Journal of Experimental Botany, Vol. 59, No. 3, pp. 705–714, 2008
doi:10.1093/jxb/erm333 Advance Access publication 4 February, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Synchronicity of thermogenic activity, alternative pathway respiratory flux, AOX protein content, and carbohydrates in receptacle tissues of sacred lotus during floral development

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Received 11 November 2007; Accepted 28 November 2007

Abstract

The relationships between heat production, alternative oxidase (AOX) pathway flux, AOX protein, and carbohydrates during floral development in *Nelumbo nucifera* (Gaertn.) were investigated. Three distinct physiological phases were identified: pre-thermogenic, thermogenic, and post-thermogenic. The shift to thermogenic activity was associated with a rapid, 10-fold increase in AOX protein. Similarly, a rapid decrease in AOX protein occurred post-thermogenesis. This synchronicity between AOX protein and thermogenic activity contrasts with other thermogenic plants where AOX protein increases some days prior to heating. AOX protein in thermogenic receptacles was significantly higher than in post-thermogenic and leaf tissues. Stable oxygen isotope measurements confirmed that the increased respiratory flux supporting thermogenesis was largely via the AOX, with little or no contribution from the cytochrome oxidase pathway. During the thermogenic phase, no significant relationship was found between AOX protein content and either heating or AOX flux, suggesting that regulation is likely to be post-translational. Further, no evidence of substrate limitation was found; starch accumulated during the early stages of floral development, peaking in thermogenic receptacles, before declining by 89% in post-thermogenic receptacles. Whilst coarse regulation of AOX flux occurs via protein synthesis, the ability to thermoregulate probably involves precise regulation of AOX protein, most probably by effectors such as α-keto acids.

Key words: Alternative oxidase, alternative pathway respiration, *Nelumbo nucifera*, plant thermogenesis, starch.

Introduction

Although body heat is usually associated with birds and mammals, some plants are able to produce heat in their flowers. Since the first report of thermogenesis in *Arum* by Lamarck in 1778 (cited in Vanlerberghe and McIntosh, 1997), thermogenic activity has been reported in the reproductive organs of a diverse range of plant taxa including the Cycadaceae, the basal angiosperm family, Nymphaceae, the monocot family, Araceae, and the eudicot family, Nelumbonaceae. Heat production is assumed to be of importance in the pollination biology of these species, by scent volatilization (Meeuse, 1975) and/or by the provision of a thermal reward to insect pollinators (Seymour, 1997). Thermogenesis may also prevent low temperature damage (Knutson, 1974) or ensure an optimum temperature for floral development (Seymour and Schultze-Motel, 1998). The capacity for respiratory heat production varies markedly among thermogenic species, ranging from 2–3 °C to almost 40 °C above ambient, for example, in the inflorescences of *Philodendron selloum* (Nagy *et al*., 1972). Respiratory heat production in most species is unregulated; however, in a small number of species, such as *P. selloum*, *Symplocarpus foetidus*, and the sacred lotus *Nelumbo nucifera*, heat production is regulated so that a constant temperature is maintained across a wide range of ambient temperatures (Seymour, 2001). These thermogenic species are thus capable of thermoregulation, sensing external temperature changes, and generating heat at the cellular level.

The alternative respiratory pathway has long been assumed to be the source of heating in thermogenic plants due to the strong correlation between heat production and cyanide-resistant respiration, where the alternative oxidase

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(AOX) is the terminal electron acceptor (Nagy *et al*., 1972; Meeuse and Raskin, 1988). Inhibiting the cytochrome oxidase (COX), however, shunts all electrons to the AOX and thus does not allow accurate quantification of actual *in vivo* AOX flux. In contrast, stable oxygen isotope discrimination techniques enable quantification of flux in the absence of inhibitors, and are now the accepted methodology (Millar *et al*., 1995; Ribas-Carbo *et al*., 1995; Robinson *et al*., 1995). To date, the role of the AOX in heat production *in vivo* has only been confirmed for one species, the sacred lotus (Watling *et al*., 2006), and remains to be demonstrated for other thermogenic species. The AOX is a nuclear-encoded protein that is present as a homodimer in the inner mitochondrial membrane of all plants (Vanlerberghe and McIntosh, 1997). In addition, the AOX is also present in fungi, protists, and many animal lineages (McDonald and Vanlerberghe, 2006). Electron transport to the AOX branches from the main mitochondrial electron transport chain at ubiquinone and, in contrast to COX, is largely uncoupled from ATP production and so energy is released as heat (Moore and Siedow, 1991). In addition to the AOX, it is also possible that heat production in plants could result from the activity of plant uncoupling proteins (pUCPs), which would result in high fluxes through the COX pathway (Ito, 1999). In most plants, pUCPs are present in much lower quantities than the mammalian UCP1, which is responsible for non-shivering thermogenesis in brown adipose tissue (Vercesi *et al*., 2006). However, in thermogenic skunk cabbage, *S. foetidus*, two pUCPs, SfUCPA and SfUCPB, are expressed in spadix tissue with higher expression of the SfUCPB form, which lacks the fifth transmembrane domain (Ito, 1999). It may be that both AOX and pUCPs play a role in thermogenesis, depending on the species. In the sacred lotus, however, alternative pathway flux increases significantly with heating and accounts for up to 75% of electron transport in the hottest flowers. In contrast, there is no relationship between COX flux and heating in thermogenic lotus receptacles (Watling *et al*., 2006), suggesting that pUCPs do not play a significant role in this species.

The protogynous flowers of sacred lotus regulate their temperature with remarkable precision (between 30 °C and 35 °C) against fluctuations at ambient temperature from 8 °C to 45 °C during the 2–4 d of floral receptivity (Seymour and Schultze-Motel, 1998). These and other thermoregulating plant tissues achieve constant temperatures without the complex neural and hormonal systems found in some animals. Thus, thermoregulation in these plants must occur at the cellular level, but almost nothing is known about this regulation. In particular, in sacred lotus, it is still unknown whether AOX regulation occurs at the level of gene expression or is post-translational. In the non-regulating thermogenic plants that have been examined, AOX protein synthesis precedes thermogenic activity by several days, suggesting post-translational control of AOX (Rhoads and McIntosh, 1992; Chivasa *et al*., 1999; Skubatz and Haider, 2004). Salicylic acid (SA) treatment induces both AOX gene expression and thermogenesis in pre-thermogenic tissues of *Sauromatum guttatum* and *Arum* lilies (Raskin *et al*., 1987; Rhoads and McIntosh, 1992); however, the role of SA in post-translational regulation of the AOX is unclear. In contrast, regulation of AOX activity in isolated mitochondria from non-thermogenic plants has been investigated extensively (Lambers *et al*., 2005; for a review, see Millenaar and Lambers, 2003). Expression of AOX increases in non-thermogenic plants exposed to high and low temperatures, water stress, phosphate deficiency, SA, herbicides, and inhibitors of the cytochrome pathway (Vanlerberghe and McIntosh, 1992; Aubert *et al*., 1997; Finnegan *et al*., 1997; Lennon *et al*., 1997; Ribas-Carbo *et al*., 2000, 2005*a*; Gonzalez-Meler *et al*., 2001; Huang *et al*., 2002; Zottini *et al*., 2002; Gaston *et al*., 2003; Rachmilevitch *et al*., 2007); however, changes in AOX protein levels are not always correlated with activity *in vivo* (Lennon *et al*., 1997; Millenaar *et al*., 2001; Gaston *et al*., 2003; Guy and Vanlerberghe, 2005; Ribas-Carbo *et al*., 2005*a*; Vidal *et al*., 2007). This may be because a number of factors influence post-translational regulation of AOX activity, including the redox state of the ubiquinone pool (Dry *et al*., 1989), a regulatory disulphide bond that modulates the redox state of AOX (Umbach and Siedow, 1993; Umbach *et al*., 1994; Vanlerberghe *et al*., 1999), and α-keto acids such as pyruvate (Millar *et al*., 1993) that can further increase the activity of the reduced form (Rhoads *et al*., 1998; Vanlerberghe *et al*., 1999). Based on respiratory quotient studies, the respiratory substrate in lotus appears to be carbohydrate (Seymour and Schultze-Motel, 1998); however, substrate limitation of AOX during thermogenesis has yet to be investigated.

Flowers of the sacred lotus have a developmental sequence during which pre-thermogenic, thermogenic, and post-thermogenic stages can be clearly distinguished. This well-defined floral sequence was used to investigate the extent to which thermogenesis in sacred lotus is regulated by AOX protein synthesis, both throughout the developmental sequence and during the thermogenic stages. It was hypothesized that coarse regulation would occur across the developmental sequence, such that AOX protein levels would be significantly higher in thermogenic stages than in non-thermogenic stages, and that regulation during thermogenesis would be post-translational, i.e. AOX protein levels would not change in relation to the degree of heating. In this study, respiratory flux, the contribution of AOX and COX to respiration, AOX protein content, and the availability of respiratory substrates in relation to heating were characterized during floral development in sacred lotus.

Materials and methods

Plant material

Lotus flowers (*N. nucifera* Gaertn.) were collected from an outdoor pond in the Adelaide Botanic Gardens, South Australia, between December and February, 2005–2006 and 2006–2007. Flowers were categorized into stages according to Seymour and Schultze-Motel (1998) with the addition of stages 0 and 4 to include the non-thermogenic stages of floral development. The sequence of development from stage 0 through to stage 4 is completed in 4–5 d. The five stages, shown in Fig. 1, are: small green pre-thermogenic bud (stage 0); larger bud with the petals closed and pointed (stage 1); petals open by 2–12 cm, immature stamens closely appressed to the receptacle (stage 2); petals horizontal revealing mature stamens (stage 3); and petals and stamens senesce and abscize, leaving a greening, post-thermogenic receptacle (stage 4). Thermogenesis occurs during stages 1–3, with maximum heating during stage 2. The temperature of each receptacle (t_r) was measured with a needle thermocouple and a Fluke model 52 digital thermometer. The temperature of a nearby non-thermogenic bud (t_n) was also measured at this time. For laboratory respiration and mass spectrometry measurements, flower stems were cut underwater, ~15 cm below the flower base, and were taken back to the laboratory in containers of pond water. Receptacles for mitochondrial protein isolation were placed on ice and immediately taken to the laboratory. A portion of each receptacle was excised for carbohydrate analysis, frozen in liquid nitrogen, and stored at –80 °C until analysis.

Respiration and discrimination analysis

Discrimination during respiration was determined in freshly harvested lotus receptacles of each stage. The steady-state flux of electrons through the cytochrome and alternative pathways in lotus receptacles was determined using the oxygen isotope technique established by Guy *et al.* (1989) and subsequently developed to measure the gas phase on-line (Robinson *et al.*, 1992, 1995). A full review of the theoretical and practical aspects of this technology can be found in Ribas-Carbo *et al.* (2005*b*).

Respiration rates and differential uptake of oxygen stable isotopes were measured simultaneously in six sequential samples taken from the gas phase surrounding the respiring receptacle tissue using the method of Robinson *et al.* (1995) as described by Watling *et al.* (2006). Small sections (~1.5 cm^3) of freshly harvested lotus receptacle tissue were weighed and placed inside a 25 ml gas-tight syringe. Air samples (100 µl) were withdrawn from the syringe at ~6 min intervals and injected into a GC-MS system (NA 1500 Carlo-Erba Instrumentazione, Italy; Optima, Micromass, UK). The fraction of O_2 remaining and its isotopic composition were measured, and the isotopic discrimination factors (D) and partitioning of electrons between the cytochrome and alternative pathways were calculated essentially as previously described (Guy *et al.*, 1989; Henry *et al.*, 1999). The r^2 of all unconstrained linear regressions between $-\ln f$ and $\ln (R/R_o)$, with a minimum of six data points, was at least 0.991.

To establish the discrimination end-points for the alternative (Δ_a) and cytochrome (Δ_c) oxidases, receptacle tissue was vacuum infiltrated with either 16 mM KCN or 25 mM SHAM [made from a 1 M stock solution in 0.5% dimethylsulphoxide (DMSO)], respectively, prior to measurement. The end-points obtained (Δ_c=17.0±2.0‰ and Δ_a=26.5±1.3‰), based on stage 1 and 2 receptacles, were then used to calculate the flux through the alternative and cytochrome pathways in uninhibited tissues as described in Ribas-Carbo *et al.* (2005*b*). The reproducibility of measurements of O_2 concentration and fractionation was determined using air samples withdrawn from the empty syringe and was ±2% and ±0.01%, respectively.

Isolation of mitochondrial proteins

Isolation of washed mitochondrial protein was based on the method of Day *et al.* (1985) with minor modifications. Briefly, receptacle tissue was blended with cold grinding buffer [0.4 M mannitol, 25 mM MOPS-KOH, pH 7.2, 2 mM EDTA, 10 mM KH_2PO_4, 1% (w/v) PVP-40, 20 mM ascorbic acid, 4 mM cysteine, 2 mM pyruvate, 1% (w/v) bovine serum albumin (BSA), and 2% (w/v) polyvinylpolypyrrolidone], filtered through two layers of Miracloth (Merck, Australia), and centrifuged at 1000 *g* for 10 min. The supernatant was then centrifuged for 20 min at 12 000 *g*. After resuspension of the pellet in cold washing buffer [0.4 M mannitol, 25 mM MOPS-KOH, pH 7.2, 2 mM pyruvate, and 0.1% (w/v) BSA], the sample was centrifuged at 1000 *g* for 10 min. The supernatant was decanted and spun at 12 000 *g* for 20 min. The mitochondrial fraction (pellet) was washed again with cold washing buffer, spun at 1000 *g* for 10 min, and then the supernatant was spun for a further 20 min at 12 000 *g*. The final mitochondrial protein pellet was resuspended in ~250 µl of washing buffer. Protein concentration was estimated by the method of Bradford (1976) with BSA as the standard.

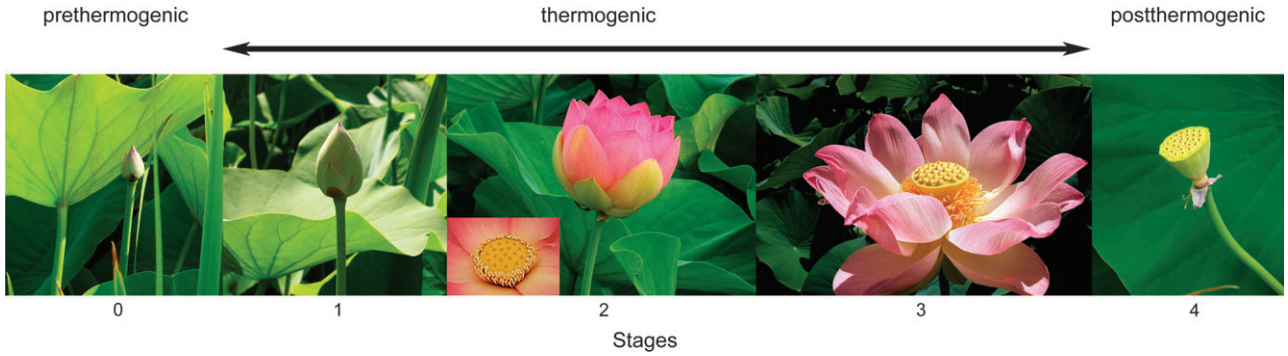

Fig. 1. Developmental sequence for sacred lotus. Stage 0, small green pre-thermogenic bud; stage 1, larger bud, petals closed and pointed, turning pink at the tip; stage 2, petals fully pink, open between 2 cm and 12 cm, revealing the receptive stigmas and the immature stamens which are closely appressed to the receptacle (inset); stage 3, petals horizontal, mature stamens falling away from the receptacle; and stage 4, petals and stamens senesce and abscize, leaving a post-thermogenic yellow/green receptacle. The majority of heat is produced by the large, central receptacle. Heating commences in stage 1, and continues through stages 2 and 3 (see Fig. 2A).

SDS–PAGE and immunoblot analysis

Equal amounts of mitochondrial protein and sample buffer [100 mM TRIS-HCl, pH 6.8, 2% (w/v) SDS, 20% (w/v) glycerol] were mixed and boiled for 10 min. Separation of proteins by SDS–PAGE analysis was performed by the method of Laemmli (1970) using 15% acrylamide resolving and 4% acrylamide stacking gels and the Mini-PROTEAN 3 System (Bio-Rad Laboratories, Richmond, CA, USA). An equal amount of mitochondrial protein (15 μg) was loaded into each lane. The separated proteins were transferred to a PVDF membrane (Millipore Immobilon 0.45 μm) using methods similar to those described by Harlow and Lane (1988). Briefly, after electrophoresis, the gels were washed for 10 min in transfer buffer containing 23 mM TRIS, 192 mM glycine, 3.5 mM SDS, and 20% (v/v) MeOH, and transferred to a PVDF membrane using a Mini Trans-Blot Cell (Bio-Rad). The transfer occurred at a constant current of 0.35 A for 1 h in transfer buffer. After transfer the membrane was washed in TRIS-buffered saline-Tween (TBST) buffer [137 mM NaCl, 2.5 mM KCl, 25 mM TRIS-HCl, pH 7.4, and 1% (v/v) Tween-20] for 10 min. The membrane was covered in 5% skim milk TBST and rocked gently for 2 h to block non-specific binding. Western blot analysis was used to detect AOX, COX, and porin (an outer membrane voltage-dependent anion channel protein not associated with the electron transport chain) abundances using a 1:500 dilution of the monoclonal antibody 'AOA' raised against *S. guttatum* AOX (Elthon *et al*., 1989), a 1:1000 dilution of anti-COXII (Agrisera) raised against subunit II of cytochrome *c* oxidase, and a 1:10 000 dilution of monoclonal antibody reacting with porin (PM035, Dr T Elthon, Lincoln, NE, USA). To check for the presence of pUCP, membranes were probed using pUCP antibodies raised against *S. foetidus* (Ito, 1999) and soybean (Considine *et al*., 2001). After primary antibody incubation, the membrane was washed three times in TBST for 5 min and incubated for 1 h in a 1:2000 dilution of secondary antibody with a horseradish peroxidase (HRP) conjugate (Pierce goat anti-mouse HRP or Pierce goat anti-rabbit HRP). The protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) by a Fluorchem 8900 Gel Imager (Alpha Innotech, San Leandro, CA, USA) with subsequent analysis using Fluorchem IS-8900 software (Alpha Innotech). A serial dilution was carried out to ensure there was a linear relationship between the amount of protein loaded and densitometry results. AOX and COX protein levels are expressed relative to porin throughout.

Soluble carbohydrate and starch determination

Receptacle tissue from all developmental stages was assayed for soluble carbohydrates and starch using a method similar to that of Scholes *et al*. (1994) with modifications by Caporn *et al*. (1999). Soluble carbohydrates were extracted by heating wedges of receptacle tissue (0.08–0.2 g FW) in aliquots of 80% ethanol (solvent:tissue, 80:1, v/w) at 70 °C for 10 min. A subsample of the total extract was dried under vacuum, and resuspended in 1 ml of distilled water. Glucose (glc), fructose (fru), and sucrose (suc) were determined sequentially following the addition of hexokinase (0.5 U; Roche 1426362), phosphoglucose isomerase (0.6 U; Roche 127396), and invertase (8 U; Sigma I-4504), respectively. Absorbance was measured at 340 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Starch was determined from the remaining tissue which was ground in H_2O, autoclaved, and incubated with α-amylase (20 U; Sigma A-3176) and amyloglucosidase (14 U; Fluka 10115) at 37 °C for 4 h to convert starch to glc. An aliquot was then assayed as for glc above.

Statistical analysis

Changes in respiratory pathways and relative AOX and COX proteins with respect to developmental stage were investigated by one-way analysis of variance (ANOVA) using JMP 5.1 (SAS Institute Inc.). Where ANOVA revealed significant interactions, Tukey HSD *post hoc* tests were applied in order to identify significantly different means. Data were tested for normality using the Shapiro–Wilk W test. Bartlett's test was applied to ensure homogeneity of variances. Respiratory and AOX flux data were log or arc sin transformed, respectively, to satisfy the assumptions of ANOVA. Starch and soluble carbohydrate data did not meet the assumptions of ANOVA and were analysed using the non-parametric Kruskal–Wallis test using JMP 5.1, and multiple comparisons were performed using the Nemenyi test (Zar, 1999). Significant differences are at $P \leqslant 0.05$, unless otherwise stated.

Results

Respiratory flux and temperature across lotus developmental stages

Five distinct morphological phases were identified in the development of *N. nucifera* flowers (Fig. 1), that are similar to those previously reported (Seymour and Schultze-Motel, 1998). Thermogenic activity was detected in stages 1–3, but not in either stage 0 (pre-thermogenic) or stage 4 (post-thermogenic) receptacles. Mean receptacle heating increased up to stage 2 and then decreased to stage 4. Significantly more heating was observed in stage 2 receptacles than pre- and post-thermogenic receptacles (Fig. 2A, ANOVA, $F_{4,58}=10.5498$, $P<0.0001$).

Total respiratory flux in receptacles increased from stage 0 to stage 2, followed by a decrease to stage 4 (Fig. 2B). Mean total respiration in stage 2 receptacles (0.0621 μmol O_2 g $FW^{-1}s^{-1}$) was 2–3 times higher than that in stages 0 and 4 (ANOVA, $F_{4,59}=15.9064$, $P<0.0001$). Mean total respiration in stage 1 was significantly higher than in stage 4 receptacles. Mean flux through the AOX, measured using stable oxygen isotopes, showed a similar pattern of response across development to that observed for total respiration (Fig. 2B). Mean AOX flux increased >4-fold between stages 0 and 1, remained significantly high during stage 2, and then decreased to stage 4 (ANOVA, $F_{4,59}=3.6883$, $P=0.0096$). Mean AOX flux was 19% of total respiration in stage 0, increasing to >40% in stage 2 (Table 1). By stage 4 the proportion of the respiratory flux attributed to AOX had declined to 34%. Across all stages, the contribution of AOX flux to total respiration ranged from 0% to 93%, with the largest contributions and range in stage 2 receptacles (Table 1). In contrast, flux through the COX pathway exhibited much less variation during floral development than AOX (cf. Fig 2B), although COX flux declined significantly between stages 2 and 4 (ANOVA, $F_{4,59}=6.41$, $P=0.0002$).

There was a significant positive relationship between the amount of heating in thermogenic lotus receptacles (t_r–t_n) and both total respiration ($r^2=0.47$, $P=0.0012$, data not shown) and AOX flux ($r^2=0.43$, $P<0.0001$, Fig. 3A), but not with COX flux ($r^2=0.16$, $P>0.05$, data not shown).

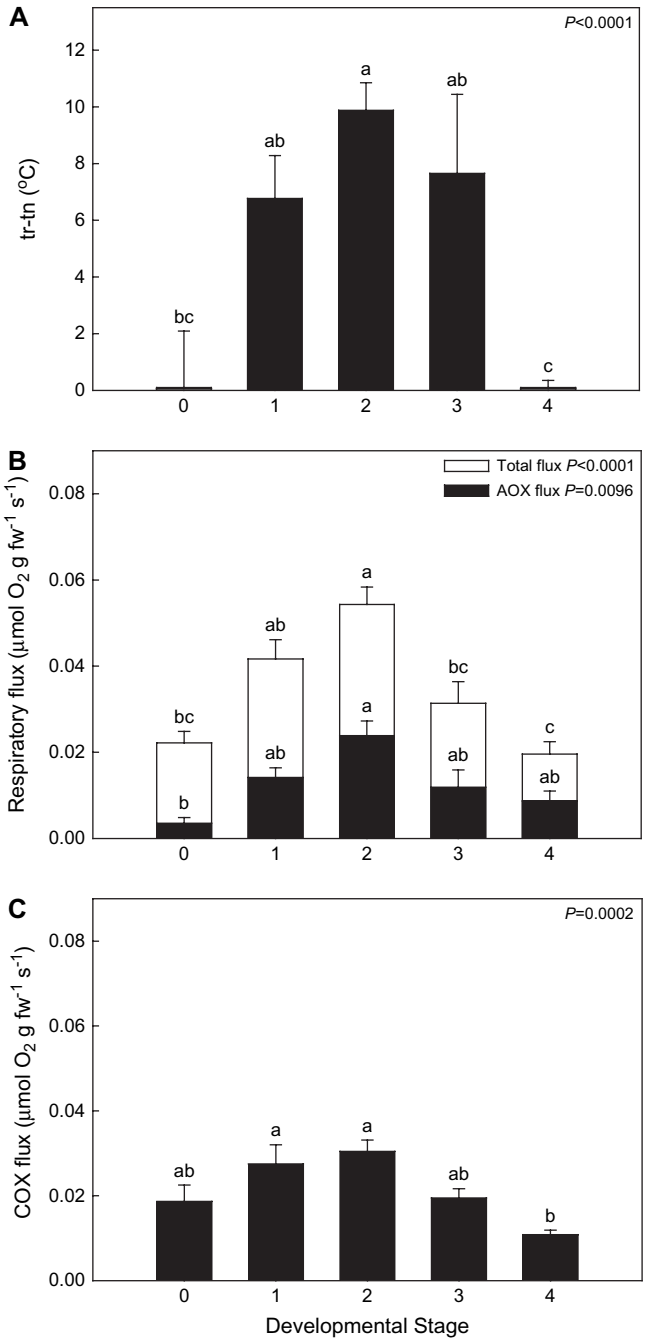

Fig. 2 Changes in (A) extent of heating, (B) total respiratory flux (open bars), and flux through the alternative pathway (AOX, black bars), and (C) flux through the cytochrome (COX) pathway, in sacred lotus receptacle tissue throughout the developmental sequence. Heating was determined in the field as the difference in temperature between the measured receptacle (t_r) and a nearby, non-thermogenic receptacle (t_n). Significant differences are indicated by different letters. Data are means ±SE, n=5–30.

Table 1. *Mean proportion and range (%) of respiratory flux through the alternative (AOX) pathway of sacred lotus receptacles for each developmental stage*

Stage	Mean contribution of AOX to total flux (% ±SE)	Range of AOX contributions to flux (%)
0 (pre-thermogenic)	19.1±7.5	1–42
1	39.8±7.2	12–74
2	41.1±4.2	11–93
3	37.5±8.8	6–69
4 (post-thermogenic)	33.6±6.2	0–63

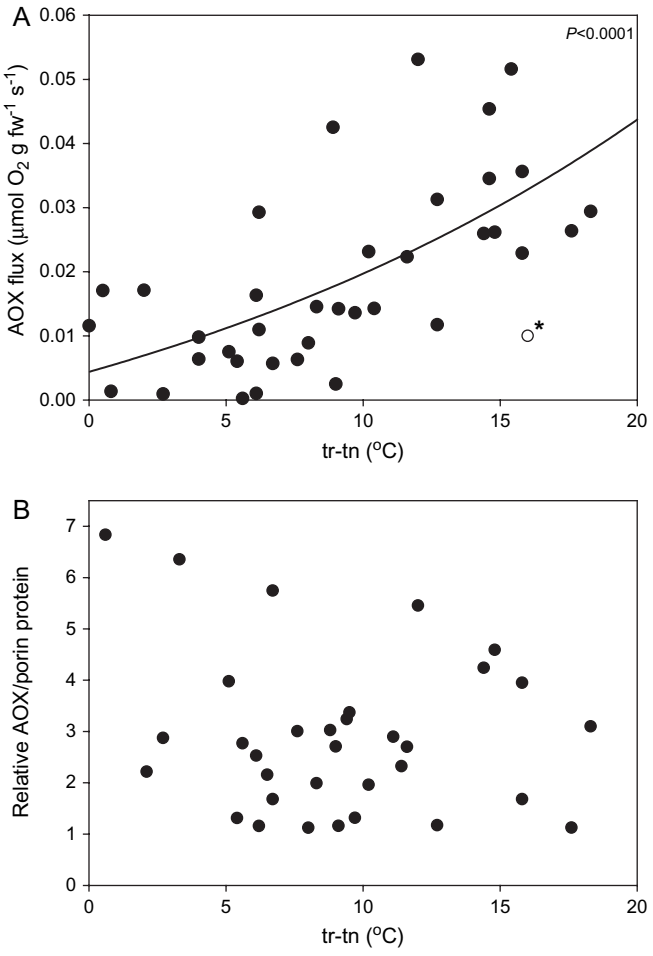

Fig. 3. Relationship between the degree of heating in thermogenic lotus receptacle (stages 1–3) and (A) AOX flux through the pathway, and (B) the relative amount of AOX protein. Regressions, where significant, are plotted (AOX flux, r^2=0.43, y=0.269e$^{0.451x}$–0.0225, P <0.0001). One outlier (*) is excluded from the regression in (A) (if included, AOX flux, r^2=0.37, y=0.0.0371e$^{0.0333x}$–0.0225, P <0.0003). Heating was determined in the field as the difference in temperature between the measured receptacle (t_r) and a nearby, non-thermogenic receptacle (t_n).

Synthesis of AOX protein during development in lotus receptacles

The presence of an ~32 kDa protein was detected in receptacle tissues of *N. nucifera* using the AOA monoclonal antibody against the AOX protein (Fig. 4A).

Relative AOX (i.e. AOX/porin) increased 10-fold in receptacles between stages 0 and 1, remained high during thermogenic stages 1–3, and then decreased significantly in post-thermogenic receptacles (Fig. 4B). Relative AOX was significantly higher in the three thermogenic stages, 1,

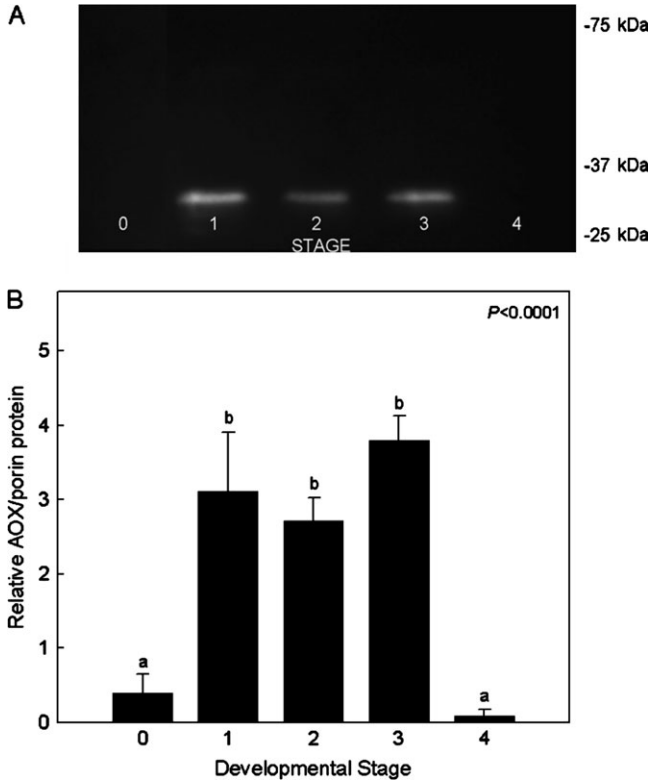

Fig. 4. A representative western blot of AOX protein detection (A) and relative amounts of AOX/porin (B) for mitochondria isolated from sacred lotus receptacle tissue throughout the developmental sequence. Significant differences are indicated by different letters. Data in (B) are means ±SE, n=5–21.

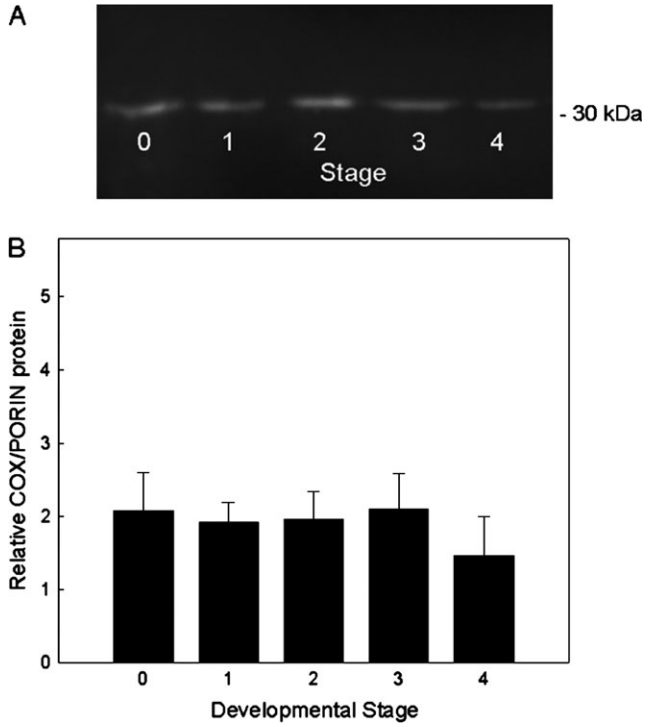

Fig. 5. A representative western blot of COX protein detection (A) and relative amounts of mitochondrial COX/porin (B) for mitochondria isolated from sacred lotus receptacle tissue throughout the developmental sequence. Means were not significantly different (see text). Data in (B) are means ±SE, n=5–21.

2, and 3, than in the non-thermogenic stages, 0 and 4, which were essentially the same (Fig. 4B; ANOVA, $F_{4,39}$= 7.84, P=0.0001). When amounts of mitochondrial protein from leaf tissue (non-thermogenic) were compared with receptacle protein, AOX was below detectable levels. During thermogenic stages there was no correlation between the amount of AOX protein and the magnitude of receptacle heating (r^2=0.09, P >0.05, Fig. 3B) or AOX flux (r^2=0.03, P >0.05, data not shown). The relative concentration of COX (i.e. COX/porin) in receptacle mitochondria did not change significantly during floral developmental in *N. nucifera* (Fig. 5A, B; ANOVA, $F_{5,39}$= 0.17, P=0.9541). Immunoblots of lotus receptacle mitochondria were probed with two different pUCP antisera, raised against *S. foetidus* (thermogenic skunk cabbage) and soybean, but no pUCPs were detected (data not shown).

Soluble carbohydrates and starch

There were significant changes in receptacle starch concentration during floral development (Fig. 6A, Kruskal–Wallis, χ^2_4=25.81, P <0.0001). Starch concentration of receptacles increased by 50% between stage 0 and stage 2 (mean stage 2, 11.29 mg g FW^{-1}), although this difference was not significant (Fig. 6A). Mean starch concentration was significantly higher in the three thermogenic stages (stages 1–3) than in stage 4 receptacles. Starch concentration declined by 89% between stage 2 and the post-thermogenic stage 4 (mean stage 4, 1.24 mg g FW^{-1}). During thermogenic stages there was no correlation between starch concentration and the magnitude of receptacle heating (r^2=0.11, P >0.05, data not shown).

In contrast to starch, total soluble carbohydrate concentration (suc+glc+fru) did not change over the developmental sequence (data not shown). There were, however, significant changes in the composition of the soluble carbohydrate pool during development. Most notably, sucrose content was highest and similar in non-thermogenic stages (0 and 4), with mean concentrations of 2.58±0.11 and 2.45±0.11 mg g FW^{-1}, respectively (Fig. 6B, Kruskal–Wallis, χ^2_4=14.01, P=0.007). In contrast to suc, glc+fru concentration was lowest in stage 0, and increased significantly throughout thermogenesis, peaking in stage 3 receptacles (Fig. 6C, Kruskal–Wallis, χ^2_4= 16.31, P=0.003).

Discussion

A significant relationship was observed between receptacle heat production and AOX flux during floral development in *N. nucifera*. Heat production was not observed until

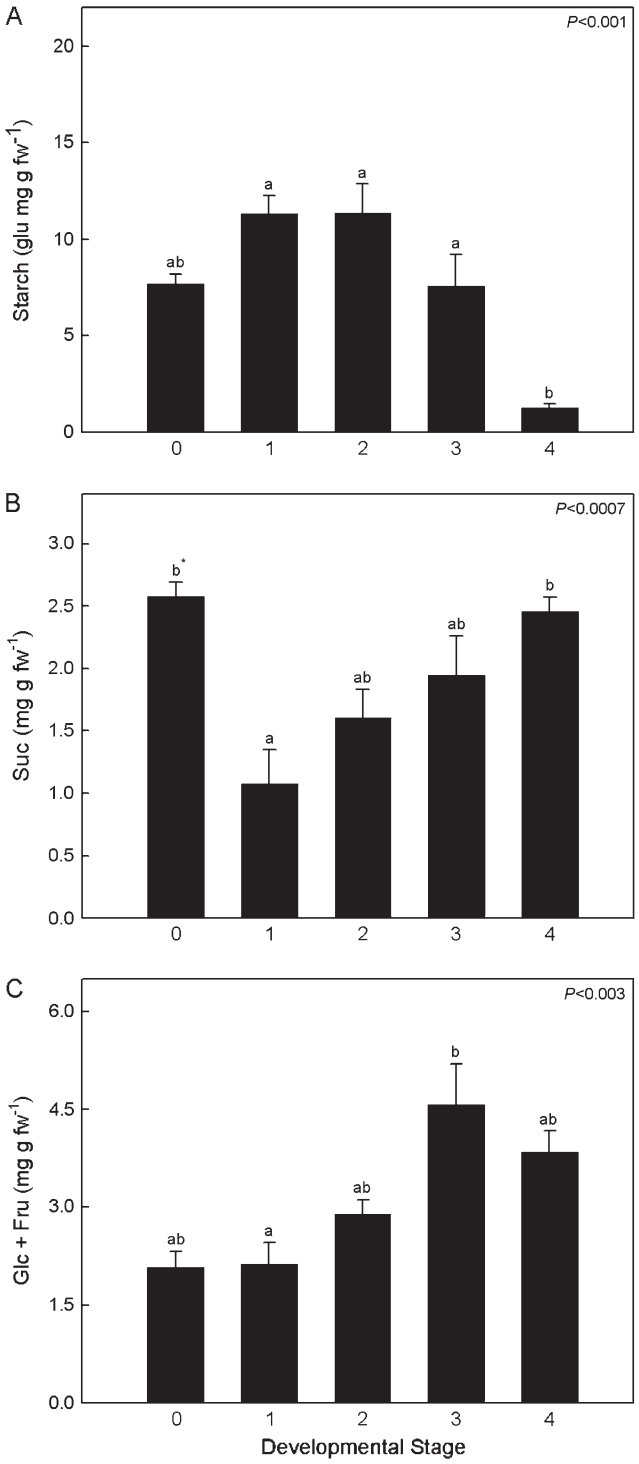

Fig. 6. Changes in tissue concentrations of (A) starch, (B) suc, and (C) glc and fru for sacred lotus receptacles throughout the developmental sequence. Significant differences are indicated by different letters. The asterisk in (B) indicates significance at $P \leq 0.06$. Data are means ±SE, n=4–28 samples. A smaller subset of the starch samples were analysed for soluble carbohydrates.

stage 1, increased through stage 2, declined through stage 3, and had ceased by stage 4 (Figs 1, 2A). Total respiration increased up to stage 2, and then declined to stage 4 in line with the pattern of heating (Fig. 2A, B). This is similar to previously reported patterns of heating and total respiration during floral development of this species (Seymour and Schultze-Motel, 1996). Stable oxygen isotope measurements confirmed that the increased respiratory flux supporting heat production was largely via the AOX pathway, with little or no contribution from the COX pathway (Figs 2, 3A). During stage 2, AOX flux accounted for between 11% and 93% of total respiratory flux, depending on the amount of heating (Table 1). This contribution is somewhat higher than the maximum reported in a previous report (Watling *et al*., 2006), and confirms that sacred lotus has the highest proportion of AOX flux of any plant measured to date (cf. Robinson *et al*., 1995; Ribas-Carbo *et al*., 2005*b*). The high mean AOX flux during heating further supports the previous conclusion that pUCPs are unlikely to be significantly involved in heat production in the sacred lotus (Watling *et al*., 2006). This is also strengthened by the stable COX flux levels during heating which would be predicted to increase if pUCPs played a role in heating in this species. Furthermore, efforts to detect pUCPs in these sacred lotus receptacles using *S. foetidus* or soybean antibodies have so far been unsuccessful (our data; K Ito and Y Onda, personal communication). Based on these respiratory fluxes, it is calculated (according to Seymour and Schultze-Motel, 1998) that receptacle AOX is responsible for approximately half the total heat produced by the thermogenic flowers. The measurements of AOX flux in sacred lotus petals suggest that these tissues, which also show very high AOX flux, account for the remaining heat observed in the flower (NM Grant *et al*., unpublished data). This concurs with Seymour and Schultze-Motel's analysis of total respiration in the different flower parts (Seymour and Schultze-Motel 1998).

The importance of the AOX in heat production in this species was further confirmed by measurements of AOX protein content of receptacles during the developmental sequence. AOX protein content in the thermogenic stages 1–3 was at least 10-fold higher than that of stage 0 and 4 receptacles (Fig. 4), and AOX in all receptacles was higher than that found in sacred lotus leaves. Assuming that sacred lotus leaves contain similar levels of AOX protein to that found in non-thermogenic tissues in other species, the levels found in these thermogenic receptacles are extremely high. This is further supported by the fact that the amount of protein loaded onto the gels was 4–6 times lower than those typically used in other studies to detect AOX by western blotting (Ducos *et al*., 2001; Gonzalez-Meler *et al*., 2001). The synchronicity between the onset of thermogenic activity and the increase in expression of AOX protein in stage 1 receptacles of

sacred lotus contrasts with the accumulation of AOX protein 3 d prior to the thermogenic burst in *S. guttatum* (Rhoads and McIntosh, 1992), and *Arum italicum* (Chivasa *et al*., 1999), and up to 8 d prior to that in *Victoria cruziana* (Skubatz and Haider, 2004). There was also synchronicity between the loss of thermogenic activity and AOX protein in sacred lotus receptacles at stage 4 (Figs 2, 4). Although low by comparison with thermogenic tissues, mass specific rates of AOX flux during stages 0 and 4 are comparable with those observed in non-thermogenic plants, such as soybean cotyledons (Ribas-Carbo *et al*., 2000) which are likely to contain similar levels of AOX protein. Such rates, however, are insufficient to produce measurable heating of tissues (Breidenbach *et al*., 1997; see Fig. 2A)

Although there was a tight relationship between the presence of AOX protein and the ability to thermoregulate across the developmental series, within the thermoregulatory stages 1–3 no quantitative relationship was found between AOX protein content and thermogenic activity (Fig. 3B) or AOX flux, suggesting that regulation of thermogenesis is post-translational. Thus, fine regulation of AOX activity during the thermogenic stages (Fig. 3A) is likely to occur within the mitochondrial electron transport chain. For example, AOX activation can be modulated either through the reduction status of a disulphide bond or via effectors, such as pyruvate and other α-keto acids (Millar *et al*., 1993; Umbach *et al*., 1994; Vanlerberghe *et al*., 1999). In many plants AOX is only active once the Q pool reaches 40–50% reduction (Moore *et al*., 1988; Dry *et al*., 1989); however, in thermogenic *Arum* mitochondrial AOX activity occurs at very low Q pool reduction states (Moore and Siedow, 1991), suggesting that this mechanism may not be responsible for modulating AOX activity in thermogenic species. Previously it has been reported that isolation of mitochondria results in complete oxidation of AOX protein, making it difficult to assess the reduction state of the AOX *in vivo* (Umbach and Siedow, 1997). However, all detectable AOX protein from the mitochondrial preparations of sacred lotus receptacles was present in the reduced form, making it unlikely that the AOX reduction state is a regulatory mechanism (see also Onda *et al*., 2008). Thus, it seems likely that regulation of AOX in sacred lotus receptacles is occurring via effectors such as α-keto acids.

Starch concentrations in sacred lotus receptacles peaked in stage 2 and were largely exhausted by stage 4, making this the likely substrate for thermogenesis (Fig. 6A). An earlier study showed that starch was present in the parenchyma tissues of lotus receptacles during stage 2, and had disappeared by the end of stage 3 (Vogel and Hadacek, 2004). Rapid loss of starch during thermogenesis has also been shown in *Arum maculatum* (ap Rees *et al*., 1977). The respiratory quotient for sacred lotus receptacles is also reported as 1, consistent with carbohydrate being the respiratory substrate (Seymour and Schultze-Motel, 1998). There was no evidence that carbohydrate concentration limited thermogenesis throughout the thermoregulatory period in sacred lotus; however, the possibility that starch was limiting respiration towards the end of this phase cannot be ruled out. High concentrations of sucrose in stage 0 are consistent with import of carbohydrates from other organs for the development of fuel reserves to support subsequent thermogenesis (Fig. 6B). This is further supported by the decrease in sucrose concentration between stage 0 and stage 1, and the concurrent increase in starch concentration between these two stages. It is possible that this early sucrose import and starch accumulation is not sufficient to support respiration for the entire thermogenic period, as sucrose concentrations increased, although not significantly from stages 1 to 3. The high concentration of sucrose in stage 4 receptacles was coupled with a significant decrease in starch. This is most probably explained by the transformation in receptacle function that occurs during this post-thermogenic stage, when receptacles become photosynthetic, presumably to support fruit and seed development.

Conclusion

Thermogenic activity in sacred lotus receptacles is mediated by the AOX pathway fuelled by starch. Three distinct physiological phases were identified during development of sacred lotus flowers. The shift from pre-thermogenic stage 0 to thermogenic stage 1 was associated with a rapid increase in AOX protein. The transition from thermogenic stage 3 to post-thermogenic flowers was characterized by a similarly rapid decrease in AOX protein content. During the thermogenic phase (stages 1–3), heat production was correlated with AOX flux but, as AOX protein remained constant during this period, regulation is likely to be post-translational. The synchronicity between AOX protein content and thermogenic activity contrasts with other thermogenic plant species where AOX protein increases some days prior to the onset of thermogenesis.

Acknowledgements

Thanks are due to the Adelaide Botanical Gardens for access to their lotus pond, Professor Kikukatsu Ito and Yoshi Onda for advice and helpful discussions, David Bruce, Tiffany Barlow, and Layla Tucker for technical assistance, and David Hollingworth for the photography and image layout in Fig. 1. AOX antibody was kindly supplied by Assistant Professor Kathleen Soole (Flinders University, South Australia), Professor James Whelan (University of Western Australia, Western Australia) provided porin and soy pUCP antibodies, and Professor Kikukatsu Ito (Iwate University, Japan) provided the *S. foetidus* pUCP antibody. This work was supported by the Australian Research Council (grant no. DP0451617), and NMG is in receipt of an APA studentship.

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