den Ursprung und der Vielfalt von Ringmeristemen zusammengefasst. Ringmeristeme bilden mehrere Wirtel von Staubblättern, die in Ranunculales weit verbreitet sind und mehrere Initiationsrichtungen aufweisen. Ich untersuchte die Blütenmorphogenese von *Pteridophyllum racemosum* (Papaveraceae, Ranunculales), einer Schwesterart aller anderen Papaveraceae-Arten. Ihre Blütenorgane sind relativ einfach und ihre Entwicklung wurde bisher noch nicht beschrieben. Im Gegensatz zu vielen anderen Vertretern der Ranunculales bildet diese Art kein Ringmeristem. Unsere Ergebnisse zeigen, dass *P. racemosum* Blüten mit zwei Kelchblättern, vier in zwei Wirbeln angeordneten Blütenblättern, vier Staubblättern und einem synkarpen Gynoecium mit zwei Samenanlagen bildet, eine Kombination, die innerhalb der Papaveraceae selten ist, aber mit den für die Stammblume der Familie rekonstruierten Merkmalen übereinstimmt.

Im zweiten Teil habe ich mich auf die Transkriptomik der Fruchtknotenentwicklung bei Eudikotyledonen konzentriert. Transkriptome von Fruchtknoten wurden für *Arabidopsis thaliana*, *Eschscholzia californica* und *Solanum lycopersicum* in je vier Entwicklungsstadien erstellt. Der Vergleich von Orthogruppen ergab, dass die meisten Regulatoren der Fruchtknotenentwicklung auf Genomebene in allen drei Arten vorhanden sind, sich ihre Expressionsmuster jedoch häufig unterscheiden. Nur wenige Regulatoren, wie *HEC*s und *FUL*, folgen konservierten Expressionsmustern. Eine detaillierte Kartierung von OGs zu

veröffentlichten Regulationsmodulen zeigte, dass der NGATHA(NGA) für die Stigma- und Griffelentwicklung über die Arten hinweg konserviert ist, während andere Netzwerke, wie diejenigen, die die Polaritätsbildung betreffen, divergieren. Diese Ergebnisse deuten darauf hin, dass die Karpelentwicklung sowohl von Kernregulatoren als auch von flexiblen Komponenten abhängt, deren evolutionäre Rolle möglicherweise durch ihre Expression vermittelt wird.

Zusammenfassend lässt sich sagen, dass diese Arbeit morphologische Studien mit vergleichender Transkriptomik integriert, um die Genomik und Expression der Blütenorganentwicklung bei Eudikotyledonen zu untersuchen. Die Ergebnisse zeigen, dass konservierte Regulatoren im Genom erhalten bleiben, während flexible Expressionsmuster zur Diversität beitragen können. Diese Ergebnisse liefern wertvolle Genressourcen für zukünftige Funktionsstudien, sobald stabile Transformationssysteme in Nicht-Modellsystemen etabliert sind.

List of Abbreviations

A. majus	*Antirrhinum majus*
A. thaliana	*Arabidopsis thaliana*
AG	*AGAMOUS*
ALC	*ALCATRAZ*
ANT	AINTEGUMENTA
AP2	APETALA2
ARFs	Auxin Response Factors
ARR	*ARABIDOPSIS RESPONSE REGULATOR*
ATX1	ARABIDOPSIS HOMOLOG OF TRITHORAX 1
BIA	benzylisoquinoline alkaloid
CAF	CARPEL FACTORY
Calpop	California Poppy
C. sempervirens	*Capnoides sempervirens*
CLF	CURLY LEAF
CLV3	*CLAVATA3*
CMM	carpel margin meristem
CRC	CRABS CLAW
CUC1	*CUP-SHAPED COTYLEDON 1*
CUC2	*CUP-SHAPED COTYLEDON 2*
CZ	central zone
DCL1	DICER-LIKE1
DZ	dehiscence zone
E. californica	*Eschscholzia californica*
FIE	FERTILIZATION-INDEPENDENT ENDOSPERM

FIL	*FILAMENTOUS FLOWER*
FM	floral meristem
FUL	*FRUITFULL*
GA	gibberellin
GOA	GORDITA
GRNs	gene regulatory networks
HDA19	histone deacetylase 19
HEC	*HECATE*
IM	inflorescence meristem
IND	*INDEHISCENT*
IPT	*ISOPENTENYL TRANSFERASE*
ISWI	Interaction with the Imitation Switch
JAG	*JAGGED*
KAN	KANADI
KNOX	KNOTTED1-like homeobox
KNOX I	Class I KNOTTED1-like homeobox
KNU	*KNUCKLES*
LFY	*LEAFY*
LMD	laser microdissection
LUG	LEUNIG
miR172	microRNA172
miRNAs	microRNAs
MRCA	most recent common ancestor
NGA	*NGATHA*
NPA	Naphthylphthalamic Acid
OC	organizing center
OG	orthogroup
P. racemosum	*Pteridophyllum racemosum*
PAN	PERIANTHIA
PCA	Principal Component Analysis

PcG	polycomb-group
PIN1	*PIN-FORMED1*
PRC	Polycomb Repressive Complex
PZ	peripheral zone
RBL	REBELOTE
REM11	REPRODUCTIVE MERISTEM 11
REM13	REPRODUCTIVE MERISTEM 13
RPL	REPLUMLESS
S. lycopersicum	*Solanum lycopersicum*
SAM	shoot apical meristem
scRNA-seq	single cell RNA sequencing
SEM	scanning electron microscope
SEU	SEUSS
SHI	SHORT INTERNODES
SPT	SPATULA
SQN	SQUINT
STM	SHOOT MERISTEMLESS
STY	STYLISH
SUP	SUPERMAN
SVP	SHORT VEGETATIVE PHASE
TAA1	*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1*
TF	transcription factor
TOE3	TARGET OF EAT 3
TPL	TOPLESS
TRN2	TORNADO2
trxG	trithorax group
TSL	TOUSLED
ULT	ULTRAPETALA
VIGS	Virus-Induced Gene Silencing
WGCNA	Weighted Gene Co-Expression Network Analysis

WGD	whole genome duplication
WUS	*WUSCHEL*
Y2H	Yeast Two-Hybrid
YAB3	*YABBY 3*
YUC4	*YUCCA4*

Contents

1 Introduction

1.1 The ABCE model of flower development

The genetic basis of flower development can be explained by the ABCE model (Coen & Meyerowitz, 1991; Theissen & Saedler, 2001).

Through studies on floral organ homeotic mutants in *Arabidopsis thaliana* (*A. thaliana*) and *Antirrhinum majus* (*A. majus*), Coen et al. (1991) developed the ABC model of floral organ development, which represented the first model of floral organ regulation by the MADS-box gene family (Coen & Meyerowitz, 1991). Flowers are organized into four concentric whorls: sepals in the outermost (1st) whorl, petals in the 2nd whorl, stamens in the 3rd whorl, and carpels in the central (4th) whorl. A common feature of the homeotic mutants studied in these two species is that the development of neighboring whorls of floral organs is affected simultaneously. Based on the spatial arrangement of the transformed organs, these mutants are classified into three types. 1) Sepals convert into carpels, and petals convert into stamens, resulting in a floral structure arranged as carpels-stamens-stamens-carpels. 2) Petals convert into sepals, and stamens convert into carpels, producing flowers arranged as sepals-sepals-carpels-carpels. 3) Stamens convert into petals, and carpels convert into sepaloid organs. This leads to flowers arranged as sepals-petals-petals-sepals, with indeterminate floral organs. Moreover, in such homeotic mutants, indeterminate floral organs resembling sepals and petals are continuously produced within the carpels (Theissen & Saedler, 2001; Theissen et al., 2000). Studies on the homeotic genes responsible for these mutations classified them into three categories, A, B, and C, leading to the establishment of the ABC model (Coen & Meyerowitz, 1991, Figure 1).

The A-function is defined by mutations in which sepals convert into carpels in the 1st whorl, and petals into stamens in the 2nd whorl (Figure 1B). The B-function is characterized by mutations where petals convert into sepals in the 2nd whorl and stamens into carpels in the 3rd whorl. Mutations resulting in flowers with only perianth but no reproductive structures characterize the C-function (Bowman & Moyroud, 2024, Figure 1C, D).

The ABC model is based on three fundamental principles. First, each type of homeotic gene functions in two adjacent floral whorls, and mutations in these genes result in altered floral organ phenotypes in the affected whorls. Second, the combinatorial action of floral

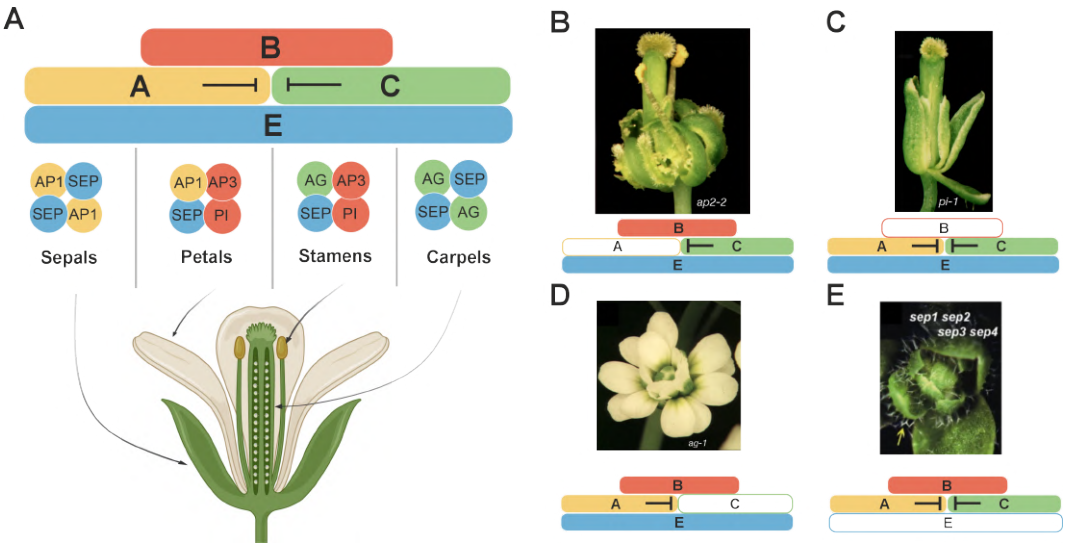

Figure 1. Schematic representation of the ABCE model of flower development in *Arabidopsis thaliana* and homeotic transformations in the respective mutants (modified from Bowman and Moyroud, 2024). **A)** Floral quartet model. The MADS-box proteins APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), AG, and SEPs act in combination to specify the identity of each floral organ. The combination of A and E functions (AP1, SEPs) is required to specify sepal identity, AP1-AP3-PI-SEP specifies petal identity, AP3-PI-AG-SEP specifies stamen identity, and AG-AG-SEP-SEP specifies carpel identity. **B)** A class mutant (*ap2*) flower consists of carpels in the first whorl, stamens in the second and third whorls, and carpels in the fourth whorl. **C)** B class mutant (*pi*) flower consists of sepals in the first and second whorls, carpels in the third and fourth whorls. **D)** C class mutant (*ag*) flower consists of sepals in the first whorl, petals in the second and third whorls, and reiterations of perianth organs in the interior whorls. **E)** E class mutant (*sep1 sep2 sep3 sep4*) flower consists of whorls of leaf-like organs.

homeotic genes determines the development of specific floral organs. Third, the expression domains of A and C-class genes are mutually exclusive and do not overlap. The classic ABC model has been widely accepted as it effectively explains the expression patterns of floral homeotic genes, elucidates the molecular mechanisms underlying floral organ mutations, and accurately predicts the phenotypes of single, double, and triple mutants.

By manipulating the expression of ABC genes, it is possible to artificially control the developmental fate of floral organs in each whorl. However, such manipulations cannot induce the conversion of leaves into floral organs (Krizek & Meyerowitz, 1996; Mizukami & Ma, 1992). This implies that, although these genes are critical for floral organ development, they are not sufficient to drive the transition from the vegetative phase to floral organ development.

During the search for proteins interacting with ABC genes, Yeast Two-Hybrid (Y2H) experiments revealed several *AGAMOUS* (*AG*)-like family genes expressed in the floral meristem, namely *AGL2*, *AGL4*, and *AGL9*, which are expressed earlier than the B- and C-class genes (Fan et al., 1997). *agl2, agl4, agl9* triple mutants produce floral organs in all whorls, but these are sepaloid in structure. Consequently, these genes were renamed: *AGL2* as *SEPALLATA1* (*SEP1*), *AGL4* as *SEP2*, and *AGL9* as *SEP3* (Pelaz et al., 2000). The phenotype of *sep1 sep2 sep3* triple mutants closely resembles that of B- and C-class gene mutants. However, the expression of B- and C-class genes remains unaffected. Similarly, in B- and C-gene double mutants, *SEP1*, *SEP2*, and *SEP3* are still expressed, indicating that there is no upstream or downstream regulatory relationship between the B- and C-class genes and *SEPs* (Theißen, 2001). A fourth member of the *SEPs*, *SEP4*, was later identified, which shares extensive sequence similarity and overlapping expression domains with *SEP1-3*. Although *sep4* single mutants resemble the wild type, *sep1 sep2 sep3 sep4* quadruple mutants exhibit severe floral defects, with all floral organs converted into leaf-like structures (Ditta et al., 2004, Figure 1E). This demonstrates that *SEP4* functions redundantly with the other *SEP*s and that all four *SEP*s collectively provide essential organ identity functions in *Arabidopsis* flowers.

The identification of *SEP*s, later classified as E-class genes, revealed another class of genes involved in this transition. *SEP*s can cooperate with other classes to facilitate the transition of vegetative organs into reproductive organs. As described before, *SEP3*, in combination with B- and C-class genes, can change leaves into stamens (Honma & Goto, 2001; Pelaz et al., 2001). Theißen subsequently proposed the "quartet model" (2001), which integrates the E-function into the classical ABC framework, leading to the widely recognized ABCE model (Figure 1) that provides a comprehensive genetic basis for explaining floral organ development (Castillejo et al., 2005; Pelaz et al., 2000; Theißen, 2001; Theissen & Saedler, 2001). In this model, A- and E-class genes determine sepal formation, A-, B-, and E-class genes regulate petal development, B-, C-, and E-class genes specify stamen development,

and C- and E-class genes define carpel identity (Pinyopich et al., 2003; Theissen & Saedler, 2001).

1.2 Ranunculales exhibit innovations in flower morphology

Ranunculales, a core member of the basal eudicots and sister group to all other eudicots, comprises approximately 202 genera and 4,500 species distributed across seven families (APG, 2016). The order exhibits remarkable diversity in floral morphology and has therefore become an important system for studying floral evolution in a comparative framework (Damerval & Becker, 2017)(Figure 2).

Across the order, flowers vary in organ number, organ arrangement, symmetry, and the degree of differentiation between sepals and petals. Floral organs may be arranged in whorls, in spirals, or in patterns that are difficult to assign clearly to either condition. Likewise, the perianth ranges from undifferentiated petaloid organs to clearly distinct sepals and petals, and in some lineages certain organs are reduced, modified, or lost altogether (Becker et al., 2023, 2024; Carrive et al., 2020; Damerval & Becker, 2017; Endress, 1995; Soza et al., 2012)(Figure 2B)

This diversity is especially evident in traits that are crucial to flower development. In Ranunculales, transitions in merism, floral phyllotaxis (whorled or spiral arrangements), organ fusion, and floral symmetry have occurred repeatedly; several of these traits exhibit homoplastic distributions (Becker et al., 2023, 2024; Carrive et al., 2020; Damerval & Becker, 2017; Endress, 1995; Soza et al., 2012). Floral phyllotaxis, for instance, ranges from clearly whorled to spiral or irregular patterns (Endress, 2011). The ancestral flower was likely whorled at anthesis, a condition retained in most families, whereas spiral or partially irregular patterns occur in Circaeasteraceae and some Ranunculaceae (Carrive et al., 2020). While reproductive organs are typically spirally arranged, exceptions exist, such as in *Aquilegia* (Tucker & Hodges, 2005). Additionally, irregular phyllotaxis may arise in the Ranunculaceae through incomplete parastichy formation during stamen development (Zhao et al., 2012). This variation suggests that Ranunculales floral diversity stems from repeated modifications in organ initiation, identity, and developmental timing.

The perianth also shows considerable variability across Ranunculales. Some families, such as Eupteleaceae, lack a perianth entirely, whereas others possess either a bipartite perianth with distinct sepals and petals or a unipartite perianth composed of sepaloid or petaloid organs (Ren et al., 2007). In Menispermaceae and Lardizabalaceae, sepals are usually persistent and often petaloid, while petals, when present, frequently function

in nectar secretion (Endress, 1995). In Papaveraceae, by contrast, floral organization differs markedly between major lineages: Papaveroideae typically bear caducous sepals and nectarless petals, whereas Fumarioideae often retain more persistent petaloid sepals and possess highly specialized spurred petals associated with nectar presentation or collection (Sauquet et al., 2015). These differences indicate repeated shifts in organ identity and function and illustrate how perianth evolution in Ranunculales is closely linked to floral specialization and pollination biology (Damerval & Becker, 2017).

Within Ranunculales, the poppy family (Papaveraceae) represents one of the monophyletic lineages. It comprises four subfamilies: Papaveroideae, Fumarioideae, Hypecoideae, and Pteridophylloideae (see Figure 1 in Publication 1 for comparison of their floral architectures). Molecular clock analyses date the origin of the Papaveraceae stem lineage to approximately 112–139 million years ago (Mya), and diversification most likely occurred during the Cretaceous Terrestrial Revolution (Peng et al., 2023). The family diverged early into two major clades, one leading to the Papaveroideae and another giving rise to Hypecoideae, Fumarioideae, and Pteridophylloideae.

The phylogenetic position of the Pteridophylloideae has long been debated. Earlier analyses combining chloroplast and nuclear ribosomal DNA data placed it as the sister subfamily to the remaining Papaveraceae (Hoot et al., 2015; Kong et al., 2024). More recent phylogenomic studies based on plastid genomes have resolved it as sister to both Hypecoideae and Fumarioideae (Becker et al., 2024; Peng et al., 2023). The subfamily contains only a single species, *Pteridophyllum racemosum* (*P. racemosum*) (Siebold & Zucc.), an evergreen perennial herb endemic to central and northern Japan. Its highly reduced and atypical floral morphology makes *P. racemosum* a morphologically and phylogenetically exceptional representative of Papaveraceae. This species provides an excellent opportunity to explore the developmental reduction and specialization of floral organs and to infer ancestral states of floral architecture within Ranunculales. These questions are further explored in section 3.2, which investigates the floral development of *P. racemosum* and its implications for character reduction and floral evolution within Papaveraceae.

In summary, As Ranunculales bridges the evolutionary gap between basal angiosperms and core eudicots, its floral structural diversity reflects a transitional phase from primitive to more advanced (Endress, 1994)(Figure 2A). The order serves as an informative framework for studying transitions in floral morphology and the emergence of novel characters. These features provide valuable opportunities to investigate the genetic and molecular mechanisms underlying the evolution of floral innovation in angiosperms.

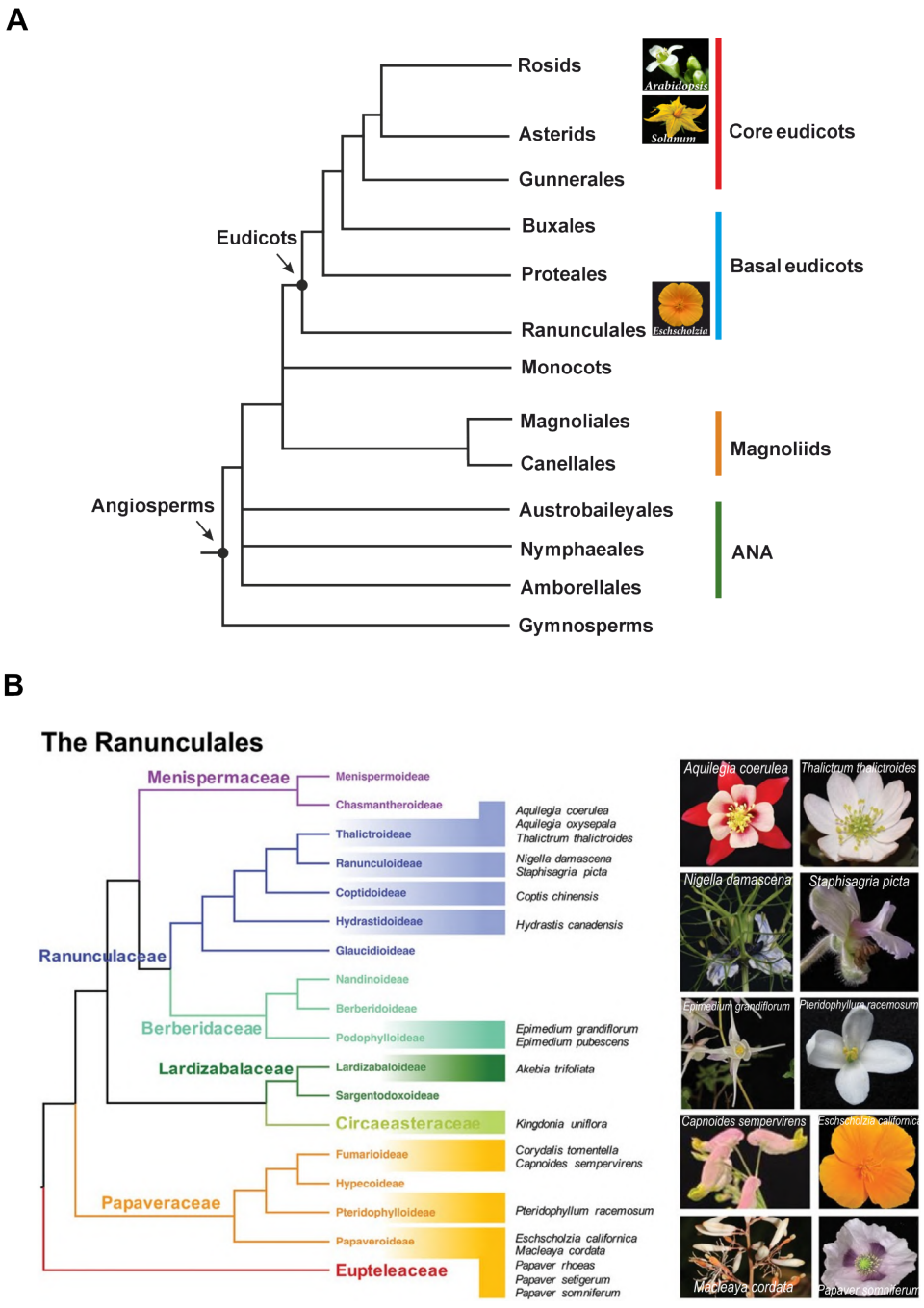

Figure 2. A simplified phylogeny of angiosperms with the study species in this study **A)** and **B)** a simplified phylogeny of Ranunculales with representative photos of Ranunculales flower (Becker et al., 2024)

1.2.1 *Eschscholzia californica* as an emerging model for Evo-Devo

Eschscholzia californica, well known as the California Poppy (Calpop) (Papaveraceae, Ranunculales) (APG, 2016, Figure 2A), is an annual to perennial herb native to the west coast of North America (Cook, 1962). It typically grows to a height of 20-40 cm and has a life cycle of ca. three months. Each fruit can produce 80 to 100 seeds. Moreover, Calpop is easy to cultivate year-round in greenhouses and requires minimal maintenance. Its large flowers make it convenient for floral organ sampling and phenotyping (Becker et al., 2005).

From the perspective of genomic resources, the draft genome sequence of Calpop has been published in 2018, with an estimated genome size of approximately 503.8 Mb (Hori et al., 2018, Eschscholzia Genome Database, http://eschscholzia.kazusa.or.jp), and a reference quality genome sequencing effort in combination with a transcriptome atlas is completed by the Open Green Genome initiative with the data being available on Phytozome (Two haplotypes; https://phytozome-next.jgi.doe.gov/info/EcalifornicavarAurantiacaOrangeKingPlant1_1HAP1_v1_1; https://phytozome-next.jgi.doe.gov/info/EcalifornicavarAurantiacaOrangeKingPlant1_1HAP2_v1_1; estimated genome sizes are 385 Mb for haplotype 1 and 375 Mb for haplotype 2;)(Rössner et al., 2026).

Besides the transcriptome atlas (Rössner et al., 2026), we have generated RNA-seq data covering different developmental stages of floral meristem (FM) and the carpel using laser microdissection (LMD) (Kivivirta et al., 2019).

In addition to sequence resources, functional analysis of candidate genes can be performed by reducing gene expression through Virus-Induced Gene Silencing (VIGS). This method has been repeatedly utilized to elucidate the roles of transcription factors involved in both vegetative and reproductive development (Becker et al., 2024; Lange et al., 2013; Lotz et al., 2024; Orashakova et al., 2009; Stammler et al., 2013). Furthermore, the floral morphogenesis of wild-type Calpop has been thoroughly characterized, facilitating comparisons between wild-type and gene-silenced individuals (Becker et al., 2005, 2024).

Those sequence resources and silencing method provide the toolkit for Calpop to serve as an emerging model system for evo-devo research.

1.3 Development of floral organs requires maintenance and termination of floral meristem stem cell activity

Meristems are essential for organ development and differentiation, serving as storage areas of stem cells. There are two types of plant meristems, the shoot meristems and root

apical meristems, with shoot meristems further developing into shoot apical meristem (SAM) and lateral/axillary meristems. During reproductive growth, the SAM converts into an IM, and the IM subsequently produces FM either at its apex or along its flanks (Bowman et al., 1989; Kwiatkowska, 2008).

The FM plays an important role in flower formation, maintaining a balance of stem cell activity to sequentially release floral organ primordia at certain times. The regulation of stem cell number and positioning within these primordia is precisely tuned, ensuring the successful differentiation into various floral organ types. Ultimately, the FM terminates, securing the correct morphology of floral organs and enabling the formation of a fully functional flower. This process relies on complex gene regulatory networks (GRNs) that integrate transcription factor (TF)s, microRNAs (miRNAs), DNA methylation, and plant hormones (Chang et al., 2020). These genetics governing FM activity have been extensively described in Arabidopsis. With advancements in molecular tools, some of these genetics have been characterized across eudicots (Becker et al., 2023, 2024; Li et al., 2024; Stammler et al., 2013; Wang et al., 2024).

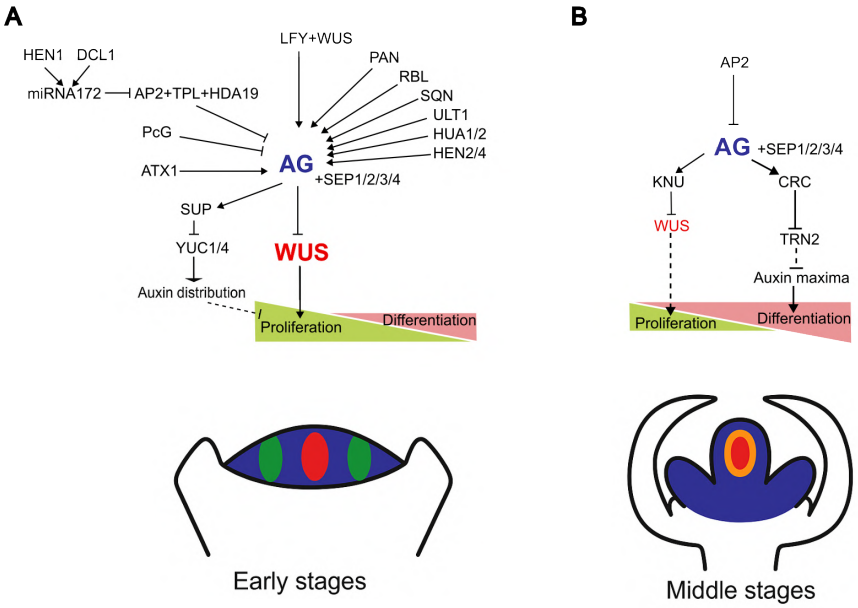

Figure 3. summary of different regulators that are involved in controlling floral meristem activity and termination in early (left) and late (right) stages, from Xu et al. (2019).

1.3.1 Molecular genetics of FM determinacy and maintenance

1.3.1.1 WUSCHEL-CLAVATA negative feedback loop

In *A. thaliana*, the WUS-CLV negative feedback loop is a key regulatory mechanism for maintaining stem cell activity in the FM. Like shoot meristems, FMs can be subdivided into distinct zones, with a central zone (CZ) harboring the stem cells and an underlying organizing center (OC) providing signals, while organ initiation occurs in the peripheral zone (PZ) (Mayer et al., 1998). *WUS* expressed in the OC and diffuses into the CZ to promote the proliferation and activity of stem cells. *wus* mutants are unable to initiate and maintain FM activity, with only 1–2 stamens and no carpels developing (Laux et al., 1996; Schoof et al., 2000). *CLAVATA3* (*CLV3*), a stem cell marker gene, encodes a peptide signal that binds to the membrane-bound CLV1-CLV2 receptor complex, thereby repressing *WUSCHEL* (*WUS*) expression (Brand et al., 2000; Lenhard & Laux, 2003). The interplay between *WUS* and *CLV3* depends on the concentration of WUS, which activates *CLV3* expression with low levels but represses it with high levels, thereby achieving a dynamic balance in the stem cell number within the meristem (Perales et al., 2016; Snipes et al., 2018; Zhou et al., 2018).

1.3.1.2 SHOOT MERISTEMLESS in meristem maintenance

SHOOT MERISTEMLESS (STM), a Class I KNOTTED1-like homeobox (KNOX I) transcription factor, is essential for maintaining stem cell activity in the shoot and floral meristems (Clark et al., 1996; Long et al., 1996; Stammler et al., 2013). In *stm* mutants, floral meristem activity is severely reduced, resulting in flowers with fewer floral organs. STM maintains meristem activity partly by promoting cytokinin biosynthesis through activation of *ISOPENTENYL TRANSFERASE* (*IPT*) (Jasinski et al., 2005; Yanai et al., 2005), and by directly binding to the *CLV3* promoter to sustain its expression in stem cells (Su et al., 2020).

Only very limited information is available about floral KNOX I loss-of-function phenotypes in species outside core eudicots. In *Zea mays*, *knotted1* mutants show delayed or absent gynoecium formation, and occasionally an increase in carpel number (Kerstetter et al., 1997). Nevertheless, there is evidence that the function of STM is conserved in basal eudicots (Stammler et al., 2013). There are two orthologs of *STM* in *Eschscholzia californica*, *EcSTM1* and *EcSTM2*, which are predominantly expressed in floral tissues. Knockdown of gene expression by VIGS revealed that both *EcSTM* genes are required for the formation of reproductive organs. Silencing of *EcSTM1* resulted in the loss of the gynoecium and a reduced number of stamens. *EcSTM2*-VIGS treated flowers have reduced and defective gynoecia and a stronger reduction in the number of stamens than observed

in *EcSTM1*-VIGS treated plants. Double silencing of both genes led to more pronounced phenotypes. scanning electron microscope (SEM) and tissue sections demonstrated that the FM is centrally flat and lacked the initiation of carpels in *EcSTM1+2* silenced plants (Stammler et al., 2013). This is consistent with the phenotype observed in Arabidopsis.

1.3.1.3 Regulation of *AGAMOUS* expression for floral meristem determinacy and maintenance

AGAMOUS, a C-class gene in Arabidopsis, is necessary for the specification and development of stamens and carpels, and for floral meristem determinacy. These roles can be genetically separated, as shown by the phenotypes of different *ag* mutant alleles. Strong alleles (*ag-1* to *ag-3*) cause both a loss of determinacy and a homeotic transition of stamens into petals, whereas weak alleles (*ag-4* and *AG-Met205*) largely retain floral organ identity but still fail to maintain a determinate FM (Bowman et al., 1989; Sieburth et al., 1995; Yanofsky et al., 1990). Similarly, partial *AG* knockdown results in delayed FM determinacy without organ identity changes, suggesting that maintaining FM activity requires relatively high *AG* expression levels (Chuang & Meyerowitz, 2000; Mizukami & Ma, 1995). Any defects in the transcription, RNA processing, or protein function of *AG* therefore impact meristem maintenance.

Several upstream regulators control *AG* expression. In the center of the FM, *AG* is activated starting from floral stage 3 by *LEAFY* (*LFY*) and *WUS* (Lenhard et al., 2001; Lohmann et al., 2001). The trithorax group (trxG) protein ULTRAPETALA1 (ULT1) promotes *AG* expression by altering its chromatin state. Loss of *ULT1* leads to delayed FM determinacy and reduced *AG* expression (Carles & Fletcher, 2009; Fletcher, 2001). The bZIP transcription factor PERIANTHIA (PAN) also acts as an activator, and *pan* mutants show *ag-like* defects under short-day conditions (Maier et al., 2009). There are other regulators, including REBELOTE (RBL) and SQUINT (SQN), that work redundantly with *ULT1* to maintain high *AG* expression for FM determinacy (Prunet et al., 2008).

AG expression is also spatially restricted by APETALA2 (AP2), which prevents its transcription in the outer floral whorls (Drews et al., 1991). *AP2* itself is repressed by microRNA172 (miR172). Defects in miR172 biogenesis, such as those found in HUA ENHANCER1 (HEN1) or DICER-LIKE1 (DCL1), also known as CARPEL FACTORY (CAF) mutants, lead to elevated AP2 protein levels and a failure to properly terminate FM activity (Jacobsen et al., 1999). RNA-processing factors, including HUA1/2 and HUA ENHANCER2/4 (HEN2/4), also contribute to FM maintenance by ensuring correct *AG* mRNA maturation (Chen & Meyerowitz, 1999; Cheng et al., 2003; Li et al., 2001).

To execute its full biological function, *AG* requires the SEPs as enhancers (Ditta et al., 2004; Pelaz et al., 2000; Xu et al., 2019). According to the 'floral quartet' model, these

complexes specify floral organ identity (Honma & Goto, 2001; Theissen & Saedler, 2001). Although these dual *AG* roles can be separated genetically, it is unclear whether there are distinct AG complexes responsible for individual functions. *sep1 sep2 sep3* triple mutants, for example, fail to maintain FM determinacy despite normal *AG* expression, implicating SEP proteins in the determinacy function (Ditta et al., 2004; Pelaz et al., 2000). Two studies show that tetramerization between AG and SEP3 is critical for this process, and that AP2 can antagonize AG function at the protein level (Huang et al., 2017; Hugouvieux et al., 2018).

1.3.1.4 APETALA2 antagonizes AG activity in the regulation of floral meristem determinacy

In the ABC model of flower development (Coen & Meyerowitz, 1991), *AP2* is an A-class gene that functions antagonistically to *AG*, specifying perianth organs and restricting *AG* expression to the inner two whorls (Drews et al., 1991). AP2 directly binds to the second intron of *AG* to repress its transcription in the outer two whorls (Dinh et al., 2012; Wollmann et al., 2010; Yant et al., 2010). *AP2* activity is mainly regulated by miR172. Moreover, AP2 expression never expands uniformly into the center of *ag* mutant flowers, while miR172 is largely unaffected by loss of *AG* activity. Misexpression of *AG* under the 35S promoter can counteract AP2 activity in the outer whorls (Wollmann et al., 2010; Zhao et al., 2007).

In addition to AP2, miR172 also targets other AP2-like transcription factors such as TARGET OF EAT 3 (TOE3), which also contribute to floral patterning by repressing *AG* expression. TOE3 binds to the second intron of *AG* and interacts with AP2 in the nucleus. Transgenic plants expressing miR172-resistant *AP2* or *TOE3* variants display severe FM indeterminacy and floral organ identity defects (Zhao et al., 2007). AP2 physically interacts with TOE3 and represses both *KNUCKLES* (*KNU*) and *AG*, thereby sustaining *WUS* expression and FM activity (Huang et al., 2017; Yant et al., 2010; Zhao et al., 2007). Thus, AP2 acts as a negative regulator of AG-mediated FM termination, integrating transcriptional repression and protein-level antagonism to modulate the timing of stem cell termination in the floral meristem (Chang et al., 2020).

1.3.1.5 Epigenetic regulation in floral meristem determinacy

In addition to transcriptional regulation, *AG* expression is restricted by polycomb-group (PcG)-mediated chromatin silencing within the floral whorls. The PcG complexes contain core components such as CURLY LEAF (CLF) and EMBRYONIC FLOWER 1/2 (EMF1/2) (Calonje et al., 2008; Wu et al., 2018). Loss of PcG activity, for example in *clf* mutants, results in ectopic *AG* expression in vegetative tissues and the SAM (Goodrich et al., 1997).

The PcG-mediated repression is antagonized by trxG complexes containing ARABIDOPSIS HOMOLOG OF TRITHORAX 1 (ATX1), which maintain an active chromatin state at the *AG* locus (Alvarez-Venegas et al., 2003). In parallel, *AP2* can suppress *AG* transcription by recruiting the co-repressor TOPLESS (TPL) and histone deacetylase 19 (HDA19) (Krogan et al., 2012).

AG promotes FM determinacy partly by repressing *WUS* once activated. Both direct and indirect repression mechanisms have been proposed. In polycomb mutants such as *clf* and *terminal flower 2 (tfl2)*, which affect Polycomb Repressive Complex (PRC) core components, the FM indeterminacy phenotype of weak *ag* alleles (*ag-10*) is strongly enhanced (Liu et al., 2011). Chromatin immunoprecipitation analyses indicate that AG binds to the *WUS* locus, and that *TFL2* occupancy at this locus depends on AG, suggesting that AG may recruit PRC1/2 to stably silence *WUS* (Liu et al., 2011).

Epigenetic regulation also modulates hormone genes that influence FM maintenance. The transcription factor SUPERMAN (SUP) works with *AG* to ensure the proper timing of FM termination; *ag sup* double mutants develop fasciated meristems and additional reproductive organs (Bowman et al., 1992; Breuil-Broyer et al., 2016). SUP plays spatial and temporal roles in FM regulation by restricting stem cell proliferation at the boundary between whorls 3 and 4 (Xu et al., 2019). SUP mechanistically recruits PcG components, such as CLF and potentially TFL2, to deposit repressive H3K27me3 marks on the auxin biosynthesis genes *YUCCA1* and *YUCCA4 (YUC1/4)*. In *sup* mutants, the loss of this repression increases local auxin levels, thereby prolonging stem cell activity and delaying FM determinacy (Xu et al., 2019).

1.3.2 Molecular genetics of FM termination

FM termination marks the final step in stem cell activity of the FM, ensuring that organ production ceases once the floral whorls are established. This process is coordinated by several transcription factors and regulatory modules, many of which are downstream of *AG* (Figure 3).

1.3.2.1 Indirect repression of *WUSCHEL* by *AGAMMOUS* through *KNUCKLES*

KNUCKLES (KNU) is a C2H2-type zinc-finger transcription factor that acts downstream of *AG* to control FM termination. In Arabidopsis, AG binds to CArG-box motifs in the *KNU* promoter at floral stage 3. However, *KNU* expression begins at stage 6, when *WUS* expression is repressed. A loss of *knu* results in prolonged FM activity, whereas premature or delayed activation disrupts normal carpel formation (Sun & Ito, 2015). *KNU* represses *WUS* by binding to its promoter and recruiting PcG proteins such as FERTILIZATION-

INDEPENDENT ENDOSPERM (FIE). This leads to the enrichment of the repressive histone mark H3K27me3 at the *WUS* locus (Sun & Ito, 2015; Xu et al., 2019).

In addition to repressing *WUS*, KNU influences hormone pathways during FM termination. It directly downregulates the auxin transporter *PIN-FORMED1* (*PIN1*) and the cytokinin biosynthesis gene *IPT7* via PcG-mediated silencing, thereby modifying auxin and cytokinin distribution at stage 6 (Wang et al., 2024).

In *S. lycopersicum*, *SlKNU* represses *SlWUS* as well as the stem cell marker gene *SlCLV3* and its receptor *SlCLV1* to ensure FM determinacy (Li et al., 2024). Loss of *SlKNU* prolongs FM activity and increases fruit size, while overexpression reduces shoot and floral meristem activity without affecting carpel development. These results indicate that although some downstream effects differ between *S. lycopersicum* and *A. thaliana*, the core role of *KNU* in FM determinacy is conserved.

1.3.2.2 CRABS CLAW coordinates floral meristem termination with gynoecium development

CRABS CLAW (CRC), a YABBY family transcription factor and a direct target of *AG*, contributes to FM determinacy during later stages of flower development (around stage 6) (Bowman et al., 1999; Gómez-Mena et al., 2005; ó'Maoiléidigh et al., 2013; Prunet et al., 2008). Although *crc* single mutants display minimal FM determinacy defects, the *crc knu* double mutant exhibits a much stronger indeterminacy phenotype than *knu* single mutants (Bowman et al., 1999; Breuil-Broyer et al., 2016; Lee et al., 2005; Yamaguchi et al., 2017). This enhancement is associated with sustained *WUS* expression at stage 6, and *wus* is epistatic to *crc* or *knu* in floral meristems, indicating that *WUS* repression is downstream of CRC-KNU regulation. In *crc* mutants, auxin levels in the medial domain are reduced and auxin maxima are disrupted. Inhibition of auxin transport or ectopic expression of auxin under the control of the *CRC* promoter restores auxin maxima and partially rescues FM determinacy defects in both *crc* and *crc knu* mutants.

By comparing the expression profiles of *crc knu* and *knu* mutants, Yamaguchi et al. (2017) highlighted six candidates associated with polar auxin transport. A member of this group is *TORNADO2 (TRN2)*, a tetraspanin family gene that likely functions as an anchoring protein via its membrane spanning domain (Xu et al., 2019). CRC represses *TRN2* expression via a YABBY-binding site. Mutation of these binding sites leads to ectopic *TRN2* expression after floral stage 6 and in sepals, thereby disrupting auxin homeostasis. Overexpression of *TRN2* under the *CRC* promoter results in a *crc*-like phenotype in the wild type and a *crc knu*-like phenotype in a *knu* background. Conversely, *trn2* loss of function mutant reduces auxin transport, lowers *WUS* expression, and partially rescues

FM determinacy in *crc knu* mutants. These findings indicate that CRC-mediated repression of *TRN2* is required for establishing proper auxin maxima and promoting FM termination.

In addition, CRC acts together with AG to activate the auxin biosynthesis gene *YUCCA4* (*YUC4*) (Yamaguchi et al., 2018). *YUC4* is a common direct target of both transcription factors, and ectopic *YUC4* expression partially rescues the indeterminate phenotype of *crc* mutants. AG binds to the *YUC4* locus as a pioneer factor and modulates chromatin accessibility through interaction with the Interaction with the Imitation Switch (ISWI)-type chromatin remodeling proteins CHROMATIN REMODELING11 (CHR11) and CHR17. This feed-forward activation of *YUC4* by AG and CRC facilitates a chromatin state transition that supports the developmental shift from floral stem cell maintenance to gynoecium formation (Xu et al., 2019).

In *E. californica*, the ortholog of *CRC* exhibits a divergent function compared to that in Arabidopsis. Silencing of *EcCRC* results in the duplication of the 4th whorl, leading to a gynoecium surrounding an additional inner gynoecium. Normally, carpel formation in *E. californica* begins at stage 5 of floral development, while FM activity terminates (Becker et al., 2005). However, in *EcCRC* knock down flowers, carpels initiate properly at stage 5, but the meristem fails to terminate, continuing to produce additional whorls of carpels. In Arabidopsis, strong *crc-1* mutants also exhibit defects in floral meristem termination, although this phenotype is observed only in combination with *ag +* mutants. An additional inner whorl of carpels has not been observed in any *crc* mutant (Alvarez & Smyth, 1999; Alvarez & Smyth, 2002; Lee et al., 2005; Orashakova et al., 2009).

Other transcription factors, such as SUP, PERIANTHIA (PAN), and ULT, also play roles in the maintenance and termination of FM activity, although their contributions are relatively minor and partially redundant (Xu et al., 2019).

1.4 Evolution and development of the carpel

The evolutionary origins of carpels are closely linked to the molecular toolkit governing their development. Genetic studies on model plants, such as *A. thaliana*, have revealed the critical roles of *CRC* and *TOUSLED (TSL)* genes in carpel development, with these genes being conserved across basal angiosperms, including *Amborella trichopoda* and *Cabomba aquatica* (Becker, 2020; Fourquin et al., 2005). Consequently, it can be deduced that the genetic underpinnings of carpel development were already in place in the earliest angiosperms. Furthermore, molecular phylogenetic studies indicate that AG and CRC may have played a pivotal role in initiating carpel formation by regulating floral organ identity and determinacy (Liu et al., 2022). Those are hypothesized to have originated from ancestral regulatory networks that predate the divergence of angiosperms from gymnosperms,

underscoring the importance of gene duplication and functional diversification in carpel evolution (Pfannebecker et al., 2017).

A series of character reconstruction studies have combined an increasing amount of morphological and molecular data from fossils and extant species to reconstruct the ancestral flower of angiosperms. The latest and most comprehensive study, Sauquet et al. (2017), concluded that the ancestral flower of angiosperms had multiple free carpels, which were simple and opened distally by secretory closures. This result is consistent with previous studies by Endress and Doyle (2009).

Comparative genomic studies indicate that genetics of adaxial-abaxial polarity, meristem termination, and tissue differentiation were recruited and repurposed during carpel evolution (Becker, 2020). Furthermore, studies in *Anaxagorea* suggest that the carpel vasculature retains axial homologs, lending support to the hypothesis that carpels arose from an integration of axial and foliar structures (Li et al., 2020). Additionally, heterotopy (positional shifts of ovules within the carpel) has been identified as a key mechanism driving carpel diversity, reinforcing the idea that carpel evolution was not a linear process but rather a mosaic of structural innovations (Sattler, 2024).

1.4.1 The carpel: a key innovation in angiosperms

The carpel, the female reproductive organ of angiosperms, is both their most complex morphological innovation and the defining feature distinguishing them from gymnosperms (Endress, 2001). In the most ancient living seed plants, the gymnosperms, male (male cone) and female (female cone) reproductive organs develop on separate plants. By contrast, the evolutionarily younger angiosperms typically bear carpels and stamens together within a bisexual flower.

In most angiosperms , carpels are fused to form a gynoecium. Fusion can be congenital, occurring from the inception of organ development, or postgenital, occurring secondarily during development (Becker, 2020; Endress, 2001). The evolutionary dominance of angiosperms is largely attributed to the advantages provided by the carpel, which offers a protective enclosure for ovules that contrasts with the exposed ovules of gymnosperms (Orashakova, 2011; Scutt et al., 2006). Beyond physical protection, the carpel holds a controlled environment optimized for fertilization and pollination through specialized tissues (Scutt et al., 2006). Following fertilization, the ontogeny of the carpel shifts toward fruit development; these resulting structures not only protect the seeds but also drive diverse strategies for dehiscence and dispersal, factors widely credited for the vast diversification of angiosperms (Orashakova, 2011).

The gynoecium comprises all carpels, whether free or fused, forming the terminal part of

the fruit-producing flower (Figure 4B). Most gynoecia consist of three main regions along the longitudinal axis: the basal ovary, a style, and an apical stigma connected to the floral base by the gynophore. Gynoecium-specific tissues include the pollen tube transmitting tract, which guides pollen tube growth, and the placenta, where ovule primordia develop. These structurally and functionally distinct tissues make the gynoecium one of the most complex organs in angiosperms (Becker, 2020).

1.4.2 Morphology and morphogenesis of the carpel in *Arabidopsis thaliana* and *Eschscholzia californica*

Over the last two decades, *A. thaliana*, a member of Brassicaceae, has been established as a model system for studying floral development. Smyth et al. (1990) divided flower development into 12 stages based on a series of landmarks. Reyes-Olalde and Folter (2019) further subdivided flower development into 20 more specific stages, from the initiation of the inflorescence to seed maturation.(Figure 4,Table 1).

Flower development begins with the initiation of the FM in the IM. From stage 1, floral organ primordia appear in sequence from the outermost to the innermost (centripetally). Sepals, petals, and stamens initiate during stages 2 to 5. At stage 6, the central cells of the FM begin to form the carpel primordia (Larsson et al., 2013). Then, at stage 7, the carpel margin meristem (CMM) emerges, and the CMM eventually develops the carpel margin tissues: placenta, ovule, septum, transmitting tract, style, and stigma (Wynn et al., 2011). At stage 9, the two CMMs meet and form a septum; at the same time, placenta forms as well. At stage 11, the gynoecium is completely closed and the stigma with papillae. At stage 12, the style and transmitting tract differentiate (Reyes-Olalde et al., 2013). At stage 13 (anthesis), fertilization of the gynoecium occurs. Following this event , the gynoecium undergoes a big developmental transition. While the ovules mature into seeds, the ovary itself begins to transform into a fruit. This process is initiated by a phase of rapid cell division and proliferation in the ovary, ultimately resulting in the formation of a specialized dehiscence zone (DZ) at the valve-replum interface. This allows the fruit to disperse and release the seeds (Lotz et al., 2024; Orashakova, 2011; Robles & Pelaz, 2005).

The gynoecium of *A. thaliana* consists of different parts which are organized into an axis-like structure (de Folter, 2020). It exhibits three main tissue organization axes: the apical-basal, abaxial-adaxial, and medial-lateral axes (Figure 4B). When viewed longitudinally, the apical-basal axis runs from the top to the base of the gynoecium (Balanzá et al., 2006; Ferrándiz et al., 1999). Along this axis, distinct structures can be identified: at the apex is the stigma, followed by a short style that connects the stigma to the ovary. At the bottom lies a small stalk called the gynophore, linking the ovary to the floral base (Figure 4B). The

upper region, including the stigma and style, forms the apical part of the gynoecium, while the ovary and gynophore belong to the basal part.

The stigma consists of elongated cells known as stigmatic papillae, which are specialized for capturing pollen. The ovary is externally marked by a replum, which separates it into two valves, corresponding to the two fused carpel walls. Inside the ovary, a septum develops through postgenital fusion. From this septum, a transmitting tract forms and extends from the style through the stigma and down into the ovary. This tract serves as a pathway for pollen tubes, guiding them toward the ovules where fertilization occurs.

In cross section, the gynoecium displays a layout along the medial-lateral and adaxial-abaxial axes as well (Figure 4B). The two valves, which represent the lateral domains, are connected to the central replum region by narrow cell strips known as valve margins. Within the medial domain, the replum develops on the abaxial side, while the placenta arises on the adaxial side. The placenta bears the ovules at their tips. The stigma, style, septum, and transmitting tract all arise from the CMMs, which are positioned along the medial plane of the gynoecium wall.

Like *A. thaliana*, *E. californica* flowers consist of four concentric whorls: two sepals in the first whorl, four petals in the second, a variable number of stamens in the third, and a central gynoecium composed of two congenitally fused carpels (Becker et al., 2005). A longitudinal view of the gynoecium reveals a continuous transition between stigma, style, and ovary. In transverse section, the two valves are connected at their margins by the presumptive replum region (Figure 4C). This region differentiates into an abaxial replum and an adaxial placenta, from which two placental outgrowths arise, bearing ovules at their tips and projecting inward into the gynoecium cavity. Along the medial-lateral axis, carpel walls occupy lateral positions, while the replum, placental outgrowths, and ovules are situated medially. Unlike in *A. thaliana*, the septum and transmitting tract are absent in *E. californica*, and pollen tubes instead grow directly through the placenta (Becker et al., 2005).

Gynoecium morphogenesis in *E. californica* initiates at floral stage 5, when a single primordium arises at the center of the floral meristem. At stage 6, the gynoecium elongates and the placental regions grow inward, producing a central hollow and separating the structure into two carpel cylinders with free apical tips. Stage 7 is characterized by ovule primordia initiation, and lateral growth of the gynoecium continues. By stage 8, each carpel develops five longitudinal ridges on the abaxial side. Ovule primordia elongate within the ovary, and narrow lignified strips form along the valve-replum borders, marking the future dehiscence lines. Anthesis occurs at stage 11. Following fertilization, the gynoecium develops into a fruit that protects the seeds. At stage 12, the capsules elongate, reaching maturity and drying by stage 13. Finally, at stage 14, the dry capsules dehisce explosively

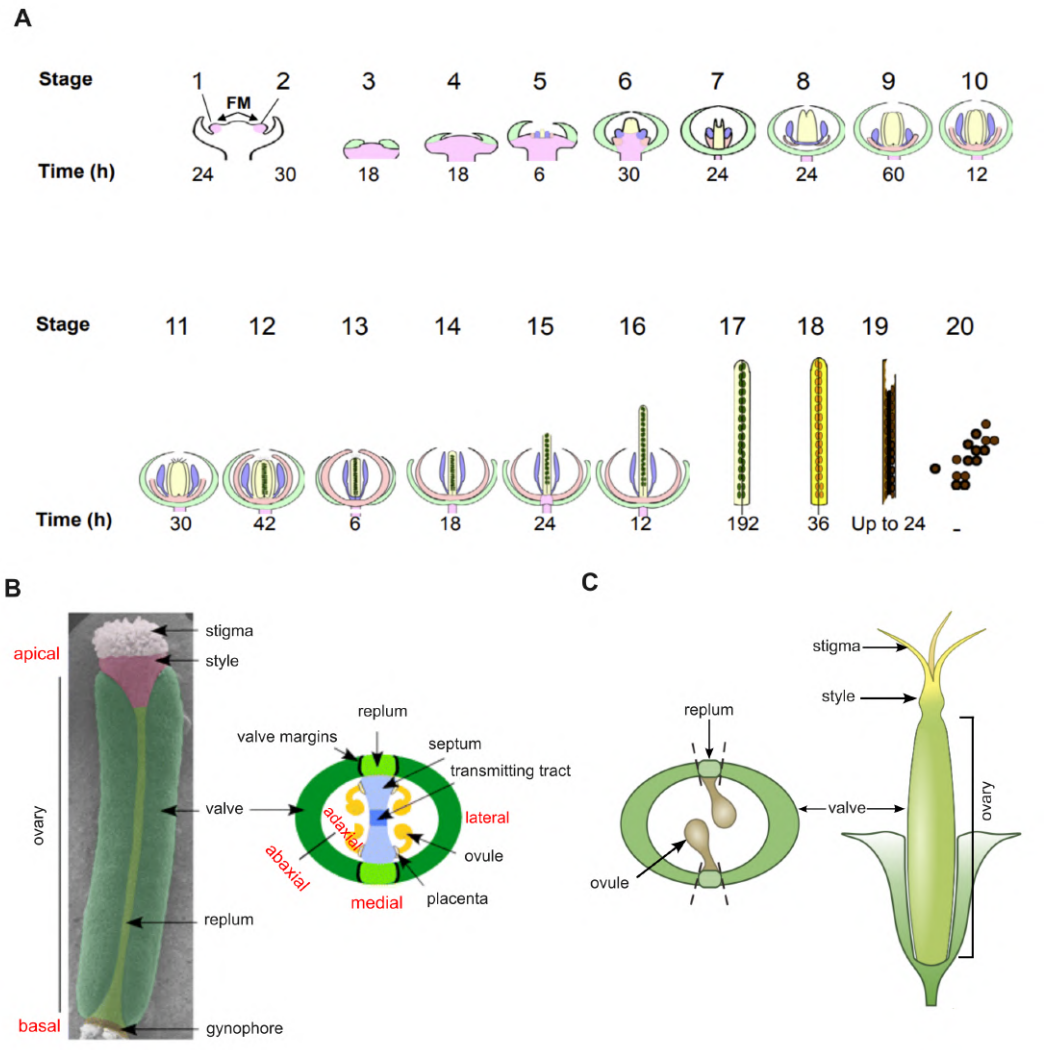

Figure 4. Flower developmental stages and tissue organization of the gynoecium in *A. thaliana* and *E. californica*. **A)** Schematic representation of the 20 developmental stages of *A. thaliana* flowers, from floral meristem initiation (stage 1) to seed dispersal (stage 20). The time (h) between stages is indicated below each diagram. FM, pink; sepals, green; petals, bright pink; stamens, blue; gynoecia, yellow; ovules, dark green; seeds, orange and brown. (adapted from Alvarez-Buylla et al., 2010). **B)** Tissue organization of the mature *A. thaliana* gynoecium. Left: longitudinal view along the apical-basal axis showing ovary, style, stigma, and gynophore. Right: cross section illustrating the medial-lateral and abaxial-adaxial domains, including valves, valve margins, replum, septum, transmitting tract, placenta, and ovules (modified from Krizek, 2011). **C)** Tissue organization of the *E. californica* gynoecium. Left: cross section through the ovary showing the arrangement of gynoecium tissues. Right: longitudinal view showing stigma, style, ovary, and valves.

Table 1. Floral developmental stages in *A. thaliana* and *E. californica*. Based on Alvarez and Smyth (2002), Becker et al. (2005), and Smyth et al. (1990).

Key events in flower development	Stages in *A. thaliana*	Stages in *E. californica*
Meristem formation	Stage 1	Stage 1
Sepal primordia appear	Stage 3	Stage 2
Petal primordia appear	Stage 5	Stage 3
Stamens initiate	Stage 5	Stage 4
Gynoecium initiate	ca. Stage 5-6	Stage 5
Placenta inception	Stage 8	Stage 6
Septum inception	Stage 8	-
Ovule primordia initiation	Stage 9	Stage 7
Male meiosis	-	Stage 8
Female meiosis	-	Stage 9
Style and stigma appear	Stage 11	Stage 11
Replum differentiation, transmitting tract develops	Stage 11-12	-
Anthesis	Stage 13	Stage 11
Capsule formation and elongation	Stage 17	Stage 12
Fully elongated capsule dries out	Stage 18	Stage 13
Capsule opens and seeds disperse	Stage 19-20	Stage 14

The dash (-) indicates absence of the event or lack of data.

from base to apex, while both valves remain attached to the style (Becker et al., 2005; Cook, 1962). For comparison, Table 1 summarizes the landmark events during floral morphogenesis in *A. thaliana* and *E. californica*.

1.4.2.1 Carpel molecular regulation in *A. thaliana*

As described in section 1.4.2, the morphogenesis of the gynoecium is a highly complex process regulated by a highly coordinated and genetically regulated network that integrates transcription factors, phytohormonal signals, and post-transcriptional regulation. Much of the current understanding of these GRNs comes from studies in *A. thaliana*, where the genetic and molecular basis of gynoecium development has been extensively characterized. The GRNs define carpel identity and guide tissue development along the apical-basal, medial-lateral, and abaxial-adaxial axes (Balanzá et al., 2006). Although many genes contribute to domain-specific organogenesis, their individual roles are often limited by redundancy and overlapping functions. Nevertheless, their combined activity is essential for the development of a functional gynoecium (Alvarez & Smyth, 1999; Azhakanandam et al., 2008).

Initiation of carpel

As described in section 1.3.1.3, the WUS-CLV feedback and the floral meristem identity factors LFY and AP1 activate *AG*. In the gynoecium, *AG* expression is spatially restricted to the central primordium through *WUS* activity, ensuring proper carpel initiation. In the outer whorls, *AG* expression is repressed by SEU, a transcriptional adaptor that interacts with MADS-box proteins, including AP1, SEP3, AGL24, and SHORT VEGETATIVE PHASE (SVP), via the co-repressor LEUNIG (LUG) (Sridhar et al., 2004, 2006).

Apical-basal patterning

The apical domain of the gynoecium, which develops into the style and stigma, is shaped by a set of key regulators, most notably SPATULA (SPT) and the SHORT INTERNODES (SHI)/STYLISH (STY) family. Both play key roles in apical tissue formation. Members of the SHI/STY family act downstream of the AINTEGUMENTA (ANT)-LUG-SEUSS (SEU)-YAB pathway and are among the most studied regulators of auxin biosynthesis in leaf and flower development (Kuusk et al., 2006). All SHI/STY proteins share a highly conserved zinc-finger domain, indicating that they likely target similar promoters (Eklund et al., 2010). Together with *NGATHA* (*NGA*) genes, SHI/STY members are expressed in the apical gynoecium from stage 6 of floral development through stages 9–10 (Kuusk et al., 2006). In particular, STY1 and STY2 are important auxin-dependent regulators of style

formation. STY1 activates the auxin biosynthesis gene *YUC4* in the apical domain, and *sty1 sty2* double mutants show reduced auxin levels and split styles (Kuusk et al., 2002).

SPT, a bHLH transcription factor, is expressed throughout gynoecium development in the medial domain and is required for the formation of the transmitting tract and other medial tissues (Reyes-Olalde, Zúñiga-Mayo, Marsch-Martínez, & de Folter, 2017; Reyes-Olalde, Zúñiga-Mayo, Serwatowska, et al., 2017). It mediates both auxin and gibberellin (GA) responses, and SHI/STY family members act as repressors of GA responses (Fridborg et al., 2001). During floral stages 6–7, new auxin sources are established in the medial domain, while cytokinin signaling is mainly confined to its adaxial side (Müller et al., 2017; Reyes-Olalde, Zúñiga-Mayo, Serwatowska, et al., 2017). SPT also activates *ARR1* and *ARR12*, which then induce the auxin biosynthesis gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) and the transport gene *PIN3*.

The HECATE (HEC1-3) transcription factors work closely with SPT, both genetically and physically (Schuster et al., 2015). HECs promote auxin transport by activating *PIN1* and *pin*. In the lateral domain, *SPT* and *HECs* suppress cytokinin signaling by activating A-type *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, which prevents medial-like growth in inappropriate regions. AHP6, a negative regulator of cytokinin signaling, provides an additional layer of control (Larsson et al., 2013; Müller et al., 2017).

Auxin Response Factors (ARFs) also contribute to apical-basal patterning. *ARF3/ETTIN* (*ETT)* promotes ovary development partly by repressing *SPT* in the basal domain; *spt* is epistatic to *ett* and suppress apical and basal defects seen in *arf3/ett* mutants (Heisler et al., 2001). ETT also works with KANADI (KAN) to define abaxial identity in apical tissues (Nemhauser et al., 2000; Sessions et al., 1997). Other auxin transport and signaling mutants, such as *pinoid/pid* and *pin*, show severe apical-basal defects, including overgrowth of style/stigma tissues and loss of ovary structures (Xu et al., 2019). Mutants of *arf3/ett* or *arf5/monopteros(mp)* display carpel wall defects and abnormal proliferation of style, stigma, and basal internode-like tissues, phenotypes similar to those produced by the polar auxin transport inhibitor Naphthylphthalamic Acid (NPA) (Li et al., 2020; Odat et al., 2014).

Abaxial-adaxial and medial-lateral patterning

In early gynoecium development, the primordium is divided into an abaxial outer domain, which develops into the carpel walls, and an adaxial inner domain, which gives rise to the medial tissues including the septum. The medial domain retains meristematic properties that later contribute to placenta and septum formation. Several shoot apical meristem maintenance genes, such as KNOTTED1-like homeobox (KNOX) family members, and boundary genes like *CUP-SHAPED COTYLEDON 1* (*CUC1*) and *CUP-SHAPED COTYLEDON 2* (*CUC2*), are expressed in this region (Ikezaki et al., 2010; Lie et al., 2012). The *cuc1 cuc2* double mutant lacks fully developed medial tissues, highlighting the role

of CUC in meristem maintenance and boundary formation (Ishida et al., 2000). CUCs act in organ primordium initiation by interacting with KNOX proteins such as STM and regulating auxin distribution through PIN1-dependent mechanisms (Gonçalves et al., 2015).

Within the KNOX I class, STM and KNAT2 act together to initiate carpel development independently of the WUS-AG pathway, while *KNAT1/BREVIPEDICELLUS* (*BP*) interacts with *REPLUMLESS* (*RPL*), *FRUITFULL* (*FUL*), and *AP2* genes to control medial tissue development. *RPL* promotes replum identity by restricting valve margin factors *SHP1/2*, *INDEHISCENT* (*IND*), and *ALCATRAZ* (*ALC*) expression to the margins, and lateral factors including *JAGGED* (*JAG*), *FILAMENTOUS FLOWER* (*FIL*), *FUL*, and *YABBY 3* (*YAB3*) to the valves (Arnaud & Pautot, 2014; Roeder et al., 2003). *BP* acts with *RPL* in replum formation, while *ASYMMETRIC LEAVES1/2* (*AS1/2*) restricts *BP* to the replum region. In turn, *AP2* maintains replum and valve margin growth by repressing *BP* and *RPL* activity in the replum, and *SHP1/2* and *IND* activity in the margins.

In the lateral domain, valve tissue identity is promoted by *JAG*, *FIL*, and *YAB3*, which also activate *SHP* genes in the valve margins. These genes function in parallel to *AG* (Dinneny et al., 2004; Gonzalez-Reig et al., 2012). *AG* is necessary for carpel identity, but some aspects of gynoecium patterning occur through *AG*-independent pathways, as revealed by *ap2 ag* double mutants that form carpels without valves (Liljegren et al., 2000). *SHP1/2*, together with *IND* and *ALC*, specify the valve margin and DZ (Rajani & Sundaresan, 2001; Sehra & Franks, 2017), while *FUL* represses *SHP* in the valves to maintain proper spatial expression. *RPL* also prevents *SHP* activation in the valves, restricting its activity to the margin. In the medial domain, *LUG*, *SEU*, *ANT*, and *FIL* form a transcriptional complex that regulates medial domain development (Azhakanandam et al., 2008; Sridhar et al., 2004). The interaction between *SEU* and *ANT* is critical for ovule formation; in *seu ant* double mutants, ovules are completely absent (Azhakanandam et al., 2008). The *CLV* genes help cells to differentiate at the outer edges of the CMM while preventing excessive proliferation in the center (Clark et al., 1996; Durbak & Tax, 2011).

KANs act together with ARF3/ETT and ARF4 in the lateral gynoecium to establish abaxial identity (Pekker et al., 2005). In *arf3/ett arf4* double mutants, valve tissues are largely absent, and ectopic ovules form at the apex. This phenotype is similar to the defects observed in *kan1 kan2* double mutants (Kelley et al., 2012; Lora et al., 2015). The KANs influence auxin distribution by repressing the expression of the auxin efflux carrier *PIN1*, and simultaneous loss of ARF3/ETT and KAN4 function disrupts auxin homeostasis, leading to a failure in maintaining the boundary between the inner and outer integuments (Ilegems et al., 2010; Turchi et al., 2015).

Studies have shown that ARF3/ETT can form a complex with the bHLH transcription factor IND to regulate auxin homeostasis by modulating the transcription of *PID* (Simonini

et al., 2017). In the early stages of gynoecium development, when auxin concentrations at the apex are low, the ETT-IND complex represses *PID* expression, allowing auxin to accumulate. By stage 8, when auxin levels reach their peak, the complex dissociates, allowing *PID* transcription and enabling the redistribution of auxin required for proper tissue differentiation (Moubayidin & Østergaard, 2017).

1.5 Decoding developmental programs through transcriptome profiling

Transcriptome profiling, most commonly performed using RNA-seq, allows the nearly complete characterization of transcriptomic events of genes occurring in a specific time. By taking the full set of active genes in a sample, the transcriptome provides a snapshot of cellular activity that is both quantitative and comprehensive. In the study of plant developmental biology, especially within model systems such as *A. thaliana*, transcriptome profiling has enabled the identification of GRNs, the inference of gene function, and the tracing of developmental trajectories with big scale and depth (Palovaara et al., 2017). Comparative transcriptome analysis is a powerful tool for understanding the similarities and differences in gene expression patterns across different species, developmental stages, tissues, or biotic/abiotic stresss. Comparative transcriptome across distant lineages allowed identification of shared patterns of co-expression and estimatedthe contribution of gene expression to phenotype for specific lineages (Rebecca M. Davidson et al., 2012). Moreover, transcriptomes provide temporal resolution by allowing the tracking of the activation and repression of regulatory processes across different developmental stages. This is particularly valuable in organ development, where the timing of gene expression changes can be just as important as the presence or absence of the genes involved (Sreedasyam et al., 2023).

However, although such global expression profiling provides a valuable foundation for both gene discovery and functional analysis, the first step typically involves the homogenization of entire organs, or even whole plants, before the extraction of RNA. This masks the cellular heterogeneity present in intact tissue. Consequently, there is an increasing focus on methodologies that can interrogate cell- and tissue-specific transcriptomes. By employing technologies like LMD, spatial transcriptomics, or single cell RNA sequencing (scRNA-seq), it is now possible to elucidate the GRNs mediating localized development while maintaining the spatial integrity of the expression profiles (Martin et al., 2016).

LMD is the method of obtaining target tissues or cells directly from frozen or paraffin-embedded tissue sections without destroying the structure of the tissues, and with as much

morphological integrity of the section to be dissected as possible. Several reliable protocols have been published previously, e.g. for *A. thaliana*, *Citrus clementina*, *S. lycopersicum*, and *Zea mays* (Anjam et al., 2016; Casson et al., 2005; Harrop et al., 2016; Kivivirta et al., 2019; Martin et al., 2016; Matas et al., 2010; Wuest & Grossniklaus, 2013).

In *A. thaliana*, LMD RNA-seq has been effectively applied to study the developmental program of the gynoecium. Kivivirta et al. (2021) generated a spatial-temporal transcriptomic atlas by LMD from gynoecia across four developmental stages. The comparison of gynoecium transcriptomes across time points reveals a temporal sequence of gene expression events, beginning with auxin-related genes, followed by transcription factors involved in tissue identity determination, and finally regulatory factors controlling meristematic tissue determination and differentiation. This study demonstrates that LMD RNA-seq can reveal spatial specificity and temporal order in gene regulatory network activity during flower organogenesis.

In monocots, Harrop et al. (2016) applied LMD RNA-seq to profile the early inflorescence meristems of *Oryza sativa* (rice). They characterized an initial expression switch between apical and axillary meristems, followed by progressive transcriptomic shifts during identity transitions, ultimately identifying several candidates involved in branching morphogenesis.

2 Aims and Objectives

This thesis aims to integrate morphological and transcriptomic approaches to understand the evolutionary diversification of floral structures in eudicots. In particular, it focuses on how morphological innovations, such as ring meristems and variations in carpel organization, evolved and how these changes are reflected at the transcriptional level across representative eudicots.

Aim 1: Morphological innovations in Ranunculales

This aim investigates floral morphological diversity in early-diverging eudicots, with particular emphasis on the origin and types of ring meristems, which are involved in initiating multiple stamen whorls (Polystemy). As part of this work, we present the first detailed study of floral morphogenesis in *Pteridophyllum racemosum* (Papaveraceae, Ranunculales), a species without polystemy, documenting a series of key developmental landmarks throughout its floral development.

Aim 2: Comparative transcriptomics of gynoecium development

This aim integrates morphological findings with comparative transcriptomic profiling of gynoecium development in *A. thaliana*, *E. californica*, and *S. lycopersicum* to distinguish between conserved and species-specific transcriptional programs. *E. californica* (Papaveraceae, Ranunculales) is used as a comparative model system for gynoecium development. Although its gynoecium shares major features with *A. thaliana*, *E. californica* lacks a septum and transmitting tract. This offers a contrasting developmental framework for identifying conserved and lineage-specific mechanisms.

3 Morphological investigations of floral organ development

3.1 Publication 1: Then there were plenty-ring meristems giving rise to many stamen whorls

This publication is a review entitled "Then there were plenty - ring meristems giving rise to many stamen whorls" in the journal Plants. The paper was accepted on 28th May, 2021 and published online on 3rd June, 2021 with the DOI: https://doi.org/10.3390/plants10061140.

Author contribution The study was conceived by the corresponding author (Annette Becker). I prepared the original draft of the manuscript, investigated part of the morphological data, prepared all visualizations, and interpreted the results.

In this paper, we first summarize recent progress on the regulation of floral meristem activity in *A. thaliana*, which has served as a model to uncover genetics underlying meristem maintenance and determinacy. We then broaden the perspective by investigating ring meristem morphology and occurrence across dicots, with special emphasis on taxa producing multiple stamen whorls. Ring meristems represent a distinct and, so far, largely overlooked type of meristem, separate from the central floral meristem, and their regulation remains poorly understood. By compiling morphological evidence and integrating it with current developmental knowledge, this review puts forward open questions on the genetic and evolutionary basis of ring meristems. Ultimately, this article aims to provide a basis for future understanding of the balance between male and female resource allocation in plant reproduction.

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Review

Then There Were Plenty-Ring Meristems Giving Rise to Many Stamen Whorls

Doudou Kong and Annette Becker *

Institute of Botany, Justus-Liebig-University, Heinrich-Buff-Ring 38, 35392 Gießen, Germany; Doudou.Kong@bot1.bio.uni-giessen.de
* Correspondence: Annette.becker@bot1.bio.uni-giessen.de; Tel.: +49-641-9935200

Abstract: Floral meristems are dynamic systems that generate floral organ primordia at their flanks and, in most species, terminate while giving rise to the gynoecium primordia. However, we find species with floral meristems that generate additional ring meristems repeatedly throughout angiosperm history. Ring meristems produce only stamen primordia, resulting in polystemous flowers (having stamen numbers more than double that of petals or sepals), and act independently of the floral meristem activity. Most of our knowledge on floral meristem regulation is derived from molecular genetic studies of *Arabidopsis thaliana*, a species with a fixed number of floral organs and, as such of only limited value for understanding ring meristem function, regulation, and ecological value. This review provides an overview of the main molecular players regulating floral meristem activity in *A. thaliana* and summarizes our knowledge of ring primordia morphology and occurrence in dicots. Our work provides a first step toward understanding the significance and molecular genetics of ring meristem regulation and evolution.

Keywords: floral meristem; polystemony; numerous stamens; evo–devo; ring meristem

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1. Introduction

Flowers are among the most beautiful examples of mutually beneficial relationships between animals and plants. Flowers are the major innovation of angiosperms, and their astonishing diversification is thought to be the major driver of the enormous success of the flowering plants. Even though most flowers are composed of only four different types of organs, their variability in structure, color, and scent is amazing and fine-tuned for attracting insect or bird pollinators or for allowing effective wind pollination [1–3].

In this review, we first outline the general morphogenesis of the floral meristem and describe the functions of the key genes involved in regulating maintenance and termination of the floral meristem. We then explain the concept of polystemony and identify lineages with independent origin of polystemony.

In many species, flower primordia arise from the flanks of the inflorescence meristem in a precise pattern (phyllotaxy), and, in few cases only, the shoot apical meristem turns into a floral meristem. For several species, the positioning of the flower primordia follows a localized auxin maximum via PIN1-mediated auxin transport in the epidermal L1 layer [4]. After initiation of the flower primordium, a meristem-to-organ boundary forms, separating the floral primordium from the rest of the plant body and the floral meristem enters its growth phase [4,5]. Auxin transport is now reversed, away from the primordium, to create an auxin sink in the central tissues of the shoot or inflorescences mediating the connection of the floral primordium to the vasculature [6].

In *Arabidopsis thaliana*, all floral organs arise from the floral meristem which, unlike the shoot or root meristem, undergoes a genetically defined succession of initiation, maintenance, and termination such that stem cell activity ceases once all floral organs are formed [5]. The floral meristem releases floral organ primordia at its flanks, and the number and position of stem cells is tightly regulated ensuring meristem stability,

as well as allowing cells to accumulate before organogenesis. Thus, the meristem is a defined structure even though its constituent cells are constantly changing. Cells exiting the meristem adopt their future cell fate according to their position within the meristem, while remaining in their original cell layers. Floral organs arise as primordia, composed of undifferentiated cells, which adopt a floral organ identity, and they subsequently grow and differentiate into the floral organs. In most flowers, floral organs are arranged in whorls, with sepals being the outermost organs, the second whorl involving the petals, followed by the pollen grain-producing stamens, and, in the center of the flower, the gynoecium is formed, harboring the ovules. After fertilization, the ovules develop into seeds, and the gynoecium is transformed into the fruit [7]. The development of floral organs follows a genetically determined blueprint, which was elucidated by molecular genetics studies in model species such as *Arabidopsis thaliana*, *Antirrhinum majus*, and grasses. Several recent reviews summarized our knowledge on floral organ identity, its variation and origin, and remaining open questions [8–11], which are not covered here.

Floral meristems are, thus, dynamic systems, balancing proliferation and termination in a species-specific manner, leading to a species-specific size and a number of different floral organ types. The shoot and inflorescence meristems are often and, to at least some extent, indeterminate, but stem cell maintenance in the floral meristem ends after the initiation of the gynoecium, rendering the floral meristem determinate [12]. *Arabidopsis thaliana* has a fixed number of floral organs and is typical for the canalized floral ground plan of many core eudicots. However, deviations from this ground plan are numerous, and little is known about the conservation of the gene regulatory network (GRN) balancing floral meristem action. While it seems reasonable to assume that some degree of conservation exists in floral meristem GRNs, this genetic system also allows extreme examples of variation of floral organ number (merosity), the floral meristem of the Ranunculaceae *Laccopetalum giganteum* generates up to 10,000 small carpels [13], while the bisexual Hydatellaceae (sister to all other Nymphaeales) flowers consist of either a single stamen or a single carpel [14].

2. Regulation of Stem Cell Activity in the Floral Meristem

The genetic networks regulating flower development have been characterized to the largest extent in the genetic model organism *Arabidopsis thaliana*. Molecular data from other species are largely missing; thus, we show the *A. thaliana* way of modulating floral meristem fate, keeping in mind that this floral meristem generates a fixed number of whorls and organs per whorl.

The regulation of floral meristem termination involves a highly complex regulatory interplay of transcription factors, microRNAs, receptor-like kinases and their ligands, MAP kinases, and chromatin remodeling (comprehensively reviewed in [15]); the homeodomain-containing transcription factors WUSCHEL (WUS) and SHOOT MERISTEMLESS (STM) function synergistically during shoot, inflorescence, and floral meristem development and maintenance. In *wus* and *stm* mutants, the floral meristem ceases prematurely because a smaller number of stem cells in the central zone is formed, which cannot keep up with the production of a larger number of cells in the periphery required for floral organ formation [16–19]. Conversely, overexpression of *WUS* leads to a conversion of cells in organ primordia into cells with stem cell characteristics [20]. *WUS* activation is limited by the CLAVATA (CLV) signaling loop, where the small apoplastic signaling peptide CLV3, activated by WUS in L1 and L2, moves toward L3 where it binds to the CLV1 and CLV2 receptors and other receptor-like kinases to indirectly repress *WUS* expression [21]. When any of the *CLV* genes is nonfunctional, the meristem's central zone increases due to an overexpression of *WUS* [22,23]. WUS also activates the floral homeotic gene *AGAMOUS* (*AG*) by direct binding to its second intron; thus, *WUS* not only regulates stem cell maintenance but is also required for floral organ initiation [24]. Once the floral meristem has produced all floral organ primordia, it is consumed in the process of carpel development, and stem cell activity ceases. For this process, the floral homeotic C function protein AG is required to repress *WUS* by directly binding to its genomic locus and by the recruitment of polycomb

group proteins that methylate chromatin to silence the *WUS* genomic locus [19,25]. In addition, AG activates *KNUCKLES (KNU)* by replacing repressing histone marks with activating ones in a time-dependent manner, and *KNU* then represses *WUS* expression [26]. Moreover, the floral homeotic A function gene *APETALA2* (*AP2*), which also promotes stem cell fate in the floral meristem by directly antagonizing *AG*, is translationally repressed by miR172d to achieve floral determinacy [27,28]. Furthermore, *AP2* not only negatively regulates the expression of *AG*, but also independently regulates the activity of floral meristem [29]. At the same time, there is evidence that *AP2* negatively regulates the *CLV* signaling pathway [30].

Moreover, additional transcription factors, such as SUPERMAN (SUP), CRABS CLAW (CRC), PERIANTHA (PAN), and ULTRAPETALA (ULT), also regulate floral meristem maintenance and termination, but to a lesser extent and in a partially redundant way, suggesting a high level of genetic robustness in the floral meristem termination network [31–33].

More recently, auxin signaling was reported to play a major role in floral meristem termination on several levels. For example, SUP recruits chromatin remodelers to suppress auxin biosynthesis genes at early stages of floral meristem development. If this function is lacking, the floral meristem is enlarged and active for a prolonged time [34]. The AG target *CRC* connects floral meristem termination with gynoecium development by activating the putative auxin transporter TORNADO2 to promote gynoecium formation while the floral meristem terminates [35]. The auxin response factor ARF3 is regulated by auxin levels, as well as by *AG* and *AP2*, and it is required to terminate *WUS* activity in the floral meristem [36]. However, it remains unclear how auxin biosynthesis and transport contribute to floral meristem termination on the molecular level.

While several meristem regulators in other species such as *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), and *Zea mays* (corn) have already been identified and functionally characterized, they all turned out to be orthologs of the genes analyzed in *A. thaliana* [37] except for *BEARDED-EAR* and *MOSAIC FLORAL ORGANS*1 from corn and tomato [38,39], respectively. Interestingly, many components of the floral organ identity and floral meristem termination regulatory network are conserved among seed and even non-seed plants. For example, *WUS* homologs are required for the initiation of cell growth during stem cell formation in the moss *Physcomitrella patens* [40], and several *WUS* homologs were also identified in gymnosperms [41].

3. How to Generate Multiple Stamen Primordia

Considering variations in the floral ground plan, the number of stamens is especially labile, and we concentrate on this trait in this section. The flower of the most recent common ancestor (MRCA) of all angiosperms most likely had more than 10 stamens arranged in three stamens per whorl, suggesting at least four whorls generating stamen primordia [42]. In recent species, the most common stamen arrangement in monocots and core eudicots involves two whorls with the outer stamens in alternate position to the petals [43]. In the ANITA grade, whose members are sister to all other angiosperms, stamen numbers are especially labile and range from one in Hydatellaceae and Chloranthaceae to up to 300 in Schisandraceae [43]. In species with an increased number of stamens, these are often not arranged spirally, but rather an androecial ring primordium forms, generating whorls of stamens. The direction of stamen primordia initiation can be toward the center of the flower (centripetal), toward the periphery (centrifugal), or bidirectional (Figure 1). The ring primordium sometimes subdivides into sectors and is, thus, fragmented [43]. This additional ring meristem provides a mean to uncouple stamen initiation from carpel initiation, such that the last stamens initiate after the carpels are already far advanced in their development (Figure 1).

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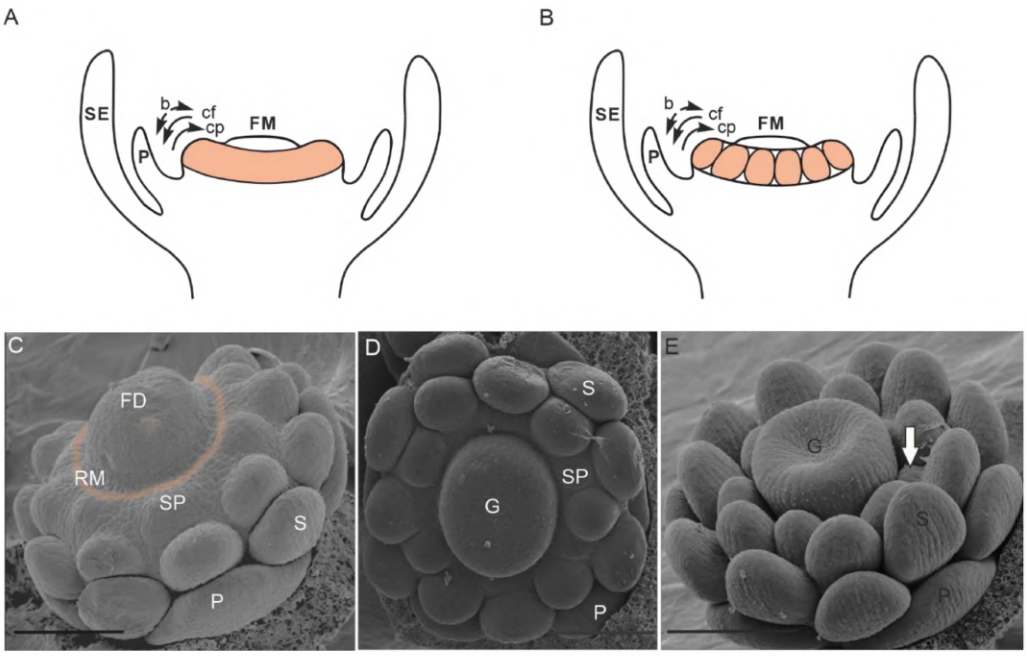

Figure 1. Flowers with ring meristems: (**A**) closed ring meristem and (**B**) fragmented ring meristem; the ring meristem is shown in orange. Black arrows indicate the direction of stamen primordia initiation. (**C**–**E**) Ring meristem of *Eschscholzia californica* forming stamen primordia. (**C**) Bud of a late stage 4 flower in which the floral dome separates from the ring meristem (indicated by a blurred orange line). (**D**) Stage 5 bud showing the continuous formation of stamen primordia while the gynoecium has already initiated. (**E**) Stage 6 bud showing the formation of new stamen primordia (white arrow) at the time when the early stamens already form a flat surface for microsporangia initiation. The sepals were removed in all images, and staging was done according to Becker et al. (2005) [44]. Abbreviations: b, bidirectional; cp, centripetal; cf, centrifugal; FD, floral dome; FM, floral meristem; G, gynoecium; P, petal; RM, ring meristem; SE, sepal; SP, stamen primordium; S, stamen. Scale bar in C = 86 µm, in D = 100 µm, in E = 120 µm ((**A**,**B**) modified from Endress, 2011; (**C**–**E**) from Becker, 2016 with permission) [7,43].

4. Ring Meristems in Eudicots

Eudicots comprise 44 orders, and ring meristems producing extra stamen whorls are found in 13 of them (APG, 2016 and Figure 2) [45]. Interestingly, the fraction of families with ring meristems per order is unequally distributed across the phylogeny. In Ranunculales, all families have members with ring meristems, and almost all combinations of closed/fragmented architecture and directions of stamen primordia initiation occur. Asterales consist of 11 families and a staggering number of almost 27,000 species, but include only the genus *Taraxacum* within the Asteraceae that has a ring meristem. In most orders, only a fraction of members develop ring meristems. For example, in Proteales, Fabales, Brassicales, and Myrtales, only a single family includes members with ring meristems. Interestingly, most ring meristems are of the closed type, and the number of orders with centripetal and centrifugal initiation of primordia is similar. However, bidirectional initiation is rarely found (Figure 2). Interestingly, centripetal primordia initiation is predominant in the non-core eudicot orders of Ranunculales and Proteales; however, in the core eudicots, centrifugal initiation is found more often. In several groups, a closed ring meristem is associated with centripetal primordia initiation, for example, in the Ranunculales, Protetales, Fabales, Hamamelidaceae, and Portulacaceae. In contrast, all combinations of primordia initiation direction and closed/fragmented ring meristem occur within the Malvales. According to our data, there seems to be no preference in the co-occurrence between closed

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and fragmented ring meristems and the direction of stamen primordia initiation. However, data compilation on the species level are lacking so far.

One of the well-known floral abnormalities is represented by double-flower phenotypes. These develop extra petals, most often at the expense of stamens, and are selected for in many ornamental species of high economic value. Interestingly, this phenomenon often correlates with mis regulation of *AG* orthologs or *APETALA2*-like genes [46], and a modification of ring meristem activity was not reported in these cases.

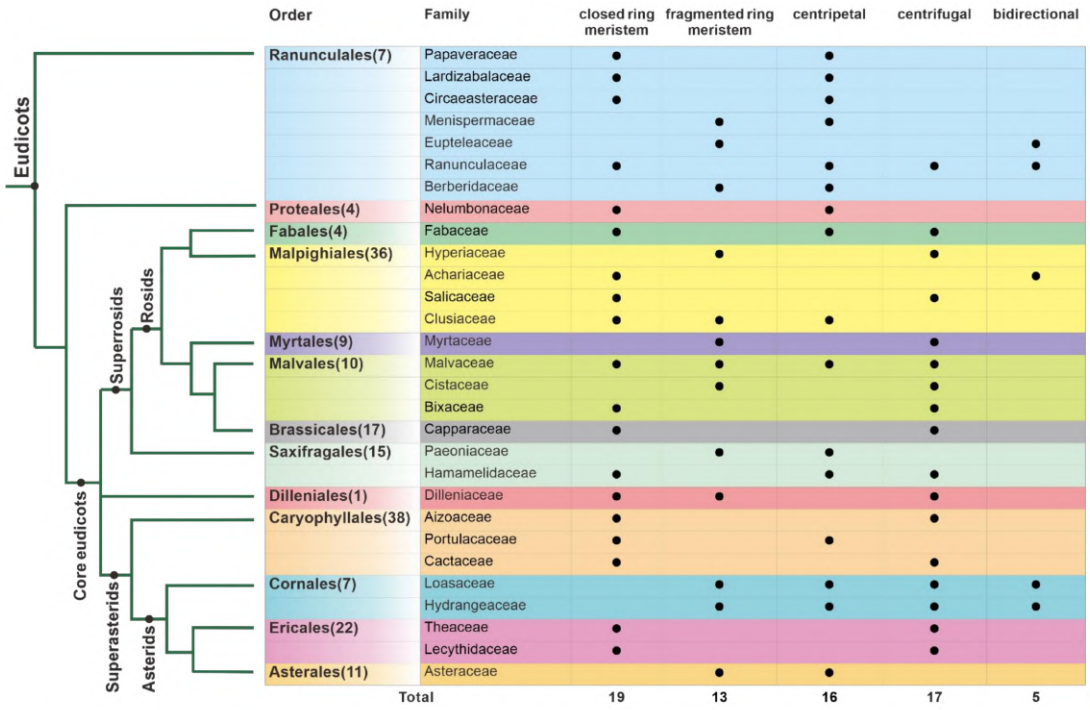

Figure 2. Distribution and types of ring meristems across eudicots. On the left, a simplified phylogeny shows only orders and families with ring meristem occurrence. Numbers in brackets next to the orders indicate the number of families in the respective order. On the right, the type of ring meristem is indicated. Numbers below summarize occurrences of morphological traits. Based on [43,45,47–57].

5. Are Ring Meristems in Dicots of Independent Origin?

In the Proteales and Ranunculales, which are sister to all other eudicots, ring meristems are found in all (Ranunculales) or at least some (Proteales) families; however, in Buxales and Trochodendrales, also non-core eudicots, ring meristems are missing. It, thus, remains unclear if the MRCA of eudicots had a floral ring meristem, and, if this was the case, we need to propose losses of ring meristems in the majority of eudicot orders. Generating more stamen and, in consequence, more costly pollen grains require a selective advantage for trait maintenance. If a high stamen number is not advantageous, independent losses may be rather frequent. In contrast, it seems also reasonable to propose that the eudicot MRCA had no ring meristem; however, according to this hypothesis, at least 13 independent gains of ring meristem activity need to be postulated. Nevertheless, the diversity of ring meristem architectures in dicots may be a hint toward several independent gains, as an evolutionary trend from an ancestral to a more derived condition in ring meristem structure cannot even be safely derived in the Ranunculales.

6. Conclusions and Outlook

Here, we compiled recent findings on transcription factors, signaling pathways, and phytohormone action, all involved in floral meristem termination of *Arabidopsis*. We further demonstrated that at least some components of the floral meristem regulatory systems are conserved between eudicots and mosses. However, the molecular regulation of floral meristem termination has mainly been analyzed in *Arabidopsis*, and even knowledge on this process in the monocot model species rice is lagging behind. Meristematic activity regulation in species with ring meristems has, to our knowledge, not been studied so far, leaving many questions open. This raises the question related to how the meristem activity in the central floral meristem and the ring meristem is differentiated and correlated in space and time, when presumably at least partially similar developmental mechanisms govern this activity. Unlike the ring meristem, which gives rise to only stamen primordia, the floral meristem releases lateral organs whose order and identity are precisely regulated. In most species, termination of the floral meristem activity is coupled to gynoecium initiation. However, ring meristems give rise to several whorls of stamen, and it remains unclear how these meristems are initiated, separated from the floral meristem, and then terminated. Furthermore, in addition to open questions related to their developmental regulation remaining unanswered so far, their ecological significance is unclear. For example, it would be interesting to test whether ring meristem occurrence and polystemony are correlated to specific pollination modes and pollinator preferences or how fast polystemony disappears when selfing becomes more prominent. Moreover, ring meristems producing varying numbers of stamen may shift the balance between male and female contributions toward sexual reproduction. Thus, the regulation of ring meristem activity termination, at least in some species [44], may be more flexible than that of the central floral meristem. For example, it may depend on environmental cues, on the accumulation of sufficient reserves, or on other metabolic changes during a plant's maturation process.

Learning about the mechanisms generating and regulating ring meristems requires new genetic model species with ring meristems, if possible, closely related to those without a ring meristem, for example, from the Ranunculales family. This allows comparative transcriptomics of flowers that develop ring meristems with those that do not. In addition, ring meristem tissue may be analyzed separately from the central floral meristem to reveal genes differentially expressed between ring and central floral meristems. The role of these candidate genes in ring meristem establishment and regulation may then be revealed by knockout or knockdown approaches.

Thus, our review provides only a first glance into a morphological structure that is enigmatic in terms of the molecular mechanism of its regulation and with respect to its origin, evolution, and ecological significance.

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3.2 Publication 2: Floral morphology and development of *Pteridophyllum racemosum* Siebold & Zucc. (Papaveraceae)

This publication is a research article entitled "Floral morphology and development of *Pteridophyllum racemosum* Siebold & Zucc. (Papaveraceae)" published in the journal *Botany Letters*. The paper accepted on 4th May 2024, and published online on 16th May 2024 with the DOI: https://doi.org/10.1080/23818107.2024.2352773.

Author contribution The study was drafted by the corresponding author (Annette Becker). I investigated the floral morphology of *P. racemosum*, including histological sectioning and scanning electron microscopy, prepared all visualizations, and interpreted the results.

In this study, we provide the first detailed description of the habitus and floral morphogenesis of *P. racemosum*. *P. racemosum* is the only species of the Pteridophylloideae, which was just recently shown to be sister to the Papaveraceae subfamilies Hypecoideae and Fumarioideae, and is thus integral for comparing morphologies within the Papaveraceae and Ranunculales. We illustrate the floral morphogenesis and clarify a series of landmarks during flower development. Our results show that *P. racemosum* produces flowers with two sepals, four petals arranged in two whorls, four stamens, and a syncarpous gynoecium with two ovules, a combination that is rare within Papaveraceae but consistent with traits reconstructed for the ancestral flower of the family. These findings clarify the floral architecture of *P. racemosum* and provide concrete morphological evidence to support its placement as an early-branching lineage in Papaveraceae.

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Floral morphology and development of *Pteridophyllum racemosum* Siebold & Zucc. (Papaveraceae)

Doudou Kong, Katrin Ehlers and Annette Becker

Institute of Botany, Plant Developmental Biology group, Justus-Liebig-University, Giessen, Germany

ABSTRACT

Papaveraceae are known for their often showy flowers and diverse morphologies. *Pteridophyllum racemosum* (Siebold & Zucc.) is the only member in the Pteridophylloideae within the Papaveraceae, and its phylogenetic position has long been controversial, and a comprehensive analysis of its floral morphology was lacking. Our study focuses on the floral morphogenesis of *P. racemosum*. Histological sections complemented with scanning electron microscopy allowed a detailed characterization of *P. racemosum* floral development including description of the vegetative morphology, showing (1) the fine structure of the unusual pinnate leaves; (2) the well-defined floral structure and disymmetry; (3) a series of landmarks in floral development stages, with emphasis on the development of the gynoecium. Interestingly, the *P. racemosum* floral architecture is similar to that of the proposed ancestral Papaveraceae flower supporting the phylogenetic placement of *P. racemosum* as an early branching lineage within the Papaveraceae. Our comprehensive overview adds to the colorful library of floral diversity in Papaveraceae, providing a solid base for comparative analyses.

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CONTACT Annette Becker annette.becker@bot1.bio.uni-giessen.de Institute of Botany, Plant Developmental Biology group, Justus-Liebig-University, Heinrich-Buff-Ring 38, Giessen 35392, Germany

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Introduction

The order Ranunculales is sister to all other eudicots and includes > 4500 species distributed among seven families (APG 2016). Ranunculales emerged approx. 130 million years ago (Magallón et al. 2015; Peng et al. 2023), and they are characterized by a large diversity in life-history traits, growth habit, leaf shape, flower and fruit forms, etc. The most common figures of merism of the floral whorls range from two to five, the perianth can be absent (Eupteleaceae) or, when present, differentiated into petals and sepals (bipartite) or composed of a single type of organ (unipartite), either sepaloid or petaloid. Additionally, floral organs vary extensively in form, size, color, and floral symmetry is also diverse, all in relationship with pollination modes (RanOmics Group 2023). In Ranunculales, several specific traits are found that are poorly represented among classical core eudicot or monocot model species, such as changes in merism, changes between whorled vs spiral phyllotaxy, or formation of novel organ types. Other traits that are found in Ranunculales are also known from core eudicots or monocots, such as radial vs. bilateral symmetry, spur formation, and transition between sexual systems in closely related taxa (Jensen and Kadereit 1995; Soza et al. 2012). Such homoplasious characters provide the opportunity to study the conservation of genetic mechanisms involved in the origin of homologous vs. convergent traits (RanOmics Group 2023). However, studying the origin and evolution of traits on a morphological or molecular scale requires precise knowledge of traits in a broad range of species. Key positions for comparative analyses hold those taxa that are sister to major lineages as they are indispensable for ancestral trait inference.

The Papaveraceae (poppy) family is one of the monophyletic families of the Ranunculales and they comprise four subfamilies: Fumarioideae, Hypecoideae, Papaveroideae, and Pteridophylloideae (Figure 1 for comparison of their floral architecture). Molecular clock analyses date the age of poppy family stem group at 112–139 MYA and they have most likely diversified during the Cretaceous Terrestrial Revolution (Peng et al. 2023). Papaveraceae split already around 120 MYA into the Papaveroideae and the lineage that led to Hypecoideae, Fumarioideae and Pteridophylloideae. Interestingly, the majority of poppy family genera are biogeographically strictly restricted (Peng et al. 2023).

The phylogenetic placement of the Pteridophylloideae has been controversial, such that they were placed as the sister subfamily to the remaining Papaveraceae in a phylogeny that combined data on chloroplast DNA and nuclear 26S ribosomal DNA (Hoot et al. 2015). However, recent research has provided a comprehensive phylogenetic framework for Papaveraceae using full plastid sequences of 10 Papaveraceae species complemented with six plastid loci from 106 taxa. In this analysis, the Pteridophylloideae are placed as the sister group to both, the Hypecoideae and Fumarioideae (Peng et al. 2023; RanOmics Group 2023).

Pteridophyllum racemosum (Siebold & Zucc.) is the only species in the Pteridophylloideae subfamily. It is

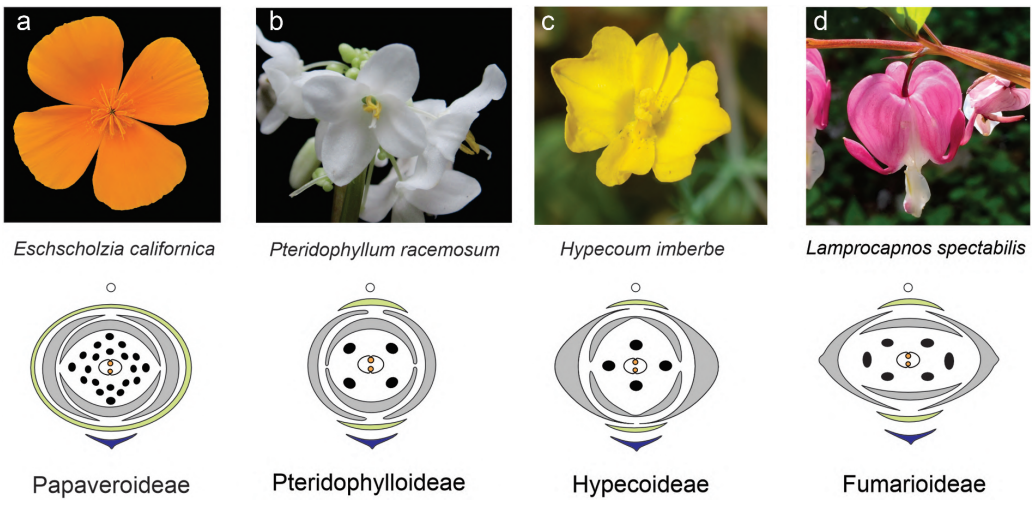

Figure 1. Floral morphologies in the Papaveraceae. (a) *Eschscholzia californica* (California poppy, Papaveroideae). (b) *Pteridophyllum racemosum* (pteridophylloideae). (c) *Hypecoum imberbe* (sicklefruit hypecoum, hypecoideae). (d) *Lamprocapnos spectabilis* (bleeding heart, Fumarioideae); floral diagrams are below the pictures with sepals colored in green, floral bract in dark blue, petals in grey, stamens in black, and the gynoecium as oval in the center. C, D referred to Sauquet et al. (2015) and Hidalgo and Gleissberg (2010); photos C, D, copyright free from Naturalist.org, C, by Quentin Groom, D, by Askalotl.

an evergreen perennial plant herb endemic to the central and northern regions of the Japan mainland (Honshu island) where it grows in the understorey of mountainous and subalpine forests (Tani 2001; Endo et al. 2011). They survive the winters by maintaining one- and two-year old leaves and winter buds, which develop right after flowering season and are surrounded by thick, protective bracts to allow fast reproduction in a short vegetative season, in contrast to most perennial herbaceous perennials that overwinter with complete shedding of old leaves (Tani et al. 2003; Endo et al. 2011). *Pteridophyllum racemosum* is self-compatible and reproduces mainly by seeds.

Ranunculales are notorious for their synthesis of diverse secondary metabolites, with benzylisoquinoline alkaloids (BIAs) being the economically most important. BIAs were also identified in root and aerial tissue of *P. racemosum*, among them protopine, sanguinarine, dihydrosanguinarine, oxysanguinarine, chelerythrine, magnoflorine, α- and β-allocryptopine (Ikuta and Itokawa 1976).

Despite the stunning morphology of *P. racemosum*, with the unusual combination of fern-like leaves and bell-shaped flowers and its distinct phylogenetic position in the Papaveraceae, a comprehensive analysis of its floral morphology is lacking. Here, we introduce the habitus of *P. racemosum*, and provide a detailed description of its floral morphogenesis by combining histological sectioning and scanning electron microscopy.

Materials and methods

Pteridophyllum racemosum plants were purchased from Dix Export bv (Heemstede, Netherlands), "A Touch of Green" Garden Webshop (Netherlands), and Pépinière AOBA nursery (La Touche au Burgot, France), transferred to the greenhouse and to a shady spot at the Botanical Garden, Giessen, Germany. The unique identifier given by the Giessen Botanical Garden for all plants is an IPEN number: XX-0-GIESS-2019-J-756. Photos were taken using a Leica DFC450 camera (Leica, Wetzlar, Germany) and a Leica M165 stereo microscope (Leica, Wetzlar, Germany).

Pteridophyllum racemosum buds develop during winter below the soil surface. For sampling, the soil surface was gently ruffled with a brush to collect the inflorescences. Fixing, embedding, sectioning and staining for histological analyses was done as described in (Becker et al. 2005). Briefly, ten intact inflorescences were fixed in freshly prepared cold FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid), vacuum infiltration was followed by overnight fixation at 4 ℃ (~16 h) and then dehydration in a graded ethanol series. Then they were embedded in Paraplast Plus (Roth, Karlsruhe, Germany) and cut into 8 µm thick sections using a Leica 2125RTS microtome (Leica, Wetzlar, Germany). After dewaxing, the sections were stained with 1% safranin and 0.2% fast green and analysed using a Leica DM 5500 microscope equipped with the above-mentioned camera (Leica, Wetzlar, Germany). The size of all floral buds was measured by ImageJ (1.53e; Wayne Rasband and contributors National Institutes of Health, USA).

For SEM, four intact inflorescences were fixed for two hours in freshly prepared glutaraldehyde fixative (2% glutaraldehyde, 0.5% paraformaldehyde, 0.2 M phosphate buffer, pH = 7.2) for 2 h at RT. After dehydration in an ethanol series, the samples were incubated twice in acetone for 1 h. Finally, samples were critical-point dried and sputter-coated at 35 mA for 35 sec, and then scanned with a LEO 982 scanning electron microscope (Zeiss, Jena, Germany) at 3kV.

Results

Morphology of the vegetative and reproductive organs

Pteridophyllum racemosum is a perennial, evergreen herb, with leaves organized in a rosette Figure (2a). The leaves appear hardy and they are pinnate, reminiscent of a fern frond. Each leaf consists of around 25–30 leaflet pairs and multicellular trichomes form on the adaxial surface of the leaflet, while stomata emerge from the abaxial surface Figure (2b-d). Winter buds contain a young inflorescence composed of many flowers. They form at or close to the center of the vegetative rosette and they are covered by several layers of protective bracts (Endo et al. 2011). The main inflorescence axis produces spirally arranged, subtending bracts and their axillary buds will give rise to lateral, secondary inflorescences with very short axes. This secondary inflorescence meristem will terminate in a single flower that opens first and produces subtending bracts with axillary buds from which secondary flowers emerge. 1–2 cm long pedicels connect the flowers with the inflorescence axis. Thus, the inflorescence type of *P. racemosum* is a thyrse, with lateral monochasia or dichasia. Flowers are disymmetric with two minute sepals, two whorls of two morphologically similar petals each, four fertile stamens in a single whorl and a syncarpous gynoecium composed of two carpels. Nectaries are absent. The petals are white, slightly shiny and oval in shape. The two outer petals occupy the outer petal whorl and two inner petals occupy the inner whorl. The stamens consist of a short, transparent filament and long anthers with two thecae Figure (2f,g). Towards the end of floral bud development, the stamens extend above the gynoecium. At anthesis, stigmatic protrusion appear at the gynoecium apex and the gynoecium extends above the stamens Figure (2e, g).

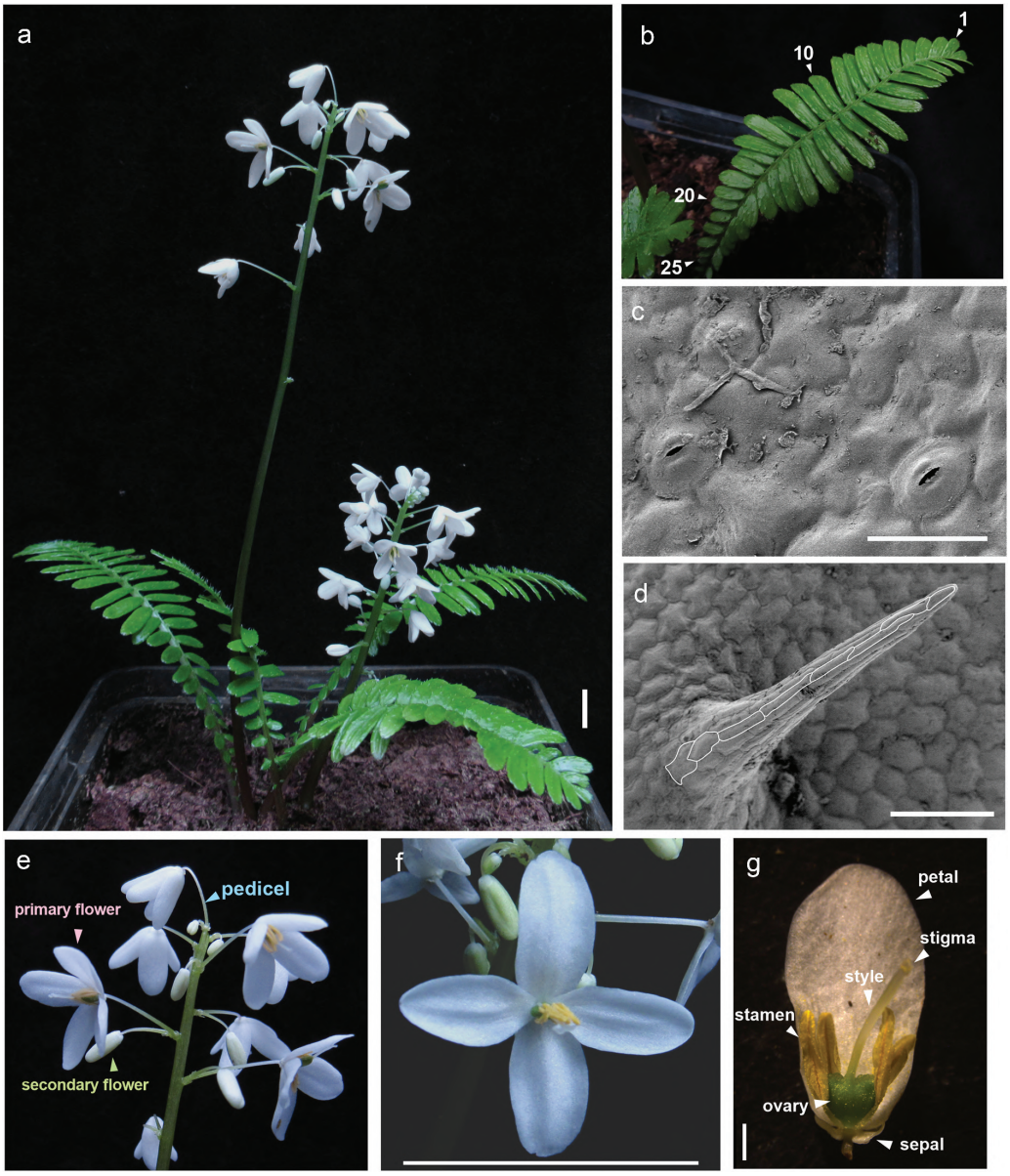

Figure 2. *Pteridophyllum racemosum* vegetative and floral morphology. (a) habitus of a flowering *P. racemosum* plant. (b) Pinnate leaf with leaflet numbers indicated. (c) scanning electron micrograph of the abaxial leaf epidermis including stomata. (d) SEM of multicellular adaxial trichomes, white lines indicated cell walls. (e) side view of the apical part of the inflorescence. (f) top view of a flower at anthesis. (g) view into the flower at anthesis with one petal removed. One sepal was falsely colored in yellow for clarity. Size bars correspond to (a, f) = 1 cm; (c) = 50 µm; (d) = 200 µm; (g) = 1 mm.

Floral morphogenesis

Floral morphogenesis commences with the initiation of a secondary inflorescence meristem at the flank of the primary inflorescence meristem with older secondary inflorescences forming basally Figure (3a-c). The secondary inflorescence produces a terminal floral meristem and a second (and rarely third) floral meristem at its flank, both connected by the same peduncle to the primary inflorescence stalk Figure (3b, c). Floral initiation occurs already under ground, before the main inflorescence axis expands. The two floral buds connected by one peduncle develop successively and our observations are all based on the later formed flower. Figure 3d shows a floral bud at stage 1, surrounded by a subtending bract and with emerged sepal primordia that will eventually encircle the dome shaped floral meristem (Table 1 summarizes the staging of flower development). Stage 2

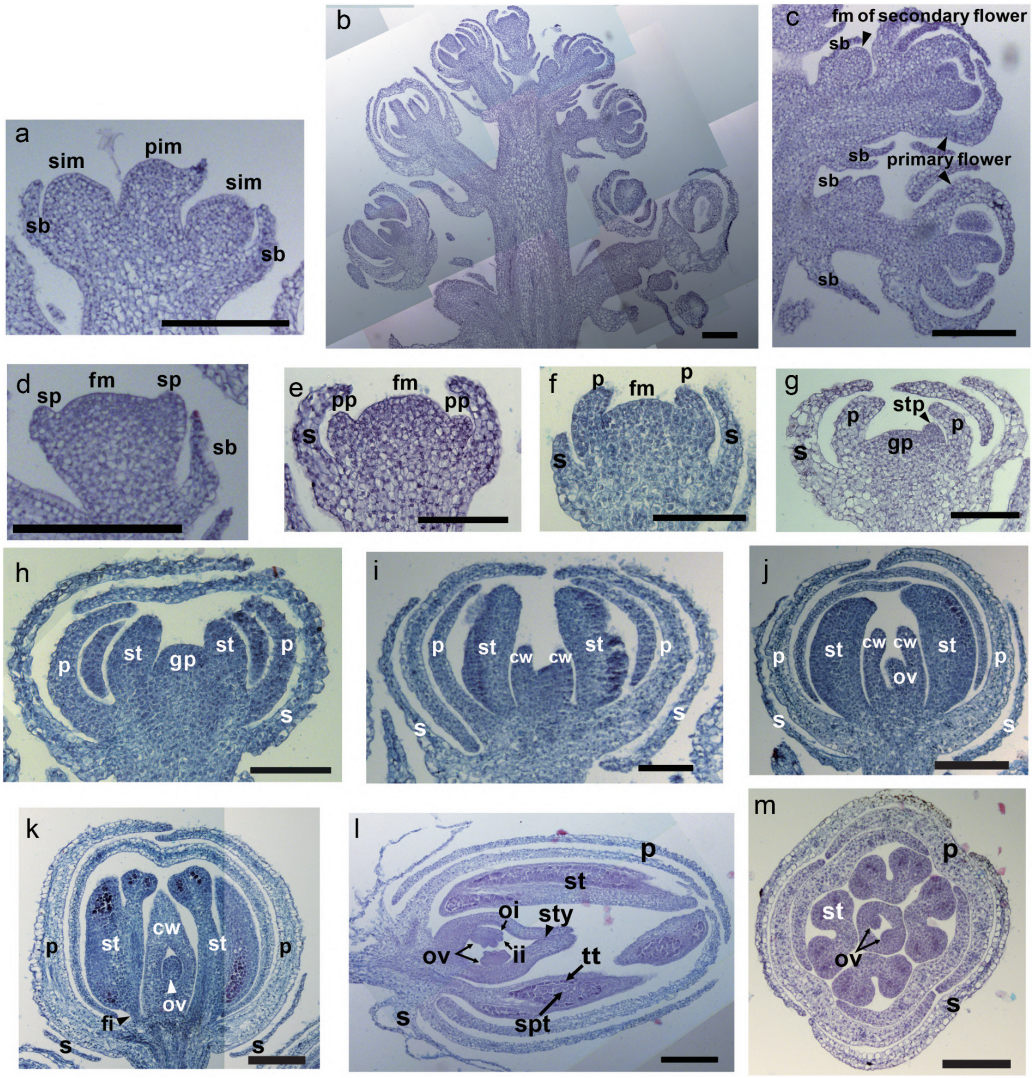

Figure 3. Histological sections showing different stages of *P. racemosum* floral development. (a) inflorescence meristems at the primary inflorescence apex. (b) longitudinal section through an inflorescence, subfigure assembled from multiple photos. (c) details of secondary inflorescence organization. (d) floral bud at stage 1 with sepal primordia initiated. (e) floral bud at stage 2, with petal primordia initiated. (f) floral bud at stage 2, when petals elongate. (g) floral bud at stage 3, when stamen primordia initiate. (h) floral bud at late stage 3, when stamen elongate and the gynoecium primordium becomes dome-shaped. (i) floral bud at stage 4, when the carpel walls initiates. (j) floral bud at stage 5, when carpel walls elongate and ovules initiate. (k) floral bud at stage 6, when carpels are fused at the apex. Longitudinal section subfigure assembled from two photos. (l) floral bud at stage 7, when the style elongates, integuments initiate, and male sporogenous tissue develops into pollen. (m) cross-section of floral bud in stage 7. Abbreviations: cw, carpel wall; fi, filament; fm, floral meristem; gp, gynoecium primordium; ii, inner integument; oi, outer integument; ov, ovule; p, petal; pim, primary inflorescence meristem; pp, petal primordium; s, sepal; sb, subtending bract; sim, secondary inflorescence meristem; sp, sepal primordia; stp, stamen primordium; st, stamen; sty, style; spt, sporogenous tissue; tt, tapetum tissue. Size bars correspond to (a, c, d, i-m) = 200 µm; (b) = 1 mm; (e-h) = 100 µm.

floral buds show elongating, inward curving sepals and emerging petal primordia that elongate. The sepals have not covered the bud yet Figure (3e, f). In stage 3, sepal primordia cover the floral bud, petals continue to elongate, and stamen primordia emerge and expand in length and width, surrounding the gynoecium primordium that becomes dome-shaped during stage 3 Figure (3g, h). In stage 4 floral buds, petals and stamens continue to expand longitudinally, but the sepals discontinue their expansion, causing the petals to cover the floral bud at the apex. The gynoecium primordium gives rise to the carpel walls Figure (3i). Two ovules emanate inside the gynoecium which elongates rapidly in stage 5 Figure (3j). In stage 6, the two carpels fuse

Table 1. Floral developmental stages of *Pteridophyllum racemosum*.

Stage	Developmental landmarks	Bud diameter	Bud length
Stage 1	Floral primordium emerges, subtended by a bract	180–250 µm	100–150 µm
Stage 2	Sepals elongate, petal primordia emerge.	230–300 µm	130–200 µm
Stage 3	Petals elongate, stamen primordia initiate, gynoecium initiates and becomes dome-shaped in late stage 3	280–420 µm	200–310 µm
Stage 4	Stamens elongates rapidly, carpel walls initiate.	400–550 µm	350–410 µm
Stage 5	Ovules initiate, carpel walls elongate.	500–640 µm	480–650 µm
Stage 6	Fusion of carpels, male sporogenous tissue forms.	600–870 µm	630–840 µm
Stage 7	Integuments initiate from ovules.	800 µm -	950 µm -

postgenitally at their apices and sporogenous tissue forms in the anthers Figure (3k). In stage 7, the style elongates, inner and outer integuments form at the ovules, and pollen development continues in the anthers. All floral organs elongate, except for the sepals Figure (3l, m). The bud extends in width from around 180 µm to more than 870 µm and in length from 100 µm to 950 µm during stages 1 to 7.

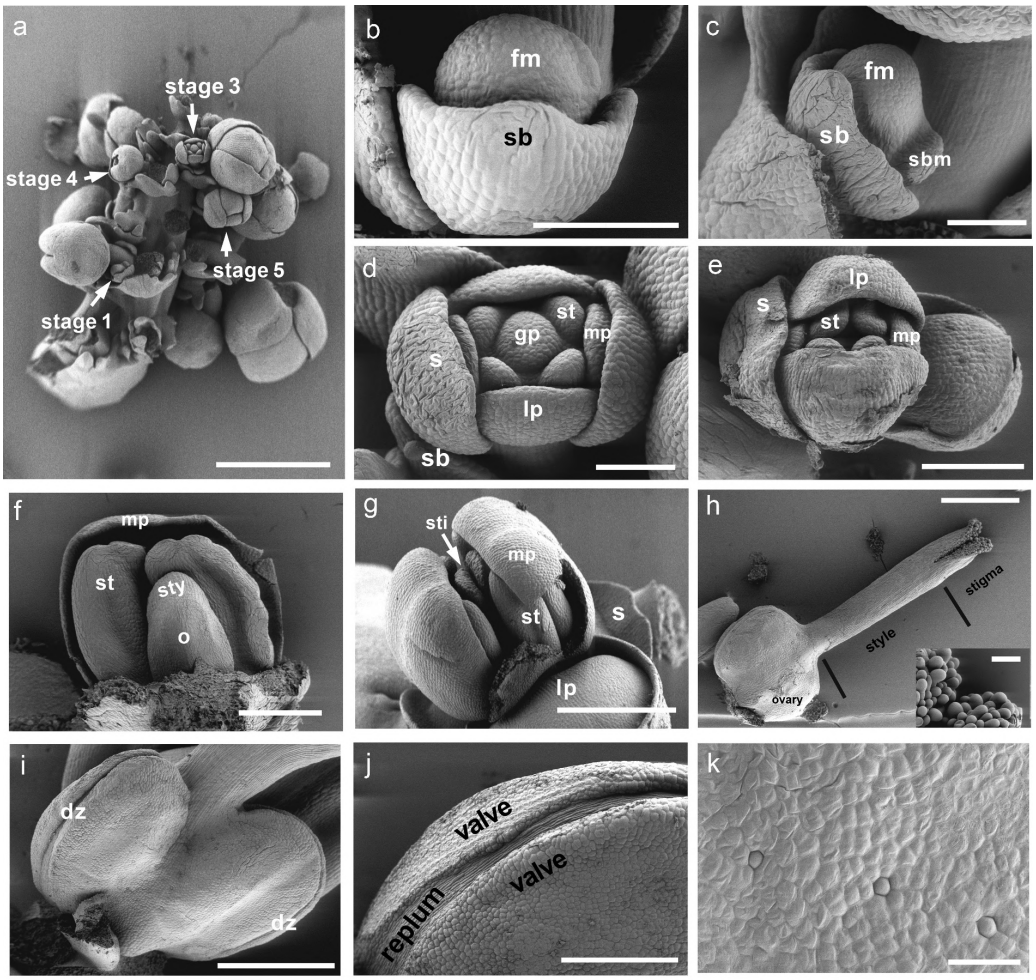

Figure 4. Scanning electron micrographs of *P. racemosum* floral development. (a) top view on an inflorescence with some buds removed. (b) floral bud at stage 1, encircled by a bract. (c) floral bud at stage 1, surrounded by a bract, with a subtending bract initiating. (d) floral bud at stage 3 with all floral organs initiated. (e) floral bud at late stage 4 with elongated outer petals. (f) floral bud at stage 6 with some petals and stamen removed to reveal the fused gynoecium. (g) Stigma and stamens grow up to the same height in late stage 6. (h) side view on the gynoecium with all other floral organs removed. The inset shows the stigmatic papillae at higher magnification. (i) side view on the ovary. (j) enlargement of the future dehiscence zone. (k) outer surface of the ovary wall. Abbreviations: b, bracts; dz, dehiscence zone; fm, floral meristem; gp, gynoecium primordia; ip, inner petal; o, ovary; op, outer petal; sb, subtending bract; sbm, subtending bract meristem; sti, stigma; sp, sepal primordia; s, sepal; st, stamen; sty, style. Scale bars (a) 1 mm; (b-d) 100 µm; (e,f,j) 200 µm; (g-i) 500 µm, enlarged fig in h: 50 µm; (k) 50 µm.

Spatial organization of floral organs and gynoecium morphology

The concurrence of floral developmental stages and the spatial arrangement of floral buds is shown in Figure 4a, where all terminal buds fully covered by petals are accompanied by buds of early developmental stages, and protective bracts are present within the inflorescence (Supplemental Figure). The base of the floral meristem is located between the primary flower and the flower subtending bract Figure (4b). Later, the subtending bract meristem emerges and initiates laterally from the floral meristem Figure (4c,b shows the secondary inflorescence meristem at inception). In stage 3, two sepals of equal size cover part of the bud. In opposing position to the sepals, the outer whorl petals are initiated and the inner whorl petals form adjacent to the sepals. The four stamen primordia occupy the gaps between the developing petals, and from the center of the bud the dome shaped gynoecium primordium has already emanated Figure (4d). In stage 4, the outer petals elongate and cover the inner whorl petals, stamens and initiated gynoecium Figure (4e). In late stage 6, the gynoecium initiates the style Figure (4f). Following carpel fusion, the stigma shows a slit positioned parallel to the inner whorl petals Figure (4g).

The gynoecium consists of a short and wide superior ovary harbouring two ovules, a style around twice the length of the ovary and a stigmatic region. The latter has a deep slit in its center with stigmatic protrusions extending into the cleft Figure (4h). As in the vast majority of the Papaveraceae, the dry dehiscent capsule of *P. racemosum* is derived from the syncarpous gynoecium. The replum is positioned at the outer sides of the mature fruit Figure (4i,j). Conspicuous cells are regularly space across the ovary wall that have cell walls that do not reach the plane of surrounding cell's cell walls Figure (4k).

In summary, we describe the floral architecture and morphogenesis of *P. racemosum*, showing a symmetrical arrangement of floral organs in five concentric whorls. Unlike in most eudicots, the floral bud is not covered by the sepals as they stop elongating early in bud development (as observed for *Capnoides sempervirens*, data not shown). However, their protective function is taken over by the outer whorl petals which cover the inner whorl petals, stamens and gynoecium. The sepals remain on the pedicel when petals and stamens have dehisced already. Further, *P. racemosum* flowers include only four stamens, and only two seeds develop in each capsule, and both are rather low numbers when compared to other members of the Ranunculales order.

Discussion

Our detailed morphological analysis of *P. racemosum* shows the development of a textbook-like flower, in that we observed little deviation from the canonical flower architecture described for model plants like *Arabidopsis thaliana* with four sepals, four petals, six stamens and a two-carpellate gynoecium. We observed only minor differences: (1) only two sepals emerge from the floral primordium that cease expansion at an early developmental stage and persist on the flower after abscission of the other floral organs, (2) the four petals are arranged in two whorls that increase in size continuously throughout flower development, (3) four stamens emerge from a single whorl and (4) only two ovules are formed in the gynoecium.

Pteridophyllum racemosum flower is highly similar to the Papaveraceae ancestral flower

Ancestral state reconstructions of floral traits are available for the Ranunculales (Carrive et al. 2020), and because the Papaveraceae are sister to the Lardizabalaceae, Berberidaceae, Menispermaceae, and Ranunculaceae, their floral morphology is especially meaningful when deriving ancestral states. Only the Eupteleaceae branch earlier from all other Ranunculales, but as this family includes only two species with possibly derived traits, they are generally omitted from analysis (Carrive et al. 2020). Thus, Papaveraceae ancestral floral traits are in many cases similar to the Ranunculales ancestral traits. The ancestral Papaveraceae flower was reconstructed as follows: whorled, dimerous, disymmetrical perianth with 5–10 organs and three or more whorls. The perianth whorls were unfused, differentiated with a constant number of organs, and the inner perianth organs were petaloid. The ancestral number of stamens was less than six and two unfused, unilocular carpels in the center. Spurs and nectaries are absent (Hoot et al. 2015; Carrive et al. 2020). Interestingly, the flower of *P. racemosum* matches with the reconstructed ancestral trait flower in most aspects. However, it deviates from the ancestral Ranunculales flower, which was supposedly actinomorphic, with at least 12 free carpels (Carrive et al. 2020) in that it is dissymmetric with no differentiation between the two planes and has only two but fused carpels.

However, as an exception to typical Papaveraceae, *P. racemosum* does not develop crystal bearing idioblasts or laticifers and does not incorporate calcium oxalate crystals in the inner epidermis of the outer ovule integument (Hoot et al. 2015), but it remains unclear if these traits are ancestral states of the Papaveraceae.

Changes to zygomorphy in the sister lineage occurs late in flower development

In the most recent Papaveraceae phylogeny, *P. racemosum* is sister to both, the Hypecoideae and the Fumarioideae (Peng et al. 2023), but substantial changes to the ancestral Papaveraceae floral state occurred only in the Fumarioideae (Figure 1), such as strongly modified perianth parts, zygomorphy, dissymmetry, and the development of nectaries at the stamen base (Damerval et al. 2013; Carrive et al. 2020). Interestingly, many stages of flower development are similar between *P. racemosum* and the zygomorphic Fumarioideae species *Capnoides sempervirens*: initiation of all floral organs is disymmetric in both flowers, but six stamens develop, of which four are monothecal and two dithecal in *C. sempervirens* (Damerval et al. 2013). Zygomorphic characters are achieved by unequal growth, organ fusion of stamens, and addition of a nectary spur in *C. sempervirens* later in development (Damerval et al. 2013).

Hypecoideae flowers are also disymmetric and with two sepals that are not enclosing the bud, they have four petals arranged in two whorls, four stamens that are arranged differently in that they are opposite of the inner whorl petals (Figure 1) and they develop nectaries. However, they are more proliferous with 30–100 seeds per capsule.

In comparison with the Papaveroideae, *P. racemosum* shares more characters with the Hypecoideae and Fumarioideae, as the Papaveroideae have more than 16 stamens, the sepals generally enclose the buds, most species have a higher number of petals, and many have more than two carpels (Hoot et al. 2015). Both *Hypecoum* and *Pteridophyllum* are characterized by actinomorphic androecia of four dithecal stamens (Sauquet et al. 2015). Thus, the floral characters support the recent phylogeny of *P. racemosum* being sister to the Hypecoideae and Fumarioideae (Peng et al. 2023). However, the absence of laticifers is unique to *P. racemosum* (Hoot et al. 2015), but could be a character that was lost in this species.

The presented work was limited by the availability of plant material, which is rarely grown in botanical gardens throughout Europe and the lack of protocols for growing the specimens. Further, access to the buds is challenging as they slowly develop after the flowering season below the substrate surface and when the inflorescence axis elongates, most stages of flower development have already passed. Additional analyses on the timing of bud development and dehiscence of the capsules would be very interesting, but requires more plant material.

Conclusions and outlook

This work provides a comprehensive overview of *P. racemosum* floral morphogenesis and detailed description of the floral morphology, which is identical to the reconstructed ancestral Papaveraceae flower. The key position of *P. racemosum* in the Papaveraceae family as sister to the dissymmetric Hypecoideae and the Fumarioideae in which zygomorphy originated once or twice, contributes to its importance for comparative analysis in terms of floral morphogenesis, but also for specialized secondary metabolites.

While *P. racemosum* is difficult to grow under controlled greenhouse conditions without a harsh winter, it can be maintained in cold frames or Botanical gardens in temperate regions, allowing for more extensive studies regarding its physiology, specialized metabolism, and unusual leaf morphogenesis.

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Disclosure statement

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Notes on contributors

Mr. Doudou Kong is a graduate student of Biolog under the supervision of Prof. Dr. Annette Becker, whose primary are of interest is comparative expression analysis in Ranunculales.

Dr. Katrin Ehlers is a cell biologist, whose primary interest is in the development and evolution of plasmodesmata and diverse microscopic techniques.

Prof. Dr. Annette Becker is a plant geneticist, whose primary interest is in the origin and evolution of flowering plants.

ORCID

Doudou Kong http://orcid.org/0000-0002-8058-7168
Katrin Ehlers http://orcid.org/0000-0001-7110-1595
Annette Becker http://orcid.org/0000-0002-3229-2162

Author contributions

AB drafted and supervised this research. DK performed specimen photography, paraffin sectioning, and SEM. KE provided and optimized the SEM protocol and supervised

SEM. DK and AB wrote the manuscript. All authors read and agreed to publish the final manuscript.

Data availability statement

Original data have been deposited here: http://dx.doi.org/10.22029/jlupub-18415.

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4 Comparative transcriptomics of carpel development across eudicots

This chapter is based on an unpublished manuscript that is still being prepared. The text has been expanded into separate sections that include detailed descriptions of methods, results, and discussion.

4.1 Brief introduction

In this chapter, we present a comparative transcriptomic analysis of carpel development across three eudicots: *A. thaliana* (Brassicaceae, Rosids), *E. californica* (Papaveraceae, Ranunculales), and *S. lycopersicum* (Solanaceae, Asterids). We sampled carpels at four successive developmental stages in each species to generate transcriptomes that capture the temporal dynamics of gene expression, and used OGs as a common framework for interspecific comparison. Our results reveal (1) sets of genes with conserved expression across species that may represent core regulators of carpel morphogenesis and (2) carpel regulators with species-specific expression patterns that may underlie evolutionary divergence in carpel development.

4.2 Materials and methods

4.2.1 Transcriptome data sources

We analyzed carpel transcriptome datasets from three eudicot species: *A. thaliana* (Col-0), *E. californica* (cv. Aurantiaca Orange King), and *S. lycopersicum* (cv. Sweet Cherry), which occupy distinct phylogenetic positions within the eudicots (Figure 2A). Carpels were sampled at four developmental stages of the gynoecium in each species. For *A. thaliana* and *E. californica*, carpels were isolated by LMD as described in Kivivirta et al. (2019); for *S. lycopersicum*, LMD was not applicable due to tissue incompatibility, so carpels were dissected manually under a stereomicroscope at equivalent stages (Brukhin et al., 2003). In all cases, at least four independent biological replicates were collected per stage.

For simplicity, the four developmental stages are referred to as S1-S4 throughout this

study, with early stages of carpel development corresponding to S1 and S2, and late stages of carpel development to S3 and S4. The correspondence between these stages and previously described floral stages and morphological landmarks is summarized in Table 2.

Table 2. Correlation of the four developmental stages of species used in this study with floral developmental stages described elsewhere. Staging for *A. thaliana* and *E. californica* is based on Kivivirta et al. (2019).

Species	Stage 1: carpel initiation	Stage 2: elongation of carpel walls	Stage 3: during meiosis	Stage 4: after meiosis	Reference
A. thaliana	0.3 mm BL; stage 5, sepals enclose bud	0.4 mm BL; stage 9, petal primordia stalked at base	0.5 mm BL; stage 11, stigmatic papillae appear	0.7-1 mm BL; stage 12, petals level with stamens	(Armstrong & Jones, 2003; Smyth et al., 1990)
E. californica	0.39-0.65 mm BD; carpel initiation	1.65-2.25 mm BD; microspo-rangia initiate	2.3-2.8 mm BD; male meiosis	3.5-5.5 mm BD; female meiosis	(Becker et al., 2005)
S. lycopersicum	~0.6 mm BL; carpel initiation	0.8-1.5 mm BL; carpel wall elongation	2-3.5 mm BL; pre-meiosis	4-6 mm BL; post-meiosis	(Brukhin et al., 2003)

* BD: Bud Diameter; BL: Bud Length.

For *A. thaliana*, we re-assembled the carpel transcriptome using the dataset published by Kivivirta et al. (2021), with the TAIR10 genome as reference. For *E. californica*, we reassembled and re-annotated raw data using the chromosome-level reference genome (haplotype 1 of section 1.2.1). For *S. lycopersicum*, raw data were assembled and annotated against the ITAG3.2 reference genome (Tomato Genome Consortium, 2012, ITAG 3.2). The carpel transcriptome datasets of *E. californica* and *S. lycopersicum* are deposited in JLUpub, with doi: https://doi.org/10.22029/jlupub-19864.

4.2.2 OrthoFinder calculation and expression profiling

We identified groups of orthologs (Orthogroups, OGs) across the three species using OrthoFinder v2.5.5 (Emms & Kelly, 2019). For each species, we prepared the complete set of predicted protein sequences (.fasta). OrthoFinder inferred OGs by reconstructing phylogeny for each group and reconciling them with the species tree, thereby producing a hierarchical orthogroup classification.

By definition, an OG is the set of genes from multiple species that descended from a single gene in the MRCA of those species. Thus, each OG represents an extension of the orthology concept from pairwise to multispecies comparisons. OrthoFinder infers these relationships by grouping genes that most likely share common ancestry (Emms & Kelly, 2019). While this automated approach cannot fully substitute for individually detailed gene phylogenetic reconstruction, it offers an approximation of orthologous relationships and allows the identification of single- and multi-copy OGs, thereby capturing gene duplication patterns among the analyzed species. Each OG is assigned a unique identifier (OG_ID) to serve as the reference index for linking gene expression across species in subsequent analyses.

4.2.3 Clustering and gene regulatory network inference

All analyses and visualizations of this section were performed in R 4.4.3, unless otherwise stated. All scripts are deposited on GitHub (Appendix). First, we compared OGs to establish a baseline for shared and lineage-specific gene sets. Using the ggVennDiagram package (Gao et al., 2024), we generated Venn plots to illustrate overlaps and species-specific components of the OGs obtained in section 4.2.2 across the three species. Due to our interest in carpel regulators, we extracted OGs containing TFs that were previously shown to be involved in gynoecium development of *A. thaliana*. The list of TFs was compiled from published studies (Herrera-Ubaldo et al., 2023, Supplementary Table 1). It should be noted that because we used carpel TFs of *A. thaliana* as an index for filtering OGs, this framework is inherently Arabidopsis biased. Next, we mapped these TFs to their corresponding OGs and visualized their conservation across species.

Principal Component Analysis (PCA) was used as an initial quality control and to summarize transcriptome variation across developmental stages and species. For each species, we kept genes with TPM > 1 in at least one sample and non-zero variance. The raw TPM was $\log_2(\text{TPM} + 1)$ and Z-score normalized across samples. Principal components (PCs), representing orthogonal linear combinations of the original expression variables that capture the major sources of variance in the dataset, were computed using the prcomp() function and visualized with the ggplot2 package (Wickham, 2016). For the interspecies

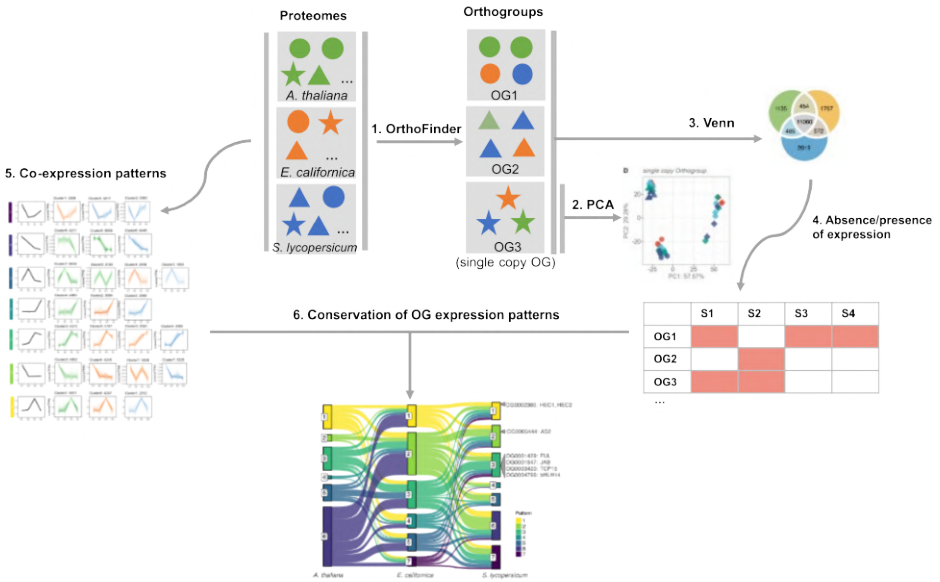

Figure 5. Brief workflow of clustering and gene regulatory network inference. The OG calculations follow Emms and Kelly (2019).

PCA, we used scaled expression of single-copy OGs shared by all three species, identified with OrthoFinder and processed with a custom pipeline in R. The variance explained by PC1 and PC2 was extracted for visualization.

For expression analyses, we evaluated transcriptomics at the OG level first rather than at the level of single genes. This step checked whether an OG showed transcriptional activity at a given developmental stage. This is because OGs can contain multiple paralogs, which have partially redundant or overlapping functions. This means that assessing their collective activity can show whether the gene family as a whole is expressed, even if there are differences in the number of copies in different species. Then, we defined OG expression status as binary (0/1) to distinguish between presence and absence of expression. We considered an OG to be expressed at a given stage if at least one of its genes had a TPM >1 in all replicates at that stage. Otherwise, we assigned the OG as showing an absence of expression. Based on this, we constructed stage-specific presence/absence matrices of OG expression, which allowed us to identify shared and lineage-specific OG expression across species. To visualize the overlap in OG expression among species at each stage, we also generated Venn diagrams using ggVennDiagram.

Next, we analyzed dynamic patterns of OG expression with the TCseq package (Wu & Gu, 2025). For each species, we averaged biological replicates within each of the four de-

velopmental stages to build an OG × stage matrix. We then applied the same normalization as used for the PCA.

The OGs for each species are independently clustered using the C-means soft clustering algorithm (algo = "cm") from TCseq, with membership scores ranging from 0 to 1. We evaluated the optimal number of clusters, setting $k = 8$ a priori based on within-cluster variance and profile interpretability. For subsequent steps, we retained OGs with a membership score of > 0.7. For the interspecies comparison, OGs were used as the index. We computed Pearson correlation coefficients (r) between cluster centroids (the mean z-scored trajectories across the four stages) from different species, then hierarchically clustered the resulting correlation matrix to define the expression patterns across species. We calculated the Pearson correlation coefficient between all cluster centroids. Then, we performed hierarchical clustering on the resulting correlation matrix (Figure 10, Figure 11A). By cutting the dendrogram at a defined height, seven expression patterns were obtained, each of which was represented by clusters from two or three species. Visualization of co-expression clusters and patterns was also performed using ggplot2 (Wickham, 2016).

To visualize the conservation and divergence of OG expression patterns across species, we used Sankey plots implemented with the ggalluvial package (Brunson & Read, 2023). The Sankey plot is particularly suitable as it can capture the "flow" of OGs across species, enabling us to track shifts or conservation in expression patterns. First, we constructed a long-format dataset by combining co-expression cluster assignments with the seven cross-species expression patterns. For each OG, we recorded the assigned pattern in *A. thaliana*, *E. californica*, and *S. lycopersicum*. Only OGs with an assignment in all three species were retained. We treated each OG as a trajectory moving from one species to another and counted the number of OGs for each At-Ec-Sl combination. These counts were then used as flow weights in the Sankey plots so that the width of each ribbon directly reflected the number of shared OGs transitioning between patterns.

4.3 Results

4.3.1 Principal component analysis

We performed PCA for each species and for the conserved single-copy OGs shared across species (Figure 6D) to examine the transcriptomic variation.

In *A. thaliana* (Figure 6A), PC1 explained 22.39% of the total variation and clearly separated early (S1, S2) and late stages (S3, S4) of gynoecium development. PC2 accounted for an additional 11.04%, mainly explaining finer-scale differences among replicates and

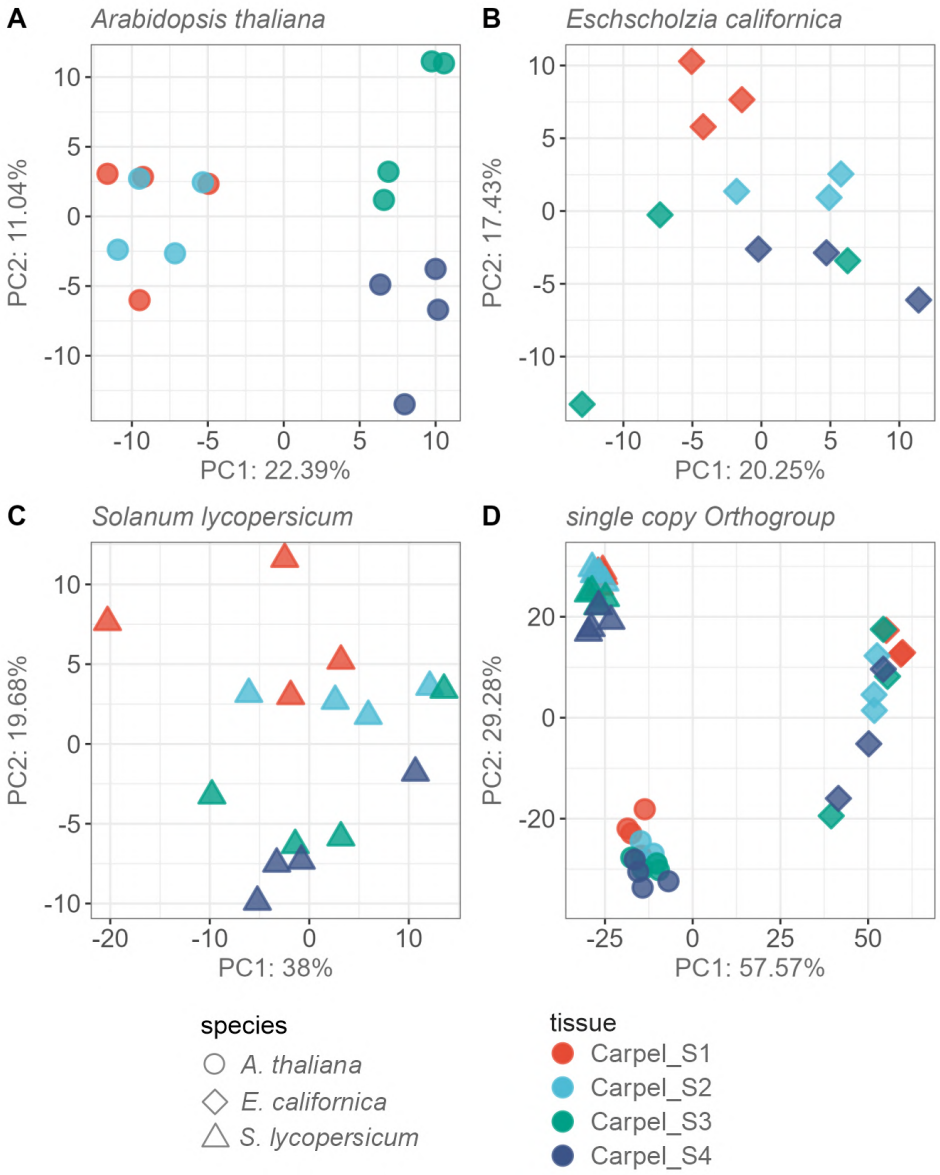

Figure 6. PCA of carpel transcriptomes **A–C)** PCAs of *A. thaliana*, *E. californica*, and *S. lycopersicum* based on species-specific expression profiles. **D)** PCA of single-copy OGs across three species. Every dot shows a biological replicate; shapes indicate species and colors correspond to developmental stages (Carpel stage1-stage4, abbreviated as Carpel_S1 to Carpel_S4).

partially separating S3 from S4. Although the replicates generally clustered by stage, some overlap was observed between early stages (S1, S2), likely reflecting a rapid developmental transition and a narrower expression window during early gynoecium development. In *E. californica* (Figure 6B), PC1 and PC2 explained 20.25% and 17.43% of the variation, respectively. PC2 mainly separated the early stages (S1, S2) from the later stages (S3 and S4). In *S. lycopersicum* (Figure 6C), PC1 explained 38% of the total variance but did not clearly separate developmental stages. Instead, samples from different stages were distributed along both axes. PC2, which explained 19.68% of the variance, more effectively separated early (S1, S2) and late (S3, S4) stages, highlighting transcriptional changes occurring along PC2.

Next, we focused on single-copy OGs that were conserved across all three species. Single-copy OGs represent orthologs inherited from the MRCA and are less likely to have experienced lineage-specific changes in copy number. Although they cover only a small portion of the transcriptome, they are more likely to retain ancestral roles and shared regulatory functions (Mantica & Irimia, 2025; Tegenfeldt et al., 2025). PC1 explained 57.57% of the variance, mainly separating samples by species and suggesting strong evolutionary divergence in expression profiles. PC2 explained 29.28% of the variance and slightly separated early and late stages, showing that developmental differences are still explained despite lineage divergence.

Overall, the PCAs indicate that developmental stage is the major source of variation within each species. Restricting the analysis to conserved single-copy OGs makes species differences more apparent, though stage-specific patterns remain.

4.3.2 Core carpel regulators common to eudicots

Across the union of the three proteomes, we recovered 17,592 OGs (Figure 7A). Of these, 11,060 are shared by all three species (62.9%), forming a large conserved core. Pairwise OGs are comparatively few (454/485/672; 2.6 –3.8%), while each species retains a unique set (*A. thaliana* 1,135; 6.5%; *E. californica* 1,767; 10.0%; *S. lycopersicum* 2,019; 11.5%). This distribution points to a broadly shared backbone with room for lineage-specific innovation through duplication and loss. The OrthoFinder outputs were post-analyzed with custom scripts to assemble the Venn sets, and to extract the single-copy OGs shared by all three species that underlie the interspecies PCA and carpel regulator analysis.

As described before, we use carpel regulatory TFs of *A. thaliana* as an index for filtering OGs, and this framework is inherently Arabidopsis biased. As a result, genes without orthologs in Arabidopsis may be underestimated in the considered OGs.

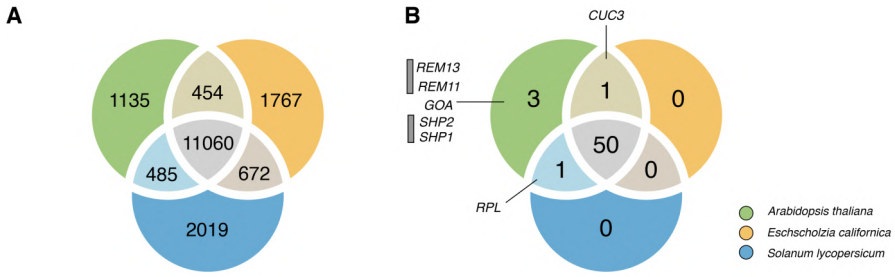

Figure 7. Comparisons of OGs in the genomes of *A. thaliana*, *E. californica*, and *S. lycopersicum*. **A)** Genome-wide distribution of predicted OGs shared among three species; **B)** OGs that contain known *A. thaliana* carpel regulators, collected from Herrera-Ubaldo et al. (2023). The gray line next to the gene name indicates that the two belong to the same OG.

Next, we mapped the published Arabidopsis carpel regulatory TFs (eighty-two TFs in total, Supplementary Table 1) to fifty-five OGs (Figure 7B); sixty-five of these TFs are assigned to forty-seven OGs with reported phenotypes, which are listed in Supplementary Table 2. When these OGs were mapped across the three species, most were found to be conserved: fifty out of fifty-five (91%) were present in all three species (Figure 7B). This conserved set covers the main regulators of carpel development, including regulators involved in meristem identity and determinacy (AG, SEP3, FUL, WUS, and WOX13) as well as regulators of auxin-mediated patterning (ETT, ARF8, MP, NGA1/2/3, STY/SRS, SPT/ALC, IND), components of the ARR family , and regulators involved in polarity and boundary specification (KAN, YAB3/FIL, and CUC1/2). Other well-known regulators, such as SEU, LUG, and TCP15, are also part of this conserved core (Supplementary Table 2).

Only five OGs fall outside of the conserved set. Three of them, GORDITA (GOA), REPRODUCTIVE MERISTEM 11 (REM11), and REPRODUCTIVE MERISTEM 13 (REM13), are found only in Arabidopsis, while two are missing from one species each (CUC3 from *S. lycopersicum* and RPL from *E. californica*). In contrast, the remaining fifty OGs are present in all three species. Despite differences in genome size, annotation quality, and evolutionary history, this broad conservation points to a stable regulatory core for carpel development in eudicots.

4.3.3 Core carpel regulators are conserved across eudicots

After establishing a core set of fifty carpel regulators, we asked whether their expression is conserved during gynoecium development across eudicots. To investigate this, we examined the overlap of expressed OGs across species at four developmental stages (Figure 8).

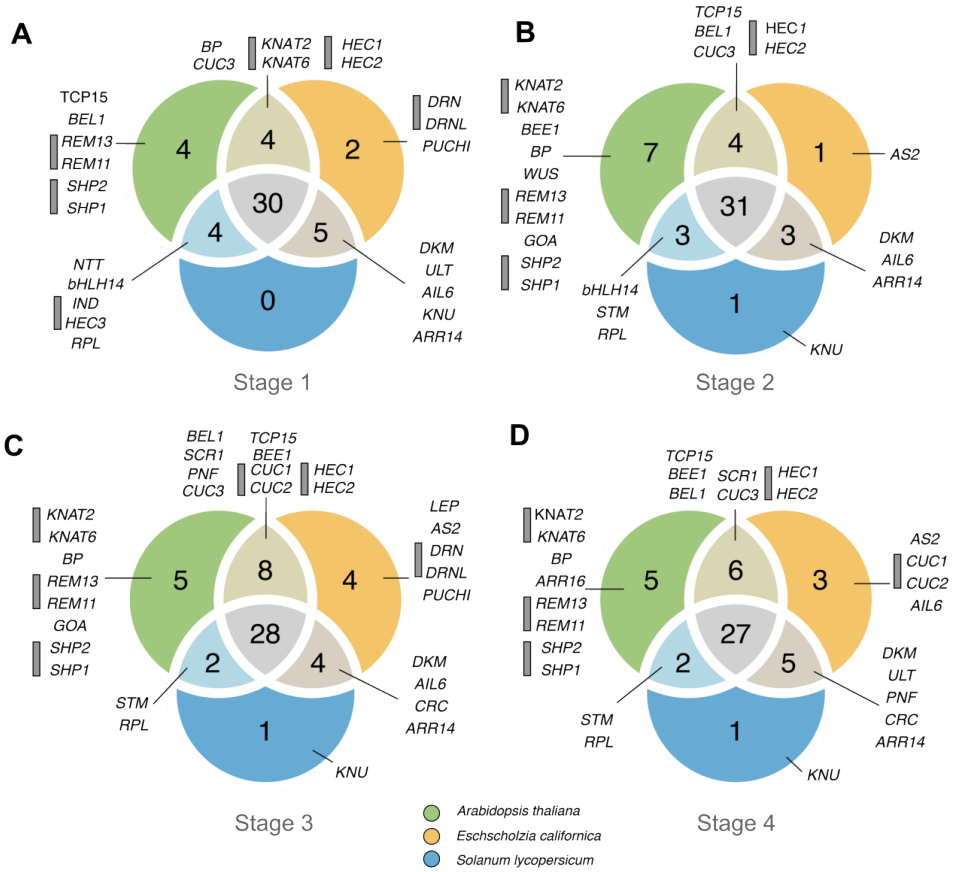

Figure 8. Comparison of the expression absence/presence of OGs in *A. thaliana*, *E. californica*, and *S. lycopersicum*. **A-D)** Presence or absence of expressed orthogroup members in gynoecium samples across four developmental stages. The gray line next to the gene name indicates that the two belong to the same OG.

The results revealed a stable transcriptional backbone. In the early stages (Figure 8A,B), thirty and thirty-one OGs are expressed in all three species, representing ca. 65% of the core set. A similar pattern was observed at later stages (Figure 8C,D), with twenty-eight and twenty-seven OGs shared, respectively. Thus, at every stage of development, between 60% and 70% of the regulators are co-expressed across species.

Although Venn diagrams provide an overview of the number of expressed OGs shared among species and stages, they fail to capture the dynamics of individual OGs. To address this, we extracted the expression matrix of these fifty-five OGs (see methods in section 4.2.3) and summarized them in a table showing dynamic expression (Supplemental Table 3). This confirmed that the majority of regulators were expressed throughout development but also revealed a small subset with stage-specific expression. For instance, *LEP* was only detected at stage 3; *ARR16* appeared exclusively at stage 4; and *WUS* and *PUCHI* were confined to early stages (Karim et al., 2009; Lohmann et al., 2001). These temporally restricted profiles align with the known functions of these genes and demonstrate that, in addition to the transcriptomic core, a smaller group of regulators acts independently. By contrast, lineage-specific expression was comparatively rare. Depending on the stage, only one to seven OGs were unique to a single species, while two to five OGs were shared between pairs. These cases may imply regulatory divergence, including subfunctionalization or neofunctionalization following duplication (Duarte et al., 2006). At present, however, their roles cannot be confirmed, as functional evidence is lacking and stable transformation systems are not yet available for *E. californica*.

4.3.4 Orthogroup members exhibit species-specific expression patterns

Although most carpel regulators are expressed across species and throughout development (see section 4.3.3, Supplementary Table 3), the timing and intensity of their expression can differ. Firstly, we calculated the co-expressed clusters of all expressed genes across the four stages in the three species, and then we investigated the distribution of core carpel regulators in these clusters. In each species, eight clusters were obtained (Figure 9). In *A. thaliana*, cluster 6 was the largest group with 8,969 genes (ca. 21% of all clustered genes), which showed a broad downregulation from early to late stages. In contrast, cluster 3, which showed a strong upregulation from early to late stages, contained only 4,239 genes (ca. 10%). Clusters 4 and 8 together accounted for 9,100 genes (ca. 20%) and showed very different expression patterns. Cluster 4 shows a significant upregulation from Stage 3, peaking at Stage 4. In contrast, Cluster 8 shows a significant downregulation at stage 3, with the lowest expression at Stage 4. These clusters likely represent transcriptional events linked to tissue differentiation and fruit morphology establishment, marking the

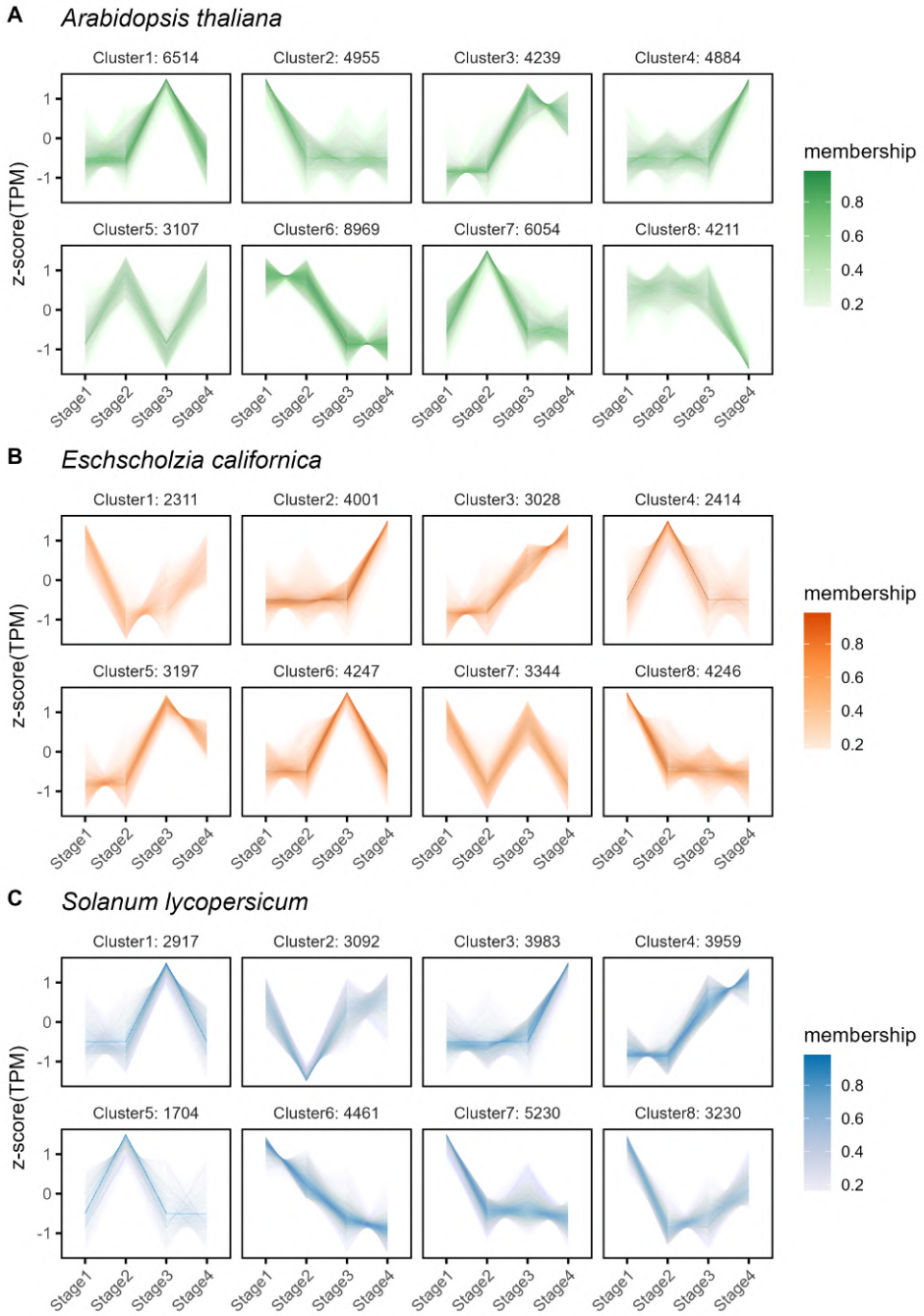

Figure 9. Co-expression clusters identified by TCseq in the three species. Cluster membership strength is illustrated by the scale bar (0-1), with darker shades indicating higher assignment to a given cluster.

transition from early meristematic regulation to extensive growth and maturation. In *E. californica*, cluster sizes were more evenly distributed than in Arabidopsis. Clusters 2, 6, and 8 each contained around 4,000 genes (15–16% of the total), with expression peaking in middle to late stages. By contrast, cluster 1 and cluster 4 were comparatively smaller, with 2,300–2,400 genes (9%). Unlike Arabidopsis, most *E. californica* clusters (Cluster3, 5, 6, 7) exhibited their strongest upregulation around stage 3.

In *S. lycopersicum*, several clusters showed broadly similar expression patterns but differed in cluster size, which might result from parameter settings, as the same cluster number (k = 8) was used in TCseq for all species to ensure comparability.

Clusters 3 and 4 both exhibited a gradual increase in expression from stage 2 onwards, with expression peaking at stage 4, representing late-activated transcriptional events in carpel development. Together, these clusters accounted for nearly 8,000 genes (ca. 27%). Clusters 6, 7, and 8 followed a related pattern. Cluster 6 contained 4,461 genes (ca. 15%), which are downregulated throughout. Cluster 7 contained the largest number of genes (ca. 18%), which are downregulated consistently from stage 1 and showed flat expression at stages 2–4. Clusters 7 and 8 differ in that transcriptional events are reactivated at stages 3 and 4, which may be related to the development of the final fruit morphology. While the gene quantity differs between clusters in *A. thaliana*, *E. californica*, and *S. lycopersicum*, we checked whether the full set of clusters could be grouped into broader expression patterns across species. The heatmap shows the correlation among clusters (Figure 10), and the corresponding profiles show the mean trajectories of the seven patterns across the four stages of gynoecium developmentFigure 11A.

As shown in Figure 11A, an interesting question is whether individual orthogroups remain in the same expression pattern across lineages or shift between them. To address this, we visualized OG membership across species using a Sankey diagram (Figure 11B), which shows that only a small group of OGs are assigned to the same pattern across species, while most are distributed across different patterns. Among the conserved cases, OG0002360 (HEC1/2) was placed in a mid-stage upregulation pattern in all three species, consistent with studies in *A. thaliana* showing that *HEC1* and *HEC2* act during style and transmitting tract formation (Gremski et al., 2007). Additionally, it aligns with recent reports on partially conserved HEC-SPT interactions in *S. lycopersicum*, although these studies lacked expression data (Martínez-Estrada et al., 2025). OG0001429 (FUL) remained in a late-expression pattern across all three species. In Arabidopsis, *FUL* regulates valve identity and fruit elongation (Ferrándiz et al., 2000). In *E. californica*, its homologs, *EscaFUL 1/2*, exhibit a similar expression pattern, demonstrating robust expression in developing carpel walls and ovules, as well as sustained activity during fruit wall development (Pabón-Mora et al., 2012). In *S. lycopersicum*, the *FUL* homologs *SlFUL 1/2* are also expressed during fruit development, aligned with *A. thaliana* (Bemer et al., 2012; Jiang et al., 2025). OG0001947 (JAIBA, JAB) showed stable assignment to an early-mid upregulation, reflecting its role

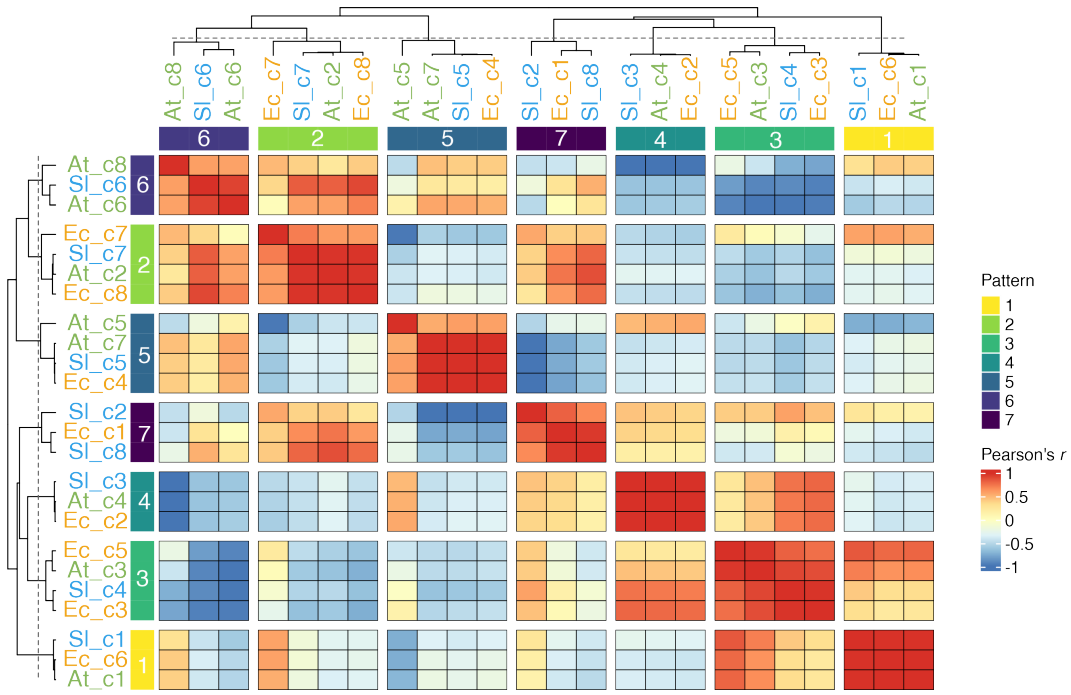

Figure 10. Correlation heatmap of co-expression clusters from carpel transcriptomes of *A. thaliana*, *E. californica*, and *S. lycopersicum*. Rows and columns represent abbreviated cluster IDs (e.g., Cluster 3 from *A. thaliana* as At_c3). Pearson correlation coefficients (*r*) between all clusters are visualized by a gradient color bar. Hierarchical clustering groups clusters with similar expression patterns, and the resulting groups are indicated by colored bars on the top and left, corresponding to predicted seven expression patterns (1–7).

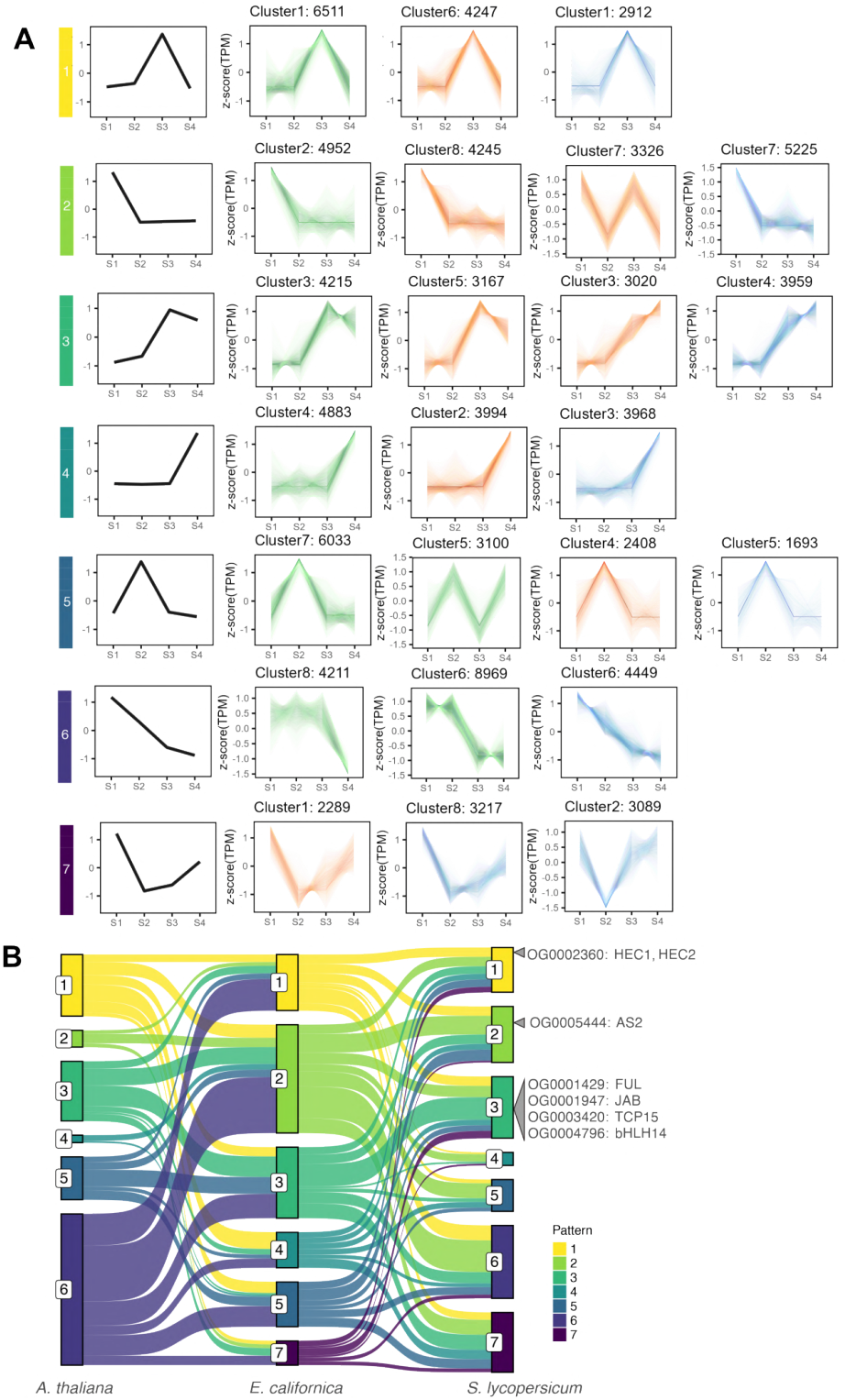

Figure 11. Expression patterns of carpel orthogroups across three species. **A)** Species-specific clusters grouped by expression pattern. Each row represents one of the seven major expression patterns. The left black line plot shows the average trajectory of each pattern, while the right panels display the original TCseq clusters of the studied species that contribute to the respective pattern. **B)** A Sankey plot showing conservation and divergence of carpel OG expression dynamics across the three species. Numbered blocks represent the seven expression patterns defined in **A)**. Ribbons connect shared carpel OGs between species, with ribbon width proportional to the number of shared OGs. Selected carpel OGs that retain the same expression pattern in all three species are labelled on the right.

in floral meristem determinacy and medial tissue development in *A. thaliana* (Zúñiga-Mayo et al., 2012), although orthologs in *E. californica* or *S. lycopersicum* have not yet been reported. Similarly, OG0005444 (AS2) exhibited a consistent expression pattern across species, which is consistent with its known role in regulating adaxial-abaxial polarity during early gynoecium development (Iwakawa et al., 2007).

By contrast, the majority of OGs did not remain in the same pattern but shifted between species, with *A. thaliana* clusters often mapping to different patterns in *E. californica* or *S. lycopersicum*. The Sankey diagram also highlights differences in how expression patterns are distributed among species (Figure 11B). In *A. thaliana*, half of the OGs are assigned to pattern 6, which indicates a dominance of broadly downregulated profiles during carpel development. By contrast, the majority of OGs in *E. californica* fall into patterns 1, 2, and 3. Patterns 1 and 3 show broad upregulation, while pattern 2 shows downregulation. The largest set of OGs in *S. lycopersicum* is found in patterns 6 and 7, which correspond to downregulation from early to late stages. There are comparatively fewer genes in early-upregulated pattern 4. These differences demonstrate that, although the patterns are present in all three species, their relative contributions differ considerably.

4.3.5 conservation of carpel gene regulatory networks

To probe the conservation of carpel gene regulatory networks, we focused on the NGA-HEC pathway that regulates stigma development. In Arabidopsis, *NGA* acts together with *HECs* to promote *IND*, which then activates *SPT* and downstream regulators such as *PID* and *WAG2* (Figure 12A). Expression profiles showed that *NGA* orthologs are consistently expressed at late stages in all three species (Figure 12B-D), following highly similar trajectories. In situ hybridization confirmed that *EcNGA* and *NGAs* share expression domains. Functional evidence further demonstrated their necessity. VIGS-treated inactivation of *E. californica* and *Nicotiana benthamiana* resulted in plants with phenotypes that in severe defects in style and stigma formation that directly paralleled the phenotypes observed in Arabidopsis (Fourquin & Ferrándiz, 2014, Figure 12E). These data show that *NGAs* play an essential and conserved role in stigma and style specification in eudicots.

In contrast to the conserved *NGA* expression, the downstream part of the stigma regulatory network showed lineage-specific losses. In *E. californica*, no clear link between *EcNGA/EcHEC* and *EcSPT* could be established. Similarly, in *S. lycopersicum*, the connection to *IND* and *SPT* appeared absent, and the expression of downstream targets such as *SlPID* and *SlWAG2* was strongly reduced. These results suggest that while NGAs are essential and conserved regulators of stigma identity, the network architecture downstream of NGA-HEC has diverged, with parts of the Arabidopsis module missing or replaced in other eudicots.

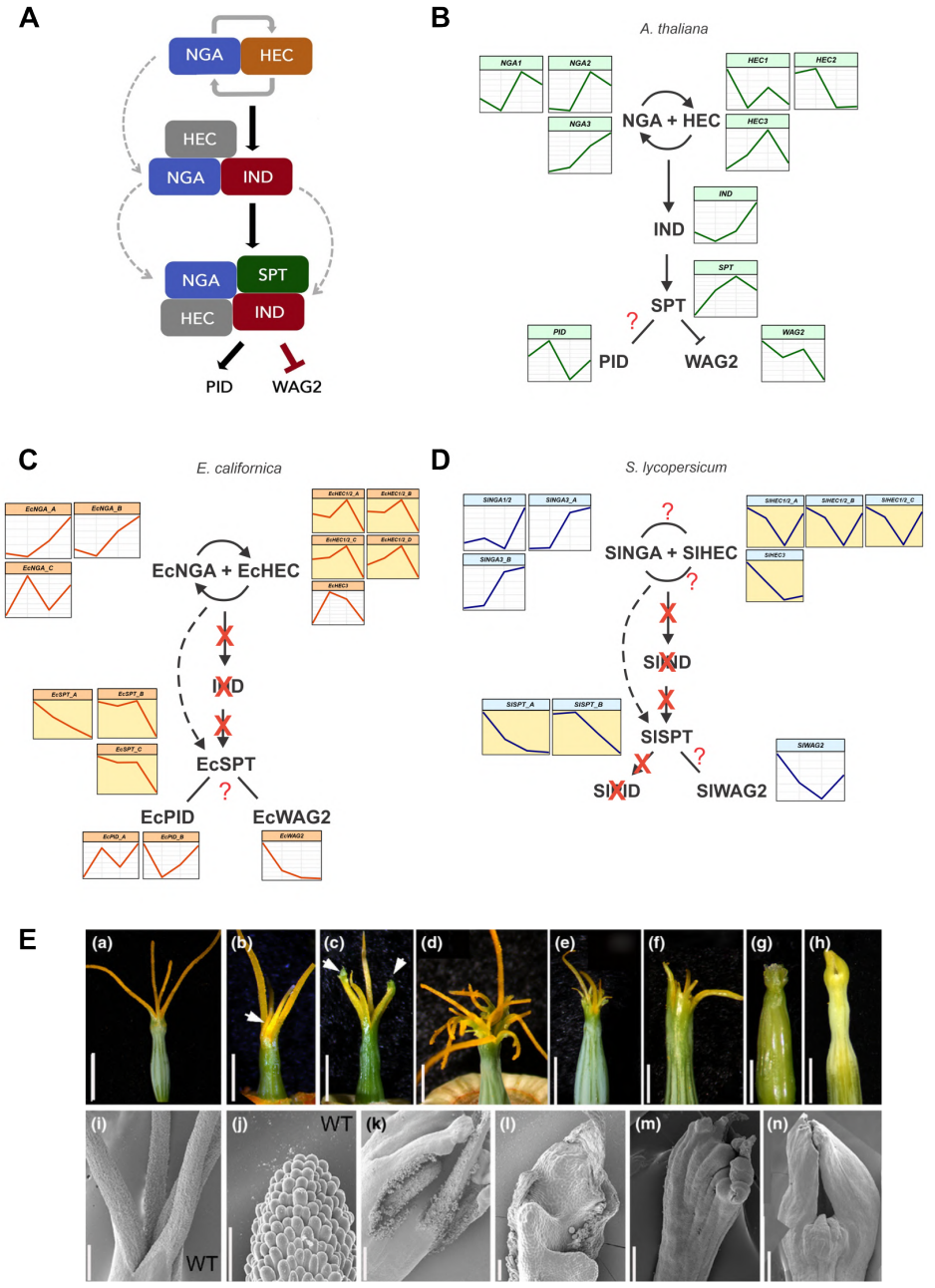

Figure 12. Regulatory model for style and stigma development and interspecies OG expression mapping. **A)** Sequential activation model in *A. thaliana*: NGA and HEC co-express and activate IND; IND with NGA activates SPT; the complex then regulates PID and WAG2 (Ballester et al., 2021). **B-D)** OG expression mapped onto the pathway in **A)** for *A. thaliana* **B)**, *E. californica* **C)**, and *S. lycopersicum* **A)**. Red X indicates that the ortholog and thus the corresponding pathway step is absent. Red question marks indicate uncertain activating/repressing relationships. The small windows next to each regulator show the expression trajectory of its OG members across stages; yellow background highlights a pattern different from Arabidopsis. **E)** Functional analysis of *EcNGA* by VIGS (Fourquin & Ferrándiz, 2014). (a–h) stigma at anthesis showing a gradient of phenotypes from weak (extra or uneven stigmatic protrusions) to intermediate (irregular protrusion distribution or enlarged style) to strong (complete loss of stigmatic tissue). (i–n) SEM images of apical regions. WT stigmatic protrusions are fully covered by papillae (i, j), while *EcNGA*-silenced pistils show partial coverage (k), absence of stigmatic tissue (l), or complete loss of papillae (m, n).

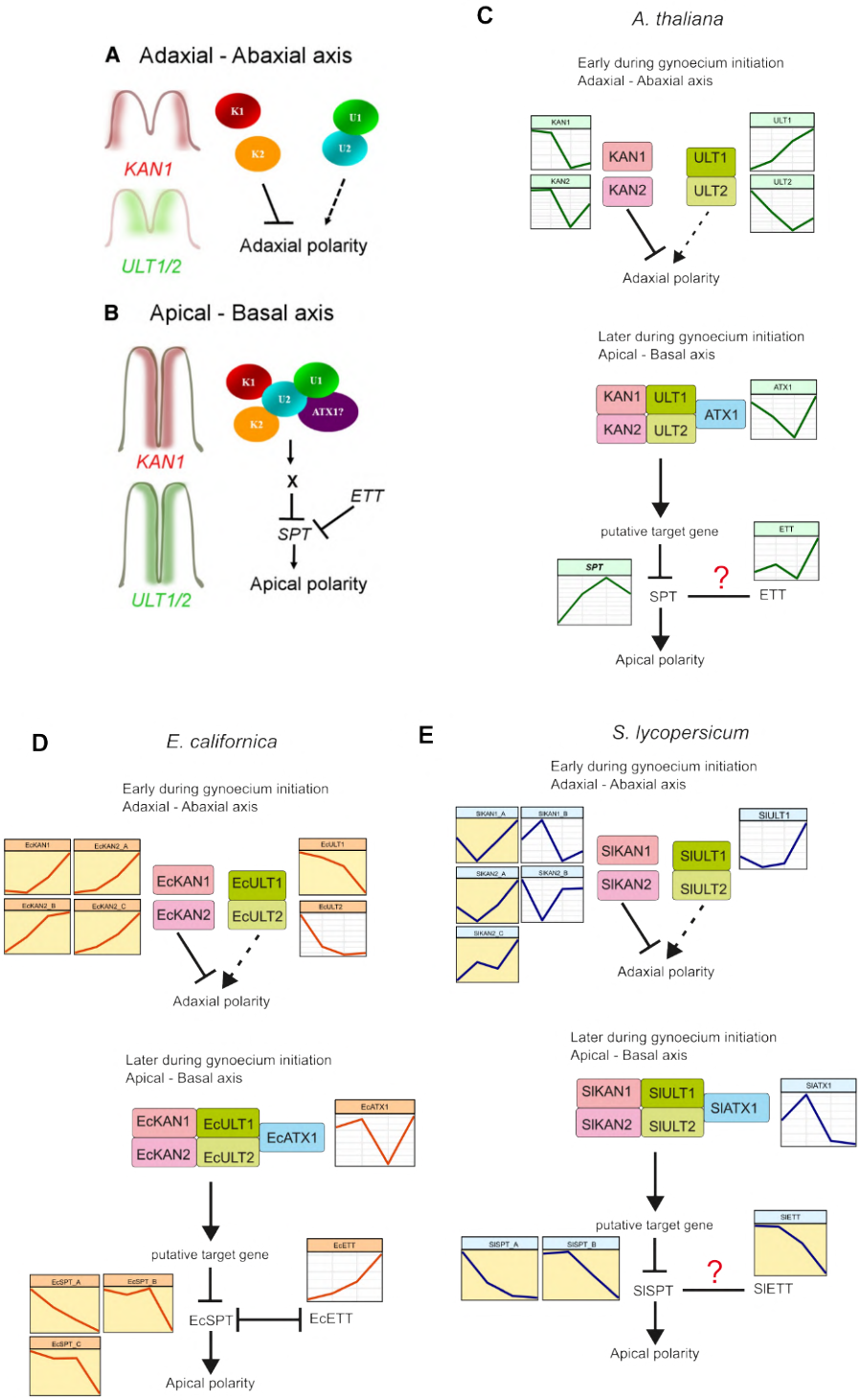

Figure 13. Regulatory model for gynoecium polarity in Arabidopsis and interspecies OG expression mapping.**A)** During early gynoecium initiation, *ULT1/2* and *KAN1/2* are expressed in complementary domains and act antagonistically to establish the adaxial–abaxial polarity, with *KAN* conferring abaxial and *ULT* conferring adaxial identity (Pires et al., 2014). **B)** Later, *ULT1/2* and *KAN1* are co-expressed, and their proteins likely act together to pattern the apical–basal axis. This complex may activate downstream targets that repress *SPT* expression in the basal abaxial region via a pathway independent of *ETT* (Pires et al., 2014). **C–E)** OG expression mapped onto the pathways in **A-B)** for *A. thaliana*; **C)**, *E. californica*; **D)**, and *S. lycopersicum*; **E)** Red X indicates that the ortholog and thus the corresponding pathway step is absent. Red question marks indicate uncertain activating/repressing relationships.

Unlike the NGA pathway, the carpel polarity network did not show clear conservation in eudicots (Figure 13). In *A. thaliana*, carpel polarity is established by *KAN* and *ULT*, which provide input to *ETT* and *SPT* (Pires et al., 2014, Figure 13A, B). However, when comparing *E. californica* and *S. lycopersicum*, although orthologs of *KAN*, *ULT*, and *ETT* are present, their temporal expression profiles differ (Figure 13B-D). In *S. lycopersicum*, the *SlKAN*s and *SlULT*s are downregulated early but rapidly upregulated from stage 2 and do not clearly co-express with *SlETT*. In *E. californica*, *EcKAN*s and *EcULT*s are expressed at different stages, and *EcSPT*s are expressed oppositely to those in Arabidopsis. These results suggest that, although polarity regulation is an essential feature of carpel morphogenesis, the timing and network structure are not highly conserved, indicating that lineages have adopted distinct solutions for establishing the adaxial-abaxial and apical-basal axes of the gynoecium.

4.4 Discussion

In this chapter, we built a comparative framework for carpel development in three eudicots using stage-specific transcriptomes. At the genome level, most carpel regulators are present across species, indicating that most carpel developmental programs are inherited from the MRCA. Comparatively, only a few sets of these OGs show conserved temporal expression patterns. This suggests that while the regulatory toolkit is largely shared, its expression during development can differ. We defined seven expression patterns that reflect differences in the transcriptional programs of the three species. In *A. thaliana*, large gene sets were rapidly downregulated; in *S. lycopersicum*, dominant clusters showed late and sustained activity; and in *E. californica*, clusters were more evenly distributed across stages (Figure 9B).

The regulatory network comparison illustrates the same pattern. The NGA pathway shows strong conservation in expression, as *NGA*s are expressed with similar timing across species and knock-down experiments in *E. californica* confirmed that the function is conserved. These results suggest that NGAs are essential carpel regulators in eudicots. In contrast, the polarity regulatory pathway shows divergent expression patterns, suggesting that even though polarity regulation is essential, it is not implemented through a conserved transcriptional program.

Although we obtained continuous transcriptomes from carpels, the resolution can only go so far with bulk LMD RNA-seq. Finer-scale differences within cell populations are likely not covered. scRNA-seq could in principle resolve these populations, but its application in gynoecia is technically constrained by the lack of marker lines and the low abundance of relevant cell types. In addition, transcript levels alone do not fully capture regulatory activity, as proteins can persist well beyond their mRNA expression (Kivivirta et al., 2021). This is especially relevant for regulators like HEC, which are expressed at low transcript levels but cause striking phenotypes when mutated (Gremski et al., 2007; Schuster et al., 2015). Finally, our analysis is biased towards Arabidopsis, since OGs of TFs are defined using Arabidopsis references (Herrera-Ubaldo et al., 2023). This bias may underestimate divergent orthologs in *E. californica* or *S. lycopersicum*, and thus represents a limitation to consider in the interpretation of the results.

Despite these limitations, the results presented here provide a valuable resource by allowing us to identify core regulators from lineage-specific parts of the GRNs of carpel development. Importantly, OGs in which individual members show different expression patterns provide promising candidates for future functional analysis.

4.5 Contributions to other publications

Besides the two first-author publications included as Chapter 3 of this thesis and the 3rd manuscript currently in preparation (Chapter 4), I also contributed to other publications. Below, I summarize the main findings of these studies and my contributions.

1. Lotz, D., Roessner, L. H., Ehlers, K., **Kong, D.**, Roessner, C., Rupp, O., & Becker, A. (2024). Conservation of the dehiscence zone gene regulatory network in dicots and the role of the *SEEDSTICK* ortholog of California poppy (*Eschscholzia californica*) in fruit development. *EvoDevo*, 15(1), 16.

This paper studied the evolution of fruit development by comparing dry dehiscent fruits of *A. thaliana* and *E. californica*. Transcriptome profiling of valve and replum-like tissues in *E. californica* identified the STK ortholog as a candidate regulator of dehiscence. Expression analyses in legumes further supported a role of STK orthologs in fruit development, and functional assays using VIGS in *E. californica* demonstrated premature fruit rupture, revealing both conserved functions in seed filling and seed coat formation as well as a novel role in restricting valve cell proliferation. These findings show that, despite morphological similarities, the gene regulatory network of Arabidopsis differs significantly from that in other dicots, where STK has been recruited into a pathway ensuring capsule stability and seed dispersal. My contribution to this work is in data visualization, including the preparation of heat maps, phenotypic analyses, and qPCR expression profiles.

2. Rössner, L. H., Rössner, C., **Kong, D.**, Lotz, D., Weisert, A., ... & Becker, A. (2026). Gene and genome duplications have contrasting impacts on biosynthetic and flower developmental pathways in California poppy. *The Plant Cell*, koag039

This study provides a haplotype-resolved genome assembly and transcriptome atlas of *E. californica*, revealing extensive lineage-specific expansions of benzylisoquinoline alkaloid (BIA) biosynthesis genes and contrasting evolutionary trajectories of other pathways such as carotenoid biosynthesis and floral regulators. My contributions included the analysis and visualization of Weighted Gene Co-Expression Network Analysis (WGCNA), visualization of evolutionary trajectories of BIA, carotenoid, and floral homeotic MADS-box genes by summarizing their duplication history and expression divergence, and the preparation of a Venn diagram of hierarchical OGs across five species.

5 Conclusion and Discussion

Briefly, this thesis integrates morphological and transcriptomic perspectives to study the evolution of floral organs in eudicots. The first part first investigates the types and distribution of ring meristems across eudicots. It then describes the floral morphogenesis of *P. racemosum* (Papaveraceae, Ranunculales) and proposes that its floral morphology reflects ancestral traits of Papaveraceae. The second part integrates cross-species transcriptomic analyses, revealing that, despite the remarkable morphological diversity of floral structures such as stamens and carpels, the genomic toolkit governing carpel development remains largely conserved. Transcriptome comparisons indicate that temporal expression of these regulators is often flexible and lineage-specific. We inferred known carpel modules, revealing the presence of a mixture of conserved modules (e.g., NGA is a key component for stigma and style development.) and highly differentiated modules (e.g., polarity regulators). These results suggest that carpel formaton arises from the interplay between conserved genetic components and flexible regulatory mechanisms.

5.1 Morphological diversity of floral organs

Angiosperm flowers are typically composed of four basic organ types: sepals, petals, stamens, and carpels. While this organ set can be recognized across most flowering plants, the number, arrangement, and degree of differentiation of each organ type vary widely among lineages (Endress, 2008, 2011).

5.1.1 Perianth

The perianth is the collective term for the sepals and petals of a flower (Endress, 2008), shows big morphological diversity across angiosperms. In the common model, sepals of the outer whorl protect the floral bud, while petals of the inner whorl provide attraction in the open flower. However, both whorls display a wide range of forms and functions, and many deviations from this pattern exist (Endress, 2010; Ronse De Craene, 2008). In eudicots, petals often show delayed development, with major growth occurring only shortly before anthesis (Endress, 2008). This strategy minimizes bud size and contrasts with the ANA

grade and magnoliids, in which perianth organs are structurally less differentiated and are often referred to as tepal (Soltis et al., 2009). In some lineages, however, petals have taken over the protective function in buds, such as in rosids (Gerrath & Posluszny, 1988; Schönenberger & Conti, 2003) and in several asterid clades (Endress, 2010). In many monocots, sepals and petals are less clearly differentiated, and both whorls often function in bud protection (Weber, 1980).

The arrangement of perianth organs in the bud also contributes to diversity. Sepals in eudicots are mostly quincuncial (Endress, 2008), reflecting their spiral initiation sequence, while petals frequently show contort aestivation, especially in rosids and Gentianales (ENDRESS, 2005; Endress, 2006, 2010). In monosymmetric flowers such as Lamiales, sepals and petals are often cochlear. Valvate aestivation occurs more often in protective whorls, commonly sepals, and is widespread in eudicots but rarer in monocots (Endress, 2011; Rudall, 2008). Fusion patterns also vary: in monocots, the six organs of both whorls often act as a coordinated unit and may fuse into a tube, while in eudicots the two whorls behave more independently (Endress, 2011).

Elaboration, reduction, and loss further increase perianth diversity. Petals have evolved, disappeared, and re-evolved multiple times in angiosperms (Brockington et al., 2009). Complete loss of the perianth has occurred repeatedly in basal groups, such as Hydatellaceae (Rudall et al., 2007), Piperales (Tucker et al., 1993), and in several monocot and eudicot clades (Barabé & Lacroix, 2008; Endress, 2011; Vrijdaghs et al., 2010). In some families, sepals have been reduced when petals or bracts took over the protective role, as in Apiaceae, Araliaceae, and Rubiaceae (Endress, 2010). In Asteraceae, sepals are often modified into the pappus, serving dispersal rather than protection (Semple, 2006).

Within Ranunculales, perianth identity ranges from complete absence to diverse combinations of sepals and petals, ranging from absence to undifferentiated tepals, or differentiated petaloid sepals and nectariferous petals (Becker et al., 2024). Flowers of Eupteleaceae are perianthless (Ren et al., 2007), whereas Circaeasteraceae usually have tepals, and in *Kingdonia* these occur with nectariferous petals of staminodial origin (Ren et al., 2004; Tian et al., 2005). Menispermaceae and Lardizabalaceae typically bear persistent petaloid sepals and sometimes nectariferous petals (Endress, 1995). In Papaveraceae, Papaveroideae have caducous sepals and nectarless petals, while Fumarioideae possess spurred, nectariferous petals (Sauquet et al., 2015). In Berberidaceae and Ranunculaceae, flowers can be perianthless or bear petaloid sepals and nectariferous petals (Endress, 1995).

5.1.2 Stamen and gynoecium

The number of stamens is among the most labile traits in the floral ground plan. Reconstructions suggest that the flower of the MRCA of angiosperms likely bore more than ten

stamens, arranged in at least four whorls with three stamens each (Crane et al., 1995). In extant species, diplostemony with two alternating whorls is the predominant arrangement in monocots and core eudicots (Kong & Becker, 2021). By contrast, in the ANA grade, stamen numbers vary widely, from a single stamen in Hydatellaceae and Chloranthaceae to several hundred in Schisandraceae (APG, 2016; Endress, 2011; Kong & Becker, 2021).

A key innovation associated with high stamen numbers is the ring meristem. Instead of a strictly spiral or simple whorled arrangement, a primary androecial ring primordium can form, from which additional whorls of stamens are generated. Stamen primordia may then initiate centripetally, centrifugally, or in both directions (Kong & Becker, 2021). The ring primordium itself may remain closed or fragment into several sectors, each producing stamens. This additional growth zone uncouples stamen initiation from carpel initiation, allowing late-forming stamens to appear after carpel primordia are already advanced in development.

Ring meristems are phylogenetically widespread, occurring in 13 of the 44 eudicot orders (APG, 2016), but their distribution is highly uneven. In Ranunculales, ring meristems are especially common, with all families containing species that display them, and with nearly all combinations of closed versus fragmented architecture and primordia initiation directions represented. By contrast, in other large orders such as Asterales, they are extremely rare and reported only in *Taraxacum* (Asteraceae). In most other lineages, ring meristems are confined to single families, as in Proteales, Fabales, Brassicales, and Myrtales (APG, 2016).

Across eudicots, centripetal initiation is most frequent in non-core groups such as Ranunculales and Proteales, whereas centrifugal initiation predominates in the core eudicots. Bidirectional initiation is rare overall. Interestingly, a closed ring meristem often coincides with centripetal initiation, as seen in Ranunculales, Proteales, Fabales, Hamamelidaceae, and Portulacaceae, whereas Malvales exhibit nearly every possible combination (APG, 2016; Kong & Becker, 2021).

The gynoecium is the central and most complex floral organ system, formed when the floral apex is transformed into a gynoecium primordium. Its structural units are the carpels, which may be ascidiate (cup-shaped), plicate (folded), or a combination of both, with ovules usually arising near the margins. During development, carpels close and enclose the ovules, which defines angiospermy. Closure can occur by secretion or by postgenital fusion of carpel flanks, and comparative evidence suggests that ascidiate carpels and secretion-based closure were ancestral states since they dominate in the ANA grade (Doyle & Endress, 2000; Endress, 2010).

A major evolutionary transition in gynoecium morphology was the origin of syncarpy, where carpels are congenitally fused. Syncarpy enables the formation of a compitum, a shared transmitting tract that connects pollen tubes from different carpels. This allows

even distribution of pollen tubes and centralized pollen tube competition, which represents a key innovation of angiosperms (Armbruster et al., 2002; Endress, 1982). Syncarpy and compitum formation evolved independently in monocots and eudicots (Buzgo & Endress, 2000), and today more than 80% of angiosperm species are syncarpous (Endress, 1982). By contrast, most ANA lineages, magnoliids, and many basal eudicots remain apocarpous. Secondary apocarpy has also arisen in both monocots and eudicots, but in many of these cases, alternative mechanisms such as postgenital fusion of carpel tips or secretion bridges recreate a compitum (Endress, 2010).

Carpel number is also highly variable. In monocots and core eudicots, gynoecia usually have two to five carpels, a stabilization that is likely linked to syncarpy and compitum function (Endress, 2006). In basal lineages, numbers vary more widely, from a single carpel in Hydatellaceae or Berberidaceae to hundreds or thousands in Monimiaceae and Ranunculaceae (Endress, 2010; Lorence, 1985). Extreme reductions can lead to unicarpellate gynoecia, which are frequent in Lauraceae and Fabaceae (Endress, 2011; Tucker et al., 1993).

5.2 Floral morphology and phylogeny of *Pteridophyllum racemosum*

Detailed morphological analysis of *P. racemosum* shows the development of a textbook-like flower, in that we observed little deviation from the canonical flower architecture described for model plants like *A. thaliana*, with four sepals, four petals, six stamens, and a two-carpellate gynoecium. We observed only minor differences: (1) only two sepals emerge from the floral primordium, which cease expansion at an early developmental stage and persist on the flower after abscission of the other floral organs, (2) the four petals are arranged in two whorls that increase in size continuously throughout flower development, (3) four stamens emerge from a single whorl, and (4) only two ovules are formed in the gynoecium.

Ancestral state reconstructions of floral traits are available for the Ranunculales (Carrive et al., 2020). Thus, Papaveraceae ancestral floral traits are in many cases similar to the Ranunculales ancestral traits. The ancestral Papaveraceae flower was reconstructed as follows: whorled, dimerous, disymmetrical perianth with 5-10 organs and three or more whorls. The perianth whorls were unfused, differentiated, with a constant number of organs, and the inner perianth organs were petaloid. The ancestral number of stamens was less than six, and there were two unfused, unilocular carpels in the center. Spurs and nectaries are absent (Carrive et al., 2020; Hoot et al., 2015). Interestingly, the flower of *P.*

racemosum matches with the reconstructed ancestral trait flower in most aspects. However, it deviates from the ancestral Ranunculales flower, which was supposedly actinomorphic, with at least 12 free carpels (Carrive et al., 2020) in that it is dissymmetric with no differentiation between the two planes and has only two but fused carpels. However, as an exception to typical Papaveraceae, *P. racemosum* does not develop crystal-bearing idioblasts or laticifers and does not incorporate calcium oxalate crystals in the inner epidermis of the outer ovule integument (Hoot et al., 2015).

In the most recent Papaveraceae phylogeny, *P. racemosum* is sister to boththe Hypecoideae and the Fumarioideae (Peng et al., 2023). Interestingly, many stages of flower development are similar between *P. racemosum* and the zygomorphic Fumarioideae species *Capnoides sempervirens* (*C. sempervirens*): initiation of all floral organs is disymmetric in both flowers, but six stamens develop, of which four are monothecal and two dithecal in *C. sempervirens* (Damerval et al., 2013). Zygomorphic characters are achieved by unequal growth, organ fusion of stamens, and addition of a nectary spur in *C. sempervirens* later in development (Damerval et al., 2013). Hypecoideae flowers are also disymmetric and with two sepals that are not enclosing the bud. They have four petals arranged in two whorls, four stamens that are arranged differently in that they are opposite to the inner whorl petals, and they develop nectaries. In comparison with the Papaveroideae, *P. racemosum* shares more characters with the Hypecoideae and Fumarioideae. Thus, the floral characters support the recent phylogeny of *P. racemosum* being sister to the Hypecoideae and Fumarioideae (Peng et al., 2023). However, the absence of laticifers is unique to *P. racemosum* (Hoot et al., 2015), but could be a character that was lost in this species.

5.3 Conservation in genome of carpel regulators

OrthoFinder is one of the most widely used tools in comparative genomics and has been repeatedly benchmarked across diverse taxa to produce reliable OG assignments. The most recent version (Emms et al., 2025)improved ortholog prediction accuracy by ca. 7%, providing greater confidence in identifying conserved developmental regulators across species at the genome scale. However, given the focus of this thesis, we will not discuss algorithmic details here.

Before the development of tools such as OrthoFinder, conservation of carpel regulators was mainly investigated through single-gene phylogenetic reconstructions. Phylogenies of MADS-box and bHLH transcription factors, for example, indicated that several lineages such as AG and FUL originated early in angiosperm evolution and were retained in most extant lineages, providing evidence for a core regulatory repertoire established in the MRCA of flowering plants (Becker & Theissen, 2003; Endress & Doyle, 2009; Pfannebecker

et al., 2017). However, it provided only a fragmented view of conservation and did not allow for genome-wide assessments. Using OrthoFinder across three species, we found that most carpel regulators implicated in Arabidopsis carpel development are shared by all three species. In total, 91% of the carpel regulators are present in all species, indicating that the genetic toolkit for carpel development is largely conserved at the genome level.

Beyond the simple presence or absence of OGs, our previous detailed comprehensive framework of whole genome duplication (WGD) provides insight into how gene familyies have been shaped during evolution. Core eudicots share an ancient γ triplication followed by additional, lineage-specific duplications, including two WGDs in Brassicaceae, a triplication in Solanaceae, and a single WGD in Fabaceae (Vanneste et al., 2014). In Fabaceae, most duplicates were rapidly lost, leaving many gene families represented by a single copy (Pfannebecker et al., 2017, Figure 14). In Solanaceae, most families retain only one or two copies, with NGA being the exception where all expected copies are maintained.

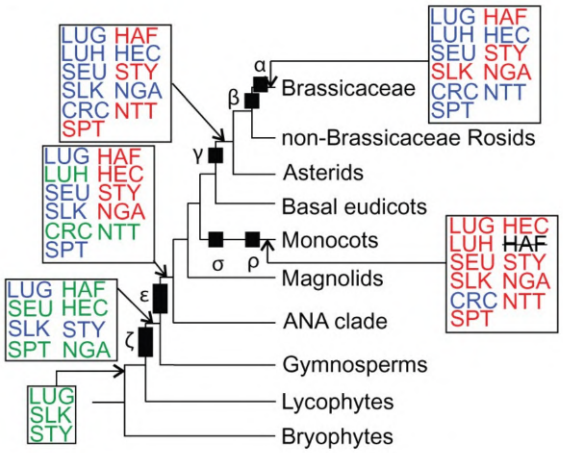

Figure 14. A simplified phylogenetic tree of land plants with carpel GRN member retention or losses after WGDs plotted onto the tree. Green letters symbolize homologs at their first appearance, blue letters symbolizes loss of one gene copy after WGD, and red letters indicate retention of both copies after WGD. A crossed out letters indicate loss of the gene lineage. (Pfannebecker et al., 2017)

The apparent absence of specific OGs in *E. californica* or *S. lycopersicum* should be interpreted cautiously. In *E. californica*, for example, the *AG* homologs (*EScaAG1* and *EScaAG2*) are recent paralogs that have diverged considerably from Arabidopsis AG, illustrating how lineage-specific duplication and sequence divergence can obscure orthology relationships rather than indicate true loss (Zahn et al., 2006). Similarly, comparative genome analyses have shown that ortholog detection is strongly affected by annotation quality: many loci appear species-specific simply due to incomplete or inconsistent annotation or the inability to detect highly diverged homologs (Ambrosino et al., 2018). More generally,

divergence after gene duplication can produce homologs that are difficult to recognize as orthologs with similarity-based methods, a problem that has motivated the development of approaches to distinguish primary from secondary orthologs (Lafond et al., 2018). Phylogenetic surveys in tomato further demonstrated that apparent absences of conserved orthologs often reflect methodological limitations or incomplete sampling rather than genuine loss (Wu et al., 2006).

Briefly, our findings show that at the genome level, the regulators of carpel development are deeply conserved across eudicots. OrthoFinder confirms the shared presence of most regulators, and previous work revealed distinct patterns of duplicate retention and loss that further shaped lineage-specific regulator sets. These conserved carpel regulators bring up the question of whether they work mainly through expression patterns, which we discuss in the following chapter.

5.4 Species-specific divergence in orthogroup expression

Although most carpel regulators are present in all three species, our results show that their temporal expression differs often. Co-expression clustering analysis revealed that OGs of carpel regulators rarely maintain a conserved expression pattern across lineages. Instead, the majority shifted between patterns, indicating that the deployment of regulators during gynoecium development is flexible. A few OGs, including HEC1/2, FUL, JAB, and AS2, remained stable in their expression trajectories, consistent with their conserved function reported in Arabidopsis (Ferrándiz et al., 2000; Gremski et al., 2007; Iwakawa et al., 2007; Zúñiga-Mayo et al., 2012) and in some cases verified in *E. californica* or *S. lycopersicum* (Bemer et al., 2012; Jiang et al., 2025; Martínez-Estrada et al., 2025; Pabón-Mora et al., 2012). These represent a small set of core regulators that appear to be deeply constrained in both sequence and expression timing.

For the majority of regulators, however, shifts in cluster assignment were the rule rather than the exception. This highlights that OG presence at the genomic level does not translate directly into conserved temporal regulation. In Arabidopsis, a dominance of a downregulated pattern (pattern 6) indicates that stem cell activity is progressively terminated as carpel development advances, reflecting that the morphological framework of the gynoecium is largely established at early stages. In contrast, *E. californica* showed upregulation around stage 3 (pattern 1 and 3), reflecting its extended maintenance of the meristem, while tomato displayed late upregulation (pattern 1 and 3) associated with fruit maturation. These differences suggest that lineage-specific developmental strategies are reflected at the transcriptomic level. While functional studies outside Arabidopsis remain limited, our data provide a set of candidates where altered timing or dosage may regulate

gynoecium development, even in species where stable transformation systems are lacking, since knock-down may be suitable to test functions.

In summary, we describe the regulatory patterns during gynoecium development as lineage-specific, while only a few sets of regulators retain highly conserved expression patterns. Then there is an interesting question of whether such shifts in expression patterns are a general feature of TFs or whether they are particularly significant in the gynoecium because of its high degree of morphological complexity. We have obtained all OGs of all TFs across three species, but have not yet done further analysis due to time limitations. A broader comparison will be done in future.

5.5 Inferred regulatory modules

Comparing regulatory modules across species reveals both similarities and differences. In Arabidopsis, *NGA* works with *HEC* to promote *IND*, which activates *SPT* and targets *PID* and *WAG2*. *NGA* orthologs are expressed at late stages in all three species. In situ hybridization confirmed that the expression domains of NGA are similar in Arabidopsis and Eschscholzia. Functional analysis also supports this pattern. Knocking down *NGA* in *E. californica* results in strong defects in the style and stigma, similar to the Arabidopsis phenotype. These results demonstrate that NGA plays a conserved role in stigma and style development. However, the downstream network is more variable. In Arabidopsis, *NGA* and *HEC* activate *IND*, which connects to *SPT* and auxin regulators. However, in *E. californica*, *IND* is absent and no clear alternative to *SPT* has been identified. *S. lycopersicum* lacks *IND* in this pathway as well. These findings suggest that parts of the style and stigma module are missing or have been replaced in other eudicots. NGA is a core component sufficient for style and stigma formation; its downregulation causes severe defects, as demonstrated in the basal eudicot species *E. californica*.

The gynoecium polarity module is another example of divergence. In Arabidopsis, the adaxial-abaxial and apical-basal axes are established by *KAN* and *ULT*, which then interact with *ETT* and *SPT*. However, the orthologs in *S. lycopersicum* and *E. californica* have different expression patterns. In *S. lycopersicum*, *SlKAN*s and *SlULT* are initially downregulated and then upregulated at stage 2 but do not coexpress with *SlETT*. In *E. californica*, the expression of *EcKAN*s and *EcULT*s differs from that in Arabidopsis. These results suggest that, although polarity establishment is important in all species, the manner in which it occurs varies.

In summary, our results support a model where carpel GRNs include both stable and flexible components. NGA is an example of a conserved regulator, while the polarity establishment genes show more variation. This may help explain how carpel identity is

preserved across species, even when morphology varies. It also supports the idea above that while many regulators are retained after WGDs across species, the timing and location of their expression can vary, leading to differences in developmental outcomes.

Appendix

The R pipeline for section 4.2.3 is available on GitHub: https://github.com/doudoukong/CarpelTranscriptomeAnalysis.

Supplementary Table 1. Complete list of Arabidopsis carpel regulators for Chapter 4, compiled from Herrera-Ubaldo et al. (2023).

Locus	Alias	Name	Family
AT5G10510	AIL6	PLETHORA3/AINTEGUMENTA-like6	AP2-EREBP (AP2)
AT4G37750	ANT	AINTEGUMENTA	AP2-EREBP (AP2)
AT4G36920	AP2	APETALA 2	AP2-EREBP (AP2)
AT1G12980	DRN	DORNROSCHEN	AP2-EREBP (ERF)
AT1G24590	DRNL	DORNROSCHEN-LIKE	AP2-EREBP (ERF)
AT5G13910	LEP	LEAFY PETIOLE	AP2-EREBP (ERF)
AT5G18560	PUC	PUCHI	AP2-EREBP (ERF)
AT1G59750	ARF1	AUXIN RESPONSE FACTOR 1	ARF
AT1G77850	ARF17	AUXIN RESPONSE FACTOR 17	ARF
AT3G61830	ARF18	AUXIN RESPONSE FACTOR 18	ARF
AT1G19220	ARF19	AUXIN RESPONSE FACTOR 19	ARF
AT5G60450	ARF4	AUXIN RESPONSE FACTOR 4	ARF
AT5G37020	ARF8	AUXIN RESPONSE FACTOR 8	ARF
AT2G33860	ETT	ETTIN	ARF
AT1G19850	MP	MONOPTEROS/ARF5	ARF
AT1G74890	ARR15	ARABIDOPSIS RESPONSE REGULATOR 15	ARR
AT2G40670	ARR16	ARABIDOPSIS RESPONSE REGULATOR 16	ARR
AT1G10470	ARR4	ARABIDOPSIS RESPONSE REGULATOR 4	ARR
AT1G19050	ARR7	ARABIDOPSIS RESPONSE REGULATOR 7	ARR
AT3G16857	ARR1	ARABIDOPSIS RESPONSE REGULATOR 1	ARR-B
AT4G31920	ARR10	ARABIDOPSIS RESPONSE REGULATOR 10	ARR-B
AT2G25180	ARR12	ARABIDOPSIS RESPONSE REGULATOR 12	ARR-B
AT2G01760	ARR14	ARABIDOPSIS RESPONSE REGULATOR 14	ARR-B
AT4G29080	IAA27	INDOLE-3-ACETIC ACID INDUCIBLE 27	AUX/IAA
AT2G46870	NGA1	NGATHA1	B3
AT3G61970	NGA2	NGATHA2	B3
AT1G01030	NGA3	NGATHA3	B3
AT5G67110	ALC	ALCATRAZ	bHLH
AT1G18400	BEE1	BRASSINOSTEROID ENHANCED EXP 1	bHLH
AT4G00870	bHLH14	basic Helix-Loop-Helix 14	bHLH
AT5G67060	HEC1	HECATE 1	bHLH
AT3G50330	HEC2	HECATE 2	bHLH
AT5G09750	HEC3	HECATE 3	bHLH
AT4G00120	IND	INDEHISCENT	bHLH
AT4G36930	SPT	SPATULA	bHLH
AT2G21230	DKM	DRINK ME	bZIP
AT1G68640	PAN	PERIANTHIA	bZIP
AT1G69180	CRC	CRABS CLAW	C2C2 (YABBY)
AT2G45190	FIL	FILAMENTOUS FLOWER	C2C2 (YABBY)
AT4G00180	YAB3	YABBY 3	C2C2 (YABBY)
AT1G68480	JAG	JAGGED	C2H2
AT5G14010	KNU	KNUCKLES	C2H2
AT3G57670	NTT	NO TRANSMITTING TRACT	C2H2

Locus	Alias	Name	Family
AT1G13400	NUB	NUBBIN	C2H2
AT1G43850	SEU	SEUSS	C2H2
AT1G75520	SRS5	SHI-related sequence 5	C2H2 (SRS)
AT3G51060	STY1	STYLISH 1	C2H2 (SRS)
AT4G36260	STY2	STYLISH 2	C2H2 (SRS)
AT2G35270	GIK	GIANT KILLER	Co-factor
AT4G32551	LUG	LEUNIG	Co-factor
AT5G16560	KAN1	KANADI 1	G2-LIKE
AT1G32240	KAN2	KANADI 2	G2-LIKE
AT3G54220	SCR1	SCARECROW	GRAS
AT2G27990	PNF	POUND-FOOLISH	HOMEOBOX
AT5G41410	BEL1	BELL	HOMEOBOX-BEL
AT5G02030	RPL	REPLUMLESS	HOMEOBOX-BEL
AT4G17460	JAB	JAIBA	HOMEOBOX-HD ZIP III
AT2G34710	PHB	PHABULOSA	HOMEOBOX-HD ZIP III
AT1G30490	PHV	PHAVOLUTA	HOMEOBOX-HD ZIP III
AT5G60690	REV	REVOLUTA	HOMEOBOX-HD ZIP III
AT4G08150	BP	BREVIPEDICELLUS	HOMEOBOX-KNOX
AT1G70510	KNAT2	KNOTTED-like from A. thaliana 2	HOMEOBOX-KNOX
AT1G23380	KNAT6	KNOTTED-like from A. thaliana 6	HOMEOBOX-KNOX
AT1G62360	STM	SHOOT MERISTEMLESS	HOMEOBOX-KNOX
AT4G35550	WOX13	WUSCHEL related homeobox 13	HOMEOBOX-WOX
AT2G17950	WUS	WUSCHEL	HOMEOBOX-WOX
AT4G18960	AG	AGAMOUS	MADS
AT5G60910	FUL	FRUITFULL	MADS
AT1G31140	GOA	GORDITA	MADS
AT1G24260	SEP3	SEPALLATA3	MADS
AT3G58780	SHP1	SHATTERPROOF 1	MADS
AT2G42830	SHP2	SHATTERPROOF 2	MADS
AT4G09960	STK	SEEDSTICK	MADS
AT2G37630	AS1	ASYMMETRIC LEAVES 2	MYB
AT1G65620	AS2	ASYMMETRIC LEAVES 1	MYB
AT3G15170	CUC1	CUP-SHAPED COTYLEDON 1	NAC
AT5G53950	CUC2	CUP-SHAPED COTYLEDON 2	NAC
AT1G76420	CUC3	CUP-SHAPED COTYLEDON 3	NAC
AT5G60140	REM11	REPRODUCTIVE MERISTEM 11	REM
AT3G46770	REM13	REPRODUCTIVE MERISTEM 13	REM
AT1G69690	TCP15	TEOSINTE BRANCHED, CYCLOIDEA,PCF 15	TCP
AT4G28190	ULT	ULTRAPETALA	ULT

Supplementary Table 2. Information on fourty-seven OGs, the subset of the fifty-five OGs in Figure 7B that have reported Arabidopsis carpel defects. The table lists OG_ID, members, and defects. Selection is based on Herrera-Ubaldo et al. (2023).

OG_ID	Member	Abaxial-adaxial	Apical-basal	Carpel number	Determinacy	Ovule	Replum	Septum	Style and/or stigma	Transmitting tract	Valve margin
OG0000356	NTT						■	■		■	■
OG0000458	AP2				■		■				■
OG0000555	PHB	■		■		■					
	PHV	■		■		■					
	REV	■	■			■		■	■	■	
OG0000650	NGA1		■			■		■	■	■	
	NGA2		■			■		■	■	■	
	NGA3		■			■	■	■	■	■	
OG0000696	SRS5					■		■	■		
	STY1	■	■				■	■	■	■	
	STY2	■	■				■	■	■		
OG0000898	AG			■	■	■					
	STK					■		■		■	
OG0001272	ARR10		■				■	■		■	
	ARR12		■				■	■		■	
OG0001330	KAN	■	■			■					
	KAN2	■	■			■					
OG0001429	FUL						■	■	■		■
OG0001723	SEP3			■	■	■					
OG0001947	JAB			■	■	■		■	■	■	
OG0002360	HEC1		■					■	■	■	
	HEC2		■					■	■	■	
OG0002740	LEP		■								
OG0002821	ANT	■	■	■	■	■		■	■		
OG0003114	SEU	■	■	■	■	■		■	■	■	
OG0003138	DKM		■			■		■		■	

OG_ID	Member	Abaxial-adaxial	Apical-basal	Carpel number	Determinacy	Ovule	Replum	Septum	Style and/or stigma	Transmitting tract	Valve margin
OG0003420	TCP15										
OG0003848	FIL										
	YAB3										
OG0004750	ARF8										
OG0004796	bHLH14										
OG0004820	ALC										
	SPT										
OG0004852	AS1										
OG0005127	LUG										
OG0005192	ULT										
OG0005444	AS2										
OG0005578	WOX13										
OG0005629	BEE1										
OG0006226	CUC1										
	CUC2										
OG0006765	AIL6										
OG0007441	DRN										
	DRNL										
OG0007466	HEC3										
	IND										
OG0007573	BEL1										
OG0007652	GIK										
OG0007854	STM										
OG0008340	ARR1										
OG0009209	BP										
OG0010227	ETT										
OG0010435	KNU										

OG_ID	Member	Abaxial-adaxial	Apical-basal	Carpel number	Determinacy	Ovule	Replum	Septum	Style and/or stigma	Transmitting tract	Valve margin
OG0010639	MP										
OG0010670	WUS										
OG0010767	JAG										
	NUB										
OG0011043	CRC										
OG0011138	RPL										
OG0011378	CUC3										
OG0014573	PUCHI										
OG0016336	GOA										
OG0022028	SHP1										
	SHP2										

Supplementary Table 3. Dynamic expression profiles of the 55 orthogroups across developmental stages. OGs were defined as description in section 4.2.3. Expression is indicated here by “+” and absence by “-”.

OG_ID	name/family	Stage1	Stage2	Stage3	Stage4
OG0000356	NTT	+	+	+	+
OG0000458	AP2	+	+	+	+
OG0000555	PHV, PHB, REV	+	+	+	+
OG0000650	NGA1, NGA2, NGA3	+	+	+	+
OG0000696	STY2, SRS5, STY1	+	+	+	+
OG0000898	AG, STK	+	+	+	+
OG0001272	ARR12, ARR10	+	+	+	+
OG0001326	KNAT2, KNAT6	+	+	+	+
OG0001330	KAN, KAN2	+	+	+	+
OG0001429	FUL	+	+	+	+
OG0001723	SEP3	+	+	+	+
OG0001947	JAB	+	+	+	+
OG0002360	HEC1, HEC2	+	+	+	+
OG0002726	IAA27	+	+	+	+
OG0002740	LEP	-	-	+	-
OG0002821	ANT	+	+	+	+
OG0003114	SEU	+	+	+	+
OG0003138	DKM	+	+	+	+
OG0003220	ARR4, ARR15, ARR7	+	+	+	+
OG0003420	TCP15	+	+	+	+
OG0003848	YAB3, FIL	+	+	+	+
OG0004750	ARF8	+	+	+	+
OG0004796	bHLH14	+	+	+	+
OG0004820	ALC, SPT	+	+	+	+
OG0004852	AS1	+	+	+	+
OG0005127	LUG	+	+	+	+
OG0005192	ULT	+	+	+	+
OG0005444	AS2	+	+	+	+
OG0005578	WOX13	+	+	+	+
OG0005629	BEE1	-	+	+	+
OG0006226	CUC1, CUC2	+	+	+	+
OG0006765	AIL6	+	+	+	+
OG0007441	DRN, DRNL	+	-	+	-
OG0007466	IND, HEC3	+	+	+	+
OG0007573	BEL1	+	+	+	+
OG0007652	GIK	-	-	-	-
OG0007854	STM	+	+	+	+
OG0008340	ARR1	+	+	+	+

OG_ID	name/family	Stage1	Stage2	Stage3	Stage4
OG0009209	BP	+	+	+	+
OG0009263	SCR1	+	+	+	+
OG0010115	PNF	+	+	+	+
OG0010227	ETT	+	+	+	+
OG0010435	KNU	+	+	+	+
OG0010639	MP	+	+	+	+
OG0010670	WUS	-	+	-	-
OG0010767	JAG, NUB	+	+	+	+
OG0011043	CRC	+	+	+	+
OG0011138	RPL	+	+	+	+
OG0011162	ARR16	-	-	-	+
OG0011172	REM13, REM11	+	+	+	+
OG0011378	CUC3	+	+	+	+
OG0011781	ARR14	+	+	+	+
OG0014573	PUCHI	+	-	+	-
OG0016336	GOA	-	+	+	-
OG0022028	SHP2, SHP1	+	+	+	+

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