Meiotic Defects and Premature Tapetal Degeneration Are Involved in the Low Fertility of *Oncidesa* Gower Ramsey, an Important Cut-flower Orchid

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Abstract. The dancing-lady orchid, *Oncidesa* Gower Ramsey, is an important cultivar for cut-flower production, but it has low pollen fertility in breeding programs. In this study, we compared the pollen germination in vitro, sporad type, and pollinia development of *Oncsa*. Gower Ramsey and a diploid species, *Oncidium sphacelatum* Lindl (one of its grandparents). In *Oncsa*. Gower Ramsey, the pollen germination in vitro was lower as compared with those in *Onc. sphacelatum*. In addition, the frequency of abnormal sporads in *Oncsa*. Gower Ramsey was higher than those in *Onc. sphacelatum*. In *Oncsa*. Gower Ramsey, the middle layer and the tapetum were disorganized before meiosis, and subsequently they degenerated at the early tetrad stage. In contrast, the middle layer and the tapetum of *Onc. sphacelatum* began to degenerate at the early tetrad stage and fully disappeared at the bicellular pollen stage. These results suggested that the abnormal meiosis caused by unbalanced genomes and the premature degeneration of the middle layer and the tapetum could probably result in the abnormal pollen development and the low fertility of *Oncsa*. Gower Ramsey.

The dancing-lady orchid, *Oncidium* Sw., and its relative genera within subtribe Oncidiinae native to Central and South America are important ornamental orchids in the horticultural market (Chase, 1997). Because of the long-lasting and bright yellow flowers, the cultivars of intergeneric hybrid *Oncidesa* (*Oncidium* × *Gomesa* R. Br.), for example, *Oncsa*. Gower Ramsey and *Oncsa*. Goldiana, are excellent cut-flower materials for decoration in flower arrangements. In Taiwan, *Oncsa*. Gower Ramsey has been an important cultivar for cut-flower production over the past few decades. The creation of new cultivars to meet the requirements of the floral

market is essential for maintaining a competitive advantage. In the breeding program of *Oncidium* alliance, hybridization allows the transfer of novel traits from one cultivar to another with the chances of selecting superior cultivars (Hieber et al., 2006; Moir and Moir, 1982). Although *Oncsa*. Gower Ramsey is a good cultivar for cut-flower production, the low percentage of capsule-set is commonly observed when *Oncsa*. Gower Ramsey is used as the pollen parent in our previous pollination experiments.

The causes of low fertility may be associated with abnormal meiosis in orchid hybrids. Meiotic irregularities, such as univalents, trivalents, chromosome bridges, and lagging chromosomes, are observed commonly in the interspecific or intergeneric hybrids of *Aranda* (Lee, 1987), *Dendrobium* Lindl. (Kamemoto et al., 1999), *Doritaenopsis* (Bolanos-Villegas et al., 2008), and *Paphiopedilum* Pfitz. (Lee et al., 2011), and the irregular meiotic pairing subsequently causes the formation of abnormal sporad types. In addition, it is possible that male sterility or low fertility is related to the

malfunction of anther tissue. The sporophytic anther tissues play important roles in pollen development, not only by providing physical support but also by supplying signals and materials necessary for pollen development. Especially, the tapetum, the most inner somatic cell layer of the anther locule, plays an essential role in pollen development. Tapetum secretes enzymes for the release of microspores from tetrads and provides nutrients and pollen wall materials required for pollen development and maturation (Goldberg et al., 1993; Hegde and Rudramuniyappa, 1986; Wilson and Yang, 2004). It has been observed that the abnormal development or dysfunction of tapetum may lead to the pollen degeneration in a number of plants, for example, *Arabidopsis thaliana* (L.) Heynh (Kim et al., 2010), garlic (Shemesh-Mayer et al., 2015), kiwifruit (Giuseppina et al., 2013), tomato (Jeong et al., 2014), *Solanum* Lindl. (Conicella et al., 1997), snap bean (Suzuki et al., 2001), petunia (Kapoor et al., 2002), radish (Shi et al., 2010), and rice (Ku et al., 2003; Shi et al., 2009).

The pollinia development of orchids has been reported in *Calanthe* Lindl. (Hegde and Rudramuniyappa, 1986), *Epidendrum* Lindl. (Yeung, 1987; Yeung and Blackman, 1983), *Zeuxine* Lindl. (Vijayaraghavan et al., 1987), *Peristylus* Lindl. (Zee and Siu, 1990), *Aranda* (Lee, 1987), and some intergeneric hybrids, for example, *Cattleya* Lindl., *Laelia* Lindl., and *Brassavola* Lindl. (Stort, 1984). However, limited information is available about the pollinia development of *Oncidesa*, and there are no reports about the possible roles of anther tissues in relation to the male sterility or low fertility. Histological study on pollinia development is required to provide the necessary background information before embarking on breeding programs. To get a better understanding of the low fertility in *Oncsa*. Gower Ramsey as the pollen parent, we compared the pollinia development of *Oncsa*. Gower Ramsey and its diploid parental species, *Onc. sphacelatum* (with good fertility). In addition, their sporad types after meiosis, pollen viability, and capsule setting by cross-pollination also were examined. The present study provides detailed information of the pollinia development at the light microscope level and extends our knowledge concerning pollen development in *Oncidesa*.

Materials and Methods

Plant material. Mature plants of *Onc. sphacelatum* and *Oncsa*. Gower Ramsey were cultivated in the greenhouse of Botanical Garden of National Museum of Natural Science, Taiwan. Plants were grown in pine bark medium (Orchiata; Besgrow, Christchurch, New Zealand) under 50% shade. Plants were fertigated monthly with a 20N–20P–20K Peters Professional 20–20–20 general purpose fertilizer (Scotts, Marysville, OH) at 0.5 g·L⁻¹. For histological and sporad type studies, developing flower buds of different stages were collected at 3-d intervals until anthesis. More than 100 developing

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flower buds and flowers were collected for this study.

Light microscopy. Developing flower buds were collected and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) buffered with 0.1 M sodium phosphate buffer, pH 6.8, at 4 °C overnight. After three 15-min buffer rinses, the materials were postfixed in 1% OsO₄ (Electron Microscopy Sciences) in the same buffer for 4 h at room temperature, then rinsed in three 15 min changes of buffer. Following fixation, the materials were dehydrated in an acetone series (Merck KGaA, Darmstadt, Germany), and embedded in Spurr's resin (Electron Microscopy Sciences). Serial sections 1 µm thick were cut, using a diamond knife, on an ultramicrotome (Ultracut E; Reichert-Jung, Vienna, Austria). These sections were stained with 0.1% (w/v) toluidine blue O in benzoate buffer for general histology. The sections were viewed and the images were captured digitally using a charge-coupled device camera attached to a light microscope (Axioskop 2; Carl Zeiss AG, Jena, Germany).

Sporad type observation. At anthesis, the pollinia were collected and fixed in fresh prepared Farmer's fluid (three parts of ethanol to one part of glacial acetic acid). The pollinia were macerated with 6% cellulose (Onozuka R-10; Yakult Honsha, Japan) and 6% pectinase (Sigma-Aldrich Co., St. Louis, MO) in 75 mM KCl, pH 4.0, at 37 °C for 90 min and squashed on slides with the same fixative. After drying, the slides were stained with 4',6-diamidino-2-phenylindole in VECTASHIELD (Vector Laboratories Inc., Burlingame, CA), and the images were captured digitally by a charge-coupled device camera attached to an epifluorescence microscope (Axioskop 2; Carl Zeiss AG).

In vitro pollen germination. At the time of anthesis, pollinia were collected from the flowers of *Onc. sphacelatum* and *Oncsa. Gower Ramsey*. After removing anther caps, the surface of pollinia was wiped with cotton soaked with 70% ethanol solution. In the laminar flow hood, six pollinia mass were transferred to one petri dish containing 15 mL of germination medium. The germination medium used in this study was the modified Brewbaker and Kwack medium (Brewbaker and Kwack, 1963), containing 20 g·L⁻¹ sucrose, 100 mg·L⁻¹ H₃BO₃, 200 mg·L⁻¹ MgSO₄·7H₂O, 300 mg·L⁻¹ Ca(NO₃)₂·4H₂O, 100 mg·L⁻¹ KNO₃, and solidified with 10 g·L⁻¹ agar (Sigma-Aldrich Co.). The pH value was adjusted to 5.5 with 1 N NaOH solution before autoclaving at 121 °C for 15 min. The cultures were maintained at 25 ± 1 °C in the dark chamber, and the pollinia were taken out for observation at 1, 2, 4, 6, 8, and 10 d of culture. The germinating pollinia were transferred onto slides and gently separated by forceps, then were stained with 0.04% methyl green (Sigma-Aldrich Co.) for pollen tube observation under a light microscopy. Germination of pollen was defined as emergence of the pollen tube from the pollen grain. The germination percentage was evaluated as the percentage of the number of

pollen grains germinated among the total counted number of pollen grains. Experiments were performed in a randomized design.

Capsule setting by cross-pollination. For cross-pollination experiments, *Oncsa. Gower Ramsey* was cross-pollinated with *Onc. sphacelatum* or *Gom. Java*, respectively. The reciprocal crosses were made at the same time. The flowers were labeled and manually cross-pollinated by transferring pollinia from the male parent onto the stigma of the female parent as soon as they opened. The capsule setting was calculated 2 months after cross-pollination.

Results

The pollen germination in vitro. In *Onc. sphacelatum*, the pollen tube began to grow by 2 d after culture, whereas in *Oncsa. Gower*

Ramsey, the pollen tube began to grow by 6 d after culture. By 10 d after culture, the percentage of pollen germination in vitro in *Onc. sphacelatum* was much greater (68.29 ± 5.21%) as compared with those in *Oncsa. Gower Ramsey* (2.30 ± 1.39%) (Table 3).

The pollen development. In *Onc. sphacelatum* and *Oncsa. Gower Ramsey*, at the early sporogenous stage, the sporogenous cells had nuclei of great volume with conspicuous nucleoli and the dense cytoplasm (Fig. 1A and B). The primary parietal layer underwent periclinal divisions, giving rise to multilayered microsporangial walls that differentiated into the endothecium, the middle layer, and the tapetum (Fig. 1C and D). The sporogenous cells gave rise to form microspore mother cells (MMCs) that were filled with numerous small vacuoles (Fig. 1C and D). The endothelial cells continued to enlarge by

Fig. 1. Micrographs of the microspore mother cell (MMC) development of *Onc. sphacelatum* and *Oncsa. Gower Ramsey*. In (A) *Onc. sphacelatum* and (B) *Oncsa. Gower Ramsey*, their sporogenous cells (S) have dense cytoplasm with nuclei of great volume. Cell divisions in the primary parietal layer give rise to multilayered microsporangial walls that differentiate into the endothecium (Ed), the middle layer (M), and the tapetum (T) in (C) *Onc. sphacelatum* and (D) *Oncsa. Gower Ramsey*, and the sporogenous cells give rise to form MMCs. In *Onc. sphacelatum* (E), the chromatins of MMCs further condense in preparation for meiotic divisions, and the tapetal cells have elongated and possess prominent nuclei, whereas in *Oncsa. Gower Ramsey* (F), the cells of the middle layer and the tapetum are disorganized with the appearance of condensed nuclei. Scale bar = 10 µm.

the process of vacuolation, whereas the cells of the middle layer and the tapetum remained cytoplasmic. Before meiosis, in *Onc. sphacelatum*, the tapetal cells were elongated and possessed prominent nuclei (Fig. 1E). In MMCs, the chromatin further condensed in preparation for meiotic divisions (Fig. 1E). On the contrary, in *Oncsa*. Gower Ramsey, the cells of the middle layer and the tapetum were disorganized with the appearance of condensed nuclei (Fig. 1F). In *Onc. sphacelatum* and *Oncsa*. Gower Ramsey, MMCs underwent meiotic divisions and resulted in the formation of microspore tetrads (Fig. 2A and B). In *Onc. sphacelatum*, the middle layer and the tapetum began to degenerate after meiosis (Fig. 2A). In *Onc. sphacelatum* and *Oncsa*. Gower Ramsey, haploid microspores were not released from tetrads after meiosis, and all tetrads aggregated to form the pollinium. The microspore within a tetrad underwent pollen mitosis I (Fig. 2A), giving rise to a vegetative cell and a generative cell (Fig. 2C and D). At anthesis, the mature pollen tetrads within a pollinium of *Onc. sphacelatum* contained numerous lipid bodies and protein bodies (Fig. 2E), whereas in *Oncsa*. Gower Ramsey, a number of mature pollen tetrads shrank and degenerated (Fig. 2F).

Sporad type observation. Following the process of meiosis, each PMC normally produced a tetrad with four microspores in *Onc. sphacelatum* (Fig. 3A), whereas in *Oncsa*. Gower Ramsey, a considerable number of irregular sporad types were observed (Fig. 3B–D). In *Onc. sphacelatum*, 85.8% of normal tetrads were formed, whereas in *Oncsa*. Gower Ramsey, only 59.8% of normal tetrads were formed (Table 1). The frequency of abnormal sporads (e.g., dyad, triad, and sporads with micronuclei) in *Oncsa*. Gower Ramsey (40.2%) was higher than those in *Onc. sphacelatum* (14.2%).

Capsule setting by cross-pollination. Capsule setting of 55% and 81.3%, respectively, was observed when *Oncsa*. Gower Ramsey was used as the female parent and *Onc. sphacelatum* and *Gom. Java* as the male parent. No capsule setting was observed when *Oncsa*. Gower Ramsey was used as the male parent and *Onc. sphacelatum* and *Gom. Java* as the female parent (Table 2).

Discussion

In this study, as compared with *Onc. sphacelatum*, the lower pollen viability and the higher abnormal sporads observed in *Oncsa*. Gower Ramsey (Tables 1 and 3) may be attributed to the irregularity of meiotic pairing. *Oncsa*. Gower Ramsey is an intergeneric hybrid that comprises three species, i.e., *Onc. sphacelatum* ($2n = 56$), *Gom. flexuosa* (Lodd.) M.W. Chase & N.H. Williams Sims ($2n = 56$), and *Gom. varicosa* (Lindl.) M.W. Chase & N.H. Williams ($2n = 112$) (Felix and Guerra, 2000), and the counts of chromosome number $2n = \text{ca. } 75\text{--}83$ were observed in *Oncsa*. Gower Ramsey, suggesting a polyploid or aneuploid hybrid (Hu,

Fig. 2. Micrographs of the microgametogenesis of *Onc. sphacelatum* and *Oncsa*. Gower Ramsey. Meiotic divisions (arrowhead) result in the formation of microspore tetrads (Te). In *Onc. sphacelatum* (A), the cells of middle layer and the tapetum (arrow) have become compressed, whereas in *Oncsa*. Gower Ramsey (B) the cells of middle layer and the tapetum have degenerated during meiosis. The pollen tetrads (Pt) of (C) *Onc. sphacelatum* and (D) *Oncsa*. Gower Ramsey contain a vegetative cell (arrow) and a generative cell (arrowhead) after pollen mitosis I. At anthesis, the pollen tetrads of *Onc. sphacelatum* (E) contain numerous lipid bodies (arrow) and protein bodies, whereas in *Oncsa*. Gower Ramsey (F), most mature pollen tetrads (Pt) have shrunk and degenerated. Scale bar = 10 μm . N = nucleus; Ed = endothecium.

2001). The degeneration of sporads with unbalanced genomes has been reported in a number of primary hybrids in *Phalaenopsis* Blume (Arends, 1970) and *Paphiopedilum* Lindl. (Lee et al., 2011). Lee (1987) examined the meiotic behaviors and the sporad formation of different clones of an intergeneric orchid hybrid, *Aranda* Lucky Laycock (*Arachnis hookerana* Rchb. $\times$ *Vanda tricolor* Lindl.) and found that the fertile clones produced a higher frequency of dyads and tetrads without micronuclei, whereas the infertile clones produced more dyads and tetrads with micronuclei. Similarly, in *Doritaenopsis* orchids, the cultivars with high fertility were well correlated with the high frequency of normal tetrads (Bolanos-Villegas et al., 2008).

Like many orchids in the subfamily Epidendroideae, species within subtribe Oncidiinae have a compact pollinium that is

composed of numerous tetrads (Pacini and Hesse, 2002). In our observations, at the time of anthesis, the pollinia within the anther locule of *Oncsa*. Gower Ramsey often appeared shriveled. To provide information about the pollinia abortion, histological studies were initiated to investigate the possible timing and tissues/cells involved in the abortion of pollinia during anther development. An interesting feature of this study revealed that the early degeneration of the middle layer and the tapetum may play an important role in the shriveled pollinia of *Oncsa*. Gower Ramsey. The tapetum is the innermost layer of the anther walls that contacts directly with the developing pollen and provides necessary nutrients and elements for pollen development and maturation (Pacini, 2010). In general, the tapetum begins to degenerate at the tetrad stage and is crushed completely by the bicellular pollen stage (Zhang et al., 2011) or

Fig. 3. The sporad type observation in *Onc. sphacelatum* and *Oncsa. Gower Ramsey*. The presence of nuclei is confirmed by the 4',6-diamidino-2-phenylindole stain when viewed with a fluorescence microscope. The normal tetrad (A) of *Onc. sphacelatum*, whereas a dyad (B), an irregular tetrad (C) with micronuclei (arrows), and a triad (D) containing two large nuclei and one small nucleus (arrow) are commonly observed in *Oncsa. Gower Ramsey*. Scale bar = 10 μ m.

Table 1. The number and percentage of sporad type observed in *Onc. sphacelatum* and *Oncsa. Gower Ramsey*.

Taxon	No. of sporad observed	Dyad (%)	Dyad ^z (%)	Triad (%)	Triad ^z (%)	Tetrad (%)	Tetrad ^z (%)
<i>Onc. sphacelatum</i>	225	18 (8) ^y	3 (1.3)	4 (1.8)	0 (0)	193 (85.8)	7 (3.1)
<i>Oncsa. Gower Ramsey</i>	239	32 (13.4)	9 (3.8)	17 (7.1)	8 (3.3)	143 (59.8)	30 (12.6)

^zSporads with micronucleus.

^yThe data in bracket are percentages.

Table 2. Capsule setting of *Oncsa. Gower Ramsey* by cross-pollination.

Combinations		No. of flower pollinated	No. of capsule setting	Capsule setting (%)
Female	Male			
<i>Oncsa. Gower Ramsey</i>	<i>Onc. sphacelatum</i>	20	11	55
<i>Oncsa. Gower Ramsey</i>	<i>Gom. Java</i>	32	26	81.3
<i>Onc. sphacelatum</i>	<i>Oncsa. Gower Ramsey</i>	20	0	0
<i>Gom. Java</i>	<i>Oncsa. Gower Ramsey</i>	5	0	0

Table 3. In vitro pollen germination of *Onc. sphacelatum* and *Oncsa. Gower Ramsey*.^z

Cultivar	Germination (%)					
	Days after culture					
	1	2	4	6	8	10
<i>Oncsa. Gower Ramsey</i>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.01 $\pm$ 0.004	0.15 $\pm$ 0.07	2.30 $\pm$ 1.39
<i>Onc. sphacelatum</i>	0.00 $\pm$ 0.00	1.67 $\pm$ 0.31	24.02 $\pm$ 2.97	40.49 $\pm$ 4.54	58.49 $\pm$ 6.18	68.29 $\pm$ 5.21

^zData are mean $\pm$ SE of four replicates and eight pollinia from four flowers for each. The pollinia were cultured in modified Brebaker and Kwack medium with 2% sucrose and 1% agar.

the vacuolated microspore stage (Mizelle et al., 1989). Judging from our histological sections, the middle layer and the tapetum of *Oncsa. Gower Ramsey* became disorganized before meiosis (Fig. 1F). At the early tetrad stage, the middle layer and the tapetum of *Oncsa. Gower Ramsey* already disappeared (Fig. 2B). On the contrary, the middle layer and the tapetum of *Onc. sphacelatum* began to degenerate at the early tetrad stage (Fig. 2A) and fully disappeared at the bicellular pollen stage (Fig. 2C).

In our results of cross-pollination, 55% to 81.3% of capsule setting could be obtained when *Oncsa. Gower Ramsey* was used as the female parent, whereas no capsule setting could be observed when *Oncsa. Gower Ramsey* was used as the male parent (Table 2). It has been broadly reported that premature degeneration of tapetum usually causes male sterility. For example, in the somatic hybrids of *Solanum commersonii* Dun. and *S. tuberosum* Lindl., the early breakdown of tapetum and meiotic defects result in the male sterility (Conicella et al., 1997). In petunia, silencing of the tapetum-specific zinc finger gene may result in the precocious degeneration of tapetum and the subsequent pollen abortion (Kapoor et al., 2002). In the photoperiod-sensitive genic male sterility rice, the pollen abortion is associated with the premature degeneration of tapetum at the PMC stage (Shi et al., 2009). In snap bean, pollen sterility is caused by the premature degeneration of tapetum under the condition of high temperature (Suzuki et al., 2001). Tapetum is a short-lived cell layer of anther wall, and the progression of cell death in tapetum is an orderly and essential process for the normal pollen development, anther dehiscence, and pollen release and consequently guarantees the pollen fertility (Varnier et al., 2005; Wu and Cheung, 2000). The premature or delayed programmed cell death (PCD) of tapetum will result in the male sterility (Papini et al., 1999). In kiwifruit, male sterility was caused by a delayed PCD in the middle layer and tapetum (Giuseppina et al., 2013), whereas the premature PCD is detected in tapetum of the male-sterile lines of rice (Ku et al., 2003) and sunflower (Balk and Leaver, 2001). In *Oncsa. Gower Ramsey*, the middle layer and the tapetum lost their regular cell shape before meiosis, then followed by cell condensation, which is similar to the progression of premature PCD in tapetum observed in the male-sterile lines of rice (Ku et al., 2003). Further studies are needed to identify the possible role of PCD involved in the abnormal pollen development of *Oncsa. Gower Ramsey*.

In conclusion, we compared the ontogeny of the anther development in *Oncsa. Gower Ramsey* and the fertile diploid species, *Onc. sphacelatum*, and provide both the histological and cytological marks of the events causing the low fertility of *Oncsa. Gower Ramsey*. We propose that the abnormal meiosis caused by unbalanced genomes and the premature degeneration of the middle

layer and the tapetum could probably result in the abnormal pollen development and the low fertility of *Oncsa*. Gower Ramsey.

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