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Effects of light on respiration and oxygen isotope fractionation in soybean cotyledons

M. RIBAS-CARBO,[1,2]* S. A. ROBINSON,[3]* M. A. GONZÀLEZ-MELER,[2] A. M. LENNON,[4] L. GILES,[2] J. N. SIEDOW[2] & J. A. BERRY[1]

[1]*Carnegie Institution of Washington, Department of Plant Biology, 260 Panama Street, Stanford, CA94305, USA,* [2]*Department of Botany, Duke University, Durham, NC27708, USA,* [3]*Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia, and* [4]*Department of Biochemistry, School of Medical Sciences, University of Bristol BS8 1TD, UK*

ABSTRACT

Light effects on electron flow through the cyanide-resistant respiratory pathway, oxygen isotope fractionation and total respiration were studied in soybean (*Glycine max* L.) cotyledons. During the first 12 h of illumination there was an increase in both electron partitioning through the alternative pathway and oxygen isotope fractionation by the alternative oxidase. The latter probably indicates a change in the properties of the alternative oxidase. There was no engagement of the alternative oxidase in darkness and its fractionation was 27‰. In green cotyledons 60% of the respiration flux was through the alternative pathway and the alternative oxidase fractionation was 32‰. Exposing previously illuminated tissue to continuous darkness induced a decrease in the electron partitioning through the alternative pathway. However, this decrease was not directly linked with the low cellular sugar concentration resulting from the lack of light because 5 min of light every 12 h was sufficient to keep the alternative pathway engaged to the same extent as plants grown under control conditions.

Key-words: Alternative oxidase; cyanide-resistant pathway; *glycine max*; greening; light; oxygen isotope fractionation; respiration; soybean.

INTRODUCTION

The effects of light on plant respiration have been studied for many years (Azcón-Bieto, Lambers & Day 1983; Azcón-Bieto & Osmond 1983; Kromer, Stitt & Heldt 1988; Azcón-Bieto *et al*. 1989; Gardeström, Zhou & Malmberg 1992; Hill & Bryce 1992; Kromer 1995). Light is known to regulate gene expression of several key respiratory enzymes through the action of phytochrome, including cytochrome *c* oxidase (Hilton & Owen 1985) and phospho*en*olpyruvate carboxylase (Sims & Hague 1981). It has also been suggested that blue light can cause an increase in total respiration (Kowallik 1982). Furthermore, there is a differential expression of alternative oxidase genes between light-grown and dark-grown tissues (Obenland *et al*. 1990; Finnegan *et al*. 1997). Other workers have reported an indirect effect of light and photosynthesis on respiration, with the cellular concentration of sugars suggested to play an important role in the regulation of respiration in leaves (Azcón-Bieto *et al*. 1983) and roots (Bingham & Farrar 1988).

Studies of the greening process in plants have focused primarily on chloroplast and photosynthetic development with only a few studies addressing the behaviour of respiration (Harris, Mackender & Smith 1986; Azcón-Bieto *et al*. 1989).

The presence of two terminal oxidases, branching from the ubiquinone pool in the mitochondrial electron transfer chain has been known for many years, along with the fact that the cyanide-resistant, alternative respiratory pathway is not coupled to the synthesis of ATP (Moore & Siedow 1991). Studies of the kinetics of ubiquinone oxidation by the alternative oxidase and complex III of the cytochrome pathway supported the notion of Bahr & Bonner (1973) that the redox level of the ubiquinone pool is a major factor controlling the electron partitioning between the alternative oxidase and the cytochrome pathway (Dry *et al*. 1989). However, this behaviour can be modified by effectors which presumably function in the metabolic regulation of the alternative oxidase. These effectors include a redox-sensitive disulfide bond (Umbach & Siedow 1993; Ribas-Carbo *et al*. 1997) and α-keto acids (Millar *et al*. 1993; Ribas-Carbo *et al*. 1997). Several workers have suggested that soluble sugars could regulate electron flow to the alternative pathway (Azcón-Bieto *et al*. 1983). Studies used inhibitors to show that the flux through the alternative pathway in wheat leaves was higher at the beginning of the night, when the soluble sugar concentration was higher, than at the end of the night (Azcón-Bieto *et al*. 1983). In light of these results we were interested in observing the effects of an extended period of darkness, which would lead to a depletion of cellular sugar, on the electron partitioning between the two respiratory pathways in soybean cotyledons. As we were interested in separating the effects of light and sugar,

Correspondence: Miquel Ribas-Carbo. Fax: +1 650 3256857; e-mail: mribas@biosphere.stanford.edu

**These authors contributed equally to this work*

we also measured electron partitioning in plants growing under continuous darkness but supplemented with 5 min of light every 12 h.

The differential fractionation of ^{18}O by the two plant oxidases has allowed the development of an oxygen isotope fractionation technique that permits measurement of electron partitioning between the cyanide-sensitive cytochrome and cyanide-resistant alternative respiratory pathways in the absence of added inhibitors (Guy *et al*. 1989; Robinson *et al*. 1992, 1995; Ribas-Carbo *et al*. 1995, 1997; Peñuelas, Ribas-Carbo & Giles 1996; Lennon *et al*. 1997). Previous work showed there was a markedly different level of engagement of the alternative oxidase between etiolated and green cotyledons (Robinson *et al*. 1995). Here the changes in electron partitioning between the alternative and cytochrome pathways during the process of greening are examined.

Previous work has also shown that the oxygen isotope fractionation value for the alternative oxidase itself (Δa) is markedly higher in green tissues (cotyledons and leaves), with Δa values around 30‰, than in non-green tissues (roots and etiolated cotyledons), which have a fractionation value around 25‰ (Guy *et al*. 1989; Robinson *et al*. 1992; Ribas-Carbo *et al*. 1995). These differences in oxygen isotope fractionation by the alternative oxidase have been confirmed at the mitochondrial level (Ribas-Carbo *et al*. 1995). Therefore, it seems likely that the change in fractionation represents a change in the alternative oxidase protein or the conditions under which it operates. Obenland *et al*. (1990) observed differences in the pattern of electrophoretic bands that reacted with antibodies against the alternative oxidase during development in soybean cotyledons and these patterns differed between etiolated and green cotyledons. Most recently, the existence of a small family of alternative oxidase (AOX) genes in soybean (Finnegan *et al*. 1997) and *Arabidopsis thaliana* (Saisho *et al*. 1997) has been reported, and Finnegan *et al*. (1997) reported changes in the relative amounts of different AOX mRNAs in etiolated soybean cotyledons during the process of greening. In the present study, we examined the changes in electron partitioning between the cytochrome and alternative pathways in response to greening and following transfer to darkness.

MATERIALS AND METHODS

Plant material

Soybean (*Glycine max* L. cv. Ransom) seeds were treated with 0·5% NaOCl for 10 min and swelled in distilled water for 2 h with continuous bubbling of air. Seeds were planted in a 1 : 1 mixture of sand and perlite.

For the greening experiments, seeds were germinated and grown for 6 d in total darkness at 25 °C. These etiolated plants were then transferred to a growth chamber at 25/23 °C on a 12 h light : 12 h dark regime at 600 μmol photons m^{-2} s^{-1}. For the variable light regimes, seeds were germinated and grown at 27/23 °C on a 14 h light : 10 h dark regime at 350 μmol photons m^{-2} s^{-1}. After 5 d, a subset of plants were moved to a growth chamber and kept in darkness ('light-starved') at 25 °C whereas the control plants remained under the standard growth conditions. Light-pulse experiments were carried out on a subset of 'light-starved' plants. These plants received a 5 min pulse of light (350 μmol photons m^{-2} s^{-1} PAR) once every 12 h.

Oxygen isotope analysis, fractionation and partitioning calculations

Cotyledons were removed from three to five plants, weighed and either placed directly into a totally dark 4 mL closed cuvette at room temperature (approximately 23 °C) or treated with inhibitor. Inhibitor treatments were carried out as described in Ribas-Carbo *et al*. (1997). After at least 20 min in the cuvette, oxygen isotope analysis was conducted as described in Robinson *et al*. (1995) and isotope fractionation and electron partitioning were calculated as described in Guy *et al*. (1989) without forcing the relationship between $-\ln f$ and $\ln(R/R_o)$ to go through zero. We also discarded all experiments in which the r^2 of the latter slope was below 0·995 with a minimum of six data points. This corresponds to an error in estimation of Δ of approximately ± 0·5‰.

Determination of soluble sugars

Soluble sugars were determined by the method of Loewus (1952) with modifications (Cerning-Beroard 1975). Samples of previously frozen (liquid nitrogen) tissue (approximately 1 g fresh weight) were ground with 3–4 mL of 60% ethanol (chilled below 0 °C). The ethanol slurry was centrifuged at 2800 *g* for 4 min. The pellet was resuspended in 2 mL of ethanol and centrifuged again. The pellet was kept frozen for starch analysis. An aliquot (0·1 mL) of the extract was added to 1·8 mL of anthrone reagent (7·7 mM anthrone dissolved in 70% sulphuric acid), the mixture was incubated at 40 °C for 30 min, and the samples were cooled to room temperature. Absorbance at 630 nm was read using a Lambda 3B (Perkin-Elmer Analytical Instruments, Norwalk, Conneticut, USA) spectrophotometer. Sugar standards were obtained from a stock of 1 mg each of sucrose, glucose and fructose in 1 mL ethanol.

Starch determination

Starch concentrations were determined by the amyloglucosidase enzymatic method (Cerning-Beroard 1975). Starch concentration was assayed by adding 2 mL of water to the ethanol-insoluble pellet obtained during the determination of soluble sugars. Samples were boiled for 2 min and maintained warm for 10 min. After cooling to room temperature, 0·1 mL of 2·5 M citrate buffer (pH 4·5) and 375–400 units/mL of amyloglucosidase (reconstituted in 0·05 M citrate buffer) were added to the samples and starch standards (obtained from a stock of 10 : 1, starch : water, w/v). Samples and standards were incubated at 45 °C

overnight, cooled to room temperature and centrifuged for 4 min at 2800 *g*. An aliquot (0·1 mL) of the supernatant was mixed with anthrone reagent and incubated at 40 °C for 30 min. Absorbance was read at 630 nm.

Chlorophyll determinations

The chlorophyll concentration of soybean cotyledons was measured at various times throughout the greening process. Cotyledon samples (approximately 1 g fresh weight) were collected at the same time as samples for mass spectrometry and were stored at – 80 °C prior to analysis. The cotyledons were then ground in liquid nitrogen and extracted in 80% buffered aqueous acetone. Total chlorophyll present in the extract was determined spectrophotometrically as described by Porra, Thompson & Kroiedemann (1989).

RESULTS

Effect of greening on etiolated cotyledons

Changes in the chlorophyll concentration during the greening process

The amount of chlorophyll in soybean cotyledons was measured as they progressed from an etiolated ($t = 0$) to a fully green ($t = 60$ h) state. The amount of chlorophyll increased from almost undetectable amounts to over 150 nmol g^{-1} FW (Fig. 1).

Changes in oxygen isotope fractionation by the cytochrome and alternative respiratory pathways during the greening process

To calculate the fraction of the electron transport going through the alternative oxidase the fractionation of each pathway acting by itself must be known. Oxygen isotope fractionation by the cytochrome pathway (Δc) by soybean cotyledons sampled during greening was 20·2‰ ± 0·6 ($n = 12$) and showed no significant change throughout the greening process (data not shown). This value is similar to values of Δ*c* measured previously in green soybean cotyledons and the mitochondria isolated from them (Robinson *et al*. 1995; Ribas-Carbo *et al*. 1997). Oxygen isotope fractionation by the alternative oxidase (Δ*a*) was measured in the presence of KCN in etiolated cotyledons placed in the light and sampled at different times during their greening. The value of Δ*a* changed from 26·9‰ ± 1·1 ($n = 3$) in etiolated cotyledons to 32·1‰ ± 0·6 ($n = 5$) after 36 h under a 12 h light : 12 h dark regime of 600 μmol photons m^{-2} s^{-1} (Fig. 2). The increase in Δ*a* occurred primarily during the first 12 h of illumination. Both of these values are similar to the values of Δ*a* measured previously using mitochondria isolated from etiolated (25·4‰) and green soybean cotyledons (30·9‰) (Ribas-Carbo *et al*. 1997).

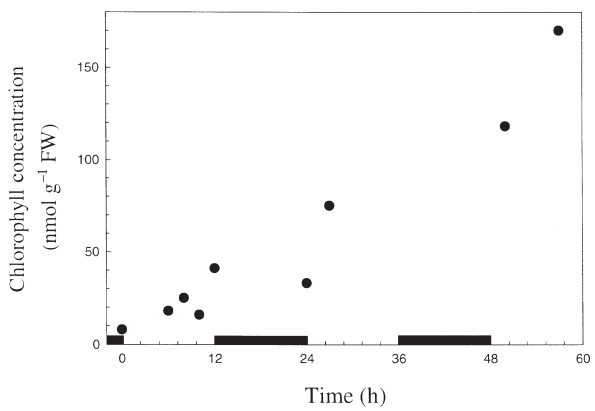

Figure 1. Chlorophyll content of soybean cotyledons during greening. Chlorophyll measurements were carried out as described in Materials and methods.

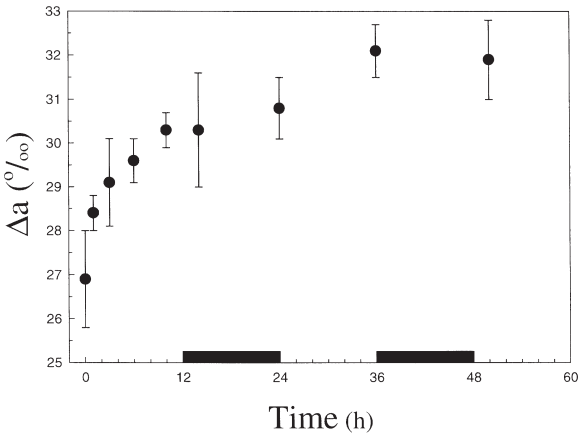

Figure 2. Changes in oxygen isotope fractionation by the alternative oxidase (Δ*a*) in soybean cotyledons during greening. Detached cotyledons were treated with 1 mM KCN for 10 min before transfer to the measuring cuvette. Results and SEM were calculated from three to seven repetitions, obtained in five different experiments. These values of Δ*a* were subsequently used to calculate the partitioning between the two pathways.

Respiratory changes during greening

Total respiration changes after exposure to light, increasing from 0·43 μmol O_2 g^{-1} FW min^{-1} before light exposure to 0·61 μmol O_2 g^{-1} FW min^{-1} during the first 10 h (Fig. 3a). This initial rise was followed by a steady decline over the next 40 h. Oxygen isotope fractionation measured in the absence of inhibitors (Δ*n*) was used to calculate electron partitioning (τ_a) between the cytochrome and alternative pathways at various times. Oxygen isotope fractionation measured in the absence of inhibitors increased from approximately 19‰ in etiolated tissues to an average value around 25‰ after 12 h in the presence of light (Fig. 3b). The value of Δ*n* remained constant between 26 and 27‰ thereafter. The electron partitioning through the alternative pathway (τ_a) changed dramatically during the first 12 h of greening, increasing from 0 to about 0·6 and main-

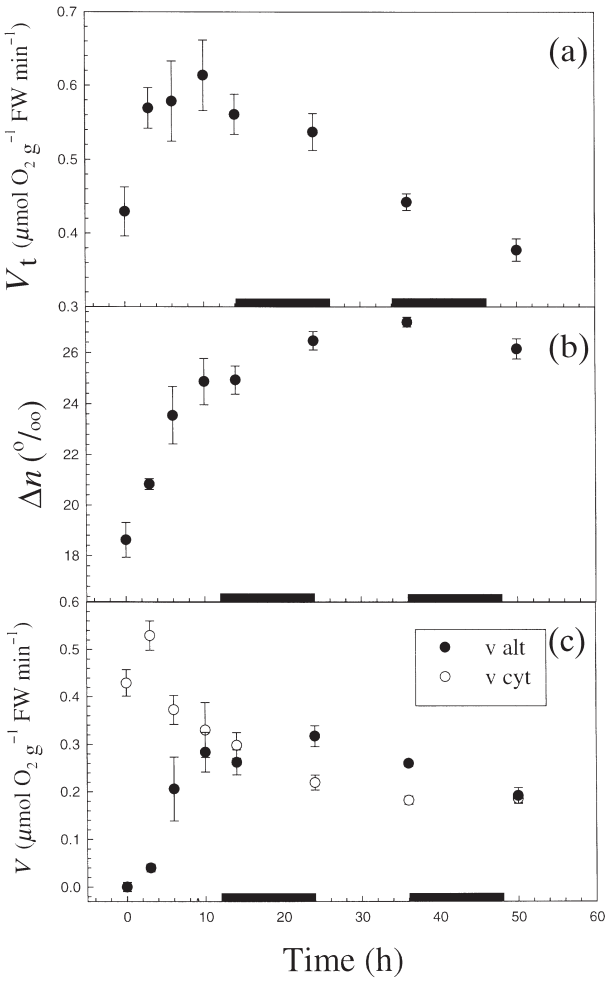

Figure 3. (a), Total respiration rate; (b), oxygen isotope fractionation and (c) activities of the cytochrome (□) and alternative (■) respiratory pathways of soybean cotyledons during the process of greening. Values and SEM are calculated from three to 11 replicated measurements on five different experiments. Rates were calculated as follows:
$\tau_a = (\Delta n - \Delta c)/(\Delta a - \Delta c)$; $v_{alt} = V_t \times \tau_a$; $v_{cyt} = V_t \times (1 - \tau_a)$.

taining this level of partitioning thereafter. It is important to remark that if the changes in Δa during this time frame were not taken into account, the electron partitioning through the alternative pathway (τ_a) would be overestimated.

Figure 3c shows the activity of the cytochrome (open symbols) and alternative (closed symbols) pathways during the greening process. The initial increase in steady-state respiration (V_t) is associated with an increase in the rate of both pathways. During the first 3 h of light there is an increase of the respiratory rate of the cytochrome pathway, from 0·43 to 0·53 μmol O_2 g^{-1} FW min^{-1}. From 3 to 12 h, the rate of the cytochrome pathway sharply decreased to 0·3 μmol O_2 g^{-1} FW min^{-1} whereas the alternative pathway rate increased to about 0·3 μmol O_2 g^{-1} FW min^{-1}.

The alternative pathway activity peaks after 24 h of greening. Thereafter, both pathways slowly decrease to around 0·2 μmol O_2 g^{-1} FW min^{-1} during the following 24 h (Fig. 3c)

Effect of variable light treatments on cotyledon respiration

In order to differentiate between the effects of light-driven metabolic activity and light signalling effects on alternative pathway activity, steady-state respiration and oxygen isotope fractionation were measured in cotyledons from light-grown seedlings placed under continuous darkness ('light-starved'), 'control' plants maintained under standard light conditions (14 h light : 10 h dark) and light-starved plants that received 5 min of light every 12 h ('flashed').

Total respiration was not significantly different between the three light treatments. In all three cases a decrease in the rate of oxygen uptake from 0·35 to 0·15 μmol O_2 g^{-1} FW min^{-1} was observed over the experimental time course (data not shown).

Electron partitioning through the alternative pathway (τ_a) was fairly high (with τ_a values ranging between 0·4 and 0·5) at the beginning and remained constant throughout the experiment in plants kept under the control light treatment (Fig. 4, open circle). Plants maintained in darkness ('light-starved') showed a continuous decline in the extent of electron partitioning through the alternative pathway, going from a value of 0·42 at $t = 0$ to a value of 0·1 after 60 h of continuous darkness (Fig. 4, closed circle). Plants that received only 5 min of light every 12 h ('Flashed'), however,

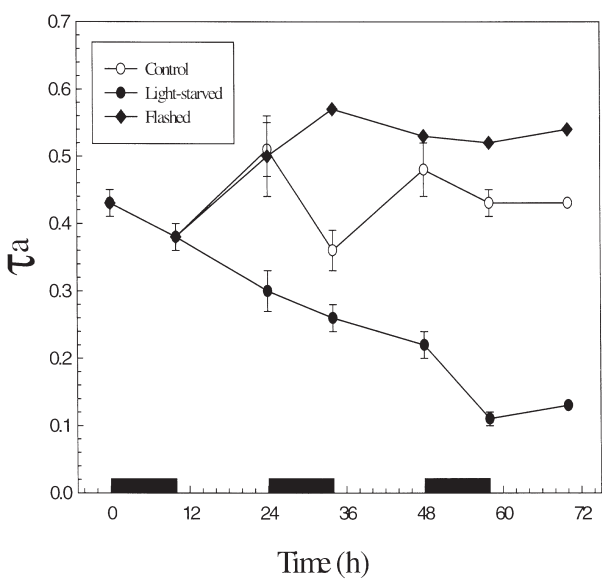

Figure 4. Partitioning (τ_a) of electrons through the alternative pathway in soybean cotyledons from plants growing under a regular 14 h light : 10 h dark photoperiod 'control' (○), moved to continuous darkness 'light-starved' (●) or moved to dark with 5 min of light every 12 h 'flashed' (♦). Results and SEM were calculated from three to seven repetitions. Experiments without error bars are the average of two experiments carried out separately.

maintained a τ_a with values ranging between 0·5 and 0·6 (Fig. 4, black diamond) throughout the 60 h treatment, similar to the 'control' plants and much higher than that seen in the 'light-starved' plants.

Soluble sugar and starch content

At the end of the experimental period, cotyledons from plants held under control conditions (14 h light : 10 h dark) had significantly higher soluble sugars and starch concentrations than cotyledons from both the 'light-starved' and 'flashed' plants (Table 1). The control cotyledons had a soluble sugar concentration ranging from 0·61 to 0·82 mg g^{-1} FW and a starch concentration ranging from 25·2 to 55·4 mg g^{-1} FW (Table 1). Plants under 'light-starved' and 'flashed' treatments had a significantly lower soluble sugar and starch concentration than controls after 24 h of treatment. However, there were no significant differences in soluble sugars and starch concentration between 'light-starved' and 'flashed' plants, indicating that 5 min of light every 12 h were not causing any significant photosynthesis production.

DISCUSSION

Our goal in these experiments was to document the short-term changes that occur in respiration during the greening process in soybean cotyledons and to determine whether any changes observed represented a response to metabolic changes and/or the response to a signal transduction pathway.

Table 1. Soluble sugar and starch concentrations in soybean cotyledons from plants grown under control light conditions ('control'), transferred from light-control conditions to continuous darkness ('light starved'), or moved to darkness with 5 min of light every 12 h ('flashed'). Values and SEM are from three different experiments except for the flash experiments, in which the values are the average of two different experiments. Measurements during the first night period (times 0 and 10 h) were combined from the three experiments

Time (h)	'Control'	'Light-starved'	'Flashed'
Soluble sugars (mg g^{-1} FW)			
0	0·61 ± 0·07	0·61 ± 0·07	0·61 ± 0·07
10	0·65 ± 0·08	0·65 ± 0·08	0·65 ± 0·08
24	0·80 ± 0·01	0·38 ± 0·02	0·44
34	0·79 ± 0·09	0·36 ± 0·07	0·479
48	0·82 ± 0·02	0·27 ± 0·02	0·287
58		0·36 ± 0·03	0·357
Starch (mg g^{-1} FW)			
0	25·2 ± 3·0	25·2 ± 3·0	25·2 ± 3·0
10	34·3 ± 4·2	34·3 ± 4·2	34·3 ± 4·2
24	55·4 ± 3·3	23·0 ± 4·4	8·8
34	54·1 ± 4·9	10·4 ± 2·0	12·1
48	36·5 ± 4·4	7·5 ± 2·9	7·52
58		7·7 ± 2·3	8·21

Our observation that the oxygen isotope fractionation value of the alternative oxidase (Δa) changed dramatically during the first hours of illumination (Fig. 2) is closely correlated with the time-course of the change in AOX gene expression observed by Finnegan *et al*. (1997). In etiolated cotyledons, the most abundant mRNA present was AOX3 with 65% mole fraction, whereas AOX2 (25%) and AOX1 (10%) were much less abundant (Figs 6 in Finnegan *et al*. 1997). After 6 h light, we observed an increase in Δa to 29·6‰, which roughly parallels the time-course of the increase in the relative amount of AOX2 transcript (35%) and the decrease in AOX3 transcript (55%) observed by Finnegan *et al*. (1997; Fig. 6). Similarly, after 12 h light we observed a Δa of 30·3‰, when the relative amount of AOX2 transcript was about 62% of the total AOX and the amount of AOX3 transcript had decreased to 30%. Although, we did not measure these changes in our own samples, there is another coincidence between AOX3 transcript (which presumably translates into levels of this isozyme) and the lower value of oxygen isotope fractionation by the alternative oxidase. Finnegan *et al*. (1997) observed a large abundance of the AOX3 gene product (over 95%) in soybean root tissues where the oxygen isotope fractionation value was also about 25‰ (Robinson *et al*. 1992, 1995; Ribas-Carbo *et al*. 1995). It is possible that the AOX3 gene was responsible for the low Δa (25–27‰) whereas AOX2 was responsible for the high Δa (30–32‰).

We observed an overall increase in respiration following the first hours of exposure to light (Fig.3a). This effect parallels a number of processes that occur during greening. A large number of genes are light-activated (Tobin & Silverthorne 1985; Kuhlemeier, Green & Chua 1987; Gilmartin *et al*. 1990) and there is a major increase in protein synthesis during the first hours of illumination (Srinivasan & Oliver 1995), particularly of those proteins concerned with chloroplast development. Consequently, it is not surprising that respiration also increases. During the first 3 h of illumination, there was an increase in both, cytochrome and alternative pathway fluxes (Fig.3c). During the following 21 h, the flux through the cytochrome pathway decreased whereas the flux through the alternative pathway continued to increase. It was in the first hours of chloroplast development that the ATP needed for transcription and protein synthesis was provided by respiration (Gregory & Bradbeer 1975; Bradbeer 1981). However, it was interesting to note that during this period of high ATP demand, electron partitioning through the alternative pathway began to increase. We do not know the basis for this shift in respiratory metabolism, but it appears to contradict the often-cited energy overflow hypothesis (Bahr & Bonner 1973; Lambers 1980; Moore & Siedow 1991). It is possible that during greening conditions the availability of ATP might not be as critical as the supply of carbon skeletons for biosynthesis. In this situation, it could be important to maintain a high rate of electron transport with a low rate of ATP synthesis. Further studies focusing on this are needed to confirm this possibility.

Moving previously light-grown plants to an extended

period of darkness caused a continuous decrease in the activity of the alternative pathway (Fig. 4). This was correlated with a decrease in the soluble sugar and starch contents (Table 1). A close relationship between the concentration of cellular sugars and activity of the alternative pathway has been suggested previously (Azcón-Bieto *et al*. 1983). However, plants that were moved to darkness but were given 5 min of light every 12 h maintained high alternative pathway activity (Fig. 4) but low soluble sugar and starch concentrations (Table 1). This result breaks the connection between sugar content and alternative pathway activity but raises another question regarding the nature of the effect of short light pulses on alternative oxidase activity. It seems clear that photosynthesis is not a sole contributing factor in this case, and that something else is acting to maintain the activity of the alternative pathway at a level similar to that seen in light-grown cotyledons. There were also no changes in the level of alternative oxidase protein per unit of mitochondrial protein over the time course of any of these three treatments (data not shown), but light could operate to maintain the reduction state of the regulatory disulphide bond associated with the alternative oxidase and/or affect the intracellular levels of pyruvate (Siedow & Umbach 1995). A more detailed study of the quality of light responsible for this effect and characterization of the receptors that are involved in this process is needed to 'shed light' on the processes responsible.

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REFERENCES

Azcón-Bieto J., Lambers H. & Day D.A. (1983) Effect of photosynthesis and carbohydrate status on respiratory rates and the involvement of the alternative pathway in leaf respiration. *Plant Physiology* **72,** 598–603.

Azcón-Bieto J. & Osmond C.B. (1983) Relationship between photosynthesis and respiration. The effect of carbohydrate status on the rate of CO_2 production by respiration in darkened and illuminated wheat leaves. *Plant Physiology* **71,** 574–581.

Azcón-Bieto J., Salom C.L., Mackie N.D. & Day D.A. (1989) The regulation of mitochondrial activity during greening and senescence of soybean cotyledons. *Plant Physiology and Biochemistry* **27,** 827–836.

Bahr J.T. & Bonner W.D. (1973) Cyanide-insensitive respiration. Control of the alternative pathway. *Journal of Biological Chemistry* **248,** 3446–3450.

Bingham I.J. & Farrar J.F. (1988) Regulation of respiration in roots of barley. *Physiologia Plantarum* **73,** 278–285.

Bradbeer J.W. (1981) Development of photosynthetic function during chloroplast biogenesis. In *The Biochemistry of Plants, Vol. 8* (eds M.D. Hatch & N.K. Boardman), pp. 423–472. Academic Press. New York.

Cerning-Beroard J. (1975) A note on sugar determination by the anthrone method. *Cereal Chemistry* **52,** 857–860.

Dry I.B., Moore A.L., Day D.A. & Wiskich J.T. (1989) Regulation of alternative pathway activity in plant mitochondria: nonlinear relationship between electron flux and the redox poise of the ubiquinone pool. *Archives of Biochemistry and Biophysics* **273,** 148–157.

Finnegan P.M., Whelan J., Millar A.H., Zhang Q., Smith M.K., Wiskich J.T. & Day D.A. (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. *Plant Physiology* **114,** 455–466.

Gardeström P., Zhou G. & Malmberg G. (1992) Respiration in barley protoplasts before and after illumination. In *Molecular, Biochemical and Physiological Aspects of Plant Respiration* (eds H. Lambers & L.H.W. van der Plas), pp. 261–266. SPB Academic Publishing. The Hague.

Gilmartin P.M., Sarokin L., Memelink J. & Chua N.-H. (1990) Molecular light switches for plant genes. *Plant Cell* **2,** 369–378.

Gregory P. & Bradbeer J.W. (1975) Plastid development in primary leaves of *Phaseolus vulgaris*. *Biochemical Journal* **148,** 433–438.

Guy R.D., Berry J.A., Fogel M.L. & Hoering T.C. (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. *Planta* **177,** 483–491.

Harris M., Mackender R.O. & Smith D.L. (1986) Photosynthesis of cotyledons of soybean seedlings. *New Phytologist* **104,** 319–329.

Hill S.A. & Bryce J.H. (1992) Malate metabolism and light-enhanced respiration in barley mesophyll protoplasts. In *Molecular, Biochemical and Physiological Aspects of Plant Respiration* (eds H. Lambers & L.H.W. van der Plas), pp. 221–230. SPB Academic Publishing. The Hague.

Hilton J.R. & Owen P.D. (1985) Phytochrome regulation of extractable cytochrome oxidase activity during early germination of *Bromus sterilis* and *Lactuca sativa* L. cv. Grand Rapids seeds. *New Phytologist* **100,** 163–171.

Kowallik W. (1982) Blue light effects on respiration. *Annual Review of Plant Physiology* **33,** 51–72.

Kromer S. (1995) Respiration during photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 45–70.

Kromer S., Stitt M. & Heldt H.W. (1988) Mitochondrial oxidative phosphorylation participating in photosynthetic metabolism of a leaf cell. *FEBS Letters* **226,** 352–356.

Kuhlemeier C., Green P.J. & Chua N.M. (1987) Regulation of gene expression in higher plants. *Annual Review of Plant Physiology* **38,** 221–257.

Lambers H. (1980) The physiological significance of cyanide-resistant respiration in higher plants. *Plant, Cell & Environment* **3,** 293–301.

Lennon A.M., Neueschwander U.H., Ribas-Carbo M., Giles L., Ryals J.A. & Siedow J.N. (1997) The effects of salicylic acid and TMV infection upon the alternative oxidase of tobacco. *Plant Physiology* **115,** 783–791.

Loewus F.A. (1952) Improvement in anthrone method for determination of carbohydrates. *Analytical Chemistry* **24,** 219–223.

Millar A.H., Wiskich J., Whelan J. & Day D.A. (1993) Organic acid activation of the alternative oxidase of plant mitochondria. *FEBS Letters* **329,** 259–262.

Moore A.L. & Siedow J.N. (1991) The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. *Biochimica et Biophysica Acta* **1058,** 121–140.

Obenland D., Diethelm R., Shibles R. & Stewart C. (1990) Relationship of alternative respiratory capacity and alternative oxidase amount during soybean seedling growth. *Plant Cell Physiology* **31,** 897–901.

Peñuelas J., Ribas-Carbo M. & Giles L. (1996) Effects of allelochemicals on plant respiration and oxygen isotope fractionation by the alternative oxidase. *Journal of Chemical Ecology* **22,** 801–805.

Porra R.J., Thompson W.A. & Kroiedemann P.E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* **975,** 384–394.

Ribas-Carbo M., Berry J.A., Yakir D., Giles L., Robinson S.A., Lennon A.M. & Siedow J.N. (1995) Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. *Plant Physiology* **109,** 829–837.

Ribas-Carbo M., Lennon A.M., Robinson S.A., Giles L., Berry J.A. & Siedow J.N. (1997) The regulation of the electron partitioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. *Plant Physiology* **113,** 903–911.

Robinson S.A., Yakir D., Ribas-Carbo M., Giles L., Osmond C.B., Siedow J.N. & Berry J.A. (1992) Measurements of the engagement of cyanide-resistant respiration in the crassulacean acid metabolism plant *Kalanchoë daigremontiana* with the use of online oxygen isotope discrimination. *Plant Physiology* **100,** 1087–1091.

Robinson S.A., Ribas-Carbo M., Yakir D., Giles L., Reuveni Y. & Berry J.A. (1995) Beyond SHAM and cyanide: opportunities for studying the alternative oxidase in plant respiration using oxygen isotope discrimination. *Australian Journal of Plant Physiology* **22,** 487–496.

Saisho D., Nambara E., Naito S., Tsutsumi N., Hirai A. & Nakazono M. (1997) Characterization of the gene family for alternative oxidase from Arabidopsis thaliana. *Plant Molecular Biology* **35**, 585–596.

Siedow J.N. & Umbach A.L. (1995) Plant mitochondrial electron transfer and molecular biology. *Plant Cell* **7,** 821–831.

Sims T.L. & Hague R.D. (1981) Light-stimulated increase of translatable mRNA for phosphoenolpyruvate carboxylase in leaves of maize. *Journal of Biological Chemistry* **256,** 8252–8256.

Srinivasan R. & Oliver D.J. (1995) Light-dependent and tissue-specific expression of the H-protein of the glycine decarboxylase complex. *Plant Physiology* **109,** 161–168.

Tobin E.M. & Silverthorne J. (1985) Light regulation of gene expression in higher plants. *Annual Review of Plant Physiology* **36,** 569–593.

Umbach A.L. & Siedow J.N. (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiology* **103,** 845–854.

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