Dynamic Association between the Vegetative Nucleus and the Generative Cell in *Tropaeolum majus* (Tropaeolaceae)

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**Summary** The behavior of the vegetative nucleus during pollen grain development and germination in *Tropaeolum majus* was examined using epifluorescence microscopy stained with 4',6-diamidino-2-phenylindole (DAPI). The vegetative nucleus exhibited gradual elongation and overall length association with the simultaneously elongated generative cell during pollen development. The elongated vegetative nucleus and generative cell in the mature pollen grains appeared in a paired structure that was stable against physically squashed pollen rupture. Electron microscopy showed that the ultrastructural relationship between the vegetative nucleus and the generative cell was discontinuous with rough endoplasmic reticulum (RER) often located in the interface and Golgi bodies appeared remarkably around the generative cell. However, the vegetative nucleus became spherical after pollen hydration and the association between the vegetative nucleus and the generative cell disintegrated markedly during pollen germination. These results suggest that the elongation of the vegetative nucleus and the physical association between the vegetative nucleus and the generative cell in the pollen grains of *T. majus* may play an important role in pollen, especially the generative cell development.

**Key words** Generative cell, Male germ unit, Pollen development, *Tropaeolum majus*, Vegetative nucleus.

A mature male gametophyte (pollen grain) of angiosperms contains two or three cells. The pollen grain that contains two cells is termed bicellular pollen and that contains three cells is tricellular pollen. In the species with tricellular pollen such as *Plumbago zeylanica* (Russell and Cass 1981, Russell 1984), *Spinacia oleracea* (Wilms and van Aelst 1983), *Brassica oleracea* (Dumas et al. 1985) and *Catanaucne caerulea* (Barnes and Blackmore 1987), it has been shown that the sperm cells and the vegetative nucleus are not spatially distinct but appear to form a structural complex named the male germ unit (MGU).

The structure and possible function of MGU are initially reviewed by Dumas et al. (1984). They suggest that the connection between the sperm cells and the association between the sperm cell and the vegetative nucleus in MGU make these components of a male gametophyte a single transmitting unit that is probably an essential prerequisite for successful fertilization. This suggestion has greatly stimulated the later cytological study on the male gametophyte. As it is reviewed by Mogensen (1992), the occurrence of MGU is confirmed in more and more angiosperm species including those with bicellular pollen, such as Hippeastrum vitatum (Mogensen 1986a, b), Rhododendron laetum (Kaul et al. 1987), Petunia hybrida (Wagner and Mogensen 1988), Cyphomandra betacea (Hu and Yu 1988), Acacia retinodes (McCoy and Knox 1988), Aloe ciliaris (Ciampolini et al. 1988), Nicotiana tabacum (Yu et al. 1989), Medicago sativa (Zhu et al. 1990) and Cymbidium

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goeringii (Yu and Russell 1992).

In the species with bicellular pollen, as the sperm cells form after germination, the appearance of the typical MGU with the connection between the sperm cells and the association between the sperm cell and the vegetative nucleus is usually confirmed in the pollen tubes (Mogensen 1986b, Kaul et al. 1987, Hu and Yu 1988, Yu et al. 1989, 1992). However, in the mature or immature bicellular pollens, it is found that the vegetative nucleus appears to have an association with the generative cell (Mogensen 1986a, McCoy and Knox 1988, Wagner and Mogensen 1988, Zhu et al. 1990, Yu and Russell 1992). This association between the vegetative nucleus and the generative cell, as is similar in structure with that between the vegetative nucleus and the sperm cell in MGU, is recognized as a two-cell typed MGU in the bicellular pollen (Mogensen 1986a, McCoy and Knox 1988, Yu and Russell 1992).

In the present study, we demonstrated a very unique behavior of the vegetative nucleus in Tropaeolum majus. The vegetative nucleus elongated in high degree and associated in overall length with the generative cell before germination. Our results suggest that the early MGU in bicellular pollen seems to function mainly for successful development of the generative cell rather than gamete transmission.

Materials and methods

Plant material

Tropaeolum majus was grown in our greenhouse in Peking University. Flowers at different developmental stages were collected and used in the experiments.

Epifluorescence microscopy

Anthers at various developmental stages were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at room temperature. Followed by 4 rinses (15 min each) in the same phosphate buffer, the anthers were dehydrated in an ethanol series and embedded in Technovit 7100 resin (Kulzer and Co., Germany) as described previously (Kuroiwa et al. 1991). Sample sections at 0.5 μm in thickness were cut with a glass knife, stained with 1 μg/ml DAPI for 10 min and observed under an Olympus BHS-RFK epifluorescence microscope. After epifluorescence examination, the sections were stained again with toluidine blue O (TBO) at pH 9.4 as described previously (O’Brien et al. 1964).

Pollen tubes were germinated in vitro by incubating the mature pollen grains in a medium contained 0.01% H3BO3, 0.03% Ca(NO3)2·4H2O, 0.02% MgSO4·7H2O, 0.01% KNO3 and 16% sucrose at room temperature. For visualization of the vegetative nucleus and the generative cell in the mature pollen grain and pollen tube, live pollen grains and pollen tubes were squashed in 1% glutaraldehyde supplemented with 1 μg/ml DAPI on a glass slide and observed directly with the epifluorescence microscope as described previously (Sodmergen et al. 1997).

Electron microscopy

Mature anthers before dehiscing were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at room temperature. After 4 times of rinse (15 min each) in the same phosphate buffer, the samples were postfixed in 1% osmium tetroxide overnight at 4°C, washed in the buffer mentioned above, dehydrated in an ethanol series and embedded in Spurr’s resin. Ultrathin sections cut by a glass knife were stained in 1% uranyl acetate and 0.2% lead citrate and then observed with a JEM-100CX electron microscope (JEOL, Japan).
Results

Behaviors of the vegetative nucleus and the generative cell

In this study, pollen grains were examined from the stages after the asymmetric mitosis when a large vegetative nucleus and a small generative cell formed (Fig. 1a1, a2). The generative cell was initially lenticular in shape with a spherical cell nucleus (Fig. 1a1, a2). The vegetative nucleus usually appeared in a position near the lenticular generative cell but did not show direct contact with the generative cell at this stage (Fig. 1a2). Soon afterwards, the generative cell came near to the vegetative nucleus when it peeled away from the intine (Fig. 1b). Elongation of the generative cell and generative nucleus proceeded at the next stages (Fig. 1c, d, e). This is a phenomenon common to the generative cells during pollen development. However, the vegetative nucleus appeared to associate closely with the generative cell and elongated as well along the generative cell (Fig. 1c, d, e).
In the mature pollen grain, both the generative cell and the vegetative nucleus became highly elongated and they integrally associated in their overall lengths (Fig. 1f1, f2). This association was physically stable and it usually remained well in the squashes of ruptured pollens (Fig. 1f1, f2).

However, the association between the generative cell and the vegetative nucleus disintegrated and the extended vegetative nucleus became spherical within 15 min after pollen hydration (Fig. 1g). The vegetative nucleus and the generative cell entered the pollen tube separately. They migrated with a distance in the pollen tube during early pollen germination (Fig. 1h). The second mitosis took place about 2 h after hydration. The generative cell came close to the vegetative nucleus just before the second mitosis (Fig. 1i).

Ultrastructural relationship between the generative cell and the vegetative nucleus

As expected, the generative cell and the vegetative nucleus exhibited close association in the mature pollen grains of T majus. The vegetative nucleus was concave and it partially surrounded the generative cell in the transverse sections (Fig. 2a). This structure continued until the tapering regions of the generative cell (Fig. 2d). The surface of the generative cell was corrugated (Fig. 2a, d) and it formed discontinuous association with the vegetative nucleus (Fig. 2a, b). Rough endoplasmic reticulum (RER) was observed in the interface between the generative cell and the vegetative nucleus (Fig. 2c). We often observed grouped Golgi bodies around the half side of the generative cell free of the vegetative nucleus (Fig. 2d).

Discussions

Close association between the vegetative nucleus and the sperm cell is initially shown in Plumbago zeylanica (Russell and Cass 1981). However, this association is usually undetectable with epifluorescence microscopy when pollen grains are squashed (such a phenomenon has been shown but not discussed, Sodmergen et al. 1995). This is because the physically squashed pollen rupture usually destroys the physical association between the vegetative nucleus and the male reproductive cell of MGU. However, in the mature pollen grains of T majus, the association exhibited good stability against pollen rupture. We propose that this is due to the great extension of the vegetative nucleus and the overall length association between the vegetative nucleus and the generative cell which may largely enhance the physical strength of the association. To our knowledge, this behavior of the vegetative nucleus is unique in angiosperms examined to date.

Another important result shown in the present report is that the association between the generative cell and the vegetative nucleus in the mature pollen of T majus disintegrated during pollen hydration and early germination but it liked reform in the pollen tube before the second mitosis. This is very similar to the result reported previously in Hippeastrum vitatum (Mogensen 1986a, b) where the author suggested that “the spatial organization among components of the male germ unit in the mature pollen grain does not necessarily reflect relationships that ultimately exist among these components within the pollen tube” (Mogensen 1986a). Another result similar to that described above is demonstrated in Cymbidium goeringii where it is reported that the association between the vegetative nucleus and the generative cell occurs during early pollen development but it loosens or even disappears in mature pollen grains (Yu and Russell 1992). From these results, we propose that although the associations between the generative cell and the vegetative nucleus in both pollen grains and pollen tubes are generally termed as MGU (Mogensen 1986a, McCoy and Knox 1988, Yu and Russell 1992), the early MGU that forms in the bicellular pollen grain before germination must have function(s) different from that of the later MGU that forms in the pollen tube. This is because that if a MGU forms simply as a transmitting unit that “may facilitate the transmission and order the arrival of the male gametes into the receptive cells of the female embryo sac” (Dumas et al. 1984), it may not need to form, disintegrate and then reform during pollen development and germi-
Fig. 2. Electronmicrographs showing the ultrastructural relationship between the vegetative nucleus and the generative cell in the premature pollens. The area boxed (in a) and that in a greater magnification (b) showed the discontinuous association (indicated with arrowheads) between the vegetative nucleus and the generative cell. Note that the concave vegetative nucleus partially surrounded the generative cell in the transverse sections (a, d), RER appeared in the interface between the vegetative nucleus and the generative cell (c) and Golgi bodies surrounded the generative cell (d). G, Golgi body; GC, generative cell; GN, generative nucleus; RER, rough endoplasmic reticulum; VN, vegetative nucleus. Bars represent 1 µm (a, d) and 0.5 µm (b, c).
nation.

In the mature pollen grain of *T. majus*, RER and Golgi bodies often occurred near the generative cell. It seemed that the generative cell was being modified by the vegetative cell at this stage. This stage-specific modification of the generative cell has been suggested previously in *Medicago sativa* from the fact that a distinct greater frequency of nuclear pores occurs in the envelope of the vegetative nucleus that faces the generative cell (Shi et al. 1991). It is proposed that there is an active transmission of substances from the vegetative cell during pollen development (Shi et al. 1991). In *T. majus*, it is apparent that the high degree elongation of the vegetative nucleus and the overall length association between the vegetative nucleus and the generative cell considerably enlarged the association area between the generative cell and the vegetative nucleus (the quantitative examination is under way). We propose that the enlargement of association area is equivalent to the occurrence of a high nuclear pore density with respects to the enhancement of substance transmission between the cells. Therefore, the close and large area association between the vegetative nucleus and the generative cell in *T. majus* may be an intercellular constitution requisite for effective providing and receiving of substances for the generative cell modification that seems occur at this stage. Within this constitution, the relatively closed space (interface) allowed efficient deposition and transmission of products originated from the vegetative nucleus and, the corrugated surface of the generative cell enlarged the receiving area of the cell, maintained the structural association with the vegetative nucleus and provided spaces for product processing. From the appearances described above, we suggest that the early MGU in the bicellular pollen grains may play an important role in pollen development rather than gamete transmission.

In the present report, we demonstrated a unique behavior of the vegetative nucleus and the independent formations of MGU in the pollen grains and pollen tubes in *T. majus*. Our results demonstrated that the physical association between the vegetative nucleus and the generative cell formed, disintegrated and then reformed dynamically. We propose that: 1) the early MGU in the pollen grain before pollination may be an essential constitution that improve the development of the generative cell while 2) the later MGU after pollination may function as it is conventionally described that facilitate the transmission of the male gametes. Further studies based on this consideration are now in progress.

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