Contribution of the Alternative Pathway to Respiration during Thermogenesis in Flowers of the Sacred Lotus[1]

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We report results from in vivo measurements, using oxygen isotope discrimination techniques, of fluxes through the alternative and cytochrome respiratory pathways in thermogenic plant tissue, the floral receptacle of the sacred lotus (*Nelumbo nucifera*). Fluxes through both pathways were measured in thermoregulating flowers undergoing varying degrees of thermogenesis in response to ambient temperature. Significant increases in alternative pathway flux were found in lotus receptacles with temperatures 16°C to 20°C above ambient, but not in those with lesser amounts of heating. Alternative pathway flux in the hottest receptacles was 75% of the total respiratory flux. In contrast, fluxes through the cytochrome pathway did not change significantly during thermogenesis. These data support the hypothesis that increased flux through the alternative pathway is responsible for heating in the lotus and that it is unlikely that uncoupling proteins, which would have produced increased fluxes through the cytochrome pathway, contribute significantly to heating in this tissue. Comparisons of actual flux, with capacity determined using inhibitors, suggested that the alternative pathway was operating at close to maximum capacity in heating tissues of lotus. However, in nonheating tissues the inhibitor data significantly overestimated the alternative pathway flux. This confirms that isotopic measurements are necessary for accurate determination of fluxes through the two pathways.

The first record of thermogenic activity in a plant was made by Jean-Baptiste Lamarck, in the European arum lily, probably *Arum italicum* (as cited in Vanlerberghe and McIntosh, 1997). Since then thermogenesis has been reported from species in the Annonaceae, Araceae, Arecaceae, Aristolochiaceae, Cycadaceae, Nymphaeaceae, Winteraceae, Illiciaceae, Magnoliaceae, Rafflesiaceae, and Nelumbonaceae (Seymour, 2001). Explanations advanced to explain the function of thermogenesis include volatilization of scent compounds to attract pollinators (Meeuse and Raskin, 1988), prevention of low-temperature damage (Knutson, 1974), and provision of a thermal reward to insect pollinators (Seymour, 1997; Seymour et al., 2003). While thermogenic capacity can be large, heating tissues by up to 40°C above ambient in some cases, flower temperature often varies considerably. In several species, however, respiratory heat production is regulated to achieve fairly constant flower temperatures in widely variable environmental temperatures (Nagy et al., 1972; Knutson, 1974; Seymour and Schultze-Motel, 1996).

The strong correlation between heat generation and cyanide-resistant respiration in thermogenic plant tissues suggests that the alternative respiratory pathway is responsible for temperature increases in these plants (Nagy et al., 1972; Meeuse and Raskin, 1988). Cyanide-resistant respiration is mediated by a nuclear-encoded, alternative oxidase (AOX) that exists as a homodimer in the inner mitrochondrial membrane. The AOX is found in all plants and some protists, fungi, and invertebrate animals (Vanlerberghe and McIntosh, 1997; McDonald and Vanlerberghe, 2004). During normal oxidative phosphorylation, electron transport is linked to the formation of a proton gradient that drives ATP production. In this instance, the terminal electron acceptor is the cytochrome *c* oxidase, and approximately three ATPs are produced per O_2 consumed (Roberts et al., 1984). In contrast, electron transport to the AOX diverges from the phosphorylating pathway at ubiquinone, and potential ATP production is reduced to about one per O_2 consumed and the extra energy is released as heat (Moore and Siedow, 1991). However, calorimetric studies of thermogenic flowers show that there is no net phosphorylation and all of the energy is released as heat (Seymour et al., 1983; Lamprecht et al., 1998).

Apart from its likely role in thermogenic tissues, the function of this apparently energetically wasteful pathway in plants was unclear until recently. However, an increasing number of studies now support a role of the alternative pathway in preventing the buildup of reactive oxygen species, by stabilizing the redox state of the mitochondrial ubiquinone pool while allowing continued operation of the citric acid cycle (Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Skulachev,

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1996; Millenaar et al., 1998; Maxwell et al., 1999; Parsons et al., 1999; Purvis, 2001; Ribas-Carbo et al., 2005b). This role in stress mediation would explain the widespread occurrence of the alternative pathway in plants. Since the alternative pathway is able to maintain respiration free from the constraints imposed by low ADP and inorganic phosphate availability, it has also been postulated that it allows high decarboxylation fluxes during C4 and Crassulacean acid metabolism photosynthesis (Robinson et al., 1992; Chivasa et al., 1999) and respiration in plants growing under phosphorus limitation (Parsons et al., 1999; Juszczuk et al., 2001; Shane et al., 2004).

Regulation of AOX activity in isolated mitochondria can occur at a number of levels (for review, see Millenaar et al., 2002; Lambers et al., 2005); however, the extent to which these operate in vivo is still unclear. Expression of the AOX has been shown to increase in plants exposed to low temperature, herbicides, and inhibitors of the cytochrome pathway, such as nitric oxide (Vanlerberghe and McIntosh, 1992; Aubert et al., 1997; Finnegan et al., 1997; Ribas-Carbo et al., 2000a; Gonzàlez-Meler et al., 2001; Huang et al., 2002; Zottini et al., 2002). However, changes in AOX protein levels do not necessarily reflect changes in activity in vivo (Millenaar et al., 2001; Guy and Vanlerberghe, 2005; Ribas-Carbo et al., 2005b). Further posttranslational regulation is mediated by the redox state of the ubiquinone pool, which determines electron partitioning between the cytochrome and AOX pathways (Dry et al., 1989), and via effectors that have a direct impact on the activation state of the AOX protein. These include a regulatory disulfide bond that modulates the redox state of AOX (Umbach et al., 1994; Vanlerberghe et al., 1999) and α-keto acids such as pyruvate (Millar et al., 1993) that can further increase activity of the reduced form (Rhoads et al., 1998; Vanlerberghe et al., 1999).

There has been surprisingly little work with thermogenic plants, even though they stimulated much of the initial interest in the alternative pathway (Nagy et al., 1972; Meeuse and Raskin, 1988). In voodoo lily (*Sauromatum guttatum*), high levels of AOX capacity (determined from inhibitor experiments) were found to coincide with spadix maturation (Rhoads and McIntosh, 1992). In dragon lily (*Dracunculus vulgaris*), expression of the AOX gene occurred in highly thermogenic male florets, but not in nonthermogenic tissues of the same plant (Ito and Seymour, 2005). Similarly, in this species and other thermogenic arum lilies, the content of AOX protein increased with increasing thermogenic activity and declined immediately after heat production (Skubatz and Haider, 2001).

Recently, the role of AOX in thermogenesis has been brought into question by the discovery of uncoupling proteins (UCPs) in most plant tissues (Vercesi et al., 1995; Laloi et al., 1997), including thermogenic spadices of skunk cabbage (*Symplocarpus foetidus*; Ito, 1999) and thermogenic receptacles of the sacred lotus (*Nelumbo nucifera*; K. Ito, personal communication). UCPs have long been known from animal tissues (e.g. brown adipose tissue), where they act as uncouplers of the mitochondrial electron transport chain, allowing energy derived from lipids to be used for heat generation instead of ATP production (Knutson, 1974; Klingenberg and Winkler, 1985). Furthermore, a number of studies have reported that the AOX and UCP co-occur in plant tissues (Sluse et al., 1998; Considine et al., 2001; Ito and Seymour, 2005). UCP may short-circuit protons that could be transferred before the AOX, accounting for the complete conversion of energy entering the electron transport chain to heat with no ATP production (Seymour et al., 1983; Lamprecht et al., 1998). Although UCP is equally expressed in both thermogenic and nonthermogenic tissues in dead-horse arum (*Helicodiceros muscivorus*; Ito et al., 2003), it may have a thermogenic function in mitochondria with high AOX activity. However, the free fatty acids that stimulate UCP activity appear to inhibit the AOX, thus it is unlikely that both will reach maximal activity simultaneously (Sluse et al., 1998). The involvement of AOX and UCP appears to depend on the substrate catabolized. UCP gene expression occurs strongly only during thermogenesis in the sterile male florets of *Philodendron selloum*, an arum lily that catabolizes primarily lipid, while the AOX gene is expressed in dragon lily, another arum lily that uses carbohydrate (Ito and Seymour, 2005).

It is now widely accepted that the use of chemical inhibitors is not an appropriate technique to measure flux through the respiratory pathways in vivo (Millar et al., 1995; Ribas-Carbo et al., 1995). Instead, stable oxygen isotope techniques have been developed (Guy et al., 1989) and improved (Robinson et al., 1992; Gonzàlez-Meler et al., 1999; Henry et al., 1999; Ribas-Carbo et al., 2005a). The stable isotope method relies on the fact that the two terminal acceptors of plant mitochondrial electron transport discriminate differentially against oxygen isotopes. Knowledge of the discrimination end points for cytochrome oxidase and AOX allows determination of the relative fluxes through the two pathways during steady-state respiration, in the absence of any chemical inhibitors. However, care must be taken to ensure that discrimination against oxygen is attributed purely to the respiratory chain, limiting the method to studies of dark respiration and to samples where diffusion of oxygen is not restricted.

These limitations in methodology have so far prevented use of the stable isotope method in studies examining the group of plants that arguably use the alternative respiratory pathway to the greatest extent, namely, thermogenic plants. Often, the thermogenic organs in these plants are structurally dense, and diffusional limitations have obscured the isotopic signature (for review, see Robinson et al., 1995). However, in the sacred lotus, strong thermoregulatory activity is found within the spongy tissues of the receptacle (Seymour and Schultze-Motel, 1998), providing us with a model system in which to examine the role of AOX and UCP in heat production.

In this study we used stable oxygen isotope techniques to determine electron partitioning between the cytochrome (and UCP) and AOXs during thermoregulatory activity in the receptacles of the sacred lotus. We have also compared these measurements with traditional methods involving inhibitors.

RESULTS

Respiration rates of whole lotus flowers, measured in the field, increased with tissue heating, although this was most apparent when the temperature difference exceeded 12°C (Fig. 1). Laboratory measurements of oxygen consumption by receptacle sections that had been excised from flowers were similar to the rates observed in the field, within a range of thermogenic intensities (see controls in Fig. 2 and total respiratory flux in Fig. 3). The slightly lower values obtained during laboratory respirometry and mass spectrometry are most likely due to the fact that these measurements were made on excised sections rather than whole flowers.

The capacities of the alternative and cytochrome pathways in lotus receptacles during the thermoregulatory phase were determined by titration with salicylhydroxamic acid (SHAM) and KCN, respectively, on both strongly heating receptacles in the morning and weakly heating tissues in the afternoon. Total uninhibited respiration was 45% higher in the mornings than later in the day (Fig. 2A). Residual respiration, in the presence of both 25 mM SHAM and KCN concentrations at or above 4 mM, was 0.007 μmol O_2 g fresh weight (fw)$^{-1}$ s^{-1}, which was 9% to 13% of the total, uninhibited rate (Fig. 2, A and B). Cytochrome pathway activity was removed by 4 mM KCN, while 25 mM SHAM was needed to block alternative pathway activity (Fig. 2, A and B). Alternative pathway capacity, measured in the presence of 16 mM KCN, was 72% of uninhibited total respiration in the morning but equivalent to total respiration in the afternoon (Fig. 2B).

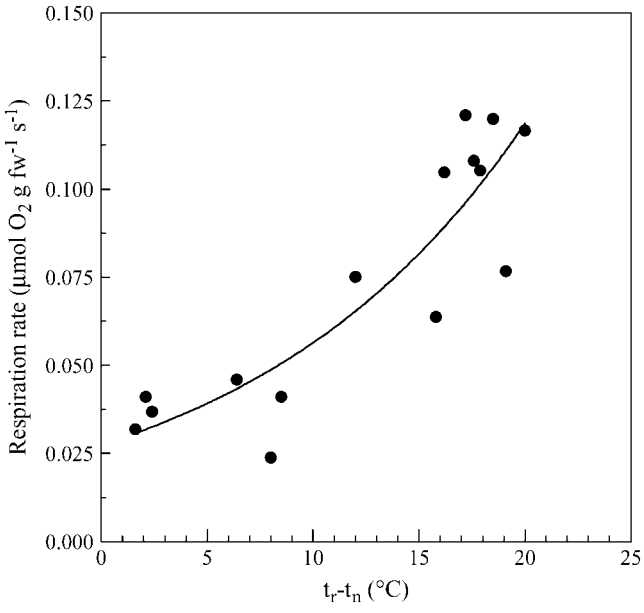

Figure 1. The relationship between receptacle respiration rate (μmol O_2 g fw^{-1} s^{-1}) and the difference between lotus receptacle temperature (t_r, °C) and temperature of a nearby nonthermogenic receptacle (t_n, °C), measured in the field (r^2 = 0.96; y = 0.0023+0.0252e$^{0.0765x}$).

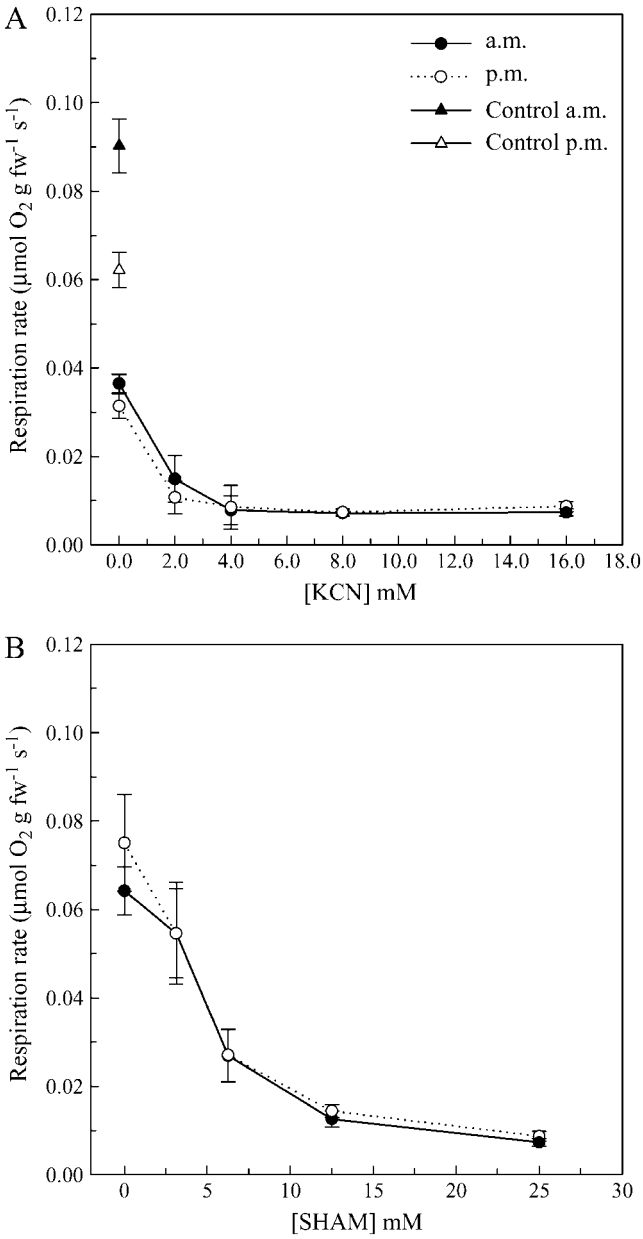

Figure 2. The effect of KCN on cytochrome pathway respiration measured in the presence of 25 mM SHAM (A) and SHAM on cyanide-resistant pathway respiration measured in the presence of 16 mM KCN (B), in lotus receptacle tissue. Total respiration rates of uninhibited receptacle tissues collected in the morning (black triangle, AM) and in the afternoon (white triangle, PM) are also shown. For inhibitor titrations, tissue sections were vacuum infilatrated with inhibitor, blotted dry, and then O_2 uptake rate measured in air at 32°C, in a closed respirometer. Respiration rate was calculated over the range 20 to 14 kPa O_2 partial pressure (approximately 15 min). Data points are means ± SE, n = 6.

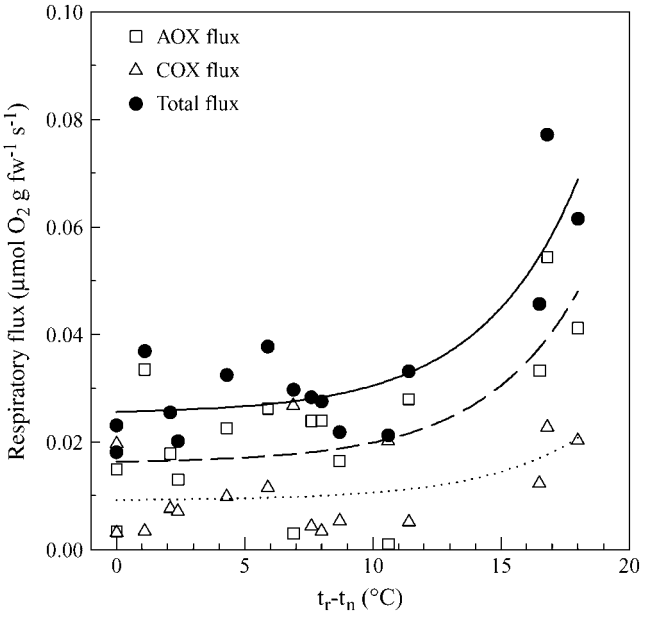

Figure 3. Total respiratory flux (μmol O_2 g fw^{-1} s^{-1}) and fluxes through the alternative (AOX) and cytochrome (COX) pathways, in lotus receptacle tissues as a function of the difference between receptacle temperature (t_r, °C) and temperature of an adjacent nonheating receptacle (t_n, °C). Total respiration (solid line, $r^2 = 0.95$; $y = 0.0253 + 0.0004e^{0.2645x}$), alternative pathway flux (dashed line, $r^2 = 0.86$; $y = 0.0161 + 0.0003e^{0.265x}$), and cytochrome pathway flux (dotted line, $r^2 = 0.75$; $y = 0.0092 + 0.0001e^{0.2613x}$) are shown. Partitioning between the two pathways was determined on the basis of $\Delta^{18}O$ measurements of intact tissues.

Cytochrome pathway capacity, measured in the presence of 25 mM SHAM, was 39% and 56% of uninhibited total respiration in the morning and afternoon, respectively.

Oxygen isotope measurements were used to determine the actual fluxes through the alternative and cytochrome pathways in the absence of inhibitors. Total respiration rates determined by gas chromatography-mass spectrometry (GC-MS) in the morning and afternoon were similar to those determined in respirometry measurements made in the laboratory and from whole flowers in the field (Fig. 3). Lotus respiration rates measured by GC-MS also showed an increase with thermogenesis, with total and alternative pathway flux increasing in parallel (Figs. 1 and 3). Analysis of the data indicated significant increases in both total respiration and alternative pathway flux in tissues that were heating to temperatures of 16°C or more above ambient (Table I). The lack of a significant increase in these two fluxes between 11°C and 15°C was likely due to the small number of flowers found heating in this range. In the least active (afternoon) tissues, alternative pathway flux accounted for 43% of total respiration but increased to 55% to 75% of the total in heating tissues. However, changes in cytochrome flux across the range of thermogenic activity were not significant.

DISCUSSION

Our results demonstrate conclusively that there is increased flux through the alternative pathway in relation to heating of thermogenic tissues of lotus and that the alternative pathway is responsible for the bulk of respiratory activity in these tissues. The use of oxygen isotope techniques in this study allowed us to confirm that previously reported high alternative pathway capacities in thermogenic tissues, measured using respiratory inhibitors, are matched by high in vivo alternative pathway fluxes. However, whereas the oxygen isotope technique allows accurate and dynamic determination of respiratory fluxes through both the alternative and cytochrome pathways, inhibitor studies can be used only to determine the maximum possible flux through each pathway (Millar et al., 1995; Ribas-Carbo et al., 1995). Until our work, oxygen isotope techniques had not been used to determine in vivo flux through the alternative pathway in thermogenic plants due to the high diffusional resistances found in most thermogenic tissues (Guy et al., 1989). This limited the use of isotopic analysis to mitochondrial extracts from such tissues, and the only reported study of this kind was for mitochondria extracted from *Symplocarpus foetidus* spadix, in which 78% of the respiratory flux was attributed to the alternative pathway (Guy et al., 1989). In contrast, the thermogenic receptacle tissue of lotus flowers contains many air spaces, making this plant an ideal model for the study

Table I. *Mean total respiration rates and fluxes through the alternative and cytochrome pathways (μmol O_2 g fw^{-1} s^{-1}) with different amounts of tissue heating in lotus receptacles*

Temperature categories represent the difference between receptacle temperature (t_r) and the temperature of an adjacent nonthermogenic receptacle (t_n). Values are means ± SE (in parentheses), $n = 2$ to 5.

	$t_r - t_n$ °C					ANOVA	
	0	1–5	6–10	11–15	16–20	$F_{4,11}$	P
Total respiration	0.021 (0.002)	0.029 (0.004)	0.029 (0.003)	0.027 (0.006)	0.061[a] (0.009)	9.24	0.0016
Alternative pathway respiration	0.009 (0.006)	0.022 (0.004)	0.019 (0.004)	0.015 (0.013)	0.043[a] (0.006)	3.99	0.031
Cytochrome pathway respiration	0.011(0.008)	0.007 (0.001)	0.010 (0.004)	0.013 (0.008)	0.018 (0.003)	0.49	NS[b]

[a]Indicates significant difference within row (Tukey test, $P < 0.05$). [b]Not significant.

of thermogenesis in vivo. The in vivo fluxes measured in our study showed that the alternative pathway contributed 55% to 75% of respiratory flux during heating, similar to the values found for *S. foetidus* mitochondria. Our inhibitor measurements indicated that alternative pathway capacity in heating tissues was 72% of total respiration. This suggests that in heating tissues of lotus the alternative pathway operates at close to full capacity. However, in nonheating tissues, our inhibitor data significantly overestimated alternative pathway flux (Fig. 2). This again demonstrates that inhibitors cannot provide reliable estimates of flux through the two respiratory pathways (Millar et al., 1995; Ribas-Carbo et al., 1995).

Alternative pathway fluxes reported for tissues from nonthermogenic plants range from 0% to 50% of total respiration (Robinson et al. 1995), similar to the 43% flux we observed in nonheating receptacles (Table I). The only report of higher alternative pathway fluxes in a nonthermogenic plant (63%) was for leaves of the Crassulacean acid metabolism plant *Kalanchoe daigremontiana* (Robinson et al., 1992). Alternative pathway fluxes higher than those reported for thermogenic plants, including those found in our study, have been observed only in mutants of *Chlamydomonas reinhardtii* lacking cytochrome oxidase (Guy et al., 1992).

Our oxygen discrimination end points for the alternative and cytochrome pathways were similar to those previously reported for nongreen tissues in a range of plants (Robinson et al., 1995). Higher end points for the alternative pathway have been reported in green tissues, with the end point increasing from 27‰ to 32‰ during greening of soybean (*Glycine max*) cotyledons (Ribas-Carbo et al., 2000b). Following pollination, the lotus receptacle greens, offering us another system in which to study the impact of greening on the isotopic fractionation by the alternative pathway. However, none of the data collected in this study were from green receptacles.

While the alternative pathway has traditionally been invoked as the most likely source of heat in thermogenic plants, the recent discovery of plant UCPs could provide an alternative mechanism for heat generation (Vercesi et al., 1995; Laloi et al., 1997). If UCPs were responsible for heat generation in sacred lotus, then we would expect an increase in flux through the cytochrome pathway during thermogenesis. However, our data show no change in flux through the cytochrome pathway during heating and thus provide no evidence for involvement of UCP in thermogenesis in lotus. This is consistent with the observation that the respiratory substrate is carbohydrate in the lotus (Seymour and Schultze-Motel, 1998) and that AOX expression is enhanced in thermogenic flowers that use this substrate, while UCP expression occurs during thermogenesis when lipid is the main substrate (Ito and Seymour, 2005).

CONCLUSION

The use of oxygen isotope fractionation has allowed us to accurately determine the flux of electrons through the cytochrome and alternative pathways in the thermogenic floral receptacle of the sacred lotus, confirming that the bulk of the heat production occurs through the alternative pathway. Flux through the cytochrome pathway did not change significantly during thermogenesis, making a significant role for UCPs in heat production unlikely in these tissues. Our results also confirm that while inhibitor titrations are suitable for assessing alternative pathway capacity, they often overestimate the actual in vivo respiratory fluxes. The suitability of the thermogenic tissues of lotus for oxygen isotope fractionation measurements makes this plant an important and fascinating model for investigating the basis for cellular thermoregulation in plants.

MATERIALS AND METHODS

Plant Material

Sacred lotus (*Nelumbo nucifera* Gaertn.) flowers were obtained from a pond in the Adelaide Botanic Gardens, South Australia. Measurements were performed during the summer flowering periods (December–February) between 2003 and 2005.

At the time of sampling, the majority of flowers were in the thermoregulatory pistillate stage of development, characterized by slightly opened or bowl-shaped petals (stage 2 according to Seymour and Schultze-Motel [1998]). A small number of nonheating flowers (stages 1 and 3) were also monitored for comparison. At each sampling time, the temperatures of the ambient shaded air, the experimental receptacle, and an adjacent, prethermogenic (stage 1) receptacle were measured with a needle thermocouple and a Fluke model 52 digital thermometer. Flowers for laboratory respiration and MS measurements were taken immediately to the nearby laboratory at the University of Adelaide. In all cases, the period between cutting a flower and the end of measurements was less than 1.5 h.

Field Respirometry

Measurements of CO_2 release were made on whole, attached flowers of sacred lotus using a flow-through system described earlier (Seymour and Schultze-Motel, 1998). Briefly, a pump drew atmospheric air through a plastic hood covering each flower and sent it through a mass flow meter that was vented to the atmosphere. Subsamples of the flows through three hoods and one reference channel of atmospheric air were drawn into a CO_2 analyzer (Anarad model AR50 IRGA). After respiration measurements were completed, receptacles were carefully dissected from each flower and weighed. Mass specific respiration rates were calculated on the basis of receptacle mass (g fw) and a proportional contribution of receptacles to total floral respiration of 54% (Seymour and Schultze-Motel, 1998).

Titration with Respiratory Inhibitors

Laboratory respirometry was performed within a 5-mL glass syringe equipped with a 3-mm-o.d. oxygen electrode (model MI-730; Microelectrodes), sealed with a Silastic sleeve in a hole at the end of the barrel, and a three-way stopcock with a needle. Four syringes were suspended horizontally in a water bath with their plungers protruding through one side and their needle tips through the other. The water bath was connected to a thermocirculator (JULABO USA), and the electrode, syringe barrel, and stopcock were thermostatted to 32°C. The oxygen electrodes were connected to a Sable Systems ReadOx-4H meter (Sable Systems International) and then to a PowerLab SP4644 interface (ADInstruments), for simultaneous recording from all four. They were calibrated with humidified, high-purity nitrogen at the beginning of a series of measurements from each flower, and with atmospheric air before each measurement.

A razor blade was used to cut vertical, pie-shaped sections of tissue from receptacles. Each section of receptacle tissue was weighed, placed in a 5-mL syringe, and then vacuum infiltrated with either buffer (TES, 0.2 mM CaCl, pH 7.2) or selected concentrations of the inhibitors KCN and SHAM. After vacuum infiltration, each section was wrapped in Whatman No. 1 filter paper

and cotton gauze and placed inside a plastic bag. Inhibitor was removed from the air spaces inside the tissue by shaking the bag. This procedure refilled the larger pore spaces with air to eliminate liquid boundary layers during subsequent respirometry. Tissue respiration rates were then measured in the respirometry syringes. There was no significant difference in respiration rates between noninfiltrated tissue and tissue that had been vacuum infiltrated with buffer, indicating that the bulk of liquid was successfully removed following vacuum infiltration.

Recordings were taken over approximately 15 min. The average slope of the decrease in O_2 partial pressure (PO_2) was measured after 1.5 min of equilibration for approximately 10 min. Minimum acceptable PO_2 was 10 kPa to avoid diffusion limitation as much as possible. The slope tended to decrease during a run in the most active controls, but the changes were less than 20%. Oxygen consumption rate was calculated from the PO_2 slope and the gas volume of the syringe, accounting for tissue volume.

Respiration and Discrimination Analysis

Discrimination during respiration was determined in freshly harvested lotus receptacles during periods of high (early morning) or low (afternoon) thermogenesis (Seymour et al., 1998). 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 online (Robinson et al., 1992). A full review of the theoretical and practical aspects of this technology can be found in Ribas-Carbo et al. (2005a).

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. Small sections (approximately 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 approximately 10-min intervals and injected into a GC-MS system (NA 1500; Carlo-Erba Instrumentazione). The fraction of O_2 remaining (f) and its isotopic composition (R) were measured, and the isotopic discrimination factors and partitioning of electrons between the cytochrome and alternative pathways were calculated as described previously (Guy et al., 1989; Henry et al., 1999; Ribas-Carbo et al., 2005a). 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 dimethyl sulfoxide), respectively, prior to measurement. The end points obtained (Δ_c = 16.97 ± 2.00‰, and Δ_a = 26.49 ± 1.25‰) were then used to calculate the flux through the alternative and cytochrome pathways in uninhibited tissues as described by Ribas-Carbo et al. (2005a, 2005b). The reproducibility of measurements of O_2 concentration and fractionation were determined using air samples withdrawn from the empty syringe and were ±2% and ±0.01%, respectively.

Statistical Analysis

Changes in flux through the respiratory pathways were investigated by analysis of variance using JMP 5.1 (SAS Institute). Respiratory flux as a function of temperature was plotted and regressions fitted and tested using SigmaPlot 9.0 (SPSS).

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