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Expression of uncoupling protein and alternative oxidase depends on lipid or carbohydrate substrates in thermogenic plants

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Thermogenesis, in which cellular respiratory activity is considerably stimulated, requires mitochondrial uncoupling protein (UCP) in mammals and an alternative oxidase (AOX) in plants. Here, we show that the genes for both proteins are expressed in thermogenic plants, but the type correlates with the respiratory substrate. A novel gene termed *PsUCPa* encoding a variant of UCP was specifically expressed in thermogenic flowers of *Philodendron selloum*, which uses lipids as substrates. However, a gene termed *DvAOX* encoding for AOX protein was expressed in thermogenic flowers of *Dracunculus vulgaris*, which presumably uses carbohydrates as substrates. These findings suggest that cellular metabolism is a major determinant in selective expression of appropriate thermogenic genes in plants.

Keywords: thermogenic plants; *Philodendron selloum*; *Dracunculus vulgaris*; respiratory substrates; uncoupling protein; alternative oxidase

1. INTRODUCTION

Aerobic respiration—a mitochondrial process common to almost all eukaryotic organisms—involves the controlled oxidation of reduced organic substrates to carbon dioxide and water. Numerous compounds can serve as substrates for respiration, including carbohydrates, lipids, proteins and organic acids. Respiration releases a large amount of free energy not only to phosphorylate ADP (Mitchell 1961), but also to support other biological processes such as transport and metabolism (Fernie *et al.* 2004).

Interestingly, dissipation of mitochondrial electrochemical potential by uncoupling protein (UCP) or redox potential by alternative oxidase (AOX) increases futile respiration, leading to an increase in temperature of the thermogenic tissue or organ (Sluse & Jarmuszkiewicz 2002). This is demonstrated in some mammals by UCP1-based non-shivering thermogenesis within brown adipose tissue (Nicholls & Locke 1984) and in some plants by AOX-mediated thermogenesis as shown in the voodoo lily (*Sauromatum guttatum*; Raskin *et al.* 1987; Rhoads & McIntosh 1992). It is reasonable to assume that mammals use UCP for adaptive thermoregulation, because they lack the *AOX* gene. It is questionable, however, whether plants that have both *AOX* and *UCP* genes (Berthold *et al.* 2000) use only the *AOX* gene during thermogenesis.

In the present study, two arum lilies, *Philodendron selloum* and *Dracunculus vulgaris*, were chosen for examination of the expression profiles for *UCP* and *AOX* genes in relation to respiratory substrate. Measurements of respiratory exchange ratios near 1.0 have shown that carbohydrates are the respiratory substrates in *Arum maculatum* (James & Beevers 1950; ap Rees *et al.* 1976, 1977) and *Symplocarpus foetidus* (Seymour & Blaylock 1999; Ito *et al.* 2003*a*,*b*). However, thermogenic *Philodendron selloum* switches to lipids, because its respiratory exchange ratio becomes 0.83 (Seymour *et al.* 1984) and $^{13}C : {}^{12}C$ ratio in respired CO_2 decreases (Walker *et al.* 1983). In this species, the lipids are in the form of glyoxysome-like bodies in the cytoplasm of the thermogenic sterile male florets (Walker 1980), and calorimetric studies show that the energy for thermogenesis is not imported during flowering (Seymour *et al.* 1983). Although the respiratory exchange ratio has not been measured in *D. vulgaris*, calorimetric studies show that it imports substrates into its thermogenic organs (Seymour & Schultze-Motel 1999). Because hydrophobic lipids are scarcely translocated through xylem sap (Fisher 2000), we assume that soluble carbohydrates support thermogenesis in this species.

2. MATERIAL AND METHODS

(a) *Plants and thermometry*

Experiments were performed during the flowering season of *Philodendron selloum* and *Dracunculus vulgaris* in suburban Adelaide, South Australia in November 2001. Thermal images were obtained using an infrared colour LSD camera (TVS-600; Nippon Avionics Ltd, Tokyo, Japan) equipped with a 35 mm lens (Ito *et al.* 2003*a*,*b*; Seymour *et al.* 2003).

(b) *cDNA cloning and expression analysis*

For the isolation of cDNAs for *AOX*s and *UCP*s, total RNAs were first isolated from flash-frozen, pulverized thermogenic tissues according to the previous reports (Ito *et al.* 1999; Ito 1999). Oligo (dT)-primed first-strand cDNAs were prepared from 1 μg samples of total RNAs using the Advantage RT-for-PCR Kit (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. Detailed procedures for the polymerase chain reaction (PCR) cloning and expression analyses are described in the electronic supplementary material.

3. RESULTS

The thermogenic sterile male flowers of *P. selloum* reached 43 °C when the ambient temperature was 15 °C (figure 1*a*). Expression analysis of isolated cDNAs encoding *UCP* and *AOX*, termed *PsUCPa* and *PsAOX*, revealed that the expression of *PsUCPa* was specifically detected only in thermogenic sterile male flowers (figure 1*b*), whereas that of *PsAOX* was found in all tissues examined, including pre-thermogenic, post-thermogenic and thermogenic sterile male flowers. Control experiments without a reverse-transcriptase reaction confirmed that there was no genomic DNA contamination in either RNA (figure 1*b*).

In *D. vulgaris*, the temperature of the thermogenic male flowers increased to as high as 23 °C, even when the ambient air temperature was 8 °C (figure 1*c*).

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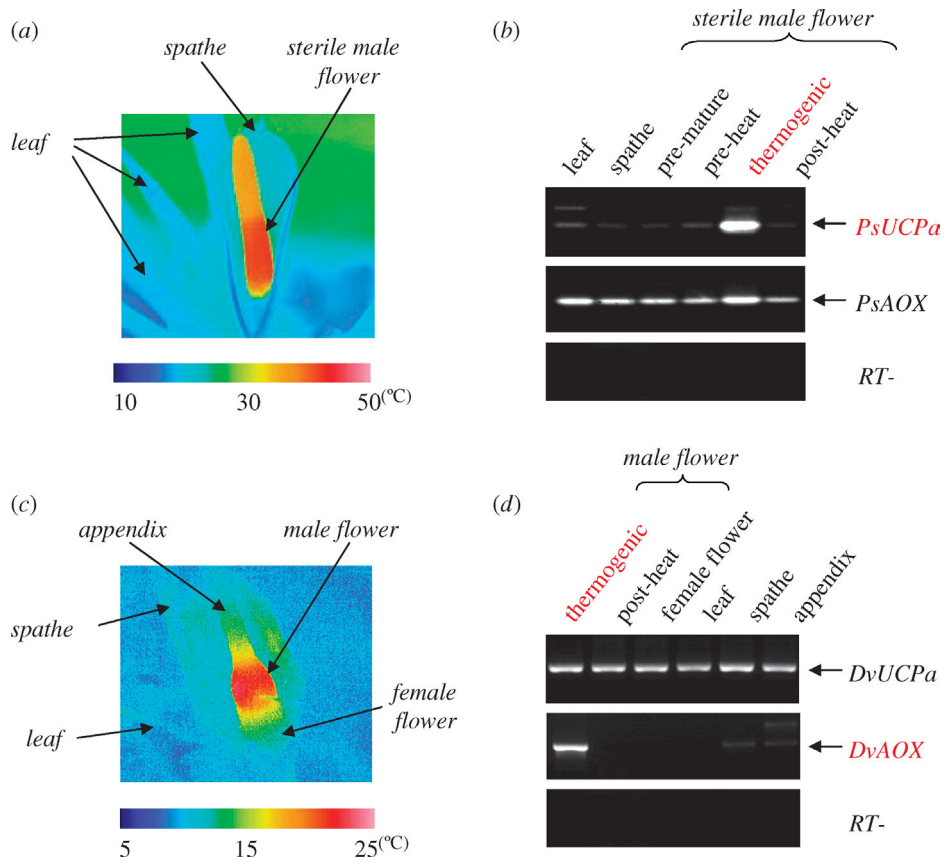

Figure 1. Thermogenesis and expression patterns of genes for uncoupling protein (UCP) and alternative oxidase (AOX) in *Philodendron selloum* and *Dracunculus vulgaris*. (*a*) Infrared thermal analysis of thermogenesis in a sterile male flower of *P. selloum*. (*b*) Expression patterns of *PsUCPa* and *PsAOX* from *P. selloum* in leaves, spathes and pre-thermogenic, thermogenic and post-thermogenic sterile male flowers. (*c*) Infrared thermal analysis of male flower-specific thermogenesis in *D. vulgaris*. (*d*) Expression patterns of *DvUCPa* and *DvAOX* from *D. vulgaris* in thermogenic and post-thermogenic male flowers, female flowers, leaves, spathes and appendices. RT-PCR products using gene-specific primers were visualized by Southern hybridization with probes of full-length *UCP* or *AOX* for each species. Reactions without reverse transcriptase (RT−) were also carried out to confirm the absence of DNA contamination in each RNA sample.

cDNA cloning of the expressed genes for UCP and AOX led to their identification, termed *DvUCPa* and *DvAOX*, respectively. Expression of *DvAOX* was specific to the thermogenic male flowers, whereas that of *DvUCPa* was ubiquitous in thermogenic and post-thermogenic male flowers, female flowers, leaves, spathes and appendices (figure 1*d*). Again, it was confirmed that there was no genomic DNA contamination in any of the RNA (figure 1*d*). These results suggest the significance of *AOX* gene expression during thermogenesis in plants.

The activities of AOX have been shown to be affected by redox modulation and α-keto acid activation (Vanlerberghe *et al.* 1998). A cysteine residue termed Cys_I, which has been shown to be required for such a regulation (Berthold *et al.* 2000), was conserved in the amino acid sequences in both DvAOX and PsAOX (data not shown). PsUCPa, however, lacked the sixth transmembrane spanning region and the purine nucleotide-binding domain (PNBD; supplementary figure 1), which potentially shows an inhibitory effect on its uncoupling activity (Bouillaud *et al.* 1994). On the contrary, like the UCPs of other plants and mammals, DvUCPa contained six transmembrane domains and a conserved PNBD (supplementary figure 1).

4. DISCUSSION

Our results clearly undermine the common dichotomy that thermogenesis depends on UCPs in mammals and AOXs in plants. *P. selloum* and *D. vulgaris*, two thermogenic plants used in this study, apparently express both *UCP* and *AOX* genes in their thermogenic tissues simultaneously. However, the specificity of the expression patterns of *UCP* and *AOX* seems to depend on the substrates for thermogenesis; that is, *PsUCPa* for lipids and *DvAOX* for carbohydrates.

Because free fatty acids (FFA) activate UCPs in plant mitochondria (Sluse *et al.* 1998), the lipid-based thermogenesis found in *P. selloum* seems to confer the advantage of using its metabolites including FFA to activate UCPs within the cells. This idea is also supported by the fact that mammalian non-shivering thermogenesis, which is stimulated by FFA-mediated UCP1 activities, uses lipids as a major respiratory substrate in brown adipocytes (Lowell & Spiegelman 2000). Furthermore, our finding of a novel gene encoding for PsUCPa, which lacks both the sixth transmembrane region and the purine-nucleotide binding domain, suggests that PsUCPa would exert its uncoupling function without any negative control by the purine nucleotides. It should also be noted that UCP3S, a mammalian homologue of PsUCPa,

which also lacks both the sixth transmembrane region and the purine-nucleotide binding domain, has been shown to exhibit more active uncoupling activities than those of UCP3L, the long type of UCP3, in a yeast heterologous expression system (Hinz *et al.* 1999). Therefore, it is probable that PsUCPa, a short variant of the UCP family, supports the high level of mitochondrial respiration in the sterile male flowers of *P. selloum*.

In our study, the thermogenic tissue-specific expression of the *AOX* gene was found in *D. vulgaris*, which is consistent with the previously proposed view of the importance of *AOX* gene expression in thermogenic tissues in plants (Rhoads & McIntosh 1992). However, expression of a gene encoding for DvUCPa, which contains six transmembrane-spanning regions and the purine-nucleotide binding domain, was found to be ubiquitous. Because similar ubiquitous expression of a gene encoding HmUCPa, which belongs to a longer type of the UCP family, has been found in the thermogenic dead horse arum *Helicodiceros muscivorus* (Ito *et al.* 2003*a*,*b*; Seymour *et al.* 2003), it seems that UCPs with six transmembrane regions are not primarily involved in tissue-specific thermogenesis in plants.

Our present results further suggest that carbohydrate-based thermogenesis, which requires specific *AOX* gene expression, has been developed to use its metabolic intermediates such as pyruvate to stimulate AOX activity (McDonald *et al.* 2002). Because pyruvate is synthesized as an end-product of glycolysis and further oxidized in the TCA cycle within mitochondria, pyruvate would be a one of the ideal activators of AOX when glycolysis is a major metabolic pathway during thermogenesis.

The two species of this study exhibit increasing respiration rates with decreasing ambient and tissue temperatures, a pattern termed 'thermoregulation' (Nagy *et al.* 1972; Seymour *et al.* 1984; Seymour & Schultze-Motel 1999). Clear upregulation of UCP and AOX suggests that these enzymes may play a crucial role in thermoregulation in plants. However, the two species exhibit a distinct diversity in their respiratory substrates and specificity of thermogenic genes, so it is important to elucidate the detailed molecular mechanisms of tissue- and substrate-specific gene expression in thermogenic plants.

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