Abolition of the Tapetum Suicide Program Ruins Microsporogenesis

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Keywords: Arabidopsis thaliana — Microsporogenesis — Pollen development — Programmed cell death — Tapetum.

The development of pollen grains (microsporogenesis) has long interested plant biologists. Microspore and pollen development takes place within the anthers of angiosperm flowers (reviewed by Mascarenhas 1989, McCormick 1993, Scott 1994). Microspores are surrounded by a layer of cells, i.e. the tapetum. The tapetum is known to provide nutrition to developing microspores and pollen grains, as well as exine, the main structural components of the pollen wall. The tapetum degenerates during the later stages of pollen development.

It has been speculated that tapetum degeneration is a programmed cell death (PCD) event (for a review, see Wu and Cheung 2000). Based on ultrastructural observation of tapetal cells in two angiosperms (Lobivia rauschii and Tillandsia albida), Papini et al. (1999) have reported that the degradation of tapetal cells shows cytological features characteristic of PCD. These include cell shrinkage, condensation of chromatin, swelling of the endoplasmic reticulum (ER) and the persistence of mitochondria. Wang et al. (1999) have reported that toward the end of the unicellular stage of pollen development in barley, oligonucleosomal DNA cleavage was observed. They have also reported that the nuclei of tapetal cells and the tissues of the anther wall were TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling) positive, which indicated that the fragmentation of DNA was detected in situ through the incorporation of fluorescein 12-dUTP at the free 3′-OH ends of the fragmented DNA. Similar features of PCD together with the release of cytochrome c, which is characteristic of animal apoptosis, have been also reported in sunflower in conjunction with the study of cytoplasmic male sterility (Balk and Leaver 2001). The role of PCD and an onset signal for PCD in the tapetum, however, have not yet been elucidated.

In animal systems, studies of apoptosis have revealed pathways where proteins of the Bel-2 family play key roles. The Bel-2 family includes pro-apoptotic (e.g. Bax, Bak and Bid) and anti-apoptotic (e.g. Bel-2, Bel-xl and Ced-9) members that appear to control the initiation of apoptosis through mitochondria (Gross et al. 1999). When Bax is translocated from cytosol to the outer membrane of mitochondria, it induces the release of proteins, such as cytochrome c, into the cytosol, and consequently triggers apoptosis (Liu et al. 1996, Jurgensmeier et al. 1998). A Bax gene has been shown to induce PCD in plant cells (Lacomme and Cruz 1999, Kawai-Yamada et al. 2001), although comparative genomics has revealed that Bel-2 family-like proteins are absent in plants (Aravind et al. 1999). A suppressor of Bax-induced cell death has been identified in plants as well as in humans. Bax inhibitor-1 (BI-1) is such an anti-apoptotic protein that localizes to the ER membrane, and is conserved in both animal and plant species (Chae et al. 2003). Overexpression of AtBI-1, a homolog of mammalian BI-1 identified in Arabidopsis thaliana, has been shown to inhibit Bax-induced cell death in A. thaliana (Kawai-Yamada et al. 2001).

To determine the critical developmental stage when the PCD signal commences in the tapetum and to elucidate the role of PCD of the tapetum for microsporogenesis, we employed Bax and AtBI-1 genes to alter PCD in the tapetum.

A mouse Bax gene or the AtBI-1 gene of A. thaliana was connected to the tapetum-specific promoter of the Osg6B gene or the LTP12 gene (Tsuchiya et al. 1994, Ariizumi et al. 2002). The commencement of promoter activation is different between them. The LTP12 promoter becomes active starting in the uninucleate microspore stage (Ariizumi et al. 2002), while
Osg6B becomes active at an earlier stage, the tetrad stage, and continues until the bicellular pollen stage, as determined by promoter–β-glucuronidase (GUS) fusion experiments in transgenic A. thaliana (T. Kawanabe, T. Ariizumi and K. Toriyama, unpublished data). In the current study, four chimeric constructs, LTP12::Bax, LTP12::AtBI-1, Osg6B::Bax and Osg6B::AtBI-1, were introduced into A. thaliana. Expression of the Bax gene is expected to cause cell death resulting in pollen abortion. Expression of the AtBI-1 gene is expected to cause the inhibition of PCD of the tapetum, and the surviving tapetum would affect pollen development.

Transgenic plants with LTP12::Bax, Osg6B::Bax and Osg6B::AtBI-1 were male-sterile. In contrast, transgenic plants with LTP12::AtBI-1 were fertile and set seeds normally (Table 1).
Table 1 Sterile and fertile plants were obtained depending on the active stage of the promoter

<table>
<thead>
<tr>
<th>Promoter (active stage in anther)</th>
<th>BAX</th>
<th>AtBI-1</th>
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<tbody>
<tr>
<td>LTP12 (uninucleate to bicellular)</td>
<td>6/6</td>
<td>0/15</td>
</tr>
<tr>
<td>Osg6B (tetrad to bicellular)</td>
<td>4/4</td>
<td>8/11</td>
</tr>
</tbody>
</table>

The number of male-sterile plants/total number of kanamycin-resistant transgenic A. thaliana with LTP12::Bax, LTP12::AtBI-1, Osg6B::BAX and Osg6B::AtBI-1 are presented.

1). Tapetum and microspore development were observed by light and electron microscopy in at least three transgenic lines for each construct. In the mature anther of the wild type, the complete disappearance of the tapetum was clearly observed (Fig. 1A). In contrast, in the tapetal cells of Osg6B::AtBI-1 plants, tapetum degeneration was not observed even at the mature stage (Fig. 1B). The tapetal cells began to enlarge with large vacuoles at the uninucleate microspore stage (Fig. 1F). Electron-dense and organelle-rich cytoplasm and vacant vacuoles were well contrasted in the enlarged tapetal cells (Fig. 1F). Exine formation was highly defective in the Osg6B::AtBI-1 plants (Fig. 1J), as shown by the fact that the bacula were clearly shorter than those of the wild type (Fig. 1I) and almost no tectum was formed. The microspores collapsed and were finally crushed by the enlarged tapetum (Fig. 1B).

In the case of the Osg6B::Bax plants, vacuolation of the tapetum at the mature stage was also observed as in the Osg6B::AtBI-1 (Fig. 1C). The tapetum was faintly and uniformly stained, and no cytoplasmic components were observed at the uninucleate microspore stage (Fig. 1G), indicating that the tapetal cells were completely destroyed by the action of Bax. The organelles in the microspores were not evident and the microspores were almost empty of contents (Fig. 1K).

LTP12::Bax was shown to cause male sterility similarly to the case of the Osg6B::Bax. Pollen development, however, was less severely affected than that in the Osg6B::Bax plants. Formation of exine with bacula and tectum was observed in LTP12::Bax (Fig. 1D, L), although only poorly developed bacula were observed in the Osg6B::Bax (Fig. 1K), indicating that exine formation is arrested at a later stage in the LTP12::Bax plants than in the Osg6B::Bax plants. The tapetum of the LTP12::Bax plants at the uninucleate microspore stage lacked plasids with relatively electron-transparent vesicles, plastoglobuli, which were observed in the wild type (Fig. 1E, H). The tapetum development appeared to be arrested. These features were probably caused by the fact that in contrast to the Osg6B promoter, the LTP12 promoter became active in the later developmental stages and thus had less effect on the tapetum and microspore development.

In A. thaliana, complete disappearance of the tapetum cell layer is observed after the second pollen mitosis at the tricellular pollen stage. Loss of the cell walls of the tapetum begins as early as the tetrad stage (Owen and Makaroff 1995, Zhang et al. 2002). It is likely that PCD signaling commences at the tetrad stage.

Why did AtBI-1 cause pollen abortion under the regulation of the Osg6B promoter, but not under the LTP12 promoter? It is considered that the signal pathway of PCD was inhibited by AtBI-1 at the tetrad stage and that PCD did not proceed further in the Osg6B::AtBI-1 plants. Inhibition of PCD in the tapetal cells consequently affects normal pollen development, arresting the supply of nutrients to the microspores, which results in male sterility. The appearance of some fertile plants with Osg6B::AtBI-1 (Table 1) might indicate that the function of AtBI-1 is not enough to suppress cell death completely. In contrast, when the LTP12 promoter starts to drive AtBI-1 at the uninucleate microspore stage, PCD becomes irreversible and AtBI-1 no longer inhibits PCD. In the LTP12::AtBI-1 plants, normal PCD takes place, resulting in production of fertile pollen.

There have been several reports on the use of genetic cell ablation for investigation of the role of the anther tapetum during microspore development. Using the promoters of tapetum-specific genes to express cytotoxic molecules in transgenic plants, ablation of tapetal cells in transformed anthers has been demonstrated to result in microspore abortion and complete male sterility (Mariani et al. 1990, Paul et al. 1992, Hird et al. 1993). In contrast to the killing of tapetum cells, however, an approach to alter the PCD program so as to allow the tapetal cells to live longer has not been reported so far. Our results demonstrated, for the first time, that the PCD signal commences at the tetrad stage and that the proper timing of PCD in the tapetum is essential for normal microsporogenesis.

Materials and Methods

cDNAs of mouse BAX (accession no. L22472) and AtBI-1 (accession no. AB025927) were cloned into pGEM-3Zf(–) (Promega). A fragment of the Osg6B promoter (Tsuzukiya et al. 1994) or the LTP12 promoter (Ariizumi et al. 2002) was ligated to the HindIII–SpeI site or HindIII–XbaI site. The HindIII–SalI fragment was then ligated to the same site of pB1101. The resulting binary vectors were transferred into Agrobacterium tumefaciens strain C58. Transformation of A. thaliana ecotype Columbia used floral dip methods (Clough and Bent 1998), and transformants were selected using 20 mg liter−1 kanamycin. For sectioning, the wild-type and transgenic flowers at different stages were fixed overnight in 3% glutaraldehyde in 100 mM phosphate buffer (pH 7.0) and dehydrated in a graded ethanol series. The subsequent procedures were carried out as previously described (Ariizumi et al. 2003, Ariizumi et al. 2004)

Acknowledgments

This research was supported by a Research for the Future grant and a Grant-in-Aid from the Japan Society for the Promotion of Science.
References


(Received January 11, 2006; Accepted March 17, 2006)