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Changes in protein and amino acid content during anther development in fertile and cytoplasmic male sterile *Petunia*

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Summary. Development of anthers in cytoplasmic male sterile (CMS) *Petunia* diverges from the normal sequence of events early in meiosis. Quantitative and qualitative changes in morphology, proteins and free amino acid contents correlate with this divergence. In anthers of the fertile line (5719), total protein content increases, and SDS-PAGE protein patterns change as the anthers mature. Enhanced levels of three polypeptides with molecular weights of 64,000, 63,000 and 45,000 daltons characterize premeiosis in fertile anthers. Protein levels and patterns from anthers of the CMS line (5707) show little alteration during anther development. Protein synthesis seems to be at least partially blocked in the CMS microspore. The 63,000 and 45,000 dalton proteins are not present, and the absence of any unique protein(s) in the CMS line argues against a virus as the causal agent of CMS in *Petunia*. Analysis of free amino acids from anthers of the fertile line shows levels of proline and pipecolic acid 2–3 and 10–20 fold higher, respectively, than in the CMS line. The amino acids incorporated into proteins show no such differences; analysis of protein hydrolysates shows similar levels of each amino acid in both fertile and CMS lines at every developmental stage examined.

Key words: Amino acids and proteins – Microsporogenesis – Cytoplasmic male sterility – *Petunia* anther development

Introduction

The morphologies of fertile and cytoplasmic male sterile (CMS) *Petunia* flowers are indistinguishable except for the anthers. Sterile anthers are not as yellow or as plump as their fertile counterparts at maturity. CMS which affects anther morphology and presumably causes *Petunia* sterility is carried throughout plant development (as well as from generation to generation) and appears to be activated only in the anther tissue when the plant flowers. The purpose of this work is to examine whether CMS can be correlated with the presence, types and amounts of proteins and free amino acids in developing *Petunia* anthers and whether CMS in *Petunia* is caused by a virus or virus-like agent.

Although CMS has been used extensively in plant breeding of many crops, the mechanisms affecting CMS are still not understood. Several reviews summarize available data and attempt to elucidate the mechanisms which result in CMS (Edwardson and Corbett 1961; Laser and Lersten 1972; Nakashima 1978; Pearson 1981). In *Petunia*, the only reported mechanism involves the faulty timing of callase activity during microsporogenesis (Frankel et al. 1969). CMS in *Petunia* has been reported to be graft transmissible (Edwardson and Corbett 1961; Pearson 1981). This would seem to implicate a virus or virus-like agent as the possible cause of CMS; however, previous workers have not reported the presence (or absence) of viral nucleic acid or protein from tissues of CMS lines of *Petunia*. Since proteins play an essential role in both the structure and function of viruses, cells and tissues, the failure of the anthers to form fertile pollen may be associated with quantitative and/or qualitative changes of certain proteins. We report here the protein patterns seen in various stages of *Petunia* anther development and the absence of any abundant novel proteins in the CMS line.

The free amino acid pool found in any plant organ or tissue changes with the balance of protein synthesis and degradation and may be characteristic either of the normal developmental pattern or of an environmental stress response. Plants may accumulate specific amino acids such as proline in response to environmental stress (Dungey and Davis 1982; Stewart and Lahrer 1980). Pollen normally contains high levels of proline accumulated during development, but pollen from CMS lines of maize (Palfi et al. 1981) and tobacco (Gerstel 1980) show much lower levels of proline. Abnormal fluctuations in the amounts and ratios of free amino acids, such as proline/histidine in apple (Tupy 1963), may be used to

differentiate between fertile and sterile plants or varieties. Since free amino acids account for 6% of the dry weight of *Petunia* pollen (Stanley and Linskens 1974), the levels of free amino acids may be closely related to pollen production and, therefore, anther development.

In this report we compare the amino acids in soluble fractions and in protein hydrolysates during *Petunia* anther development in fertile and CMS lines.

Materials and methods

Stock seeds of *Petunia parodii*, fertile lines 5719 (Izhar) and 3699 (Sink) and their respective isogenic CMS lines 5707 and 3688, were provided by Shamay Izhar. Seeds were germinated and grown in Jiffy mix in a greenhouse maintained at 22–27 °C (day) and 16–22 °C (night) with natural light. Although variable growing conditions were inevitable due to change in day length and available sunlight, consistent results were obtained when flowers for experiments were taken only on sunny days between 10–12 AM.

Determination of developmental stages of anthers

Flowers were gathered according to length measured from the bottom of the sepals to the tip of the petals. Eight size classes were created, and single anthers from each size class were squashed in acetocarmine and examined on a Leitz Orthoplan Microscope. Developmental stages were related to stage of microsporogenesis or microgametogenesis (Table 1). The four remaining anthers from each flower were analyzed for free amino acids according to the procedure below.

Protein determination

Anthers from a particular flower size class were collected and homogenized with a glass homogenizer in a solubilization buffer containing 70 mM tris-HCl, pH 6.8; 2% sodium lauryl sulfate (SDS); 2 mM benzamidine; and 50 µg/ml phenylmethylsulfonylfluoride (PMSF). The homogenates were sonicated briefly and centrifuged in an Eppendorf microfuge for 3 min to remove all debris. The supernatants were 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 (–20 °C) and solubilized in the above buffer with SDS concentration reduced to 0.5%. Protein concentration was determined using the Lowry method (Lowry 1951).

Table 1. Developmental stages of *Petunia* flowers collected for amino acid and protein analysis

Class	Size (mm)	Stage
1	< 2	Premeiosis
2	2 – 5	Meiosis I
3	5 – 10	Meiosis II
4	10 – 15	Microgametogenesis
5	15 – 20	Microgametogenesis
6	20 – 40	Microgametogenesis
7	40 – 60	Microgametogenesis
8	>60	Anthesis

Electrophoresis of proteins

Anthers were collected in prechilled 10% TCA solution (0 °C) and homogenized in a glass homogenizer. The homogenates were centrifuged, and the precipitates were washed once with 8% TCA and twice with 90% acetone. Precipitants were dried and then resuspended in solubilization buffer containing 5% B-mercaptoethanol (Wu and Wang 1984). The samples were sonicated before being subjected to a 6–12% gradient polyacrylamide gel electrophoresis (PAGE; Laemmli 1970). Two dimensional methods (McLellan et al. 1983) were hampered by extremely high levels of proteolytic activity in the *Petunia* tissues examined.

Amino acid analysis of protein hydrolysates

Anther proteins were extracted, and 100 µg of protein were precipitated as described above. After washing with acetone, the protein pellets were dried and taken up in 0.2 ml of 0.4% NaOH. After 0.5 ml of 5.5 N HCl was added, the samples were hydrolyzed for 16 h at 115 °C. Excess HCl was evaporated, and the samples were derivatized as N-heptafluorobutyryl isobutyl esters. Residue was dissolved in 50 µl of ethyl acetate : acetic anhydride (1 : 1 v/v) and analyzed and quantified by gas chromatography (GC) as described by Rhodes et al. (1981), excluding pipecolic acid as internal standard for these applications.

Analysis of free amino acids

Anthers were collected and extracted with 0.5 ml of methanol for 24 h. After adding 0.5 ml chloroform and 0.5 ml water, the upper phase was removed and rotary evaporated to dryness. Residue was redissolved in 0.4 ml water and applied to Dowex 50W H+ resin. The column was eluted with 6M NH_4OH, and the sample was evaporated to dryness, derivatized, reconstituted in ethylacetate: acetic anhydride and analyzed by GC as described above. Some of the samples were analyzed by GC-mass spectrometry for proline and pipecolic acid content (Rhodes et al. 1981), monitoring ions 266.1 and 280.1, respectively.

Results

Size and protein content of Petunia anthers during development

Flower size does not vary between fertile and CMS lines during development. Anther size is comparable through microsporogenesis, with < 1.0 mm at premeiotic stages to 1.5 mm at the tetrad stage. During microgametogenesis, fertile anthers continue to grow reaching 2.3 mm length while the anthers of the CMS line grow only slightly. Premeiotic anthers of both fertile and sterile lines are fairly equivalent in protein content (4 µg/anther); however, anthers of the CMS line show little increase in total proteins during subsequent stages. Total protein content increases rapidly in fertile *Petunia* anthers averaging 42 µg/anther at anthesis (Fig. 1). Plumpness and coloration are more pronounced in mature anthers of the fertile line.

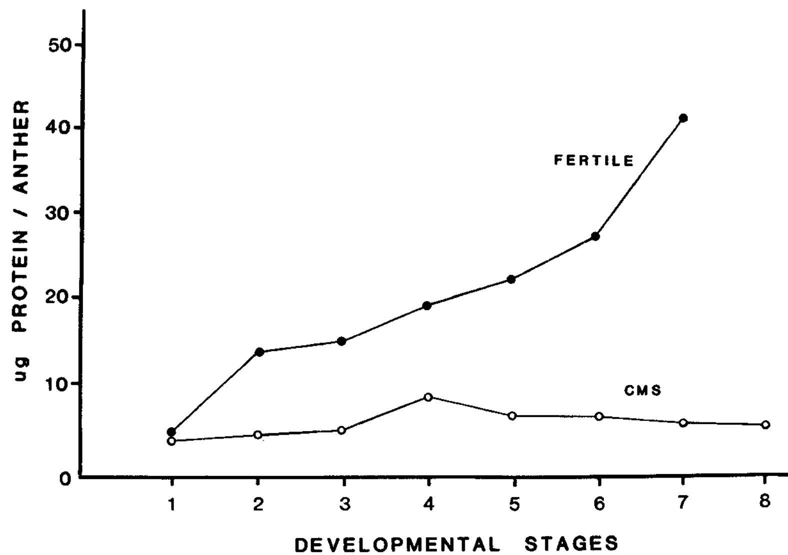

Fig. 1. Quantitation of SDS-extracted proteins during different developmental stages of *Petunia* anther. The stages are classified cytologically as listed in Table 1

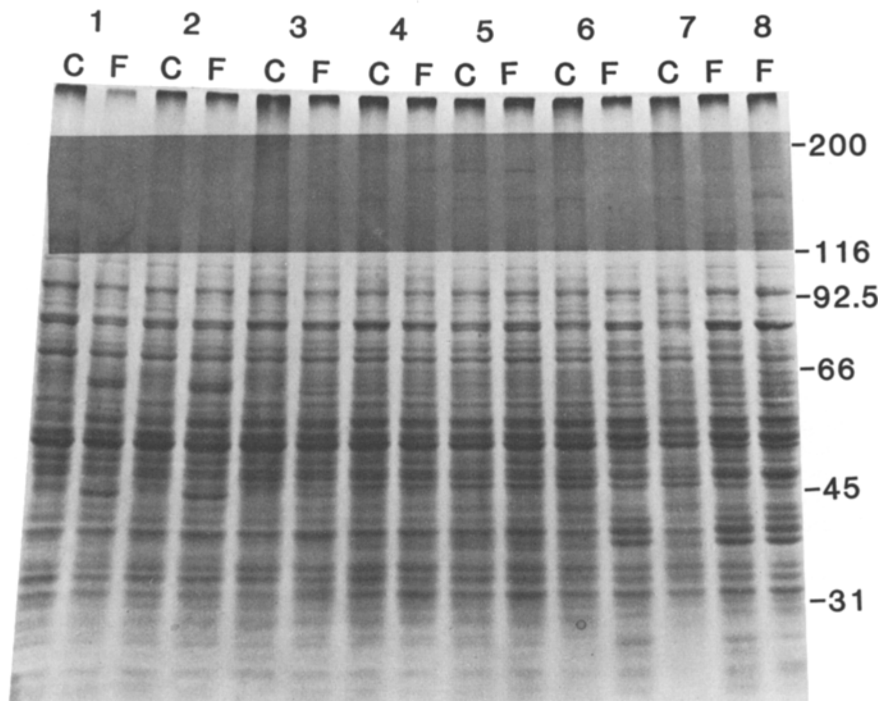

Fig. 2. SDS-PAGE of proteins extracted from *Petunia* anthers at different developmental stages (Table 1; C = CMS and F = fertile). The high molecular weight area (*shadowed*) was overexposed during printing to reveal the minor protein bands. Molecular weights in $\times 10^3$ daltons of marker proteins are indicated

Changes in SDS-PAGE protein patterns during development

When anther proteins are analyzed in SDS-PAGE, differences in protein pattern are obvious (Fig. 2). At premeiosis, three polypeptides with molecular weights of 64,000, 63,000 and 45,000 d are abundant in the fertile anthers. The amount of these proteins increases during microsporogenesis. In fertile anthers, the 64,000 d protein reaches its maximum concentration during meiosis. Anthers of the CMS line show a greatly reduced amount of the 64,000 d protein and lack the 63,000 and 45,000 d proteins through all developmental stages. CMS anthers contain enhanced levels of 180,000 and 66,000 d proteins during premeiotic stages. The 180,000 d protein remains higher in the anthers of the CMS line than in fertile anthers during development. The 66,000 d protein appears to maintain equivalent levels in fertile and sterile lines following microsporogenesis.

The differences in SDS-PAGE protein patterns between anthers of the CMS and fertile lines become

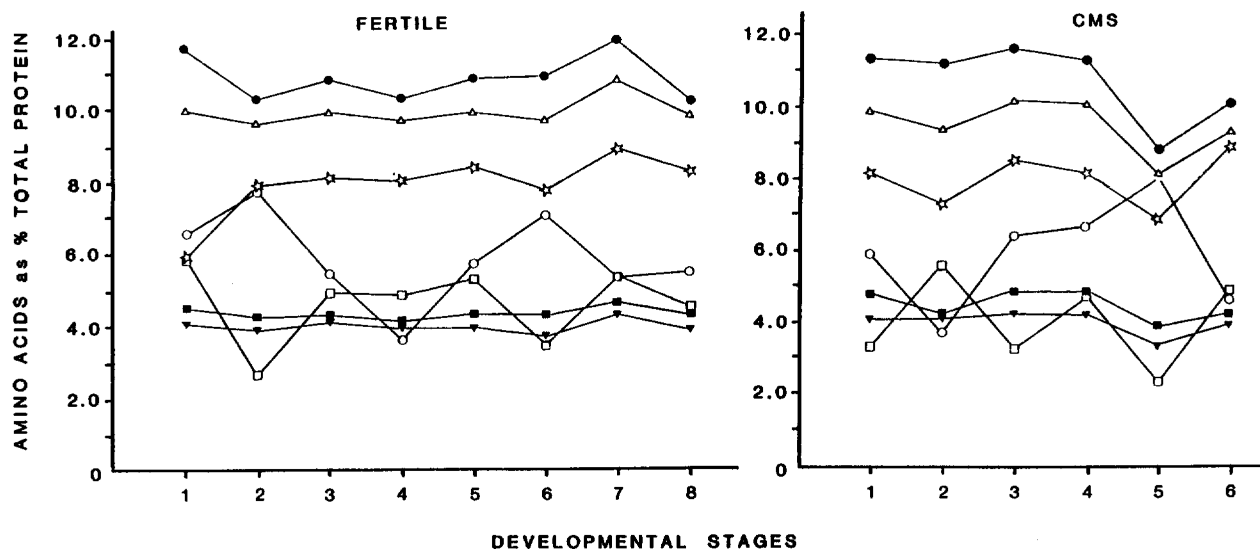

Fig. 3. GC analysis of representative amino acids from the protein hydrolysate of anthers from fertile and CMS lines of *Petunia*. Other amino acids showed no differences between fertile and sterile lines over the stages (Table 1) surveyed. ● = Glu, △ = Asp, ✧ = Leu, ○ = Ala, □ = Lys, ▼ = Pro, ■ = Thr

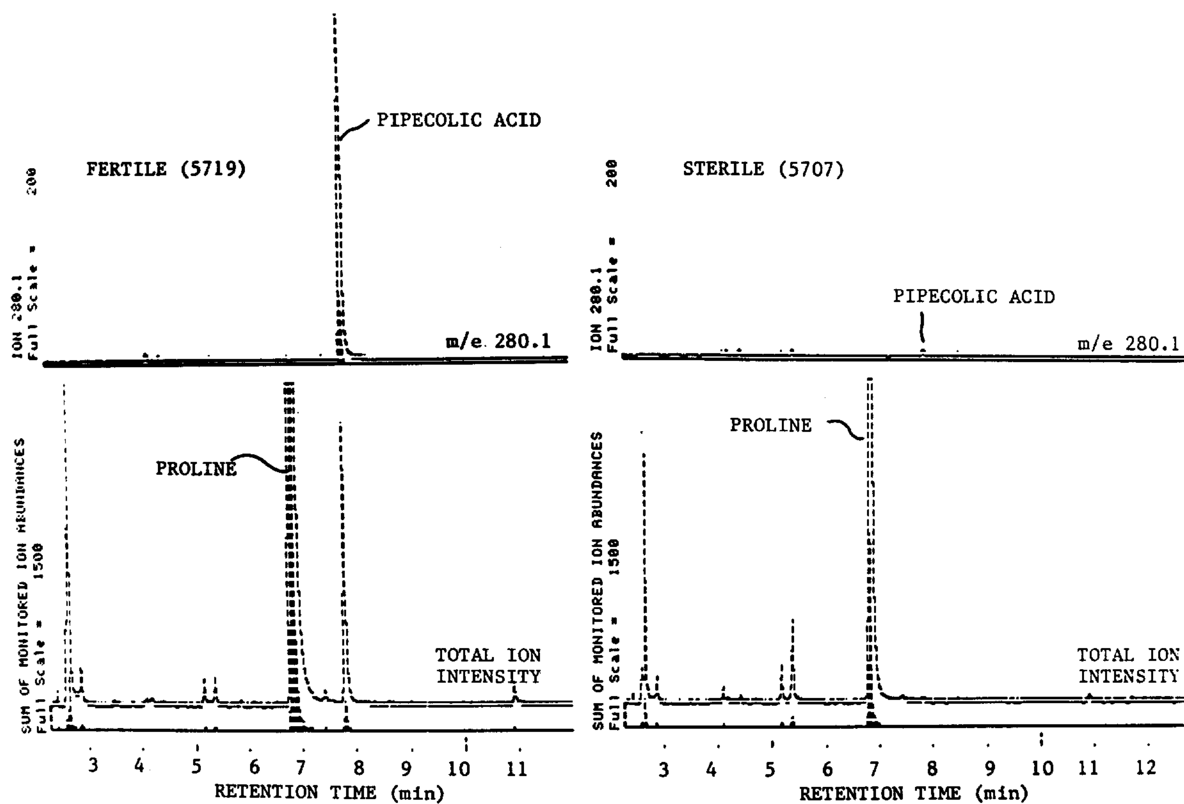

Fig. 4. GC-MS selected ion monitoring of N-heptafluorobutyryl isobutyl derivatives of free amino acids of mature anthers of fertile and CMS lines of *Petunia*. Total ion intensities (*lower panels*) and the selected ion 280.1 (*upper panels*) are shown

Table 2. Free amino acid composition of representative stages of *Petunia* anthers from fertile and cytoplasmic male sterile lines

Amino acid	Stage 1		Stage 4		Anthesis			
	nmoles/anther						% Total	
	CMS	F	CMS	F	CMS	F	*	**
Alanine	0.28	0.22	0.77	2.66	5.63	2.83	4.00	8.75
Glycine	0.30	0.10	0.28	0.71	1.01	1.65	2.34	0.74
Valine	0.13	0.04	0.22	2.84	1.90	0.55	0.78	1.70
Threonine	0.10	0.06	0.23	1.84	1.43	0.46	0.65	7.75
Serine	0.44	0.14	0.72	3.12	3.56	1.49	2.11	3.39
Leucine	0.05	0.05	0.16	0.75	1.08	0.47	0.66	0.23
Isoleucine	0.05	0.04	0.14	1.42	1.29	0.32	0.45	0.46
GABA	0.02	0.11	0.04	0.29	0.22	0.18	0.25	0.00
Proline	0.27	0.32	2.15	34.72	26.10	39.26	55.57	54.90
Methionine	0.05	0.05	0.08	2.14	0.37	1.92	2.72	0.36
ASN+ASP	0.37	0.22	1.65	10.66	5.17	4.50	6.37	6.24
Phenylalanine	0.03	0.02	0.09	1.26	0.92	0.39	0.55	0.13
GLN+GLU	1.36	0.80	8.98	9.00	11.82	7.60	10.76	11.11
Lysine	0.02	0.04	0.07	0.18	0.32	0.49	0.69	0.77
Histidine	0.03	0.06	0.10	0.90	0.24	0.58	0.82	1.23
Pipecolic Acid	0.05	0.21	0.11	6.60	0.60	6.90	9.77	0.00
Tyrosine	0.02	0.09	0.11	0.69	0.88	0.61	0.86	0.13
Arginine	0.11	0.18	0.40	0.70	0.23	0.44	0.62	2.00
Totals	3.68	2.75	16.30	80.48	62.77	70.64	99.97	99.89

* Calculated from the preceding column
** Calculated from Zhang et al. 1982

more pronounced in the later stages of pollen development. Through time, more proteins are seen in the fertile anthers, and it is likely that these are pollen-specific proteins. Figure 2 shows a preferential loss of low molecular weight proteins in anthers of the CMS line as development proceeds. No new proteins which might represent viral capsid material are observed.

The proteins of the stigmas from flowers of the fertile and CMS lines were extracted and analyzed in SDS-PAGE (data not shown). No differences in protein patterns are evident over those stages that parallel the development of the anthers.

Amino acid analysis of TCA precipitable proteins in Petunia anthers

When *Petunia* anthers are precipitated with TCA and analyzed for amino acid composition, most amino acid levels, including that of proline, are quite constant through development in both fertile and CMS lines (Fig. 3). Those fluctuations seen in alanine and lysine compensate one another and are ascribed to experimental variability.

Comparison of free amino acids in anthers of the fertile and CMS lines

Analysis of free amino acids from representative developmental stages of anthers from fertile and CMS lines is shown in Table 2. At premeiosis, free amino acid content is comparable in the fertile and sterile lines. As development proceeds, the amount of free amino acid found in sterile anthers is slightly less than that seen in fertile anthers. The most striking differences are in amounts of the imino acids, proline and pipecolic acid, where levels are 20 and 70 fold higher, respectively, in fertile anthers at the beginning of microgametogenesis, and 2–3 and 10–20 fold higher, at anthesis. These differences in proline and pipecolic acid levels were confirmed by electron impact GC-MS selected ion monitoring of ions 266.1 and 280.1 (Fig. 4).

Discussion

The only morphological expression of CMS in *Petunia* involves the development of a less plump, less colorful anther in the CMS flower. Quantitative and qualitative changes in proteins during abortive microspore development are observed. At the beginning of meiosis, anthers of the CMS and fertile lines are similar in total protein contents, but SDS-PAGE reveals a clear difference in protein patterns. Fertile anthers are enriched in three polypeptides with molecular weights of 64,000, 63,000 and 45,000 d. Anthers of the CMS line lack the 63,000 and 45,000 d proteins but are enriched in two polypeptides with molecular weights of 180,000 and 66,000 d. These two polypeptides are present in fertile anthers but at lower levels.

After microsporogenesis, protein content (Fig. 1) of sterile anthers changes little in comparison to fertile anthers of *Petunia*. Figure 2 shows that protein patterns of CMS material vary little during microgametogenesis except in the later stages when there is a preferential loss of low molecular weight polypeptides. In contrast, the protein patterns of anthers from the fertile line change constantly, and at pollen maturity, are significantly different from premeiotic patterns. 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 abortive microspores.

The graft transmissability of CMS in *Petunia* has led some investigators to implicate a virus (or virus-like entity) as the causal agent (Edwardson and Corbett 1961). The failure of CMS anthers to increase in protein content and to complete development could suggest the existence of such an agent. Numerous mechanisms could be postulated, but a simple explanation might involve the insertion of the virus into the genome of the host plant in a position which would prevent normal protein synthesis. It is well known that virulent animal viruses shut off host cell protein synthesis upon infection (Wu and Lucas-Lenard 1980); and it is possible that a similar mechanism might be operating in plants. Unfortunately, efforts to find any nucleic acid of a virus or virus-like agent in the CMS line of *Petunia* have not been successful (unpublished data). The complete absence of novel proteins from anthers of the CMS line during microsporogenesis or microgametogenesis suggests that any possible viral agent does not take over the cellular machinery of the host for active synthesis of its own proteins. The lack of discernible viral symptoms and the absence of proteins for encapsidation makes it rather unlikely that the graft transmissible agent is a classical plant virus. We acknowledge the possibility that a graft transmissible virus-like entity may cause CMS in *Petunia,* but at this time we find no evidence to support such a hypothesis.

Analysis of amino acid composition of protein hydrolysates of anthers of both fertile and CMS lines show that there is very little difference between them. Analysis of free amino acids in *Petunia* anthers, indicates marked differences in amounts of the imino acids, proline and pipecolic acid, between fertile and CMS lines. In general our free amino acid data agrees with that reported by Zhang et al. (1982) for fertile *Petunia* pollen (Table 2). However, we note that their threonine values exceed ours, and they do not report on pipecolic acid content. The increase in proline found in the free amino acid pool of fertile *Petunia* anthers is common to many other plant species (Stanley and Linskens 1974). Proline represents about 50% of the amino acid pool of *Petunia* pollen (Table 2), and it is utilized for protein synthesis during pollen germination (Zhang et al. 1982). Proline may also function as a solute protectant in developing pollen (Zhang and Croes 1983) preserving the grain against dessication or other unfavorable environmental conditions during dispersal and prior to germination. It may be that pipecolic acid serves a similar function.

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