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Mechanism of Male Sterility in *Petunia*

II. Free Amino Acids in Male Fertile and Male Sterile Anthers during Microsporogenesis [1]

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Summary. The free amino acid contents in the anthers of male fertile, cytoplasmic male sterile (*cms*) and genic male sterile (*gms*) petunia lines were compared at different developmental stages of the male gametophyte. Quantitative differences in the amounts of free amino acids were found between the fertile and male sterile lines and between the *cms* and *gms* lines. The differences between the sterile lines were correlated with the different developmental stages at which the breakdown in microsporogenesis occurred. In the Rosy Morn (*RM*) *cms* line, where breakdown of microsporogenesis occurred at the end of prophase 1, there was an associated increase in asparagine and decrease in the other amino acids. In the *RM gms* line, in which breakdown occurred at the tetrad stage, an accumulation of asparagine in the anthers corresponded with an accumulation of glutamine beginning at prophase 1. Compared with fertile anthers, the sterile anthers accumulated much proline at the early meiotic stages, but no γ-aminobutyric acid. Comparison of the free amino acids of the fertile and the male sterile lines indicates that certain biochemical events leading to breakdown of microsporogenesis precede the observed cytological breakdown. The results from adding asparagine and glutamine to extracts of anthers at different developmental stages suggest that the amino acid balance may contribute to the changes in pH in the fertile and male sterile anthers which we observed previously.

Introduction

Several comparative studies of the free amino acids in the anthers of male fertile and male sterile plants have been carried out to find whether a specific difference in a certain amino acid might play a part in the sterility phenomenon. Most of these studies showed a characteristic decrease or lack of proline in sterile anthers, compared with fertile anthers, e.g. Fukasawa (1954) using wheat and maize, Khoo and Stinson (1957) with maize, Ozaki and Tai (1961) in rice, and Briticov, Musatova, Vladimirtseva and Protsenko (1964) in poppy and lily. Abnormal accumulation of alanine was reported by Khoo and Stinson (1957) in male sterile maize anthers. Brooks (1962) showed an abnormal increase of glycine in male sterile anthers of sorghum and Fukasawa (1962) showed an abnormal increase of amides in male sterile wheat anthers. Differences in the composition of total amino acids (both free and contained in proteins) between sterile and fertile corn anthers were demonstrated by Sarvella, Stojanovic and Grogan (1967), who also reported that fertility was occasionally restored by injecting proline into male sterile corn stalks.

The studies cited above reported on male sterility resulting from a postmeiotic breakdown in microsporogenesis, but in our petunia material the breakdown occurred during meiosis. Sterility was complete, i.e., no pollen grains were produced or microspores released from the tetrads (see Izhar and Fran kel, 1971).

The present study was undertaken with the following primary objectives:

1. To find whether there are differences in the free amino acids between fertile and male sterile petunia anthers and whether such differences could be correlated with the breakdown of microsporogenesis in the sterile anthers.

2. To examine the possibility that differences in free amino acids between the fertile and the sterile anthers, and between the *cms* and *gms* anthers, may play a part in specific changes in the *pH* and the activity of a phase-specific enzyme (callase) in the anthers. The latter phenomena had been found to be closely associated with the breakdown of microsporogenesis in the sterile anthers (see Izhar and Frankel, 1971).

Materials and Methods

Plant material — We used the following *Petunia hybrida* Hort. Vilm. lines:

1. 'Rosy Morn' (*RM*) — line 434. A normal male fertile line.

2. *RM* cytoplasmic male sterile (*cms*) — line 450. Both cytoplasm and genome are of *RM* origin.

3. *RM* genic male sterile (*gms*) — line 451-S. Sterility is monogenic recessive. These three lines can be considered isogenic apart from the sterility factor (Frankel, 1970; Izhar and Frankel, 1971).

Methods — The plants were grown in the greenhouse during summer and winter. In the summer, temperatures were about 25°–30 °C during the day and 15–20 °C during the night. In the winter, the temperature in the greenhouse was controlled by central heating and kept at

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25 °C during the day and 17 °C during the night. The plants were exposed to 16 hours' day length supplemented, as required, by artificial light in both summer and winter.

Collection of anthers and determination of the developmental stage – Floral buds of different sizes were collected on ice. Since the five anthers of each bud were found to develop in synchrony (Izhar and Frankel, 1971), the developmental stage of each bud was determined by an orcein-stained squash of a single anther of that bud.

Extraction of amino acids – Extraction was carried out in three ways:

1. Fresh anthers were squeezed on a Whatmann No. 3 filter paper and immediately dried with forced dry air.

2. The anthers were dried in an oven at a temperature of 65 °C for 24 hours and extracted in cold 80% ethanol.

3. The fresh anthers were extracted in cold 80% ethanol. The three methods yielded similar results, so were used interchangeably according to convenience.

Chromatography – Both one- and two-dimensional ascending paper chromatography were used to separate the free amino acids. Whatmann No. 3 paper (46 × 27 cm) was used. The solvent system for the two dimensions was 2-butanol: 3% ammonium hydroxide (75:30) for the first direction, and 2-butanol: formic acid: H_2O (75:15:10) for the second. Other solvent systems were tried but were not as efficient. For the one-dimension separation, the second solvent was used.

The dried papers were dipped in 0.25% ninhydrin in acetone. The quantitative evaluation of the spots on the one- and two- dimensional papers was carried out using a "Photovolt" densitometer according to Redfield (1953). The set of free amino acids for each chromatogram is referred to in this paper as a profile of free amino acids.

Titration of glutamine and asparagine – Anthers at known developmental stages were squeezed into a set volume of glass-distilled water or a saturated *KCl* solution. Glutamine or asparagine (anhydrous preparations from several sources) was added and the pH changes of the extract were recorded.

Results

Anthers for the amino acids analysis were collected and assayed in each month from August 1970 to July 1971. Fertility in the fertile lines and male sterility in the sterile lines were consistent throughout the year, whereas partially sterile lines, which were grown concurrently in the greenhouse, showed sterility in summer and partial fertility in the autumn, winter and spring. Samples of material from *RM* fertile, *RM gms* and *RM cms* were collected and tested each month during one year. The profiles of free amino acids were found to be similar for each type regardless of the sampling date. Additional genotypes and plasmatypes yielded free amino acids profiles according to their developmental pattern e.g. a *RM* genic male fertile line (heterozygous for the sterility allele) behaved similarly to *RM* fertile. A Ewart *cms* line (cytoplasm from a source other than *RM* but with *RM* genome and a breakdown time as in *RM cms*) gave free amino acids profiles similar to those of the *RM cms*.

Table 1 presents the data on all the amino acids which appeared in measurable quantities in the *RM*

Table 1. *Free amino acids content in fertile and sterile anthers at different developmental stages of the male gametophyte. Each figure in the table when multiplied by* $\times 10^8$ *gives the quantity of a certain amino acid in moles per 10 anthers.* (+) (++) (+++) *means increasing quantities of proline, lysine and histidine, and γ-aminobutyric acid. Different letters* (*a*) (*b*) *means significant difference (by t-test) in quantities of a certain amino acid between lines at a certain developmental stage. Each figure is a mean of more than 12 sampling dates and at least three chromatography runs each time*

Developmental stages	Plant material (lines)	Cystine	Asparagine	Glutamine	Aspartic acid	Serine	Glutamic acid	Threonine	Alanine	Lysine & histidine	Proline	γ-aminobutyric acid
Premeiosis	*RM* fertile	.76	7.00(a)	1.23	.04	.60	2.71	.16	.70	++	+	++
	RM gms	.56	2.24(b)	2.86	.05	.68	1.49	.15	1.85	+	+	+
	RM cms	.64	3.95(b)	.80	.03	.42	1.52	.13	2.72	+	++	+
Prophase 1	*RM fertile*	1.78(a)	9.04(a)	1.47(a)	.05	.67	3.94(a)	.26	1.86(a)	+	+	+++
	RM gms	1.30(a)	7.44(a)	8.40(b)	.07	1.11	3.97(a)	.30	4.33(b)	+	+++	+
	RM cms	.38(b)	11.35(b)	1.53(a)	.04	.66	1.39(b)	.09	1.96(a)	+	++	+
Metaphase 1	*RM fertile*	3.29(a)	14.25(a)	3.51(a)	.09	1.40	4.26(a)	.35	2.05(a)	+	+	+++
	RM gms	1.72(a)	10.97(a)	10.60(b)	.08	1.53	2.71(a)	.23	5.49(b)	+	+++	+
	*RM cms**	.61(b)	30.27(b)	3.28(a)	.12	1.56	1.35(b)	.10	1.85(a)	+	++	+
Tetrads	*RM fertile*	2.01(a)	6.81(a)	1.36(a)	.15	1.55	6.79(a)	.47	6.17(a)	+	++	+
	RM gms	.57(b)	13.24(b)	10.94(b)	.11	1.37	2.20(b)	.12	6.73(a)	+	+++	+
	*RM cms***	.57(b)	33.30(c)	2.46(a)	.12	1.04	1.35(b)	.07	1.68(b)	+	+++	+
Tetrads releasing microspores	*RM fertile*	1.30(a)	6.43(a)	2.10(a)	.21	3.72	6.11(a)	.44	5.05	+	+++	+
	*RM gms****	.41(b)	27.92(b)	12.47(b)	.14	1.71	2.24(b)	.14	4.38	+	+++	+
Young microspores	*RM fertile*	1.38	10.97	7.18	.81	7.13	10.87	.81	3.59	+++	+++	+++

* Stage in which first signs of cytological breakdown are evident.
** Stage in which a total collapse of the tissue is evident.
*** There is no release of microspores from tetrads; tissue is broken down.

fertile, *RM cms* and *RM gms* anthers. The following amino acids, which appeared in small amounts and with no detectable differences in all the lines, were not measured quantitatively: tyrosine, valine, phenylalanine, isoleucine and leucine. Lysine and histidine were present in small amounts and their appearance was not consistent, except in the fertile anthers at the young microspore stage when they appeared in relatively large quantities. Proline was found in relatively small quantities in the fertile anthers during meiosis; at the beginning of the tetrad stage, proline increased considerably. In comparison with the fertile anthers, large amounts of proline appear at an early stage in the *RM gms* and in lesser amounts in the *RM cms*. The detection of both proline and γ-aminobutyric acid was somewhat difficult because both run close to alanine and their appearance was less consistent than that of the other amino acids.

Fig. 1 describes the accumulation of the free amino acids which appeared consistently in large quantities and whose contents in the fertile anthers changed during microsporogenesis. The progress of asparagine and glutamine during microsporogenesis in the *RM* fertile was similar: they both had peaks at metaphase 1 and a strong increase at the young microspore stage. Cystine and alanine reached peaks at the metaphase 1 and tetrad stage, respectively, and decreased after meiosis was completed. Glutamic acid content increased steadily except for a slight drop at the tetrads-releasing microspores stage.

In *RM gms*, in contrast to the *RM* fertile, there was a steady increase in asparagine and glutamine during meiosis. After signs of breakdown in microsporogenesis became evident, asparagine increased markedly while glutamine did not. Alanine appearance in *RM gms* was similar to that in *RM* fertile, but its level during meiosis was significantly higher. Glutamic acid started to decrease in prophase 1 in the *RM gms* and becomes even lower than in the *RM* fertile.

In the *RM cms*, cystine, glutamic acid and alanine were lower than in the other two lines at the start of prophase 1. A small change in glutamine was observed in the *RM cms*. The marked increase in asparagine was associated with early signs of breakdown of microsporogenesis at metaphase 1. It can be seen in fig. 1 that different profiles of free amino acids are associated with normal microsporogenesis in the *RM* fertile, and abnormal microsporogenesis in the *RM gms* and the *RM cms*.

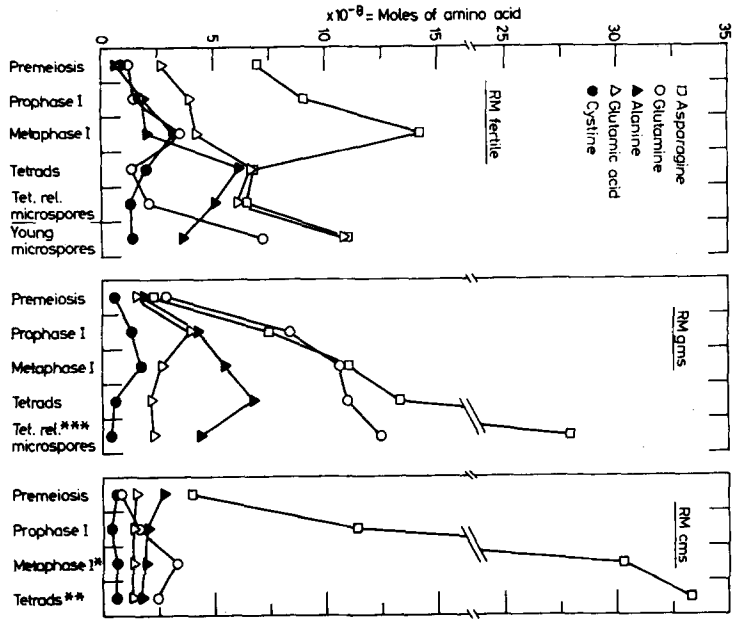

Fig. 1. Accumulation of free amino acids in anthers of *RM* fertile, *RM gms* and *RM cms* at different developmental stages of the male gametophyte, *, **, *** are stages of sterile anthers equivalent in size to the *RM* fertile (see footnotes to table 1)

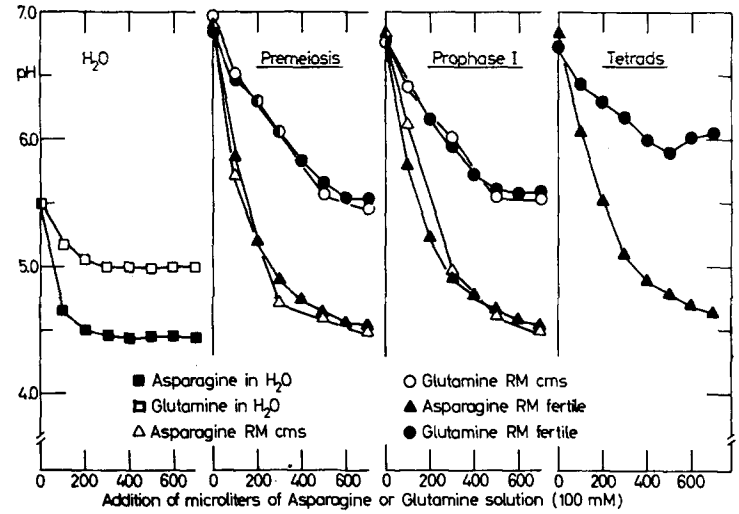

Fig. 2. Titration of asparagine and glutamine into extracts of anthers of *RM* fertile and *RM cms* at different developmental stages. Titration is in set volumes of 100 μl. of 100 mM of asparagine or glutamine

The results of the free amino acids analysis indicate (fig. 1) that asparagine is associated with changes in the *pH* of the anther locule in the male sterile lines (concerning *pH* changes, see Izhar and Frankel, 1971). In fertile anthers the *pH* normally drops at the tetrads-releasing microspores stage. In the *RM gms* and the *RM cms*, *pH* drop precedes the first signs of cytological breakdown of the sporogenic tissue. On the basis of this correlation between aspa-

ragine and the *pH* change, we titrated asparagine into extracts of anthers at different developmental stages and measured the effect on the *pH*. We also titrated glutamine, which appeared in large amounts in the *RM gms* (fig. 1).

Fig. 2 presents the titration curves of asparagine and glutamine into extracts of *RM* fertile and *RM cms* anthers. Asparagine considerably lowered the *pH* of the extract: the addition of 600—700 µl. of 100 mM asparagine solution brought the *pH* level to that of the original asparagine solution. Compared with asparagine, glutamine was better buffered by the extracts: addition of 600—700 µl. of 100 mM glutamine (a) brought the *pH* of the extracts to equilibrium at premeiosis and prophase 1 of both *RM* fertile and *RM cms* and (b) caused a rise in the *pH* of the metaphase 1 (not seen in the fig.) and tetrads extracts of the *RM* fertile. The first 100 µl. addition of asparagine is of the same order of magnitude as the quantity of asparagine in the *RM cms* at the metaphase 1 stage.

Discussion

The first two observations described in the results section suggest that the free amino acids profile of anthers at a certain developmental stage is independent of changes in environmental conditions and of certain genome and plasmon influences, in both fertile and male sterile phenotypes. In other words, the data indicate that certain developmental patterns, normal or abnormal, are associated with a specific profile of free amino acids at each developmental stage regardless of the growing conditions or the general genetic background. These findings permit us to consider free amino acids analysis as a useful tool for studying alterations in metabolic pathways associated with sterility.

As far as the free amino acids are concerned, premeiosis is a more or less equal starting point for all the lines, although there is more asparagine, lysine and histidine, and the first signs of γ-aminobutyric acid, in the *RM* fertile compared with the *RM gms* and *RM cms* anthers. The interesting phenomenon at this stage is the lack of γ-aminobutyric acid in the sterile lines which later is coupled with high proline content. On the other hand, proline appears in low quantities in the *RM* fertile anthers during meiosis and increases at the tetrad stage when γ-aminobutyric acid decreases. The relationship between these two acids should be further examined in the light of the fact that γ-aminobutyric acid does not enter protein (McKee, 1962) and that protein synthesis in anthers slows down during meiosis (Sauter, 1971).

Changes in the amounts of some of the free amino acids in the *RM* fertile are evident, starting with prophase 1 (these acids are described in fig. 1). A different tendency is observed in the sterile lines: in *RM cms*, asparagine increases at the expense of the other acids (with the exception of glutamine); in *RM gms*, asparagine and particularly glutamine rise to levels far above the other amino acids. Thus, the data show that asparagine accumulation is associated with the breakdown of microsporogenesis in sterile anthers. The fact that glutamine does not rise after breakdown in the *RM gms* fits the general view that glutamine, unlike asparagine, is not directly associated with breakdown of the tissue (McKee, 1962).

The results obtained by titrating asparagine and glutamine into anther extracts indicate that asparagine itself may account for the *pH* drop in the anther locule which is associated with callase activity and the breakdown of microsporogenesis (see Izhar and Frankel, 1971). The titration curves for glutamine may explain the fact that its accumulation in the *RM gms* does not cause a drop in the *pH*. On the other hand, the drop in *pH* in the *RM* fertile anthers at the tetrads-releasing microspores stage could not be explained by the slight rise in asparagine at this stage but perhaps by a rise in glutamic acid (pI 3.08) and other factors.

The balance of free amino acids in anthers is apparently subject to the action of several factors, such as protein breakdown and synthesis, specific amino acid breakdown and synthesis, and translocation into and out of the anther. Our data do not supply enough evidence to determine which of the above factors dominate in the fertile or the sterile anthers but provide hints as to the exact timing of the primary events leading to sterility. The data may also explain the phenomenon of *pH* drop in the anther, which is associated with the activity of a phase-specific enzyme (callase) and the breakdown of microsporogenesis (Izhar and Frankel, 1971). However, the possibility that the amino acids may influence the pH together with other factors is left open.

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