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厦　门　大　学

博　士　学　位　论　文

# 白菜核雄性不育性的细胞分子生物学研究

# Cytological and molecular biological research on a genic male sterile Chinese cabbage (*Brassica campestris L.*)

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# Abstract

A male sterile Chinese cabbage *(Brassica campestris L. ssp. chinensis* Makino) was examined using cytological, cytochemical, calcium-potassium antimonite depositional, ATPase-lead nitrate depositional, and programmed cell death methods to characterize the development of fertile anther and the mechanism of pollen abortion in sterile anther of this plant. Some molecular biological methods were also used to clone the gene segment which correlation with the male sterility. The results as following:

(1). Comparing the structure of fertile and sterile anthers using light microscopy, we found that there was no difference before microspore mother cell meiosis stage. After MMC meiosis, however, pollenkitt substance was unevenly accumulated on the surface of early microspores of sterile anthers. As the abnormal microspores continue to grow, their cytoplasm becomes less and shrinks. Subsequently, the plasmolysis occurred and cytoplasm of aborting microspore separated with its abnormal exine. Finally, only some exine vestiges left in locule of sterile anther. The pollen abortion occurs in the early microspore stage in this male sterile plant when observed by light microspore.

(2). Polysaccharides and lipid was characterized during fertile and sterile anther development using cytochemical methods. There were no differences in distribution of starch grains and no lipids in fertile and sterile anthers before the meiosis of microspore mother cell. After the early microspore stage, the number of starch grains in the connective tissue and anther wall decreased in fertile anthers and some lipid droplets appeared in tapetal cells. Then the lipid droplets also appeared in pollen. The tapetal cells of sterile anthers, however, appeared red color which suggested some polysaccharides in the cell, but no lipid droplets appeared in the cells. The result indicated that the tapetal cells can absorb the polysaccharides from

the connective tissue and anther wall, and transform them into lipid to supported pollen development. The tapetal cells of sterile anthers, however, didn't transform polysaccharides into lipid because of the pollen abortion.

(3). The differences of ultrastructure between fertile and sterile anthers was also conducted. The earliest abnormal phenomenon in sterile anther was nucleolus of sporogenous cells locating in the edge of nucleus. During microspore mother cell development, callus wall surrounding the cell displayed uneven in the thick and discontinuous, and then some cytoplasm leaked out of the cell from some rifts in the wall. After meiosis of microspore mother cells, the cells of tetrad were irregular and some of them contained more than one nucleus in the cell. The evident disorder during exine formation in the sterile pollen occurred during its primexine formation and then the sporopollenin was irregularly deposited to form a layer of uneven and discontinuous pollen exine. Cytoplasm of aborting microspores contracted and finally degenerated after they released from tetrad. During the late microscope stage, the tapetal cells appeared disorder in synthesizing lipid material. Therefore, aborting microspore induced the functional default of tapetal cells synthesizing lipid material.

(4). Calcium distribution in the fertile and sterile anthers was tested using Potassium antimonite. During fertile anther development, calcium granules increase evidently in anther wall cells after meiosis, and then they appear in locule, suggesting calcium influx into locule from anther wall cells. Calcium granules also appear in microspore cytoplasm at the early stage, and then they are mainly accumulated on the membrane of small vacuoles which are fusing to form a large vacuole, making a polarity in the cell and preparing for asymmetry division of microspore. After microspores division, the big vacuole decomposes, and many calcium granules again accumulate on the membrane of small vacuoles, displaying calcium regulating vacuole formation and decomposition during pollen development. In sterile anthers abnormal calcium

distribution first appears in callus wall of microspore mother cell. However, only a few calcium granules appear in the cytoplasm of early microspores which then can not form small vacuoles and a big vacuole. The aborting microspores degenerate by cytoplasm shrinking. The different patterns of distribution of calcium granules in the fertile and sterile anther indicated that anomalies in the distribution of calcium accumulation correlate with the failure of pollen development and pollen abortion.

(5). $Mg^{2+}$-ATPase in the fertile and sterile anthers was located by Lead nitrate. During fertile anther development, the ATPase deposits increase evidently in the cytoplasm and nucleolus of MMC. After the meiosis of MMC, the ATPase deposits reach the most in the tetrad stage, and then decrease in the late microspore development, at last fully disappear in the bicellular pollen. The tapetal cells of anther wall have functions of synthesizing and transporting the nutrition material into locule, so there are much ATPase deposits in the tapetal cells during the early stages of microspore development. After that, the ATPase deposits in the tapetal cells decreased evidently. Furthermore, ATPase deposits in the inner surface of tapetum are more than the outer surface, suggesting that the primary function of tapetum is to transport the nutrition materiel into locule. There was no large difference in the distribution of $Mg^{2+}$-ATPase between the fertile and sterile anther.

(6). Comparing the DNA electrophoresis of fertile and sterile anthers during both developments, we found that the fertile anthers DNA appear ladder's distribution after early microspore stage. The ladder's distribution of DNA is a character of programmed cell death. However, the DNA of sterile anthers starts to appear ladder's distribution in the MMC stage. From the results of ultrastructure and DNA electrophoresis, we confirmed that the ladder's distributed DNA in fertile anthers was arose by the programmed cell death of tapetal cells, but the ladder's distributed DNA in sterile MMC stages anthers was arose by the programmed cell death of

MMC.

(7). The technology of PCR was used to clone the gene which controls male sterility in this plant. A gene segment about 890bp was differentially screen out from the sterile genome. Comparing the sequence of the gene segment with the *Arobidopsis* genebank, we found the gene segment was about 33% homologous to the gene of *Arobidopsis* MS5. The MS5 gene controls the cell meiosis, and its mutation ms5 displays abnormal process of meiosis in anther. Furthermore, we used the gene segment cross with the fertile and sterile genome respectively, we found that the gene segment coexists on the fertile and sterile genome, but its copys and location are differences between both anthers. The difference of location maybe was resulted by the mutation and recombination, and the difference of copys may affect the gene expressive production. Therefore, it is the gene segment that controls male sterility of the Chinese cabbage.

Key words: Chinese cabbage; Male sterility; Cytochemictry; Ultrastructure; $Ca^{2+}$ localization; ATPase localization; Male sterile gene clone

# 摘　要

本研究以白菜核雄性不育两用系为材料，采用细胞学观察、组织化学方法对多糖和脂类物质的定位、焦锑酸钾沉淀钙的技术、ATPase-磷酸铅沉淀技术、细胞程序性死亡研究技术以及超微结构观察等方面探讨可育花药和不育花药发育过程中在细胞水平上的差异，并且通过 PCR 克隆技术克隆得到了白菜核雄性不育性相关的基因片段,同时对该基因片段进行了序列分析及 Southern 杂交验证。通过比较系统的研究，结论如下：

1. 通过比较可育株和不育株各个时期花药的结构差异，确定了在减数分裂之前两种花药的母细胞没有明显差异。减数分裂完成以后，不育花药中的小孢子显现出了异常：从四分体中释放出的小孢子外壁不完整。随后不育花药中小孢子的细胞质逐渐发生质壁分离现象，细胞质不断收缩，最终小孢子只剩下一圈空的外壁而完全败育。该种白菜核雄性不育花药中小孢子发生败育的时间是在小孢子发育早期。

2. 用组织化学的方法研究了可育株和不育株花药发育过程中的多糖和脂类的分布动态，发现在减数分裂前，可育花药和不育花药的药隔细胞中都储藏了大量的淀粉粒，二者没有明显差异。在小孢子发育晚期，可育花药药隔细胞的淀粉粒消失，同时绒毡层细胞中出现大量的脂滴类物质，暗示着绒毡层细胞将药壁细胞中的淀粉粒多糖吸收并转化成脂类。接着二胞花粉中也积累了大量的脂类储藏物质。在不育花药中，虽然减数分裂后药隔细胞中的淀粉粒也消失了，但在绒毡层细胞中仍显示含有很高多糖类物质的红色，而显示脂类物质的黑色颗粒很少，表明不育花药绒毡层细胞将糖类转化为脂类的功能发生变异。同时在败育小孢子细胞质中也没有脂类积累。这一结果说明不育花药中，由于小孢子的败育，无法正常吸收绒毡层合成的物质，因此花药绒毡层细胞虽然将药隔组织中多糖颗粒吸收了进来，但并不将其转换成脂类。由此可见白菜不育花药中小孢子的异常也会影响到绒毡层细胞功能的正常发挥，而以往将小孢子的败育主要归因于绒毡层功能异常的观点就值得进一步的探讨。

3. 用电镜研究了可育和不育花药发育过程中超微结构的变化，进一步发现在不育花药的花粉母细胞中常出现核仁靠近核膜的边移现象。另外，包围花粉母细胞的胼胝质壁的不完整导致了花粉母细胞在减数分裂时发生紊乱，结果常发生细胞核分裂但并不伴随细胞壁形成的现象。四分体时期的异常现象还包括花粉外壁原基的异常，导致了败育小孢子外壁沉积的异常。超微结构研究的结果也验证了前面组织化学的研究结果，进一步肯定了不育花药的绒毡层细胞不将多糖物质转化为脂类物质的原因并不是绒毡层细胞功能的异常，而是由于药室内败育小孢子不吸收绒毡层分泌物质的反馈结果。此外还对可育株花药发育过程中的各个关键时期和部位的超微结构变化进行了详细的描述，明确了小孢子的内外壁发育模式以及绒毡层细胞在小孢子发育过程中的功能等。

4. 用焦锑酸钾沉淀法研究了可育和不育花药发育过程中钙的动态变化，发现在可育花药发育过程中的钙分布特征，根据花药内在特定时间和特定位置上的钙分布规律推测其生理功能：花药药室中的大量钙积累可能使药室中的渗透压较高，从而使药室成为吸收营养物质的中心，使体内的营养物质源源不断地运往药室中。在小孢子发育过程中细胞质内的钙含量增加，钙作为渗透调节物质或者液泡膜代谢调控物与小孢子形成大液泡和二胞花粉中大液泡的消失有密切关系。在不育花药中，钙的分布显示出了异常：在母细胞时期胼胝质壁内部以及绒毡层细胞内却分布着大量的钙，推测很可能是由于质膜上的钙通道异常阻止了钙进入花粉母细胞中而积累在质膜外的胼胝质壁中。减数分裂后，由于小孢子中的钙一直很少，使游离小孢子不能形成大液泡，因而小孢子发生质壁分离现象，最终小孢子通过细胞质收缩方式败育。结合前人的研究结果可以认为：在高等植物花药发育过程中需要较高钙含量具有一定的普遍性，而钙以特殊的时间和空间动态分布变化在调控花药发育中具有一定功能。同时当花药中有关钙代谢发生异常时也与雄性不育密切相关。

5. 用磷酸铅沉淀法比较研究了可育和不育花药发育过程中 $Mg^{2+}$-ATPase 的动态变化，发现各个时期可育与不育花药内 ATPase 的分布基本一致，没有表现

出明显的差异。因此不育花药的败育同 ATPase 及能量代谢的关系不紧密。在可育花药的发育过程中，小孢子母细胞的细胞质、细胞核中都表现出了较强的 ATPase 活性。减数分裂完成后，四分体细胞内的 ATPase 活性达到最高。以后药室内的 ATPase 活性就逐渐下降，到了二胞花粉后，ATPase 活性已明显下降。由于绒毡层细胞担负着合成及转运小孢子发育所需营养物质的功能，因此绒毡层的细胞质在其发育早期一直都表现出较强的 ATPase 活性，到了小孢子发育早期，绒毡层细胞质中的 ATPase 活性达到最高，随后开始下降。此外我们还发现整个发育过程中，绒毡层细胞内切向面细胞膜上的 ATPase 活性要远高于外切向面，说明绒毡层行使的主要功能是向药室内分泌营养物质。

6. 用 DNA 梯状电泳技术比较研究了可育及不育花药各个发育时期的总 DNA 电泳情况，发现可育花药的总 DNA 在小孢子发育早期开始出现细胞程序死亡的梯状 DNA 条带特征，而不育花药在小孢子母细胞时期的总 DNA 就提前出现了这种梯状 DNA 条带现象。由此我们认为在可育花药中小孢子发育早期出现的梯状 DNA 条带是由绒毡层细胞的程序性死亡造成的，而不育株花药在花粉母细胞时期提前出现的梯状 DNA 条带则很可能是花粉母细胞发生细胞程序死亡造成的。根据不育花药败育过程中的超微结构特征以及 DNA 电泳实验的结果我们认为白菜核雄性不育两用系不育株花药的败育是一种细胞程序死亡过程。

7. 用 PCR 克隆技术从不育株基因组总 DNA 中分离得到了一条 890bp 的特异基因片段，对该基因片段进行测序并用 Blast 软件分析的结果表明该基因片段与拟南芥 MS5 蛋白的基因有 33%同源。拟南芥 Ms5 基因被认为是与小孢子减数分裂过程相关的一个基因，它的突变会导致小孢子母细胞减数分裂过程的紊乱。同时该基因片段通过地高辛标记作为探针分别同可育株及不育株基因组进行杂交验证，发现该基因片段在可育株及不育株基因组中均有分布，但在所处的位置和拷贝数上存在着明显的差异，所处位置不同可能跟该基因的突变和重组有关，而拷贝数不同则会影响该基因的表达结果。

关键词：白菜；雄性不育；细胞化学；超微结构；$Ca^{2+}$定位；ATPase 定位；不育基因克隆