CHAPTER III

CHARACTERIZATION OF ANther PROTEINS DURING MICROSPOROGENESIS FROM NORMAL AND CYTOPLASMIC MALE STERILE COTTON

(Gossypium hirsutum L.)
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INTRODUCTION

Developmental processes in plants are believed to be controlled by selective gene expression which exerts its effects, in part through the synthesis and/or activation of enzymes and other proteins. It is reasonable to assume, that any structural changes observed during the ontogeny of anther must be preceded by or concomitant with changes in the activities of various enzymes and proteins. CMS provides one of the few systems in which the role of proteins in the phenomenon of CMS can be studied. MS is defined as inability of plants to produce functional anthers, pollen or male gametes. It is a maternally inherited character. CMS plants are valuable in investigating the gene controlled mechanisms in stamen development. Microsporogenesis and pollen development takes place in the stamen. It is a complex process controlled by several genes (Mascarenhas 1993). The anther itself is a complex structure which consists of several tissues comprising heterogenous cell types. Proper coordination and communication between these different anther tissues is very much essential for the formation of viable pollen grains. Any deviation or abnormality in one, or more than one, process would inevitably lead to Pollen abortion. Pollen expressed genes have been successfully isolated and identified in maize, tomato, barley, Brassica, Tradescantia, tobacco (Mc Cormick 1991, 1993; Ursin et al 1989; Davies et al 1992; Zaki and Dickinson 1992; Mascarenhas 1992; Albani et al 1992).
In general, there are more genes expressed in gametophytic tissues (Pollen grains) than in sporophytic tissues (PMCs, tapetum) (Zaki and Dickinson 1992). Nearly 20,000 different mRNAs are found in mature pollen and any aberration or variation in those genes may lead to pollen abortion as in Tradescantia (Willing et al 1988). It is estimated that about 15,000 different genes are expressed in the developing tobacco anther and several of the corresponding mRNAs are spatially restricted to tapetum (Vergne and Dumas 1988).

At present it is clearly evidenced that the phenomenon of CMS is controlled by the mitochondrial genome (Levings and Pring 1976; Pring and Levings 1978; Levings 1983; Kemble et al 1980; Kool et al 1982; Mikami et al 1984; Dewey et al 1986; Rottmann et al 1987; Wise et al 1987; Young and Hanson 1987; Nivison and Hanson 1989; Hanson 1991; Johns et al 1992; Krishnasamy and Makaroff 1994). Development and cytological studies have revealed that the time of expression of these genes varies from species to species (Kaul 1988). According to Abad et al (1995) Vicia faba CMS genes encoded by mitochondria express only in anther tissues. It is also known that male sterility is also associated with several biochemical and molecular changes in plant tissues (Kaul 1988).

Hitherto very few studies have been made concerning to the characterization of anther proteins in CMS and fertile lines. A number of proteins are produced within the mitochondria (Dillon 1981). Deviations in the synthesis of one of the proteins may ultimately result in abortion of the tapetal and sporogenous cells. Several unique polypeptides from mitochondria are associated with male sterile cytoplasm. This distinct polypeptide composition possibly influence sterile microsporogenensis and may ultimately result in abortion of the tapetal and sporogenous cells (Kool et al 1982, Bino et al 1988). Thus differences in specific polypeptides have been observed in normal
and sterile systems. Alam and Sandal (1969) made electrophoretic analysis of anther proteins from CMS and fertile lines of sudan grass, and they noticed differences in banding patterns for peroxidase, cytochrome oxidase and total protein. They observed fewer bands in comparison to fertile line. Similarly, in the normal and a CMS line of barley, specific protein differences were observed at later stages of stamen development (Ahokas 1980). Absence of 20KDa protein at meiocytes stage in sterile anthers of Capsicum has been observed (Manoharan et al 1993). Some workers have observed differences in various amino acids between male fertile and sterile stocks while others have found no differences (Kaul 1988). Differences in amino acids were found between sterile and fertile plants of cotton (Sarvella and Stojanovic 1968). Abnormal accumulation of alanine in maize (Khoo and Stinson 1957) and glycine in Sorghum sterile anthers have been reported. More accumulation of amino acids namely proline and piperolic acid was higher in fertile anthers than in CMS line of Petunia (Wu and Murry 1985). The authors reported that both CMS and fertile lines are similar in total protein contents, but SDS-PAGE revealed a clear difference in protein patterns (Wu and Murry 1985). In the normal and CMS lines of Capsicum Markova and Daskaloff (1974, 1976) have reported differences in protein banding pattern. In several studies, sterility appears to be also associated with alterations of number of isoenzyme patterns (Watson et al 1977; Dixon and Lever 1982; Hohler and Bomer 1980; Hanson et al 1984; Karim et al 1984; Van Marrewijk and Suurs 1985; Bino et al 1985; Nave and Sawhney 1986). Having assessed developmental and histochemical changes in both CMS and normal lines of cotton anthers (see Chapter-II), we have focused on biochemical variation during development of CMS and fertile anthers of cotton. The present study has as a main objective the analysis of protein content in CMS and fertile anthers during microsporogenesis.
MATERIAL AND METHODS

CMS and maintainer lines of cotton (Gossypium hirsutum L. Variety CAK 32 A and B) were grown in University greenhouse.

Anther Separation:

Anthers of flower buds of different sizes were classified according to development stage. Each flower bud contains many anthers which develop synchronously. One or two anthers were removed for stage determination and the rest were utilized for SDS-PAGE of proteins. The development stages recognized cytologically in the CMS line were:

1. Sporogenous; 2. pollen mother cells; 3. tetrads and 4. free microspores; and in the CMS line: i) sporogenous and ii) abortive stage of anthers.

Protein Extraction:

Anthers were homogenized with a glass rod homogenizer in a solubilization buffer containing 0.1 M Tris - HCl, pH 7.4, 5 mM - mercaptoethanol, 5mM EDTA and 5% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was precipitated with trichloroacetic acid (TCA) at a final concentration of 10%. After 30 min in an ice bath, the precipitants were collected by centrifugation, washed two times with prechilled 90% acetone and once with 100% acetone. Precipitants were dried and stored at -20°C.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS - PAGE):

SDS - PAGE was carried out at room temperature with a slightly modified Laemmli buffer system (Laemmli 1970) using 75 mm slab gel with 4% stacking gel and 8 to 16% gradient resolving gel. The anther pellets were resuspended in a sample buffer consisting of 62.5 mM Tris-HCl, (pH 6.8) 2% (w/v) SDS, 2% (v/v) B - mercaptoethanol.
and 10% (v/v) glycerol and heated at 100°C for 5 min and centrifuged. The supernatant was recentrifuged and protein content determined according to Lowry et al. (1951) with BSA as a standard. Before electrophoresis, the proteins were precipitated again with ice cold acetone, redissolved in the sample buffer and boiled again for 5 min. Electrophoresis was carried out with a constant current of 15 mA for 12 to 14 hr. The co-electrophoresed molecular weight markers (Sigma, USA) were: albumin (Bovine) 66 KDa, albumin (egg) 45 KDa, carbonic anhydrase 19 KDa, and lactalbumin 14.2 KDa.

**Gel staining**

After electrophoresis, the gel was removed and fixed for 6 hours with acetic acid/methanol/water (10:40:50, v/v/v). Subsequently, the gel was stained overnight with 0.2% Coomassie Brilliant Blue in acetic acid/methanol/water (10:25:65, v/v/v) and destained in acetic acid/methanol/water (10:25:65, v/v/v).

**RESULTS**

SDS-PAGE of anther proteins shows numerous bands during microsporogenesis (Fig.1). The fertile anthers showed an additional polypeptide of 12 Kd throughout sporogenous, PMCs and tetrad stages (Fig.1). Sterile anthers lack this specific protein during both sporogenous and aborted stage of microsporogenesis.

**DISCUSSION**

In the present study, an attempt has been made to correlate developmental and histochemical changes with biochemical changes that occur during anther development in both CMS and fertile lines of cotton. Developmental studies revealed the time of action of male sterility genes. In CMS cotton PMCs undergo degeneration before the initiation of meiosis. Understanding the stage at which the cytological aberration occurs in CMS plants is very important. We propose the degeneration of PMCs in cotton is due to
FIGURE 1: A and B

Photographs and line diagram of electrophoretic patterns of the gels stained for proteins are shown in Figure 1 A and 1 B.

SDS - PAGE of anther proteins from normal and CMS lines of cotton (*Gossypium hirsutum* L.) at different stages of microsporogenesis. Electrogram is drawn after observing the bands in gel. The lane 2(Bi) and 4 (B2) show an extra protein band of 12 KD molecular weight. Molecular weights of marker proteins (in kilo Daltons) are indicated in left side.

B1 - Normal line at sporogenous stage.

B2 - PMC stage.

A1 - Sterile line at sporogenous to early PMC stage.

A2 - Aborted sterile anther.

MW - Molecular weight markers.
precocious activity of enzyme callase which dissolves the callose present around PCMs (see Chapter -II). Characterization of anther proteins at different stages of anther development in CMS and fertile lines of cotton revealed that CMS anthers lack a 12 Kd protein which is present in fertile anthers throughout microsporogenesis. We hypothesize that this protein is related to the normal deposition and dissolution of callose which is altogether a new substance synthesized and deposited around meiocytes during normal microsporogenesis. In cotton there are several reports on amino acid content of CMS and fertile lines (Sarvella and Stojanovic 1968). The leaves and flowers of several genetic and CMS lines were examined. Fewer amino acids were observed in the leaves in the free amino acid fraction than in the protein fraction. In both samples of leaves of all lines have a similar number and quantity of aminoacids. Differences in aminoacids between normal and sterile flowers were more qualitative than qualitative. Presence of asparatic acid and arginine in the leaves of alloplasmic line Gossypium hirsutum, these amino acids are absent in normal lines. The amount of acid soluble aminoacids in the sterile buds having G. anomalum cytoplasm is nearly same as in the normal buds containing G. hirsutum cytoplasm (Sarvella and Stojanovic 1968). Differences in the various aminoacids between the male sterile and fertile stocks were observed whereas in some cases no differences were observed. Analysis of free aminoacids from anthers of Petunia fertile line shows levels of proline and pipelicolic acid 2-3 and 10-20 fold higher respectively, than in the CMS line (Wu and Murry 1985). In maize (Sarvella et al (1967) similar to cotton found no differences in specific aminoacids but the differences between the normal and sterile flowers and buds were of more quantitative than qualitative. Differences in specific polypeptides have been reported in other normal and male sterile systems. In CMS Sorghum, Alam and Sandal (1969) reported 20 and 9 protein bands in fertile and in sterile lines, respectively. Similarly, in the normal and a CMS line of barley, specific protein differences were observed at later stages of stamen development (Ahokas 1980). In petunia (Wu and Murry 1985) at the
beginning of meiosis, anthers of the CMS and fertile lines are similar in total protein contents, but SDS - PAGE revealed a clear difference in protein patterns. Fertile anthers are enriched in three polypeptides with molecular weight of 64-, 63- and 45 Kd proteins which characterize pre-meiosis stage. Anthers of the CMS line lack the 63 and 45 Kd proteins but are enriched in two polypeptides with molecular weight of 180 and 66 Kd proteins. These two polypeptides are present in fertile anthers but at lower levels (Wu and Murry 1985). The fact that two major proteins are missing and that no new polypeptides are found in the CMS line may indicate a possible block in protein synthesis in the CMS microspore. In *Capsicum*, as evidenced by SDS - PAGE, Manoharan et al (1993) reported the absence of 20 Kd protein in CMS anthers and this difference is maintained until the completion of pollen abortion.

Correlation of occurrence of certain enzymes and their multiple forms - isoenzymes pattern-have been made from the anther tissues of fertile and sterile plants (Hohler and Borner 1980; Karim et al 1984; Bino 1985; Van Marrewijk and Suurs 1985; Nave and Sawhney 1986; Sawhney and Bhadula 1988). Enzymatic aberrations during the ontogeny of CMS anthers in a few cases would inevitably lead to pollen abortion. Differentiation of normal pollen is known to be controlled by selective gene expression which exerts its effects, in part through the synthesis and/or activation of proteins and enzymes. It is reasonable to assume, therefore, that any changes in their activities may result in abnormality in the anther which inevitably disrupt normal pollen formation.

The difference in anther protein bands of CMS and fertile lines of cotton during anther development may be interpreted as indicative of aberrations in physiological functions of different anther tissues. However, since these aberrations do not appear to be the same for all plant species exhibiting CMS, we propose that alterations in the activity of biochemical substances in fertile and sterile plants are the consequences
rather than a primary cause of CMS. This is a reasonable assumption based on the results obtained on cotton. In contrast, protein contents of normal and sterile anthers of tomato do not show much difference during stamen development (Sawhney and Bhadula 1988). The authors found other structural and enzymatic aberrations in the tapetum of tomato.

It is well established that the CMS is an mitochondrially inherited trait (Levings and Pring 1979; Kemble et al 1980; Kool et al 1982; Levings 1983; Mikami et al 1984; Hanson et al 1984; Hanson and Conde 1985; Dewey and Levings 1986; Rottmann and Bears 1987; Wise et al 1987; Young and Hanson 1987; Turpen et al 1988; Levings and Brown 1989; Johns et al 1992; Krishnasamy and Markaroff 1994). Mitochondria of CMS lines synthesize specific polypeptides which may be involved in the expression of CMS. Several recent studies on number of crop plants confirmed the involvement of mitochondria in the mechanism of CMS. In the case of CMS - T maize and Petunia 13 Kd and 25 Kd proteins have been associated with CMS (Hanson 1991). Presumably these specific polypeptides in CMS line bring about biochemical alterations which lead to pollen abortion. Accordingly it seems relevant to analyze mitochondrial translational products from CMS and normal lines of cotton seeking to identify differences between them, which may be related to male sterility.