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Oxidation of Proline and Glutamate by Mitochondria of the Inflorescence of Voodoo Lily (*Sauromatum guttatum*)[1]

Hanna Skubatz*, Bastiaan J. D. Meeuse, and Arnold J. Bendich

Departments of Botany (H.S., B.J.D.M., A.J.B.) *and Genetics* (A.J.B.), *University of Washington, Seattle, Washington 98195*

ABSTRACT

In appendices of *Sauromatum guttatum* that are developing thermogenicity, mitochondria isolated from successive developmental stages of the inflorescence show an increase in the oxidation rates of proline and glutamate. A similar rise in the oxidation rates of these compounds is observed in mitochondria obtained from the spathe, a nonthermogenic organ of the inflorescence. Changes in oxidative metabolism were also observed in mitochondria isolated from sections of immature appendix treated with salicylic acid (SA) at 0.69 microgram per gram fresh weight indicating that they are induced by SA. At that concentration, however, SA has no effect on oxygen consumption by mitochondria in the presence of glutamate, proline, or malate. Furthermore, oxygen uptake by mitochondria in the presence of proline or glutamate is partially sensitive to salicylhydroxamic acid (SHAM) at concentrations greater than 2 millimolar when in the presence of 1 millimolar KCN. For NADH, succinate, and malate a high capacity of the alternative (cyanide-resistant) pathway is found that is completely sensitive to SHAM at 1.5 to 4 millimolar. The increase in the mitochondrial capacity to oxidize either amino acid is also found in four other Araceae species including both thermogenic and nonthermogenic ones. After anthesis, the rates of proline and glutamate oxidation decline.

The inflorescence of *Sauromatum guttatum* consists of a spadix (a fleshy stalk bearing male and female flowers) and a leaflike spathe surrounding it. The naked upper part of the spadix is known as the appendix (17). One day before D-day (the first day of flowering), the level of SA[2] increases in the inflorescence resulting in the characteristic thermogenic respiration (4, 23). The chemical nature of the agent that triggers SA synthesis is unknown but it can be released by floral injury (17). On D-day, the spathe unfolds and the thermogenic, cyanide-insensitive respiration in the appendix reaches a peak (17). The thermogenic activity serves to volatilize ammonia and amines that attract pollinators (27).

Since the levels of ammonia and amines are high during the thermogenic activity, we wondered whether appendix mitochondria are involved in the production of nitrogen compounds. We report that proline and glutamate are respiratory substrates for these mitochondria. Thus, mitochondria may play a role in the mobilization of nitrogen during anthesis.

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[2] Abbreviations: SA, salicylic acid; GDH, glutamate dehydrogenase; SHAM, salicylhydroxamic acid; GOT, glutamate:oxaloacetate aminotransferase; OAA, oxaloacetate; α-KG, α-ketoglutarate; TCA, tricarboxylic acid; DNP, 2,4-dinitrophenol.

MATERIALS AND METHODS

Plant Material

Sauromatum guttatum and *Amorphophallus campanulatus* were grown in the greenhouse, while *Dracunculus vulgaris* and *Arum italicum* were raised in an outdoor garden. *Lysichitum americanum* (Western Skunk Cabbage) inflorescences were collected in the field.

Corms of *S. guttatum* were stored in the dark at 10°C. Growth of the inflorescence resumed once the corms were placed in a growth chamber under 15-h light/9-h dark periods with a light-intensity of 150 $\mu E/m^2/s$ at 19°C. The developmental stage of *S. guttatum* inflorescences was determined relative to the appearance of thermogenicity. In *A. italicum*, a white appendix and white pistillate flowers in a closed spathe were taken to indicate preanthesis. An opened spathe, a yellow appendix, and white pistillate flowers were taken to indicate anthesis. In *D. vulgaris* the developmental stage of the faintly thermogenic inflorescence was determined only by the degree of spathe opening.

Isolation of Mitochondria

The isolation was carried out at 2°C by a modification of an earlier method (21). One appendix (or one spathe) was cooled for 0.5 h in distilled water at 4°C, and then it was homogenized in an Oster Juicer run at low speed at a ratio of 40 mL grinding buffer to 1 g fresh weight. The grinding buffer contained 0.4 M sucrose, 25 mM Hepes, 10 mM K monophosphate, 1 mM EDTA, 0.3% (w/v) BSA, and 0.4% (w/v) PVP-40 (mol wt 40,000, pharmaceutical grade) adjusted to pH 7.5 with KOH. The brei was filtered through several layers of cheesecloth and the filtrate was centrifuged at 500*g* for 5 min. The resulting supernatant was centrifuged at 27,000*g* (Beckman, JA-14 rotor) for 6 min. The pellet was suspended in the grinding buffer, recentrifuged at 3,000*g* for 5 min and then layered on 10 mL of a 0.6 M sucrose cushion and centrifuged for 10 min at 17,000*g*. These are termed 'washed mitochondria.' The mitochondria were suspended in the grinding buffer at a concentration of 20 mg protein/mL. Two mL were layered onto 30 mL of 29% (v/v) Percoll (Pharmacia, ref. 18) in the grinding buffer and centrifuged for 35 min at 40,000*g* (Beckman, JA-20 rotor). The mitochondrial band was collected, diluted with wash buffer (the grinding buffer without

PVP-40 and EDTA), and the mitochondria were pelleted at 10,000*g* for 10 min and suspended again in the same buffer. One g of fresh weight yielded about 30μg of mitochondrial protein.

Integrity of Isolated Mitochondria

The intactness of the mitochondria was evaluated by measuring Cyt *c* oxidation in the presence of 8 mM ascorbate and 30 μM Cyt *c* (20). The percentage intactness was calculated from the relative rates of KCN-sensitive Cyt *c*-dependent oxygen-uptake before and after bursting the mitochondrial outer membrane by treating for 10 s in 40 mM sucrose. The degree of contamination with microbodies and cytoplasmic proteins was evaluated by the activity of catalase (24) and by observing the mitochondrial structure seen in the transmission electron microscope. Appendix tissue is essentially free of Chl.

Transmission Electron Microscopy

Percoll-purified mitochondria were fixed in 0.4 M sucrose, 10 mM K-phosphate buffer (pH 7.5), and 0.25% glutaraldehyde for 2 h. Electron microscopy was conducted as described previously (20) except that phosphate buffer was used instead of cacodylate buffer, and tannic acid was omitted from the fixation solution. The samples were viewed in a JEOL 1003 microscope at 60 KV.

Protein Determination

Mitochondrial proteins were precipitated in 10% (w/v) trichloroacetic acid at 4°C overnight, after which they were centrifuged and resuspended in 0.5 N NaOH; 80% of the protein was recovered. Protein amount was determined by the Bradford procedure (3), with BSA (fraction V) as the standard.

Measurement of Oxygen-Uptake

Oxygen consumption was monitored with a Clark oxygen electrode (Yellow Springs Instrument Co.) in a stirred, 3 mL reaction volume at 30°C. The oxygen content of air-saturated water was 227 μM at 30°C (8). The reaction medium contained 0.3 M mannitol, 1 mM $MgSO_4$, 5 mM K-phosphate, and 10 mM Tris, adjusted to pH 7.2 with KOH.

Mitochondrial protein per assay was adjusted to give initial rates of about 100 natom 0/min. The amount of mitochondrial protein per assay was about 30 μg with succinate and NADH, 70 μg with malate, and 150 μg with proline or glutamate as substrates.

The final substrate concentration in the reaction mixture was as follows: 30 mM malate, 15 mM succinate, 15 mM α-KG, 15 mM proline, or 15 mM monosodium glutamate.

Application of SA to Sections of Appendix

The application procedure followed that of Raskin *et al.* (23). Two d before anthesis, and 1 h after the beginning of the light-period, the appendix and spathe were sliced transversely into sections of about 3 g. The inflorescence with part of the appendix was saved to establish retroactively the first day of anthesis. Ten μL of 0.015 mM, 0.15 mM, and 1.5 mM SA were pipetted on top of each section so that the SA concentrations in μg/g fresh weight were 0.0069, 0.069, and 0.69, respectively. Water was pipetted onto the control sections. The sections were placed on a moist filter paper and incubated overnight in the growth chamber at 19°C. The next morning, 4 h after the beginning of the light period and at the peak of heat production (23), mitochondria were prepared from the appendix and spathe sections treated with various concentrations of SA, and the respiratory activities were examined.

RESULTS

Purity and Quality of Isolated Mitochondria

On a protein basis, the rate of oxygen-uptake in the presence of malate, glutamate, and proline was twofold higher for Percoll-purified mitochondria than for washed mitochondria (Table I). The integrity of Percoll-purified mitochondria was determined by several criteria. First, they were more than 99% pure as judged by the rates of Cyt *c* oxidation. These rates corresponding to 100% damage for washed and Percoll-purified mitochondria were 15 and 32 nmol O_2/min/mg protein. The low rates were also obtained when mitochondria were isolated by a different method (26). Second, these mitochondria were not contaminated with microbodies as judged by catalase activity. Third, transmission electron microscopy revealed that the mitochondrial preparation was free from cellular contamination (Fig. 1) and that more than 85% of the mitochondria were intact. Fourth, oxygen-uptake by mitochondria was completely abolished in the presence of KCN+SHAM with NADH as a respiratory substrate (Fig. 4A).

According to Van Herk (30, table III, average of line 4), *Sauromatum* appendix tissue consumes oxygen at the rate of 0.374 μL O_2/min/mg dry weight at the peak of heat production (we find that 1 g fresh weight yields about 230 mg of dry weight). From this, we estimate that 4.4 μmol O_2/min/g fresh weight are being respired by the tissue. Appendix tissue of *Arum maculatum* consumes oxygen at the rate of 6.7 to 22.3 μmol O_2/min/g fresh weight (1). Washed mitochondria isolated from that tissue consume 900 nmol O_2/min/mg protein in the presence of malate and 4000 nmol O_2/min/mg protein with NADH (16). Therefore, the rates of oxidation observed in our mitochondrial preparation, although high, are in accordance with the level of respiration observed in the tissue and with isolated mitochondria.

Table I. *Properties of Isolated Mitochondria*

Washed mitochondria were prepared from D-day appendices and then fractionated on a Percoll gradient.

	Catalase	Malate	Proline	Glutamate
	nmol O_2/min/mg protein			
Washed	180	600	50	50
Percoll-purified	0	1250	100	100

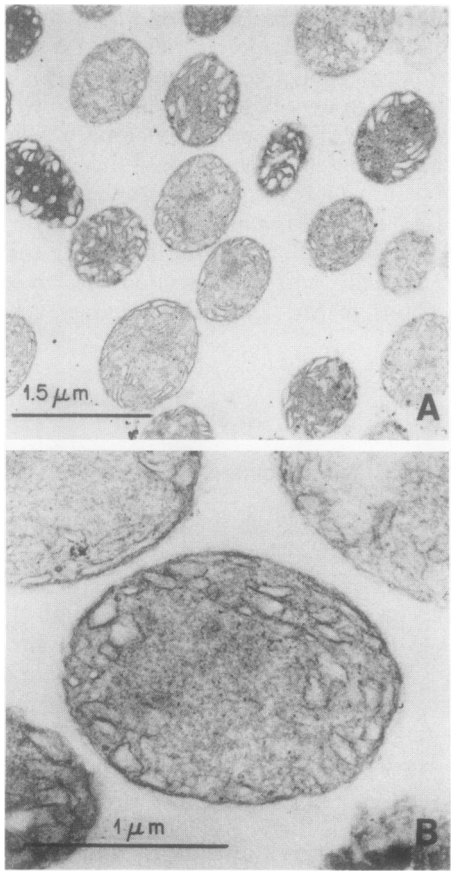

Figure 1. Electron micographs of Percoll-purified mitochondria isolated during the thermogenic phase depicted in 0.4 M sucrose medium.

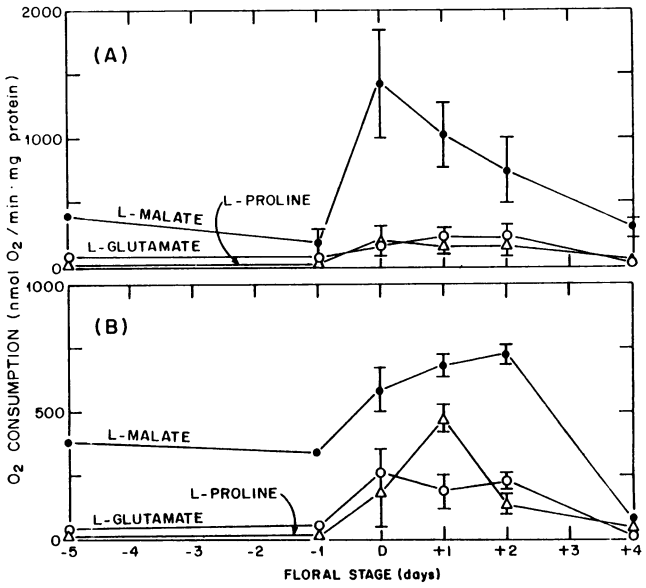

Figure 2. Developmental changes in respiratory rates of mitochondria from *S. guttatum*. A, Appendix mitochondria; B, spathe mitochondria. Oxygen consumption was measured by using various substrates. The reactions were carried out with saturating levels of substrates (data establishing the optimal concentrations are not shown): 30 mM malate, 15 mM proline, and 15 mM Na-glutamate. After establishing the rate of oxygen consumption, DNP (2.5 μM) was added to eliminate the electrochemical gradient allowing maximal rates of oxidation. The abscissa indicates days before or after D-day on which mitochondria were isolated. Each point represents the mean values of 1 or 3 to 6 separate determinations with the standard deviation indicated by vertical bars.

Respiratory Changes during Development

The appendix of *Sauromatum guttatum* develops a high level of thermogenic, cyanide-insensitive respiration when the spathe unfolds on D-day (17). On D-day, appendix mitochondria reached a peak of respiratory activity with proline or glutamate as well as with malate as respiratory substrates (Fig. 2A). Mitochondria isolated from spathes exhibited high rates of oxidation of proline and glutamate over a period of 3 d, from D-day to D+2, without a steep increase in malate oxidation (Fig. 2B). After D-day, the organs of the inflorescence begin to senesce and this may account for the decline in respiration after the thermogenic phase.

The degree of heat production varies among appendices (from 28–34°C), an indication that mitochondrial activity is slightly different among appendices. This may be the reason for the variations in respiratory rates obtained among several mitochondrial preparations.

Other *Arum* Lilies

Mitochondria from thermogenic inflorescences of three other species, *Amorphophallus campanulatus*, *Arum italicum* and *Dracunculus vulgaris* and from the nonthermogenic species *Lysichitum americanum* (Western Skunk Cabbage), were examined for their capacity to oxidize proline and glutamate. Table II shows that during anthesis a stimulation of oxygen uptake in the presence of proline and glutamate was observed with *D. vulgaris* and *A. italicum* mitochondria. The mitochondria of *A. campanulatus* appendix and *L. americanum* spadix also had the capacity to oxidize proline and glutamate during anthesis (preanthesis stage was not studied). These data suggest a common induction mechanism, shared by several members of this family.

Table II. *Developmental Expression of Proline- and Glutamate-Oxidizing Systems in Mitochondria of D. vulgaris and A. italicum*

Washed mitochondria were assayed for oxygen uptake in the presence of proline, glutamate, and malate. Maximum capacities of oxygen uptake with various substrates were determined after addition of 2.5 μM DNP. With malate as respiratory substrate, 95% of oxygen consumption by appendix mitochondria of *A. italicum* was sensitive to SHAM, whereas this value was 57% for *D. vulgaris*.

Substrate	*D. vulgaris*		*A. italicum*	
	Preanthesis	Anthesis	Preanthesis	Anthesis
	nmol O_2/min/mg protein			
None	25	25	20	15
Malate	60	250	1350	1350
Proline	25	60	25	70
Glutamate	25	60	30	50

Induction by SA

Application of SA to sections of appendix had two effects on inflorescence development: (a) reduction of the time that

elapsed before inflorescence opening and (b) alteration of mitochondrial respiratory activities. The latter normally occurs at a later time, during anthesis. Table III shows that 1.5 mM SA (0.69 μg/g fresh weight) roughly triples the rate of oxidation of proline and glutamate. Spathe injury had the same effect as SA on appendix sections. Two to 3 d before D-day (age estimated from color and size of the inflorescence) 5 to 7 scalpel blade cuts were made on the spathe still attached to the inflorescence. One d later the spathe unfolded and the appendix exhibited a thermogenic response. Mitochondria isolated from the appendix showed the same increase in oxidative capacity as did the normal appendix on D-day (data not shown). The accelerated inflorescence opening and the increase in mitochondrial oxidation caused by floral injury cannot be due solely to damage *per se* since mitochondria prepared from sections of spathe or appendix treated with water do not exhibit induced oxidation capacity.

Since by calculation 1 g of fresh appendix contains about 0.8 mg of mitochondrial protein, 0.69 μg of SA was applied to approximately 0.8 mg of mitochondrial protein in the tissue. When added to isolated mitochondria at this concentration (0.86 μg of SA/mg mitochondrial protein) SA had no effect on the oxidation of proline, glutamate or malate (data not shown).

Metabolism of Proline and Glutamate

Proline undergoes two oxidation reactions when it is converted to glutamate, and glutamate undergoes one oxidation reaction when it is converted to α-KG (see Fig. 3). Therefore, the rate of oxygen consumption for proline might be expected to be higher than that for glutamate. However, *S. guttatum* mitochondria oxidized proline or glutamate by the same rate (Table IV). This suggests that the rate of proline-oxidation is limited either by its transport system or at the level of its oxidizing enzymes. Furthermore, the TCA cycle is very active since a high rate of oxidation was obtained with succinate and malate. This suggests that glutamate oxidation is also limited either by its transport system or by GDH activity. When mitochondria were supplied with both amino acids, the rate of oxygen consumption was the same as that obtained with either substrate added alone. A likely explanation is that the oxidation of proline was completely inhibited by the presence of its product, glutamate, and GDH activity was essentially unchanged. A competition at the transport level or at the electron-transport chain level would result in a combined rate lower than the sum of the two rates and higher than the rate with either amino acid.

The rate of oxygen-uptake remained unchanged when glutamate or proline was added to mitochondria already utilizing malate. A likely explanation for this is that OAA, a product of malate oxidation, underwent very little transamination with glutamate via GOT. Thus, the contribution of the aminotransferase route toward glutamate and proline oxidation may be minimal (see Fig. 3).

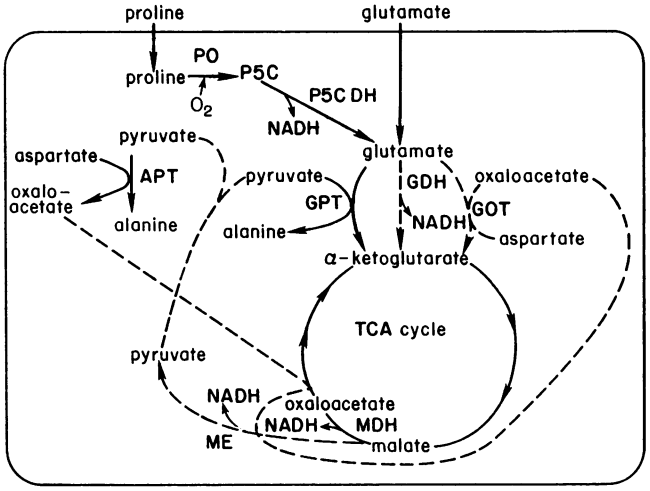

Figure 3. Schematic diagram of glutamate and proline metabolism by mitochondria of *S. guttatum* appendix. Solid arrows indicate metabolic pathways in plant mitochondria; dashed arrows indicate metabolic pathways in mitochondria of *S. guttatum* inflorescence suggested by our data and by other studies (1, 22). Abbreviations used: APT, aspartate:pyruvate aminotransferase; GDH, glutamate dehydrogenase; GOT, glutamate:oxaloacetate aminotransferase; GPT, glutamate:pyrvuate aminotransferase; MDH, malate dehydrogenase; ME, malic enzyme; PO, proline oxidase, P5CDH, Δ′-pyrroline-5-carboxylic dehydrogenase.

Table III. *SA Induction of the Ability of Appendix Mitochondria of S. guttatum to Oxidize Proline and Glutamate*

Washed mitochondria were isolated from appendix treated overnight with various concentrations of salicylic acid. The rates of oxidation of proline and glutamate were determined. Each datum is the mean of two experiments.

SA	Malate	Proline	Glutamate
mM	*nmol O_2/min/mg protein*		
Water	340	55	40
0.015	380	25	25
0.15	220	50	35
1.5	690	140	120

Table IV. *Oxidation of Glutamate and Proline by Mitochondria of S. guttatum Isolated during the Thermogenic Phase*

Respiration rates of Percoll-purified mitochondria were first determined in the presence of each of the respiratory substrates. Next, additional substrates (as indicated) were added and the rates of oxygen-uptake were determined again. The oxidation rate of succinate was determined in the presence of 100 μM ATP.

Substrate	Respiration
	nmol O_2/min/mg protein
Malate	1050
Succinate	1800
Glutamate	200
Proline	225
Proline + glutamate	225
Malate + glutamate	1100
Malate + proline	1050

Cyanide-Insensitive Respiration

The capacity for alternative respiration was determined by using KCN and SHAM. It was highest with NADH as a substrate (Fig. 4, curve A), and lower with succinate (curve B) or malate (curve C). In each of these cases SHAM com-

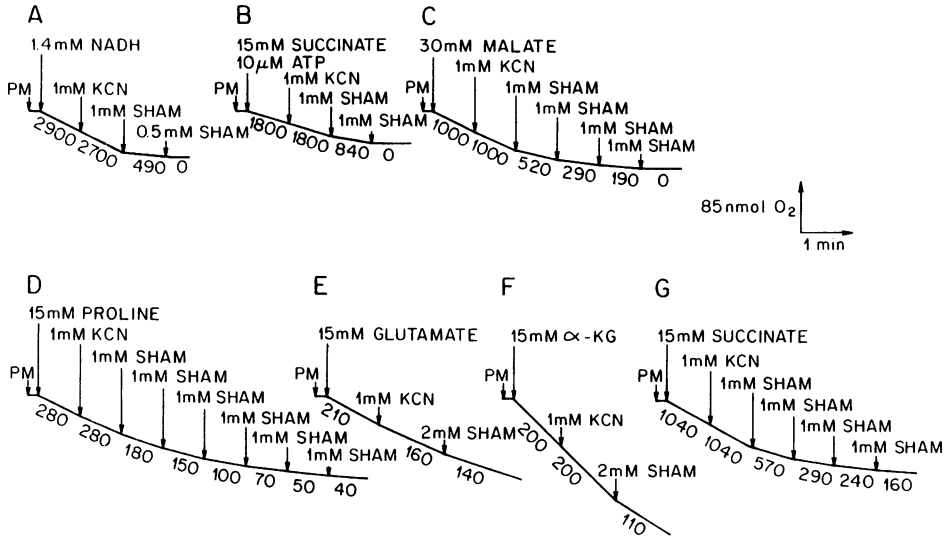

Figure 4. Capacity for alternative respiration in the presence of various respiratory substrates. Percoll-purified mitochondria (PM) isolated from *S. guttatum* appendix during the thermogenic phase were incubated with each of the six substrates: NADH, succinate, malate, proline, glutamate, and α-KG. After establishing the rate of oxygen uptake, KCN was added and thereafter SHAM was added. Rates shown on traces are expressed as nmol O_2/min/mg protein.

pletely abolished respiration. A much lower capacity was obtained with proline, glutamate, and α-KG (curves D, E, and F). With proline, the degree of inhibition of the alternative pathway was not proportional to the amount of added SHAM. *n*-Propyl gallate (150 μM) was not more effective than SHAM at 2 mM as an inhibitor of proline and glutamate oxidation (data not shown). When succinate dehydrogenase was fully active (in the presence of ATP, curve B) 2 mM SHAM completely inhibited oxygen consumption. However, when succinate dehydrogenase was only partially active (in the absence of ATP, curve G), a decrease of 43% in the rate of oxidation was observed and even at 4 mM SHAM did not abolish oxygen consumption. This suggests that when a rate-limiting step exists before ubiquinone (at the level of succinate dehydrogenase, for example) the ability of SHAM to inhibit respiration is decreased. For proline, glutamate, or α-KG oxidation, a rate-limiting step at the level of transport or substrate-oxidation may also result in a respiration that is partially resistant to SHAM.

DISCUSSION

A principal and novel result of this work is the demonstration that the ability of mitochondria to oxidize proline and glutamate increased during anthesis. This increase can be triggered by addition of SA, presumably the natural inducer of the respiratory change in the inflorescence. Furthermore, the capacity for alternative pathway respiration is lower with proline, glutamate, and α-KG than with some other respiratory substrates.

For mitochondria of the thermogenic aroid species *A. italicum* the level of the alternative pathway is nearly constant irrespective of substrate that is oxidized. Our data show that in thermogenic mitochondria, as in other plant mitochondria (25), the capacity for the alternative pathway is determined by the nature of the respiratory substrate.

Proline is a respiratory substrate in the mitochondria of many plant species (2, 6). In plant mitochondria studied so far, glutamate is poorly oxidized via GDH (9) and usually undergoes transamination with OAA (12). In mitochondria of *S. guttatum* inflorescence, however, glutamate is probably oxidized via GDH during anthesis. Another aspect that distinguishes these mitochondria from other plant mitochondria is the presence of proline and glutamate oxidizing systems at the same time. Whether these metabolic pathways characterize inflorescence mitochondria in general is yet to be determined. However, it does seem that mitochondria from the inflorescence of several aroid species exhibit these novel metabolic pathways.

It has been shown that SA induces the activity of the alternative oxidase in the appendix of *S. guttatum* (7), and our data show that the oxidation of proline and glutamate is also induced by SA. It is also known that SA affects the induction of flowering in several plant species (5, 13). It is therefore reasonable to posit that mitochondrial activities induced by SA are involved in flowering.

Three questions arise from our observations. First, what is the significance of proline and glutamate metabolism? It is unlikely that proline and glutamate serve as fuel for heat production because of their small contribution to the burst of respiration and because their oxidation is also triggered in nonthermogenic spathe tissue and in the nonthermogenic inflorescence of *L. americanum*. The ammonia that is released during their oxidation may serve as a nitrogen source for the synthesis of the volatile amines that attract pollinators (27).

The second question is whether the induced oxidation of proline and glutamate is generally associated with floral development in plants. The importance of proline in reproductive organs has been noted in several studies. Proline is the most abundant free amino acid in pollen grains (28), and its amount in the grains is correlated with pollen viability (29). Also, in anthers and pollen of cytoplasmic male-sterile corn, wheat, petunia, and sorghum the level of proline is lower than in the male-fertile counterparts (14). Since alternations in the mitochondrial genome appear to be the basis for cytoplasmic male sterility in plants in general (10), it is possible that a mitochondrially encoded product is involved in the catabolism or transport of proline. Unlike proline, glutamate is

usually not one of the abundant amino acids in pollen and a function specific to reproduction has not been indicated.

The third question is whether mitochondria that exhibit a high level of cyanide-insensitive respiration also oxidize proline and glutamate. We are unaware of studies linking the level of cyanide-insensitive respiration to either aminotransferase or glutamate dehydrogenase activities. It has been shown, however, that during the climacteric respiration in apple fruit the level of glutamate dehydrogenase in the mitochondria is high (11). Thus, we would like to learn whether other storage organs (19) and C4 (15) and CAM (15) plants oxidize both amino acids during their cyanide-insensitive respiration.

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