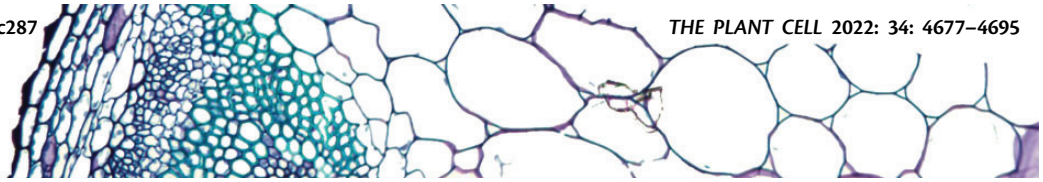
https://doi.org/10.1093/plcell/koac287 THE PLANT CELL 2022: 34: 4677–4695



Review

Anther development—The long road to making pollen

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Abstract

Anthers express the most genes of any plant organ, and their development involves sequential redifferentiation of many cell types to perform distinctive roles from inception through pollen dispersal. Agricultural yield and plant breeding depend on understanding and consequently manipulating anthers, a compelling motivation for basic plant biology research to contribute. After stamen initiation, two theca form at the tip, and each forms an adaxial and abaxial lobe composed of pluripotent Layer 1-derived and Layer 2-derived cells. After signal perception or self-organization, germinal cells are specified from Layer 2-derived cells, and these secrete a protein ligand that triggers somatic differentiation of their neighbors. Historically, recovery of male-sterile mutants has been the starting point for studying anther biology. Many genes and some genetic pathways have well-defined functions in orchestrating subsequent cell fate and differentiation events. Today, new tools are providing more detailed information; for example, the developmental trajectory of germinal cells illustrates the power of single cell RNA-seq to dissect the complex journey of one cell type. We highlight ambiguities and gaps in available data to encourage attention on important unresolved issues.

Introduction

The alternation of generations from diploid sporophyte to haploid gametophyte and back to sporophyte is a central tenet of plant biology. While enormous variation in the longevity, interdependence, and structure of these alternating, multicellular life phases exist, the reproductive processes transitioning between them share universal principles. Sporophytes develop reproductive organs, or sporangia, which contain cells that undergo sporogenesis via meiosis to produce haploid spores (Figure 1). Spores develop into gametophytes, in which some cells can undergo gametogenesis to produce male (sperm) and/or female (egg) gametes. The male and female gametes then fuse via syngamy (fertilization) to develop into a diploid sporophyte. All seed plants are heterosporous, producing separate male (micro-) and female (mega-) sporangia, spores, and gametophytes. In flowering plants, megasporangia are housed within the carpel and microsporangia within the anther. Because plants lack predestined germlines, unlike animals, the reproductive organs and cell types must arise de novo from nonreproductive tissues. The development and evolution of the angiosperm carpel have been reviewed in detail (Skinner et al., 2004; Cucinotta et al., 2014; Pfannebecker et al., 2016; Becker, 2020) as has meiosis (Harrison et al., 2010; Lenormand et al., 2016; Lei and Liu, 2020; Böwer and Schnittger, 2021; Wang et al., 2021). To complement this coverage, we focus on the architecture, development, and evolution of the anther from inception through meiotic

Received July 11, 2022. Accepted August 29, 2022. Advance access publication September 22, 2022

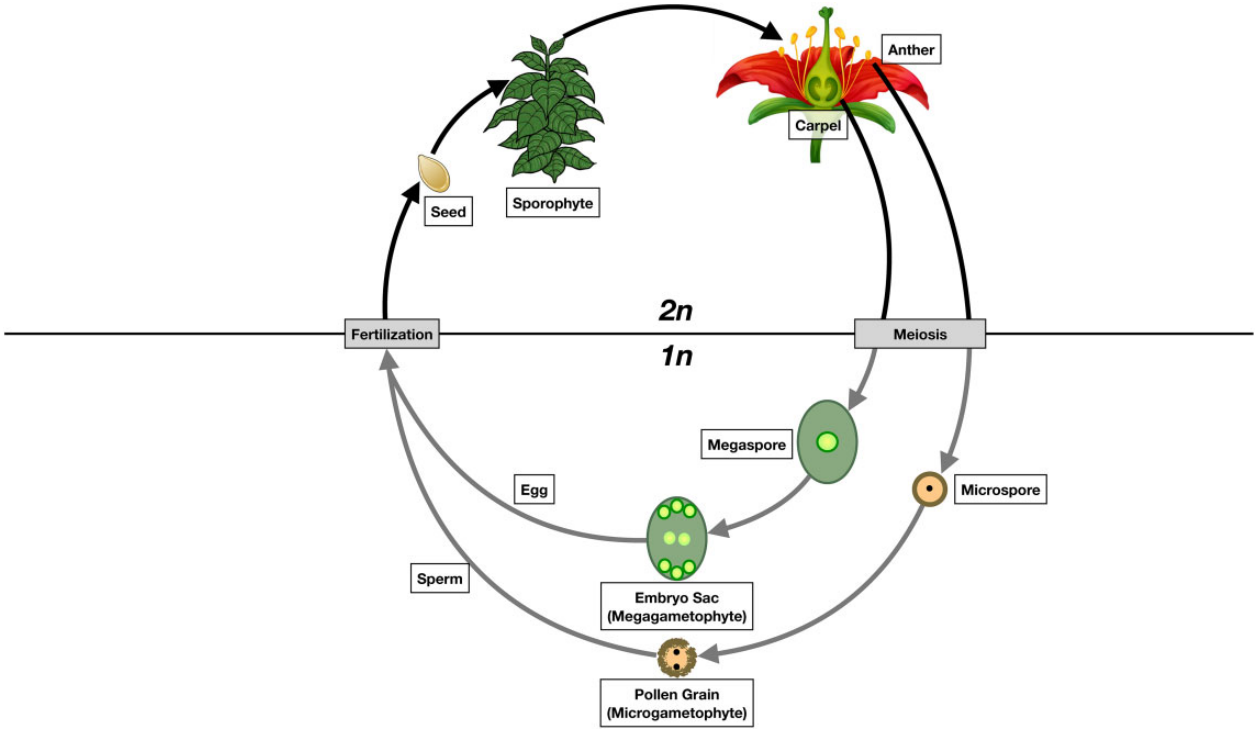

Figure 1 The alternation of generations. In angiosperms (flowering plants) the sporophyte dominates the lifecycle, terminating in meiosis of two types of germinal cells. Within the carpel, meiosis generates megaspores, and these formed the multinucleate (or multicellular) megagametophyte. One haploid cell within the embryo sac differentiates as the egg. Anthers support the maturation of germinal cells that undergo meiosis to form haploid microspores; after two mitotic divisions, the microgametophyte. This matures into pollen, containing two sperm cells and a vegetative cell. The fertilization of an egg cell by a sperm cell results in a new sporophyte.

entry, with inclusion of the entire developmental trajectory of the germinal lineage from specification as archesporial cells through mature pollen.

Understanding anther development is essential in the study of plant reproduction, controlled crop breeding, and hybrid seed production as anthers are the source of pollen and thus male gametes in flowering plants. Architecturally, anthers are bilaterally symmetrical with two theca that each consists of an abaxial (lower) and adaxial (upper) lobe (Figure 2). Each lobe is a microsporangium, housing and supporting the developing germinal cells. There are four distinct somatic cell layers in most angiosperm anthers surrounding the germinal cells at the start of meiosis: the epidermis, endothecium, middle layer, and tapetum, from outermost to innermost. The intermicrosporangial stripe (IMS) 1 and 2 regions contain epidermal and endothecial cells that reorganize anther structure before anthesis to permit pollen dispersal (Cheng et al., 1979). Joining the four lobes is the connective tissue, which surrounds the central vascular tissue; the connective and vascular columns extend along the *Y*-axis of the anther into the filament, which attaches the anther to the base of the flower (Figure 2). Together, the filament and anther form a stamen.

This review will largely center on maize (*Zea mays*) anther development as maize has been used as a model system for studying anthers for decades and is a crop dependent on hybrid seed production. Maize is the most prolific US crop in terms of tons produced, acreage harvested, and crop value. As such, advances in maize reproductive output, breeding efficiency, or environmental tolerances can have profound effects on US food security and the economy. Structurally, maize is ideal for investigating anther development as the plants are monecious, with separate male and female inflorescences (tassel and ear, respectively). The tassel comprised hundreds of male-only spikelets, each spikelet encompassing an upper and lower floret. Each floret contains three developmentally synchronous anthers. The upper- and lower-floret anthers differ by about a day in development; in addition, spikelet development varies by location along the main spike and side branches of the tassel (Egger and Walbot, 2015), allowing the dissection and isolation of anthers that span about 7 days of developmental stages, which can be charted by anther length, all from a single plant (Figure 3). Finally, maize has a sizeable collection of male-sterile mutant genotypes, which have been instrumental in understanding the genes regulating male fertility (Figure 3 and Table 1).

Initiation of the stamen primorida and cell fate specification

The ABCE model of flower development dictates floral architecture based on differential expression of combinations of at least four factors to initiate the four whorls of flower organogenesis. In the case of maize stamen primordia, the

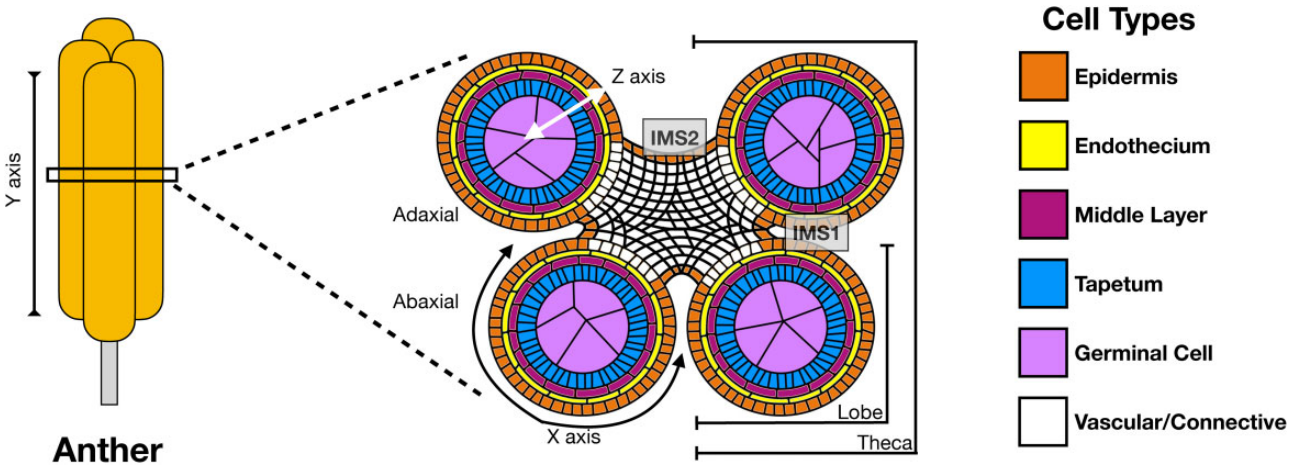

Figure 2 Transverse section of 2.0-mm maize anther. On the left is a schematic of an anther, with the long axis designated as the *Y*-axis. In transverse view, the schematic anther is composed of four lobes; the adaxial lobes are closer to the original plant apex, and the abaxial lobes are more distant, using the same terminology applied to the upper and lower surfaces of leaves. Each lobe is a microsporangium. Initially, the anther consists of two theca, and these each partition into an abaxial and an adaxial microsporangia/lobes. In the central diagram the *X*-axis (circumferential around each lobe) and the *Z*-axis (transverse across a lobe) are indicated. The intermicrosporangial regions (IMS1 and IMS2) are indicated; the IMS1 regions are utilized late in anther development to rejoin lobe pairs into a single unit prior to pollen shed.

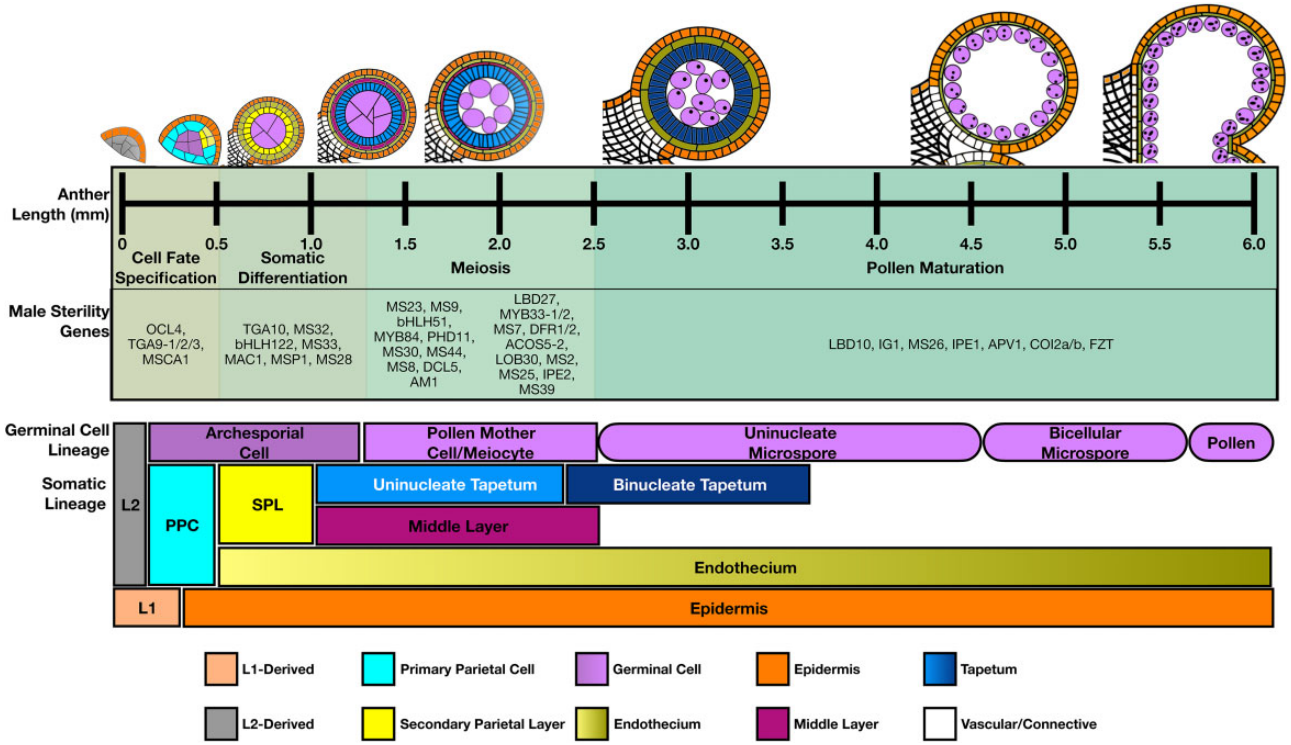

Figure 3 Developmental series of maize anther. Across the top of the diagram, the sequential stages of anther development are displayed in cartoons. Note the increase in complexity of cell types initially and the gradual increase in size. In maize these stages represent ~30 days of development. Developmental progression is highly regular, such that anther length is a reasonable guide to the stage of cell development within the anther. Indicated below the length (mm) is the term used to describe a group of stages: these successive major stages are colored in different shades of green. Within the stage boxes are lists of male-sterility genes in maize. Below the staging system, there is a schematic representation of the emergence of cell types initiating with just Layer 1 cells (L1) and L2 cells present. The L1 differentiates as the epidermis, but all of the other anther lobe cell types derive from the L2 cells in an ordered set of events. Periclinal cell divisions generate additional layers within the lobe, that is, the primary parietal cells divide periclinally to generate the endothecium and the secondary parietal layer cells.

Table 1 Maize male-sterility genes

Peak expression stage	Gene name	Maize gene model	Rice gene	Arabidopsis gene	Protein	Mutant fertility status	Anther cytology	References
Initial anther specification	*OCL4*	*Zm00001d030069*			HD-ZIP TF	Full sterile	Additional periclinal divisions in subepidermal cell layer	Vernoud et al. (2009); Yadava et al. (2021)
	TGA9-1	*Zm00001d052543*	*OsTGA9*	*AtTGA9*	TGACG (TGA) motif-binding subclade of bZIP TF	Redundant	Not Available (NA)	Jiang et al. (2021); Liu et al. (2022)
	TGA9-2	*Zm00001d042777*		*AtTGA9*	TGACG (TGA) motif-binding subclade of bZIP TF	Redundant	NA	Jiang et al. (2021); Liu et al. (2022)
	TGA9-3	*Zm00001d012294*		*AtTGA9*	TGACG (TGA) motif-binding subclade of bZIP TF	Redundant	NA	Jiang et al. (2021); Liu et al. (2022)
	MSCA1	*Zm00001d018802*	*OsMIL1*	*ROXY1/2*	Glutaredoxin	Full sterile	Does not form archesporial cell (AR), anther wall layers do not develop and locules are filled with parenchyma-like cells; nonfunctional vascular strands are present in each lobe	Chaubal et al. (2003); Timofejeva et al. (2013)
Somatic differentiation	*TGA10*	*Zm00001d020938*	*OsTGA10*	*AtTGA10*	TGACG (TGA) motif-binding subclade of bZIP TF	Full sterile	Anthers exerted and pollen grains appear normal, but anthers failed to dehisce. Scanning electron micrograph (SEM) showed fragmentary materials were pasted on the pollen grain and smaller Ubisch bodies	Jiang et al. (2021)
	MS32	*Zm00001d006564*	*OsUDT1*	*AtDYT1*	bHLH TF	Full sterile	Extra TAP periclinal divisions	Chaubal et al. (2000); Moon et al. (2013); Nan et al. (2016, 2022)
	bHLH122	*Zm00001d017724*	*OsEAT1*		bHLH TF	Full sterile	Extra tapetal (TAP) periclinal divisions; SEM showed smooth inner and outer surfaces of anther wall without knitting cuticle and Ubisch bodies	Jiang et al. (2021); Nan et al. (2022)
	MS33; GPAT6	*Zm00001d007714*	*Os11g45400*		Glycerol-3-phosphate acyltransferases	Full sterile	Inhibits the biosynthesis of glycolipids and phospholipids and disrupts the development and function of endothecium (EN) chloroplasts resulting in the formation of abnormal EN chloroplasts containing numerous starch granules	Zhang et al. (2018b); Zhu et al. (2020)
	MAC1	*Zm00001d023681*	*OsTDL1a/MIL2*	*AtTPD1*	Small, secreted protein ligand	Full sterile	Failure of somatic cell specification; over proliferation of AR cells	Sheridan et al. (1999); Kelliher and Walbot (2012); Wang et al. (2012)

(continued)

Table 1 Continued

Peak expression stage	Gene name	Maize gene model	Rice gene	Arabidopsis gene	Protein	Mutant fertility status	Anther cytology	References
	MSP1	*Zm00001d042362*	*OsMSP1*	*AtEMS1*	LRR-RK	Full sterile	Failure of TAP and middle layer (ML) cell specification	Timofejeva et al. (2013); van der Linde et al. (2018)
	MS28	*Zm00001d013063*	*OsMEL1*	*AtAGO5*	Argonaute	Full sterile	Premature vacuolated TAP, defective Ubisch bodies, and nonviable pollen grains	Li et al. (2021a)
Early meiosis	*MS23*	*Zm00001d008174*	*OsTIP2*		bHLH TF	Full sterile	Extra TAP periclinal divisions	Chaubal et al. (2000); Nan et al. (2016, 2022)
	MS9	*Zm00001d028777*	*OsTDF1*	*AtTDF1*	MYB TF	Full sterile	Pollen mother cell (PMC) degeneration begins prior to meiosis; TAP develop thin walls between the two nuclei	Beadle (1932); Greyson et al. (1980); Albertsen et al. (2016)
	bHLH51	*Zm00001d053895*	*OsTDR*	*AtAMS*	bHLH TF	Full sterile	Extra TAP periclinal divisions; SEM showed smooth inner and outer surfaces of anther wall without knitting cuticle and Ubisch bodies	Jiang et al. (2021); Nan et al. (2022)
	MYB84	*Zm00001d025664*	*OsMYB80*	*AtMYB80*	MYB TF	Full sterile	SEM showed denser cuticle layer, while the inner surface of anther wall was smooth due to lack of Ubisch bodies	Jiang et al. (2021)
	PHD11	*Zm00001d013416*	*OsTIP3*	*AtMMD1*	PhD Finger TF	Full sterile	SEM showed lack the knitting cuticle and Ubisch bodies	Jiang et al. (2021)
	MS30	*Zm00001d052403*			GDSL lipase	Full sterile	Defective anther cuticle, irregular foot layer of pollen exine	An et al. (2019)
	MS44	*Zm00001d052736*			Type C nonspecific lipid transfer protein	Dominant full sterile	NA	Fox et al. (2017)
	MS8	*Zm00001d012234*			Beta-1,3-galactosyltransferase	Full sterile	Excess number of smaller than normal epidermis (EPI) and fewer but larger TAP as meiosis starts. Callose remodeling around the meiocytes is also abnormal	Wang et al. (2013, 2010)
	DCL5	*Zm00001d032655*	*OsDCL3B*		Dicer-like protein	Temperature-dependent sterile	TAP development is delayed or arrested	Teng et al. (2018)

(continued)

Table 1 Continued

Peak expression stage	Gene name	Maize gene model	Rice gene	Arabidopsis gene	Protein	Mutant fertility status	Anther cytology	References
	AM1	*Zm00001d013659*	*OsAM1*	*AtSWI1/DYAD*	Unknown	Full sterile	Meiocytes continue undergoing mitotic divisions	Pawlowski et al. (2009)
Late meiosis	*LBD27*	*Zm00001d013732*		*AtLBD10*	Lateral organs boundary domain TF	Partial sterility (9.53% sterile), 32% sterile with LBD10	Largely wild-type (WT)	Jiang et al. (2021)
	MYB33-1	*Zm00001d012544*	*OsGAMYB*	*AtMYB33/65*	MYB TF	Redundant	SEM showed outer and inner surfaces of anther wall were smooth and glossy	Jiang et al. (2021); Li et al. (2021b)
	MYB33-2	*Zm00001d043131*	*OsGAMYB*	*AtMYB33/65*	MYB TF	Redundant	SEM showed outer and inner surfaces of anther wall were smooth and glossy	Jiang et al. (2021); Li et al. (2021b)
	MS7	*Zm00001d020680*	*OsPTC1*	*AtMs1*	PhD Finger TF	Full sterile	Poorly developed, thin microspore wall and aperture as well as abnormal TAP development	Albertsen and Phillips (1981); Morton et al. (1989); Zhang et al. (2018a)
	DFR1	*Zm00001d031488*	*OsTKPR1*	*AtTKPR1*	Dihydroflavonoid reductase	Partial sterility (10.25% sterile), full sterile with DFR2	NA	Liu et al. (2022)
	DFR2	*Zm00001d020970*	*OsTKPR1*	*AtTKPR1*	Dihydroflavonoid reductase	Partial sterility (32.5% sterile), full sterile with DFR1	NA	Liu et al. (2022)
	ACOS5-2	*Zm00001d039276*	*OsACOS12*	*AtACOS5*	Acyl-CoA Synthetase	Full sterile	NA	Liu et al. (2022)
	LOB30	*Zm00001d036435*		*AtLBD2*	Lateral organs boundary domain TF	Full sterile	SEM showed smooth cell walls	Li et al. (2021b)
	ABCG26; MS2	*Zm00001d046537*	*OsABCG15*	*AtABCG26*	ATP-binding cassette (ABC) transporter	Full sterile	Irregular lipid metabolism; anther walls were smooth and glossy without knitting cuticle and Ubisch bodies	Jiang et al. (2021); Xu et al. (2021)
	FAR1; MS6021; MS25	*Zm00001d048337*	*OsDPW*	*AtMs2*	Fatty acyl reductases	Full sterile	Irregular lipid metabolism; lacked the knitting cuticle and Ubisch bodies	Tian et al. (2017); Jiang et al. (2021); Zhang et al. (2021)
	IPE2	*Zm00001d015960*	*OsGELP34*	*AtGELP77*	GDSL-lipase	Full sterile	Delayed degeneration of TAP and ML, leading to defective formation of anther cuticle and pollen exine	Huo et al. (2020)
	MS39	*Zm00001d043909*	*OsGSL2*	*AtGSL5*	Callose synthase	Full sterile	Vacuolization of the TAP, an irregular arrangement of the TAP layer, and degradation of the tetrads	Zhu et al. (2018)

(continued)

Table 1 Continued

Peak expression stage	Gene name	Maize gene model	Rice gene	Arabidopsis gene	Protein	Mutant fertility status	Anther cytology	References
Postmeiosis	*LBD10*	*Zm00001d033335*		*AtLBD10*	Lateral organs boundary domain TF	Partial sterility (2% sterile), 32% sterile with LBD27	Largely WT	Jiang et al. (2021)
	IG1	*Zm00001d042560*	*OsIG1*	*AtAS2*	Lateral organs boundary domain TF	Full sterile	NA	Evans (2007)
	MS26	*Zm00001d027837*	*OsCYP704B2*	*AtCYP704B1*	Cytochrome P450 monooxygenase	Full sterile	Outer surface of anthers was smooth at the mature pollen grain stage, and pollen exine did not form the typical three-layer structure at the late uninucleate microspore stage	Chen et al. (2017)
	IPE1	*Zm00001d029683*		*AT1G12570*	GMC oxidoreductase	Full sterile	Pollen wall undeveloped	Chen et al. (2017)
	MS45	*Zm00001d047859*			Strictosidin synthase	Full sterile	Anther outer surface was not smooth but lacked the regular reticular structure, important in cuticle maturation and pollen exine thickening	Cigan et al. (2001); Chen et al. (2017)
	APV1	*Zm00001d024712*	*OsCYP703A3*	*AtCYP703A2*	cytochrome P450 monooxygenase	Full sterile	SEM showed anther EPI surface was smooth, shiny, and plate shaped without crowded epicuticular ridges, fewer unevenly distributed Ubisch bodies on the inner anther surface	Somaratne et al. (2017)
	COI2a	*Zm00001d028543*	*OsCOI2*	*AtCOI1*	F-box protein; receptor for perceiving jasmonoyl-isoleucine	Redundant	Nondehiscent anthers, short filaments, delayed anther development	Qi et al. (2022)
	COI2b	*Zm00001d047848*	*OsCOI2*	*AtCOI1*	F-box protein; receptor for perceiving jasmonoyl-isoleucine	Redundant	Nondehiscent anthers, short filaments, delayed anther development	
	FZT	*Zm00001d027412*	*OsDCL1*	*AtDCL1*	Dicer-like	Full sterile	Microspores become vacuolated and the TAP is completely degraded; fails to accumulate significant levels of starch	Thompson et al. (2014); Field and Thompson (2016)
Unknown	*TMS5*	*Zm00001d053351*	*OsTMS5*		RNase ZS1	Temperature-dependent sterile	NA	Li et al. (2017)

Class B factors needed for stamen development are STERILE TASSEL SILKY EAR1 and MALE-STERILE SILKY (Ambrose et al., 2000; Bartlett et al., 2015). The necessary Class C factors are ZEA AGAMOUS1 and ZEA MAYS MADS2 (Schmidt et al., 1993; Mena et al., 1996); BEARDED EAR is the putative Class E factor (Thompson et al., 2009). The expression of these five MADS-box transcription factors and the presence of specific phytohormones are essential for the initiation of stamen primordia from the floral meristem (reviewed in Li and Liu, 2017). Upon specification, maize stamen primordia have an outer cell layer [Layer 1 (L1)-derived], which eventually differentiates into the epidermis, and an inner bilayer [Layer 2 (L2)-derived]. Historically, it was assumed that each lobe in an angiosperm anther had a single L2-derived cell, the hypodermal cell, from which the remaining somatic and germinal cell types arose through predetermined asymmetric cell divisions (Figure 4); this model was based on observations in ferns, which do contain single large cells at the center of the apical meristem, and 2D microscopic observations of anther primordia in which the surface area but not the volume of cells could be measured. When applied to the origin of germinal cells in anthers, however, this assumption had several flaws. Foremost is the presence of multiple, pluripotent L2-derived cells per anther lobe upon initiation of the stamen primordia. By confocal observation, these cells have a similar volume. Consequently, a large hypodermal cell could not be found (Figure 4B) (Kelliher and Walbot, 2011). Finally, the means by which a ring of differentiated somatic cells could form around the germinal cell was unclear. Instead, Kelliher and Walbot (2012) found that the multi-potent L2-derived cells proliferate until the reduction in oxygen levels triggers the glutaredoxin MALE STERILE CONVERTED ANTHER1 (MSCA1) action to start the differentiation of the innermost L2-derived cells into archesporial cells, the initial cell type in the germinal cell lineage (Kelliher and Walbot, 2012). By altering the oxygen levels surrounding maize anthers, the authors could overcome the sterility defect of *msca1* anthers, demonstrating that sufficient reducing conditions can substitute for the MSCA1 glutaredoxin. They also observed ectopic differentiation of archesporial cells throughout the anther, including the innermost connective and vascular tissue in nonoxidizing treatments and surprisingly even L1-derived cells could become archesporial cells. The assay for archesporial cell specification was the formation of a bilayered ring of L2-derived cells around each archesporial cell. In wild-type anthers the maize archesporial cells secrete MULTIPLE ARCHESPORIAL CELLS1 (MAC1) protein, the homologs of which are TAPETUM DETERMINANT (TPD)-like 1A/MICROSPORELESS2 (TDL1A/MIL2) in rice (*Oryza sativa*) and TPD1 in Arabidopsis (*Arabidopsis thaliana*). MAC1 is perceived by neighboring L2-derived cells via the leucine-rich repeat receptor kinase MULTIPLE SPOROCYTE1 (MSP1) in maize (MSP1 in rice and EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS in Arabidopsis), and this interaction results in their differentiation to primary parietal cells, as discussed in more detail below (van der Linde et al., 2018). In maize *mac1* mutant anthers, hypoxia treatment resulted in archesporial cell specification in the normal locations and in ectopic sites, but no bilayered rings formed (Kelliher and Walbot, 2012).

Zhao et al. (2021) recently reported that in rice anthers both the archesporial cells and the neighboring L2-derived cells (and later the primary parietal cells) are required to permit stabilization of meiotic fate acquisition. This insight comes from observing mutants in both pregerminal and somatic cells and in double mutants. It is not surprising as cell fate setting is a process not an instantaneous, discrete event. Recall that the *mac1* mutant is aptly named, because in the absence of MAC1 there are excess archesporial cells and later excess meiocytes. Because the mutant fails to establish the neighboring somatic niche, there are two hypotheses to explain the excess archesporial cells: MAC1 is an inhibitor of archesporial cell division (acting on or within the cells that synthesize it) or MAC1 stimulates somatic specification and then the primary parietal cells repress archesporial cell division by an unknown pathway. The observations of Zhao et al. (2021) favor the second hypothesis.

Similar studies modulating oxygen levels have not been attempted in any other species; however, the lack of a meristem in anthers suggests environmental triggers, such as hypoxia, could be the norm among flowering plants for dictating whether there are enough cells to fully commit to organogenesis. In addition, the presumably hypoxic conditions maintained by sealed buds would suppress reactive oxygen levels and therefore lower the risk of mutations in the germinal cells. How ubiquitous is the use of hypoxia as the primary trigger for archesporial specification across flowering plants? Could this adaptation be conserved across all land plants, as every derivation of sporangia has defined somatic cell layers surrounding the germinal cells? Are there other environmental or external triggers, such as a surge in sugar delivery or a hormone to trigger archesporial cell fate in the anther primordium? In Arabidopsis, the transcription factor SPOROCYTELESS/NOZZLE (SPL/NZZ) is required for archesporial specification (Schiefthaler et al., 1999; Yang et al., 1999). Zheng et al. (2021) demonstrated that a centripetal auxin gradient is first established then maintained by SPL/NZZ within Arabidopsis immature lobes; this gradient is required for the successful progress of germinal fate setting. What triggers localized expression of SPL/NZZ in immature lobes, could it be hypoxia or another signal?

There is convergence between maize and Arabidopsis archesporial specification when considering the key role played by redox regulators immediately following hypoxic conditions in maize or the auxin gradient and SPL/NZZ activation in Arabidopsis. Current understanding is that cytoplasmic glutaredoxins, such as MSCA1, when reduced under low oxygen conditions, in turn reduce TGA transcription factors, facilitating their entry into the nucleus to activate new programs of gene expression locking in archesporial specification and thus also initiating somatic differentiation events. Three paralogous

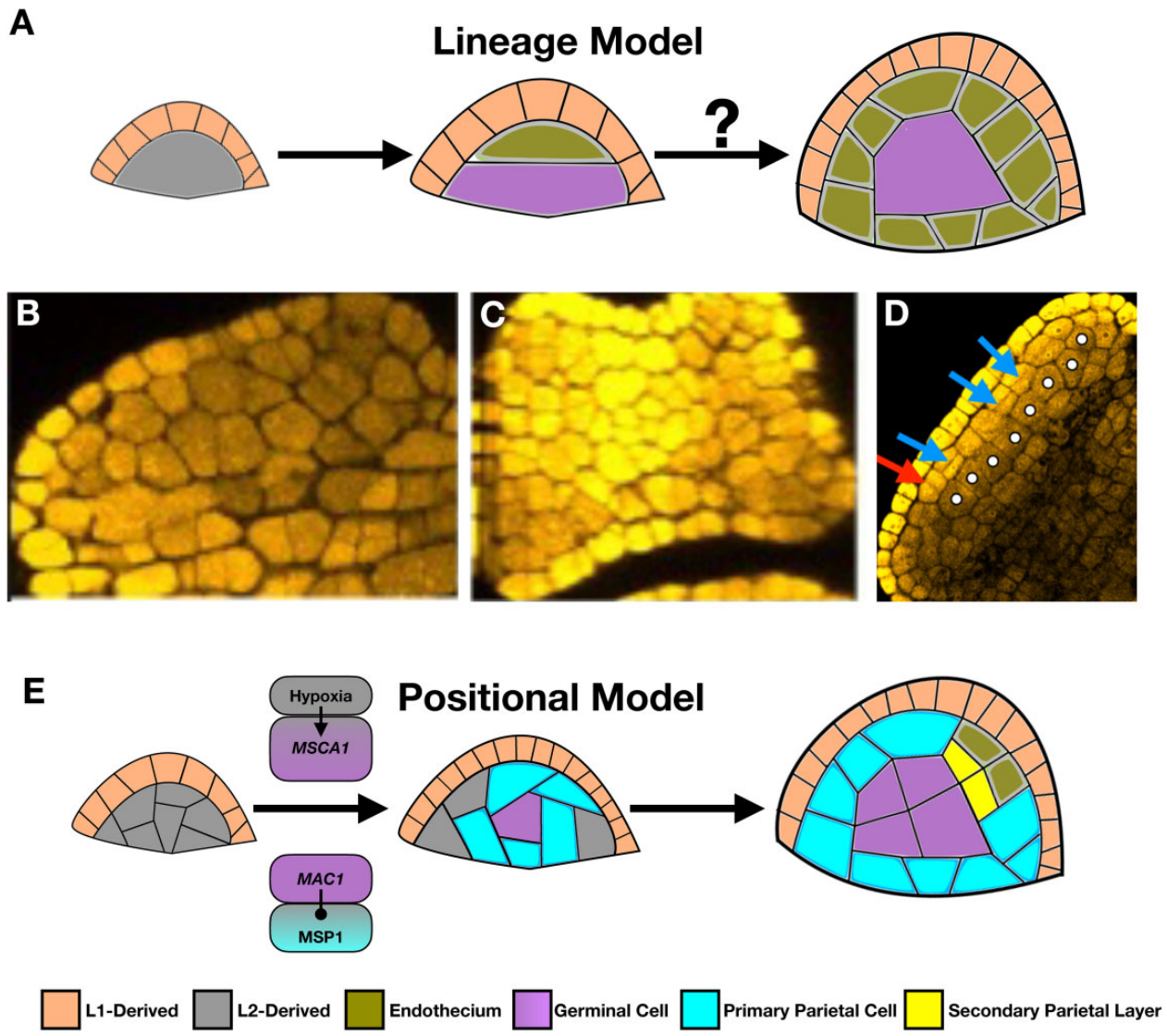

Figure 4 Lineage versus positional model. A, Until recently it was assumed that at lobe initiation there was a single hypodermal cell underlying the epidermis. This large cell was proposed to divide to make a smaller daughter cell destined to generate the somatic layers of the lobe and a larger, more interior daughter that served as the germinal lineage founder. This model had several difficulties: no marker existed for the hypodermal cell or its daughters except size, there are multiple subepidermal cells in immature lobes and by standard microscopy the cross-sectional area of one cell might appear larger than neighbors but designation was arbitrary, and the greatest difficulty was the inability to describe how a single somatic precursor cell could divide in a manner to surround the germinal cell. B–D, By confocal microscopy of immature maize lobes just prior to germinal cell specification (Kelliher and Walbot, 2011), there are multiple subepidermal cells initially as viewed from a longitudinal view of a lobe (B) or a transverse view of the entire anther (C). Germinal cell specification in maize is a process that occupies about 24 h. As viewed in a longitudinal image, individual archesporial cells are marked with a white dot (D); these cells enlarge after specification and are consistently larger than somatic cells (Nelms and Walbot, 2019). Triggered by hypoxic conditions and the expression of *msca1*, the first such cells occur in the middle of the lobe (measuring from lobe base to tip) and then are designated sequentially to form a column of 10–12 archesporial cells. None of these cells divide until the entire column has formed. E, Newly specified germinal cells secrete the MAC1 protein, which results in neighboring L2 cells becoming primary parietal cells; the mark for this differentiation is periclinal division (D, three right-most, upper arrows) to generate the endothecium and secondary parietal layer. During these cell specification events, the L1 and L2 cells continue anticlinal division (D, single left-most, lowest arrow), contributing to the doubling of anther length from the birth of the first to the final archesporial cell. Confocal images from Kelliher (2013).

TGA-type basic leucine-zipper (bZIP) transcription factors, TGA9-1/2/3, are important for early maize anther differentiation and male fertility, although they are functionally redundant only resulting in male sterility when all three genes are knocked out (Jiang et al., 2021; Liu et al., 2022). The rice glutaredoxin, TDL1A/MIL2, interacts with OsTGA1 (Hong et al., 2012; Yu and Zhang, 2019), and in Arabidopsis, two glutaredoxins homologous to MSCA1, ROXY1, and ROXY2, are required for fertility and interact with both TGA9 and TGA10 (Murmu et al., 2010). The ability of glutaredoxins to activate TGA transcription factors has also been established for other developmental contexts (Yang et al., 2021).

Considering all of the evidence, a working model is that anther L2-derived cells, and perhaps all anther lobe cells, are poised to respond to a signal by committing to the archesporial cell fate in a cascade that involves reduction of glutaredoxins and their interaction with TGA transcription factors. It is crucial to determine why only the central-most lobe cells commit to this fate normally—are central cells in a privileged location with regard to hypoxia as suggested for maize or at a specific auxin status as hypothesized for Arabidopsis? In maize, rice, and Arabidopsis, once archesporial identity is established, the germinal cells secrete a protein ligand (MAC1; MIL2; TPD1) that is perceived by neighboring L2-derived cells and triggers them to be specified as somatic cells, the primary parietal cells (Wang et al., 2012; Huang et al., 2016; Yang et al., 2016; van der Linde et al., 2018).

The germinal cell lineage is specified over approximately a 24-h period in maize, as within each lobe a column of 10–12 archesporial cells of separate origin from individual L2-derived cells (Monocot Formation Model; Figure 5). These archesporial cells undergo approximately 4 mitotic divisions to generate a population of approximately 160 cells that differentiate into pollen mother cells; the pollen mother cells are meiotically competent, depending on the *AMEIOTIC1* (*AM1*) gene (Pawlowski et al., 2009), and initiate Prophase I about 2 days later. Single-cell RNA-seq analysis of archesporial cells and pollen mother cells confirmed that there is not a stem cell(s) generating the archesporial cell population; the entire population specified early in development proliferates to generate the pollen mother cells (Nelms and Walbot, 2019).

Establishing the somatic niche

Meanwhile, the somatic cells of the maize anther remain pluripotent L2-derived cells until their differentiation is triggered by the secretion of the small protein ligand, as mentioned earlier. The newly specified maize archesporial cells secrete the protein ligand MAC1 (Wang et al., 2012), which is detected by the surrounding L2-derived cells, via the leucine-rich repeat receptor kinase MSP1 (van der Linde et al., 2018). The binding of MAC1 by MSP1 in the L2-derived cells trigger their differentiation into primary parietal cells which divide periclinally to produce the endothecium and secondary parietal layer while the L1-derived cells differentiate into the epidermis. The HD-ZIP IV transcription factor OUTER CELL LAYER4 (OCL4), which is localized to the epidermis, is needed for proper early somatic differentiation as the mutant allele of *ocl4* results in a second ectopic layer of endothecium in the outer lobe hemisphere (farthest from the connective tissue) and causes male sterility (Vernoud et al., 2009). One interpretation of these results is that the MAC1 signal stimulates differentiation and periclinal division while an OCL4-dependent signal from the epidermis suppresses periclinal division of the endothecium to ensure formation of a single cell layer. Recently, a derived lineage of male-sterile *ocl4* was found to be fertile during environmental conditioning, namely a heat wave, despite the presence of the ectopic endothecium bilayer (Yadava et al., 2021). Such fertility restoration was linked to 21-nt, phased, small interfering RNA (phasiRNA) accumulation; these small RNAs are known to accumulate during initial cell fate acquisition and are essential for male fertility in maize and rice (Nonomura et al., 2007; Zhai et al., 2015; Araki et al., 2020).

Following subsequent rounds of anticlinal divisions in all four cell types, the secondary parietal cells divide periclinally to produce the final two somatic cell types, the middle layer and tapetum. With the four somatic cell layers specified, the archesporial cells cease mitosis and re-differentiate into meiotically competent pollen mother cells and the somatic cells divide anticlinally expanding anther size to produce an empty several nanometers-wide space between the neighboring pollen mother cells in the central locule. The pollen mother cells exude callose into the locule, thickly coating their cell walls and the surrounding tapetal cells release β-glucanases to remodel the callose coat into a thinner, smooth coating around each pollen mother cell. During meiosis, anthers continue to grow in length and width; sporadic cell divisions but primarily cell expansion in the epidermis and endothecium sustain anther growth, while the middle layer cells shrink in width and height.

Microsporogenesis to microgametogenesis

Interestingly, all pollen mother cells within a lobe begin microsporogenesis synchronously, entering and proceeding through meiosis concurrently despite substantial spacing between the germinal cells (Kelliher and Walbot, 2011). Interconnections between the pollen mother cells may permit an unknown mobile signal to enforce synchrony; however, such connections have yet to be identified. Lei and Liu (2020) summarize the evidence that tapetal cells are key to meiotic entry by the neighboring pollen mother cells. It will be important to define the type of signal or nutritional molecules that the tapetum provides to the germinal cells: in maize there are approximately 30 tapetal cells for each pollen mother cell at the start of meiosis, and these tapetal cells appear to be mitotic sister cells (Kelliher and Walbot, 2011). The unit of organization may be such that a group of tapetal cells is responsible for each meiotic cell. Coordination of meiotic entry would thus require understanding how the tapetal cell groups coordinate their activities.

Over 2 days, maize pollen mother cell chromosomes are replicated and remodeled for meiosis, conduct recombination, and then in the subsequent several days, the cells divide into dyads and again into tetrads. During meiosis, the middle layer degrades completely, and the tapetal cells asynchronously become binucleate. At the same time, 24-nt phasiRNAs are rapidly synthesized in the tapetum early in meiosis and reach peak abundance around mid-meiosis (Zhai et al., 2015; Zhou et al., 2022). Four basic helix–loop–helix (bHLH) transcription factors, MALE STERILE23 (MS23),

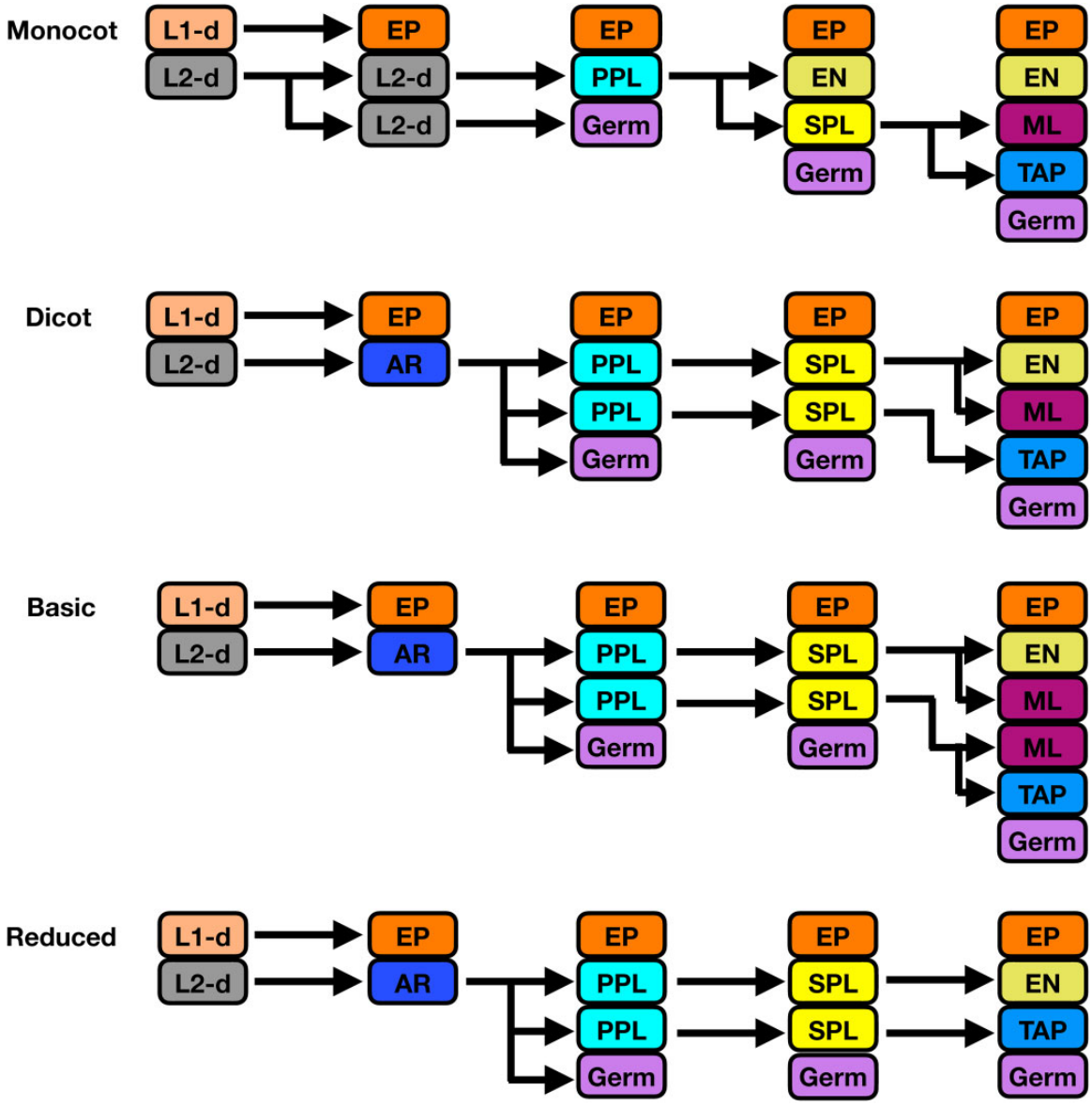

Figure 5 Anther cell layer formation models. This diagram expands on a presentation by Åstrand et al. (2021). For maize and rice and other monocots examined, the order of cell divisions and resulting cell differentiation classes are as shown and as described in the text. For dicots, cell identity and the order of events is much less clear, and there is conflicting literature. L2 derivatives are thought to proceed through the plan shown in some cases, with the initial "founder" cell called an archesporial cell that divides to make primary parietal cells as well as germinal cells (what are called archesporial cells in grasses). This interpretation of available standard microscopy contains aspect of the original lineage model in having a founder cell that divides to make the initial somatic and germinal cell. Support for or refutation of this hypothesis is needed to clarify the actual order of events in particular species. Subsequently, the somatic cell becomes bilayered primary parietal cells, and these cells become the secondary parietal layer and then daughters differentiate as the three somatic cell layers. In contrast to monocots, the tapetal cells arise directly from an existing secondary parietal layer cell rather than being generated by a periclinal division of a secondary parietal layer cell that generates middle layer and tapetal sister cells. The Basic model is a schematic to attempt to explain dicots with two middle layers, and in the Reduced model, a hypothesis for anthers with no middle layer. The variation in middle layer presence or the number of layers when present signifies that the development of the middle layer has not been canalized into a single pathway. For this reason, there could be more variation in its ontogeny than for other layers, particularly the epidermis and germinal cells. L 1-derived (L1-d); L 2-derived (L2-d); archesporial cell (AR); epidermis (EP); primary parietal cell layer (PPL); secondary parietal cell layer (SPL); germinal cell lineage (Germ); endothecium (EN); middle layer (ML); tapetum (TAP).

MS32, bHLH122, and bHLH51, are sequentially expressed in the tapetum and are essential for male fertility during meiosis; the former three transcription factors are needed for 24-nt phasiRNA production (Nan et al., 2022). In rice, the homologs of these four maize bHLH transcription factors are TAPETUM DEGENERATION REDARDATION (TDR) INTERACTING PROTEIN2 (TIP2), UNDEVELOPED TAPETUM1 (UDT1), ETERNAL TAPETUM1 (EAT1)/DELAYED TAPETUM, and TDR, respectively. Arabidopsis has three functionally redundant paralogs homologous to maize

bHLH122 and MS23 (bHLH010, bHLH089, and bHLH091), while DYSFUNCTIONAL TAPETUM1 (DYT1) is homologous to MS32 and ABORTED MICROSPORES is homologous to bHLH51.

Upon completion of meiosis, the tapetum again excretes β-glucanases degrading the callose surrounding the tetrads and releasing the haploid meiotic products from the pollen mother cell coat to become uninucleate microspores. These haploid cells contain a single nucleus and large vacuole, and their release marks the end of microsporogenesis. In the next phase, the tapetum secretes exine components such as sporopollenin-precursor macromolecules into the locule that deposit onto outer surface of the uninucleate microspores where the precursors are polymerized. During this process, the uninucleate microspores attach to the outer surface of groups of tapetal cells, a crucial step for pollen maturation (Kelliher and Walbot, 2011). After 11 days, the uninucleate microspores enter microgametogenesis undergoing an asymmetric mitotic cell division (Pollen Mitosis I) to become bicellular microspores containing a small generative cell and larger vegetative cell, as the tapetum degrades via programmed cell death. The vegetative cell essentially engulfs the smaller generative cell, surrounding it completely. The death of the tapetum releases its final cellular contents into the anther locule to cover the bicellular microspores, forming the final pollen coat. In maize, the septum between the abaxial and adaxial lobes of each theca degrade—mediated by changes in IMS1—producing bilocular anthers and the generative cells of the bicellular microspores undergo mitosis again (Pollen Mitosis II) becoming tricellular mature pollen grains with two haploid sperm cells within the cytoplasm of the vegetative cell and ending microgametogenesis. Pollen is released from anterior pores that form at the tips of the two anther theca upon dehiscence.

The anther cell layers

The germinal cell lineage

Substantial insight into the transcriptional changes when differentiating from the archesporial cells to pollen mother cells to meiocytes to uninuclear microspores to bicellular microspores to mature pollen has been provided by single-cell RNA-seq of these stages across the ∼30 days of maize germinal cell development (Nelms and Walbot, 2022, 2019). Key observations are that all archesporial cells divide during early development and that the transcriptome is relatively constant during this proliferation period. Despite their immaturity, archesporial cells express some meiosis-associated genes from their inception (Kelliher and Walbot, 2014). Early in Prophase I there is a substantial reorganization of the transcriptome at approximately the leptotene/zygotene transition, followed by another period of relative constancy. The Prophase I meiocytes contain the highest amount of RNA compared to prior and later stages. Although it has long been assumed that activation of the haploid genome would occur in uninuclear microspores, the independent gametophyte stage, transcripts synthesized in diploid cells prior to the first meiotic division persist and there is no substantial contribution from the haploid nucleus to mRNA composition until approximately the uninuclear microspore to bicellular microspore transition. At this point, there is a major reorganization of the transcriptome, which is followed by a second burst of new haploid synthesis in maturing, trinucleate pollen.

The nurse cells: the tapetum

Found in all land plant sporangia, the tapetum is the nutritive "nurse" cell layer that insulates the germinal cells from the rest of the somatic tissue. It is essential for sporogenesis as it sustains, remodels, and fortifies the germinal cells before, during, and after meiosis. While the tapetum is found in all land plants, the exact functions of the tapetum are most studied in the anthers of flowering plants. Classically, the tapetum has three roles in the anther: (1) providing nutrients to the germinal cells; (2) pollen mother cell and microspore wall reconfiguration; and (3) microspore wall deposition. More recently, it has been demonstrated that the tapetum is essential for lipid production and wax and cuticle biosynthesis as the maize gene *male sterile33* (*ms33*) is predominantly expressed in the tapetum and the *ms33* mutant has defective anther cuticle formation and early microspore degradation leading to male sterility (Zhang et al., 2018b). A subsequent analysis of *ms33* found that the mutation also led to aberrant chloroplasts in the endothecium, as these organelles rely on glycolipids and phospholipids for their membranes (Zhu et al., 2020). As the source of energy in the developing anther, these abnormal chloroplasts were ineffective at starch turnover leaving the tapetum starved for carbohydrates. These results raise a variety of questions regarding the transportation of molecules within the anther: without plasmodesmata (PM) across the tapetum and middle layer, how are carbohydrates transported from the outermost to innermost cell layers? What pathways control the transportation of lipids synthesized in the tapetum to the endothecium and epidermis?

An important and often overlooked feature of the tapetum is sequential redifferentiation for new roles. Many cell types achieve terminal differentiation for a specific function; however, tapetal cells perform distinct roles that require remodeling of the nucleus, cytoplasm, organellar composition, and cell wall to acquire new capabilities. As previously stated, the tapetum differentiates from the secondary parietal layer then rapidly proliferates to become the most abundant cell type in the premeiotic anther. Tapetal cells then undergo nuclear division without cytokinesis, producing binucleate cells dense with organelles, while the germinal cells undergo meiosis. Chen et al. (2019) found a greater than six-fold increase in the total mitochondrial volume per tapetal cell in Arabidopsis during meiosis and shortly after meiosis. During meiosis, tapetal cells begin synthesis of sporopollenin and other components essential for pollen structure. To facilitate secretion of these structural components, tapetal cells fully degrade or substantially remodel the wall facing the germinal cells (Cheng et al., 1979). During

pollen maturation, the tapetum undergoes programmed cell death to form the adhesive layer upon which the pollen grains attach in maize (Kelliher and Walbot, 2011). Similar to archesporial cells re-differentiating into meiotically competent pollen mother cells then microspores and eventually mature pollen grains, the tapetum re-differentiates to match its various roles. Clearly, a finely choreographed interplay between the tapetum, germinal cells, and other somatic cell layers is necessary for male fertility as the majority of maize male sterile mutants have tapetal defects (Skibbe and Schnable, 2005).

The tapetum has been of distinct interest among plant systematists and evolutionary biologists for decades reflecting its diverse and sequential roles in nutritional support and interdependence with germinal cells across the major lineages of land plants. Differences in tapetal structure were used to characterize land plant clades at the family and ordinal levels, leading to an abundance of classic, largely microscopy-based literature on tapetal cellular architecture and development across the land plant tree of life (reviewed in Pacini et al., 1985; Pacini, 2016). The tapetum can vary wildly in lifespan, from days (most angiosperms) to months (most gymnosperms); shape, in some cases fully invading the anther locule and enclosing the developing microspores; and features (Pacini, 2016). For example, Ubisch bodies, or orbicules, are extracellular particles of sporopollenin which are released from the tapetum to line the pollen grain wall. Based on microscopy-based characterization, these tapetal products are present in plant families spanning all of land plants, yet certain clades, such as Brassicaceae, completely lack them (Verstraete et al., 2014). Ubisch bodies have only recently been incorporated into male-sterility studies in cereals (Chen et al., 2017; Somaratne et al., 2017; Yang et al., 2019; Zhang et al., 2021), providing insight into the genes underlying the presence of these tapetal products; however, the actual function of Ubisch bodies is still unknown. Despite the historical focus on the tapetum, few studies outside of model species (e.g. Arabidopsis, rice, and maize) have genetically characterized or implemented modern comparisons of tapetum development or tapetum-specific genes.

A recent analysis of a moss (*Physcomitrium patens*) sporangium development identified two bHLH transcription factors that were essential for tapetal development and fertility (Lopez-Obando et al., 2022). Interestingly, these genes were homologous to maize *bHLH51* and *ms23* (Nan et al., 2016, 2022), which are also essential for tapetal development and fertility, demonstrating the evolutionary conservation of the tapetum and the genes controlling its development. Fully understanding the development and role of the tapetum in diverse plant systems can provide an evolutionary framework for identifying male-sterility genes in crops lacking substantial genetic resources and permit the identification of key tapetal regulatory genes to assess gene family conservation and novelty. Ideally, quantitative and spatiotemporal analyses of the tapetum, perhaps using single-cell RNA-sequencing, in diverse taxa would illuminate the successive functions, including clade-specific aspects, and associated re-differentiation steps of this critical cell layer.

Relic or barrier: the middle layer

The most enigmatic of the maize anther cell types, the middle layer is short-lived and lacks any known role or marker genes. It is sister layer to the tapetum following the periclinal division of the secondary parietal layer. In Arabidopsis anthers, the middle layer was recently shown to arise from either the inner or outer secondary parietal layer (Xue et al., 2021) and is clearly important for auxin signaling late in anther development (Cecchetti et al., 2017). In maize, the middle layer is only present in the anther until the end of meiosis then is either mechanically crushed by the rapidly expanding and dividing tapetal cells or undergoes programmed cell death. van der Linde and Walbot (2019) proposed that the middle layer may serve to separate the tapetum from direct communication and influence of the endothecium. Alternatively, the middle layer, even upon degradation, may serve as a protective layer for the tapetum from the endothecium to fortify the essential "nurse" layer from reactive oxygen species (concept provided by R. Egger, personal communication), which can cause cell death if not removed and are known to be accumulated in the endothecium as a byproduct of energy-generation (Murphy, 2015). Historical microscopy-based studies have often overlooked the middle layer, labeling it as a second endothecium or second tapetum. While certain plant taxa clearly have multiple cell layers in between the tapetum and endothecium (e.g. Hamamelidaceae, Magnoliaceae, Myristicaceae, and Stegnospermaceae) (Kubitzki et al., 2013), the developmental origins, homology, and therefore identification of these cell types as middle layers needs to be further investigated. Furthermore, is there a role for additional "middle layers" in anther development? Could there be a need for added protection or separation in these taxa or is the middle layer truly just a vestigial "relic feature" of land plant evolution (Davis, 1967)?

The powerhouse of the anther: the endothecium

The endothecium is one of the earliest cell layers to be established in the anther, originating from the primary parietal layer. These cells are essential for anther development as they are the dominant site of starch synthesis and photosynthesis in the anther. Chloroplasts are principally found in the endothecium of the anther, more specifically the endothecial cells directly adjacent to the epidermis (subepidermal endothecium) and not those adjacent to the connective tissue (interendothecium) (Murphy et al., 2015; Zhu et al., 2020). Up until the end of meiosis, endothecial chloroplasts biosynthesize, and store starch granules, which are expended as energy sources at night (Zhu et al., 2020). Upon completion of meiosis and coinciding with the programmed cell death of the tapetum, the chloroplasts begin to photosynthesize (Zhu et al., 2020). Furthermore, Nan et al. (2022) found that the tapetum may control chloroplast production and function and overall endothecium development based

on the transcriptional ramifications of the tapetum-specific *ms23* mutant. While the broad function of energy production by the endothecium is established, much of the details surrounding this cell type are still unknown. Does the inter-endothecium originate from the primary parietal cells as the subepidermal endothecium do? How distinct are their functions? Should these be classified as two separate cell types? Does the production of reactive oxygen species via photosynthesis in the endothecium trigger programmed cell death in the tapetum?

The outer defense: the epidermis

The epidermis is the first layer to be established and, alongside the endothecium, the last to persist. As previously stated, OCL4 synthesized in the epidermis is needed to repress additional endothecial divisions solely within the outermost hemisphere of an anther lobe (Vernoud et al., 2009). The transcripts are localized to the epidermis, but the localized effect of the *ocl4* mutant on the cells furthest from the connective tissue suggests some kind of gradient or variation among the epidermal cells. In addition, the epidermis does not initially produce a defensive cuticle around the anther, perhaps because the young anthers are surrounded by fluid. Little else is known regarding the subsequent functions and transcriptional regulation of the epidermis once cuticle production begins. When exactly does cuticle formation initiate? How transcriptionally distinct is the anther epidermis from leaf or stem or petal epidermis?

While investigating MSCA1, Chaubal et al. (2003) found that young, wild-type maize anthers lacked stomata; however, stomata were present in the male-sterile mutant *msca1*. Do stomata form postmeiosis once the endothecial cells start photosynthesizing? If not, how do those cells attain the needed carbon dioxide and release oxygen? Does the tapetum provide lipids to the epidermis, similar to the endothecium? The maize β-1,3-galactosyltransferase encoding gene, *male sterile8* (*ms8*), and rice gene *wax-deficient anther1* lend credence to this possibility as both of these male-sterile genes are expressed in the tapetum and affect cuticle formation and the pollen coat (Jung et al., 2006; Wang et al., 2013). In addition, the synthesis of anther cuticle and pollen exine share the same lipidic pathways in rice tapetal cells (Li et al., 2010; Shi et al., 2011, 2015). Finally, what genes are needed for anther anthesis and the opening of the epidermis and endothecium to release the mature pollen grains?

Current puzzles and future directions in anther biology

Despite intensive efforts to recover and characterize male-sterile mutants for basic research and to generate materials useful in plant breeding, there are still vast gaps in our knowledge. We close out this review by highlighting current puzzles and challenges in anther biology and areas requiring further study.

Challenges for anther analysis

Anthers form at the end of the annual plant lifecycle, requiring growing plants for a substantial period prior to study. Anthers express the most genes of any organ (Nelms and Walbot, 2022), most overlapping with other sporophyte organs along with anther-specific genes and gametophyte (pollen)-specific genes. Therefore, many genes involved in anther development and function are also likely involved in embryo and vegetative plant development; mutations in these will disrupt early stages and prevent genetic analysis in the anther unless an "anther-specific" allele is found. For this reason and the redundancy in some gene families, genetic analysis is limited to and focuses on individual genes or small families that when mutated yield male sterile, defective anthers. Both microscopy and biochemistry—including transcriptomics, proteomics, lipidomics, and metabolomics—are essential tools for analyzing normal development and comparison to mutant states. As in all developmental studies, mutants are problematic because the exact nature and timing of a defect can be unclear: cytological defects are often delayed relative to the normal time of action of a particular gene product. The identity of mutant cells is usually unclear: is the cell arrested or has it embarked on a novel developmental pathway? The identity question can be partly resolved through single cell RNA-seq, at least with regard to the transcriptome. Given the limitations of genetic and cytological methods, experimental approaches manipulating conditions and more biochemical characterization are required to understand fully the complex ontogeny of anthers and pollen. Inspired by the example of single-cell RNA-seq analysis of the germinal lineage, we can anticipate similar studies of all lobe cell types. More precise information about the transcriptomes of individual cell types should clarify where specific gene products are likely produced. If the site of action is in another cell type, it will require attention to trafficking within lobes to resolve.

Clarifying the origin of anther lobe somatic cells

For maize, the order and ontogeny of the L2-derived somatic cells is firmly established by a combination of confocal microscopy charting cell numbers and 5-ethynyl-2′-deoxyuridine (EdU) fluorescent labeling of DNA synthesis and cell division events (Monocot Formation Model; Figure 5). The primary parietal cells divide to generate the outer endothecium and inner secondary parietal layer; later secondary parietal layer cells divide periclinally to establish the middle layer and tapetum. For eudicots, a different model has been proposed (Åstrand et al., 2021) in which the primary parietal cells divide periclinally; the inner cells develop directly into tapetum and the outer cells (secondary parietal layer) divide periclinally to establish the endothecium and middle layer (Dicot Formation Model). In some species, there is no middle layer, and here it is proposed that the primary parietal cell periclinal division establishes the inner tapetum and outer endothecium directly (Reduced Formation Model). Other example eudicots have two middle layers; in this case both the primary parietal cells and secondary parietal layer

make a middle layer, resulting in two rings of middle layer cells in each lobe (Basic Formation Model). A further twist, which deserves additional investigation, is a recent report that in Arabidopsis middle layer cells originate from divisions of both the tapetum and endothecium to result in a single ring (Xue et al., 2021). This model is based on microscopic identification of new (thinner cell walls). For the eudicots, it would be very helpful to have EdU labeling evaluations, because sister cells share the same intensity of nuclear DNA fluorescence, permitting lineage reconstructions over a few days of development. Such labeling combined with confocal microscopy to measure cells in all three dimensions would clarify and hence greatly contribute to our understanding of somatic cell specification dynamics and its variations.

Intercellular communication within anther lobes

Primary PM form between sister cells during cell plate formation, and if these remain open, sister cells share cytoplasm. Secondary PM can form between neighboring cells, either nonsisters within a layer or cells can become interconnected between layers. Although PM have been observed in anther lobes, we lack a "map" of these connections for any species. To what extent do all of the somatic cells share cytoplasm? Given the pattern of periclinal cell divisions a few endothecium and middle layer + tapetal cells would be interconnected across layers, but if no secondary PM form, most connections are between cells within a layer. Do secondary PM form routinely? If so, when in development? Furthermore, does the L1-derived epidermis layer form secondary PM with the endothecium and hence communicate with the entire L2-derived soma? This point merits further investigation, given the recent report that fatty acids and other building blocks for cuticle in the epidermis and plastid lipids destined for the endothecium are synthesized in the tapetum and delivered to these outer lobe cell types (Zhu et al., 2020).

A fascinating possibility for the meiotic entry trigger is a signal from the adjacent tapetal layer. What types of connections exist between the tapetum and germinal cells, which share no immediate predecessors? Are secondary PM or other channels formed "just in time" to modulate meiotic entry and possibly supply required nutrients or other materials to the pollen mother cells and meiocytes? 24-nt phasiRNAs are primarily synthesized in the tapetum, starting at the Prophase I stage of meiosis; however, these small RNAs accumulate in the meiocytes, peaking in late Prophase I – first mitotic division (Zhou et al., 2022). Are the 24-nt phasiRNAs transported through channels? If so, what type of channels exist? Is transport via secretion and uptake, despite the presence of the callose coat around the meiocytes?

New protocols, such as small dyes introduced into individual cells could chart small molecule movement throughout the anther lobe. Of equal interest is the route and distribution of materials delivered by the central vasculature: is transport through the connective apoplastic or symplastic? Do the endothecium cells, which contain chloroplasts and accumulate copious starch (Murphy et al., 2015) take up sugar, store sugar, and also transport sugar through PM or other means to nourish other lobe cell types? Is distribution primarily endothecium to endothecium cells, resulting in equal sugar concentration in the entire ring, then delivery to internal cells? Or do the interendothecial cells (endothecium adjacent to the connective that lack green chloroplasts) take up sugar and then preferentially distribute it into the adjacent middle layer and tapetum, generating a concentration gradient across the lobe?

Trafficking

Communication between cells within a tissue and between tissue layers within the lobes integrates information (nutritional, developmental, and environmental) from signaling molecules and the redistribution of macromolecules such as proteins, lipids, small RNAs, and even mRNAs. Much work is needed to define the traffic routes and molecules and assess their rates and roles in coordinating anther development. One example serves as a reminder that the site of synthesis and the site of accumulation can differ. Zhou et al. (2022) found that maize tapetal cells in Prophase I anthers contain the long precursor transcripts for 24-nt phasiRNAs as well as miR2275, the microRNA trigger of 24-nt phasiRNA biogenesis, and transcripts for *dicer-like5* (*dcl5*) required for cleaving the precursors into the 24-nt products (reviewed in Liu et al., 2020). Similar results have been found in rice (Ono et al., 2018) and Arabidopsis (Long et al., 2021), confirming that the tapetum is the site of biogenesis for 24-nt phasiRNAs, and yet these small RNAs accumulate to much higher levels in meiocytes by late Prophase I. How are the 24-nt phasiRNAs transferred from tapetal to meiocyte cells? In addition, how evolutionarily conserved are these transfer methods across land plants?

Expanding the evolutionary sampling of anther development

More species should be examined to better understand the common and clade-specific aspects of anther development. Generating and analyzing mutants can be difficult and time-consuming. This review has focused on maize, with examples also drawn from Arabidopsis and rice. Our understanding of angiosperm anther ontogeny derives almost completely from these three species. Some principles are conserved in these two grasses and one Brassicaceae: the archesporial to soma, small protein ligand—receptor module and the importance of glutaredoxin(s) and TGA transcription factors. It would be enormously useful to have some information about a legume, a solanaceous species, and an asterid, three large families of eudicots plus a nongrass monocot. Early-diverging angiosperms should also be investigated, such as *Amborella*, and monocots with extensive genome resources such as *Pharus*.

Therefore, what may be more generally useful and more readily obtained would be analyses along the following lines.

(1) What is the developmental timeline for a species: days from anther inception to the start of meiosis, duration of meiosis, and postmeiotic development to pollen shed?
(2) Does the endothecium store starch (KI/I_2 stain) and contain chloroplasts (red fluorescent signal)?
(3) Are there four somatic wall layers in the lobe, or is the species an exceptional three layer (no middle layer) or double middle layer type?
(4) By EdU labeling, can the pattern of cell division established in the somatic wall layers be deduced?
(5) If a genome sequence exists, are there orthologs of the key genes? In a premeiotic anther transcriptome, are these orthologs expressed?

Anthers and the environment

Another frontier is the developmental resilience and limits in anther development (reviewed in De Storme and Geelen, 2014; Raja et al., 2019; Chaturvedi et al., 2021). Adverse environmental conditions can limit pollen production, to the detriment of crop yield. A number of conditional male-sterility cases are well studied in rice (Fan et al., 2016; Fan and Zhang, 2018), corn (Liu et al., 2020; Yadava et al., 2021), and other plants. What makes anther development so sensitive to particular environmental conditions? Most plants produce a huge excess of pollen, relative to embryo sacs, thus it may be that pollen production from individual anthers is dispensable. What processes in anther development manifest the environmental sensitivity? Can this sensitivity be eliminated or modified in crop plants? As Darwin pointed out with numerous examples in his book "The Different Forms of Flowers on Plants of the Same Species" (Darwin, 1897), plants often adopt alternatives to their standard reproductive strategy to ensure some seed set. Plants with open flowers, requiring wind or an animal pollinator, can switch to cleistagamous flowers if there is low seed set. Plants with initial low seed set and a strong self-incompatibility barrier, can later deploy self-compatible flowers. The modern focus on mutant analysis uncovers extreme cases and may highlight environmental sensitivity, but there may be higher tolerance of developmental variations in response to the environment in "wild type" crop plants and in undomesticated species under variable conditions.

Acknowledgments

We thank Chong Teng, Junpeng Zhan, Sebastien Belanger, Blake Meyers, and Xiangyuan Wan for their feedback on this manuscript.

Funding

This work was supported by the US National Science Foundation Plant Genome Research Program (NSF-PGRP) awards Postdoctoral Research Fellowship in Biology 1907220 (to D.B.M.) and 17540974.

Conflict of interest statement. None declared.

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