Comparative anther and pollen tetrad development in functionally monoecious *Pseuduvaria trimera* (Annonaceae), and evolutionary implications for anther indehiscence

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Title Page

**Comparative anther and pollen tetrad development in functionally monoecious
Pseuduvaria trimera (Annonaceae), and evolutionary implications for anther
indehiscence**

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Abstract

Multiple evolutionary origins and diverse morphologies of unisexual flowers in angiosperms indicate that many different developmental mechanisms (sporophytic and/or gametophytic tissues) underlie patterns of sex differentiation; yet these mechanisms, leading to unisexuality, remain largely unresolved. In *Pseuduvaria trimera*, morphologically hermaphroditic flowers are functionally female owing to indehiscent anthers, but the developmental and anatomical mechanisms preventing their dehiscence are still unknown. Anther and pollen development were compared in both male and functionally female flowers using histological observations to test whether anther indehiscence results from a sporophytic and/or gametophytic default. The epidermis, endothecium, middle layers, and pollen development were identical in the two floral morphs, but variations occurred in the tapetum and stomium regions. In male flowers, concurrently with the binucleate tapetal cell degeneration, the appearance of intercellular spaces and lysis of the stomium region cells lead to anther dehiscence. Conversely, in the functionally female flowers, trinucleate tapetum appears with delayed degradation, and the persistent cells with a highly vacuolated cytoplasm and stomium region remain intact at maturity. Sporophytic tissues with tapetum abnormalities and stomium integrity are, thus, responsible for anther

indehiscence. Lack of microspore rotation in *P. trimera* might indicate a different evolutionary origin of pollen tetrad formation in this family.

Key words: tapetum abnormalities, stomium integrity, functionally female flowers, gametophytic defect, histological observations.

Introduction

The majority of flowering plants have hermaphroditic sex expression, with flowers bearing functional male and female organs (Endress and Doyle 2009; Diggle et al. 2011), whereas approximately 10% of flowering plants produce unisexual flowers, in which organs of one sex are either absent or not functional (Mitchell and Diggle 2005; Renner 2014). If the nonfunctional sex organs are morphologically similar to the functional ones, the flowers are commonly referred to as functionally male or female flowers (Mitchell and Diggle 2005; Diggle et al. 2011). A considerable body of anatomical studies have shown that flowers may become functionally unisexual by sporophytic (epidermis, tapetum, middle layers, endothecium or stomium region tissue) and/or gametophytic (microspores and pollen grains) developmental defects, such as a sporophytic abnormality (tapetum abortion) in species from the Cactaceae (Strittmatter et al. 2002, 2006; Flores-Rentería et al.

2013), microsporogenesis and microgametogenesis abnormalities in *Vitis vinifera* (Caporali et al. 2003), and sometimes both, such as species in Sapindaceae where the developmental abnormalities of the tapetum, endothecium and microspores result in indehiscent anthers (Solís et al. 2010; Zini et al. 2012). Moreover, ontogenetic studies on a range of early divergent angiosperm families have led to an understanding of the easy occurrence of unisexuality and the diversity of their developmental pathways (Fu et al. 2009; Xu and Ronse De Craene 2010; Dong et al. 2012; Yang and Xu 2016, 2017). However, the anatomical mechanism leading to sex organ abortion and evolution of functionally unisexual flowers is not well-known in these early divergent lineages.

Annonaceae are one of the particularly interesting families in early divergent angiosperms for the study of the evolution of functionally female flowers (Couvreur et al. 2011), as they display great diversity in their reproductive morphology. Most species with (functionally) unisexual flowers have male and bisexual flowers on the same plant (andromonoecious) or not (androdioecious) (Su and Saunders 2006; Su et al. 2008; Saunders 2010). However, in *Pseuduvaria*, flowers on the same plant can be either morphologically male, or bisexual but functionally female because the anthers of the stamens fail to dehisce (Pang et al. 2013; Yang and Xu 2016). However, it is

still unclear whether sporophytic and/or gametophytic tissues are linked to the indehiscence of the anthers. The available anatomical data in Annonaceae are limited to representatives with bisexual flowers, and focused on either anther septa development (Periasamy and Thangavel 1988; Tsou and Johnson 2003), or the compound pollen development and its binding mechanism between microspores (Periasamy and Kandasamy 1981; Gabarayeva 1993; Tsou and Fu, 2002, 2007; Lora et al. 2009, 2014). In diclinous species of this family, no anatomical investigation regarding the development of anthers and pollen tetrads or abortive processes has been reported.

In this work, to identify whether the failure of anther dehiscence in functionally female flowers in *Pseuduvaria trimera* is due to sporophytic and/or gametophytic developmental defects, comparative anther wall, pollen tetrad, and stomium region development were investigated in both floral morphs. Features accompanying tetrad cohesion were also compared with those of previous studies to identify the possible evolutionary origin of pollen tetrads in this family. Special attention was given to the cytological events associated with the development of the stomium region and tapetum to provide detailed anatomical features related to anther dehiscence and non-functionality.

Materials and Methods

Between December 2012 and February 2014, fresh male and functionally female inflorescences at different developmental stages were collected from trees at the Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences, and fixed immediately in FAA (70% alcohol, formaldehyde, and glacial acetic acid in a ratio of 90:5:5).

To analyze anther development, histological sections of anthers from both male and functionally female flowers were examined from microsporogenesis to maturity. For light microscope observations, stamens and staminodes at various stages were dissected and stored in 2% glutaraldehyde with 0.1 M phosphate buffer at pH 7.2–7.4 overnight at 4°C. About 20 samples per stage were dehydrated in an ethanol series, embedded in Spurr Resin, and sectioned at 2 µm using a rotary microtome. Sections were stained with toluidine blue and observed under a light microscope (Leica, DM5500B). Herbarium materials were deposited at the South China Botanical Garden (IBSC).

Results

In *Pseuduvaria trimera*, male flowers contained only stamens and no carpels or carpel residues were observed (Figs. 1A and 1C). All morphologically bisexual flowers have indehiscent anthers, and are thus always functionally female (Figs. 1B and 1D). Nine developmental stages were identified based on distinctive cellular events of the gametophytic and anther wall development and these are described in Table 1. The anthers were tetrasporangiate in all male flowers (Fig. 1E), and most functionally female flowers (Fig. 1F), which occasionally had only two (Fig. 1G) or three differentiated pollen sacs (Fig. 1H). The stomium region located within the notch region of the two adjacent anther locules (Figs. 1E, 1F, and 1H) was the site of anther dehiscence and pollen tetrads release in anthetic male flowers (Fig. 1E).

Anther wall and pollen development

Male flowers. During the development of the anthers, 2–5 layers of cells were observed between the epidermis and the sporogenous tissue, depending on the developmental stage and region of the anther. The epidermis and endothecium layers persisted in the mature anther, whereas the middle layers and tapetum disappeared during pollen development. At the early microspore mother cell stage of development, microspore mother cells were distinguished by their large nuclei and often large nucleoli, and characterized by their polygonal shape and compact arrangement (Fig.

2A). Occasionally, the migration of the chromatin towards the periphery of the nucleus could be observed in the microspore mother cells just before meiosis (Fig. 2B). At this stage, the young anther wall was composed of an epidermis, an endothecium, two middle layers, and a tapetum. Tapetal cells were slightly polygonal in shape and their inner tangential common walls with the microspore mother cells, whereas the remaining anther wall cells were uniform in size (Fig. 2A). Large nuclei and vacuoles were evident in the tapetal cells at the late microspore mother cell stage (Fig. 2B). Upon entering meiosis, the microspore mother cells were dissociated completely from the tapetum and surrounded by a callose layer, which persisted during the entire meiosis (Fig. 2C). Cytokinesis was simultaneous and occurred through the formation of centripetal callose furrows after the second nuclear division (Fig. 2D). After meiosis, tetrahedral (Fig. 2E), tetragonal (Fig. 2L), and rhomboidal microspore tetrads (Fig. 2F) were produced. During this meiotic stage, tapetum cell cytoplasm was condensed and started to undergo karyokinesis without cytokinesis. By the end of meiosis, almost all the tapetal cells were binucleate. The middle layer cells began to disintegrate and the endothecium cells became slightly larger than its neighboring anther wall layer cells, while the epidermis did not appear to show evident changes during the meiosis stage (Figs. 2C and 2D). At the tetrad stage, the

tetrads randomly scattered in the anther locules and the nucleus of the haploid microspore was located in their center (Fig. 2E). At this stage of development, the cytoplasm of the tapetal cells became shrunken and much denser than the previous stage, and this indicated that the tapetum started to degenerate (Fig. 2E). Following the dissolution of the callose envelope that holds the four microspores together, the microspores continue to adhere to each other and form a permanent pollen tetrad at the early uninucleate microspore stage (Fig. 3H). An incipient microspore wall was observed at this stage. At the early intermediate uninucleate microspore stage, the nucleus and the cytoplasm migrated from the center of the cells towards its internal side (facing the center of the tetrad) with the formation of the large vacuole. At this stage, a well-developed microspore wall including the exine and the intine was visible, and a translucent microspore wall layer was present surrounding the condensed cytoplasm (Fig. 2F). When haploid microspores developed a big vacuole, the cytoplasm of the tapetal cells became granular, the middle layers were completely flattened and disorganized, and the radially enlarged endothecium became conspicuously larger than any of the anther wall layers (Fig. 2F). By the end of the intermediate uninucleate microspore stage, the microspores expanded greatly in size and its large vacuole developed into several small vacuoles (Fig. 2G). As small

vacuoles vanished, the nucleus moved to the external margin of the dense cytoplasm (distal side of the tetrad) at the late uninucleate microspore stage (Fig. 2H). By the late uninucleate microspore stage, the tapetal nucleus degenerated completely and its cytoplasm became thin. The endothecium cells developed strong fibrous thickenings on their radial and inner tangential walls, and the crushed middle layers and disconnected epidermis were still visible (Figs. 2G and 2H). After one asymmetric mitotic division, each microspore had a lens-shaped generative cell against the pollen wall, and a vegetative cell that occupies most of the pollen grain volume at the early bicellular pollen grain stage (Figs. 2I and 2J). The generative cell subsequently migrated to the center of the pollen grain and was completely enclosed by the cytoplasm of the vegetative cell at the late bicellular pollen stage (Fig. 2K). No remnants of the middle layers and tapetum were detectable at this late bicellular pollen stage (Fig. 2K). The pollen grains were bicellular at maturity (Fig. 2L). The endothecium and epidermis were the only remaining layers that constituted the anther wall from the early bicellular pollen stage to the mature pollen stage (Figs. 2K and 2L). Tapetal cells degraded completely at their original sites and therefore, the tapetum was of the secretory type.

Functionally female flowers. The microsporogenesis, microgametogenesis, and the

development of the epidermis, endothecium, and middle layers were identical to that of the male flower (Figs. 3A–3O), and only that of the tapetum differed from the early microspore mother cell stage onwards. Compared with male flowers, the tapetal cells started to undergo karyokinesis earlier at the early microspore mother cell stage (Fig. 3A) instead of during meiosis and were mostly binucleate in early meiosis (Fig. 3B). The binucleate tapetal cell subsequently underwent a further nuclear division, producing the trinucleate tapetum by the end of the meiosis stage (Figs. 3D and 3E), that was not observed in male flowers. Tapetal cells degraded later at the early uninucleate microspore stage, but not the tetrad stage as in male flowers (Figs. 3G and 3H). At the late bicellular pollen stage, the remaining tapetal cytoplasm was present with numerous tiny vacuoles (Fig. 3M), while the tapetal cytoplasm degenerated completely in male flowers. Thereafter, the vacuolated tapetal cytoplasm either persisted or continued degenerating. At the mature pollen stage, the vacuolated tapetal cell and cell remnants were detectable, indicating that the tapetal cytoplasm degenerated incompletely (Figs. 3N and 3O).

Stomium region development

Male flowers. No morphological differences were exhibited in the cells of the stomium region and the neighboring connective tissue at the early microspore mother

cell stage (Fig. 4A). The stomium region cells were distinguished by their smaller sizes than those of the adjacent connective tissue at the tetrad stage (Fig. 4B). During the intermediate uninucleate microspore stage, the stomium region cells become smaller in size than the adjacent connective ones, in which some druses (crystals of calcium oxalate) were present (Fig. 4C). Afterwards, intercellular spaces appeared among and within the stomium and the parenchyma of the septum within which the cells lost cell adhesion by the early bicellular pollen stage (Fig. 4D). At the late bicellular pollen stage, the septum cells disintegrated and dissociated from the connective cells (Fig. 4E). The stomium cells eventually became disrupted, leading to the dehiscence of the anther locules and the release of the pollen tetrads (Fig. 4F).

Functionally female flowers. Compared with male flowers, what occurred at the tetrad stage appeared at the early microspore mother cell stage in functionally female flowers (Fig. 5A). After the completion of the stomium region differentiation, the stomium region in both male and functionally female flowers was composed of the septum and stomium. The septum contained two cell layers contiguous with the connective cells, whereas the stomium consisted of two to three specialized epidermal cells, which clearly differed from the surrounding rectangle epidermis cells by their nearly spherical shape. However, following the differentiation of the septum and

stomium cells at the early microspore mother cell stage, the stomium region cells remained intact and compact, and became tangentially elongated till the mature pollen stage (Figs. 5B–5F). All the anther dehiscence related changes, for example, the appearance of intercellular spaces, disintegration and dissociation of the septum cells, and disruption of the stomium cells did not occur in the stomium region throughout the anther development (Figs. 5B– 5F). As a consequence, the integrity of the anther locules was preserved, and the anthers were indehiscent.

Discussion

Anther wall and pollen development

In both floral morphs of *P. trimera*, the young anther wall consisted of an epidermis, an endothecium, two middle layers, and a secretory tapetum, whereas the mature anther wall consisted of a discrete epidermis and endothecium with fibrous thickenings. Tapetum and endothecium development in *P. trimera* proceeded in a similar fashion to that of *Annona squamosa* (Periasamy and Kandasamy 1981), where the secretory tapetum was also binucleate and the endothecium developed thickenings when the uninucleate microspores became vacuolated. However, the mature anther wall in *A. squamosa* was composed of the endothecium alone (Periasamy and

Kandasamy 1981). The thickening pattern of the endothelial cell corresponds to the U-shaped type according to the four types of endothecium thickening recognized by Wilson et al. (2011). The middle layer was made up of two cell layers, compressed and disorganized during the meiosis stage, and disappeared completely by the late bicellular pollen stage. This developmental process has not been reported in any other member of the Annonaceae.

In *P. trimera*, pollen development was identical between male and functionally female flowers. Pollen was shed in tetrads in male flowers. Comparison of the pollen tetrad formation in *P. trimera* with those recorded in other species, e.g., *Annona glabra* (tetrads), *A. montana* (tetrads), *A. cherimola* (tetrads), *A. senegalensis* (tetrads), *Asimina triloba* (tetrads), and with that of octads found in *Cymbopetalum baillonii*, which all have bisexual flowers and belong to the subfamily Annonoideae (Tsou and Fu 2002, 2007; Lora et al. 2009, 2014), suggests that the key events during microspore development in these species were identical but variation in the first cohesion mechanism of young microspores occurred during meiosis. Rotation of the four microspores to flip the thick proximal wall outward to become the distal wall was observed in species of Annonoideae with tetrad and octad pollen, and this distal-proximal transition represented a widespread condition in this basal family, as

hypothesized by Lora et al. (2009). However, the rotation of microspores was not found in the pollen tetrad formation in *P. trimera* and *Mitrephora thorelii* (unpubl. data), which both belong to the subfamily Malmeoideae of Annonaceae, suggesting a different evolutionary origin of pollen tetrad formation in this subfamily. As one of the independently isolated lines for pollen tetrad evolution in Annonaceae (Doyle and Le Thomas 2012), the mechanism of microspore binding in *Pseuduvaria* is worthy of a detailed ontogenetic study since the evolution of tetrads in Annonaceae remains the most vexing unresolved problem.

Anther dehiscence process

Anther dehiscence is a complex process that involves regulated differentiation and degeneration of specific anther tissues (Sanders et al. 2005). Essential events for normal anther dehiscence involve the specification of cell types within the anther primordium (e.g., endothecium, stomium, septum), the thickening of endothelial cells, the breakage of the septum and stomium which are the two main steps within this program, and the release of pollen grains (Sanders et al. 1999; Scott et al. 2004; Sanders et al. 2005; Wilson et al. 2011). Comparative analyses of the septum and stomium structures in *P. trimera* and characterized *Arabidopsis thaliana* and solanaceous species show variations in the histological features of the septum and

stomium. In solanaceous species (Sanders et al. 2005; García et al. 2008), the specialized cell types under the stomium have been referred to as the circular cell cluster and accumulate calcium oxalate crystals, whereas in *P. trimera* and *A. thaliana* (Sanders et al. 1999), the septum is simpler in structure, with a small number of cells (two layers in the former and two or three cells in the latter). The stomium in *P. trimera* and *A. thaliana* (Sanders et al. 1999), consisting of only two to three small epidermal cells are also simpler than that observed in solanaceous species with a multi-tiered stomium (Sanders et al. 2005; García et al. 2008). Variation has also been observed in the cellular events during septum and stomium development. In *A. thaliana* and solanaceous species, the stomium becomes visible after septum degeneration (Sanders et al. 1999; Sanders et al. 2005; García et al. 2008), whereas in *P. trimera*, both the septum and stomium become visible during meiosis and intercellular spaces among and within them are visible just before their degeneration. However, compared with *Lilium hybrida* (Varnier et al. 2005), no significant difference was found in anther dehiscence and in the development and structure of the septum and stomium in *P. trimera*. Although subtle differences in septum and stomium structure and development exist between these divergent plant species, ranging from the dicot model plant *A. thaliana* to the Solanaceae, the member of an

early divergent family *P. trimera* and to the monocot *Lilium*, the comparison supports the fact that the basic processes of anther dehiscence are conserved across angiosperms, as hypothesized by Wilson et al. (2011).

The relationship between anther indehiscence and tapetum abnormality

Most studies on male-sterility of flowering plants focused on investigating the development of pollen sterility, and only a few focused on indehiscent anthers (Sanders et al. 1999; Steiner - Lange et al. 2003; Strittmatter et al. 2006; Li et al. 2010; Solís et al. 2010; Zini et al. 2012; Fei et al. 2016; Yadav et al. 2016; Yang and Xu 2016; Luo et al. 2017; present study). In *P. trimera*, pollen development in both floral morphs follows the same pattern. However, the septum, stomium and tapetum of functionally female flowers show some anomalies in their development. Compared with the stomium region of male flowers, the intersporangial septum and stomium in functionally female flowers revealed a well-organized appearance with intact and compact cells since they were differentiated at the microspore mother cell stage, and neither intercellular spaces nor degeneration were observed during the bicellular pollen stage. Ultimately, indehiscent anthers with pollens could be found in mature functionally female flowers. Obviously, the anther indehiscence in functionally female flowers is not linked to any pollen defects, but rather only to sporophytic

tissue abnormalities, e.g., a tapetal defect and failure of the septum and stomium lysis.

The same situation was also found in species of Sapindaceae, where failure in anther dehiscence of functionally female flowers is characteristic of the family (Solís et al. 2010; Zini et al. 2012; Yadav et al. 2016; Luo et al. 2017).

The tapetum, a critical sporophytic tissue that surrounds the microspore mother cells, plays important roles in microsporogenesis and pollen development, such as supplying nutrients for gametophytes, and synthesizing and releasing callose (Pacini 1997, 2010). In most plants, the degeneration of the tapetum, coordinated with those of other tissues, is essential for the occurrence of normal anther dehiscence and the subsequent release of mature pollen grains (Li et al. 2010; Gómez et al. 2015; Sharma et al. 2015). Abnormal tapetal behavior has been described in relation to male sterility in many species (Raghavan 1997; Tsai et al. 2015), e.g., in *Oxalis debilis* (Rosenfeldt and Galati 2012), *Actinidia deliciosa* (Falasca et al. 2013), and the rice TDR (TAPETUM DEGENERATION RETARDATION) mutant (Li et al. 2006). In *P. trimera*, apparent differences between male and female anthers were detected in the time of tapetum karyokinesis and degeneration, the number of tapetum nuclei, and the extent of the degeneration of the tapetal cytoplasm. In anthers of male flowers, the tapetal cells underwent karyokinesis at the onset of meiosis and began to degenerate at

the tetrad stage, while in functionally female flowers, the karyokinetic process was already evident at the early microspore mother cell stage and the degeneration occurred at the early uninucleate microspore stage. Such a process probably induced the delayed degeneration of the tapetum, which was one type of the tapetum abnormalities in onion described by Holford et al. (1991). Strittmatter et al. (2006) found that in *Consolea*, tapetal cells of male-sterile anthers in female flowers remain uninucleate throughout, but male-fertile tapetal cells were polyploid, suggesting that tapetal nuclei abnormalities correlated with male sterility in female flowers. Accordingly, the tri-nucleate tapetum in functionally female flowers of *P. trimera* could probably be related with anther indehiscence and be evolutionarily significant. In addition, following the complete degradation of tapetal cytoplasm, the septum cells in male flowers were thoroughly dissociated and crushed at the late bicellular pollen stage, producing a bilocular anther with weakened stomiums along which dehiscence occurred. However, the tapetal cytoplasm in functionally female flowers did not degenerate completely as in male flowers at this stage, but was highly vacuolated, leading to the development of the persistent tapetal cells characterized by many small vacuoles or cell remnants at the mature pollen stage. Zini et al. (2012) also found that the tapetal cells were rapidly vacuolated and persistent in indehiscent anthers of

functionally female flowers in *Meilicoccus lepidopetalus*. The authors, inferred that the abnormal events of the tapetum, and the aberrant endothelial cell wall thickening, either less visible or absent, were associated with the failure of anther dehiscence, as in other unrelated species (*Chrysanthemum morifolium*, Li et al. 2010; *Cardiospermum grandiflorum* and *Urvillea chacoensis*, Solís et al. 2010). However, in *P. trimera*, the endothecium of mature anthers in both male and functionally female flowers had well developed thickenings, and no significant differences in thickening were noted. Obviously, the thickening of the endothelial cells is not correlated to anther indehiscence of functionally female flowers in this study. Therefore, the abnormal development of the tapetum might contribute to the intact appearance of the stomium region and result in the failure of anther dehiscence in functionally female flowers in *P. trimera*.

Conclusion

Comparison of the development of gametophytic (pollen) and sporophytic (anther wall and stomium region) tissues in both male and functionally female flowers in *P. trimera* demonstrates that in our species, anther indehiscence is functionally linked to sporophytic tissue only, i.e., a tapetal defect and failure of the septum and

stomium lysis. Obviously, this aberrant tapetal behavior is commonly associated with indehiscent anthers in angiosperm taxa, although other factors, including endothelial thickening, may also be involved. Further studies should be conducted to identify the PCD mechanism that is responsible for tapetum abnormalities and to clarify its role in the development of the male sterility and functionally unisexual flowers in *P. trimera*.

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Table 1 Comparison of the major events during *Pseuduvaria trimera* anther development of male and functionally female flowers.

Anther stage	Major events and morphological markers	
	Male flowers	Female flowers
Microspore mother cell stage	Formation of microspore mother cells surrounded by a five-layered anther wall.	Same as in male flowers. Tapetal cell becomes binucleate. Septum and stomium cells can be distinguished.
Meiosis stage	Microspore mother cells undergo meiosis. Middle layers are compressed. Tapetum becomes condensed and binucleate. Septum and stomium appear.	Same as in male flowers. Tapetum becomes bi- or tri-nucleate.
Tetrad stage	Tetrads appear and anther undergoes a general increase in size. Tapetum becomes further shrunken and starts to degenerate.	Same as in male flowers. Tapetum becomes slightly condensed.
Early uninucleate microspore stage	Permanent tetrads appear.	Callose wall surrounding tetrads dissolves and permanent tetrads appear. Tapetum becomes glandular and vacuolated and starts to degrade.
Intermediate uninucleate microspore stage	The microspore becomes vacuolated and generates an exine and intine wall; its cytoplasm and nucleus locate at the center of the tetrad. Endothelial cells expand and start to develop thickenings. Remnants of middle layers present. Tapetal cells become more degenerated and are granular.	Same as in male flowers.

Table 1 (Continued).

Late uninucleate microspore stage	The microspore nucleus locates at the distal side of the tetrad and its cytoplasm becomes dense. The epidermis cells become tangentially elongated and disconnected. U-shaped thickenings appear in the endothecium. The tapetal nucleus disappears and its cytoplasm becomes thin.	Same as in male flowers.
Early bicellular pollen stage	A centrally located vegetative cell and a lens-shaped generative cell attached to the pollen wall are formed via a mitotic division. Intercellular spaces appear within the septum and stomium cells.	A centrally-located vegetative cell and a lens-shaped generative cell attached to the pollen wall are formed via a mitotic division.
Late bicellular pollen stage	The generative cell moves to the center and is surrounded by the vegetative cell cytoplasm. The tapetum almost completely degenerates into cellular debris. Degeneration and breakage of septum cells are visible.	Same as in male flowers. The tapetal cytoplasm with numerous tiny vacuoles can be seen.
Mature pollen stage	Dehiscence. The tapetum completely disappears. Breakage of the stomium cells and release of the two-cell pollen tetrads appear. Only the epidermis and endothecium layers remain intact.	Indehiscent anther locules with two-cell pollen tetrads occur. The epidermis, endothecium layers, and septum and stomium cells remain intact. Tapetal cells with vacuolated cytoplasm or tapetal cell remnants persist.

Figure captions

Fig. 1. Flower morphology and anther structure of *Pseuduvaria trimera*. (A) Male flower with compact androecium. (B) Functionally female flower, showing compact gynoecium with receptive stigma and intact stamens. (C) Detail of a male flower viewed by scanning microscopy showing uppermost stamens. (D) Detail of a functionally female flower viewed by scanning microscopy showing carpels and stamens. (E–H) Cross sections with the dorsal side of anthers oriented upwards. (E) From the male floral bud, with four pollen sacs. (F–H) From different female floral buds, with four (F), three (G), two (H) pollen sacs. Abbreviations: An, androecium; C, carpel; CT, connective tissue; E, epidermis; En, endothecium; Gy, gynoecium; IP, inner petal; L, locule; ML, middle layer; OP, outer petal; s, stamen; StmR, stomium region; Ta, tapetum; V, vascular bundle. Scale bars = 500 μm (C, D) and 50 μm (E–H).

Fig. 2. Anther wall development, microsporogenesis and microgametogenesis in male flowers from *Pseuduvaria trimera*. Cross (A–C, E–L) and longitudinal (D) sections of the microsporangia from different anthers. (A) The early microspore mother cell (MMC) stage; the anther wall is composed of the epidermis (E), endothecium (En), two middle layers (ML) and tapetum (Ta). (B) Late microspore mother cell (MMC)

stage, showing peripheral MMCs chromatin. (C, D) MMCs undergo meiosis. (C) The first meiotic division of the MMCs. Tapetal cells undergo karyokinesis (arrows). (D) The second meiotic division of the MMCs, showing the centripetal furrow (arrowhead) and binucleate tapetum. (E) Tetrad stage, showing tetrads (Td) are surrounded by a callose layer (arrowhead), a dense cytoplasmic tapetum, and compressed middle layer cells. (F) The early stage of the intermediate uninucleate microspore stage, the permanent pollen tetrad contains a condensed cytoplasm, a large vacuole, a granular tapetum and flat middle layer cells can be observed. (G) Late stage of the intermediate uninucleate microspore stage; the microspore contains several vacuoles and a peripheral nucleus, and endothecium cells develop thickenings (arrowhead). (H) Late uninucleate microspore stage, the microspore vacuoles vanish and the nucleus locates to the distal periphery of the tetrad, and the endothecium cells develop U-shaped thickenings (arrowhead). (I) Microspores start mitosis (arrowhead), and middle layer cells are crushed. (J) Early bicellular pollen stage, showing a small generative nucleus (GN) against the pollen wall, a large vegetative nucleus (VN), and a crushed tapetum. (K) Late bicellular pollen stage, showing intact endothecium and epidermis. (L) Detail of a mature pollen tetrad, showing the generative nucleus is surrounded by the cytoplasm of the vegetative cell. Abbreviations: E, epidermis; En, endothecium; GN,

generative nucleus; ML, middle layer; MMC, microspore mother cell; N, nucleus; Ta, tapetum; Td, tetrad; VN, vegetative nucleus. Scale bars = 20 μm (A–L).

Fig. 3. Anther wall development, microsporogenesis, and microgametogenesis in functionally female flowers from *Pseudevania trimera*. Cross (A, C–I, L–O) and longitudinal (B, J, K) sections of microsporangia from different anthers. (A, B) Different stages of the microspore mother cell. (A) The anther wall is composed of the epidermis (E), endothecium (En), two middle layers (ML) and the tapetum (Ta), and the tapetal cells undergo karyokinesis (arrows). (B) MMCs start meiosis. (C–E) Different stages at meiosis. (C) The first meiotic division of the MMCs. (D) The second meiotic division of the MMC, showing the centripetal furrow (arrowhead), and compressed middle layers (arrow). The binucleate tapetal cells continue undergoing karyokinesis. (E) Detail of the anther wall, showing the trinucleate tapetum (Ta). (F) Following meiosis, each tetrad is surrounded by callose at the tetrad stage. (G) Permanent tetrads are formed after the dissolution of the callose layer. (H) The early uninucleate microspore stage, each microspore of the tetrad contains a centrally located nucleus, and the tapetal cells are separate from the middle layer cells. (I) The early stage of the intermediate uninucleate microspore stage, showing the enlarged microspores with a big vacuole (Va), a peripheral nucleus (N), and a granular tapetum.

(J) Late stage of the intermediate uninucleate microspore stage, showing further vacuolated microspores and thickened endothecium cells (arrowhead). (K) Late uninucleate microspore stage; the microspore vacuoles vanish, the nucleus locates to the distal margin of the tetrad, and the endothecium has U-shaped fibrous thickenings (arrowhead). (L, M) Early bicellular pollen stage; the pollen contains a small generative nucleus (GN) against the pollen wall and a large vegetative nucleus (VN) located centrally, and the tapetal nucleus degrades completely. (N) Mature pollen stage; the tapetal cells contain shrunken cytoplasm with numerous small vacuoles. (O) Detail of a mature pollen tetrad, showing bicellular pollen and persistent tapetum debris. Abbreviations: E, epidermis; En, endothecium; GN, generative nucleus; ML, middle layer; MMC, microspore mother cell; N, nucleus; Ta, tapetum; Td, tetrad; Va, vacuole; VN, vegetative nucleus. Scale bars = 20 μm (A–O).

Fig. 4. Stomium region development in male flowers from *Pseuduvaria trimera*. (A–F)

Cross sections of the stomium region from different anthers. (A) Zone between two adjacent anther locules at the microspore mother cell (MMC) stage, showing the stomium region (arrowhead). (B) Meiosis stage, showing two layers of septum cells (Sm) and one layer of stomium cells (Stm). (C) Intermediate uninucleate microspore stage, both the septum and stomium cells are conspicuously smaller than the

neighboring cells. (D) Early bicellular pollen stage, intercellular spaces (arrowhead) are present among stomium region cells. (E) Late bicellular pollen stage, septum cells lose cell adhesion and break from the connective tissue cells. (F) Mature pollen stage, showing anther opening. Abbreviations: COC, calcium oxalate crystal; E, epidermis; En, endothecium; L, locule; ML, middle layer; Sm, septum; Stm, stomium; Ta, tapetum. Scale bars = 20 μm (A–F).

Fig. 5. Stomium region development in functionally female flowers from *Pseuduvaria trimera*. (A–F) Cross sections of the stomium region from different anthers. (A) Microspore mother cell (MMC) stage, stomium region is composed of two layers of septum cells (Sm) and one layer of stomium cells (Stm), and their cells are compact and much smaller than the adjacent cells. (B, C) Septum and stomium cells remain small at the meiosis and intermediate uninucleate microspore stage, respectively. (D) Early bicellular pollen stage, stomium region cells are devoid of intercellular spaces. (E) Late bicellular pollen stage, both the septum and stomium cells remain intact and compact, without signs of degeneration. (F) Mature pollen stage, showing intact septum and stomium cells. Abbreviations: COC, calcium oxalate crystal; E, epidermis; En, endothecium; L, locule; ML, middle layer; Sm, septum; Stm, stomium; Ta, tapetum. Scale bars = 20 μm (A–F).

Draft

Figure 1.

165x235mm (300 x 300 DPI)

Figure 2.

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Figure 3.

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Figure 4.

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Figure 5.

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