國立臺灣大學生物資源暨農學院園藝學系

碩士論文

Department of Horticulture

College of Bioresources and Agriculture

National Taiwan University

Master Thesis

番椒細胞質雄不稔系統穩定性之研究

The Stability for Cytoplasmic Male Sterile in Pepper

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中華民國 九十八 年 二 月

Feb., 2009

誌謝

首先感謝恩師 林宗賢老師與楊雯如老師於修業期間的督促與鼓勵，讓我順利完成碩士學位與論文。林老師您雖曾暫時離開園藝系至台中科博館當館長，仍抽空關心我的試驗狀況並給予我許多的意見，激盪出更多有趣的想法與觀點，而您對農業的熱忱與堅持、待人處事的方法及態度，為我努力學習的目標。楊老師您對我的指導照顧，包含生活上的瑣事、每一次的上台演出、及未來的人生，總是用您獨特的風格談笑一切，卻也都刺中核心，我總是一直放在心上並反覆思考，成為我能不斷進步的原因。

感謝亞蔬—世界中心 Dr. Gniffke 提供我試驗材料與場地，您總是非常有耐心地與我溝通並給我最大的協助，感謝林世雯小姐與謝雪琴小姐幫助我對試驗材料的熟悉，給我許多幫忙及意外的美食。感謝鍾美珠老師給我極大的自由與空間運用您的設備及論文的修改，每每一起看著顯微鏡一起討論 data，都讓我滿載而歸。感謝李勇毅博士於我試驗之初提供試驗方向，並教導切片技術。感謝黃玲瓏老師與陳香君老師帶我進入且愛上植物解剖，上妳們的課是種很棒的享受。感謝中研院植微所簡萬能老師與技術人員、及台大生科院生物影像平台(TC5 Bio- image) 提供設備及切片技術指導，讓我順利完成試驗。

謝謝大學的同窗好友芳瑀、蓓文、愛媜、祖威、紹益及紹謙，即使大學畢業後就各自在不同地方打拼，偶爾的相聚總能帶給我滿滿的開心。謝謝一蘆學長、錦興學長、元聰學長在 lab meeting 時提供各式各樣的意見，在中研院的芳綺學姊、桂瑞學姊及紹安學長的照顧與餵食(哈)，還有可愛的嵐雁、孟姿、美蘭、李廉、嘉彬、少雄、資棟、楓俊、禎陽、本忞、芳怡、恩康跟伊婷，與你們聊天及搞笑為我的研究生生活中帶來更多的歡樂。

最後，謝謝爸媽給我無限的包容與溫暖，讓我順利完成學業且不需煩惱家裡經濟，謝謝大哥跟二哥對妹妹的照顧跟疼愛。特別謝謝一蘆學長，是你帶我踏入真正的農業；謝謝李廉，你的支持與陪伴幫我度過許多難過的日子。謝謝。

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摘要

細胞質雄不稔 (cytoplasmic male sterility, CMS) 由細胞質與細胞核之間的交互作用所控制，但某些雄不稔番椒品系並非可以一直維持其雄不稔性，植株受低溫影響會部分跟完全恢復稔性，當溫度升高則植株會再回復雄不稔性。本試驗的目的為確認所收集的番椒品系之遺傳背景、溫度及季節變化對雄不稔系之影響、以及穩定與不穩定雄不稔系之解剖之差異。

所有參試雄不稔番椒品系可藉由引子組，分別擴增出預期之(S)*ψatp6-2*片段，而其辣椒維持系則無法擴增出預期產物；針對分子標記 *orf456* 亦有相似的結果。上述結果可顯示所有參試雄不稔番椒之細胞質均含有(S)*ψatp6-2* 與 *orf456*，而該辣椒維持系因具 N 型細胞質，故不會帶有(S)*ψatp6-2* 與 *orf456*。然而，不穩定雄不稔甜椒之維持系‘PBC 84 selex’ 與‘9946-2138’，可由 CMS P1 或 CMS P2 引子組擴增出 *orf456* 片段，表示在特定的維持系中，其細胞質之遺傳背景可能較複雜且異於其他維持系。

不穩定雄不稔辣椒系‘CCA 7243’，於溫室與田間之夜溫低於 21^{o}C 可恢復稔性，且於 20/15^{o}C 之人工氣候室內也有恢復稔性之現象；夜溫低於 21 ^{o}C 或 17 ^{o}C，另一不穩定雄不稔甜椒系‘CCA 7236’可恢復稔性，但日溫低於 18^{o}C 時，則該植株不易恢復稔性。此外，不穩定雄不稔系之稔性恢復並非持續表現，而是呈現規律週期。兩不穩定雄不稔系夜溫升高時均會回復雄不稔。雄不稔系‘CCA 7244’與‘CCA 7234’於一段長時間的低夜溫環境下，仍幾乎維持其雄不稔性。

不穩定雄不稔系‘CCA 7243’於高溫下表現雄不稔性，其絨氈層細胞之異常極不同於穩定雄不稔系‘CCA 7244’之絨氈層。‘CCA 7243’之花藥腔室於四分子體形成時會增大，或者不會；相較於‘CCA 7244’，其花藥腔室於該時期不會擴大。隨著小孢子細胞發育期間，‘CCA 7243’之絨氈層為濃稠的細胞質或具有一液泡於細

胞質中，相較於‘CCA 7244’之絨氈層僅呈現液泡化；此外，絨氈層細胞瓦解之時間點難以辨識，但兩品系之絨氈層細胞最終會消逝。由上述解剖結果顯示，不穩定雄不稔系之遺傳調控可能較為複雜。雄不稔不穩定系‘CCA7243’之花藥恢復稔性，表示其發育構造上與維持系‘PBC 385’無異。

關鍵字：花藥分化、*atp6*、番椒、細胞質雄不稔、小配子發生、*orf 456*、溫度

Abstract

Cytoplasmic male sterility (CMS) is the result of interaction between cytoplasm and nuclear genes. The sterility of the CMS pepper lines was not always stable. Some of them might restore their fertility partially or completely due to low temperature, and such restoration could be resumed as temperature re-elevate. The objective of this thesis is to study the genetic background of collected pepper lines, the influences of temperature and seasonal changes on CMS pepper lines, and the anatomy difference between stable and unstable CMS line.

All CMS pepper lines could generate predicted a (S)*ψatp6-2* fragment by primer sets, while their maintainer lines couldn't produce the predicted products, and similar results were obtained for *orf456* fragment. These results revealed that the cytoplasms of all the CMS pepper lines contained (S) *ψatp6-2* and *orf456*, and their maintainers of hot pepper lines, with N cytoplasm, didn't have such defected *ψatp6-2* and *orf456*. Surprisingly, 'PBC 84 selex' and '9946-2138', maintainer lines of unstable CMS sweet pepper, could generate *orf456* fragment by CMS P1 or CMS P2 primer sets, representing that the genetic background of cytoplasm in specific maintainers might be more complicated and different from other maintainers.

Unstable CMS hot pepper line ‘CCA 7243’ could restore fertility in the greenhouse and in the field while the night temperature was below 21 ^{o}C, and also could restore in the 20/15 ^{o}C phytotron. Plants of unstable CMS sweet pepper line ‘CCA 7236’ restored fertility while night temperature was below 21^{o}C or 17 ^{o}C, but plants hardly restored while day temperature below 18 ^{o}C. Besides, the fertility restoration of unstable CMS lines showed as a regular cycle, not always steady expressed. When night temperature went higher, both lines were reverted male sterile. Both CMS line ‘CCA 7244’ and ‘CCA 7234’ almost expressed male sterile even under a long period of lower night temperature.

As unstable CMS line ‘CCA7243’ expressed sterility under high temperature, abnormalities in tapetum cells were very different from the results of stable CMS line ‘CCA7244’. While tetrads formed, locule in ‘CCA 7243’ may or may not increase in size as compared with locular space did not increase in ‘CCA 7244’. As microspores development, the appearance of tapetum in ‘CCA 7243’ with dense cytoplasm or containing a vacuole was different from vacuolated tapetum in ‘CCA 7244’, and timing of tapetum cells degeneration was hardly identified, but tapetum cells in both lines were gone at the end. These anatomical data revealed that the genetic control might be more complicated in unstable CMS line. As the anthers of ‘CCA7243’ resumed the

fertility, there was no difference between ‘CCA7243’ and its maintainer ‘PBC 385’ anatomically.

Keywords: anther differentiation, *atp6*, *Capsicum annuum* L., cytoplasmic male sterility (CMS), microgametogenesis, *orf 456*, temperature

Chapter 1

Introduction

Capsicum is a genus of plants from Solanaceae family and native to the tropical and subtropical Americas. Among approximately 30 species of *Capsicum*, five major domesticated species are used widely: *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*. *C. annuum*, which contains both hot and sweet pepper, is the most cultivated and economically important species worldwide. Pepper fruits are in a tremendous variety of colors, shapes, sizes, and tastes (Bosland, 1992; Bosland and Votava, 2000; Heiser and Pickersgill, 1969; Smith et al, 1987).

C. annuum is a warm-season crop, and the optimum range of temperature for plant growth is 21-30 ^{o}C. High temperature 33 ^{o}C exposure during bell pepper floral bud at < 2.5 mm in length does not affect flower production, but reduced pollen viability and fruit set. In addition, fertilization is also sensitive to high temperature 33 ^{o}C, which inhibited fruit set (Erickson and Markhart, 2001; Erickson and Markhart, 2002). Besides, high night or low night temperature also decreased fruit production. High night temperature at 24 ^{o}C caused considerable blossom drop in bell pepper (Rylski and Spigelman, 1982). Low night temperature 10 ^{o}C and 15 ^{o}C decreased pollen viability (Mercado et al, 1997). As floral bud development under low night temperature 10±2 ^{o}C, starch accumulation in pollen grains at 3 days before anthesis

and more than two-fold in total soluble sugars in the mature pollen grains were both decreased (Pressman et al, 2006).

Pepper is also an important vegetable crop in Central and South Taiwan. Chiayi County, Pingtung County, and Kaohsiung County are the mainly produced places in hot pepper production; Nantou County, Yunlin County, and Pingtung County are in sweet pepper production. Pepper plant is typically grown as annual in Taiwan even they can be also grown as perennial in other regions. Although pepper is a warm-season crop, plant is usually grown in fall and winter in Taiwan. The commercial pepper cultivars are often sold as F1 hybrids, which were produced by three-line cytoplasmic male sterility (CMS) system. Using three-line CMS system in pepper F1 hybrid seeds producing has several advantages, as it can reduce labor costs for emasculation, generate purer hybrid seed, and protect all the proprietary while increasing crop vigor and yield through heterosis (Schnable and Wise, 1998; Wise and Pring, 2002; Eckardt, 2006).

According to previous studies, the stability of CMS pepper lines could not always maintain their sterility. Some CMS pepper lines can restore partial or completely fertility caused by low temperature, revert to sterility again when temperature elevated (Kaul, 1988; Peterson, 1958; Shifriss and Guri, 1979; Shifriss, 1997). This

phenomenon also observed in specific CMS pepper lines which ware bred by breeders in Taiwan. Two CMS sweet pepper lines of AVRDC-The World Vegetable Center, ‘CCA 7229’ and ‘CCA 7236’ showed fertility in the field in winter from 2005 to 2006 (unpublished data). A CMS hot pepper line ‘ACC. 17’, which was bred by Evergrow Seed CO., LTD, could produce normal pollens and fruit set in the growth chamber at 25/18 °C, 20/18 °C, and 20/12 °C (Hong, 2003).

Up to date, the anther structure and microgametogenesis observations by light microscopy were only compared in CMS pepper and near-isogenic line, for identifing the reasons for the production of no functional pollen by CMS pepper plant (Horner and Rogers, 1974; Luo et al., 2006; Novák and Betlach, 1970). But, few studies have worked on the unstable CMS pepper lines by comparing the anatomical differences between fertile and sterile phenotype under varied temperatures (Hong, 2003).

In present study, both identified CMS and maintainer pepper lines are studied to confirm their genetic background by previously developed markers. Next, the stability of CMS hot and sweet pepper were examined in the phytotron of NTU and in the field of AVRDC–The World Vegetable Center to figure out the critical temperature causing unstable CMS lines restored fertility. Finally, the reason of unstable CMS pepper lines restored fertility were discussed by comparing the anther structure and

microgametogenesis in the stable CMS line and the unstable CMS line which expressed fertile and sterile phenotype.

Chapter 2

Literature Review

2.1 Cytoplasmic male sterility in higher plant

Cytoplasmic male sterility (CMS) system which had been detected over 150 plant species was a maternally inherited trait (Kaul, 1988). CMS system involves interactions between nuclear and cytoplasm genes. The CMS plants fail to produce functional pollens, which is caused by cytoplasmic dysfunction in plant mitochondrial genomes (Hanson, 1991; Hanson and Bentolila, 2004; Schable and Wise, 1998; Wise and Pring, 2002). The specific nuclear genes, known as fertile restorer genes, restored male fertility of plant carrying a CMS cytoplasm (Bentolila et al., 2002; Hanson and Bentolila, 2004; Schable and Wise, 1998). CMS plants are able to generate next generation by crossing with pollens offered by fertile plants containing fertile restorer genes.

The ‘three-line’ CMS system, which was a breeding system for commercial use, consisted of a male sterile line, a maintainer line, and a restorer line. A male sterile line was maintained by a maintainer line which was propagated by self-pollinating at the same time, and crossed with a restorer line to produce F1 hybrid seeds (Kaul, 1988). This system played an important role in agronomy due to several advantages, as it

could reduce labor costs for emasculation, generate purer hybrid seed, and protect all the proprietary while increasing crop vigor and yield through heterosis (Schnable and Wise, 1998; Wise and Pring, 2002; Eckardt, 2006).

2.2 The characters of CMS-related genes

CMS-related genes, which were expressed as novel chimeric genes, had been already identified in plant mitochondrial genomes (Hanson and Bentolila, 2004; Schnable and Wise, 1998). Mitochondria were the organelles of respiration, a process of series metabolic pathways of citric acid cycle, mitochondrial electron transport chain and ATP synthesis (Browse et al., 2006). Well-evidenced CMS-related loci were found in standard mitochondrial genes associated to respiration, as in ATP synthase subunit coding sequences, cytochrome oxidase subunit gene coding regions, NADH dehydrogenase subunit, or associated to ribosomal and transfer protein. Besides, more CMS-related genes were often associated with abnormal open reading frames (*orfs*), which function was not clear, combined with sequences of standard mitochondrial genes or co-transcript with standard mitochondrial genes in some case (Hanson, 1991; Hanson and Bentolila, 2004; Schable and Wise, 1998; Wise and Pring, 2002).

Several CMS-related genes had been reported in crops. In *Brassica napus* with "Polima" (pol) cytoplasm, a rearranged region, *orf 224*, was in the upstream of *atp6* in

the CMS phenotype (Handa et al., 1995; Singh and Brown, 1991). In Solanaceae, *Petunia* was an important model for studying CMS system. The CMS-related gene, called as *pcf* (petunia CMS-associated fused gene), consisted of the 5' region of the *atp9* gene which was parts of the first and second exons of the *cox II* gene, and an unidentified sequence (*urfS*) which were co-transcribed (Conley and Hanson, 1995; Schnable and Wise, 1998). In CMS hot pepper, which was also from Solanaceae family, *atp6* and *cox II* were two candidate CMS-related genes. One copy of *atp6* genes lost one *Eco*RI site through a C-to-T mutation at 1892bp in the 5' region and had a 251 bp truncated in the 3' region. A new ORF, called *orf456,* was found at the 3' end of the *cox II* gene and co-transcribed with the *cox II* gene upstream (Kim et al, 2001; Kim and Kim, 2005; Kim and Kim, 2006; Kim et al, 2007).

2.3 The characters of restorer genes and interaction with CMS-related genes

The specific dominant nuclear genes, fertility restorer genes (*Rf*), were able to alter the male sterile phenotype by suppressing or counteracting the expression of the CMS-associated gene (Bentolila et al., 2002; Hanson and Bentolila, 2004; Schable and Wise, 1998). The restoration systems divided into two forms depending of the number of restorer genes. In one form, one or two major restorer loci conferred complete restoration. In another form, full restoration required the concerted action of multiple

genes, each one of which provided only small incremental effects (Schable and Wise, 1998).

In molecular identification, pentatricopeptide repeat (PPR) proteins, which contained degenerated repeat units of 35 amino acids, were encoded by the *Rf* genes, involving the male-fertility restoration to CMS plants. The PPR gene family was a eukaryote-specific protein family particularly expended in higher plants (Small and Peeters, 2000; Andrés et al., 2007; Bentolila et al., 2004; Chase, 2006). The typical structure of PPR family consisted of 3 parts: untargeting protein or plastids- or mitochondria-targeting protein at N-terminus, repeat structure, and additional C-terminal extensions. The character of repeat structure containing 2 to 26 repeat proteins (also called as repeat motifs), with an average of 12 proteins in plants, are as tandem arrays. Depending on the type of repeat proteins, the PPR gene family divided into P subfamily, which comprised all the same repeat motifs with 35 amino acids, and PLS subfamily, which comprised an organized pattern of triple motifs (P-L-S) repeated. The PLS subfamily of proteins was distinguished by C-terminal extensions, which contained three types of domain, E, E+, and DYW and were unrelated to the repeat motifs. These C-terminal extensions were present as E alone, E combined with E+, or E combined with E+ and DYW, but the roles of these domains were not clear (Andrés

et al., 2007; Chase, 2006; Lurin et al., 2004).

Up to date, *Rf* genes, which encoded mitochondria-targeting proteins with PPR repeat proteins, had been cloned in *Petunia*, radish, and rice (Hanson and Bentolila, 2004). PPR proteins seemed to operate the maturation of plant mitochondrial (and chloroplast) mRNAs at the post-transcriptional level, including transcript editing and transcript processing. Therefore, the decreased abundance of CMS-related transcripts, or the occurrence of internal processing events that truncate theses transcripts, often accompanied protein products of the CMS-related genes loss in the presence of restorer genes which encoded PPR proteins (Bentolila et al., 2002; Chase, 2006; Mackenzie and Mclntosh, 1999; Small and Peeters, 2000). Such as *Rf* gene encoding *Rf-PPR592* in *Petunia*, which was able to decrease *pcf* (petunia CMS-associated fused gene) mRNA expression and reduce the protein products to nearly undetectable levels, restored fertility when transferred to *rf*/*rf* CMS plants (Bentolila et al., 2002; Gillman et al., 2007).

2.4 Phenotypes and abnormalities in tapetum of CMS plants

The CMS plants of maize, petunia, and sunflower, which are no distinct morphological differences from male fertile plants but with the degenerated stamens, could not produce viable, functional pollens due to the dysfunction in tapetum (Hanson

and Bentolila, 2004; Chase, 2006). The tapetum, an inner layer of anther wall around the sporogenous tissue, played an important role in pollen development. Several functions had been attributed to the tapetum, including the supply of sporopollenin and a role in exine formation, breakdown of callose wall around tetrads, supply of nutrients to developing pollen and reserve metabolites of mature pollen; and supply of exine proteins and surface coat substances (pollenkitt/tryphine). Hence, the abnormalities in the structure and/or function of the tapetum resulted in pollen development arrested (Shivanna et al., 2005).

In some CMS plants, pollen formation is disrupted at the meiotic or post meiotic stage. Such as CMS *P. hybrid* and *P. parodii*, the sporogenous tissue of sterile anther stopped developing during early meiosis I, showing that pollen mother cells rarely completed anaphase I (Conley and Hanson, 1995). In CMS sunflower (*Helianthus annuus*), irregular tapetum cells enlarged radially and crushed tetrads, thereafter tapetum degenerated. In CMS sweet pepper lines, pollen mother cells could complete meiosis, but the tetrads failed to release microspores and were crushed by highly vacuolated tapetum cells (Horner and Rogers, 1974). In another CMS sweet pepper lines, tetrads of were able to release microspores, which failed to develop into mature pollens due to crushed by the swollen tapetum cells (Luo et al., 2006).

Chapter 3

Genetic Background Identification in Hot and Sweet Pepper Lines

3.1 Introduction

CMS system is an interaction between nuclear and cytoplasm genes. The male sterility was often associated with abnormal open reading frames (ORFs) in plant mitochondrial genomes, causing cytoplamic dysfunction (Hanson, 1991; Hanson and Bentolila, 2004; Schable and Wise, 1998; Wise and Pring, 2002). The fertility could be restored by nuclear genes known as fertile restorer genes (*Rf*) which suppressed the male-sterility phenotype to lead functional pollens produced by plants with CMS cytoplasm (Bentolila et al., 2002; Hanson and Bentolila, 2004; Schable and Wise, 1998).

Structural differences of CMS-related genes in the mitochondrial genomes between male sterility (S type) and male fertility (N type) cytoplasms were observed (Hanson, 1991; Hanson and Bentolila, 2004; Schable and Wise, 1998; Wise and Pring, 2002). In hot pepper, the mitochondrial genes that involved in CMS being identified includes *atp6* (ATP synthase subunit 6) and *cox II* (cytochrome oxidase subunit 2) (Kim et al, 2001; Kim and Kim, 2005; Kim and Kim, 2006; Kim et al, 2007), which participate in ATP synthesis and electron transport chain, respectively. In the

mitochondrial genome of pepper, two copies of *atp6* genes were identified, designed as *atp6-1* and *atp6-2*. Comparing the sequences of the (N)*atp6-2* and (S)*atp6-2*, the later one lost one EcoRI site through a C-to-T mutation at 1892bp in the 5' region and had a 251 bp truncated region at 3', and (S)*atp6-2* was thus renamed as (S)*ψatp6-2*. Besides, both (N)*atp6-2* and (S)*ψatp6-2* could be normally transcribed in pepper mitochondria (Kim and Kim, 2005; Kim and Kim, 2006). Another major difference was located at the downstream region of the stop codon of *cox II*. The sequences of the CMS pepper and the respective maintainer lines diverted from 41bp downstream of the stop codon, and an *orf456* adjacent *cox II* was identified in the CMS line which encoded for a 17-kDa protein and putatively interact with the fertility restore genes (*Rf*) (Kim and Kim, 2005; Kim et al, 2007). Five primer sets were designed according to sequence of (S)*ψatp6-2* and *orf456* for comparison the cytoplasmic genetic background of CMS pepper lines developed in TSIPS (Taiwan Seed Improvement and Propagation Station, COA) with hot pepper line 'Milyang' in our previous studies (Kim and Kim, 2005; Kim and Kim, 2006; Kim et al, 2007), and the result confirmed that the TSIPS lines contain (S)ψ*atp6-2* and *orf456* (Lee et al., 2007).

Fertility restorer genes (*Rf*) of nuclear had the ability to suppress the deleterious effects causing by CMS-related genes. In the *Petunia*, transformation of the *Rf* gene,

encoded for Rf-PPR 592, could restore the fertility of the CMS *Petunia* due to decrease mRNA expression of *pcf* (petunia CMS-associated fused) (Bemtolila et al., 2002). In hot pepper, *Rf* gene could affect the expression of *atp6* mRNA from anther and thus produce functional pollens (Kim and Kim, 2006). Several markers for detecting *Rf* genes in hot pepper lines have been developed. Two RAPD markers, $OP13_{1400}$ and $OW19_{800}$, were also able to detect the major *Rf* gene in hot pepper especially $OP13_{1400}$ which was tightly linked to *Rf* gene (Zhang et al, 2000); however, these markers could not detect *Rf* gene in sweet pepper lines even if they were male fertile (Zhang et al, 2000; Kumar et al, 2007). The restorer marker CRF-S reported by Guylas et al (2006) was specific to a major *Rf* gene in hot pepper. Moreover, a co-dominant CAPS marker conversed from AFLP marker, AFRF8CAPS, was also linked to *Rf* gene in pepper lines and could differentiate *Rf* and *rf* genes (Kim et al, 2006).

The sterility of the CMS pepper lines was not always stable. Some of them might restore fertility partially or completely due to low temperature, and such restoration could be resumed as temperature re-elevating (Hong, 2003; Kaul, 1988; Peterson, 1958; Shifriss and Guri, 1979; Shifriss, 1997). The genetic background of the unstable CMS lines was not clear yet. In the present study, all the collected CMS pepper lines and the respective maintainers would be examined by the five primer sets that we designed

previously for the identification of (S)*ψatp6-2 and orf456* (Lee et al., 2007). Besides, both stable and unstable sweet pepper lines developed by AVRDC – The World Vegetable Center would be examined as well. All the samples were also checked for the *Rf* gene.

3.2 Materials and methods

Plant materials

The CMS lines and the respective maintainer and restorer lines of hot peppers and sweet peppers used in this study were listed in Table 3.1. Seeds of hot and sweet pepper were sown in flats in growth chamber at day/night temperature of 30/25 ^{o}C with a 14h photoperiod. Seedlings were be transplanted to 3' pots until they reached six leaves, and then transplanted to 5' pots. Plants were maintained in the greenhouse of NTU. Other CMS, maintainer, and restorer pepper lines from AVRDC - The World Vegetable Center examined in this study were also listed in Table 3.1. The leaves of each line were harvested and placed into sealed plastic bags and sent to Taipei under refrigeration and then frozen immediately by liquid nitrogen. The leaf samples were stored at – 80 ^{o}C until used.

Reagents and chemicals

Extraction buffer consists of 3% CTAB (w/v), 1.4 M NaCl, 20 mM EDTA, 100

mM Tris-HCl (pH 8), and 0.2% β-mercaptoethanol (v/v) added just before use. TNE buffer consists 10 mM Tris-HCl (pH 8), 100 mMNaCl, and 0.5 mM EDTA (pH 8). TE buffer consists 100 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8). In addition, chloroform : isoamyl alcohol (24:1), 75% ethanol, and 7.5 M ammonium acetate were also prepared.

Extraction of pepper plant's genomic DNA

0.2 g young green leaves of each pepper line were harvested and ground into fine powder with a mortar and pestle in liquid nitrogen. Powder was transferred to a clean autoclaved 1.5mL eppendorf tube and 700 µl extraction buffer was added. The tube was incubated in 60^{o}C for 30-40 min with twice swirling. 700 µl chloroform: isoamyl alcohol was added into the same tube, and mixed by gentle inversion for 10 times. The tube was centrifuged at 14000 rpm for 10 min at 4 ^{o}C and supernatant was transferred to another tube. Repeat these two steps once. The supernatant was precipitated with 450 µl ice-cold isopropenol and centrifuged at 14000 rpm for 10 min at 4 ^{o}C. The supernatant was discarded and the pellet was washed twice with 700 µl 75% ethanol. The pellet was air dried at RT and dissolve in 400 µl TNE buffer. 2 µl RNaseA (10 µg/ml) was added and placed at 4 ^{o}C overnight. 400 µl chloroform: isoamyl alcohol was added to the tube, and mixed by gentle inversion for 10 times. The tube was

centrifuged at 14000 rpm for 10 min at RT or 4 ^{o}C and supernatant was transferred to another tube. 800 µl ice-cold 95% ethanol and 40 µl ice-cold ammonium acetate was added and mixed by gentle inversion. The tube was centrifuged at 14000 rpm for 10 min at RT. The supernatant was discarded and the pellet was washed with 1 mL 70% and 95% ethanol. The pellet was air dried at RT and dissolve in 200 µl TE buffer. And the DNA solution was stored at – 20 ^{o}C until used.

Genomic DNA quality and quantity analysis

The quality of extracted DNA was checked by agarose gel electrophoresis. 5 µl DNA solution and 2 µl loading dye were mixed and loaded in a 0.7% agarose gel. The gel were run at 50 mA for 20 min, and stained by ethidium bromide for 10 min and rinsed by water for 30 min. The quality was estimated by UV light (UVi-Silver GAS7308, UVi-tec, Violet Bioscience INC.).

The quantity of extracted DNA was estimated by spectrophotometry (U-2001 Spectrophotometer, HITACHI Instrument, INC.) by calculating $A_{260/280}$ ratio, and the DNA concentration was estimated by measuring absorbance at 260 nm. DNA concentration (ng/µl) = O.D.$_{260}$ x 50 µg/ml x 100 (dilution factor). The concentration of each sample was adjusted to10 ng/µl, and stored the DNA solution at -20 ^{o}C until used.

PCR amplification to genetic background identification

In order to identify genetic background of each pepper line, primer sets listed in table 3.2 for the identification of CMS-related genes and fertile restorer gene were used in this study. PCR amplification was performed in RoboCycler GRADIENT 96 (STRATAGENE INC.) Each PCR reaction mixture of 25 µl consisted of 60ng genomic DNA, 2.5 µl 10X reaction buffer, 3 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, 0.5µl of 10 µM primer set, and 0.5 µl *Taq* DNA polymerase. For identification of CMS-related genes, the PCR reaction mixtures were heated at an initial step of 92 ^{o}C for 2 min, and then subjected to 35 cycles of following program: 92 ^{o}C for 1min, 52 ^{o}C for 45 s, 72 ^{o}C for 1 min. After the last cycle, temperature was maintained at 72 ^{o}C for 10 min, and then cooled at 6 ^{o}C for 10 min. For identification of fertile restorer gene (Gulyas *et al.*, 2006), The PCR reaction mixtures were heated at an initial step of 94 ^{o}C for 3 min, and then subjected to 35 cycles of following program: 94 ^{o}C for 30s, 58 ^{o}C for 1 min, 72 ^{o}C for 1 min. After the last cycle, temperature was maintained at 72 ^{o}C for 5 min, and then cooled at 6 ^{o}C for 10 min. The amplified DNA were stored – 4 ^{o}C until used. The amplified DNA was electrophorised in a 1.2% agarose gel containing 0.5 mg/ml ethidium bromide at 50 mA for 25-30 min. The results were recorded by UV light (UVi-Silver GAS7308, UVi-tec, Violet Bioscience INC.).

3.3 Results and discussion

All the CMS pepper lines could generate predicted (S)ψ*atp6-2* fragments by each primer set, and their maintainer lines didn't produced the predicted products (Fig. 3.1, Fig. 3.3 and Table 3.3). For *orf456*, similar results were obtained (Fig. 3.2 and Fig. 3.4) and summarized in Table 3.3. These results revealed that the cytoplasms of all the CMS pepper lines including hot and sweet pepper lines contained (S)ψ*atp6-2* and *orf456*, and their maintainers of hot pepper lines, with N cytoplasm, didn't have such defected ψ*atp6-2* and *orf456* as the maintainer of CMS hot pepper line 'Milyang' (Kim and Kim, 2005; Kim and Kim, 2006). Surprisingly, 'PBC 84 selex' and '9946-2138', maintainer lines of unstable CMS sweet pepper 'CCA 7236' and 'CCA 7231' respectively, could generate *orf456* fragment by CMS P1 or CMS P2 primer set (Fig. 3.2, Fig. 3.4 and Table 3.3). For '9847-4754', the other maintainer line of stable CMS line 'CCA 7234', did not generate *orf456* fragment. (Fig. 3.5 and Table 3.3). These results revealed that the genetic background of cytoplasm in maintainers of unstable CMS sweet pepper lines might be more complicated and different from it in maintainers of CMS hot pepper lines and of stable CMS sweet pepper line. In previous studies, CMS-related genes, (S)ψ*atp6-2* and *orf456*, were cloned only in hot pepper line 'Milyang' (Kim et al, 2001; Kim and Kim, 2006; Kim et al, 2007), and it was not

clear that which one of CMS-related genes mainly caused male sterile phenotype (Kim et al, 2007). All of the restore lines including hot and sweet peppers did not generateψ*atp6-2* fragment by ATP 1 primer set, but only two restorer sweet pepper line 'C03804' and 'PBC1419-Y', as an unstable restorer and a stable restore respectively, generated *orf456* fragment by CMS P1 primer set (Fig. 3.3, Fig. 3.4 and Table 3.3). These results showed that cytoplasm of restorer lines containing *orf456* fragment was not related to the stability of fertile expression.

For *Rf* gene, all the CMS lines and maintainers did not generate predicted *Rf* fragment by CRF-S, and a control 'A5', a restorer hot pepper line with identified genetic background *Rf Rf* from TRIS, generated predicted product (Fig. 3.5, Fig. 3.6 and Table 3.3). These results revealed that the nuclei of all the CMS and maintainers including hot and sweet pepper lines did not have *Rf* as the results of a CMS line (Gulyas et al., 2006). Surprisingly, only restorer hot pepper line 'PBC 142' and restorer sweet pepper line '9852-131' could generate predicted product (Fig. 3.6 and Table 3.3). The result reveals that this primer set might not be tightly linked to *Rf* gene. Another two RAPD markers, $OP13_{1400}$ and $OW19_{800}$, were also able to detect the major *Rf* gene in hot pepper, especially $OP13_{1400}$ which was tightly linked to *Rf* gene (Zhang et al, 2000); however, the markers could not detect *Rf* gene in some restorer lines with

identified genetic background *Rf Rf* or *Rf rf* (Zhang et al, 2000; Kumar et al, 2007).

The markers for detecting CMS-related genes were necessary for early genetic background identification, and reducing costs for waiting plants grown up to examine floral characteristics. Besides, the analysis results could be convinced even if the unstable sterility expression of CMS plants influenced by environment (Kim and Kim, 2005). According to the data in this study, the S cytoplasm of CMS lines including hot and sweet peppers were able to examine by each primer sets while plants was young, but N cytoplasm of maintainer sweet pepper lines need identify by fertility phenotype expression.

Table 3.1 The pedigree of CMS, maintainer or near-isogenic lines, and restorer pepper lines derived from AVRDC- The World Vegetable Center.

No.	CCA Code	Pedigree	hot/sweet pepper	line of CMS system	Stability of sterility
	CCA7243	Seungchon(cms)/8*Arunalu = CCA4758/PBC 483	hot	CMS	Unstable
	CCA7244	Seungchon(cms)/8*9907-9611 =CCA4759/9907-9611	hot	CMS	Stable
	PBC 483	Arunalu	hot	maintainer of CCA 7243	
	9907-9611	PBC 385 sle.	hot	maintainer of CCA 7244	
	CCA7236	PBC385-Aline/9*Jin's Sweetie selex	sweet	CMS	Unstable
	CCA7234	PBC385-Aline/9*Mito Lee Selex	sweet	CMS	Stable
	PBC 84 selex	Jin's Sweetie	sweet	maintainer of CCA 7236	
	9847-4754	Mito Lee selex	sweet	maintainer of CCA 7234	
SW-A1	CCA7234	PBC385-Aline/9*Mito Lee Selex	sweet	CMS	Stable
SW-A2	CCA7236	PBC385-Aline/9*Jin's Sweetie selex	sweet	CMS	Unstable
SW-A3	CCA7231	Suwon cms//9*Maor/Perennial	sweet	CMS	
SW-A4	CCA7229	PBC385-Aline/8*White King sel.	sweet	CMS	Unstable
SW-A5	CCA7235	PBC385-Aline//9*HDA120/MI-Gold	sweet	CMS	Unstable
SW-B1	9847-4754	F1 Mito Lee selex	sweet	maintainer of CCA 7234	
SW-B2-1	PBC 84 selex*	Jin's Sweetie	sweet	maintainer of CCA 7236	
SW-B2-2	PBC 84 selex*	Jin's Sweetie	sweet	maintainer of CCA 7236	

*Same line from different individual plant.

Table 3.1 (cont.) The pedigree of CMS, maintainer or near-isogenic lines, and restorer pepper lines derived from AVRDC- The World Vegetable Center.

No.	CCA Code	Pedigree	hot/sweet pepper	line of CMS system	Stability of sterility
SW-B3	9946-2138	Maor/Perennial-1	sweet	maintainer of CCA 7231	
SW-B4	9852-174-3	F1 BlueStar/ECW-30R	sweet	near-isogenic line	
HP-C1	9955-15	9955-15	hot	restorer	
HP-C2	PBC 142	Pant C-1	hot	restorer	
SW-C1	PP0037-7645	Yellow#1	sweet	restorer (unstable)	
SW-C2	9852-131	CCA 215A	sweet	restorer	
SW-C3	0407-7094	0407-7094	sweet	restorer (unstable)	
SW-C4	C03804	403 7499	sweet	restorer (unstable)	
SW-C5	PBC1419-Y	C01757 SELEX	sweet	restorer	

Table 3.2 Primer sets used in this study.

Primer set	**5` forward primer**	**3` reverse primer**	**size of predicted amplification (bp)**	**name of fragments**
ATP-1	ATGAAGACAGCACGGGTTTT	TATGCACTCCACTCGCTGTC	650 bp	*ψatp6-2*
ATP-2	CGATCACCAGTCCACTTGAA	TATGCACTCCACTCGCTGTC	550 bp	*ψatp6-2*
ATP-3	GAACCCGGTAAACGAACAAA	TCGCTCTGCTTCGTAGACAA	420 bp	*ψatp6-2*
CMS P1	GTCCCATGTATTTCTGGTTAAA CAAACC	CAGCCCTAATATTCGTTCCCT CAC	400 bp	*orf 456*
CMS P2	TGGAAGAGCAAGAAGCGGAA CTAC	GAGTCAGCCCTAATATTCGTT CCC	350 bp	*orf 456*
CRF-S	ATTTTCAGATTGTGGCGACG	CGACCATCACGACGAGG	850 bp	*Rf*

Table 3.3 Genomic pepper DNA analysis by ATP-1, ATP-2, ATP-3, CMS P1, CMS P2, and CRF-S primer sets.

No.	CCA Code	hot/sweet pepper	line of CMS system	Stability of sterility	primer set[a]					
					ATP-1	ATP-2	ATP-3	CMS P1	CMS P2	CRF-S
	CCA7243	hot	CMS	Unstable	+	+	+	+	+	–
	CCA7244	hot	CMS	Stable	+	+	+	+	+	–
	PBC 483	hot	maintainer of CCA 7243		–	–	–	–	–	–
	9907-9611	hot	maintainer of CCA 7244		–	–	–	–	–	–
	CCA7236	sweet	CMS	Unstable	+	+	+	+	+	–
	CCA7234	sweet	CMS	Stable	+	+	+	+	+	–
	PBC 84 selex	sweet	maintainer of CCA 7236		–	–	–	+	+	–
	9847-4754	sweet	maintainer of CCA 7234		–	–	–	–	–	–
SW-A1	CCA7234*	sweet	CMS	Stable	+			+		–
SW-A2	CCA7236*	sweet	CMS	Unstable	+			+		–
SW-A3	CCA7231*	sweet	CMS		+			+		–
SW-A4	CCA7229*	sweet	CMS	Unstable	+			+		–
SW-A5	CCA7235*	sweet	CMS	Unstable	+			+		–
SW-B1	9847-4754*	sweet	maintainer of CCA 7234		–			–		–
SW-B2-1	PBC 84 selex*	sweet	maintainer of CCA 7236		–			+		–
SW-B2-2	PBC 84 selex*	sweet	maintainer of CCA 7236		–			+		–
SW-B3	9946-2138*	sweet	maintainer of CCA 7231		–			+		–

[a] (+) Presence and (–) absence of bands.

*CMS-related genes analysis by primer set ATP6 1 and CMS P1.

Table 3.3 (cont.) Genomic pepper DNA analysis by ATP-1, ATP-2, ATP-3, CMS P1, CMS P2, CRF-S primer sets.

No.	CCA Code	hot/sweet pepper	line of CMS system	Stability of sterility	primer set[a]					
					ATP-1	ATP-2	ATP-3	CMS P1	CMS P2	CRF-S
SW-B4	9852-174-3*	sweet	near-isogenic line		–			+		–
HP-C1	9955-15*	hot	restorer		–			–		–
HP-C2	PBC 142*	hot	restorer		–			–		+
SW-C1	PP0037-7645*	sweet	restorer (unstable)		–			–		–
SW-C2	9852-131*	sweet	restorer		–			–		+
SW-C3	0407-7094*	sweet	restorer (unstable)		–			–		–
SW-C4	C03804*	sweet	restorer (unstable)		–			+		–
SW-C5	PBC1419-Y*	sweet	restorer		–			+		–
	A5[b]	hot	restorer							+

[a](+) Presence and (–) absence of bands.

[b]Genomic DNA of hot pepper plant with identified genomic background *Rf Rf* from TRIS.

*CMS-related genes analysis by primer set ATP6 1 and CMS P1.

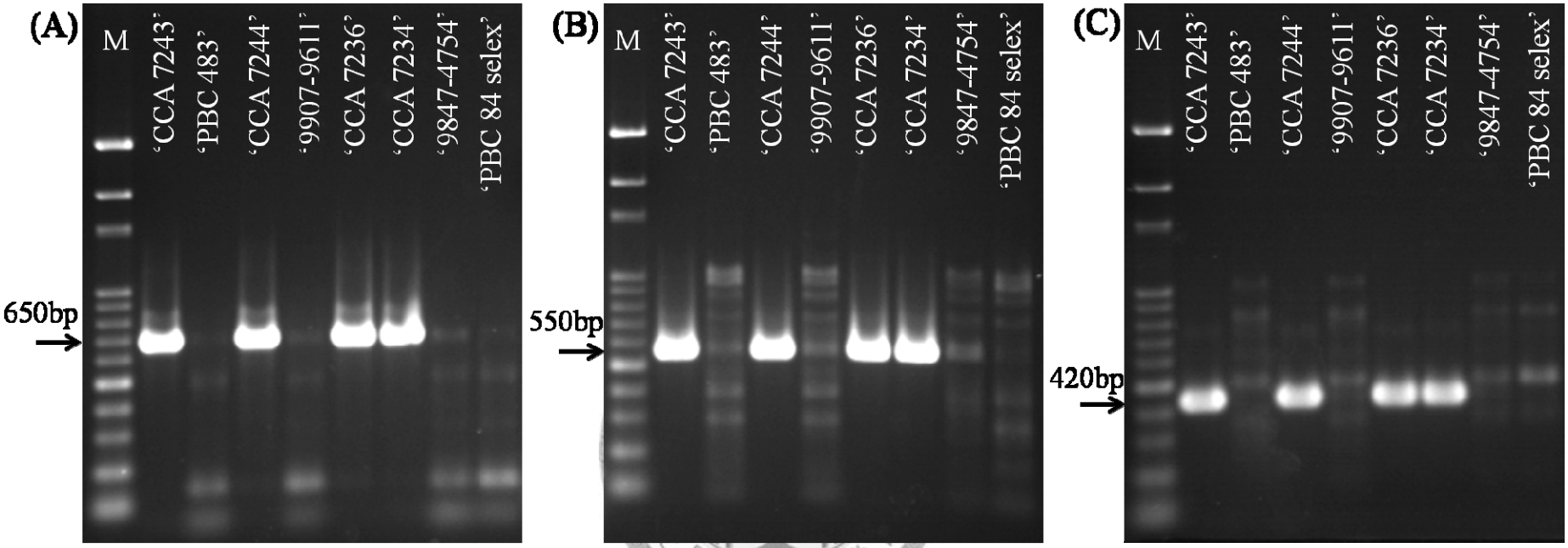


Fig. 3.1 PCR amplification of ψ*atp6*-2 fragment from pepper leaf genomic DNA by (A) ATP-1, (B) ATP-2, and (C) ATP-3 primer sets.

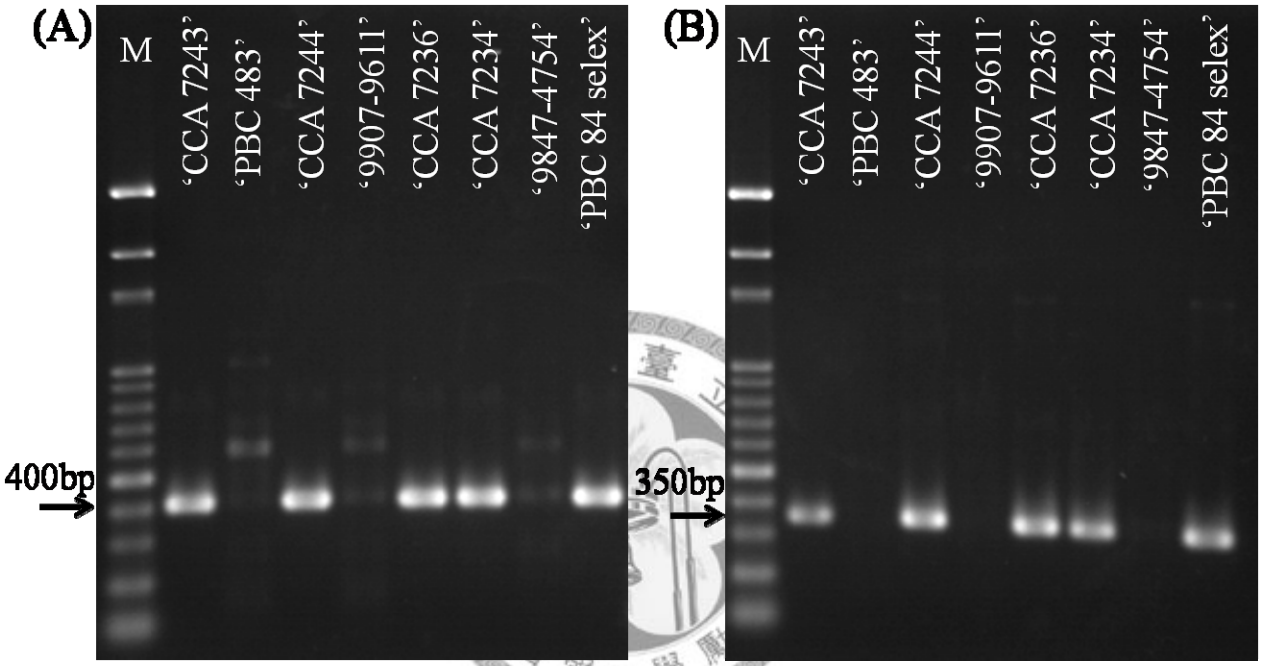


Fig. 3.2 PCR amplification of *orf456* fragment from pepper leaf genomic DNA by (A) CMS P1, and (B) CMS P2 primer sets.

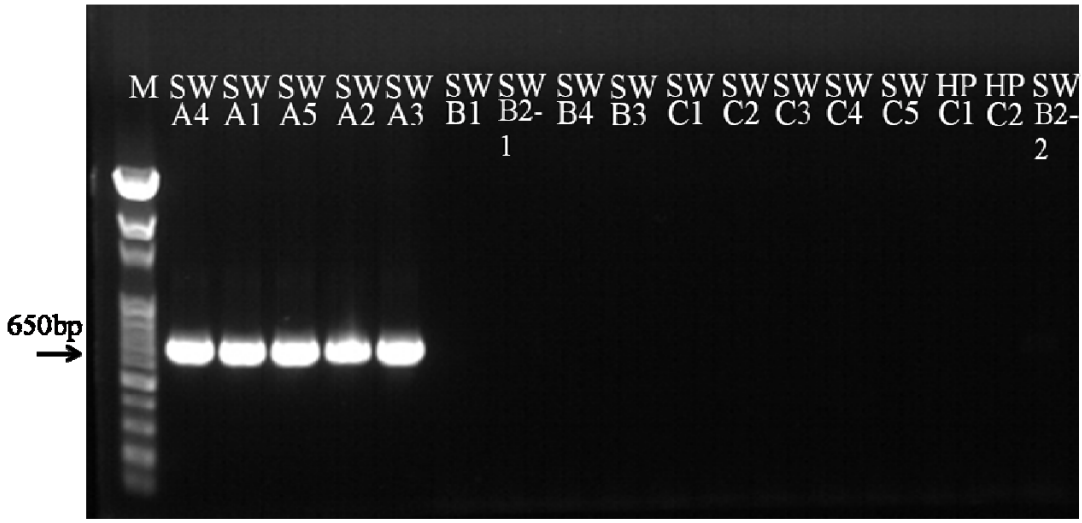


Fig. 3.3 PCR amplification of *ψatp6*-2 fragment from pepper leaf genomic DNA by ATP-1 primer set.

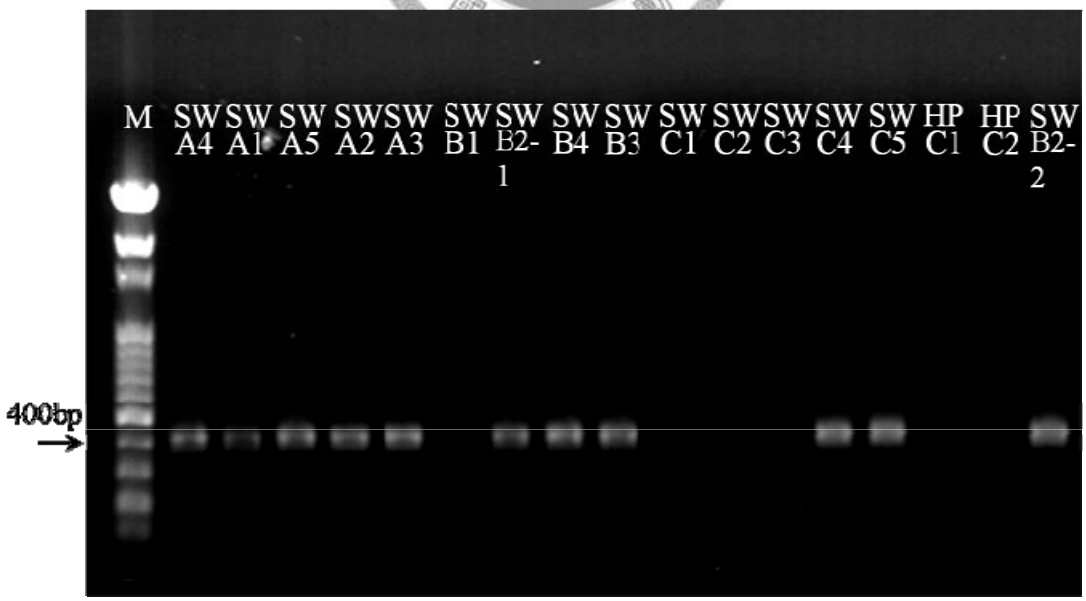


Fig. 3.4 PCR amplification of *orf456* fragment from pepper leaf genomic DNA by CMS P1 primer set.

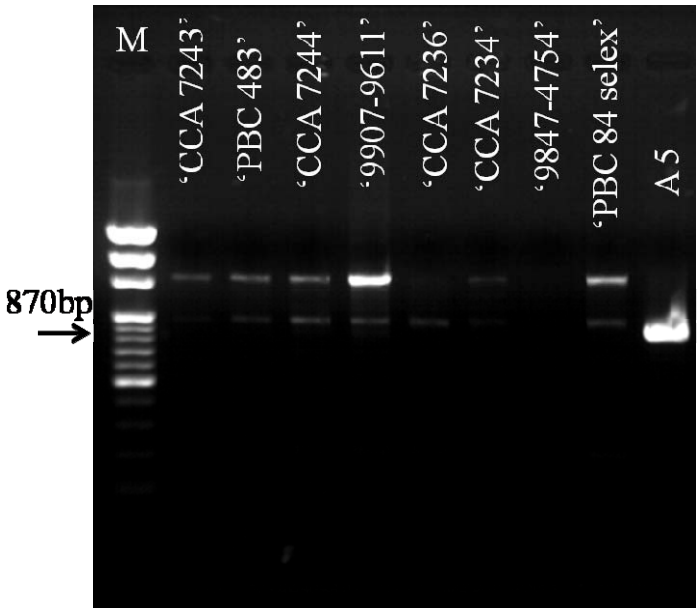


Fig. 3.5 PCR amplification of *Rf* fragment from pepper leaf genomic DNA by CRF-S primer set.

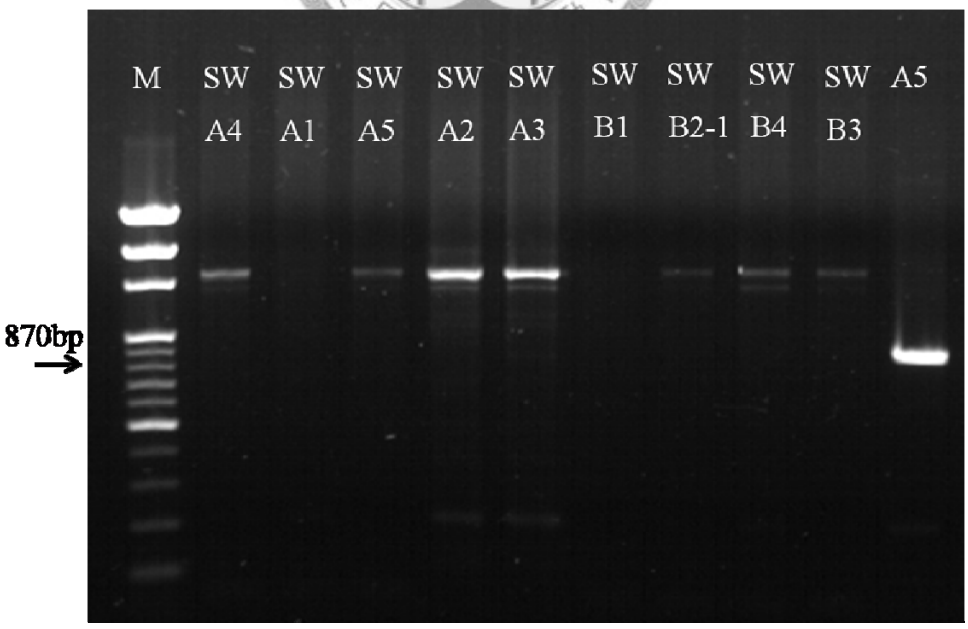


Fig. 3.6 PCR amplification of *Rf* fragment from pepper leaf genomic DNA by CRF-S primer set.

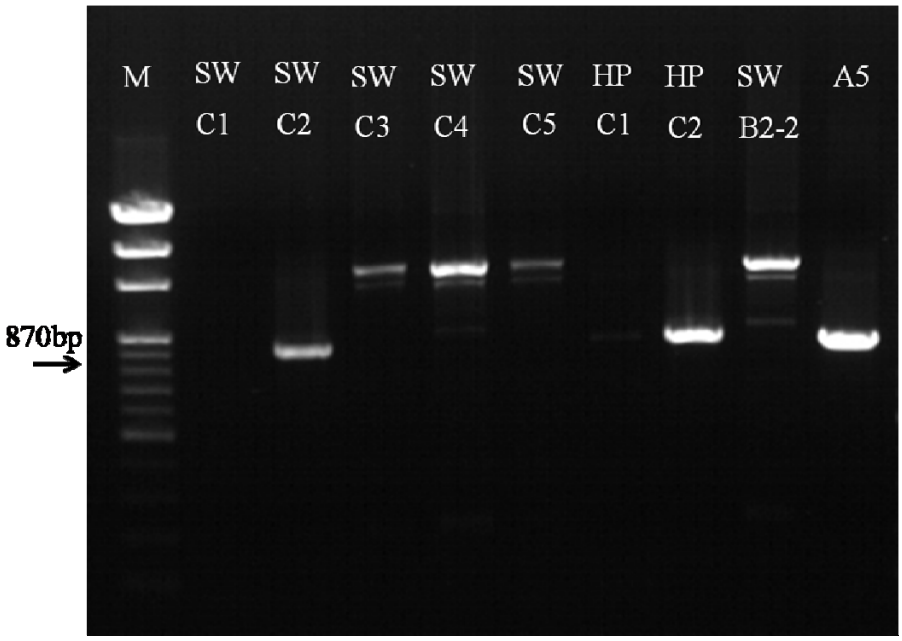


Fig. 3.6 (cont.) PCR amplification of *Rf* fragment from pepper leaf genomic DNA by primer set CRF-S.

Chapter 4

The Influences of Temperature and Seasonal Changes on Cytoplasm Male Sterile Pepper Lines

4.1 Introduction

The sterility of cytoplasm male sterile (CMS) pepper lines were not always expressed, some CMS lines could be affected by some environmental factors and restored their fertility partial or completely and thus produce functional pollens leading to self-pollination. And the sterility could resume as the temperature raise up again (Kaul, 1988; Peterson, 1958; Shifriss and Guri, 1979; Shifriss, 1997). The environmental factors that might affect the restoration include temperature, photoperiod, light intensity, soil micronutrients and soil pH, and of which temperature and photoperiod were two major environmental factors (Kaul, 1988). In *Brassica napus* L., the Polima cytoplasmic male sterility (Pol CMS) was highly dependent on temperature and thus could be divided into three types: high temperature CMS lines, low temperature CMS lines and stable CMS lines. Photoperiod did not affect the stability of Pol CMS *Brassica napus* L. lines (Fu et al. 1990). However, photoperiod-sensitive cytoplasmic male sterility (PCMS) of wheat was caused by the interaction between *Aegilops crassa* cytoplasm and *Triticum aestivum* cv. Norin 26 nucleus. It expressed almost complete male sterility under long-day conditions of 15 h or longer, but restored

fertility under short-day conditions of 14.5 h or less. The male sterility of PCMS wheat was not sensitive to temperature (Murai and Tsunewaki, 1993). Peterson (1958) observed that CMS sweet pepper would not produce pollens in summer, but produced 20% to 30% pollens in late October. The fertility of these set of plants was completely restored during the whole winter in the greenhouse. However, male sterility would be resumed when temperature went higher. In CMS sweet pepper line 'Bikura' and 'Zohar', plants restored fertility as night temperature dropped to 15°C in winter, but resumed their male sterility about 6 days after plants moving to growth chamber at 32/25°C (Shifriss and Guri, 1979). The CMS sweet pepper lines of AVRDC–The World Vegetable Center, 'CCA 7229' and 'CCA 7236', showed fertility restoration in the field in winter from 2005 to 2006, which suggested that these two CMS sweet pepper line were unstable lines which could produce functional pollens when the temperature below 18 °C. Besides, 'CCA 7229', 'CCA 7235', and 'CCA 7236' could restore fertility 17 days after transferring form the summer field to the growth chamber at 23/20 °C (unreported data). In CMS hot pepper line, 'ACC. 17' could produce viable pollens and set fruit in the growth chamber at 25/18 °C, 20/18 °C, and 20/12 °C, and the viable pollen numbers produced by 'ACC. 17' showed no significant difference with those produced by fertile lines (Hong, 2003). Besides, Hong (2003) also suggested that 20

days low temperature period before anthesis was required for fertility restoration.

In the present study, we would confirm the stability of cytoplasmic male sterility of hot and sweet pepper lines both in the phytotron and in the field of AVRDC–The World Vegetable Center.

4.2 Materials and methods

Plant materials

The CMS lines and maintainers of hot peppers and sweet peppers (Table 4.1) were used in this study. Plants were grown in the phytotrons and greenhouse at National Taiwan University from January 2007 to July 2007 and in the field of AVRDC – The World Vegetable Center from December 2007 to July 2008.

Seedlings of hot pepper being grown at NTU were sown on Jan. 3 to Feb. 9, 2007 in flats in growth chamber at day/night temperature of 30/25 ^{o}C with a 14h photoperiod. Seedlings were transplanted into 3' pots as they reached six leaves and moved to the greenhouse of NTU for 1 month then they were transplanted into 5' pots. One to three plants of each line were moved to the phytotron of NTU at 30/25 ^{o}C, 25/20 ^{o}C and 20/15 ^{o}C under natural photoperiod on May 15 for hot pepper and on Mar. 18 for sweet pepper. Remaining plants were maintained in the greenhouse. During the growth period, lateral

leaf buds and fruit need to be thinned. Temperature in the greenhouse was recorded by HOBO every day (Appendix 1).

Seedlings that grown at AVRDC – The World Vegetable Center were sown in flats on August 15, October 1, and January 1, in order to maintain plant vigor during the observation. Seedlings were transplanted to the field at AVRDC - The World Vegetable Center as they reached six leaves. During the growth period, fruits need to be thinned. Temperature in the field was recorded by LogTag Humidity and Temperature Records (LogTag Records Ltd) every day (Appendix 2).

Percentage of pollen viability

Pollen viability investigation was started from the first flower bloomed. In NTU, pollen viability of each line was recorded once per week, one anther of a pre-bloomed bud per line was sampled, and final pollen viability recorded was the average of counts from two light fields. In the AVRDC - The World Vegetable Center, pollen viability was recorded once every two weeks, 6 plants per line and of which one pre-bloomed bud per plant were sampled for pollen viability, every anther was inspected and pollen viability recorded was the average of every plant.

Pollen viability was determined by the percentage of stained pollen grains from

one anther with 0.5% acetocarmine under 100X light microscope. The percentage of pollen viability was calculated with “No. of fertile pollens / No. of fertile plus sterile pollens.”

4.3 Results

The phenotype of CMS lines and maintainers in the field of AVRDC – The World Vegetable Center

The morphological phenotypes of CMS pepper plants were similar to that of maintainer lines (Fig. 4.1 and Fig. 4.3) except their anthers. Comparing the phenotype of anthers during anthesis, the anthers of CMS line were smaller than those of the maintainers (Fig. 4.2 and Fig. 4.4). Although the anther of CMS line could dehisce, no light yellow pollen was observed on the surface of anther. When unstable CMS line ‘CCA 7243’ restored fertility, the phenotype of anthers was similar to that of the maintainer ‘PBC 385 (Fig. 4.2 B and C).

The influences of temperature and seasonal changes on CMS hot pepper lines and the maintainers

To determine the critical range of temperature that caused unstable CMS pepper lines restore fertility, plants were grown in the greenhouse of NTU and the phytotron of NTU at 30/25 ^{o}C, 25/20 ^{o}C and 20/15 ^{o}C under natural photoperiod. Since all the plants

were grown in the greenhouse before moved to the phytotrons at May 15, 2007, most of CMS hot pepper line ‘CCA 7243’ expressed fertility restoration from April 29 to May 8, 2007 (Fig.4.5A). In the present study, the night temperature in the greenhouse was below 20 ^{o}C before Apr. 21 and there was a front at Apr. 5 lowering both day and night temperature to below 15 ^{o}C while the day temperature maintained mostly above 25 ^{o}C. Such long period of lower temperature might explain the fertility restoration in the greenhouse. The highest restoration of ‘CCA 7243’ was appeared on May 8 and went down thereafter and finally reverted their male sterility in the greenhouse. ‘CCA 7243’ plant moved to 20/15 ^{o}C phytotron kept expressing the fertility while those moved to 30/25 ^{o}C and 25/20 ^{o}C phytotrons resumed to male sterile (Fig.4.5A). In the stable CMS line ‘CCA 7244’, all plants remained male sterility in every location (Fig. 4.5B). Both maintainer line ‘PBC 483’ and ‘9907-9611’ expressed fertile in the greenhouse and in the phytotron, especially in the phytotron at 25/20 ^{o}C and 20/15 ^{o}C, the percentage of pollen viability maintained more than 70% (Fig. 4.5 C and D). Due to server aphid problem, plants in the phytotron could not flower continuously and resulted in no data (Fig. 4.5).

For the field observation in AVRDC – The World Vegetable Center, CMS line ‘CCA 7243’ had restored 5 times of fertility from Dec. 7, 2007 to May 8, 2008, and the

fertility could restore about 50% as compared to the maintainer line, ‘PBC 385’. The percentage of pollen viability in maintainers ‘PBC 385’ and ‘9907-9611’ was about 60% or more, except for the last observation in ‘9907-9611’ which that only 18% (Fig. 4.6). Surprisingly, ‘CCA 7244’ observed some fertility twice in this field experiment. During the experimental period, the night temperature in the field was lower than 21 ^{o}C before Apr. 8 while day temperature maintained mostly higher than 26 ^{o}C, and long period of lower temperature might also explain the fertility restoration in the field.

The influences of temperature and seasonal changes on CMS sweet pepper lines and the maintainers

In sweet pepper lines, since all the plants of CMS line ‘CCA 7236’ were grown in the greenhouse before transferred into phytotron at May 18, 2007, most plants restored fertility from Apr, 6 to May 8, 2007 (Fig 4.7 A). The temperature changes in the greenhouse of NTU showed in Appendix 1, and long period of lower temperature might also caused the fertility restoration of unstable CMS sweet pepper line ‘CCA 7236’ in the greenhouse. The highest rate of the restoration of ‘CCA 7236’ was appeared on Apr. 22 and which went down thereafter and finally male sterility was reverted in the greenhouse. ‘CCA 7236’ plants which were moved to 20/15 ^{o}C phytotron kept their fertility while those were moved to 30/25 ^{o}C and 25/20 ^{o}C phytotrons resumed to male

sterile (Fig.4.7 A). In another CMS sweet pepper line ‘CCA 7234’, all plants remained male sterility in every location (Fig. 4.7 B). Both maintainer lines ‘PBC 84 selex’ and ‘9847-4754’ expressed fertility in each location, but plants could hardly develop flowers in the 30/25 ^{o}C phytotron (Fig. 4.7 C and D). Due to server aphid problem, plants in the phytotron could not flower continuously and resulted in no data (Fig. 4.7).

For the field observation in AVRDC in CMS ‘CCA 7236’, plants restored fertility three periods from Dec. 7, 2007 to Apr. 10, 2008, and each restoration period could maintain 2 weeks or more, and the fertility could restore about 35% to 60% as compared to the maintainer line, ‘PBC 84 selex’. Maintainers ‘PBC 84 selex’ and ‘9847-4755’ maintained fertile but pollen viability was not steady, which was about 20% to 95%. Another CMS line ‘CCA 7234’ remained male sterility in the field (Fig. 4.8). During the experimental period, the night temperature in the field was lower than 17 ^{o}C before Mar. 10 while daily temperature maintained mostly higher than 26 ^{o}C. There was a front on Jan. 30 causing day temperature to drop to 18 ^{o}C or even lower. Several sweet pepper plants suddenly wilted due to the infection of *Sclerotium rolfsii* from Apr. 2008.

4.4 Discussion

Peterson (1958) indicated that the floral organ of CMS pepper was similar to that of fertile pepper, but its stamens were approximate one-half of the size of fertile anthers and just produced few viable pollen grains occasionally. The color and size of anthers between CMS line while plants expressed male sterility and their maintainers was distinctly different. When unstable CMS hot pepper line 'CCA 7243' restored fertility, the phenotype of anthers in unstable CMS plants was as same as it in maintainer 'PBC 385' (Fig. 4.2 B and C), but sometimes no pollen grains were obtained in anthers of unstable CMS line and the fertility restoration needed investigate by light microscope.

The unstable CMS sweet pepper lines of AVRDC – The World Vegetable Center were recorded to restore their fertility approximately after 17 d incubated in 23/20^{o}C growth chamber (unreported). Hong (2003) reported that the temperature at 20 d before anthesis could cause CMS hot pepper fertility restoration. According to the greenhouse and the field observations in this study, the process of pepper floral bud development stages from the visual bud to anthesis needed in almost 30 d, we suggested that the temperature at 30 d before unstable CMS plants showed fertility was related to plant fertility restoration.

Previous reports did not show the critical range of temperature that caused unstable CMS lines restore fertility. Shifriss (1997) suggested that CMS pepper lines restored

fertility when the temperature dropped below the optimal for pepper production (day/night temperature was 25/17°C). The other unstable CMS hot pepper line 'AAC. 17' could restore fertility at 25/18°C, 20/18 °C, and 20/12 °C growth chamber (Hong, 2003). The unstable CMS sweet pepper lines of AVRDC restored fertility when minimum temperature less than 18 °C in the field, and also restored at 23/20 °C or at 23/15 °C growth chamber (unreported).

In this study, our data showed that night temperature is the critical factor caused unstable CMS lines restore fertility. CMS hot pepper line 'CCA 7243' could restore fertility in the greenhouse and in the field while the night temperature was below 21 °C, and also could restore in the 20/15 °C phytotron (Fig. 4.5 A and Fig. 4.7). Plants of CMS sweet pepper line 'CCA 7236' restored fertility while night temperature was below 21°C or 17 °C, but plants hardly restored while day temperature below 18 °C (Fig. 4.6 A and Fig. 8). Besides, the fertility restoration of unstable CMS lines showed as a regular cycle, not always steady expressed. When night temperature went higher, both lines were reverted male sterile. Therefore, 'CCA 7243' and 'CCA 7236' both belonged to unstable CMS line.

In other CMS hot pepper line 'CCA 7244', plants had ever restored two times of fertility in the field of AVRDC (Fig. 4.6), but remained as male sterile in the greenhouse

and each phytotron (Fig. 4.5 B), and these results representing that ‘CCA 7244’ were less sensitive to temperature changes than ‘CCA 7243’. CMS sweet pepper line ‘CCA 7234’ always expressed male sterile even under a long period of lower night temperature. Therefore, ‘CCA 7244’ and ‘CCA 7234’ are stable CMS lines.

The reasons why unstable CMS plants could restore fertility as environment changes could be classified into two categories. One, the genetic background of CMS plants might be related to fertility restoration expressed under specific conditions. The level of male sterility expression of PCMS wheat lines was determined by the genotype of the nuclear donor (Murai, 2001a). Certain temperature could suppress or delay the recessive restorer gene action in some CMS plants, such as onion, alfalfa, pearl millet and rye (Kaul, 1988). The variation among CMS pepper lines in the expression of sterility presumably was caused by differences in number and nature of male-sterility modifying genes (Peterson, 1958; Shifriss, 1997). In cytological aspects, the PCMS wheat at floret development stage was sensitive to photoperiod, such as pistillate stamens under long-day condition (17h) transferring into normal stamens under short-day condition (13h) and producing functional pollens to self-pollination (Murai and Tunewaki, 1993). On the other hand, the breakdown during the process of microsporogenesis or microgametogenesis in CMS plants might block under the

specific conditions. Because malfunction might occur in early microsporogenesis in CMS pepper (Peterson, 1958), meiotic breakdown in unstable CMS pepper lines is either stopped or delayed when the temperature drop below the optimal temperature for pepper production and plants could produce viable pollens (Shifriss, 1997).

The fertile expression in maintainers was also affected by temperature. In hot pepper maintainers ‘PBC 483’ and ‘9907-9611’, the pollen viability maintained 60% and higher in the 20/15 ^{o}C and 25/20 ^{o}C phytotron, but fewer flowers bloomed and pollen viability decreased in the 30/25 ^{o}C phytotron at (Fig. 4.5 C and D). However, ‘PBC 483’ and ‘9907-9611’ in the field still maintained higher fertility even if the day temperature was higher more than 30 ^{o}C but night temperature was lowerthan 25 ^{o}C (Fig. 4.6). The same results were in maintainer sweet pepper line ‘PBC 84 selex’ and ‘9847-4755’. (Fig. 4.7 C and D and Fig. 4.8). According to previous reports, the highest night temperature (24 ^{o}C ± 1) caused sweet pepper ‘Ma'or’ considerable blossom drop, but the highest tested day temperature (28 ^{o}C) did not cause increased blossom drop (Rylski and Spigelman, 1982). It could explain that the pollen viability of maintainers decreased in the 30/25 ^{o}C phytron at caused by high night temperature.

In tradition three-line CMS system, CMS plant was crossed by maintainer line to maintain their progeny, and crossed by restorer line to produce F1 seeds. The unstable

CMS plant which was sensitive to environment was also useful. The unstable CMS plants maintained their progeny by self-pollination while plants restored fertility, and produced hybrid seeds by cross-pollination with restorer while CMS plants remained sterile. It is so-called ‘two-line system’ (Murai and Tsunewaki, 1993; Murai et al, 2008; Shifriss, 1997; Murai, 2001; Yang et al, 2006). Therefore, unstable CMS pepper lines used in this study also could develop into ‘two-line system’, and plants maintained their progeny from Dec. to Jun while night temperature was below 21 ^{o}C and produced hybrid seeds during Jul. to Nov. while night temperature was above 21 ^{o}C.

Table 4.1 The pedigree of CMS lines and maintainers from AVRDC-The World Vegetable Center.

CCA Code	**Pedigree**	**hot/sweet pepper**	**line of CMS system**	**Stability of sterility**
CCA7243	Seungchon(cms)/8*Arunalu = CCA4758/PBC 483	hot	CMS	Unstable
CCA7244	Seungchon(cms)/8*9907-9611 =CCA4759/9907-9611	hot	CMS	Stable
PBC 483	Arunalu	hot	maintainer of CCA 7243	
9907-9611	PBC 385 sle.	hot	maintainer of CCA 7244	
CCA7236	PBC385-Aline/9*Jin's Sweetie selex	sweet	CMS	Unstable
CCA7234	PBC385-Aline/9*Mito Lee Selex	sweet	CMS	Stable
PBC 84 selex	Jin's Sweetie	sweet	maintainer of CCA 7236	
9847-4754	Mito Lee selex	sweet	maintainer of CCA 7234	

Fig. 4.1 CMS and near-isogenic hot pepper plants grown in the AVRDC field. (A) unstable CMS line ‘CCA 7243’; (B) near-isogenic line ‘PBC 385’ of ‘CCA 7243’; (C) stable CMS line ‘CCA 7244’; (D) near-isogenic line ‘9907-9611’ of ‘CCA 7244’.

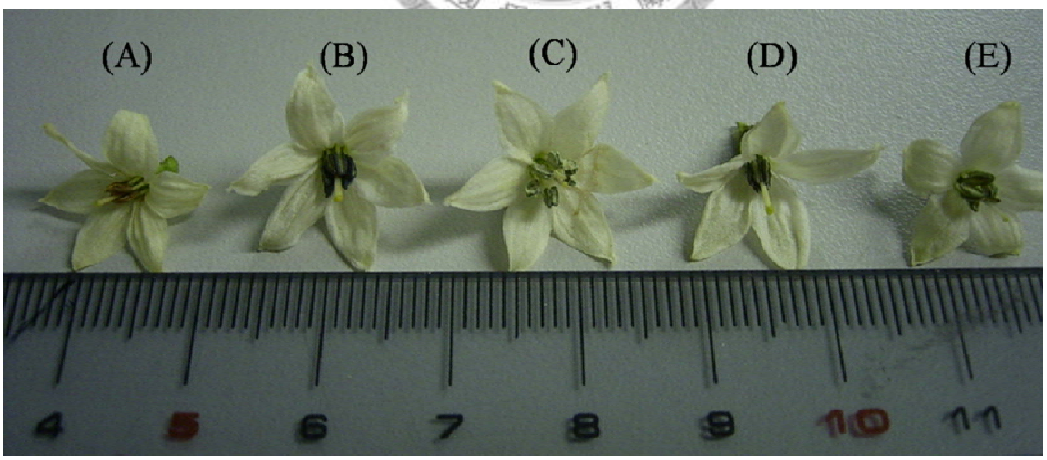


Fig. 4.2 CMS and near-isogenic hot pepper flowers harvested from plants in the AVRDC field. (A)sterile flower of unstable CMS line ‘CCA 7243’(B) restored fertile flower of unstable CMS line ‘CCA 7243’; (C) fertile flower of near-isogenic line ‘PBC 385’; (D) sterile flower of stable CMS line ‘CCA 7244’; (E) fertile flower of near-isogenic line ‘9907-9611’.

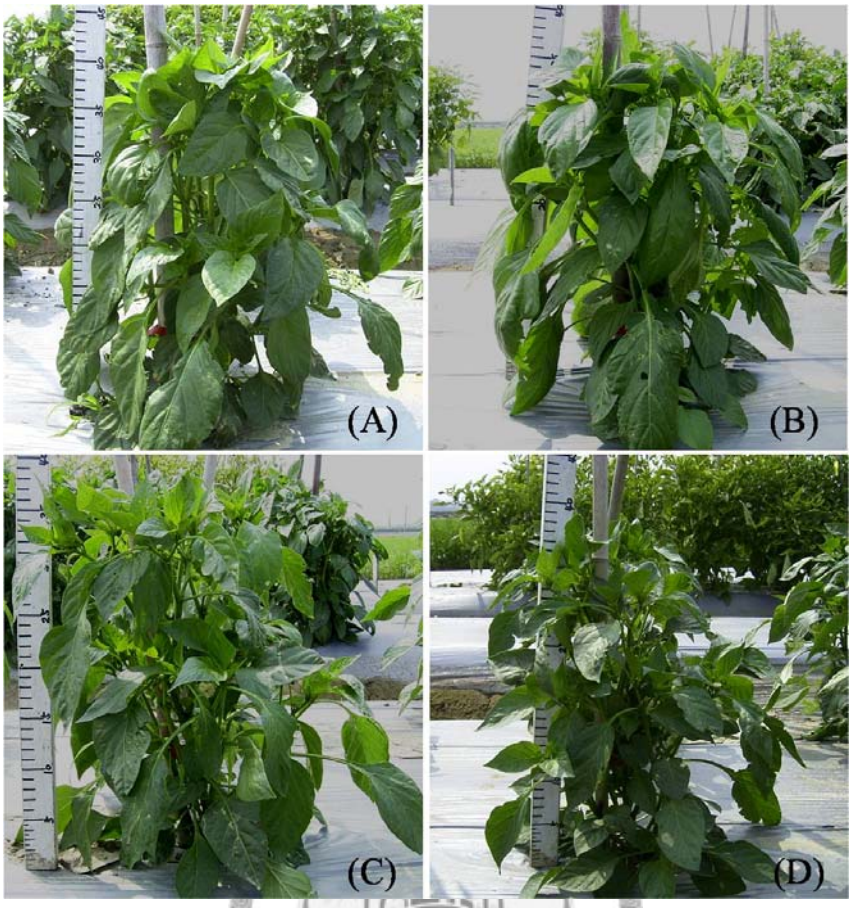


Fig. 4.3 Sweet pepper plants grown in the AVRDC field. (A) unstable CMS line ‘CCA 7236’; (B) near-isogenic line ‘PBC 84sel’ of ‘CCA 7236’; (C) stable CMS line ‘CCA 7234’; (D) near-isogenic line ‘9847-4754’ of ‘CCA 7234’.

Fig. 4.4 CMS and near-isogenic sweet pepper flowers harvested from plants in the AVRDC field. (A)sterile flower of unstable CMS line ‘CCA 7236’; (B)fertile flower of near-isogenic line ‘PBC 84sel’; (C) sterile flower of stable CMS line ‘CCA 7234’; (D) fertile flower of near-isogenic line ‘9847-4754’ of ‘CCA 7234’.

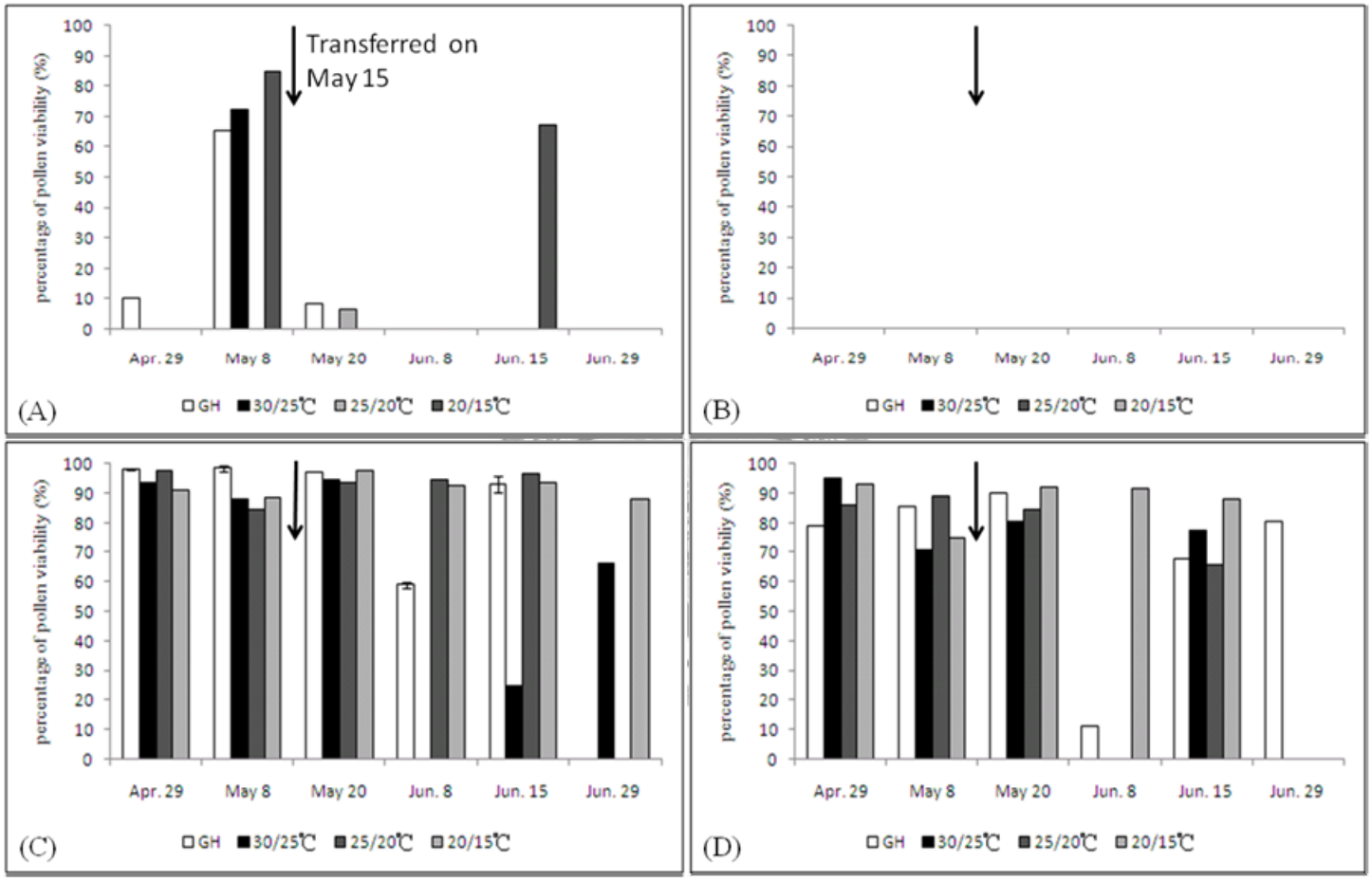


Fig. 4.5 The trend of pollen viability percentage in hot pepper lines of (A) 'CCA 7243' (B) 'CCA 7244' (C) 'PBC 483' (D) '9907-9611' in the greenhouse (GH) and in the phytotron of NTU at 30/25 ^{o}C, 25/20 ^{o}C, and 20/15^{o}C from Apr. 29 to Jun. 29, 2007.

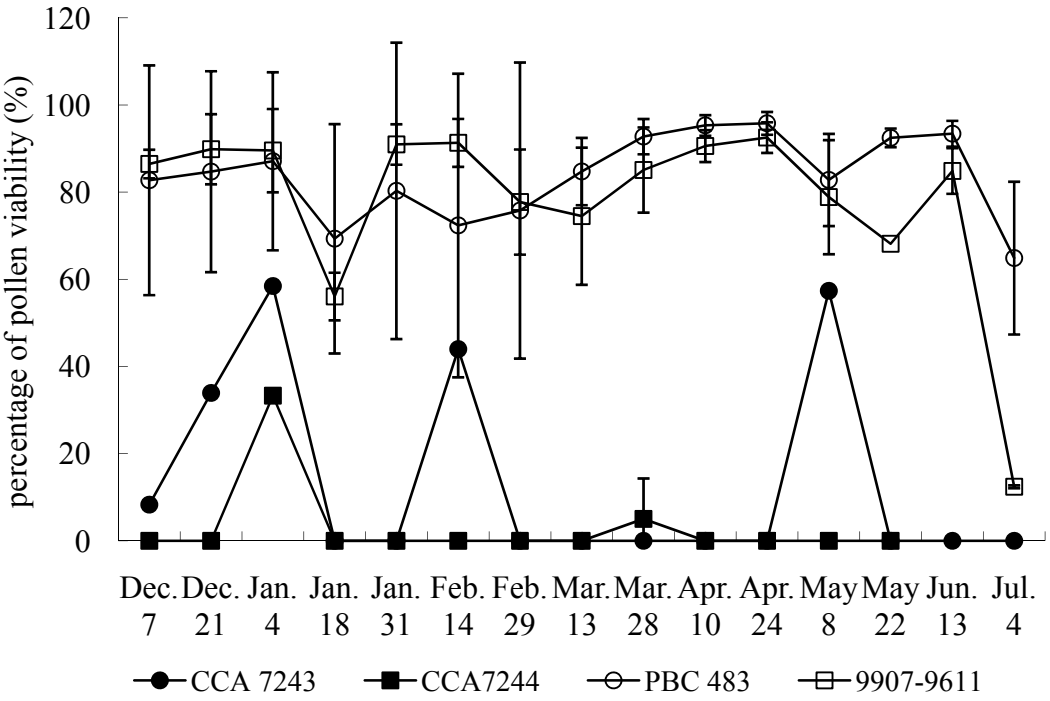


Fig. 4.6 The trend of pollen viability percentage in hot pepper lines in the field of AVRDC-The World Vegetable Center from December 7 to July 4.

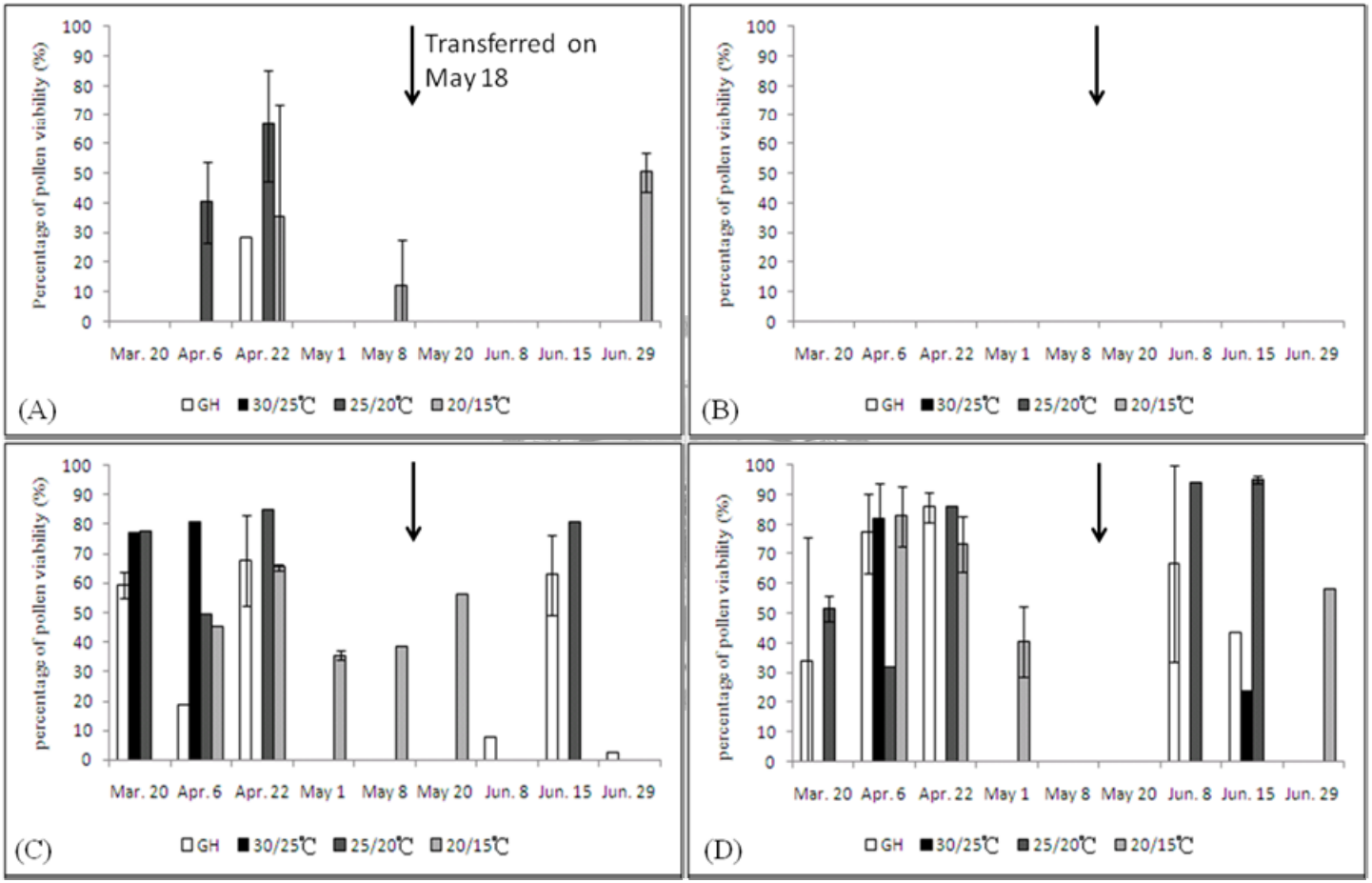


Fig. 4.7 The trend of pollen viability percentage in sweet pepper lines (A) 'CCA 7236', (B) 'CCA 7234', (C) 'PBC 84 selex', and (D) '9847-4754' in the greenhouse (GH) and in the phytotron of NTU at 30/25 °C, 25/20 °C, and 20/15°C from Mar. 20 to Jun. 29, 2007.

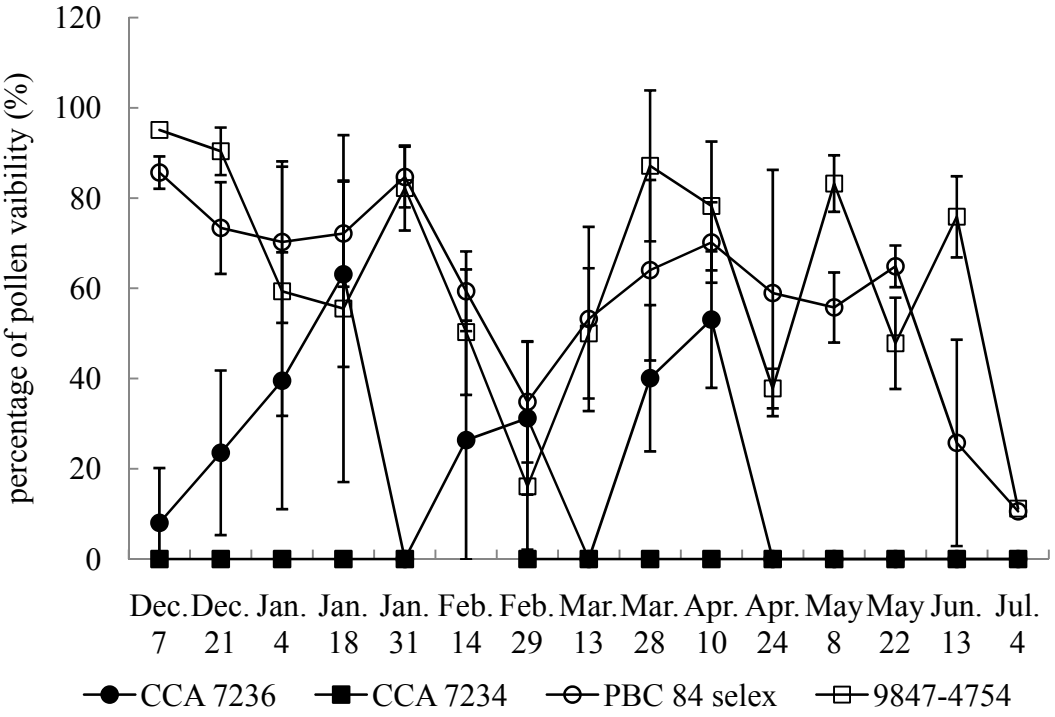


Fig. 4.8 The trend of pollen viability percentage in sweet pepper lines in the field of AVRDC-The World Vegetable Center from December 7 to July 4.

Appendix 4.1

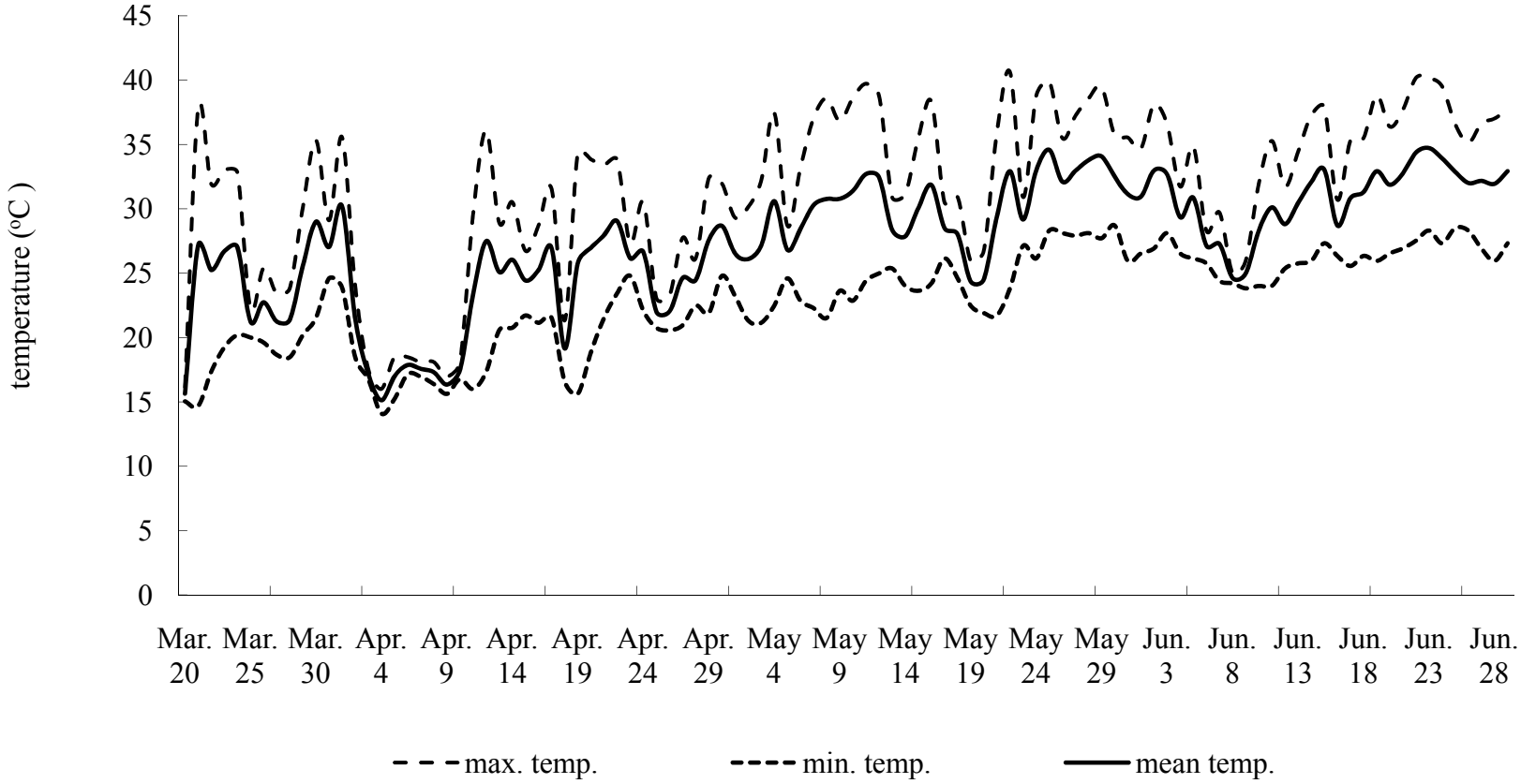


Fig. Maximum, minimum, and mean temperature in the greenhouse of NTU from Mar. 20 to Jun. 29, 2007.

Appendix 4.2

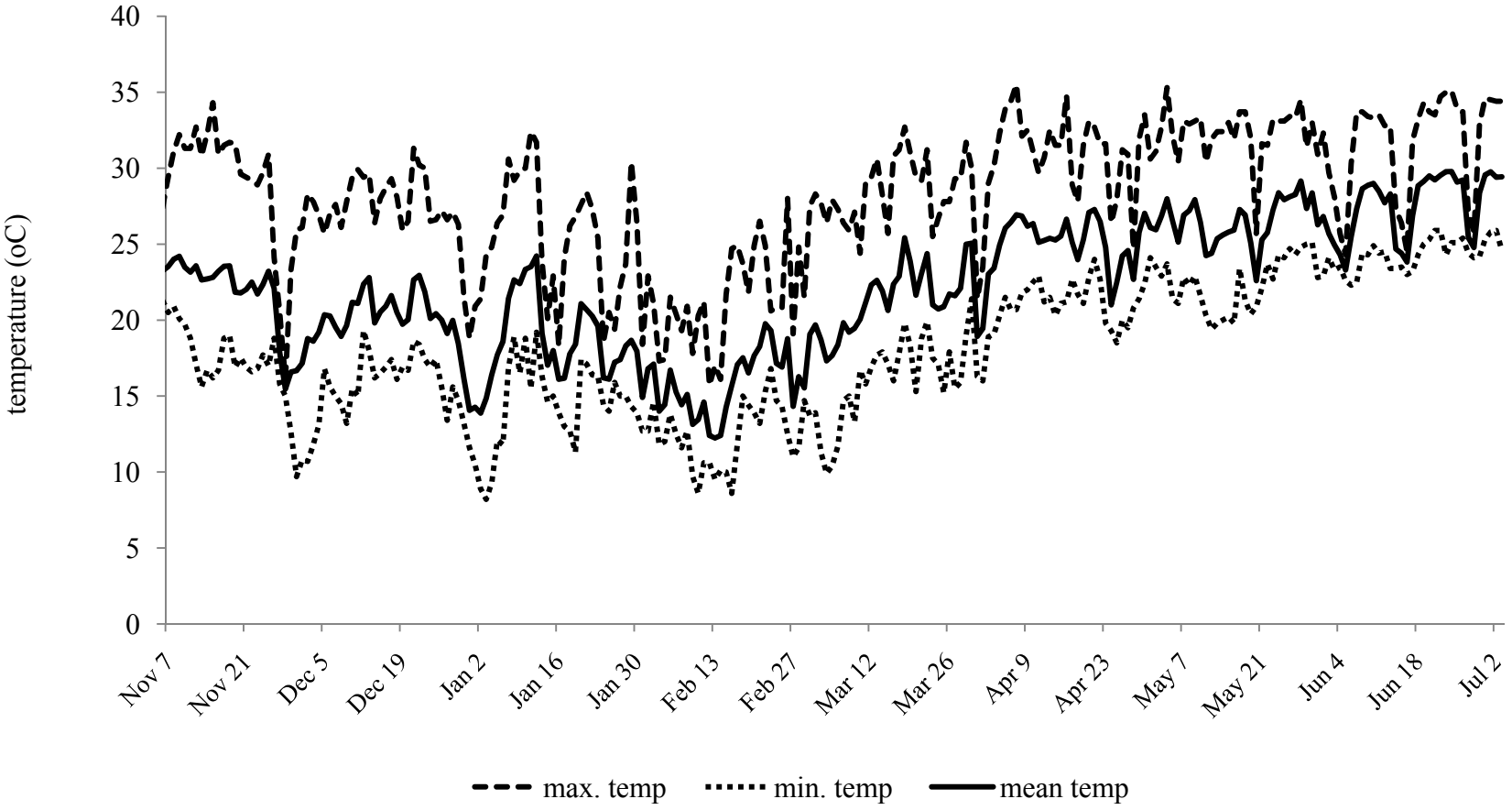


Fig. Maximum, minimum, and mean temperature in the field of AVRDC-The World Vegetable Center from Nov. 7, 2007 to Jul. 3, 2008.

Chapter 5

The Influences of Temperature on Anther Wall Differentiation and Microgametogenesis of CMS Pepper Lines

5.1 Introduction

The CMS plants could not produce viable, functional pollens due to the abnormalities of morphology or functions of male reproductive organs. In some CMS species, male sterility was associated with apparent homeotic changes in floral tissue. For example, stamens transformed into carpels (a carpeloid phenotype) in CMS tobacco, or transformed into petals (a petaloid phenotype) in CMS carrot. In another CMS species, such as maize, petunia, and sunflower, there was no distinct morphological differences from male fertile plants, except the degenerated stamens (Hanson and Bentolila, 2004; Chase, 2006).

CMS pepper was first reported by Peterson (1958). The flower of CMS pepper was similar to that of fertile pepper, but stamens were approximate one-half the size of fertile anthers and could produce just a few viable pollens. Up to date, some anatomical evidences revealed the reasons of failure in producing viable pollens of CMS pepper lines. In fertile sweet pepper lines, pollen mother cells complete meiosis and form tetrads, and then microspores are released from tetrads and developed into mature pollens. Tapetum cells, the innerest layer of anther wall which offer nutrients

for microspore development, would degenerate progressively around the process of microspores releasing (Novák and Betlach, 1970; Luo et al., 2006) or from vacuolated microspore stage (Horner and Rogers, 1974), and the degeneration is almost complete at immature pollen stage. In CMS sweet pepper lines, abnormalities took place in the process of microsporogenesis or the timing of tapetum degeneration. Pollen mother cells could complete meiosis, but the tetrads failed to develop into microspores. Besides, tapetum cells became highly vacuolated and gradually eliminated locular cavity and thus crush meiocytes (Horner and Rogers, 1974). Evidence showed that tetrads of CMS pepper line were able to develop into microspores has been observed (Luo et al.., 2006), and the microspores failed to develop into mature pollens due to crushed by the swollen tapetum cells. In addition, no sporopollenin accumulation on the microspores wall also caused them easily collapsed by swollen tapetum cells.

According to previous reports, some unstable CMS pepper lines restored fertility due to low temperature (Hong, 2003; Peterson, 1958; Shifriss, 1997; Shifriss and Guri, 1979). Hong (2003) compared the anatomical difference of the anther structures between the sterile and fertile phenotypes of the unstable CMS hot pepper line‘Acc. 17’. While ‘Acc. 17’ expressed sterile in the 28/20 ^{o}C growth chamber, the swollen and vacuolated tapetum cells crushed tetrads and degenerated after anther wall secondary

thichness; and while ‘Acc. 17’ expressed fertile in the 25/18 ^{o}C growth chamber, the appearances and degeneration timing of tapetum cells were similar as typical tapetum in fertile CMS pepper lines (Horner and Roger, 1973). Although stable and unstable CMS pepper lines both express male sterility, no comparative study about the abnormality in anther structures between them has been reported. In the present study, we study the effect of temperature on the anatomical difference of anther wall differentiation and microgametogenesis among the stable CMS line ‘CCA 7244’, unstable CMS lines ‘CCA 7243’, and the maintainer line‘PBC 483’.

5.2 Materials and metohods

Plant materials

The stable CMS line ‘CCA 7244’, unstable CMS line ‘CCA 7243’, and maintainer ‘PBC 483’ of ‘CCA 7243’ were grown in the field at AVRDC - The World Vegetable Center from December 2007 to July 2008. In order to maintain plant vigor during the experiment, 10 seeds of each line were sown in flats on August 15, October 1, and January 1. Seedlings were transplanted to the field as they reached six-leaves stage. During the growth period, fruits were thinned occasionally. Temperature was recorded by LogTag Humidity and Temperature Records (LogTag Records Ltd) every day.

Preparations of semi-thin section for light microscope

According to the fertility restoration of unstable CMS line ‘CCA 7243’ (Fig. 4.6), specimens were collected on 25 Jan. and 15 Feb. as ‘CCA 7243’ expressed fertile and on June 24 and July 4 as ‘CCA 7243’ expressed sterile. In addition, specimens of stable CMS line ‘7244’ and maintainer line ‘PBC 483’ were also collected at the same time.

Floral buds of each line sizing from 2 to 8 mm in length measured from petal to the end of sepal were collected, petals were removed if needed or two anthers were picked depending on the size of floral buds, and specimens were immediately fixed in Karnovsky fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer for two days at 4^{o}C. After rinsed with 0.1M phosphate buffer for 15 min for three times, specimens were post-fixed with 1% (w/v) osmium tetroxide (OsO_4) in 0.1M phosphate buffer at 4^{o}C for overnight. After postfixative, specimens were washed with 0.1M phosphate buffer for 15 min three times. Next, samples were dehydrated by series concentration of ethanol (from 30%, 50%, 75%, 85%, 95%, and absolute ethanol), and displaced by absolute acetone three times. After dehydrated completely, specimens were infiltrated by series concentration of Spurr’s resin (ERL-4221=10.0g, DER-736=5.0g, Nonenylsuccinic anhydride (NSA)=26.0g) to pure Spurr’s resin with no dimethylaminoethanol (DMAE), and then displaced by Spurr’s resin with 0.3g DMAE a day. Samples were polymerized for 8 to 16 h at 70^{o}C.

950 nm semi-thin sections were prepared by ultramicrotome (Ultracut E), and stained by 1% Toluidine Blue O (TBO) at 50^{o}C for 5 s, and then rinsed by dist. H_2O. Sections were mounted in Entellan in xylene. Sections were observed by light microscope.

5.3 Results

Anther wall differentiation and microgametogenesis in both CMS hot pepper and maintainer lines under high temperature

Fig 5.1 showed the anther wall differentiation and microgametogenesis of the maintainer line 'PBC 483' which expressed fertile at all experimental condition in the present study. At primary sporogenous cell stage, epidermis of anther wall already formed, and primary parietal layers underwent periclinal divisions (Fig. 5.1 A), and thereafter pollen mother cells formed and anther wall developed into 4 distinct layers, containing epidermis, endothecium, middle layer and tapetum cell (Fig. 5.1 B). During prophase of meiosis I, the cytoplasm of tapetum cells was quite dense and contained many small vacuoles and would undergo karyokinesis to form bi-nucleated tepetum cells (Fig. 5.1 C). At the end of meiosis II, tetrads formed, locule continued to increase in size, and the cytoplasm of tapetum cells remained dense and contained larger vacuoles (Fig. 5.1 D). Then the tetrads developed into microspores by callose wall

dissolution (Fig. 5.1 E). At mid-vacuolated microspore stage, microspores enlarged and contained small vacuoles within dense cytoplasm, and the pollen wall thickened by the deposition of exine materials (Fig 5.1 F). As microspore enlarged, mitosis occurred and a larger vacuole was observed in the cytoplasm of each microspore. At this stage, the tapetum cells degenerated obviously, and the secondary cell wall of endothecium and middle layer were thickened (Fig 5.1 G). Finally, tapetum completely degenerated and pollens matured (Fig. 5.1 H).

In stable CMS hot pepper line 'CCA 7244', abnormality of anther wall differentiation and microgametogenesis were observed in spite of meiosis was completed (Fig. 5.2). At the secondary sporogenous cell stage, the anther wall already developed into epidermis, endothecium, middle layer, and tapetum cell; however, the layering was not as clear especially between endothecium and middle layer as compared with fertile line 'PBC 483' (the maintainer line of 'CCA 7243') (Fig 5.2 A). During prophase of meiosis I, the tapetum cell less enlarged and contained a vacuole, but the cell of endothecium and middle layers enlarged in stead (Fig. 5.2 B). At the end of meiosis II, tetrads formed but the locule remained small and the tapetum cells became vacuolate and abnormally swelled to press the tetrads (Fig 5.2 C). As anther keeps developing, microspores released and the pressing continued (Fig 5.2 D) and

contributed to microspore abortion (Fig 5.2 E). The character of the anther matured stage was secondary thickness at the radial walls of endothecium and middle layers (Fig 5.2 F), and at this stage, tapetum cell decomposed completely.

Unstable CMS hot pepper line ‘CCA 7243’ expressed male sterility under high temperature. By anatomical observation during anther development, anther wall structure was same as maintainer line ‘PBC 483’, but the the abnormality of the tapeutm cells was much more complex than that of ‘CCA 7244’ (Fig 5.2 and Fig. 5.3). At primary sporogenous cell stage, epidermis of anther wall formed, and primary parietal layers underwent perclinal divisions (Fig. 5.3 A). As pollen mother cells formed, anther wall already divided into 4 distinct layers, including epidermis, endothecium, middle layer, and tapetum. The tapetum cells enlarged and contained a large vacuole (Fig 5.3 B). In the prophase I of meiosis, the enlarging tapetum cells contained several small vacuoles or a bigger one and would unerdgo karyokinesis to form bi-nucleated tepetum cells (Fig. 5.3 C) as the maintainer line (Fig. 5.1 C). At the end of meiosis II, tetrads formed and the locule may or may not increase in size. Fig. 5.3D showed the evidence of locule increased in size and tetrads spaced out from one another. The microspore developing after released from tetrads, and the surrounding tepetum cells might swell and contain a large vacuole (Fig. 5.3E) or contain many

small vacuoles with dense cytoplasm (Fig. 5.3F). In the situation of locule remained small size, tetrads were pressed by tapetum cells which contained many small vacuoles in the dense cytoplasm and stuffed up the locule and left no space for microspore development (Fig. 5.3G). Microspores were squeezed by swollen tapetum cells with a large vacuole (Fig. 5.3 H), or by irregular tapetum cells with small vacuoles in dense cytoplasm (Fig. 5.3 I). At final stage, nearly all the microspores were crushed and tapetum decomposed (Fig. 5.3J), and secondary thickness at the radial walls of endothecium and middle layers was observed thereafter (Fig. 5.3K). Besides, a few swelled microspores sizing 43.5 μm in diameter was observed and which were normally non-viable (Fig. 5.3J and Fig. 5.3K).

Anther wall differentiation and microgametogenesis in unstable CMS line 'CCA 7243' under low temperature

As the unstable CMS line 'CCA 7243' restored its fertility, the anther wall differentiation and microgametogenesis (Fig 5.4) was similar to those of the maintainer line 'PBC 483' (Fig. 5.1). However, the number of normal microspore and viable pollens was obvious less in 'CCA 7243' (Fig. 5.1H and Fig. 5.4H).

5.4 Discussion

Compared with the anther wall differentiation and tapetum development, in stable

CMS line ‘CCA 7244’, the layering of anther wall was not clear especially between endotheicum and middle layer (Fig.5.1 B and Fig. 5.2 B), and vacuolated tapetum cells swelled abnormally in the beginning of tetrad stage and locule did not increase in size as anther development causing tetrad or microspores abortion (Fig. 5.1 D and Fig. 5.2 C). These results were similar to abnormalities in tapetum of other CMS sweet pepper lines, which swollen tapeum remained pressing tetrads or uni-nucleate microspores and eliminating any locular spaces between the latter (Horner and Rogers, 1974; Luo et al., 2006).

As unstable CMS line ‘CCA7243’ expressed sterility under high temperature, abnormalities in tapetum cells were very different from the results of stable CMS line ‘CCA7244’. The locular space and abnormal appearances of tapetum cells in ‘CCA 7243’ were observed at several developmental stages. While tetrads formed, locule ‘CCA 7243’ may or may not increase in size (Fig. 5.3 D and G) as compared with locular space did not increase in ‘CCA 7244’ (Fig. 5.2 C). As microspores development, the appearance of tapetum with dense cytoplasm or containing a vacuole (Fig. 5.3 E, F, H and I) was different from vacuolated tapetum in ‘CCA 7244’ (Fig. 5.2 C, D, and E), and timing of tapetum cells degeneration was hardly identified, but tapetum cells in both lines were gone at the end (Fig. 5.3 K and Fig. 5.2 F). These anatomical data

revealed that the genetic control might be more complicated in unstable CMS line. Besides, a few swelled microspores sizing 43.5 μm in diameter, which were normally nonviable, were observed in the locule (Fig. 5.3J and Fig. 5.3K) and were 1.5 as large as normal mature pollens of maintainer line (Fig. 5.1 H).

As unstable CMS line ‘CCA7243’ expressed sterility under high temperature, the results of abnormalities in tapetum cells at several stages and microspores abortion (Fig. 5.3 D to I) were different from the results of that vacuolated tapetum cells did not degenerate and crush tetrads in another unstable CMS line ‘Acc.17’ expressed sterility in the 28/20 ^{o}C growth chamber (Hong, 2003). Besides, the locule in‘CCA 7243’ may or may not increase in size (Fig. 5.3 D and G) as compared with locular space in ‘Acc. 17’ remained increasing (Hong, 2003).

As ‘CCA7243’ restored its fertility under low temperature, the tapetum cells remained in the parietal position until degeneration in the beginning of late-vacuolated microspore stage (Fig. 5.4 F) toward the end of pollen development (Fig. 5.4 H). While ‘Acc. 17’ expressed fertile in the 25/18 ^{o}C growth chamber, the tapetum cells departed from other layers of anther wall, thereafter they were vacuolated and degenerated (Hong, 2003). Due to the tapetum in pepper belonged to secretory type, which type of tapetum remained in the parietal position of the anther wall as anther development

(Shivanna et al., 2005), the results of Hong (2003) might be caused by artificial errors.

As the unstable CMS line ‘CCA 7243’ restored fertility under low temperature, the anther wall differentiation and microgametogenesis (Fig. 5.4) was similar to those of maintainer line ‘PBC 483’ (Fig. 5.1), but less viable pollens formed in ‘CCA 7243’ (Fig. 5.4 H) than pollens in ‘PBC 483’ (Fig. 5.1 H). These results were compared with the trend of pollen viability percentage in AVRDC’s field, the fertility in ‘CCA 7243’ could restore about 50% as compared to the maintainer line, ‘PBC 483’, which pollen viability was an average of 80% (Fig. 4.6).

The reasons of fertile restoration in unstable CMS lines were not clear yet. Previous studies had reported that another type of unstable CMS pepper line, which could produce both normal and aborted pollens that remain stuck to the inside of anther wall and did not shed even after anther dehiscence, resulting in low fruit and low seed setting, is termed as partial restoration of CMS line (Lee et al., 2008a). By genetic analysis, this partial restoration phenomenon is genetically controlled by one recessive nuclear gene, termed as partial-restoration (*pr*), which might be tightly linked to the *Rf* locus or the third allele of *Rf* locus (Lee et al., 2008b). However, unstable CMS line, which was usually male sterile but restored fertility temporarily by low temperature, might be genetically controlled by sterility-modifier genes that were affected by

temperature (Peterson, 1958; Shifriss and Guri 1979; Shifriss, 1997). Therefore, the gap between anatomical observation and genetic controlled in unstable CMS line 'CCA 7243' is still far.

Moreover, locule not increased in the size was another reason for suppressing microspores development in both CMS lines. As anther development, the size of locule in maintainer line 'PBC 483' increased continuously at the tetrad stage, allowing tetrads to separate from each other and microspores to development in enough space (Fig. 5.1 D-F). In contrast to both CMS lines, 'CCA 7244' and 'CCA 7243', small locule which was not increase in the size as anther development led tetrad or microspores to press each other (Fig. 5.2 C, Fig 5.3 E-H).

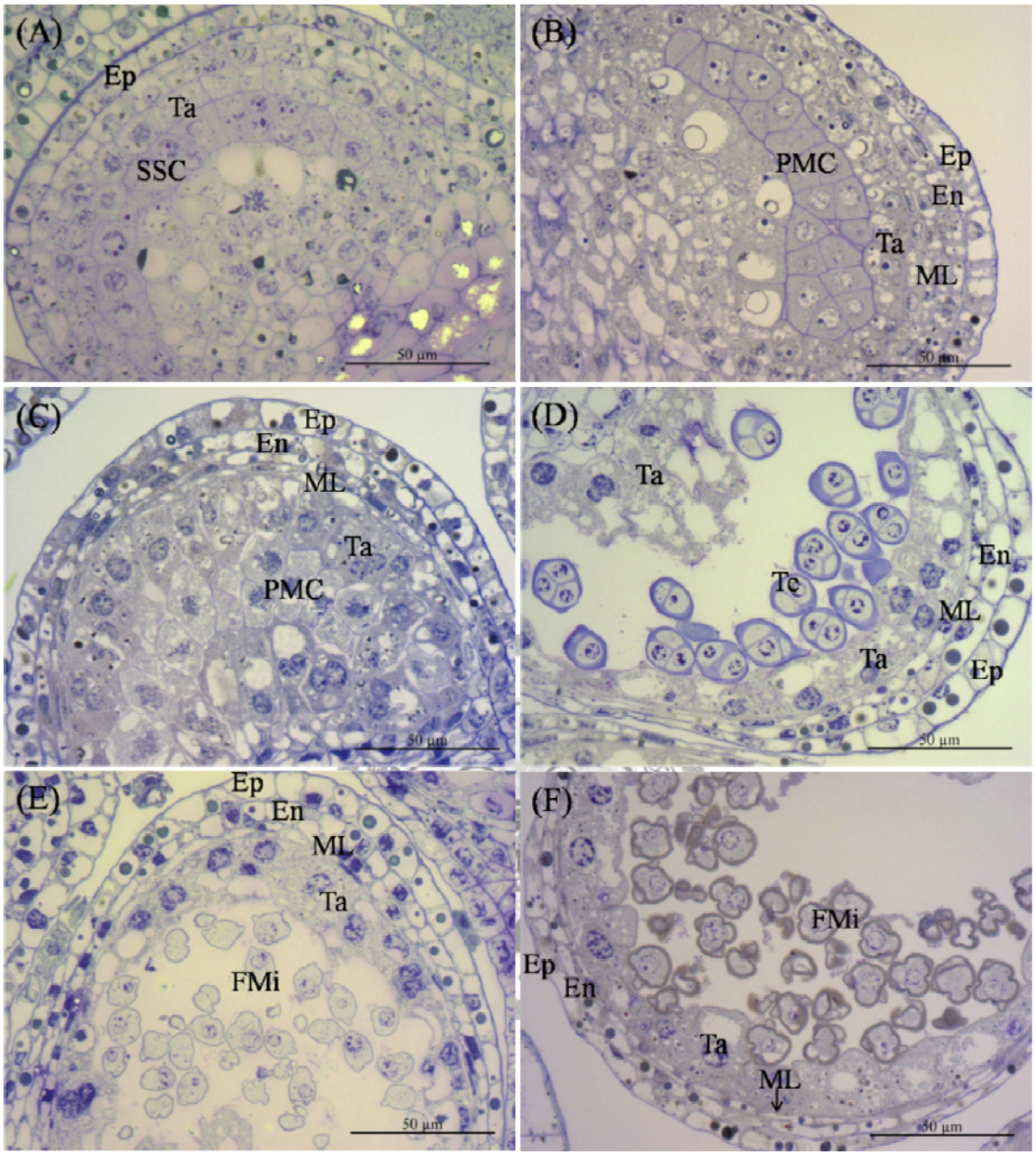


Fig. 5.1 Semi-thin transverse sections of hot pepper maintainer line 'PBC 483' anther under high temperature. Sections stained by 1% TBO. En: endothecium; AP: aperture ; Ep: epidermis; FMi: free microspore; ML: middle layer; PMC: pollen mother cell; PSC: primary sporogenous cell; Ta: tapetum; Te; tetrad.

(A) Primary sporogenous cell stage with periclinal division in endothecium and middle layer;

(B) Early pollen mother cell stage;

(C) Pollen mother cell stage during prophase I;

(D) Tetrad stage;

(E) Early released free microspore stage;

(F) Mid-vacuolated microspore stage;

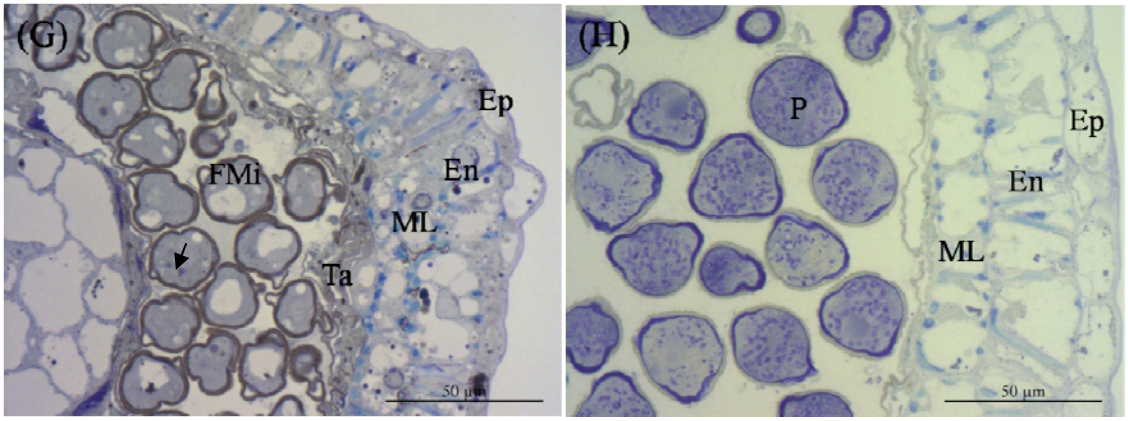


Fig. 5.1 (cont.) Semi-thin transverse sections of hot pepper maintainer line 'PBC 483' anther under high temperature. Sections stained by 1% TBO. En: endothecium; Ep: epidermis; FMi: free microspore; ML: middle layer; P: pollen; Ta: tapetum.

(G) Late vacuolated pollen stage during nucleus mitosis (arrowed);

(H) Mature pollen stage before anther wall dehiscence.

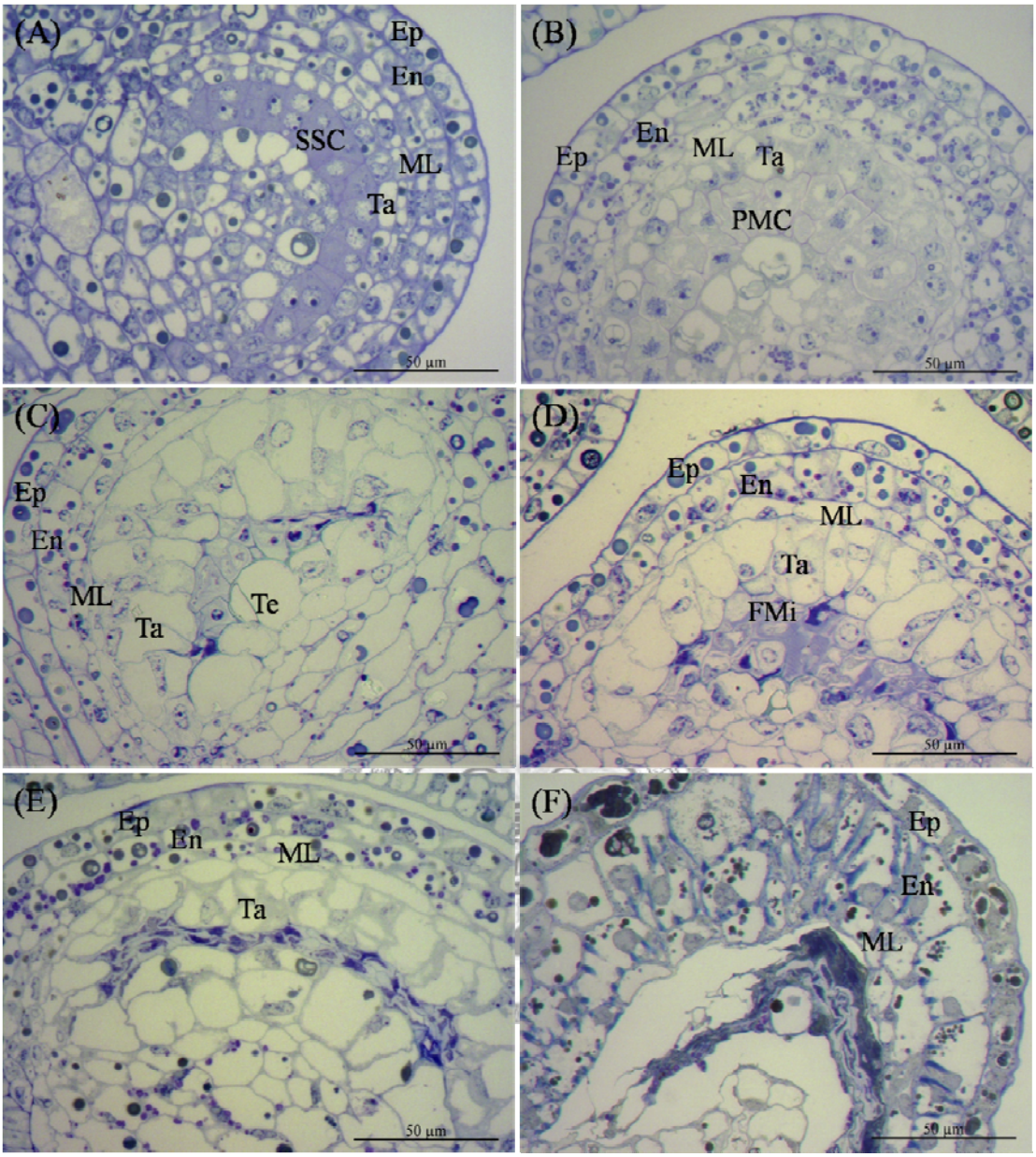


Fig. 5.2 Semi-thin transverse sections of CMS hot pepper line 'CCA 7244' anther under high temperature. Sections stained by 1% TBO. En: endothecium; Ep: epidermis; FMi: free microspore; ML: middle layer; PMC: pollen mother cell; SSC: secondary sporogenous cell; Ta: tapetum; Te; tetrad.

(A) Secondary sporogenous cell stage with anther wall differentiating into 4 distinct layers;

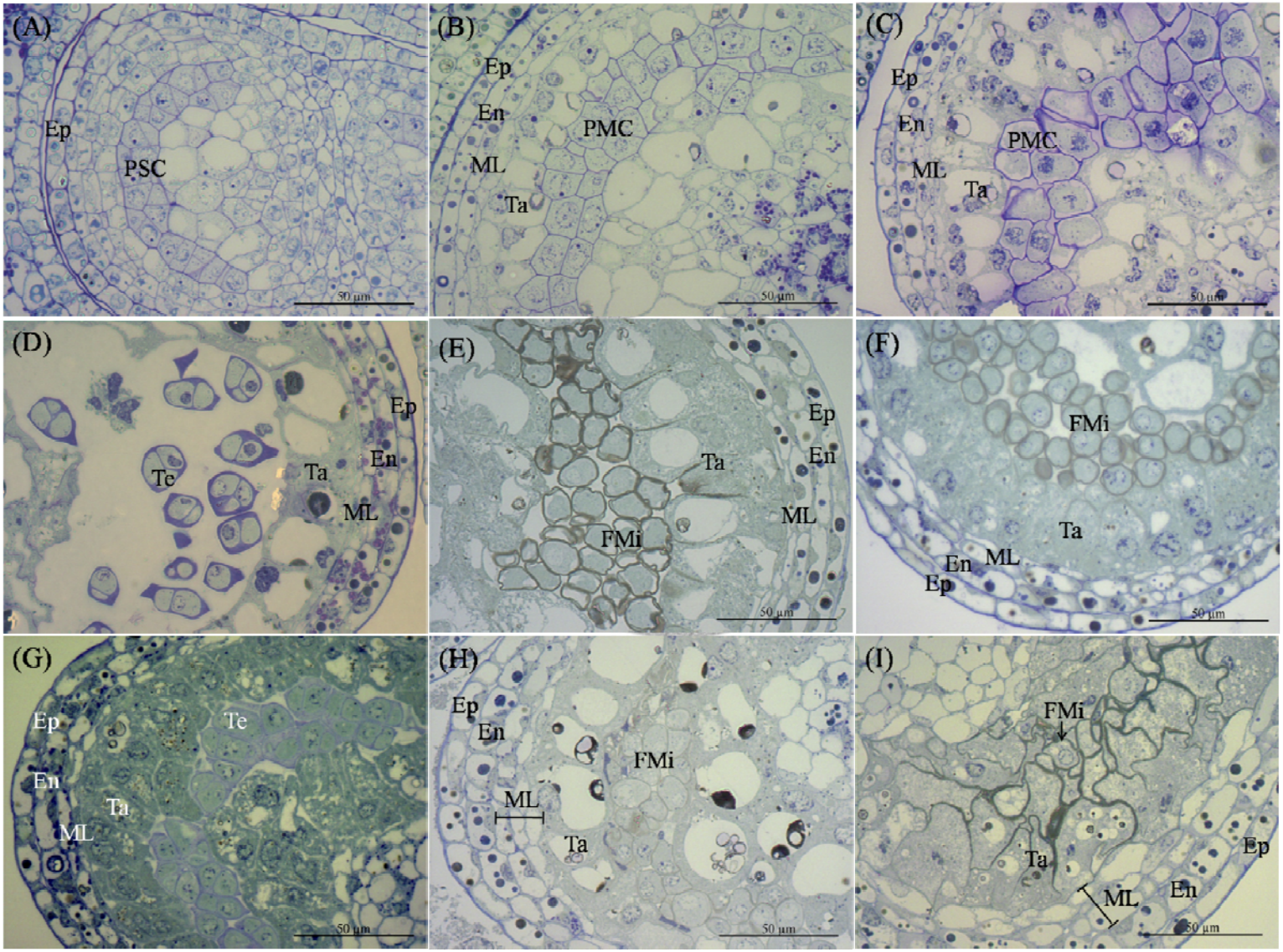
(B) Pollen mother cell stage during prophase stage;

(C) Tetrad stage;

(D) Early released free microspore stage;

(E) Relative late free microspore stage;

(F) Debris in the locule before anther dehiscence.


(A)
Ep
PSC
50 µm
(B)
Ep
En
PMC
ML
Ta
50 µm
(C)
Ep
En
PMC
ML
Ta
50 µm
(D)
Ep
Te
Ta
En
ML
(E)
Ep
Ta
En
ML
FMi
50 µm
(F)
FMi
Ta
En
ML
Ep
50 µm
(G)
Ep
Te
En
Ta
ML
50 µm
(H)
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ML
Ta
50 µm
(I)
FMi
Ta
Ep
ML
En
50 µm

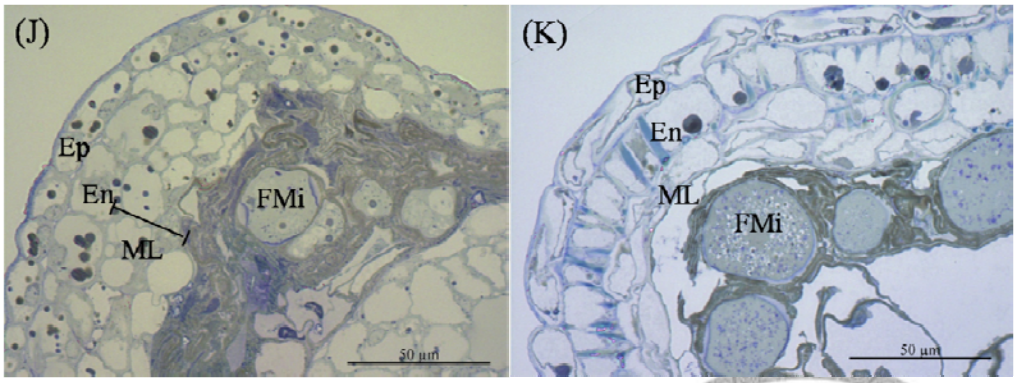

Fig. 5.3 Semi-thin transverse sections of unstable CMS hot pepper line 'CCA 7243' anther under high temperature. Sections stained by 1% TBO. En: endothecium; Ep: epidermis; FMi: free microspore; ML: middle layer; PMC: pollen mother cell; SSC: secondary sporogenous cell; Ta: tapetum; Te; tetrad.

(A) Primary sporogenous cell stage with periclinal division in endothecium and middle layer;

(B) Early pollen mother cell stage;

(C) Pollen mother cell stage during prophase I;

(D) Tetrad stage;

(E) Mid-vacuolated microspore stage with vacuolated tapetum cells;

(F) Mid-vacuolated microspore stage with remained dense cytoplasm in tapetum cells;

(G) Tetrad stage with dense cytoplasm in tapetum cells;

(H) Early released free microspore stage;

(I) Early released free microspore stage pressed;

(J) Relative late-vacuolated microspore stage with debris and abnormal microspores in the locule;

(K) Debris and abnormal microspores in the locule before anther dehiscence.

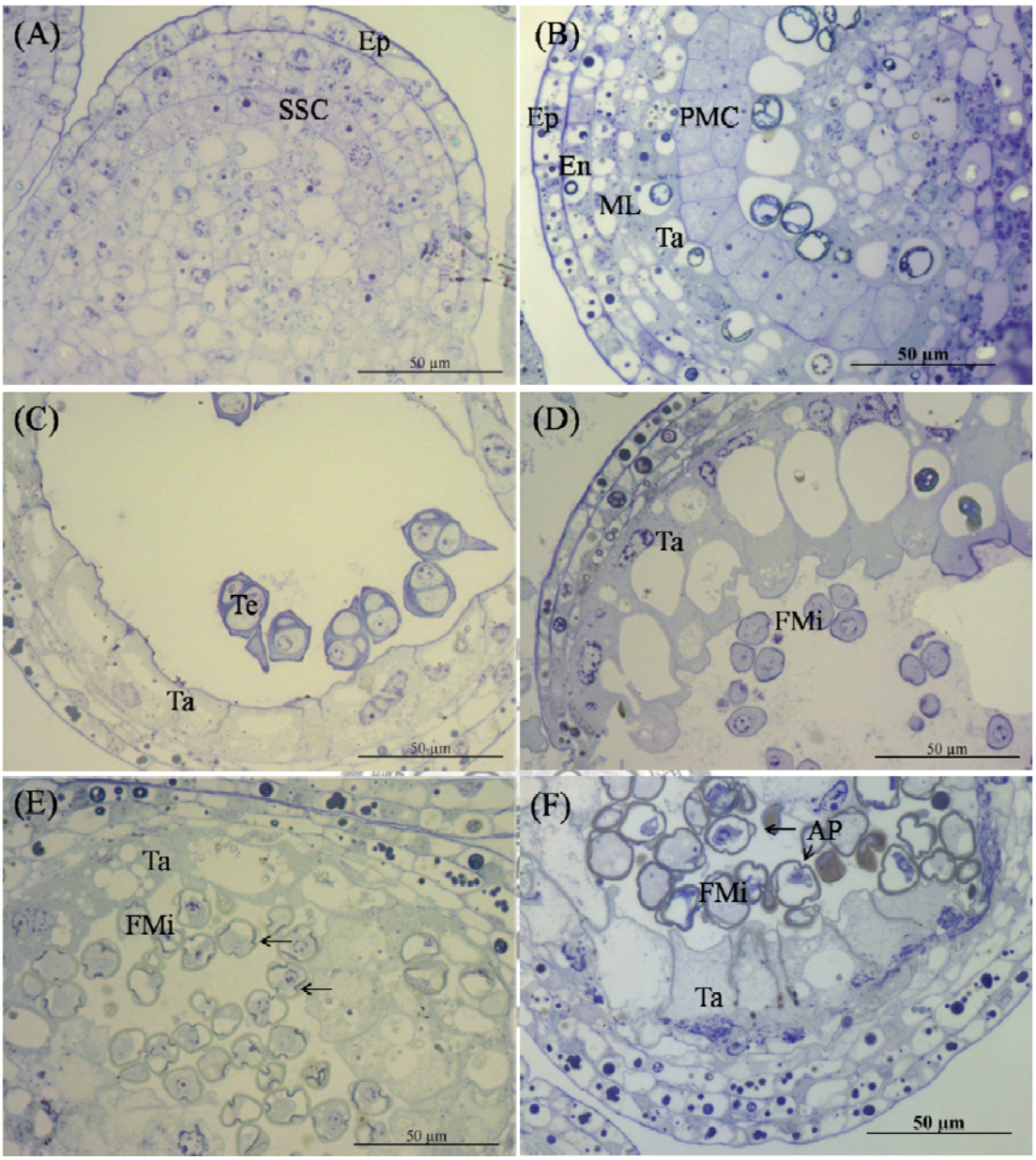

Fig. 5.4 Semi-thin transverse sections of unstable CMS hot pepper line 'CCA 7243' anther under cool temperature. Sections stained by 1% TBO. AP: aperture; En: endothecium; Ep: epidermis; FMi: free microspore; ML: middle layer; PMC: pollen mother cell; SSC: secondary sporogenous cell; Ta: tapetum; Te; tetrad.

(A) Primary sporogenous cell stage with periclinal division in endothecium and middle layer;

(B) Early pollen mother cell stage;

(C) Tetrad stage;

(D) Early released free microspore stage;

(E) Mid-vacuolated microspore stage;

(F) Late-vacuolated microspore stage;

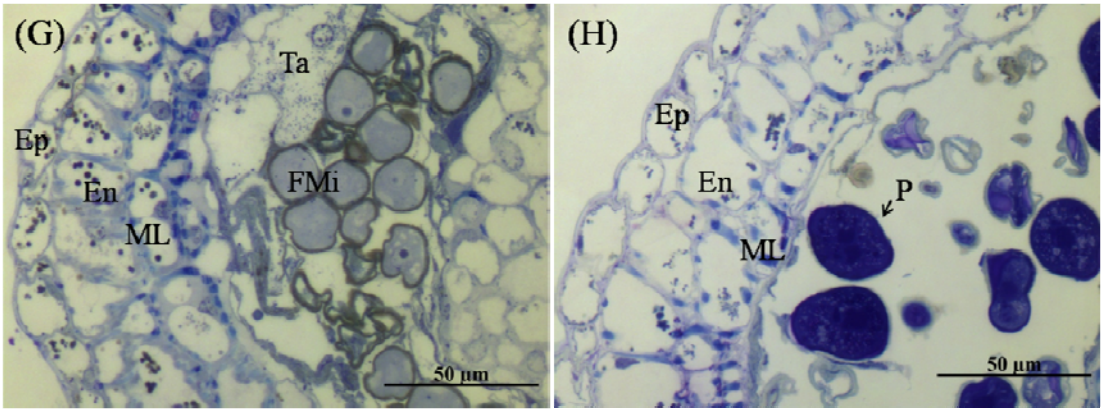

Fig. 5.4 (cont.) Semi-thin transverse sections of unstable CMS hot pepper line 'CCA 7243' anther under cool temperature. Sections stained by 1% TBO. En: endothecium; Ep: epidermis; FMi: free microspore; ML: middle layer; P: pollen; Ta: tapetum.

(G) Vacuolated pollen stage during nucleus mitosis;

(H) Mature pollen stage before anther wall dehiscence.

Chapter 6
Conclusion

Cytoplasmic male sterility (CMS) is the result caused by the interaction between cytoplasm and nuclear genes. The male sterility of the CMS pepper lines was not always stable. Some of them might restore fertility partially or completely due to low temperature, and such restoration could be resumed as temperature re-elevate.

For genetic background identification, all CMS pepper lines could generate predicted (S)*ψatp6-2* fragments by the each primer set, and their maintainer lines didn't produced the predicted products (Fig. 3.1, Fig. 3.3 and Table 3.3), and similar results were obtained for *orf456* (Fig. 3.2, Fig. 3.4 and Table 3.3). These results revealed that the cytoplasms of all the CMS pepper lines contained (S)*ψatp6-2* and *orf456*, and their maintainers of hot pepper lines, with N cytoplasm, didn't have such defected *ψatp6-2* and *orf456*. Surprisingly, 'PBC 84 selex' and '9946-2138', maintainer lines of unstable CMS sweet pepper, could generate *orf456* fragment by CMS P1 or CMS P2 primer sets (Fig. 3.2, Fig. 3.4 and Table 3.3), representing that the genetic background of cytoplasm in specific maintainers might be more complicated and different from other maintainers.

For the influences of temperature and seasonal changes on CMS plants, unstable CMS hot pepper line 'CCA 7243' could restore fertility in the greenhouse and in the

field while the night temperature was below 21 ^{o}C, and also could restore in the 20/15 ^{o}C phytotron (Fig. 4.5 A and Fig. 4.7). Plants of unstable CMS sweet pepper line ‘CCA 7236’ restored fertility while night temperature was below 21^{o}C or 17 ^{o}C, but plants hardly restored while day temperature was below 18 ^{o}C (Fig. 4.6 A and Fig. 8). Besides, the fertility restoration of unstable CMS lines showed as a regular cycle, not always steady expressed. When night temperature went higher, both lines were reverted male sterile. Both CMS line ‘CCA 7244’ and ‘CCA 7234’ almost expressed male sterile even under a long period of lower night temperature (Fig. 4.5 B, Fig. 4.6, Fig. 4.7 B, and Fig. 4.8).

For anatomy difference between unstable and stable CMS line, as unstable CMS line ‘CCA7243’ expressed sterility under high temperature, abnormalities in tapetum cells were very different from the results of stable CMS line ‘CCA7244’. While tetrads formed, locule in‘CCA 7243’ may or may not increase in size (Fig. 5.3 D and G) as compared with locular space did not increase in ‘CCA 7244’ (Fig. 5.2 C). As microspores development, the appearance of tapetum with dense cytoplasm or containing a vacuole (Fig. 5.3 E, F, H and I) was different from vacuolated tapetum in ‘CCA 7244’ (Fig. 5.2 C, D, and E), and timing of tapetum cells degeneration was hardly identified, but tapetum cells in both lines were gone at the end (Fig. 5.3 K and Fig. 5.2

F). These anatomical data revealed that the genetic control might be more complicated in unstable CMS line. As the anthers of ‘CCA7243’ resumed the fertility, there was no difference between ‘CCA7243’ (Fig. 5.4) and its maintainer ‘PBC 385’ anatomically (Fig. 5.1).

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