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The Effect of Growth and Measurement Temperature on the Activity of the Alternative Respiratory Pathway[1]

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A postulated role of the CN-resistant alternative respiratory pathway in plants is the maintenance of mitochondrial electron transport at low temperatures that would otherwise inhibit the main phosphorylating pathway and prevent the formation of toxic reactive oxygen species. This role is supported by the observation that alternative oxidase protein levels often increase when plants are subjected to growth at low temperatures. We used oxygen isotope fractionation to measure the distribution of electrons between the main and alternative pathways in mung bean (*Vigna radiata*) and soybean (*Glycine max*) following growth at low temperature. The amount of alternative oxidase protein in mung bean grown at 19°C increased over 2-fold in both hypocotyls and leaves compared with plants grown at 28°C but was unchanged in soybean cotyledons grown at 14°C compared with plants grown at 28°C. When the short-term response of tissue respiration was measured over the temperature range of 35°C to 9°C, decreases in the activities of both main and alternative pathway respiration were observed regardless of the growth temperature, and the relative partitioning of electrons to the alternative pathway generally decreased as the temperature was lowered. However, cold-grown mung bean plants that upregulated the level of alternative oxidase protein maintained a greater electron partitioning to the alternative oxidase when measured at temperatures below 19°C supporting a role for the alternative pathway in response to low temperatures in mung bean. This response was not observed in soybean cotyledons, in which high levels of alternative pathway activity were seen at both high and low temperatures.

The biochemical basis of the CN-resistant alternative respiratory pathway in plants is an oxidase in the mitochondrial electron-transport chain that transfers electrons from reduced ubiquinone to oxygen, bypassing two sites of proton translocation and releasing the resulting free energy as heat (Moore and Siedow, 1991). Although the physiological role of this nonphosphorylating, energetically wasteful pathway remains unclear, a number of factors are known to affect alternative oxidase activity (Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997). Regulation of alternative oxidase protein level through changes in gene expression and the dependence of activity on the reducing substrate, ubiquinol, have long been recognized (Moore and Siedow, 1991; Vanlerberghe and McIntosh, 1997), but more recently two additional regulatory mechanisms have been identified.

One regulatory mechanism is a redox-sensitive sulfhydryl/disulfide system, which, when oxidized, forms a disulfide bond between the subunits of the alternative oxidase homodimer, resulting in an essentially inactive enzyme (Umbach and Siedow, 1993). The second regulatory feature involves activation by α-keto acids such as pyruvate (Millar et al., 1993), and the regulatory disulfide bond must be reduced for this latter activation to occur (Umbach et al., 1994). Recent studies using sulfhydryl reagents have suggested that the site of α-keto acid action is at a sulfhydryl group, probably through formation of a thiohemiacetal (Umbach and Siedow, 1996), and site-directed mutagenesis has been used to establish that both the regulatory sulfhydryl/disulfide system and the site of activation by α-keto acids involve the same Cys residue (Rhoads et al., 1998). One of the consequences of these regulatory features is that the alternative oxidase can compete for electrons with an unsaturated Cyt pathway (Ribas-Carbo et al., 1995) rather than acting as a simple electron overflow path off the main Cyt pathway, as was postulated previously (Lambers, 1982).

Plant respiration rates are affected by numerous abiotic factors, and temperature is one of particular significance. There is a direct relationship between respiratory rate and temperature in the short term because the kinetics of most metabolic reactions are highly temperature dependent (Raison, 1980). In addition to short-term responses, plants grown at low temperatures often show higher rates of respiration than plants grown at higher temperatures when both are measured at the same temperature (Amthor, 1989; Collier and Cummins, 1990). This stimulation of respiration by growth at low temperatures has been reported to be an adaptive feature of plants grown in cold and arctic climates compared with related species or ecotypes from warmer climates (Billings, 1974; McNulty and Cummins, 1987). It has also been suggested that the increased rate of respiration at low temperatures involves a greater participation by the alternative pathway (McNulty et al., 1988; Purvis and Shewfelt, 1993).

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Abbreviation: SHAM, salicylhydroxamic acid.

Plant growth at low temperatures often results in higher CN-resistant respiratory activity (McCaig and Hill, 1977; Elthon et al., 1986; McNulty and Cummins, 1987). This increased resistance to CN appears to be due to enhanced synthesis of alternative oxidase protein (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992), as low temperature increased the steady-state mRNA levels of *aox1a* and *aox1b* genes in rice (Ito et al., 1997). Moreover, in isolated cells or in mitochondria, CN-resistant respiration is relatively insensitive to a wide range of temperatures (Yoshida and Tagawa, 1979; Stewart et al., 1990). Experiments with intact tissues have also concluded that the Cyt pathway is more sensitive to short-term changes in temperature than the alternative pathway (Collier and Cummins, 1990). Chilling stress led to lower Cyt oxidase activity and protein levels in corn seedlings transferred to 14°C (Prasad et al., 1994) and in mung bean hypocotyls chilled at 0°C (Yoshida et al., 1989). This suggests that at low temperatures the alternative pathway may be able to maintain a higher percentage of its relative activity than the Cyt pathway. Such alternative pathway activity may prevent the formation of potentially toxic active oxygen species that can result from overreduction of the ubiquinone pool following inhibition of the Cyt pathway (Purvis and Shewfelt, 1993; Wagner and Krab, 1995).

Previous attempts to measure the activity of the alternative pathway at low temperatures are suspect because the traditional use of inhibitors to assess the in vivo activities of the two electron-transfer pathways leads to inconclusive results if the pathways compete for electrons from the ubiquinone pool (Ribas-Carbo et al., 1995; Day et al., 1996). Furthermore, an increase in alternative oxidase protein levels will not necessarily lead to an increase in its activity in the absence of inhibitors. In tobacco leaves the level of the alternative oxidase protein was enhanced by adding salicylic acid, but neither the total respiratory activity nor the partitioning of electrons to the alternative pathway was affected by this treatment (Lennon et al., 1997).

In the present study we tested the hypothesis that low temperatures lead to greater alternative pathway activity in plants grown at either low (14°C or 19°C) or high (28°C) temperatures by measuring oxygen-isotope fractionation in different organs during tissue respiration over a temperature range from 9°C to 35°C. This technique allows in vivo measurements of the partitioning of electrons between the alternative and Cyt pathways in the absence of added inhibitors (Guy et al., 1989).

MATERIALS AND METHODS

Plant Material

Mung bean (*Vigna radiata* [L.] Wilczeck) and soybean (*Glycine max* L. cv Ransom) seeds were treated with 0.5% NaHOCl for 10 min, washed, and hydrated in distilled water for 2 to 4 h with continuous air bubbling. Seeds were planted in a 1:1 mixture of gravel and sand and grown at a constant temperature of 19°C (mung bean), 14°C (soybean), or 28°C (both) in growth cabinets on a 14-h/10-h light/dark regime at 350 μmol m^{-2} s^{-1}. The low-temperature treatments used in this study increased the time of germination and resulted in 2- to 3-fold slower growth relative to plants grown at the higher temperature for both mung bean and soybean. In addition, mung bean plants grown at temperatures below the 19°C used in this study were visibly damaged and did not survive beyond the first-leaf stage.

Based on the definition of stress as any external factor that exerts a disadvantageous influence, and on the fact that stress is most often measured in terms of factors that include growth (Taiz and Zeiger, 1998), both plant species were stressed when grown at the lower temperatures. Whether this specifically reflects changes in the balance between any components of the respiratory pathway is not known.

Mung bean hypocotyls were harvested at d 15 in the 19°C temperature treatment at a developmental stage (first unfolding of primary leaves) that was equivalent to d 5 in the 28°C treatment. Sliced hypocotyl sections (0.8–1 cm long) were used for respiratory measurements to minimize oxygen-diffusion limitations that may affect the isotope-fractionation measurements. Respiration of sliced hypocotyls was constant 10 to 15 min after the sections were made and remained so for several hours.

Leaf samples were taken from mung bean plants that had at least two fully expanded trifoliates at both growing temperatures. Three to four 10-cm^2 discs of fully developed mung bean trifoliates were taken from the same plant for each experiment.

Intact soybean cotyledons from plants grown at 14°C were collected at d 14 to 16, which was a developmental stage (first unfolding of the primary leaves) equivalent to 6- to 7-d-old cotyledons of plants grown at 28°C.

Respiratory and Oxygen Isotope Measurements

Plant samples (0.5–1.5 g fresh weight) were kept in the dark for 30 min before respiratory measurements were taken. Respiratory measurements were made in a 4.96-mL, stainless steel, closed cuvette. A CO_2 absorber (Ascarite, A-M Systems, Carlsborg, WA) was present during measurements because the buildup of CO_2 in the closed cuvette during the course of the experiment resulted in inhibition of respiration (Gonzàlez-Meler et al., 1996). During inhibitor treatments, either 1.0 mM KCN in water or 10 mM SHAM in water from a 1.0 M stock in DMSO was applied by sandwiching the tissues between medical wipes soaked with the corresponding inhibitor and incubating for 25 min (Lennon et al., 1997). All stocks were freshly prepared before use. No recovery from inhibitor treatment was observed, as respiratory rates remained constant throughout the experiment and the r^2 values for all unconstrained linear regressions of the fractionation values were greater than the value of 0.995 considered minimally acceptable (Ribas-Carbo et al., 1995, 1997; Lennon et al., 1997).

Oxygen extraction and isotope analysis were carried out as described by Robinson et al. (1995) with modifications. Temperature was controlled by a water bath set at the desired temperature, which was connected to a copper-plated base attached to the measurement cuvette. Temper-

ature inside the cuvette was measured using a thermocouple between experiments. Also, the mixing syringe was substituted with a plunger filled with Hg to avoid leaks during air mixing in the measurement cuvette. The gas-phase system was regularly tested for leaks by filling the cuvette with He and measuring the appearance of air in the system over time. Leaks were always negligible during measurements. Over the course of the experiment the sample consumed at least 30% but no more than 50% of the initial oxygen. Calculations of oxygen-isotope fractionation were made as described by Guy et al. (1989) and Ribas-Carbo et al. (1995) with modifications. The measured value of 1.066 was used for the argon-to-nitrogen gain factor correction instead of the theoretical value used previously. Electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy et al. (1989).

Mitochondrial Isolation

Mitochondrial mini-preparations were performed as described by Lennon et al. (1997), and full mitochondrial preparations as described in Umbach and Siedow (1993). Protein concentrations were estimated by the method of Lowry et al. (1951).

Immunoblotting

SDS-PAGE was performed using 10% to 17% gradient polyacrylamide gels and samples were prepared with 100 mM DTT as a reductant in the sample buffer (Umbach and Siedow, 1993; Umbach et al., 1994). Proteins were transferred to nitrocellulose according to the method of Towbin et al. (1979), and immunoblotting was performed as described by Lennon et al. (1997) using the alternative oxidase monoclonal antibody against the alternative oxidase protein from *Sauromatum guttatum* (Elthon et al., 1989). Bound antibodies were detected using an anti-mouse-horseradish peroxidase conjugate and alternative oxidase bands were detected with a chemiluminescence assay (DuPont NEN) according to the manufacturer's instructions. Densitometry to quantify the relative alternative oxidase protein levels was performed as described by Umbach and Siedow (1993).

RESULTS

Effects of Growth Temperature on the Levels of Alternative Oxidase Protein

The amount of alternative oxidase protein increased substantially in the mitochondria of mung bean plants grown at 19°C compared with plants grown at 28°C (Fig. 1). Growth temperature had no effect on the levels of the mitochondrial ATPase β-subunit (data not shown). The increase in protein at low temperature was greater for leaves (5-fold) than for hypocotyls (3-fold). In plants grown at 28°C, the amount of alternative oxidase protein in leaf mitochondria was less than that in the hypocotyl mitochondria, but the levels of alternative oxidase protein in leaf and hypocotyl mitochondria of plants grown at 19°C were comparable (Fig. 1). The 3-fold increase in alternative oxidase protein content seen in mung bean hypocotyl mitochondria from plants grown at 19°C was correlated with the increase in the CN-resistant respiratory activity measured at 25°C in the intact tissue from about 4 μmol O_2 g^{-1} fresh weight h^{-1} in plants grown at 28°C to over 10 μmol O_2 g^{-1} fresh weight h^{-1} in plants grown at 19°C. In mung bean leaves, the CN-resistant respiratory activity at 25°C was 6 μmol O_2 g^{-1} fresh weight h^{-1} in plants grown at 28°C and 11 μmol O_2 g^{-1}fresh weight h^{-1} in plants grown at 19°C. In soybean cotyledons no differences were observed in either the levels of alternative oxidase protein or the rate of CN-resistant respiration at 25°C in plants grown at 14°C versus 28°C (Fig. 1).

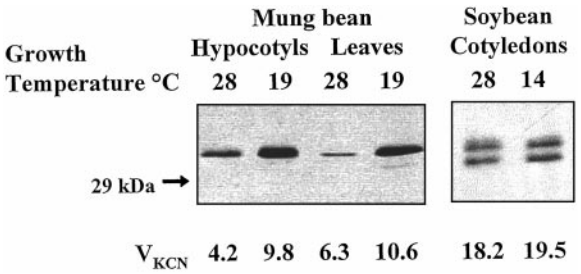

Figure 1. Immunoblots of the alternative oxidase protein in isolated mitochondria from mung bean hypocotyls and leaves and soybean cotyledons grown at low (19°C or 14°C) or high (28°C) temperatures and CN-resistant respiratory rates (V_{KCN}) in the intact tissue. Mitochondrial protein equivalent to 40 μg was loaded in each lane in the presence of DTT as a reductant. CN-resistant respiration values are the average of three to four replicates and were measured as μmol O_2 g^{-1} fresh weight h^{-1} from tissue used for isotope fractionation experiments. SEs were less than 9% of the mean value.

Effects of Temperature on Total Respiration

Total respiratory activity of mung bean hypocotyls was over 2-fold higher in low-temperature-grown versus high-temperature-grown plants whether measured at the higher (28°C) or the lower (19°C) growth temperature (Fig. 2A). However, total respiration was the same for hypocotyls from plants grown at 19°C as for plants grown at 28°C when measured at their respective growth temperature, indicating a marked acclimation response of respiration to growth temperature in hypocotyls.

In contrast, respiration rates of leaves from high- versus low-temperature-grown mung bean were the same at each measurement temperature (Fig. 3A). Consequently, when leaf respiration is compared at the respective growth temperature, the respiratory rate in plants grown at 19°C was approximately half that seen in plants grown at 28°C (Fig. 3A).

Like mung bean leaves (Fig. 3A), no differences in respiration were seen between soybean cotyledons from plants grown at 14°C and those grown at 28°C when measured at the same temperature (Table I).

Effects of Temperature on Alternative Pathway Activity

CN-resistant respiratory activity was more sensitive to temperature than the SHAM-resistant respiratory activity of the Cyt pathway (Fig. 4). In leaves of both high- and low-temperature-grown mung bean, the apparent Q_{10} for CN-resistant oxygen uptake between 10°C and 30°C averaged about 3.0, whereas it was close to 2.0 for SHAM-resistant respiration (Fig. 4). Oxygen fractionation by the alternative oxidase in mung bean hypocotyls and leaves and in soybean cotyledons maintained a constant value of 30‰ to 31‰ over this temperature range (Fig. 4).

In mung bean hypocotyls grown at 19°C, electron partitioning to the alternative pathway remained low (15% of total respiration) and constant between 9°C and 28°C (fractionation value of about 21.5‰; Fig. 2B). The oxygen isotope fractionation in hypocotyls of plants grown at 28°C was also low and constant between 28°C and 19°C, but decreased to the value of the Cyt pathway by 14°C (Fig. 2B). Respiration through the alternative pathway in hypocotyls of plants grown at 19°C was always higher than that in plants grown at 28°C (Fig. 2C).

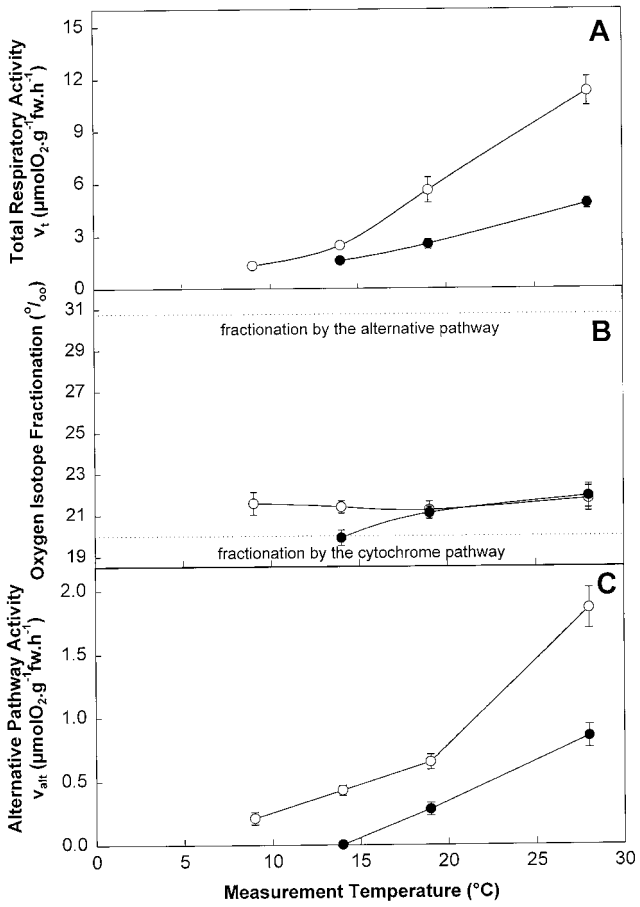

Figure 2. Effects of growth and measurement temperature on mung bean hypocotyl respiration (v_t) (A), oxygen isotope fractionation (B), and alternative pathway activity (v_{alt}) (C) of plants grown at 19°C (○) or 28°C (●). Values are means ± SE of four replicates. fw, Fresh weight.

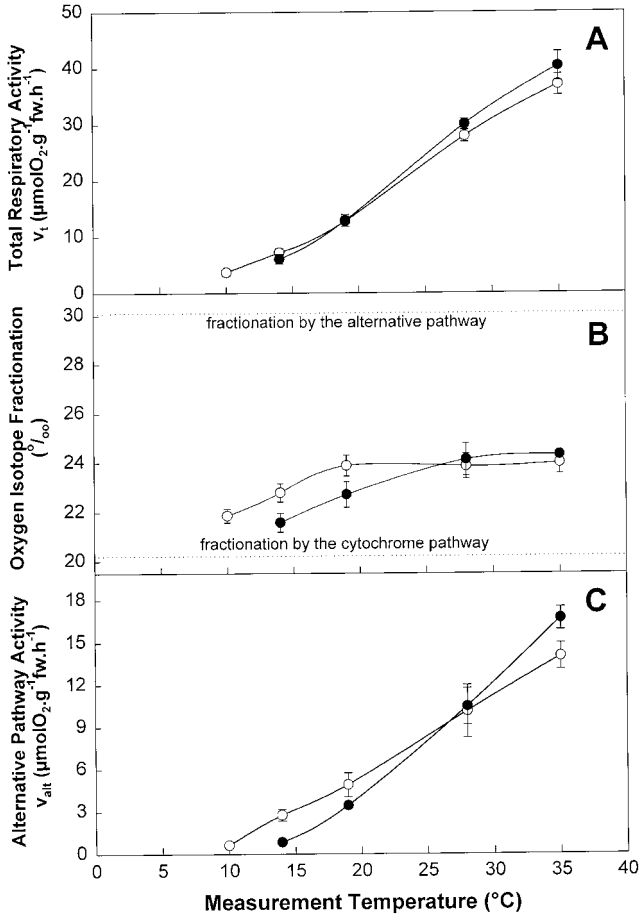

Figure 3. Effects of growth and measurement temperature on mung bean leaf respiration (v_t) (A), oxygen isotope fractionation (B), and alternative pathway activity (v_{alt}) (C) of plants grown at 19°C (○) or 28°C (●). Values are means ± SE of five to six replicates. fw, Fresh weight.

Table I. *Effect of growth temperature on oxygen-isotope fractionation, respiration (V_t), and activity of the alternative (v_{alt}) and Cyt pathways (v_{cyt}) of soybean cotyledons grown at 14°C or 28°C*

Fractionation values for soybean cotyledons were 31.1‰ for the alternative oxidase and 20.1‰ for Cyt oxidase. Values are means ± SE of five replicates.

Parameter/Measurement Temperature	14°C Growth Temp.	28°C Growth Temp.
	‰	
Fractionation		
14°C	24.8 ± 0.4	25.7 ± 0.4
28°C	23.8 ± 0.5	24.9 ± 0.6
	μmol g^{-1} fresh wt h^{-1}	
V_t		
14°C	6.0 ± 0.6	6.3 ± 0.2
28°C	20.3 ± 1.4	20.6 ± 1.3
v_{cyt}		
14°C	3.5 ± 0.4	3.2 ± 0.2
28°C	13.6 ± 0.9	11.7 ± 1.0
v_{alt}		
14°C	2.6 ± 0.1	3.2 ± 0.2
28°C	7.0 ± 0.4	8.9 ± 0.6

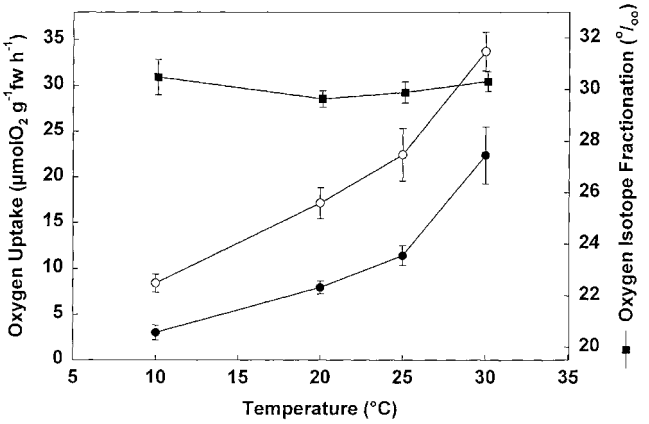

Figure 4. Effect of temperature on CN- and SHAM-resistant respiration and oxygen isotope fractionation during CN-resistant respiration in mung bean leaves. Q_{10} values for CN-resistant (●) and SHAM-resistant (○) respiration obtained from the resulting curves were 2.7 and 1.9, respectively. Oxygen-isotope fractionation values measured in the presence of CN were pooled from mung bean leaves of plants grown at 19°C and 28°C. Values are means ± SE of four to nine replicates. fw, Fresh weight.

In contrast to hypocotyls, the partitioning of electrons to the alternative oxidase in mung bean leaves was considerably higher (fractionation value of 24.0‰; Fig. 3B) and represented about 40% of total respiration when measured at the growth temperature for either low- or high-temperature-grown plants (Fig. 3B). When leaves of mung bean plants grown at 28°C were measured at 19°C, electron partitioning to the alternative pathway decreased to 25% (fractionation value of 22.6‰, Fig. 3B), whereas plants grown at 19°C maintained 40% partitioning of electrons to the alternative pathway, from 19°C to 28°C (Fig. 3B). With plants grown at either temperature, when the measurement temperature was increased to 35°C, electron partitioning to the alternative pathway was maintained at 40% (Fig. 3B). Electron partitioning to the alternative pathway decreased at measurement temperatures below 19°C in both groups of plants, but its value was always higher in leaves grown at 19°C than in those grown at 28°C (Fig. 3B). Therefore, respiration via the alternative pathway in plants grown at 19°C compared with plants grown at 28°C was higher at measurement temperatures of 19°C or below, but not at temperatures equal to or above 28°C (Fig. 3C).

In soybean cotyledons neither the oxygen isotope fractionation nor the alternative pathway respiration was significantly affected by growth temperature when low- or high-temperature-grown plants were measured at either 14°C or 28°C (Table I).

DISCUSSION

Mung bean plants maintained at a low, growth-limiting temperature (19°C) showed an increase in alternative oxidase protein levels (Fig. 1), which is consistent with previous observations made with corn seedlings (Stewart et al., 1990) and cultured tobacco cells (Vanlerberghe and McIntosh, 1992). However, this was not the case for soybean cotyledons, in which the already high protein levels seen in plants grown at 28°C were unchanged in plants held at a growth-limiting temperature of 14°C (Fig. 1).

CN-resistant respiration has been correlated with the amount of alternative oxidase protein (Obenland et al., 1990; Vanlerberghe and McIntosh, 1992, 1996; Rhoads and McIntosh, 1993; Fiorani et al., 1998). Although the observed increases in alternative oxidase protein levels in mung bean hypocotyls and leaves are related to increases in the rate of CN-resistant respiration, this relationship is not linear (Fig. 1). In mung bean leaves, alternative oxidase protein levels increased more (5-fold) than CN-resistant respiration rates (2-fold) in low- versus high-temperature-grown plants (Fig. 1). Furthermore, the respiration through the alternative pathway in the absence of inhibitors at 25°C in mung bean leaves grown at 28°C (8 μmol O_2 g^{-1} fresh weight h^{-1}; Fig. 3C) was higher than the rate of CN-resistant respiration measured at 25°C (6 μmol O_2 g^{-1} fresh weight h^{-1}; Fig. 1). Our results show that although CN-resistant respiration rates are related to protein levels, the response is not linear and CN-resistant respiration cannot be used as a quantitative estimation of the capacity of the alternative pathway.

The above observations suggest that regulatory mechanisms may act to vary alternative oxidase activity depending on metabolic conditions. However, the observation that respiration through the alternative pathway varies in response to temperature does not indicate where the regulation takes place; effects upstream of the ubiquinone pool are as likely to be involved as effects on the alternative oxidase (Krab, 1995). The presence of mechanisms that regulate respiration through the alternative pathway are also illustrated by the response of mung bean hypocotyls to growth temperature. The respiration rate of hypocotyls from plants grown at 28°C in the presence of CN (4 μmol O_2 g^{-1} fresh weight h^{-1} at 25°C; Fig. 1) was much higher than the alternative pathway activity measured in the absence of CN in plants grown at 19°C (up to 2.0 μmol O_2 g^{-1} fresh weight h^{-1} at 30°C; Fig. 2C). However, alternative oxidase protein content was increased 3-fold in plants grown at 19°C compared with plants grown at 28°C, raising the rate in the presence of CN to 10 μmol O_2 g^{-1} fresh weight h^{-1} at 25°C (Fig. 1), well above the observed activity in the absence of inhibitors (Fig. 2C). Increases in alternative oxidase protein levels by growth at low temperatures (Fig. 1) could be one of the mechanisms required to maintain a higher respiratory activity via the alternative pathway at low temperatures.

It has been suggested that the reported insensitivity of the alternative pathway to changes in temperature leads to a greater contribution of the alternative pathway to total respiration as temperature is lowered (Purvis and Shewfelt, 1993). However, in mung bean leaves, the sensitivity of CN-resistant respiration to temperatures between 10°C and 30°C was actually greater than that of SHAM-resistant respiration (Fig. 4), in contrast to what has been seen in isolated maize mesocotyl mitochondria (Stewart et al., 1990), in tissue cultures (Yoshida and Tagawa, 1979), and during respiration of some arctic plants (McNulty and Cummins, 1987). Oxygen-isotope fractionation by the al-

ternative pathway (CN-resistant activity) remained constant at approximately 30‰ and was not affected by either growth or measurement temperature.

At any given measurement temperature, the total respiratory rate of mung bean hypocotyls grown at 19°C was always higher than that seen in hypocotyls from plants grown at 28°C (Fig. 2A). This has been reported previously in other species (Billings, 1974; Amthor, 1989; Collier and Cummins, 1990). In contrast, neither mung bean leaves nor soybean cotyledons increased their respiration rates when grown at the low temperatures (Fig. 2C; Table I). Therefore, the acclimation of respiratory rate to low temperatures cannot be generalized among species (Atkin and Day, 1990) or organs within the same species.

Despite the increase in alternative oxidase protein and total respiration rate in mung bean tissues grown at 19°C, the partitioning of electrons to the alternative pathway did not increase as temperature was lowered. More generally, electron partitioning to the alternative pathway decreased as temperature was lowered below the growth temperature (except in hypocotyls of mung bean grown at 19°C and soybean cotyledons, in which partitioning remained constant). The observed decrease in electron partitioning to the alternative pathway is consistent with the greater temperature sensitivity seen for the CN-resistant respiration compared with SHAM-resistant respiration (Fig. 4). These results are not consistent with an increased contribution by the alternative pathway to total respiration as temperature is lowered (Purvis and Shewfelt, 1993). However, in addition to the short-term response to changes in temperature, cold-acclimated mung bean plants did maintain a higher percentage of alternative pathway activity at temperatures below 19°C than the plants grown at 28°C .

The 3- to 5-fold increase in alternative oxidase protein in plants grown at 19°C may enhance the activity enough to allow the plants to maintain the level of partitioning of electrons to the alternative pathway seen at low measurement temperatures. However, the mung bean response cannot be generalized for all species or tissues. Soybean cotyledons maintain a high level of electron partitioning to the alternative pathway at both high and low growth temperatures (Table I), which may be at least in part a reflection of the large amounts of alternative oxidase protein seen in cotyledon mitochondria at both temperatures.

The increase in alternative oxidase protein seen in mung beans grown at 19°C, combined with the higher apparent Q_{10} of the alternative pathway, might be expected to result in greater electron partitioning to the alternative pathway at high measurement temperatures unless the activity of alternative oxidase is attenuated accordingly. Electron partitioning to the alternative pathway reaches a maximum value (15% for hypocotyls and 40% in leaves) at near growth temperature and is maintained at this level above the growth temperature (Figs. 2B and 3B). It is possible that as temperature increases, the activation state of the alternative oxidase protein is down-regulated; this is especially true of plants grown at 19°C, in which alternative oxidase protein levels are increased (Fig. 1). This observation suggests the operation of regulatory mechanisms in vivo, possibly at the level of redox-active or α-keto-acid-reactive Cys (Umbach and Siedow, 1993; Ribas-Carbo et al., 1997; Rhoads et al., 1998), although other as yet unidentified mechanisms, including regulation of upstream dehydrogenase activity (Krab, 1995), may also play a role.

The increase in total respiration and the maintenance of oxygen-fractionation values in cold-grown mung bean hypocotyls led to a 2-fold increase in the activity of the alternative pathway at all temperatures compared with plants grown at 28°C (Fig. 2C). In leaves of mung bean plants grown at 19°C, the activity of the alternative pathway at low temperatures (9°C–19°C) was higher than in leaves of plants grown at 28°C, but equal to them at temperatures above 25°C (Fig. 3C). This observation is consistent with the idea that increases in alternative oxidase protein may be an acclimation response for maintaining alternative oxidase activity at low temperatures, at least in mung bean.

The increased partitioning of electrons to an enhanced activity of the alternative pathway at low temperatures in mung bean plants grown at 19°C compared with plants grown at 28°C may still serve to stabilize the reduction state of the ubiquinone pool to avoid the production of reactive oxygen species, as has been suggested previously (Purvis and Shewfelt, 1993; Prasad et al., 1994; Wagner, 1995; Vanlerberghe and McIntosh, 1996; Popov et al., 1997). It has also been suggested that the alternative pathway could play a local thermoregulatory role in chilling-sensitive plants exposed to low temperatures, because heat-emission rates increased significantly in leaves of several plants, including legumes, exposed to chilling temperatures (Ordentlich et al., 1991; Moynihan et al., 1995).

Because the alternative pathway is nonphosphorylating, the energy that is otherwise used to phosphorylate ADP is released in the form of heat. Breidenbach et al. (1997) used thermodynamic models to point out that any temperature increase in tissues where respiration shifts entirely to the alternative pathway would be too small to serve such a thermoregulatory role, and that the rapid rate of heat dissipation would not allow significant local heating of mitochondrial membranes. Our results with mung bean, a chilling-sensitive plant (Poehlman, 1974), show that respiration does not switch entirely to the alternative pathway and that the amount of heat generated from the alternative oxidase will actually decrease as the temperature decreases. These results support the conclusions of Breidenbach et al. (1997) and argue against a local thermoregulatory role for the alternative oxidase in mung bean. The increases in the rate of heat emission observed by Moynihan et al. (1995) in several plants exposed to low temperatures must be derived from other metabolic sources.

In summary, the relative contribution of the alternative pathway to total respiratory activity generally decreased as the temperature was lowered in the chilling-sensitive tissues of mung bean but not in soybean cotyledons. However, the observed up-regulation of alternative oxidase protein in cold-acclimated plants of mung bean does correlate with the ability to maintain a higher alternative pathway activity at low temperatures, perhaps compensating for the apparent high sensitivity of CN-resistant respiration to changes in temperature.

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