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Male Sterility of Chinese Cabbage, Wheat and Rice: Cell Biological Research on the Process of Anther Abortion

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ABSTRACT

Male sterility of higher plants is a valuable trait used to improve agricultural crops through hybridization. Pollen abortion is a complex and complicated process and the mechanisms involved are actively being studied. Recently, new data on the structure and function of the tapetal cell, changes in Ca^{2+} distribution, ATPase activity distribution and programmed cell death in anther cells were obtained using cell biology. These data have helped identify the process of pollen abortion and illuminated the mechanisms of male sterility of higher plants, and provide an important link between research on male sterility at the individual and molecular levels. This paper summarizes the recent data regarding the aborting process of male-sterile anthers of Chinese cabbage, wheat and rice obtained using cell biology.

Keywords: ATPase, calcium, programmed cell death, tapetum
Abbreviations: ATPase, adenosine triphosphatase; **PCD**, programmed cell death

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INTRODUCTION

Male sterility, or failure to produce functional anthers, pollen or male gametes, is a common phenomenon in higher plants and is found in 43 families, 162 genera, 320 species and 297 hybrids (Kaul 1988). Male sterility forms the basis for hybrid predominate crops, which are desirable because of their superior agronomic characteristics. As such, the mechanisms of male sterility have been actively studied for many years; molecular biological studies of male sterility have recently provided valuable information. Several review papers describing molecular mechanisms of male sterility and the gene inducing male sterility were published along with theories and hypotheses to explain the mechanisms (Araya *et al*. 1998; Budar and Pelletier 2001; Hanson and Bentolila 2004). Cytological studies of male sterile anthers mainly explore the process of pollen abortion, and are a means of connecting molecular studies and individual studies of pollen abortion and help us understand the mechanism of male sterility of crops. Cytological studies identify important cellular events during pollen development, and changes in cellular structure and function of sterile anthers provide a basis for recognizing the cause of sterile gene regulating pollen abortion. Recently, new data regarding pollen abortion have been obtained using modern cell biological techniques. This review presents and discusses new data on the structure and function of the tapetal cell, the abnormal distribution of Ca^{2+} and ATPase in cells, and programmed cell death in anther cells. These data may help us to understand the complex mechanisms of male sterility.

TAPETUM AND POLLEN

Tapetum is the innermost layer of the anther wall. It directly contacts the microspore mother cell (MMC) and generally attains its maximum development at the tetrad or microspore stages. Tapetal cells have considerable physiological importance for microsporogenesis because all food material is passed through them to the MMC (Pacini 1997). The tapetal cells have intercommunion with developing microspores, and male sterility occurs when this intercommunion is abnormal. Several reports suggested abnormal tapetum may induce male sterility (Aarts *et al*. 1997; Jin *et al*. 1997; Taylor *et al*. 1998; Fei and Sawhney 1999; Suzuki *et al*. 2001). The tapetum degenerated at the late microspore stage in photoperiod-sensitive genic male sterile mutant rice, and disappeared at the bicellular pollen stage in fertile anthers. The germ pore of the microspore adheres to the tapetum while it becomes attenuated during degeneration. However, the tapetum between the two microspores remains intact; suggesting late microspores with a large vacuole may induce tapetum disaggregation. In sterile anthers, the microspores abort at the late microspore stage; the tapetum does not disaggregate and will reserve until anthesis, again sug-

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gesting a relationship of intercommunion between the tapetum and microspores (Tian *et al*. 1993). Recently, inducing UDP-glucose pyrophosphorylase (UGPase) gene silencing by RNA interference resulted in rice male sterility pollen mother cells of Ugp1-silenced plants appeared normal before meiosis, but during meiosis, normal callose deposition was disrupted and pollen mother cells began to degenerate at the early meiosis stage. Meanwhile, the degeneration of tapetum and middle layer was inhibited because pollen aborted (Chen *et al*. 2007).

Taylor *et al*. (1998) found the tapetal cytoplasm disintegrates at the late vacuolate microspore stage in the *ms7* mutant of *Arabidopsis thaliana*, causing degeneration of microspores and pollen grains of the male-sterile mutant. Houman *et al*. (1999) observed the ultrastructure of *A. thaliana ms32*, and found rough endoplasmic reticulum (ER) appeared in its tapetal cells before meiosis of the microspore mother cells. The rough ER generally synthesizes and secretes callase to disaggregate the callus wall of the tetrad. In the corresponding wilt type, this rough ER appeared at the tetrad stage to disaggregate the callus wall and release microspores. Therefore, tapetum synthesized and secreted callose too early in the sterile anther, causing the MMC callose wall to disaggregate and induce pollen abortion. In two male sterile maize mutants, secondary parietal cells, which should have formed the middle layer and the tapetum, divided to form two layers of cells (t1 and t2). Both cell layers were unable to differentiate normally and abortion of the MMCs was finally induced at meiosis I (Chaubal *et al*. 2000). Katsumi *et al*. (2001) observed that the ER in the tapetum degenerated earlier than usual under heat stress, resulting in high pollen abortion. Smith *et al*. (2002) investigated anther ultrastructure of a cytoplasmic male-sterile soybean and found the first detectable change leading to cell degeneration was the degeneration of the inner mitochondrial membrane in the tapetal cells. This was followed by the formation of atypical concentric rings in the tapetal ER. Premature degeneration of the tapetum affected pollen development and finally caused pollen abortion. A loss of synchrony in the development of the tapetum and microspores altered lipid accumulation in the tapetal cells and ultimately leading to pollen abortion in a male sterile mutant *aot*1-3 of *Arabidopsis thaliana* (Zhang *et al*. 2002). Ma *et al*. (2007) found three maize male sterile mutants, two of which were related with tapetum abnormality: the *mac1* mutant has an excess of archesporial derivative cells and lacks a tapetum and middle layer; the *ms23* mutant lacks a differentiated tapetum. Taken together these studies indicate that changes in tapetal development will induce pollen abortion.

Gene engineering techniques have been used to confirm that abnormal tapetum induces pollen abortion (Li *et al*. 1999). Chimaeric ribonuclease genes expressed in the anthers of transformed tobacco and oilseed rape plants were constructed and their expression within the anther selectively destroyed the tapetal cell layer, prevented pollen formation, and resulted in male sterility (Mariani *et al*. 1990). Tsuchiya *et al*. (1995) fused a cDNA for a pathogenesis-related *endo*-β-1,3-glucanase isolated from soybean with an anther tapetum-specific promoter (*Osg6B* promoter) isolated from rice, and introduced this chimeric gene into tobacco. The *Osg6B* promoter became active in the anther tapetum during formation of tetrads and the tapetal glucanase activity in the transgenic plants caused a significant reduction in the number of fertile pollen grains. Artificial male-sterile plants created by disturbing tapetum development will further the use of hybrid crops around the world. Inducing gene silence by RNA interference or co-suppression resulted in male sterility of rice (Chen *et al*. 2007). The Arabidopsis AtMYB103 gene codes for an R2R3 MYB domain protein whose expression is restricted to the tapetum of developing anthers. Blocking the function of the AtMYB103 gene of *Arabidopsis thaliana*, employing either an insertion mutant or an AtMYB103EAR chimeric repressor construct under the control of the AtMYB103 promoter, resulted in complete male sterility. A restorer containing the AtMYB103 gene under the control of a stronger anther-specific promoter was introduced into pollen donor plants and crossed into the male sterile plants transgenic for the repressor. The male fertility of F_1 plants was restored (Li *et al*. 2007).

ATPase DISTRIBUTION

Membrane-associated ATPase is ubiquitous in organisms and catalyzes ATP hydrolysis to produce energy to support cell metabolism of material synthesis and decomposition. Energy defects may be a factor in causing pollen abortion. In early male-sterile anthers of photoperiod-sensitive genic male-sterile rice, ATP content was lower than that seen in fertile anthers, suggesting that a decrease in ATP is related with pollen abortion (Deng *et al*. 1990). The quantity of ATPase in anther cells may reflect cell energy and viability. Sane *et al*. (1997) observed the differences in kinetic properties of isolated mitochondrial F1-ATPase between the sterile and the fertility restored line. They proposed F1-ATPase kinetic property differences may play a role in the expression of the cytoplasmic male-sterile trait at the time of anther formation. Recent reports have suggested the location of ATPase in developing anthers. Yao *et al*. (2000) found there was no difference in ATPase distribution in the anther wall and the connective tissue between sterile and fertility maintenance lines of wheat, suggesting the nutritional material provided normal support. However, ATPase was distributed differently in pollen grains of each line: an increase in ATPase in pollen of the fertility maintenance line occurred with increased pollen development, but ATPase did not increase in the sterile line, suggesting abnormal ATPase affects pollen fertility. Similarly, Meng *et al*. (2000b) reported there was less ATPase in sterile microspores and epidermis cells, as well as the endothecium, the middle layer and the vascular bundle of a photoperiod-sensitive genic male-sterile wheat. They also reported the tapetum decomposed early and suggested ATPase and energy may be in short supply and lead to early microspore abortion. There was less ATPase in the connective tissues of the anthers in the male-sterile line of rice than in the fertility restored line, suggesting an abnormality in material transportation to the anther causes pollen abortion (Guan *et al*. 2000). Little ATPase appeared in fertile rice anthers before the MMC stage. However, after meiosis, ATPase in the anther cells began to increase and was accumulated in the exine, which originated from the tapetum during microspore development. During intine formation in bi-cellular pollen, abundant ATPase from vegetative cells of bi-cellular pollen also accumulated. There was more ATPase in the vegetative cells than in the generative cells, suggesting the former had higher metabolism activity (Wang *et al*. 2006). The quantity of ATPase in anther wall cells and connective tissue reflects the cell viability and transport ability of material into anther locules. The difference between ATPase in fertile and sterile pollen reflects the state of viability of the pollen itself (**Figs. 1, 2**). In various male sterile plants, the distributions of ATPase in anther somatic cells and various stages of pollen indicates that abnormal metabolism of ATP in these cells may induce pollen abortion.

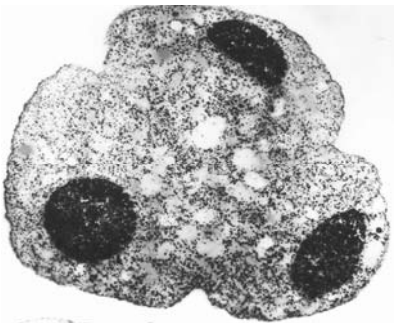

Fig. 1 Many ATPase precipitates in the tetrad of fertile anther of a male sterile Chinese cabbage. ×6700

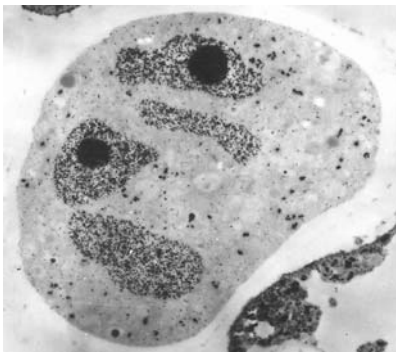

Fig. 2 Few ATPase precipitates in the tetrad of sterile anther of a male sterile Chinese cabbage. ×5000

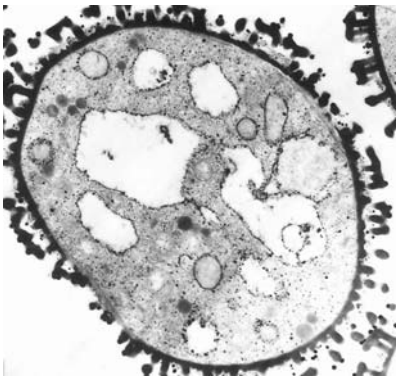

Fig. 3 Much calcium precipitates in fertile microspore of a male sterile Chinese cabbage. ×6700

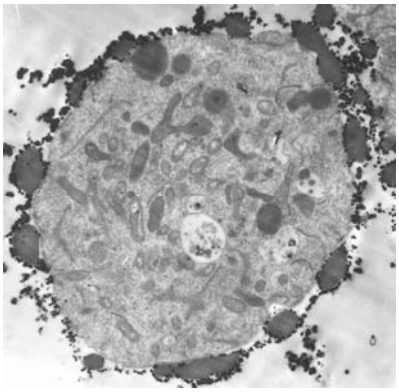

Fig. 4 Little calcium precipitates in fertile microspore of a male sterile Chinese cabbage. ×6700

CALCIUM DISTRIBUTION

Calcium is a necessary ion in plant development, with a myriad of physiological functions. The concentration of calcium in specific states of availability is highly compartmentalized within plants and is closely related to normal growth and development of plant cells. Calcium is known to interact directly and indirectly with calmodulin, regulating other proteins through signal transduction pathways as a second messenger and as a metabolic factor in various cellular activities. Studies of calcium regulation in plant physiological processes have been an active topic of research for many years; with frequent literature reviews summarizing current results (some general reviews include Bush 1995). Several studies focused on the role of calcium signaling during pollen tube elongation, while the study of calcium regulating anther development has been comparatively neglected. Tirlapur and Willemse (1992) observed higher membrane calcium in sporogenous cells than in the adjacent tapetal cells. However, during meiosis there was a significant increase in membrane calcium in the meiocytes compared to that found in the young microspores. Membrane calcium fluorescence in the sporogenous cells, meiocytes and young microspores was punctate and slightly diffused throughout the cytoplasm. In the microspores of the tetrad and the young released microspores, membrane calcium fluorescence was polarized and mainly associated with the area opposite the future colporal region. Subsequently, there was a shift in the polarity, and most of the membrane calcium fluorescence in the old microspores and pollen was regionalized towards the colporal region. The fluorescence was more diffused, indicating a change in the organellar-bound calcium. Gorska-Brylass *et al*. (1997, 1998) found the level of loosely-bound Ca^{2+} ions was higher in generative cells than in the vegetative cells of the mature pollen grain, which is one of the symptoms of metabolic differentiation of the two sister cells.

Tian *et al*. (1998) observed the calcium distribution in fertile and sterile anthers of photoperiod-sensitive genic male-sterile rice. In the late microspore stage of fertile anthers, numerous calcium precipitates appeared in the tapetum and locules, especially on the surface of microspore and Ubicsh bodies, but a few were found in the microspore cytoplasm, suggesting calcium regulates exine formation and controls movement of material into the locules. However, most calcium precipitates did not appear in the microspore, suggesting calcium channels in the plasma membrane control distribution. In sterile anthers, fewer calcium precipitates were located in tapetal cells, and more calcium accumulated in the middle layer. In addition, more calcium precipitates appeared in the aborting microspores, suggesting abnormal calcium channels were allowing calcium to move into the microspores and poison them (Tian *et al*. 1998).

Calcium precipitates accumulated in anther microspores of another male-sterile line of rice to a higher degree than in microspores of the fertility maintenance line. Additionally, more calcium precipitates accumulated in the cells of the vascular bundle of sterile anthers than in those of the fertility maintenance line, indicating abnormal calcium distribution in the vascular bundle in sterile anther affects pollen development (Meng *et al*. 2000a; Li *et al*. 2001). A recent study of a genic male-sterile Chinese cabbage in which pollen abortion occurs in the early microspore stage, showed calcium precipitates appeared in higher numbers in the fertile microspores than in the sterile microspores, which contradicts the earlier studies of rice. Some precipitates in the microspores appeared in the ER, which inflated to form small vacuoles that fused into a large vacuole to create a polarity leading to microspore inequality division (**Figs. 3, 4**). In sterile anthers, many calcium precipitates accumulated in the tapetal cells and in locules, but there were a few in the microspores that could not form vacuoles. Finally, the microspores in an environment of high calcium were aborted by plasmolization (unpublished data).

All of the results summarized above indicate it is necessary for a given content of calcium to accumulate at the right time and in the right location for anther development. High concentrations of calcium ion may create a high osmolarity, and induce material to move into the locules. However, although locules may contain high calcium content, the distribution of calcium in pollen may be different among different male sterile types of plants. For example, pollen abortion in rice and wheat occurs in late microspores that have formed a large vacuole. Therefore, after large vacuole formation, more calcium precipitates accumulated in the microspore cytoplasm will affect normal microspore metabolism and induce abortion. Pollen abortion in Chinese cabbage occurs in early microspores. Fertile microspores need to absorb large quantities of calcium to form a large vacuole; sterile microspores are not able to make a large vacuole because of the lower calcium content in the cytoplasm, resulting in pollen abortion. Therefore, calcium distribution in pollen is diverse in different plants and results in different types of pollen abortion.

ANTHER PCD

In higher plants, some organs, tissues and cells die prematurely to fulfill a special function. This programmed cell death (PCD) is a physiological developmental reaction and also a self-determination death induced by exterior signals (Pennell and Lamb 1997; Gray and Johal 1998; Drury and Gallois 2006). Early cytological studies of anthers indica-

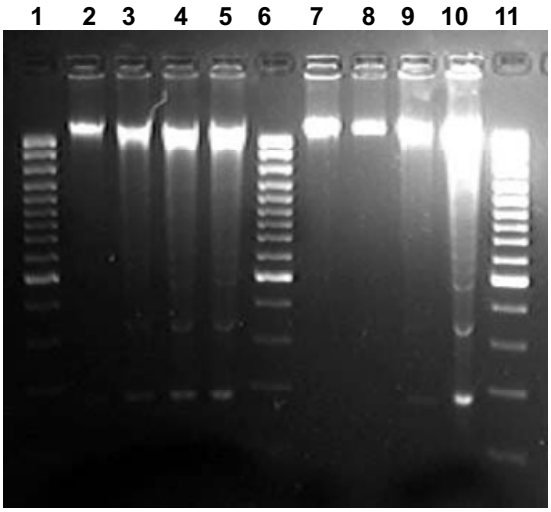

Fig. 5 Genomic DNA undergoes internucleosomal fragmentation during both fertile and sterile anther development of Chinese cabbage. 1-11 = Lane no. 1, 6, 11: molecular marker. 2-5: sterile anthers; 7-10: fertile anthers; 2: sporogenous cell stage; 3: microspore mother cell stage; 4: tetrad stage; 5: early microspore stage; 7: sporogenous cell stage; 8: microspore mother cell stage; 9: tetrad stage; 10: early microspore stage. The results indicate that laddered DNA of programmed cell death (PCD) of sterile anthers occurred during the tetrad stage. However, the laddered DNA in fertile anthers occurred during the early microspore stage, which is the tapetum PCD.

ted that as a precondition of normal anther development, the cells of the tapetum and the middle layer undergo advance death following an exact time sequence. If the sequence of cell death changes, pollen abortion will occur. Tapetum, an innermost somatic tissue located in the anther, generally dies during microspore development and does not exist in mature anthers (Piffaneli and Murphy 1998; Papini *et al*. 1999; Wu and Cheung 2000; Tian 2002). In *Lilium*, the signs of tapetal PCD first occur as early as the premeiosis stage. The signs of PCD then extended to other sporophytic tissues, leading to anther dehiscence. In pollen, no signs of PCD occur in microspore before its mitosis, then apoptotic signs display progressively in the vegetative cell (Varnier *et al.* 2005). In fertile anthers of a photoperiod-sensitive genic male-sterile rice, the parts of tapetal cells contacting the microspore first became sunken, suggesting that PCD is affected by microspores; in sterile anthers, tapetal cells can exist until anther maturation, because of microspore aborting. This further supports the proposal by Tian *et al*. (1993) that the microspore affects tapetum PCD. However, in a genic male-sterile Chinese cabbage, even microspore aborted tapetal cells degenerated on time. At anthesis the anther wall consists of two layers, the epidermis and endothecium (Xie *et al*. 2004). Using a DNA electrophoresis technique, the researchers found the fertile anther displayed an electrophoresis pattern of ladder DNA, a feature of PCD, at the early microspore stage, which was the result of tapetal cells undergoing PCD. In sterile anthers, the ladder DNA was displayed during the MMC stage, indicating PCD takes place in the MMCs (**Fig. 5**). Recently, in an Arabidopsis male sterility mutation, PCD occurred in the wild-type tapetum after microspore mitosis, but no signs of PCD are seen in the mutant tapetum. After the formation of the large autophagic vacuole in the tapetum, PCD sign of TUNEL is detected in the mutant microspores, indicating that they may go through a PCD-based breakdown as a second consequence of the observed tapetal aberrations (Vizcay-Barrena and Wilson 2006). The concept of PCD can be used to research male plant sterility and connect molecular research with cellular processes of pollen abortion in male sterility of higher plants.

CONCLUSIONS

The phenomena of male sterility in higher plants are multifaceted, and the reasons male sterility occurs also are complex. Depending on when pollen abortion occurs, the structural changes may appear in the MMC stage, the tetrad stage, the microspore stage or the bi-cellular pollen stage. Both genic male sterility and cytoplasmic male sterility can occur, driven by the genetic factors controlling male sterility. In addition, environmental factors, such as photoperiod and temperature can induce male-sterile plants. It is necessary to use diversified cell biological methods to determine the exact timing and process of pollen abortion in these different male-sterile types. Cytological analysis results combined with the results of molecular studies of male sterile genes can help improve our understanding of the mechanisms of male sterility in higher plants, and provide a theoretical basis for improving the use of hybrid-predominant crops.

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