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Expression of the Alternative Oxidase in Thermogenic and Non-Thermogenic Reproductive Organs

By

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With 4 Figures

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Summary

SKUBATZ H. & HAIDER S.T. 2004. Expression of the alternative oxidase in thermogenic and non-thermogenic reproductive organs. – *Phyton* (Horn, Austria) 44 (1): 83–94, with 4 figures. – English with German summary.

The distribution of mitochondria in fixed sections of thermogenic appendages of *Victoria cruziana* (*Nymphaeaceae*) flowers was visualized by using indirect immunofluorescence of the monoclonal antibody against the mitochondrial alternative oxidase (AOX). It revealed clustering of mitochondria at the perinuclear region. A comparative staining with anti-Cyt-actin and anti- β -tubulin subunit showed that both proteins stained the perinucleus and the periphery of the cells as well. On Western blots of acetone extracts of total appendage proteins, anti-AOX recognized two protein bands. A protein band with an apparent molecular weight of 37 kDa, and a lower band with an apparent molecular weight of 32 kDa. The level of AOX (37 kDa) stayed unchanged during floral development. The thermogenic staminal appendages of *Nelumbo nucifera* (*Nelumbonaceae*), another thermogenic plant, also expressed both polypeptides. However, the 37-kDa protein was the prominent one. The thermogenic male cones of *Encephalartos ferox* (*Zamiaceae*), also expressed the 37 kDa and 32 kDa proteins and the non-thermogenic, fragrant and non-fragrant flowers exposed the 37 kDa protein.

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Zusammenfassung

SKUBATZ H. & HAIDER S. T. 2004. Die Ausprägung der alternativen Oxidase in Wärme produzierenden und nicht Wärme produzierenden reproduktiven Organen.- *Phyton* (Horn, Austria) 44 (1): 83–94, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Verteilung der Mitochondrien in fixierten Schnitten der Wärme produzierenden Anhängsel der Blüten von *Victoria cruziana* (*Nymphaeaceae*), wurden mittels indirekter Immunofluoreszenz von monoclonalen Antikörpern gegen die mitochondriale alternative Oxidase (AOX) dargestellt. Es zeigte sich eine Anhäufung von Mitochondrien im perinuclearen Bereich. Eine Vergleichsfärbung mit anti-Cyt-actin und anti- β -tubulin Untereinheiten ergab, dass beide Proteine die Umgebung des Kerns und gleichzeitig auch die Peripherie der Zellen färbten. Auf Western-blots der Acetonextrakte von Gesamtproteinen zeigte anti-AOX zwei Proteinbanden; eine Proteinbande mit einem Molekulargewicht von 37 kDa und eine niedrigere Bande mit 32 kDa. Der Gehalt an AOX (37 kDa) blieb während der Blütenentwicklung unverändert. Die Wärme produzierenden Staubgefäßanhängsel von *Nelumbo nucifera* (*Nelumbonaceae*), einer anderen Wärme produzierenden Pflanze, zeigten ebenfalls beide Polypeptide, jedoch war das 37-kDa-Protein immer vorherrschend. Die Wärme produzierenden Zapfen von *Encephalartos ferox* (*Zamiaceae*), bildeten ebenfalls AOX wie es auch die nicht Wärme produzierenden duftenden und nicht duftenden Blüten machten.

Introduction

Heat is generated by reproductive organs of plants of at least seven families; e.g., the flowers of *Victoria* (*Nymphaeaceae*; SCHNEIDER 1976) and *Nelumbo* (*Nelumbonaceae*; HAYES & al. 2000, SEYMOUR & SCHULTZE-MOTEL 1996), and the well-studied inflorescence of the voodoo lily, *Sauromatum guttatum*, a member of the *Araceae* family (MEEUSE & RASKIN 1988). Among other thermogenic plant organs are the cones of many cycads, e.g. *Encephalartos ferox*, which are thermogenic for 6 days between 5 P.M. to 7 P.M. (TANG 1987).

The high activity of the alternative, cyanide-insensitive oxidase (AOX) in the mitochondria of the *Sauromatum* appendix coincides with the generation of heat. The AOX activity was well studied in the mitochondria of thermogenic appendix of *S. guttatum* (ELTHON & McINTOSH 1986, ELTHON & al. 1989a, SKUBATZ & al. 1989, 1991) and *Arum maculatum*, another member of the *Araceae*, family (KAY & PALMER 1985, LEACH & al. 1996, PROUDLOVE & al. 1987). Four (ELTHON & al. 1989a) to 3 forms (SKUBATZ & HAIDER 2001) of AOX can be detected by Western blots 2 days prior to heat-production by the *Sauromatum* appendix. The expression of AOX in thermogenic plants from other families has not received much attention.

The carpellary appendages of the flowers of *Victoria cruziana*, an aquatic plant, are thermogenic for two consecutive days when the flower unfolds. Their temperature rises to 5–10°C above ambient and at that

time a pleasant odor is released to attract pollinators. In *Nelumbo nucifera*, another water plant, the staminal appendages are thermogenic for two consecutive days when the flower unfolds and odorants are released. The production of heat in cycads is also accompanied by the production of a very pronounced odor. At the end of heat-production, pollen is shed in the male cones of cycads and in all other thermogenic plants. In *Victoria* carpellary appendages as in all other thermogenic organs, thermogenicity and cyanide-insensitive respiration coexist (SKUBATZ & al. 1990). The same is true for thermogenic cycads (SKUBATZ & al. 1993a).

The objectives of the present study are: 1) In many thermogenic plants in the Araceae family the mitochondria expressing AOX are clustering in the perinuclear region with no strong correlation with the distribution of two cytoskeletal proteins, actin and tubulin (SKUBATZ & HAIDER 2001). Examination of the distribution of AOX in fixed sections of the carpellary appendages of *Victoria* flowers was carried out in order to determine whether mitochondria expressing AOX are clustered in the perinuclear regions. 2) To determine the expression of AOX forms in the thermogenic organ of *Victoria* (*Nymphaeaceae*), and *Nelumbo* (*Nelumbonaceae*), and *Encephalartos ferox* (*Zamiaceae*) by Western-blotting analysis. 3) To determine whether odor release to the atmosphere requires the presence of AOX. The combination of odor- and heat-development might have a biological significance in attracting pollinating insects to the flowers. Therefore, we have examined the expression of AOX in fragrant and non-fragrant flowers that are pollinated by insects.

Material and Methods

Plant material

Thermogenic plants – *Victoria cruziana* (*Nymphaeaceae*) and *Nelumbo nucifera* 'Alba' (*Nelumbonaceae*), and *Encephalartos ferox* (*Zamiaceae*).

Non-thermogenic plants – *Nicotiana tabacum* 'Xanthii' and *Brugmansia* x 'Charles Grimaldi' (*Solanaceae*), *Stanhopea wardii* and *Bulbophyllum ornatissimum* (*Orchidaceae*). *Passiflora* 'Sunburst' (*P. gilbertiana* x *jorullensis*, *Passifloraceae*).

All thermogenic and non-thermogenic plants, except for *E. ferox*, were grown in greenhouse at the University of Washington. Sporophylls from male cones of *E. ferox* were collected at different stages of heat-production at the Fairchild Tropical Garden, Miami, FL, and kept at -80°C until further use.

For convenience, the terminology coined by MEEUSE to describe the stage of development in relation to heat-production in the *Sauromatum* appendix is used here for all other thermogenic plants. For example, the day of heat-production is D-day; one day before heat-production is D-1, etc. One day after the first day of heat-production is D+1 (CHEN & MEEUSE 1972).

Acetone extracts

Acetone powder preparation was described in details in our previous paper (SKUBATZ & al. 2000). Briefly, the fine powder was prepared by grinding fresh plant tissue in cold acetone immediately after it was cut off with the exception of the shipped frozen sporophylls of *Encephalartos ferox*. The thermogenic carpellary appendages of *Victoria* flowers were collected during floral development. The staminal appendages of *N. nucifera* were collected on the second day of heat production. The labellum of *S. wardii* was cut off on the first day of blooming. All other flowers were extracted with acetone on their first day of flowering, immediately after opening.

Monoclonal antibodies

The following three mouse monoclonal antibodies were used:

Anti-alternative oxidase (AOX) against the mitochondrial alternative oxidase of the *S. guttatum* appendix (ELTHON & al. 1989b, SKUBATZ & HAIDER 2001).

Anti- β -tubulin subunit (Amersham Corp.; N 357). This monoclonal antibody interacts specifically with β -tubulin subunit present in acetone extracts of the *Saurumatum* appendix (SKUBATZ & al. 2000).

Anti-cytoplasmic actin isoforms Cyt-actin; Amersham Corp, N.350). This monoclonal antibody interacts with actin polypeptides (SKUBATZ & al. 2000).

Indirect immunofluorescence

For immunofluorescence staining, fresh blocks of thermogenic tissue were fixed as described previously (SKUBATZ & al. 2000). A 1:1000 dilution of the monoclonal antibodies against β -tubulin subunit and Cyt-actin, and a 1:3 dilution of the hybridoma supernatant of the monoclonal antibody against AOX were used for staining.

Electrophoresis and immunoblotting

Electrophoresis and immunoblotting of proteins in the acetone powders were carried out as described previously (SKUBATZ & HAIDER 2001). Briefly, 1 mg of acetone powder was solubilized in 10 μ l of SDS-PAGE sample buffer (LAEMMLI 1970), centrifuged, and different amounts of the supernatant were fractionated on 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. After blotting, the membrane was washed with 20 mM Tris-HCl, 137 mM NaCl (TBS). Subsequently, the membrane was incubated overnight at 4°C in 3% BSA in TBS to block non-specific binding. The membrane was washed with 0.05% Tween-20 in TBS (TTBS), and incubated overnight at 4°C with a diluted primary antibody in 3% BSA in TBS. Next day, the membrane was washed 5 times with TTBS for 5 min, and incubated in the secondary antibody conjugated to alkaline phosphatase (goat anti-mouse IgG, Bio-Rad) at a 1:1000 dilution for 2 h at room temperature. The immunoreactive bands were visualized by color development with 160 μ M bromochloroindoyl phosphate, 130 μ M nitro blue tetrazolium (BCIP/NBT; Bio-Rad) in 100 mM Tris-HCl, 4 mM MgCl₂, pH 9.8. The reaction was stopped by washing with water.

Results

AOX distribution in *Victoria* carpellary appendages

The *Victoria* carpellary appendages that are regarded as odor-producing organs consist of two distinct regions: an external cortex and an internal aerenchyma. The cortex is composed of epidermis and parenchyma (Fig. 1). A collenchymatic layer that provides a mechanical support for the appendage is observed at areas where the appendages are connected to the flower base (Fig. 1B). A transitional layer with cells that appear different than the surrounding cells is found between the collenchyma and parenchyma (Fig. 1B). The aerenchyma is composed of stellate cells with a narrow cytoplasm (Fig. 1D-F). These cells occupy the interior of the appendage, and their shape seems to vary from cylinder to elongated ones. Vascular bundles are found in the cortical and the interior regions, their size is larger in the interior region.

The staining of the mitochondria in the parenchymatic cells of the *Victoria* carpellary appendages by a monoclonal antibody against AOX was readily detected in a non-homogeneous distribution (Fig. 2A-B). Higher concentration of AOX was found around the nucleus. Appendage sections were also stained with the two monoclonal antibodies against Cyt-actin (Fig. 2C-D) and β -tubulin subunit (Fig. 2E) to compare the distribution of both cytoskeletal proteins with that of AOX. Both antibodies stained the nucleus but they also stained the periphery of the cells.

Expression of AOX in thermogenic organs of *Victoria* and *Nelumbo*

Figure 3 shows the result of a comparison of the level of AOX (37 kDa) and a 32-kDa cross-reactive protein in the *Victoria* carpellary appendages during development. The amount of the 37-kDa protein was lower than that of the 32-kDa protein on D-day and D+1. Although the same amount of tissue was used in these experiments, no significant changes in the levels of the 37 kDa protein were detected during floral development. In the thermogenic staminal appendages of *Nelumbo* both polypeptides were detected, and the ratio between them was different than in *Victoria*. The staining of the lower band that corresponds to the 32-kDa protein was not as strong as the upper band that corresponds to the 37-kDa protein. When the presence of the reactive proteins was examined in the thermogenic sporophylls of *Encephalartos ferox* during the week of thermogenic activity both bands were detected (Fig. 4). The level of the 37-kDa AOX form was slightly higher on the 3rd day of heat-production than on D-day (1st day).

Expression of AOX in non-thermogenic flowers

In non-thermogenic flowers of *Bulbophyllum ornatissimum*, *Stanhopea wardii*, *Brugmansia* that release a pleasant strong fragrance the

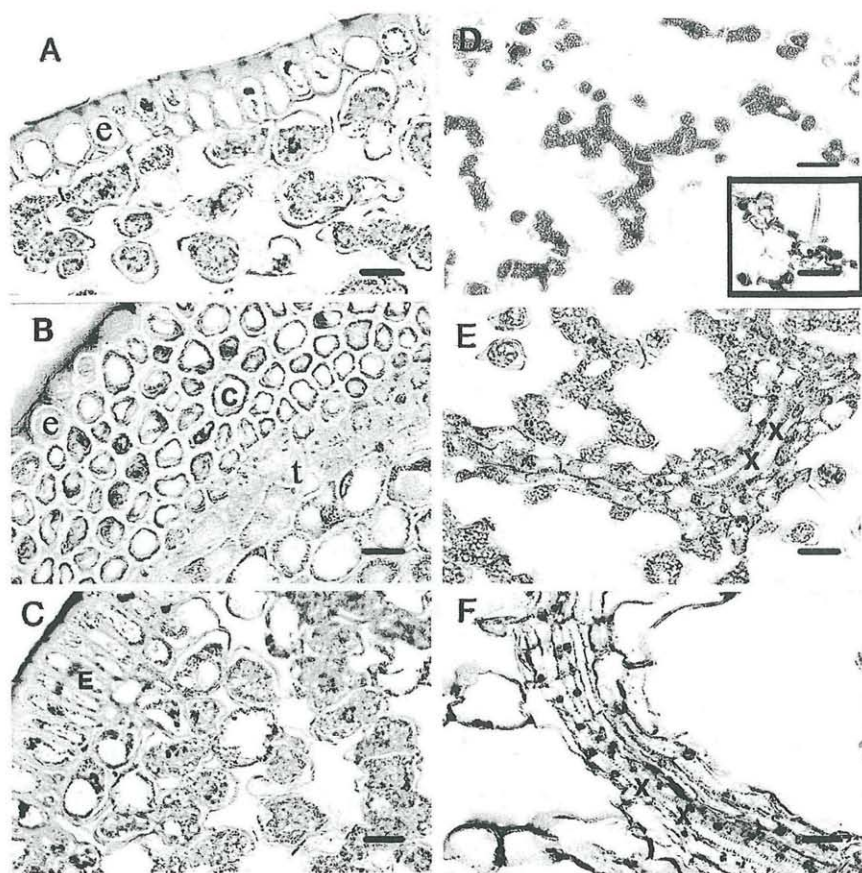


Fig. 1. The anatomy of the *Victoria cruziana* appendage. A, Cortical region composed of epidermis (e) and parenchymatic cells. B, Collenchymatic region (c) underneath the epidermis and a transitional region (t) between the collenchyma and parenchyma. These two layers are found only at the contact sites between the carpellary appendages and the base of the flower. C, Elongated epidermal cells with a thick cuticle along the appendages. D, Interior region with aerenchymatic cells loosely connected. The insert in D shows an aerenchymatic cell with a spike-like structure. E–F, Aerenchyma with xylem elements (X) and a complex cell arrangements. The sections were obtained from flowers 2 to 4 days before D-day. The appendages were longitudinally sectioned. Bars: 78 μ m.

37-kDa protein was detected at very low levels. The 37-kDa protein was also detected in *Passiflora* 'Sunburst' flowers that release an unpleasant aroma, and in *N. tabaccum* flowers that have not a noticeable odor.

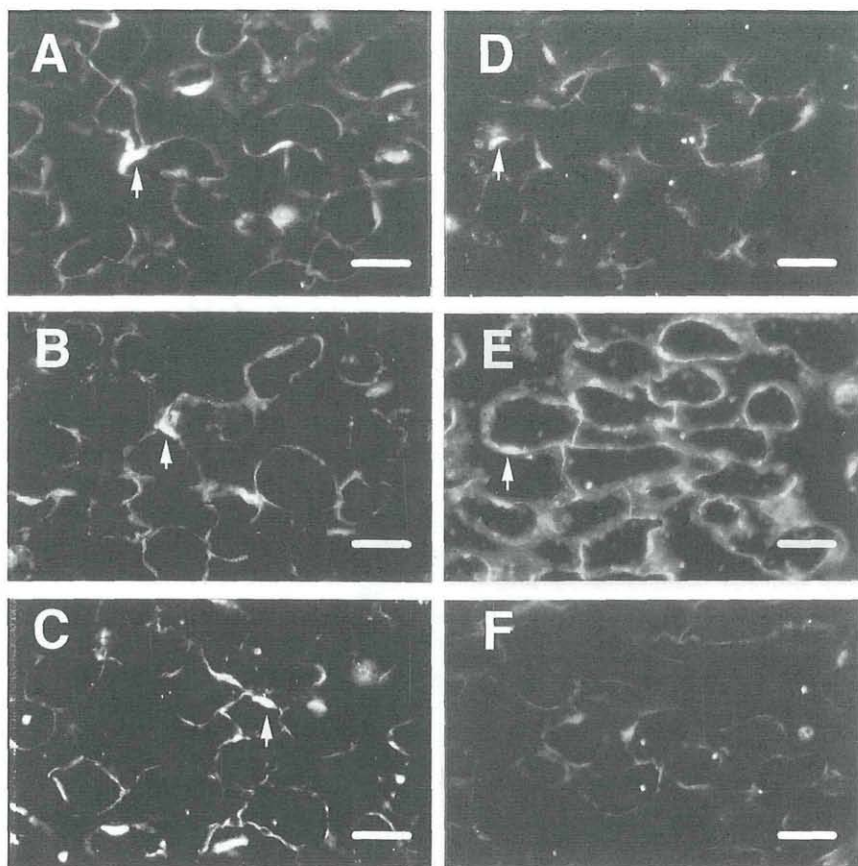


Fig 2. Immunolocalization of AOX, Cyt-actin, and β -tubulin subunit in the carpelary appendages of *Victoria* flowers. A-B, Distribution of AOX, C-D, Cyt-actin, and E, β -Tubulin subunit. F, A section stained with the secondary antibody only. Arrows point to stained nuclei. The epidermis and parenchyma are only shown because the collenchyma is strongly autofluorescent and thus cannot be examined. The sections were obtained from flowers 2 to 4 days before D-day. The appendages were longitudinally sectioned. Bars: 130 μ m.

Discussion

AOX in Thermogenic Species

The present study on 3 thermogenic species provides further support to our conclusion in the previous study (SKUBATZ & HAIDER 2001) that thermogenicity is not necessarily associated with one form of AOX. One form of AOX was detected in the thermogenic organs of *Vicotoria*, *Nelumbo*, and *Encephalartos ferox* instead of 3 forms in *S. guttatum* (SKUBATZ

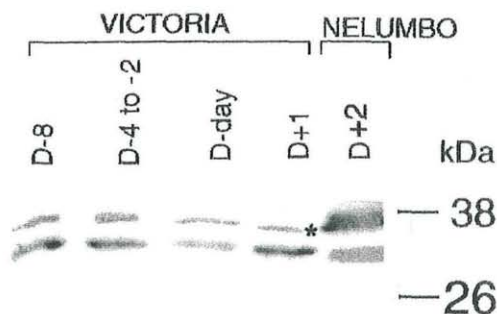


Fig. 3. Western immunoblot analysis of AOX in acetone extracts of the *Victoria* cellary appendages and *Nelumbo* staminal appendages. The lanes were loaded with 500 µg of acetone powders dissolved in SDS-buffer, loaded on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-AOX. Two major polypeptides were detected with an apparent molecular weight of 37 (asterisk) and 32 kDa, respectively. The stage of development in respect to D-day is indicated on top of each lane. The molecular weight of marker proteins is shown on the right.

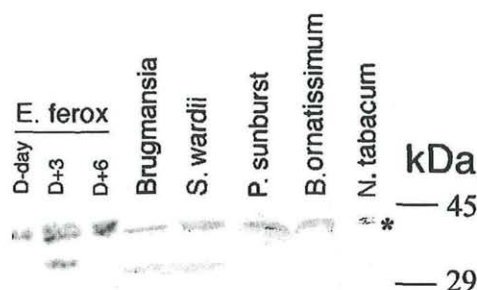


Fig. 4. Western immunoblot analysis of AOX in acetone extracts of the thermogenic sporophylls of *Encephalartos ferox* and non-thermogenic flowers. The lanes were loaded with 1.5 mg of acetone powders dissolved in SDS-buffer, loaded on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-AOX. One major polypeptide was detected in all the lanes with an apparent molecular weight of 37 kDa. The 32-kDa protein was weakly stained. The stage of development of *E. ferox* sporophylls in respect to D-day is indicated on top of the lanes. The molecular weight of marker proteins is shown on the right.

& HAIDER 2001). Unlike the case of *S. guttatum*, the level of AOX did not change during floral development of *V. cruziana*. It suggests the idea that thermogenicity is not associated with a specific form of AOX. The level of AOX in *E. ferox* also did not change during the week of heat-production. It has already been shown that any form of AOX is sufficient to obtain an active AOX in *E. coli* (KUMAR & SOLL 1992), and our data suggest that any form of AOX is also sufficient for thermogenicity.

It is interesting that all thermogenic species, except for *Victoria*, contain 1–2 µg/g fresh wt salicylic acid (SA), the natural thermogenic inducer in the *Sauromatum* appendix, in their thermogenic organs. This concentration is enough to induce heat-production in the *Sauromatum* appendix (RASKIN & al. 1990). SA in the *Victoria* carpellary appendages was not detected one day before heat-production nor during heat-production. It is, however, possible that the sensitivity of this tissue to salicylic acid is higher than the sensitivity of the *Sauromatum* appendix.

AOX in Non-thermogenic Flowers

In non-thermogenic flowers from 3 other plant families, only the 37 kDa was detected at lower levels. All the flowers examined, except for *N. tabacum*, release odorants to the atmosphere when they unfold. AOX activity reaches a peak during the opening of the fragrant orchid (HEW & al. 1978) and carnation (WULSTER & al. 1984) flowers. However, the enzyme activity is also high in unfolding flowers of *Saxifraga cernua* (COLLIER & CUMMINS 1991). In the aroid inflorescences the high level of AOX activity may allow a rapid operation of glycolysis without the adenylate control in order to produce large amounts of volatile terpenes at a specific developmental stage. In non-fragrant flowers, the activity of AOX may also allow other cell processes to continue without the mitochondrial control.

Distribution of AOX and Cytoskeletal Proteins

AOX is distributed throughout the carpellary appendages of *Victoria* suggesting that the entire tissue is thermogenic. The same conclusion was reached with other thermogenic appendices (*S. guttatum*, *Amorophallus konjac*, *Arum italicum*, *A. dioscoridis*, *Dracunculus vulgaris*). The concentrations of the mitochondria at the perinuclear region of the carpellary appendage tissue was high (SKUBATZ & HAIDER 2001) whereas Cyt-actin and β -tubulin subunit were distributed more uniformly throughout the cell. It is possible that the mitochondria were lining up on a subpopulation of Cyt-actin filaments and microtubules and at the perinuclear region. In yeast, for example, a MDM20-gene product is required for the formation of actin filaments and mitochondrial transport (HERMAN & al. 1997). The arrangement of tubulin affects the mitochondrial distribution in yeast (BERNIER-VALENTIN & al. 1983, BERGER & al. 1997, YAFFE & al. 1996). The organization of the endoplasmic reticulum (ER) can also affect the distribution of the mitochondria (RIZZOTO & al. 1998, RUTTER & RIZZOTO 2000). The ER can be attached to microtubules or other cytoskeletal proteins and consequently affect mitochondrial distribution.

General Consideration

Thermogenicity and the production of odor are intertwined in thermogenic plants. We have already done some of the ground work on the ultrastructure of odor-producing organs (SKUBATZ & al. 1993b, 1995a, 1996, SKUBATZ & KUNKEL 1999, 2000), and on changes in fatty acid composition (SKUBATZ & al. 1995b) and expression of AOX forms (SKUBATZ & HAIDER 2001) which precede the production of heat and odor in the *Sauromatum* appendix.

The study of other thermogenic species reinforced our previous data that thermogenicity is not associated with a specific AOX forms. However in non-thermogenic plants only one form is detectable. AOX expression is not induced by salicylic acid since the acid was not detected in *Victoria* flowers but AOX was. In the *Sauromatum* appendix, the appearance of AOX forms precedes the detection of SA. Mitochondria expressing AOX are clustered in the perinuclear region. This broader study of highly specialized organs demonstrates that thermogenicity cannot be explained by the variable expression and quantity of AOX.

In the present investigation we have demonstrated that: (1) AOX can be detected in the appendage tissue of *Victoria* flowers. (2). The level of AOX per gram fresh weight does not seem to change during the development of *Victoria* flowers. (3). Non-thermogenic, fragrant and non-fragrant flowers also express AOX.

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