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Dark Material Accumulation and Sclerotization During Seed Coat Formation in *Vanilla planifolia* Jacks. ex Andrews (Orchidaceae)

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Abstract *Vanilla planifolia* Jacks. ex Andrews has the sclerotic seed coat, an exceptional character state of seed coat in the Orchidaceae. We observed seed coat formation of the species in every 10 days after pollination. The ovule develops into a nucellar filament with 6 to 8 nucellar cells at the time of anthesis prior to artificial pollination. The inner integument differentiates at 20 days after pollination. The outer integument differentiates at 30 days after pollination. The cells of the outermost layer of the outer integument start to thicken at 40 days after pollination. Dark material starts to accumulate in the outer and lateral cell walls of the outermost layer of the outer integument at 50 days after pollination. The dark material accumulates further at 60 to 80 days, while the inner integument has degenerated at 80 days. The thickened cell walls with dark material occupy the whole cell cavity and the cells become sclerotic at 120 days after pollination. The inner layer cells of the outer integument have degenerated and only the outermost layer of the outer integument remains as the seed coat at 180 days after pollination when the seed matures.

Key words : integument, Orchidaceae, ovule, sclerotization, seed coat, *Vanilla*.

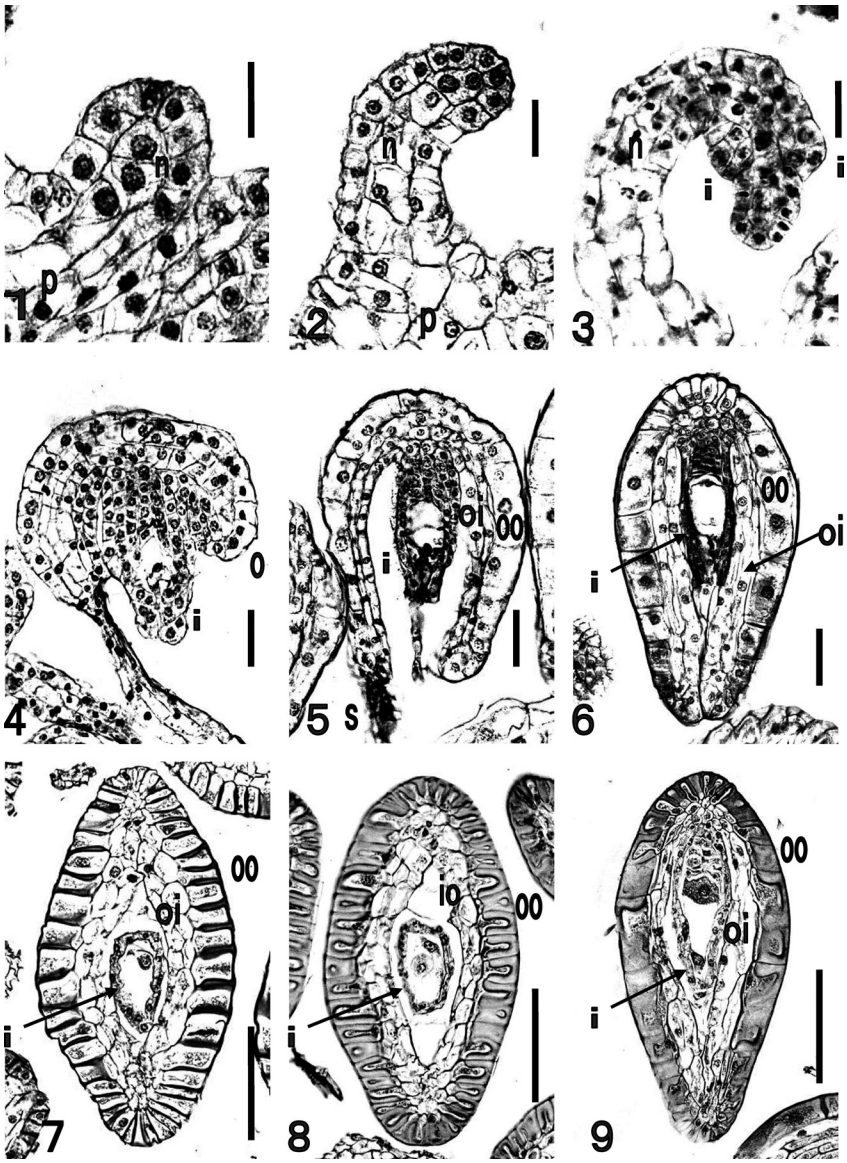
Introduction

In most orchid species the seed coat usually consists of a transparent, single cell layer (Wirth and Withner, 1959). Exceptionally, the seed coat thickens and dark material accumulates as observed in *Apostasia nipponica* Masam. (Nishimura and Tamura, 1993), *Selenipedium chica* Rchb.f. (Garay, 1960), *Vanilla planifolia* (Swamy, 1947), and *Galeola* (Burgeff, 1936). Developmental process of the opaque and sclerotic seed coat of orchids has been scarcely known. However, the following two observations indicate that the structure of the sclerotic seed coat in orchids is diversified. Nishimura and Tamura (1993) clarified that this type of seed coat in *A. nipponica* consists of two layers of the outer integument. The outer layer is thin and transparent, and the inner layer thickens and turns opaque. On the other hand, Swamy (1947) reported in *V. planifolia* that the inner and outer integument and even nucellus tissue take part in the seed coat formation and the dark material accumulates in the outermost layer of the outer integument. However, he did not show accumulating process of the dark material in detail and the observation did not incorporate time scale during the ovule development.

We therefore observed the seed coat formation of *Vanilla planifolia* every 10 days after the pollination with emphasis on the accumulating process of the dark material in the integument.

Materials and Methods

The materials of *Vanilla planifolia* were culti-

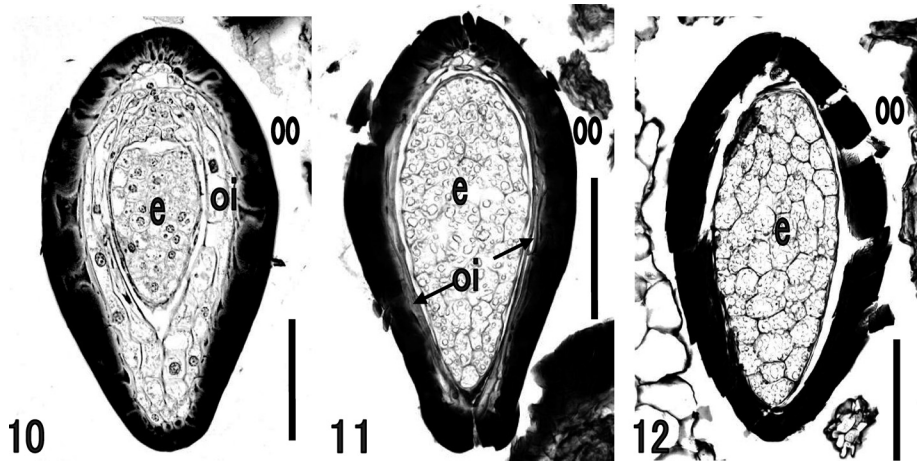


Figs. 1–9. Development of ovule in *Vanilla planifoloia* I. **1**. Development of the ovule primordium at anthesis prior to artificial pollination (longitudinal section). Note two nucellar cells covered with the epidermis. **2**. The nucellar cells arrange in line at anthesis prior to artificial pollination (longitudinal section). The nucellar filament begins to bend. **3**. Development of the inner integument at 20 days after pollination (longitudinal section). **4**. Development of the outer integument at 30 days after pollination. The embryo sac is surrounded by the inner integument (longitudinal section). **5**. Thickened outermost cell layer of the outer integument at 40 days after pollination (longitudinal section). The ovule and the suspensor are connected. **6**. Thickened outermost layer of the outer integument at 40 days after pollination. Note that the ovule is detached from the placental tissue (longitudinal section). **7**. Early stage of dark material accumulation in the outer and lateral cell walls of the outermost layer of the outer integument at 50 days after pollination (transverse section). **8**. Further accumulation of the dark material at 60 days after pollination (transverse section). **9**. The ovule at 60 days after pollination (longitudinal section). **i**, inner integument; **n**, nucellar cells; **o**, outer integument; **oi**, inner cell layers of the outer integument; **oo**, outermost cell layer of the outer integument; **p**, placental ridge; **s**, suspensor. Scale bars are 20 μm in **1–3**, 50 μm in **4–6**, and 100 μm in **7–9.**

vated at the greenhouse of Tsukuba Botanical Garden, National Museum of Nature and Science. The ovaries were collected at anthesis and every 10 days after artificial pollination from August 4 to December 24, 2005 and from March 21 to July 2, 2006. The materials were fixed in FAA solution containing 2.5% formaldehyde, 2.5% glacial acetic acid and 50% ethanol for 3 days at room temperature. The materials were then dehydrated in *n*-butyl alcohol series, and embedded in paraffin (melting point: 56–58°C). Serial sections were cut at 6 μm thick with a steel knife and stained with Delafield's haematoxylin (Nishimura and Tamura, 1993).

Results

The ovule primordium, which consists of nucellar cells and a single layer of epidermis, differentiates from the placental ridge (Fig. 1), and becomes a nucellar filament which contains 6 to 8 linear nucellar cells at anthesis. At this stage, some filaments begin to bend (Fig. 2). The inner integument differentiates at 20 days after pollination (Fig. 3). The outer integument differentiates and grows into 4–5 cell layers thick at 30 days after pollination. At this stage, the inner integument surrounds the embryo sac (Fig. 4). At 40 days after pollination, the cells of the outermost layer of the outer integument thicken (Fig. 5) and the ovule is detached from the placental tissue (Fig. 6). The dark material starts to accumulate in the outer and lateral cell walls of the outermost layer of the outer integument at 50 days after pollination (Fig. 7). The cell walls thicken further at 60 days after pollination (Figs. 8, 9). As the embryo develops into globular stage at 80 days after pollination, the cells of the inner integument have degenerated (Fig. 10). The thickened cell walls occupy the cavity of the outer integument cells, and they become sclerotic at 120 days after pollination (Fig. 11). At 180 days after pollination, the inner cell layers of the outer integument have degenerated and the thickened outermost layer of the outer integument only persists as the seed coat (Fig. 12).



Figs. 10–12. Development of ovule in *Vanilla planifolia* II. **10**. Dark material occupies most of the cell cavity of the outermost cell layer of the outer integument at 80 days after pollination. The cell layers of the inner integument have degenerated (longitudinal section). **11**. Dark material occupies the whole cell cavity of the outermost cell layer of the outer integument at 120 days after pollination (longitudinal section). **12**. The inner cell layers of the outer integument are degenerated and compressed into a thin layer enclosing the embryo proper, while the thickened outermost layer of the outer integument only persists as the seed coat at 180 days after pollination. Some cells of the outermost layer of the outer integument are broken by the edge of microtome (longitudinal section). **e**, embryo; **i**, inner integument; **oi**, inner cell layers of the outer integument; **oo**, outermost cell layer of the outer integument;. Scale bars are 100 μm in **10–12**.

Discussion

We found that the ovule of *Vanilla planifolia* develops into a nucellar filament with 6 to 8 nucellar cells at anthesis prior to pollination. The results did not support Swamy (1947)'s observation in which the ovule starts to differentiate only after pollination. Vij and Sharma (1986) classified the ovule development of orchids at anthesis into three groups. In the first group the ovule development does not occur at anthesis and takes place only after pollination. This group includes genera such as *Cymbidium* (Swamy, 1942), *Phalaenopsis* (Duncan and Curtis, 1942a; Poddubnaya-Arnoldi, 1960; Nimoto and Sagawa, 1961, 1962), *Cottonia, Dendrobium* (Swamy, 1943), *Cattleya* (Poddubnaya-Arnoldi, 1967) and *Epidendrum* (Cocucci and Jensen, 1969; Yeung and Law, 1989). In the second group, the ovule is weakly developed at anthesis. *Apostasia nipponica* (Nishimura and Tamura, 1993), *Cypripedium* (Duncan and Curtis, 1942b; Poddubnaya-Arnoldi, 1960), and *Paphiopedilum* (Duncan and Curtis, 1942b) belong to this group. In the third group, the ovule differentiates and ready for fertilization at anthesis. *Epipogium aphyllum* Sw. (Afzelius, 1954), *Gastrodia elata* Blume (Kusano, 1915), *Epipactis papillosa* Franch. & Sav. (Sato, 1974), and *Chamaegastrodia shikokiana* Makino & Maek. (Tohda, 1967) constitute this group. Our observation showed that *V. planifolia* belongs to the second group. Members of this group, viz., *Apostasia*, *Vanilla*, *Cypripedium*, and *Paphiopedilum*, belong to the earliest divergent clades in orchids (Cameron *et al*., 1999). By contrast, members of the other groups belong to more derived clades. The weakly developed ovule at anthesis thus may represent a plesiomorphic character state in the Orchidaceae.

We found that the cells of the inner integument and the inner cell layers of the outer integument have degenerated when the seed maturates, and that the sclerified outermost layer of the outer integument only persists as the seed coat. We confirmed that Swamy (1947)'s observation in which he noted the outer and inner integuments, and even the nucellar cells take part in the seed coat is due to misinterpretation. In comparison with the structure of another sclerotic seed coat species, *Apostasia nipponica*, in which the inner cell layer of the outer integument only sclerifies and deposits the dark material, it is clear that sclerified, opaque seed coat in orchids does not represent a single character state. So far as known, the dark material accumulates in the outermost cell layer of the outer integument in the order Asparagales, the sister group of the Orchidaceae (Wunderlich, 1937; Dahlgren and Clifford, 1982; Dahlgren *et al*., 1985; Wittich and Graven, 1995). These observations indicate that sclerotization in the outermost cell layer of the outer integument may represent a plesiomorphic character state in the Orchidaceae.

Chemical composition of the dark material of the sclerified seed coat in orchids has not been studied yet. Similar dark material, called phytomelan, characterizes the seed coat of Asparagales (Dahlgren and Clifford, 1982). The dark material of the orchid seed coat may be homologous with phytomelan if taking the phylogenetic position of the Orchidaceae and Asparagales into consideration. Wittich and Graven (1995) studied chemical composition of phytomelan in *Gasteria verrucosa* (Asphodelaceae) using histochemical technique and the results indicated that phytomelan cannot be stained for cellulose, pectin, lignin, carbohydrates, proteins or fatty substances, but the presence of phenolics is indicated. This suggests that the phytomelan is a type of melanin, because phenolics play role in the melanin synthesis. Dahlgren and Clifford (1982) also reported that phytomelan is a charcoal-like substance which is very rich in carbon and has a hydrogen : oxygen proportion of ca. 2 : 1 suggesting dehydration of carbohydrates. Further anatomical and histological analyses of the other orchid and Asparagales species exhibiting the sclerified, opaque seed coat are needed to elucidate the diversity and evolution of this character state.

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