Embryo Development in the Lady’s Slipper Orchid, *Paphiopedilum delenatii*, with Emphasis on the Ultrastructure of the Suspensor

YUNG-I LEE1, EDWARD C. YEUNG2, NEAN LEE3 and MEI-CHU CHUNG1,*

1Institute of Plant and Microbial Biology, Academia Sinica, 115, Taipei, Taiwan, Republic of China, 2Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada and 3Department of Horticulture, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd, 106, Taipei, Taiwan, Republic of China

Received: 22 June 2006 Return for revision: 22 August 2006 Accepted: 31 August 2006 Published electronically: 20 October 2006

- **Background and Aims** Owing to large-scale collecting, the lady’s slipper orchid, *Paphiopedilum delenatii*, is under threat of extinction. Asymbiotic germination provides a useful way to re-establish plants in the wild and for commercial propagation. A detailed study of embryo development would provide information on subsequent germination events and aid in the propagation of the species.
- **Methods** Developing capsules were collected for histochemical and ultrastructural studies by using both light and transmission electron microscopy.
- **Key Results** The suspensor of this species consists of three vacuolated cells. During the early globular stage of embryo development, structural differentiation occurs, revealing an abundance of smooth endoplasmic reticulum cisternae and wall ingrowths within the suspensor cells. These features are not present in cells of the embryo proper. Furthermore, the results of Nile red staining demonstrate that a cuticular layer is present only in the embryo proper, but absent from the suspensor. Cuticular material is also present in the inner walls of the seed coat, and persists through seed maturation.
- **Conclusions** The morphological features of the transfer cell and the absence of cuticular material in the suspensor cells indeed have structural specializations similar to those of other flowering plants. Thus, in order to support the notion that the orchid suspensor serves as the conduit for nutrient flow to the embryo proper, additional structural information is needed.

**Key words:** Cuticular material, embryology, lady’s slipper orchid, *Paphiopedilum*, suspensor.

**INTRODUCTION**

Compared with the majority of flowering plants, orchids have an atypical pattern of embryo development (Arditti, 1992; Dressler, 1993). One of the distinctive features of orchid embryos is the diverse morphology of their suspensors. In 1949, Swamy devised a classification scheme for orchid embryo development based on suspensor morphology alone. Depending on the species, the suspensor of orchids may be unicellular or else consist of a few filamentous cells (Swamy, 1949; Clements, 1999). At the light microscope level, the orchid suspensor usually appears as a vacuolated organ such as found in *Phaius* (Ye et al., 1997) and *Cymbidium* (Yeung et al., 1996). The suspensor in flowering plants is important to embryo development. Structural and experimental evidence clearly indicates that it can serve as a conduit for nutrient flow and may provide unique metabolites for the growth of the embryo proper (Yeung and Meinke, 1993; Nikitcheva, 2006; Yeung et al., 2006). One of the unique structural features of suspensor cells is that they take on a ‘transfer cell’ morphology with wall ingrowths and numerous mitochondria nearby. Endoplasmic reticulum (ER) cisternae and specialized plastids can also be abundant within the cytoplasm of suspensor cells. At present, detailed ultrastructural information concerning orchid suspensors is lacking (see Yam et al., 2002). Owing to the vacuolated nature of the orchid suspensor cells, it is uncertain whether these cells indeed have structural specializations similar to those of other flowering plants. Thus, in order to support the notion that the orchid suspensor serves as the conduit for nutrient flow to the embryo proper, additional structural information is needed.

The genus *Paphiopedilum* comprises a number of commercially important species that have been extensively cultivated and produce a wide range of attractive varieties, cultivars or hybrids as a result of intensive breeding (Cribb, 1998). *P. delenatii*, an endemic slipper orchid species of southern Vietnam, has pale pink flowers (Averyanov et al., 2003). It is of interest horticulturally, but is also considered to be a threatened species in the wild due to over-collection and habitat destruction. Similar to many terrestrial orchids, *Paphiopedilum* seeds are very difficult to germinate *in vitro* (Pierik et al., 1988). For the conservation and commercial production of this endangered species, information concerning its reproductive biology and improved methods of *in vitro* propagation are thus of great importance. Basic knowledge of embryo and seed development will aid in the design of experiments for asymbiotic seed germination studies, as shown in our previous study of *Cyripedium formosanum* (Lee et al., 2005). At present, information concerning embryo development in *Paphiopedilum* species is limited, except

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for work on *P. insigne* (Zinger and Poddubnaya-Arnoldi, 1966). In this study, the structural pattern of embryo development of *P. delenatii* from fertilization to seed maturity was investigated, as were histochemical changes during embryo development. In addition, emphasis was placed on the ultrastructural features of the suspensor to determine whether the suspensor indeed has the structural requirements to serve as a nutrient conduit for the developing embryo.

**MATERIAL AND METHODS**

**Plant material**

Plants of *P. delenatii* were maintained in the greenhouse of the National Taiwan University, Taipei, Taiwan. Anthesis occurs during March–April. To ensure a good fruit set and seed quantity, flowers were hand-pollinated. Developing fruits were harvested at regular intervals after pollination. Approximately 40 developing fruits were gathered for this study. At each developmental stage, more than 100 embryos were analysed under light microscopy to examine their morphological characteristics.

**Light microscopy**

Seeds at different developmental stages were fixed with 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, at 4°C overnight. After three 15-min buffer rinses, material was dehydrated in an graded ethanol series, and processed for Historesin embedding according to Yeung (1999). Historesin sections of 3 μm were cut using glass knives on a Reichert 2040 Autocut microtome. These sections were stained with the periodic acid–Schiff (PAS) procedure and counterstained with toluidine blue O for general histological examination. Histochemical staining of protein and total carbohydrates was performed according to Yeung (1984). The sections were stained with the PAS reaction for total carbohydrates, and counterstained with amido black 10 B (Merck KGaA, Darmstadt, Germany) for proteins. A red colour indicates carbohydrates, and blue indicates proteins. These preparations were examined and photographed with a Carl Zeiss Imager A1 light microscope (Carl Zeiss AG, Jena, Germany).

The presence of a cuticle was detected using Nile red as detailed in Yeung et al. (1996). The Historesin-embedded tissues were stained with 1 μg ml⁻¹ Nile red (Sigma Chemical Co., St Louis, MO, USA) for 10 min, briefly washed in distilled water and mounted in water containing 0.1 % n-propyl gallate (Sigma), an anti-fading compound. The fluorescence pattern was examined using an epifluorescence microscope (Imager A1, Carl Zeiss) equipped with the Zeiss filter set 15 (546/12 nm excitation filter and 590 nm emission barrier filter).

**Transmission electron microscopy**

Seeds at different developmental stages were fixed with 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, at 4°C overnight. After three 15-min buffer rinses, the material was post-fixed with 1 % OsO₄ in the same buffer for 4 h at room temperature and then rinsed in three 15-min changes of buffer. Following fixation, the material was dehydrated in a graded acetone series, and embedded in Spurr’s resin (Electron Microscope Sciences, Washington, PA, USA). Ultrathin sections 60–70 nm thick were cut using a diamond knife on an ultramicrotome (Richert-Jung Ultracut E, Vienna, Austria). These sections were stained with uranyl acetate and lead citrate. The sections were examined and photographed using a Philips CM 100 transmission electron microscope at 80 kV.

**RESULTS**

Fertilization occurred approximately 60 d after pollination (DAP). At 75 DAP, the zygotes and proembryos could be observed within the capsules. The zygote appeared as a highly polarized cell (Fig. 1A). The nucleus of the zygote was located toward the chalazal end, and a prominent vacuole was found toward the micropylar end. The cytoplasm of the zygote, distributed at the apical end, contained numerous mitochondria, plastids, small vacuoles and osmiphilic lipid bodies (Fig. 2A). These organelles tended to congregate around the nucleus. As in other orchid species, no endosperm was observed in *P. delenatii*. The polar nuclei within the endosperm cavity eventually disintegrated during embryo development (Fig. 1A).

The first division of the zygote was unequal, producing a smaller terminal cell and a larger basal cell (Fig. 1B). The terminal cell had a dense cytoplasm whereas the basal cell was elongated and vacuolated. The basal cell first divided transversely, resulting in a three-celled embryo (Fig. 1B, C). This was soon followed by an anticlinal division occurring in the cell at the terminus, giving rise to a four-celled embryo (Fig. 1D).

**Development of the embryo proper**

In the four-celled embryo, the terminal tier of cells towards the chalazal end divided and resulted in the formation of a group of four cells (Fig. 1E). These cells eventually gave rise to the embryo proper. Mitotic figures were readily observed during the early stages of formation of the embryo proper (Fig. 1F). By the early globular stage (90 DAP), additional cell divisions had occurred in the inner tiers (Fig. 1G) as well as in the surface layer (Fig. 1H, I), resulting in the growth of the embryo proper. Periclinal divisions from the surface layer of the cells gave rise to the protoderm as well as contributing additional cells to the embryo proper (Fig. 1I). A distinct protoderm layer was found at approximately 105 DAP (Fig. 3A).

Ultrastructural observations demonstrated an abundance of organelles within cells of the embryo proper at all stages of development. At the early globular stage (105 DAP), plastids with starch granules, mitochondria, lipid bodies and small vacuoles were readily seen in the cells of the embryo proper (Fig. 2B). Although the starch-containing plastids were prominent at this stage, they gradually
Fig. 1. Light micrographs of the early stages of Paphiopedilum delenatii embryo development. (A) Light micrograph of the zygote (arrows) after fertilization at 75 DAP. The zygote is highly polarized with a chalazally located nucleus and a prominent vacuole occupying the micropylar end. The polar–chalazal complex (arrowhead) includes the chalazal nuclei and the polar nuclei. Endosperm fails to develop in this species. DS, degenerated synergid; PS, persistent synergid. Scale bar = 30 μm. (B) The first cell division of the zygote results in the formation of a smaller terminal cell and a larger basal cell. The basal cell divides first transversely (arrowhead). IL, the inner layers of the seed coat; OL, the outer layers of the seed coat. Scale bar = 30 μm. (C) A three-celled embryo resulting from the transverse division of the basal cell of the two-celled embryo. The cell towards the micropylar end is highly vacuolated. Scale bar = 30 μm. (D) A T-shaped, four-celled embryo is the product of an anticlinal division occurring in the terminal cell of a three-celled embryo. Scale bar = 30 μm. (E) Additional anticlinal divisions occurring in the second derivative of the terminal cell produce a six-celled embryo. The dividing basal cell shows condensed chromosomes at metaphase (arrowhead) just before the periclinal cell division. Scale bar = 30 μm. (F) The embryo proper continues to develop by periclinal (arrow) and anticlinal (arrowhead) divisions, resulting in the formation of the globular-shaped embryo. At this stage, the suspensor (S) consists of two cells. Scale bar = 30 μm. (G) By the early globular stage (90 DAP), the cell division occurs in the inner tier of cells (arrowhead) and the hypophyseal cell (arrow), resulting in an increase in embryo size. S, suspensor. Scale bar = 60 μm. (H) Tissue differentiation begins with the formation of the protoderm by anticlinal divisions (arrowhead) in the outmost cell layer. S, suspensor. Scale bar = 60 μm. (I) Light micrograph showing the occurrence of periclinal division (arrowhead) within the embryo proper that results in the formation of the inner tier of cells. The additional cell divisions of the basal cell result in the formation of a three-celled suspensor (arrows). Soon thereafter, the suspensor enlarges and elongates towards the micropylar region of the seed. Scale bar = 60 μm.
disappeared as the embryos reached the mature globular stage of development (Fig. 2C). Wall ingrowth was absent from the walls and an electron-dense line was visible enveloping the embryo proper (Fig. 2B). As the embryo reached the mature globular stage (150 DAP), mitotic activity had ceased. The volume of large vacuoles within the cytoplasm became smaller and the cytoplasm consequentially took on a dense appearance (Fig. 3B). Storage materials, i.e. lipid and protein bodies, could be detected by histochemical staining at this stage. It is important to note that storage materials began to accumulate first in the cells near the suspensor (Figs 2C and 3B). At maturity, the embryo was only nine cells long and six cells wide (Fig. 3C). The embryo cells had abundant storage materials deposited in the form of lipid and protein bodies in the cytoplasm (Figs 2D and 3C).
Nile red staining indicated that a cuticular substance started to appear in the surface wall of cells of the embryo proper at the early globular stage and was clearly absent from the suspensor (Fig. 4A, B). As the embryo approached maturity (150 DAP), the fluorescence became brighter over the entire surface of the embryo proper and, again, the cuticular layer did not extend to the walls of the suspensor cells (Fig. 4C, D). As the seeds became fully matured (Fig. 4E; 210 DAP), prominent fluorescence enveloped the entire embryo when viewed with a fluorescence microscope (Fig. 4F). An obviously defined cuticular layer could be observed on the surface wall of the embryo proper (Fig. 2E).

Suspensor development

The cell nearest to the micropyle of a four-celled embryo gave rise to the suspensor (Fig. 1D); hence, this is regarded as the suspensor initial. This cell divided once unequally, resulting in a smaller upper cell and a larger cell near the micropyle. The smaller cell subsequently divided once more, with the resulting upper cell being incorporated into the embryo proper and the lower one becoming the uppermost suspensor cell (Fig. 1I). The larger cell near the micropyle as shown in Fig. 1E also divided once more, giving rise to two more suspensor cells. In total, three suspensor cells were present in this species. Throughout embryo development, the suspensor cells were more vacuolated as compared with cells of the embryo proper. The suspensor cells elongated after their formation and often appeared convoluted and compressed as the embryo proper expanded in size.

At the four-celled embryo stage, the suspensor initial had a prominent vacuole located at the micropylar end of the cell. The nucleus and the associated cytoplasm were located towards the embryo proper. In the cytoplasm surrounding the nucleus, mitochondria, starch-containing plastids and a few dictyosomes were readily found (Fig. 5A). Plasmodesmata were present in the common walls between the suspensor initial and its adjoining cell. Structural specializations in the suspensor could be detected beginning at the six-cell stage. At the micropylar end of the basal suspensor cell, wall ingrowths appeared as small papillae along the cytoplasmic face of the cell wall (Fig. 5B). Mitochondria were abundant and located near the wall ingrowths. Another notable feature was the abundance of smooth ER (SER) cisternae in the cytoplasm (Fig. 5B). Such a profile was absent from the cells of the embryo proper (Fig. 2B, C). The general organization of the cytoplasm in the suspensor remained the same throughout early development of the globular embryo.

By the globular stage (105 DAP), the vacuolated suspensor cells continued to elongate and often became twisted within the endosperm cavity enclosed by the seed coat (Fig. 3A). At this stage, well-developed wall ingrowths were present along the walls of the suspensor abutting onto the seed coat (Fig. 3C). SER remained abundant at this time. In addition to the SER, a notable amount of rough ER also became evident (Fig. 5C). The rough ER tended to congregate around the nucleus.
Plastids of different configurations were readily seen throughout the cytoplasm (Fig. 5C). At the final stages of seed maturation (210 DAP), the suspensor degenerated (Fig. 5D). The cytoplasmic components had broken down and appeared disorganized (Fig. 5D). The cytoplasm became less electron-dense and, except for the wall ingrowths, the organelles were not clearly defined at this stage.

Nile red staining indicated that a cuticular substance was absent over the walls of the suspensor cells throughout their development and maturation (Fig. 4B, D). Moreover, the cell wall stained pink with toluidine blue O, indicating that phenolic compounds were not present in the wall (Fig. 4C). Ultrastructural observations indicated that the electron-dense cuticular layer that had accumulated on the surface walls of the embryo proper was absent from the suspensor cell wall (Fig. 5B–D).

Changes in the integumentary tissues

The seed coat originated from the inner and outer integuments of the ovule. Both integuments were composed of two layers of highly vacuolated parenchyma
Cells of the inner layers (derived from the inner integuments) of the seed coat took on a slender shape, whereas those of the outer layers (derived from the outer integuments) were broader (Fig. 1B). By the globular stage, the outermost layer of the seed coat started to compress and shrivel gradually (Fig. 3A). At maturity, the entire seed coat became dehydrated and constricted into a thin layer that enveloped the entire embryo (Fig. 3C).

One of the notable features in the development of the seed coat was the deposition of cuticular material in the innermost walls. At the early globular stage, Nile red staining demonstrated that cuticular material was present over the innermost walls enclosing the endosperm cavity (Fig. 4B) although the fluorescence was less intensive toward the micropylar end. As the embryo matured, the fluorescence became brighter and more distinctive (Fig. 4D). The outer layers reacted negatively to the Nile red stain, indicating the absence of cuticular material in the wall. Ultrastructural observations further confirmed the presence of an electron-dense layer in the inner surface of the seed coat (Fig. 2C).
DISCUSSION

The suspensor is a short-lived embryonic organ of flowering plants. Physically, the suspensor attaches the embryo proper to the seed coat and there are no direct symplastic connections, i.e. plasmodesmata, between the embryo and the maternal seed coat. These structural features suggest that the suspensor serves as a conduit to supply the necessary nutrients for the growth of the embryo proper (Yeung and Clutter, 1978; Yeung and Meinke, 1993). Some recent findings regarding embryo development in flowering plants (e.g. Rodkiewicz et al., 1994; Lackie and Yeung, 1996; Yeung et al., 1996) have indicated the presence of cuticular substances on the surface of the embryo proper and their absence in the suspensor cell walls. In the present study, Nile red staining of developing P. delenatii embryos also indicates the absence of cuticular substances in the suspensor wall. These observations clearly indicate structural and functional differences between the two parts of the embryo. Furthermore, the absence of cuticular substance in the suspensor would allow for apoplastic continuity between the maternal tissues and the embryo. This would also facilitate nutrient movement from the maternal tissues to the embryo proper via the suspensor.

A majority of structural studies of orchid embryo development have focused on changes at the light microscope level (see Yam et al., 2002) and the suspensor generally appears as a vacuolated organ, which is similar to the suspensor of P. delenatii as reported herein. Ultrastructural studies are rare for orchid embryos and the few studies available have tended to focus on changes in the embryo proper, in particular on storage product deposition (see Yam et al., 2002). The vacuolated appearance suggests that orchid suspensors may not have further functional specializations. In this study, it is clear that structural specializations are indeed present in the suspensor of P. delenatii. The suspensor takes on a ‘transfer cell’ morphology, albeit that the wall ingrowths are not as elaborate as those reported in species such as Phaseolus coccineus (Yeung and Clutter, 1979). The ‘transfer cell’ morphology is a common structural feature found in the suspensor of flowering plants (Yeung and Meinke, 1993). The wall ingrowths are strategically located on the side walls abutting the maternal seed coat. The increase in the surface area of the suspensor cell supports the idea of enhanced nutrient uptake at this location. Furthermore, recent studies indicate that symporter proteins are localized in transfer cells of Vicia faba (Harrington et al., 1997), clearly signifying the importance of transfer cells in nutrient uptake. Thompson et al. (2001) have summarized the development and functions of seed transfer cells in a recent review, pointing out that the main function of transfer cells in seeds is the uptake of the solutes as sugars and amino acids for the production of storage product. The presence of wall ingrowths and the abundance of mitochondria near the ingrowths in P. delenatii suspensor cells therefore support the notion that the suspensor is the nutrient uptake site for the developing orchid embryo.

ER and plastids are additional structural features found in suspensor cells of flowering plants (Zhukova, 2006). In P. delenatii, the presence of a large amount of SER and the varied morphologies of the plastids and their absence in the embryo proper further indicate the unique nature of the embryo suspensor. SER appears early during embryo development, similar to that reported for suspensor cells of Stellaria (Newcomb and Fowke, 1974) and Phaseolus (Yeung and Clutter, 1979). Although the role of SER is not well understood, it is often found in a variety of gland cells secreting fats, oils and volatile essential oils (Gunning and Steer, 1996). The presence of SER suggests that besides serving as a nutrient conduit, the suspensor may provide unique nutrients that are necessary for the growth of the embryo proper.

In Cymbidium sinense (Yeung et al., 1996) as well as in P. delenatii, a cuticle is well developed in the embryo proper as early as the globular stage of development. The rapid development of a cuticle could represent an adaptation to the unique environment surrounding the developing orchid embryo. In orchids, an endosperm fails to develop during seed development. Furthermore, the seed coat is thin and may not offer protection to the embryo, especially in terms of moisture retention. The formation of a prominent cuticle on the surface of the embryo proper, as well as in the innermost layer of the seed coat, may ensure moisture retention by the embryo cells. The cuticular material may also offer physical protection to the embryo during germination. However, one of the potential drawbacks of having a well-developed cuticle and a tightly fitted seed coat with a thick cuticular layer is that the seeds are difficult to germinate in vitro. In our previous study, optimal asymbiotic seed germination of P. delenatii occurred at 150 DAP (68%), and the germination percentage decreased drastically at 210 DAP when the seeds were fully mature (31%; Lee, 1998; Fig. 3C). At 150 DAP, histological observation from this study indicates that the embryo is fully developed in size and yet the cuticular layer has not fully formed. The suspensor cells are still vacuolated and functional (Fig. 3B). As a result of these combined developmental events, this appears to be the most opportune time for in vitro germination to occur for this species. Asymbiotic seed germination is often difficult for fully mature orchid seeds, especially those of the terrestrial orchids. Knowledge of embryo development will help facilitate success in asymbiotic seed germination and the conservation of endangered orchid species.

In conclusion, the findings of the present study corroborate the hypothesis that the suspensor is the major site of nutrient uptake for the developing embryo. The presence of wall ingrowths, and the abundance of mitochondria, ER and plastids clearly indicate that the suspensor of P. delenatii indeed has structural specialization similar to those of other flowering plants. The presence of cuticular material in the walls of the embryo proper and the inner walls of the seed coat creates a protective covering of the embryo proper. Moreover, the cuticular material may also serve as a barrier for nutrient uptake. As a result, nutrient uptake is confined to the micropylar end of the seed via the suspensor.
ACKNOWLEDGEMENTS
This work was supported by grants from the Council of Agriculture of Taiwan to N.L., from Academia Sinica, Taiwan, to M-C.C., and from the Natural Sciences and Engineering Research Council of Canada to E.C.Y.

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