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In Vivo Ubiquinone Reduction Levels during Thermogenesis in Araceae[1]

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In vivo ubiquinone (UQ) reduction levels were measured during the development of the inflorescences of *Arum maculatum* and *Amorphophallus krausei*. Thermogenesis in *A. maculatum* spadices appeared not to be confined to a single developmental stage, but occurred during various stages. The UQ pool in both *A. maculatum* and *A. krausei* appendices was approximately 90% reduced during thermogenesis. Respiratory characteristics of isolated appendix mitochondria did not change in the period around thermogenesis. Apparently, synthesis of the required enzyme capacity is regulated via a coarse control upon which a fine control of metabolism that regulates the onset of thermogenesis is imposed.

UQ plays a central role in the mitochondrial respiratory chain in plants. It is reduced by several dehydrogenases and can distribute electrons to the Cyt pathway and the AOX. Consequently, in isolated plant mitochondria, the UQ redox state can be varied by modulating the activities of UQ-reducing and -oxidizing systems (Dry et al., 1989; Van den Bergen et al., 1994). In a previous paper we reported that in petunia cells the UQ pool is about 60% reduced throughout the whole batch culture (Wagner and Wagner, 1995). These cells go through a cycle of high supply of respiratory substrate at the beginning of the culture, when the cells are diluted in fresh medium with excess Glc, to the point of Glc starvation, but this variation in substrate supply apparently does not influence the UQ reduction state. It was proposed that AOX plays an important role in controlling these UQ-reduction levels by acting as an overflow for excess reducing equivalents, thereby preventing the production of reactive oxygen species by highly reduced components of the respiratory chain (Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Purvis, 1997; Wagner and Moore, 1997).

Even when the Cyt pathway is inhibited by antimycin A, in vivo UQ-reduction levels of about 60% are observed in petunia cells (measured after 2 d of culture in the presence of antimycin A), whereas the expression of AOX protein is increased and the kinetics of succinate dehydrogenase are changed (Wagner and Wagner, 1997). Apparently, in these cells the relative amounts and kinetic parameters of the components of the respiratory chains are reorganized in such a way that the reduction level of the UQ pool is not affected, whereas O_2 consumption continues at a high rate, preventing the O_2 concentration in the cells from rising to high levels, which would also favor free radical formation (Wagner and Moore, 1997). Respiration rates vary by at most a factor of 3 during batch culture of these cells.

A much more extreme situation with respect to differences in substrate supply occurs during the development of the flowers of *Arum maculatum*. The inflorescence consists of a central column (spadix) wrapped by a modified bract (spathe). The base of the spathe forms the floral chamber in which the female flowers and, above these, the male flowers are located. The upper part of the spadix consists of a club-shaped organ, the appendix. When the inflorescence emerges, the spathe is tightly wrapped around the spadix, but at the time of flowering the upper part of the spathe unfolds, exposing the appendix, which undergoes a metabolic "explosion." Respiration, which increases 100-fold, proceeds mainly via the alternative pathway, which leads to a temperature rise in the appendix tissue that may be as high as 15°C to 20°C above ambient temperature.

In an attempt to determine if the UQ-reduction levels vary during the development of the inflorescence of *A. maculatum*, we have measured in vivo UQ concentrations and reduction states and the temperature of the appendix at various developmental stages. Since there appeared to be dramatic differences in UQ reduction during the period of thermogenesis compared with pre- or postthermogenic tissues, we also studied the characteristics of mitochondria isolated from thermogenic appendices and compared them with those of nonthermogenic but otherwise morphologically indistinguishable appendices.

MATERIALS AND METHODS

Plant Materials

Inflorescences of *Arum maculatum* were collected from plants growing at various sites on the campus of the University of Sussex (Brighton, UK). A flowering *Amorphophal-*

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Abbreviations: AOX, alternative oxidase; SA, salicylic acid; UQ, ubiquinone.

lus krausei was obtained from the Rijksherbarium/Hortus Botanicus of the Rijksuniversiteit Leiden (The Netherlands).

Temperature Measurements

Appendices of *A. maculatum* were collected during various times of the day and left at room temperature (varying between 20°C and 22°C) in a shaded area for 15 min, after which time the appendix temperature was measured with a thermocouple (Electronic Thermometer model 1604, Comarck Electronics Ltd., Hertfordshire, UK) at about 1 cm below the appendix surface. For continuous temperature measurements, the upper half of the spathe of an intact plant in situ was removed to reveal the appendix, and a thermocouple connected to a recorder was inserted.

Measurements of in Vivo UQ Reduction

Pieces of appendix tissue of 0.5 to 1 g were cut and immediately dropped in liquid N_2, ground to a fine powder with a mortar and pestle, and 10 mL of 0.2 M $HClO_4$ in methanol (0°C) was added. The mixture was transferred to a tube with 10 mL of petroleum ether (boiling point 40°C–60°C) and vortexed for 1 min. After the mixture was centrifuged at 1500*g* for 2 min, the upper petroleum ether phase was removed, transferred to a test tube, and evaporated to dryness under a flow of N_2. Another 10 mL of petroleum ether was added to the lower phase, and the vortex and centrifugation steps were repeated. The upper phase was added to the one previously obtained. After evaporation, extracts could be stored for at least 1 d under N_2 at −20°C. Immediately before use, the extracted UQ was resuspended with a glass rod in 100 μL of N_2-purged ethanol, and analyzed by HPLC at the Vrije Universiteit in Amsterdam with a pump system (Gilson, Villiers le Bel, France) and detector (model 811, Perkin-Elmer), and at the University of Sussex with an absorbance system (model 160, Beckman), a solvent-delivery module (model 110B, Beckman), and a reverse-phase column (model 10-RP 18 Lichrosorb, Chrompack, Bergen op Zoom, The Netherlands; 4.6 × 250 mm in size). The column was equilibrated with N_2-purged ethanol-methanol (3:2, v/v) and this mixture was used as the mobile phase. Detection of UQ was performed at A_{290}. The amounts of UQ10 and $UQH_2$10 were calibrated from the peak areas (as determined by weighing).

Isolation of Mitochondria and Measurements of Mitochondrial Respiration

Mitochondria from *A. maculatum* appendices were isolated and purified on Percoll gradients as described by Moore et al. (1993). O_2 consumption was measured at 20°C in 2 mL of reaction medium containing 0.3 M mannitol, 1 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM KCl, and 20 mM Mops, pH 7.2, in a glass vessel housing a Rank O_2 electrode. Succinate (20 mM) or NADH (2 mM) in the absence or presence of 5 mM pyruvate were added as substrates. State 3 measurements were performed in the presence of 0.15 mM ADP. KCN (0.1 mM) was added to inhibit respiration via the Cyt pathway, and 2 μM octyl gallate was added to inhibit AOX.

Mitochondrial protein was determined by the method of Bradford (1976), using BSA for calibration.

RESULTS AND DISCUSSION

Classification of the Developmental Stages of *A. maculatum* Inflorescences

James and Beevers (1950) described six stages during the development of the *A. maculatum* inflorescence: the α-stage, in which the inflorescence is just emerging and the spathe is tightly folded; the β-stage, in which the spathe is still tightly folded; the γ-stage, in which the spathe is loosening from the appendix; the δ-stage, in which the spathe is half-open; the ε-stage, in which the spathe is fully open and the pollen is shed; and the ζ-stage, in which the spadix withers after the female flowers are pollinated and the pollen of the male flowers is shed. It was reported that respiration reached the highest rates in the β-stage and the γ-stage. ap Rees et al. (1977) reported that thermogenesis takes place late in the γ-stage, although it was noted that the extent and timing of thermogenesis varied considerably.

Other literature describes the onset of thermogenesis to occur at the time of opening of the spathe (Prime, 1960), or even when the spathe is partly unfolded (Bermadinger-Stabentheiner and Stabentheiner, 1995), which would suggest that thermogenesis occurs in the δ-stage, when the spathe is half-open. In the present study we measured appendix temperatures from *A. maculatum* inflorescences at various developmental stages. We classified the inflorescences according to the stages β through ε as defined by James and Beevers (1950), with the exception that we also distinguished between fully open inflorescences before (ϵ^-) and after (ϵ^+) pollen is shed.

Appendix Temperature and Developmental Stage

Figure 1 shows appendix temperatures in a total of 101 inflorescences of *A. maculatum.* With the exception of the inflorescences in which pollen was shed (stage ϵ^+) the average temperature of the appendix was higher than the ambient temperature, with the highest temperatures found in the δ-stage. In this stage, as in the γ-stage, the ϵ^--stage, and, to a lesser extent, the β-stage, a large variation in appendix temperature was observed, including appendix temperatures that did not differ from ambient temperatures, and temperatures below ambient temperatures, which may be caused by the high transpiration rates in the spadix (Skubatz et al., 1990; Bermadinger-Stabentheiner and Stabentheiner, 1995). Inflorescences at all stages could be found irrespective of the time of day, and no correlation was found between time of harvesting and appendix temperature at any stage (data not shown).

Temporal Dynamics of the Development of Thermogenesis

The observed large variation in appendix temperature at each developmental stage of *A. maculatum* inflorescences

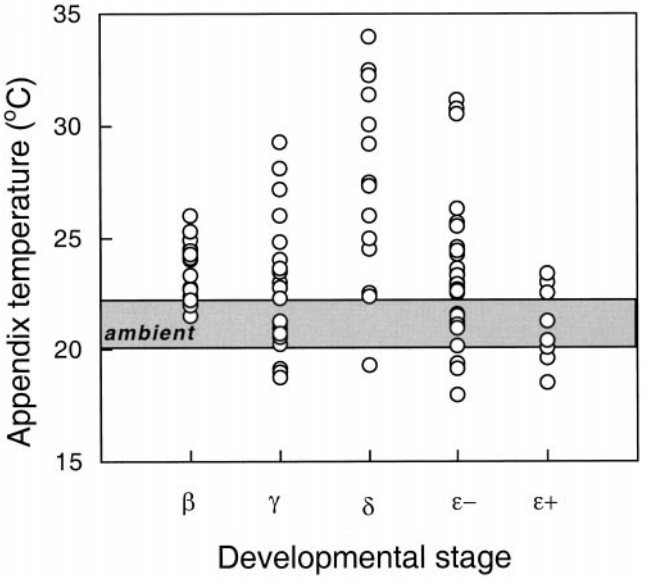

Figure 1. Appendix temperatures during the various stages of development of Arum lily inflorescences. Appendices were collected during various times of the day and left at ambient temperature (varying between 20°C and 22°C, shaded area) for 15 min, after which time the appendix temperature was measured with a thermocouple at about 1 cm below the appendix surface. For definitions of the developmental stages, see "Materials and Methods."

could be explained either by a large variation in the timing of thermogenesis (not related to a certain degree of spathe opening), to variations in the final temperature the appendix achieves, or to the length of the thermogenic period. With respect to the temporal dynamics of spadix development, Prime (1960) reported that there is no definite time of day that all plants open, and that the sequence and timing of events varies considerably with the site of the plant and the weather at the time of flowering. It was reported that if the spathe is sheltered and the weather is cool, the pollen may not be shed until the 3rd d after opening of the spathe, and in cold periods the spathe may not even fully unfold. When *A. maculatum* was observed growing at various sites on the campus of the University of Sussex, a large variation in timing of the opening process was found and one particular specimen even remained half-open (δ-stage) for more than 2 d.

To estimate the variation in length of the thermogenic period and the variation in maximal temperatures reached, the temperatures in three appendices of *A. maculatum* plants were continuously recorded. They were measured in situ on different days at ambient temperatures varying from 12°C to 19°C. The upper half of the spathe was removed to reveal the appendix, and a thermocouple connected to a recorder was inserted. Table I shows that the onset of the thermogenic period was not confined to a certain time of day, but that the total length of the thermogenic process and the maximal temperatures reached were not markedly different in the three plants. It would therefore appear that it is mainly the variation in timing of the onset of thermogenesis that causes the large variation in appendix temperature at each developmental stage.

In Vivo UQ Reductions and Developmental Stages

The temperature measurements at the various developmental stages show that the onset of thermogenesis in *A. maculatum* is not necessarily confined to a certain morphological stage. Consequently, when inflorescences are collected by morphological stage only, a mixed population of prethermogenic, thermogenic, and postthermogenic inflorescences will be present. We therefore measured in vivo UQ reductions in appendices for which the temperature was determined individually. The spathe was removed, the temperature was measured, and close to the point where temperature was measured, a piece of tissue was cut from the appendix and UQ was extracted. HPLC traces showed that UQ9 and UQ10 were both present in the appendix tissue, with UQ10 being the major UQ present at every stage. Expressed on a fresh-weight basis, UQ10 concentration increased from 0.26 ± 0.07 nmol/g fresh weight (n = 6) at the β-stage to 0.72 ± 0.18 nmol/g fresh weight (n = 12) at the δ-stage. This may have been due to an increase in mitochondrial protein during development. Indeed, this appears to be the case, since when UQ amounts were measured in isolated mitochondria from the β- and δ-stages, the difference between these two stages expressed on the basis of mitochondrial protein was less than that expressed on a fresh-weight basis (1.74 ± 0.45 to 2.48 ± 0.68 nmol/mg protein; n = 6). The amount of UQ9 also increased during development of the inflorescence, but to a somewhat larger extent than UQ10, and the UQ9:UQ10 ratio changed from 1:11 to 1:7 (data not shown). In accordance with previous results (Ribas-Carbo et al., 1995), it was found that UQ9 and UQ10 had the same reduction level under the various conditions investigated. Since both UQ9 and UQ10 showed similar variations in reduction levels, all further results are expressed in UQ10 reduction levels only.

Figure 2 shows the levels of in vivo UQ10 reduction of 31 appendices at various developmental stages. In contrast to all tissues measured previously (petunia [Wagner and Wagner, 1995, 1997] and bean roots, potato, and potato callus [A.M. Wagner, unpublished data]), in vivo UQ reductions in *A. maculatum* did not remain constant, but varied from 40% to almost 100%. The highest UQ10 reduction levels were generally observed in the δ- and ε-stages, although the UQ10 pool in one spadix from a γ-stage plant was approximately 90% reduced.

In Figure 3 the relationship between appendix temperature and UQ10 reduction is plotted. At temperatures above

Table I. *Characteristics of the thermogenic period in A. maculatum appendices*

Time of day at which the temperature started to rise, duration of the thermogenic period, and the maximal temperatures of three intact plants measured in situ.

Parameter	Plant No.		
	1	2	3
Ambient temperature (°C)	12–14	15–19	13–18
Time of onset of thermogenic period (h)	13.45	17.00	19.30
Maximal temperature (°C)	28.2	26.4	27.9
Length of thermogenic period (h)	12.0	10.2	10.9

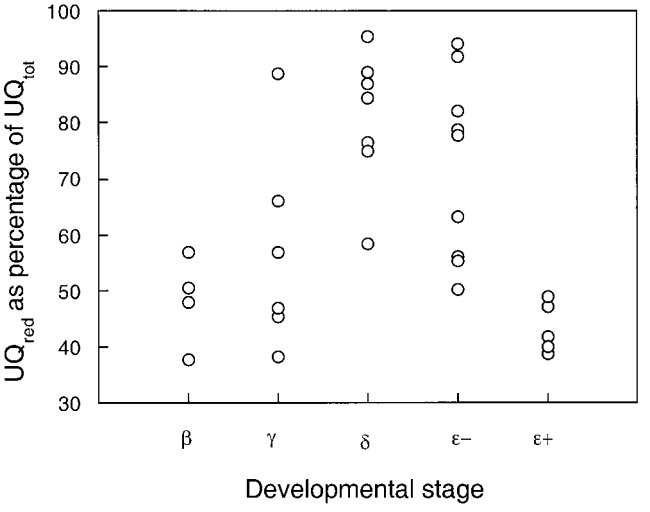

Figure 2. In vivo UQ10 reduction expressed as UQ reduced (UQ_{red}) and as the percentage of total UQ (UQ_{tot}) in the appendix during the various stages of development of *A. maculatum* inflorescences. For definitions of the developmental stages, see "Materials and Methods."

25°C, the level of UQ10 reduction was always around 90%. In the temperature region between 20°C and 25°C, the extent of UQ10 reduction was highly variable, probably representing a mixture of appendices that were either warming up or cooling down. The very high reduction levels found at high appendix temperatures can be explained in two ways: (a) the input of substrate into the respiratory chains may exceed the electron transport capacity of the oxidizing pathways, or (b) O_2 may become a limiting factor.

James and Beevers (1950) reported that respiration of the appendix of *A. maculatum*, measured both in tissue slices and in a cell-free extract, was not saturated at 100% O_2. Likewise, Wedding et al. (1973) described a K_m for O_2 of appendix slices of 1.3 μM, whereas the K_m in isolated appendix mitochondria was 0.3 μM. Therefore, it is possible that with the extremely high respiratory rates found in thermogenic tissues, O_2 diffusion becomes limiting, especially within the massive structure of the *A. maculatum* appendix.

The inflorescence of *A. krausei*, another member of the Araceae, has a hollow appendix and the thermogenic tissue consists of a layer of less than 0.5 cm. Therefore, it was important to measure in vivo UQ-reduction status during the thermogenic period of this plant. In the appendix of *A. krausei*, UQ10, the main UQ present, became completely reduced during thermogenesis (91% ± 8%; mean value ± SD of four samples from the same plant, taken when temperature had reached a stable level of 38°C). When the temperature had returned to room temperature, the UQ pool became more oxidized (65% ± 8%; mean value of three samples from the same plant ± SD). Although O_2 will diffuse more rapidly into the hollow spadix of *A. krausei* than into the massive spadix of *A. maculatum*, it is still possible that with the very high respiration rates during thermogenesis, O_2 diffusion also becomes a limiting step in the *A. krausei* appendix. The regulation and control of O_2 consumption and UQ reduction in thermogenic tissues will be the subject of further studies.

Mitochondrial Characteristics of Thermogenic and Nonthermogenic Appendices in the δ-Stage

Since very different in vivo metabolic conditions can occur within one developmental stage, the question arises whether mitochondria isolated from thermogenic appendices differ from those present in nonthermogenic appendices at the same developmental stage. Appendices in the δ-stage were therefore collected and divided into two groups, one with appendix temperatures below 24°C (which may have been either pre- or postthermogenic), and one with appendix temperatures higher than 26°C. Table II shows the mitochondrial parameters of these two groups. In both preparations, NADH was respired at a faster rate than succinate, and in both samples respiration was entirely CN resistant, with the CN-resistance rate being stimulated by pyruvate only when NADH was the substrate. This stimulation was only about 20%, which is low compared with other tissues, a phenomenon previously observed in Araceae (Leach et al., 1996; Umbach and Siedow, 1996).

The severe inhibition of O_2 uptake upon addition of octyl gallate indicates that in both preparations the Cyt pathway activity is low. Leach et al. (1996) reported that no significant changes occur with respect to the characteristics of the Cyt pathway during the development of the inflorescence of *A. maculatum*, but found that AOX became more active at low UQ-reduction levels when the inflorescence developed from the β- and γ-stages to the δ-stage. Our results show

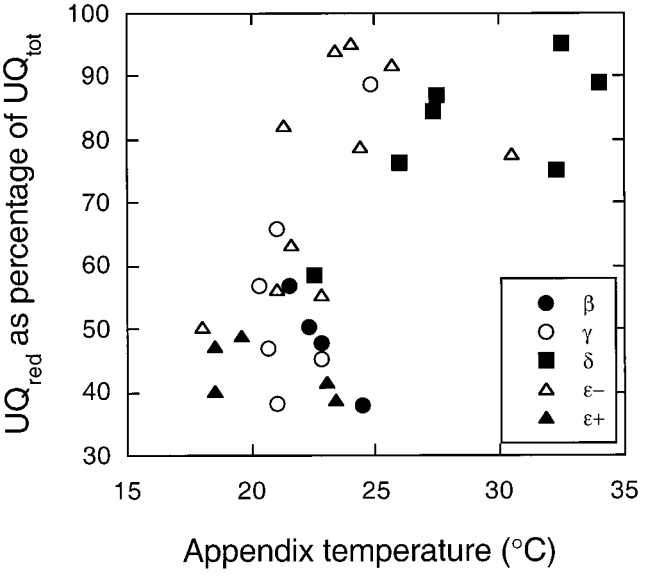

Figure 3. Relationship between appendix temperatures and UQ10 reduction (UQ_{red}) levels as a percentage of total UQ (UQ_{tot}) at the various stages of development of *A. maculatum* inflorescences. For details of temperature measurements, see legend to Figure 1. For definitions of the developmental stages, see "Materials and Methods."

Table II. *Characteristics of A. maculatum mitochondria isolated from appendix tissue of inflorescences in the δ-stage with an appendix temperature of >26°C or <24°C*

Succinate (20 mM) or NADH (2 mM) were given as respiratory substrates in the absence or presence of 0.15 mM ADP. CN-resistant respiration (+ 0.1 mM CN) was measured in the absence or presence of 5 mM pyruvate. Cyt pathway activity was measured in the presence of 2 μM octyl gallate (OG). Data are mean values ± SD of triplicate (O_2 uptake) or single (UQ10 reduction) measurements of one mitochondrial isolation.

Assay Condition	Appendix Temperature >26°C		Appendix Temperature <24°C	
	O_2 uptake	UQ10 reduction	O_2 uptake	UQ10 reduction
	nmol min^{-1} mg^{-1} protein	*% of total*	*nmol min^{-1} mg^{-1} protein*	*% of total*
Succinate				
+ ADP	197 ± 22	nd[a]	185 ± 24	nd
+ ADP + CN	199 ± 20	nd	186 ± 20	nd
+ CN + pyruvate	209 ± 19	48	193 ± 15	51
+ ADP + OG	51 ± 21	45	41 ± 15	46
NADH				
+ ADP	777 ± 35	nd	782 ± 56	nd
+ ADP + CN	776 ± 34	nd	783 ± 55	nd
+ CN + pyruvate	931 ± 34	93	914 ± 32	89

[a] nd, Not determined.

that once the inflorescence has reached the δ-stage, no further changes take place with respect to AOX kinetics. Independent of whether a δ-stage inflorescence is actually thermogenic, O_2 uptake rates under various conditions and accompanying UQ reductions are the same, indicating that kinetic parameters of UQ-reducing (dehydrogenases) and UQ-oxidizing (Cyt pathway and AOX) enzymes were similar in the two mitochondrial preparations (Wagner and Krab, 1995). This suggests that in the period around thermogenesis, the mitochondrial "machinery" in *A. maculatum* does not change anymore.

This is in good agreement with what has been found for the capacities of glycolytic enzymes such as phosphofructokinase (ap Rees et al., 1976), which increase during the early developmental stages, but are the same in prethermogenic and thermogenic appendices. Conversely, the situation in *A. maculatum* appears to be different from what has been described for *Sauromatum guttatum*, in which a marked decrease is observed in the amount of Cyt oxidase protein and activity as the appendix reaches "D-Day" (Elthon et al., 1989). In *S. guttatum*, these changes in Cyt oxidase activity are correlated more with the onset of thermogenesis than the increase in AOX protein, which occurs well before thermogenesis sets in. Also, in *A. maculatum* increasing amounts of AOX protein, and especially the appearance of a 35- to 36-kD polypeptide, are already seen at the β-stage and the early γ-stages, which may be several days away from thermogenesis (Leach et al., 1996).

The results found for *A. maculatum* suggest that the mitochondrial "equipment" is put in readiness for the thermogenic respiratory burst via a coarse control. We can still only speculate about the exact nature of the fine control signal(s) that initiates and regulates the metabolic "explosion" in *A. maculatum*. In the appendix of *S. guttatum*, synthesis of SA has been suggested to play a role, since concentrations of SA increase 100-fold and reach final levels of over 1 μg/g fresh weight on the day before flowering (Raskin et al., 1987, 1989). High levels of SA have also been found in thermogenic tissues of several other Araceae species from the genera *Arum* and *Amorphophallus* (Raskin et al., 1990). It has been reported that SA can induce AOX protein expression in nonthermogenic tissues (Kapulnik et al., 1992; Rhoads and McIntosh, 1993; Lennon et al., 1997); however, the extreme rise in concentration of SA in *S. guttatum* occurs only shortly before thermogenesis, when the amount of AOX protein has already increased.

Concluding Remarks

In petunia cell suspensions, in vivo UQ-reduction levels are maintained at a stable level of about 60%, and we have argued that this may be part of a strategy by the plant cell to prevent free radical formation, which is likely to occur during high reduction states of the respiratory chain, particularly with respect to UQ (Wagner and Wagner, 1995, 1997). In this paper, however, we show that during development of the inflorescence of *A. maculatum*, the UQ pool in the appendix tissue becomes reduced to very high levels in vivo. Apparently, the regulatory mechanisms that normally control or buffer the redox levels of the UQ pool do not function in the thermogenic appendix. The reason for this may be related to the specialized function of the appendix tissue compared with normal, growing cells. The sole function of the appendix is to produce heat to attract insects for pollination. A highly reduced UQ pool will give maximal activity of the heat-producing AOX (Leach et al., 1996). Following pollination, the appendix tissue senesces, so the need to protect the appendix against free radical damage, which would occur at such high reduction levels of the respiratory chain components, is removed.

Thermogenesis in the appendix is characterized by high respiratory activity and loss of control of redox regulation, which may result in the formation of reactive oxygen species, increased SA concentrations, and, finally, the death of the appendix tissue. This is similar to the description of programmed cell death (apoptosis), as occurs in the hypersensitive response in plant-pathogen interactions (Greenberg, 1997). The order of events and the nature of the signal

molecules in the program that causes apoptosis in plants are unclear at present. There is agreement that an oxidative burst precedes cell death, but the role of SA is still under debate (Lamb and Dixon, 1997). Therefore, more insight into the signals triggering both the onset of thermogenesis and programmed cell death is required before we can decide whether thermogenesis in the Arum lily spadix can indeed be regarded as an apoptotic event.

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