Theor Appl Genet (1992) 84:92–96

THEORETICAL AND APPLIED GENETICS

Differences in amino acid transport in isonuclear lines of cytoplasmic male-sterile and male-fertile petunia

M. Perl, Dvora Swartzberg, and S. Izhar**

Department of Plant Genetics, ARO, The Volcani Center, Bet Dagan 50250, Israel

Received September 9, 1991; Accepted October 9, 1991
Communicated by H. F. Linskens

Summary. Two pairs of isonuclear lines of cytoplasmic male-sterile (CMS) and fertile (F) petunia cells grown in suspension culture in the presence or absence of amino acid sources were examined for uptake of 11 amino acids and adenosine. Cells from CMS lines exhibited a significant lower rate of uptake than F cells. These differences, for various amino acids, are a result of lower affinity (high Km) values and of lower maximal velocities. Although the uptake of most of the amino acids examined was affected by the availability of energy in the cell, the differences in uptake seem to be less dependent on the energy status of the cell.

Key words: Amino acids – Cytoplasmic male sterility – Petunia – Transport

Introduction

Cytoplasmic male sterility (CMS), a maternally inherited trait, leads to the abortion of the pollen mother cells during gametogenesis and microsporogenesis.

The alterations in the mitochondrial genome and the interaction with nuclear genomes, as well as the functional biochemistry of the CMS phenomenon, have been investigated extensively (see Hanson and Conde 1985). The main biochemical changes occurring in anthers that characterize CMS lines may be divided into three groups: (1) the accumulation or deficiency of metabolites in floral organs (amino acids: see Pearson 1981; pyrimidins: Saini and Davis 1969; polyamines: Rastogi and Sawhney 1990; hydroxycinnamic acids: Tanguy et al. 1982); (2) enzyme activities (callase: Izhar and Frankel 1971; RNase: Mariani et al. 1990; adenine-phosphoribosyl-transferase: Regan and Mofatt 1990); and (3) mitochondrial oxidation activities, ATP production and ADP/ATP translocation (Musgrave et al. 1986; Connett and Hanson 1990; Glab et al. 1990; see also Kaul 1988).

The biochemical activities of anthers that characterize CMS plants may be a result of impaired membrane structure and function (Bino 1985; Evenor 1988), followed by inefficient transport abilities, with the result finally being an imbalance in nutrient concentrations. A disturbed nutrient balance in highly active tissue (reproductive organs) may lead to metabolic deficiences and finally to incomplete development of the tissue.

In this article we describe studies on the uptake of amino acids by cell cultures of CMS and fertile (F) lines.

Materials and methods

Cell suspension cultures of petunia (*Petunia hybrida*) lines 3699 and 3704 (F cells) and 4544 and 11727 (CMS cells) were generated by growing surface-sterilized stem segments in liquid UM1A medium as described by Connett and Hanson (1990). Cells were also cultured on media in the absence of casein hydrolyzate (9S).

For uptake, release and incorporation measurements, the cells were collected on a large glass fiber filter (47 mm) under moderate suction. About 10^4 cells per mg/wet weight were found in both CMS and F cell cultures.

About 100 mg [wet weight (ww)] cells were transferred to glass vials containing 1 ml 9S medium (UM1A medium without casein hydrolyzate), 100 μM single amino acids (10^5 CPM/ml, if not otherwise stated) or ^{14}C-uniformly labeled amino acid, and other additions as indicated in the Results. The cells were incubated on a gyrotory-shaker at 150 rpm at 25 °C for the indicated time periods. They were then collected on a glass fiber filter and washed 3 times with 3 ml cold water. The cells were divided into two portions and weighed: one portion was counted for radioactivity, and the other portion was transferred to 3 ml cold 5% trichloroacetic acid. After at least 30 min on ice, the acid-insoluble fraction was collected on a glass fiber filter, washed 3 times with 3 ml cold acid and counted for radioactivity (amino acid incorporation). In release experiments, the cells were loaded

* Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. No. 3351-E, 1991 series

** Correspondence to: M. Perl

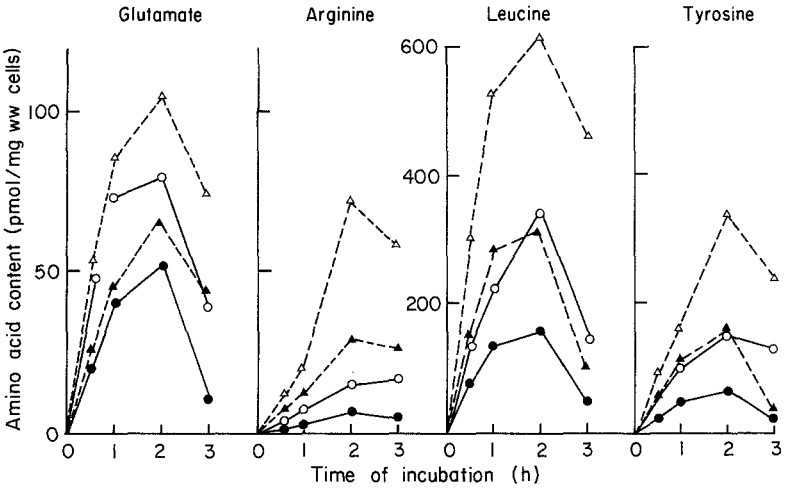

Fig. 1. The accumulation and release of amino acids in CMS and F cells (11727 and 3740) grown on UM media. CMS (●; ▲) and F (○; △) cells were incubated with 100 μ*M* radioactive amino acids (2×10^5 CPM/ml). At the indicated times, cells were removed and examined for total and acid-insoluble radiactivity. After 2 h the last portion of cells was washed, resuspended in 9S medium, and re-incubated for 1 h; then total and acid-insoluble radioactivity was quantitated. For details, see the Methods section. The acid-soluble concentrations were calculated from the data (●, ○ acid-insoluble; ▲, △ total radioactivity)

Table 1. The accumulation of amino acids and adenosine in the acid-insoluble and acid-soluble fractions of CMS and F cells grown on UM media. Cells were incubated with the indicated ^{14}C-amino acids or adenosine (100 μ*M*) for 1 h. The radioactivity accumulated in the cells and in the acid-insoluble fraction was quantitated as described under Methods. The radioactivity in the acid-soluble fraction was calculated. The data are from four experiments (SD in parenthesis) for lines 11727 and 3704 and the average of two experiments for lines 4544 and 3699

	Radioactivity accumulated (pmol.mg^{-1} cells)											
	Acid-insoluble						Acid-soluble					
	11727 (CMS)	3704 (F)	CMS/F (%)	4544 (CMS)	3699 (F)	CMS/F (%)	11727	3704	CMS/F (%)	4544	3699	CMS/F (%)
Glutamic ac.	30 (13)	62 (21)	48	18	22	82	9 (5)	27 (11)	33	7	48	15
Arginine	9 (5)	20 (12)	45	61	400	15	20 (4)	73 (18)	27	15	122	12
Proline	14 (6)	108 (15)	13	28	161	17	129 (11)	62 (18)	47	111	750	15
Leucine	148 (42)	387 (63)	38	286	464	62	140 (38)	318 (40)	44	164	290	57
Phenylalanine	103 (40)	300 (90)	34	392	673	58	69 (30)	278 (82)	25	132	756	17
Tyrosine	62 (24)	200 (75)	31	185	276	67	94 (50)	278 (72)	34	120	415	29
Aspartic ac.	16 (4)	39 (4)	41	89	110	81	9 (4)	8 (2)	113	74	120	62
Alanine	52 (12)	112 (4)	46	87	88	100	11 (3)	30 (5)	37	25	53	47
Lysine	70 (11)	110 (8)	64	132	214	62	86 (30)	142 (16)	61	88	204	43
Valine	47 (8)	161 (12)	29	99	419	24	32 (4)	83 (9)	39	25	41	61
Histidine	27 (8)	61 (14)	44	43	150	29	13 (4)	23 (8)	57	19	103	18
Adenosine	72 (6)	75 (29)	96	731	666	110	25 (4)	35 (11)	71	711	770	92

with labeled amino acids for 2 h, collected on a filter and washed as described above. The washed cells were resuspended in 9S medium: one portion was treated for amino acid content and incorporation immediately, and the other portion was treated after 1 h incubation on the shaker (as described above). For metabolite tests, labeled cells were collected, washed with water and extracted with cold 5% perchloric acid. The soluble fraction was neutralized with KOH, and the insoluble salt was eliminated by centrifugation.

The supernatant was subjected to thin layer chromatography (butanol:ethanol: H_2O; 4:4:2). Non-labeled amino acids were co-chromatographed, and the amino acids were visualized by ninhydrine. The plates were divided into 1-cm segments, and the gel was scraped off and counted for radioactivity.

Results

The uptake of some amino acids in CMS cells incubated in 9S medium containing 100 μ*M* of amino acid was 2–3 times lower than in F cells (Table 1 and Fig. 1). These differences were reduced when the amino acid concentrations were increased to 10 m*M* (Table 2). At 100 μ*M*, the accumulation of the amino acids in the acid-insoluble fraction did not differ significantly between CMS and F cells in at least half of the examined substrates. In the acid-soluble fraction the accumulation was significantly higher in F cells for most of the amino acids studied. This

indicated that the differences in uptake were not the result of the cell's energy status, a possibility which was examined in a more direct experiment: the relation between the depletion of amino acid incorporation (which is an energy-dependent process) to amino acid accumulation in the acid-soluble fraction under a partial inhibition of the oxidative-phosphorylation-energy-providing system by 2 m*M* KCN. Table 3 presents data on total amino acid accumulation in CMS and F cells and their partition (percentage) in the acid-soluble and acid-insoluble fractions. The incorporation rate into the acid-insoluble fraction reflects the energy status of the cells. Thus, the ratio between the inhibition by KCN in the accumulation of amino acid (in acid-soluble fraction) to the inhibition of amino acid incorporation (the acid-insoluble fraction) may indicate the level of dependence of the uptake on the energy availability in the cell. A ratio of 1 or higher would indicate an energy-dependent change in uptake. The data in Table 3 shows that the uptake of most of the amino acids studie was partially affected by the energy status of the cell, and to an equal degree in both CMS and F cells. Thus, the differences in uptake between CMS and F cells seem not to be a result of cell energy.

Table 2. Amino acid and adenosine accumulation in CMS (4544) and F (3699) cells cultured in UM media (in the presence of casein hydrolyzate) examined at 10 m*M* radioactive substrate concentration. For details, see Table 2. The data are averages of two experiments

	Radioactivity accumulated (nmol.mg^{-1} cells)					
	Acid-insoluble			Acid-soluble		
	CMS	F	CMS/F (%)	CMS	F	CMS/F (%)
Glutamic ac.	3.0	2.3	130	0.0	4.7	0
Arginine	1.0	0.9	115	0.7	1.2	58
Proline	1.4	1.6	88	2.1	7.9	27
Leucine	1.4	2.3	61	2.5	3.8	66
Phenylalanine	1.5	3.9	36	7.6	13.3	57
Tyrosine	2.1	1.9	110	1.7	3.4	50
Aspartic ac.	0.9	1.2	75	1.0	1.3	77
Alanine	2.2	3.3	67	0.9	1.1	82
Lysine	1.6	1.9	84	0.6	1.5	40
Valine	2.1	2.0	105	1.7	2.5	68
Histidine	1.1	0.6	180	0.5	0.4	125
Adensine	1.8	1.4	129	3.3	3.0	110

Comparisons of uptake in the presence of high and low amino acid concentrations may reveal possible differences between CMS and F cells in their affinity to the substrates as well as differences in the uptake velocities at maximal substrate concentrations. We used cells cultured on amino acid-deficient media (no casein hydrolyzate). Table 4 shows that when amino acid-defficient cells were used and the substrate concentration was 100 μ*M*, 5 amino acids and adenosine showed a significant increased uptake by F cells as compared with CMS cells, as expressed by high accumulation in the acid-soluble fractions.

In the acid-insoluble fraction no significant differences were noted, except for higher incorporation of glu-

Table 3. The effect of KCN (2 m*M*) on accumulation of amino acids and adenosine in the acid-soluble and acid-insoluble fractions of CMS (11727) and F (3704) cells grown on UM media. Cells were incubated with the indicated amino acids or adenosine (100 μ*M*) for 45 min in the presence or absence of 2 m*M* KCN. The total and the acid-insoluble accumulation of the radioactive substrates was examined as described under Methods. The acid-soluble percentage was calculated

	Cumulative radioactivity Total/acid-soluble (pmol.h^{-1} mg^{-1} cells)		Percent inhibition					
			Acid-soluble		Acid-insoluble		Acid-soluble/ Acid-insoluble	
	CMS	F	CMS	F	CMS	F	CMS	F
Proline	30/7	182/63						
Proline + KCN	8/3	61/20	58	68	78	65	0.74	1.05
Leucine	230/154	508/228						
Leucine + KCN	95/36	290/157	58	31	61	52	0.95	0.60
Phenylalanine	86/21	387/220						
Phenylalanine + KCN	67/14	249/159	18	28	33	46	0.55	0.67
Tyrosine	96/78	325/82						
Tyrosine + KCN	68/53	152/73	17	11	32	59	0.53	0.19
Alanine	74/16	133/112						
Alanine + KCN	16/10	31/25	89	78	38	45	2.3	1.7
Valine	83/30	250/194						
Valine + KCN	20/5	98/81	72	58	83	70	0.87	0.83
Adenosine	51/12	81/64						
Adenosine + KCN	32/8	50/41	38	36	33	47	1.2	0.77

Table 4. The accumulation of amino acids and adenosine in the acid-insoluble and acid-soluble fractions in CMS and F cells grown in the absence of casein hydrolyzate and examined at 100 μ*M* and 10 μ*M* substrate concentrations. Cells (CMS, 4544; F, 3699) cultured on media in the absence of casein hydrolyzate were examined for amino acid and adenosine accumulation as described under Methods, at 100 μ*M* (three experiments) and 10 m*M* (two experiments) of labeled substrate concentrations (SD in parentheses)

	Cumulative radioactivity											
	Acid-insoluble						Acid-soluble					
	At 10 m*M* (nmol.mg^{-1} cells)			At 100 μ*M* (pmol.mg^{-1} cells)			At 10 m*M* (nmol.mg^{-1} cells)			At 100 μ*M* (pmol.mg^{-1} cells)		
	CMS	F	CMS/F (%)	CMS	F	CMS/F (%)	CMS	F	CMS/F (%)	CMS	F	CMS/F (%)
Glutamic ac.	4.5	7.3	62	112 (8)	41 (11)	273	3.0	6.2	48	30 (5)	89 (18)	34
Arginine	1.7	1.0	170	212 (18)	204 (21)	104	5.0	4.1	122	220 (25)	408 (20)	54
Proline	2.5	1.4	179	341 (29)	170 (15)	200	11.0	9.0	122	363 (33)	272 (30)	133
Leucine	4.3	2.7	160	305 (20)	319 (37)	96	1.8	2.1	86	92 (21)	86 (12)	107
Phenylalanine	2.0	2.2	91	265 (25)	255 (16)	104	4.9	4.3	114	445 (37)	484 (48)	92
Tyrosine	0.8	0.6	133	235 (12)	188 (11)	125	4.3	1.4	307	105 (8)	302 (21)	35
Aspartic ac.	1.1	1.1	100	116 (15)	58 (8)	200	0.9	2.0	45	53 (12)	49 (11)	108
Alanine	2.0	1.9	105	182 (40)	169 (17)	108	4.0	3.9	103	32 (16)	164 (20)	195
Lysine	2.4	2.5	96	315 (27)	258 (25)	122	4.4	5.8	76	135 (15)	252 (22)	54
Valine	1.9	1.7	112	334 (17)	299 (26)	112	1.2	2.0	60	55 (5)	251 (24)	22
Histidine	0.9	0.6	150	128 (36)	102 (28)	125	0.7	0.7	100	274 (52)	252 (40)	109
Adenosine	3.2	3.0	96	136 (16)	175 (20)	78	1.5	2.4	63	205 (19)	465 (25)	44

tamate proline and aspartate in the CMS cells. This may be a result of metabolic changes of the amino acid and the incorporation of their products. The presence of high concentrations of amino acids during the experiment reduced the difference between CMS and F cells to 2 or 3 amino acids in the acid-soluble fraction. With the exception of glutamate, no differences were found in the acid-insoluble fractions. This experiment points to the possibility that at an amino-acid-deficient stage (high protein synthesis) the translocation of amino acids (as well as adenosine and other metabolites) may be impaired, resulting in degeneration processes.

Examination of half-maximal-velocity concentrations (apparent Km values) revealed that for 4 of the 6 amino acids examined, affinity values were higher for F cells than for CMC cells (Table 5) when both were grown on amino acid-rich media (UM).

The differences in the accumulation of amino acids, as measured, may result from metabolic processes that serve as a trapping system of their radioactive products. This possibility was excluded by evaluating the composition of the acid-soluble radioactive material extracted from cells after their incubation for uptake experiments with thin layer chromatography (TLC). From both CMS and F cells only alanine and proline showed 20–40% of their radioactivity on their marker spot on the TLC plate. The radioactivity of other amino acids was concentrated at 80–90% on the respective marker spot (data not shown). Adenosine was found to be changed, probably phosphorylated, to a similar extent in both CMS and F cells. It should be noted that during the incubation time of this experiment, no release of radioactive CO_2 could be detected from any of the amino acids examined.

Table 5. Apparent "Km" values for the total accumulation of amino acids and adenosine by CMS (11727) and F (3704) cells. Cells were grown on UM media and examined for the accumulation of radioactive substrate of various concentrations (0.2–3.0 m*M*) during a 30-min incubation. For details, see Methods

Substrate	Apparent "Km" values (m*M*)[a]	
	CMS	F
Proline	4.0	2.0
Leucine	1.3	1.6
Phenylalanine	4.0	2.5
Tyrosine	5.0	1.1
Alanine	0.7	2.0
Valine	2.0	0.6
Adenosine	1.0	1.2

[a] Data obtained from double-reciprocal Lineweaver-Burke plots

Discussion

The data presented indicate that CMS cells have a lower rate of amino acid uptake than F cells. The differences in the accumulation of some amino acids seem to be a result of greater affinities and higher velocities rather than of available energy. This points to possible changes in the cytosole membrane structure of the cell.

It is well known that flowering, a biochemically active stage, is affected by amino acids. Moreover, external glutamate has a strong flowering-promoting effect at very low concentrations, although this amino acid is found in the plant body in large quantities (Tanaka and Takimoto 1977). This leads to the conclusion that the natural effect of amino acids on flowering is regulated by their translocation abilities within the plant organs and plant cells, rather than by their biosynthesis. This conclusion is also supported by the hormone effect on the formation of buds (Conrad et al. 1986; Doonan et al. 1987; Traas 1990), probably as a result of the hormone influence on cytoskeleton functioning.

Likewise, photoperiodicity and temperature affect flowering, apparently through their effect on the membrane architecture resulting in nutrient transfer (Moss and Heslop-Harrison 1968; Tanaka and Takimoto 1977; Lichter 1978; Pearson 1981; Cabanne et al. 1981).

Male sterility is accompanied by a variety of biochemical phenomena, all of which may result from an altered membrane structure with the consequences being sequentially depleted membrane activities (Connett and Hanson 1990; Rastogi and Sawhney 1990) and impaired nutrient translocation (Liu et al. 1988), which leads to a disturbed amino acid balance (Wu and Murry 1985; see also Pearson 1981) and finally to detrimental metabolites of amino acids (Tanguy et al. 1982; Rastogy and Sawhney 1990).

Our data support the hypothesis that the alteration in the membrane caused by the CMS genome occurs in the whole plant but the physiological consequences are apparent only in metabolically highly active tissues under marginal stress of environmental and nutritional conditions. One of the main primary effects seems to be amino acid translocation triggering biochemical and physiological reactions related to imbalanced amino acid concentrations.

Since the transport system seems – for most amino acids – to be saturable, impaired uptake will be apparent only at relatively low concentrations (Table 4). This is supported by the fact that "local" application of external precursors may enhance fertility in CMS flowers (Saini and Davis 1969). This may also explain the ability of CMS plants to complete their vegetative growth and development, but their failure to develop metabolically highly active tissues when subjected to environmental and nutritive stress conditions.

References

Bino SR (1985) Ultrastructural aspects of cytoplasmic male sterility in *Petunia hybrida*. Protoplasma 127:230–240

Cabanne F, Dalebroux MA, Martin-Tanguy J, Martin C (1981) Hydrocinnamic acid amides and ripening to flower of *Nicotiana tabacum* var. *xanthi* c.c. Physiol Plant 53:399–404

Connett MB, Hanson MR (1990) Differential mitochondrial electron transport through the cyanide-sensitive and cyanide-insensitive pathways in isonuclear lines of cytoplasmic male sterile, male fertile and restored petunia. Plant Physiol 93:1634–1640

Conrad PA, Stencec GL, Hepler PK (1986) Bud formation in *Funaria:* organelle redistribution following cytokinin treatment. Protoplasma 131:211–223

Doonan JH, Cove DJ, Corke FMK, Lloyd CW (1987) Preprophase band of microtubes, absent from tip-growing moss filaments, arises in leafy shoots during transition to intercalary growth. Cell Motil Cytoskel 7:138–153

Evenor Dalia (1988) Studies on the genetics and ultrastructure of male sterility in petunia. Ph.D. thesis submitted to the Hebrew University, Israel

Glab N, Wise RP, Pring DR, Jacq C, Slonimski P (1990) Expression in *Saccharomyces cerevisiae* of a gene associated with cytoplasm male sterility from maize: Respiratory dysfunction and uncoupling of yeast mitochondria. Mol Gen Genet 223:24–32

Hanson MR, Conde MF (1985) Function and variation of cytoplasmic genomes: lessons from cytoplasmic-nuclear interactions affecting male fertility in plants. Int Rev Cytol 94:213–267

Izhar S, Frankel R (1971) Mechanism of male-sterility in *Petunia:* The relationship between pH, callase activity in the anthers, and the breakdown of the microsporogenesis. Theor Appl Genet 41:104–108

Kaul MLH (1988) Male sterility in higher plants. In: Monograph on theoretical and applied genetic, vol 10. Springer, Berlin Heidelberg New York, pp 221–232

Lichter R (1978) The restoration of male fertility in cytoplasmic male sterile sugar belt by heat treatment and mersitem culture. Z Pflanzenzücht 81:159–165

Liu XC, Jones K, Dickinson HG (1988) Cytoplasmic male sterility in *Petunia hybrida:* factors affecting mitochondrial ATP export in normal and cytoplasmically male sterile plants. Theor Appl Genet 76:305–310

Mariani C, De Beuckeleer M, Truettner J, Leemans J, Goldberg RB (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature 347:737–741

Moss GI, Heslop-Harrison J (1968) Photoperiod and pollen sterility in maize. Ann Bot 32:833–846

Musgrave ME, Antonovics J, Siedow JN (1986) Is male-sterility in plants related to lack of cyanide-resistant respiration in tissue? Plant Sci 44:7–11

Pearson DH (1981) Nature and mechanism of cytoplasmic male sterility in plants. HortScience. 16:482–487

Rastogi R, Sawhney VK (1990) Polyamines and flower development in the male sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum* Mill.). Plant Physiol 93:439–445; 446–452

Regan SM, Moffatt BA (1990) Cytochemical analysis of pollen development in wild-type *Arabidopsis* and male-sterile mutant. Plant Cell 2:277–280

Saini SS, Davis GN (1969) Male sterility in *Allium cepa* and some species hybrids. Econ Bot 23:37–49

Tanaka O, Takimoto A (1977) Flowering-promoting effect of some amino acids and amides in *Lemma pancicostata* 6746. Plant Cell Physiol 18:27–34

Tanguy JM, Perdrizet E, Prevost J, Martin C (1982) Hydroxycinnamic acid amides in fertile and CMS lines of maize. Phytochemistry 21:1939–1940

Traas JA (1990) The plasma membrane associated cytoskeleton. In: Larson C, Moller IM (eds) The plant plasma membrane: structure, function and molecular biology. Springer, Berlin Heidelberg New York pp 285–287

Wu FS, Murry LE (1985) Changes in protein and amino acid content during anther development in fertile and cytoplasmic male sterile *Petunia*. Theor Appl Genet 71:68–73