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# The alternative oxidase in roots of *Poa annua* after transfer from high-light to low-light conditions

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**Summary**

**The activity of the alternative pathway can be affected by a number of factors, including the amount and reduction state of the alternative oxidase protein, and the reduction state of the ubiquinone pool. To investigate the importance of these factors *in vivo*, we manipulated the rate of root respiration by transferring the annual grass *Poa annua* L. from high-light to low-light conditions, and at the same time from long-day to short-day conditions for four days. As a result of the low-light treatment, the total respiration rate of the roots decreased by 45%, *in vitro* cytochrome *c* oxidase capacity decreased by 49%, sugar concentration decreased by 90% and the ubiquinone concentration increased by 31%, relative to control values. The absolute rate of oxygen uptake via the alternative pathway, as determined using the $^{18}O$-isotope fractionation technique, did not change. Conversely, the cytochrome pathway activity decreased during the low-light treatment; its activity increased upon addition of exogenous sugars to the roots. Interestingly, no change was observed in the concentration of the alternative oxidase protein or in the reduction state of the protein. Also, there was no change in the reduction state of the ubiquinone pool. In conclusion, the concentration and activity of the alternative oxidase were not changed, even under severe light deprivation.**

**Keywords: alternative oxidase, root respiration, sugars.**

## Introduction

The respiratory electron-transport pathways of plant mitochondria comprise the cytochrome pathway and the alternative pathway; beyond the branch point (ubiquinone), the alternative pathway does not contribute to the generation of a proton-motive force, in contrast to the cytochrome pathway (Vanlerberghe and McIntosh, 1997). The alternative pathway consists of only one protein, the alternative oxidase (AOX). In the past few years, we have gained insight into biochemical mechanisms that affect the activity of the alternative oxidase in isolated mitochondria, including activation by reduction of an intermolecular disulphide bond and by α-keto acids, such as pyruvate (Hoefnagel *et al.*, 1995; Millar *et al.*, 1993; Millar *et al.*, 1996; Umbach and Siedow, 1993; Umbach *et al.*, 1994). Information on the significance of these activating mechanisms *in vivo*, however, is still scarce. Simons *et al.* (1999) found no oxidized form of the AOX protein in leaves of *Arabidopsis thaliana*, despite large changes in total respiration rate, AOX protein and mRNA encoding the alternative oxidase. In the roots of *Poa annua*, the alternative oxidase is invariably in the reduced form during the light period (Millenaar *et al.*, 1998). During the day, a relatively large amount of carbohydrates is imported into the roots, so that there is probably a need for an overflow of the cytochrome pathway, which would require the active, reduced form of the alternative oxidase. Millenaar *et al.* (1998) concluded that the alternative pathway activity can stabilize the ratio of reduced ubiquinone to total ubiquinone ($Q_r/Q_t$) *in vivo*, and consequently prevent production of reactive oxygen species (e.g. Purvis and Shewfelt, 1993). Pyruvate, which activates the alternative oxidase in isolated mitochondria, probably does not play a role *in vivo* (Millenaar *et al.*, 1998).

If the alternative oxidase indeed acts as an overflow of electrons for the cytochrome pathway, one might expect that the activity of the alternative oxidase would decrease with decreasing sugar concentration in the roots. The activity of the alternative oxidase may decrease by (i) lowering the amount of alternative oxidase, (ii) decreasing the fraction of the oxidase that is in the reduced state, or (iii) decreasing the reduction state of its substrate, ubiquinone ($Q_r/Q_t$).

The question therefore arises as to what will happen to the concentration and reduction state of the alternative oxidase when the sugar supply to the roots is lowered. Azcón-Bieto *et al.* (1983) concluded that the level of respiratory substrates in leaves of *Spinacia oleracea*, *Triticum aestivium* and *Pisum sativum* determines the rate of respiration and the salicylhydroxamic acid (SHAM) sensitivity. In the roots of *Cucumis sativus*, a diurnal fluctuation in the sugar concentration correlates with the SHAM sensitivity of root respiration (Lambers *et al.*, 1996). In recent years, however, it has become clear that SHAM sensitivity provides, at best, an indication of whether the alternative oxidase was active before the addition of inhibitors; absence of inhibition is no proof of absence of alternative path activity (Day *et al.*, 1996; Hoefnagel *et al.*, 1995; Ribas-Carbo *et al.*, 1995; Wagner and Krab, 1995).

In order to investigate the relation between sugar supply to the roots and the regulation of alternative pathway activity in the annual grass *Poa annua*, plants were exposed to a long night (16 h instead of the normal 10 h) and to a low light intensity (70 instead of 450 $\mu$mol $m^{-2}$ $sec^{-1}$ photosynthetically active radiation (PAR). The regulation of the activity of the alternative oxidase was studied by measuring the amount and reduction state of the protein as well as the reduction state of the ubiquinone pool ($Q_r/Q_t$). Total respiration, SHAM-resistant respiration and KCN-resistant respiration were determined, and $^{18}O$-isotope fractionation was used to calculate the contribution of the alternative and cytochrome pathways (Lennon *et al.*, 1997; Robinson *et al.*, 1995). During the low-light treatment, sugar concentrations and cytochrome *c* oxidase capacity were measured. In addition, *Poa annua* plants were placed in complete darkness for 4 and 7 days to severely reduce the sugar supply to the roots.

## Results

### *Carbohydrates*

To change the endogenous sugar concentration in the roots of *Poa annua*, we transferred plants from a 10 h to a 16 h night period, and at the same time to a lower light intensity during the day (70 instead of 450 $\mu$mol $m^{-2}$ $sec^{-1}$, PAR). During the following 4 days, the concentrations of glucose, fructose, sucrose, inositol, malate and citrate were measured (Figure 1). The sugar concentrations in the roots were not significantly changed after the normal 10 h period in the dark, and recovered when no low-light treatment was given (data for time 0 are the average of data from several days with normal light conditions). After transfer to a light regime with 16 h of darkness, however, the concentrations of glucose, fructose and sucrose did not recover to their initial concentration in the light; they decreased significantly, compared with the initial concentration at time 0 and 10 h (6.2, 10.5 and 1.6 $\mu$mol $g^{-1}$ FW, respectively, Figure 1A–C). After two long nights, glucose and fructose concentrations were only 14% of their initial concentration at time 0, while the sucrose concentration had decreased by 94%. A small, but significant, increase in glucose, fructose and especially sucrose was observed during nights 3 and 4. Inositol and malate concentrations decreased more gradually with time, to 24 and 22% of their initial concentrations (Figure 1D,E). The citrate concentration ($1.0 \pm 0.3$ $\mu$mol $g^{-1}$ FW) did not change significantly during the low-light treatment (Figure 1F).

### *Respiration*

Root respiration (oxygen electrode) did not significantly decrease during the 10 h of normal darkness compared with the initial respiration rate in the light period ($5.3 \pm 0.6$ nmol $O_2$ $g^{-1}$ FW $sec^{-1}$; data for time 0 are the average from several days with normal light conditions). However, when the night period was extended to 16 h, root respiration decreased by 45%. During the following 3 days, respiration decreased further, reaching a value of 27% of the initial control respiration (Figure 2A). After this time, the respiration did not show much more of a decrease; even after 4 and 7 days of complete darkness, respiration was 26 and 20%, respectively, of its original value. The KCN- or SHAM-resistant respiration decreased during the low-light treatment (Figure 2B), when compared with plants grown under initial conditions. The residual respiration did not change with time.

The alternative pathway activity was measured using the $^{18}O$-isotope fractionation method in the absence of inhibitors. The oxygen fractionation by the alternative oxidase (in the presence of KCN) was $26.6 \pm 0.10$‰, and that for cytochrome *c* oxidase was $19.5 \pm 0.32$‰ (with SHAM); for the residual respiration it was $21.4 \pm 0.24$‰ (with KCN and SHAM, mean $\pm$ standard error, $n = 3$). The fractionation values are in agreement with values for isolated mitochondria (Ribas-Carbo *et al.*, 1997). The fractionation of respiration in the absence of inhibitors increased during the first 16 h of the first long night

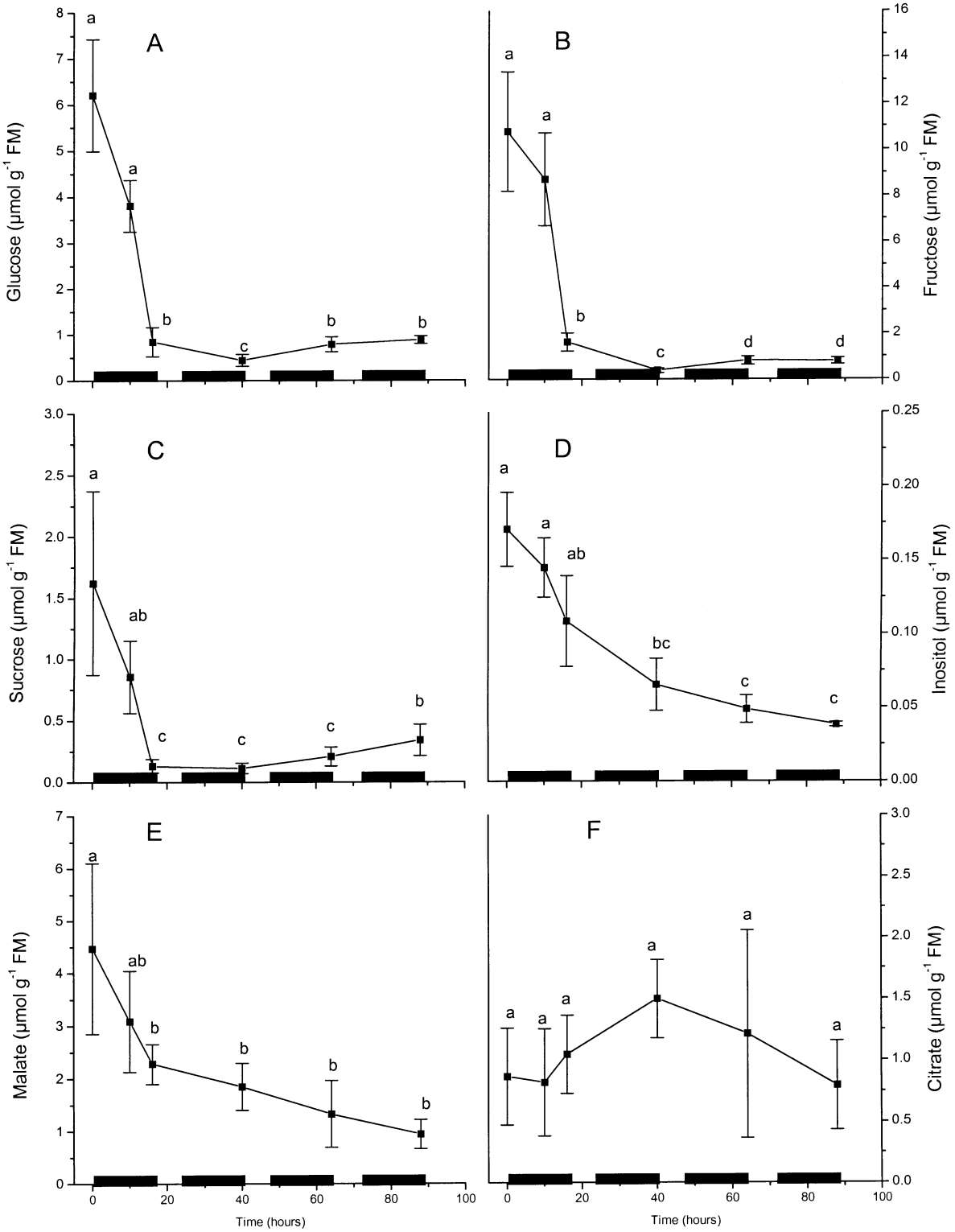


**Figure 1.** Carbohydrate, sugar alcohol and organic acid levels in the roots of *Poa annua* plants during 4 days of exposure to low-light conditions. Error bars represent standard deviation and the number of replicates was at least three, based on different plants and plant batches; points with a different letter are significantly different. All concentrations are in µmol $g^{-1}$ FW. (A) glucose; (B) fructose; (C) sucrose; (D) inositol; (E) malate; (F) citrate.

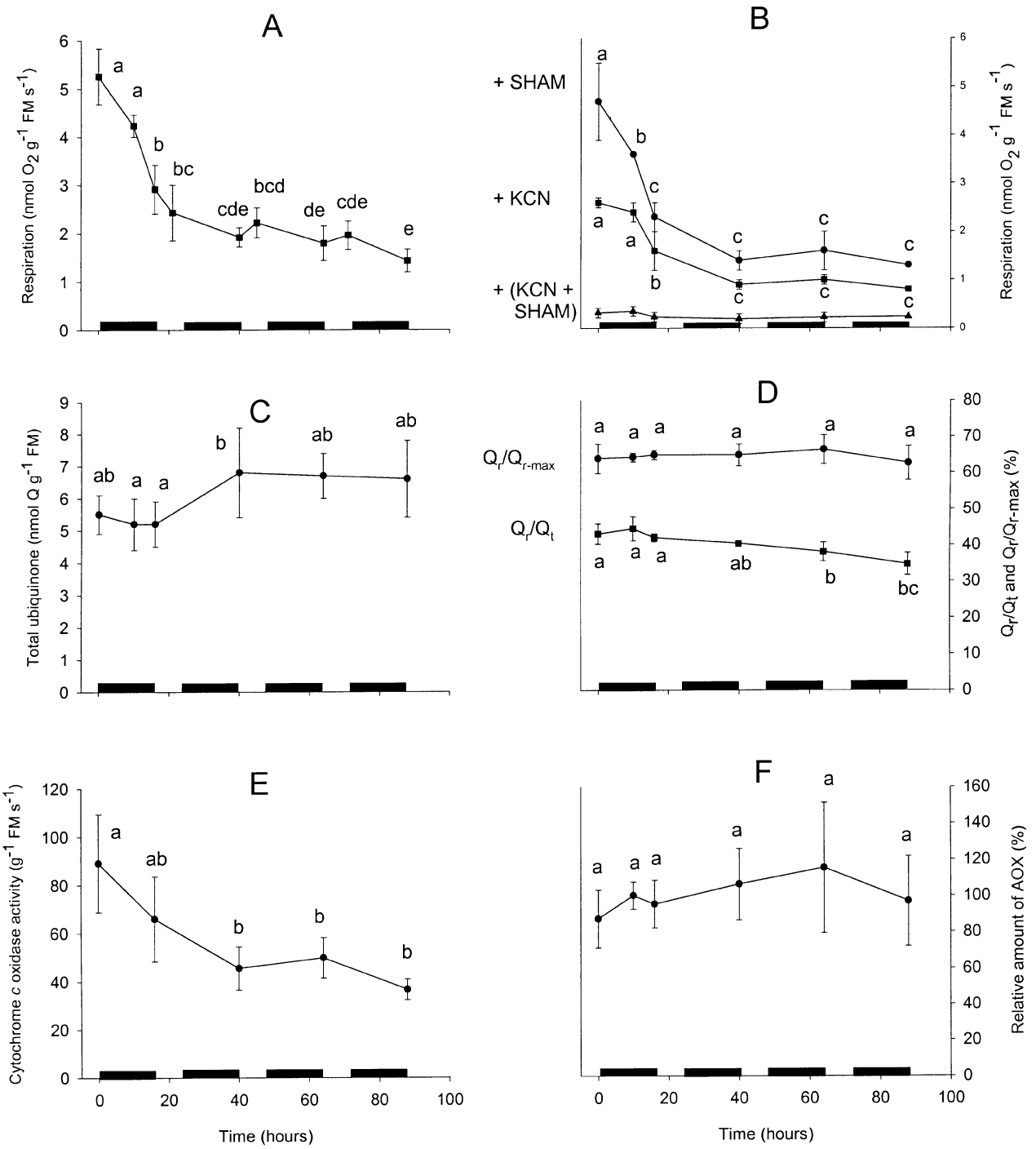


**Figure 2.** Respiration, ubiquinone levels, cytochrome *c* oxidase and alternative oxidase in the roots of *Poa annua* plants during 4 days exposure to low-light conditions.

Error bars represent standard deviation and the number of replicates was at least three, based on different plants and plant batches; points with a different letter are significantly different. (A) Respiration (nmol $O_2$ $g^{-1}$ FW $sec^{-1}$, oxygen electrode); (B) SHAM (●), KCN (■) and SHAM + KCN (▲) resistant respiration (oxygen electrode); (C) total ubiquinone pool (nmol $g^{-1}$ FW); (D) $Q_r/Q_t$ (■) and $Q_r/Q_{r\text{-}max}$ (●); (E) cytochrome *c* oxidase capacity ($g^{-1}$ FW $sec^{-1}$); (F) relative alternative oxidase protein (%), detected with monoclonal antibodies.

compared with the control (Table 1). The absolute activity of the alternative pathway, calculated from the fractionation data combined with the total oxygen uptake, was constant (Figure 3). In contrast, the activity of the cytochrome pathway decreased in response to the low-light treatment. When roots of *Poa annua* were

**Table 1.** Fractionation (Δ,‰; as defined by Farquhar and Richards, 1984), the percentage alternative respiration (% alternative), the percentage total respiration (% total) and absolute total respiration, versus time (h) for the low-light experiment, with or without sucrose addition just before the measurement

| Time (h) | Sucrose present | *n* | Δ (‰) | % alternative | % total | Absolute total respiration (nmol $O_2$ $g^{-1}$ FW $sec^{-1}$) |
|---|---|---|---|---|---|---|
| 0 | No | 5 | 21.6 ± 0.44 | 29.9 ± 6.3 | 100.0 ± 6.7 | 4.0 ± 0.3 |
| 10 | No | 2 | 22.2 ± 0.81 | 38.4 ± 11.6 | 96.7 ± 13.8 | 3.6 ± 0.7 |
| 16 | No | 4 | 23.6 ± 0.43 | 57.9 ± 6.2 | 57.5 ± 3.1 | 2.2 ± 0.2 |
| 64 | No | 3 | 22.9 ± 0.29 | 48.1 ± 4.1 | 40.3 ± 2.5 | 1.5 ± 0.2 |
| 0 | Yes | 4 | 20.9 ± 1.49 | 19.8 ± 10.6 | 118.5 ± 5.4 | 4.4 ± 0.4 |
| 64 | Yes | 3 | 22.1 ± 0.72 | 36.8 ± 5.9 | 52.2 ± 3.0 | 2.0 ± 0.2 |

Mean and standard error; *n* = number of replicates. The fractionation was 19.5 ± 0.32 for the cytochrome pathway and 26.6 ± 0.10 for the alternative pathway.

fed sucrose at times 0 and 64 h, respiration increased by 19 and 30%, respectively. This increase in respiration upon addition of sucrose was completely due to an increase in the activity of the cytochrome pathway, while the activity of the alternative pathway remained constant (Figure 3).

### *Ubiquinone*

One of the factors that determines the activity of the alternative pathway is the amount of available substrate ($Q_r$) (Wagner and Krab, 1995), and therefore the total amount of ubiquinone as well as $Q_r/Q_t$ was measured. The total concentration of ubiquinone (mainly $Q_9 + Q_8$; Millenaar *et al.*, 1998) before transfer to the long-night treatment was 5.5 ± 0.6 nmol $g^{-1}$ FW, and this increased during the second long night to 131% of the control values. No further changes occurred during the last 3 days (Figure 2C). The percentage of $Q_8$ did not change during the course of the dark treatment (16, 14, 10, 14 and 11% of the total amount of ubiquinone at times 0, 16, 40, 64 and 88 h, respectively).

The maximum obtainable $Q_r/Q_t$ (with KCN and SHAM) was 68 ± 2.1, 69 ± 3.8, 65 ± 1.8, 62 ± 3.8, 57 ± 0.9 and 60 ± 0.9% at times 0, 10, 16, 40, 64 and 88 h, respectively (mean ± standard deviation, *n* = 3), suggesting that a gradually increasing part of the ubiquinone pool could not be reduced and seemed to be inactive. A significant correlation was found between the maximum obtainable $Q_r$ ($Q_{r\text{-}max}$) and $Q_r/Q_t$. The reduction state of the ubiquinone pool, corrected for the maximal obtainable reduction state ($Q_r/Q_{r\text{-}max}$), had a constant value of 65% during the treatment (Figure 2D). The concentration of reduced ubiquinone was also constant during the low-light experiment (2.2 ± 0.2, 2.4 ± 0.4, 2.2 ± 0.3, 2.4 ± 0.7, 2.5 ± 0.4 and 2.2 ± 0.5 nmol $g^{-1}$ FW at time 0, 10, 16, 40, 64 and 88 h, respectively; mean ± standard deviation, *n* = 3), since the total concentration of ubiquinone increased as much as the $Q_r/Q_t$ decreased.

### *Alternative oxidase*

In addition to effects of changes in substrate availability ($Q_r$), the activity of the alternative oxidase is also influenced by changes in the amount and reduction state of the alternative oxidase protein. There was no change, however, in the amount of AOX (Figure 1F) and all of the AOX was in the reduced form (Figure 4) during the low-light treatment.

Also, when plants were in complete darkness for 4 and 7 days, the concentration did not change (scanned blots, data not shown), and only the reduced form of AOX was found (Figure 5). The relative concentration of the combined bands did not change; however, an extra band appeared, with a higher molecular mass, and the main AOX band decreased.

### *Cytochrome c oxidase*

The cytochrome *c* oxidase capacity (control 89 ± 20.6 $g^{-1}$ FW $sec^{-1}$, first-order rate constant) decreased by 26% during the first night, and 49% during the following night; it did not change any further during the last two days (Figure 2E). The cytochrome *c* oxidase capacity after 4 and 7 days of darkness was 48 ± 13.6 and 38 ± 2.6 $g^{-1}$ FW $sec^{-1}$, which was not different from the values for roots in low light during the last two days.

## Discussion

### *Sugars, cytochrome c oxidase and respiration*

Plants that were transferred from short to long nights (from 10 to 16 h) and, at the same time, from high to low

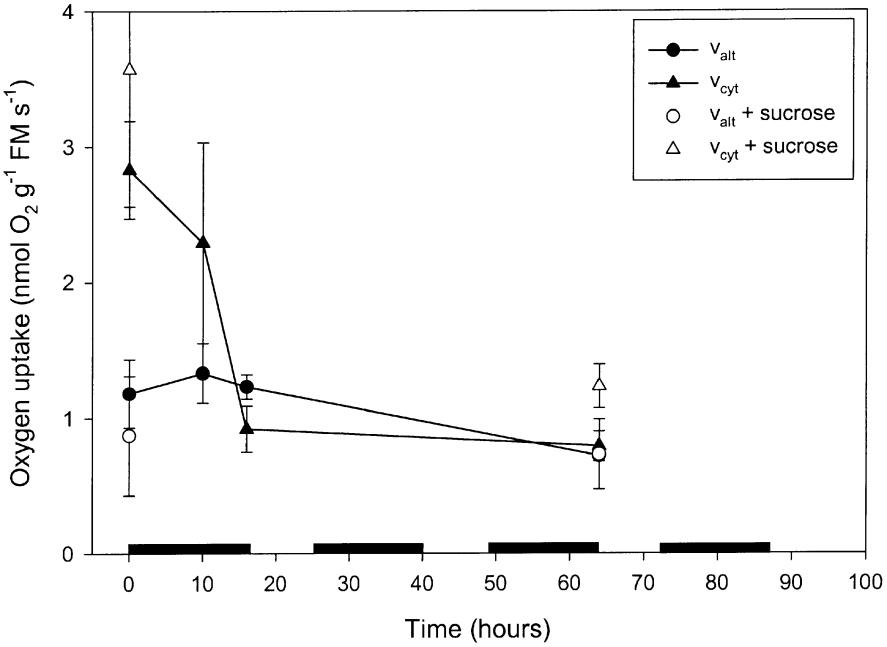


**Figure 3.** Activity (nmol $O_2$ $g^{-1}$ FW $sec^{-1}$) of the alternative (circles) and cytochrome pathways (squares) with sucrose (open symbols) or without sucrose (closed symbols), measured using the $^{18}O$ isotope fractionation technique.
Error bars represent standard error; $n = 5$, 2, 4, 3 for 0, 10, 16, 64 h without sucrose, and $n = 4$ and 3 for 0 and 64 h with sucrose, respectively, based on different plants.

light intensity (from 450 to 70 $\mu$mol $m^{-2}$ $sec^{-1}$), showed a dramatic decrease in sugar concentration (by 90%). The respiration rate decreased (by 59%) during the low-light treatment and was similar to that after 4 and 7 days of darkness. The aim of the treatments, namely to lower the sugar concentration and the respiration, was therefore achieved. There were large changes in oxygen-isotope fractionation. Possible regulatory mechanisms that account for this are discussed below.

The total respiration measured with the gas chromatograph (GC) during the activity measurements ($^{18}O$ fractionation) at Duke University was 22% lower compared with the respiration measured with the oxygen electrode in previous experiments at Utrecht University. The growing conditions may have been slightly different; moreover, the duration of the measurements was about twice as long with the GC as compared with the oxygen electrode. During the measurements, the roots are detached from the plant, and the respiration decreases during the measurement. After correction for the duration of the measurement, the difference in total respiration is much smaller; however, the GC measurements are still 11% lower compared with the oxygen electrode. The decrease in total respiration does not influence the partitioning between the pathways, since the regression lines obtained to calculate the partitioning have $r^2$ values higher than 0.995. The lines would, in fact, have been curvilinear if the partitioning had changed during the measurement.

While the rate of respiration decreased by 59% after 4 days of low-light treatment, the *in vitro* capacity of cytochrome *c* oxidase decreased by 73%. McDonnell and

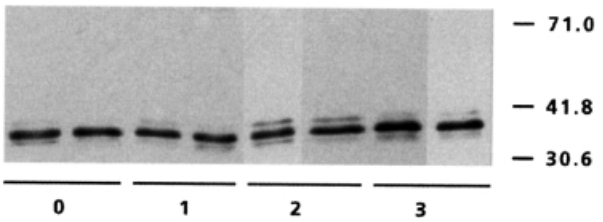


**Figure 4.** Immunoblot of alternative oxidase in whole-root extracts of *Poa annua* at different times during the low-light experiment detected with monoclonal antibodies.
Lanes 1 & 2, day 0, with the normal light conditions; lanes 3 & 4, after one long night; lanes 5 & 6, after 2 long nights; lanes 7 & 8, after 3 long nights.

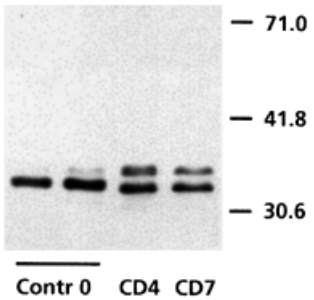


**Figure 5.** Immunoblot of alternative oxidase in whole-root extracts of *Poa annua* at different times in complete darkness detected with monoclonal antibodies.
Lanes 1 & 2, day 0, with the normal light conditions; lane 3, after 4 days of darkness; lane 4, after 7 days of darkness.

Farrar (1992) also found a decrease of 28% in cytochrome *c* oxidase capacity and 35% in fumarase capacity, in

roots of *Hordeum vulgare* plants that were shaded for 24 h, while the respiration decreased by 34%.

### *The substrate ubiquinone*

The concentration of total ubiquinone increased during the low-light treatment. Apart from being a component of the mitochondrial electron-transport chain, ubiquinone can also act as an anti-oxidant (Nohl *et al.*, 1998), which raises the question as to whether the increase in the ubiquinone concentration during the low-light treatment is perhaps a result of an increase in reactive oxygen species. An increase in ubiquinone concentration under stress conditions (low temperature) has been reported in green bell pepper (Wagner and Purvis, 1998).

Reduced ubiquinone is the substrate for both the cytochrome and the alternative pathway. $Q_r/Q_t$ decreases during the low-light treatment; however, the maximum obtainable $Q_r/Q_t$ also decreases, i.e. the fraction of the ubiquinone pool that cannot be reduced increases during the low-light treatment. It has been found before (in isolated mitochondria) that a fraction of the ubiquinone pool is not redox-active (Ribas-Carbo *et al.*, 1995; Van den Bergen *et al.*, 1994). This inactive component of the ubiquinone pool cannot accept electrons from the mitochondrial dehydrogenases and hence cannot act as a substrate for the cytochrome and alternative pathways. In order to make valid comparisons, $Q_r/Q_t$ was corrected accordingly. Therefore, to compare $Q_r/Q_t$ values obtained from pools that differ in the size of the active pool, $Q_r$ is divided by $Q_{r\text{-}max}$ (maximal obtainable $Q_r$), instead of by $Q_t$. In isolated mitochondria, there is also a part of the ubiquinone pool that cannot be fully oxidized; however, it is not known whether such a pool also exists *in vivo*, since this part cannot be measured *in vivo*. $Q_r/Q_{r\text{-}max}$ (65%) does not change during the low-light treatment, when sugar concentration and respiration rates decrease. In conclusion, the available substrate concentration is stable during the low-light treatment, as also seen during soybean development (Millar *et al.*, 1998) and during a cell-culture cycle (Wagner and Wagner, 1995).

$Q_r/Q_{r\text{-}max}$ does not change during the low-light period, in contrast with the total electron flux through the respiratory chain, which decreases. Because $Q_r/Q_{r\text{-}max}$ is not altered, whereas the total electron flux decreases, this suggests that the activity of the combined dehydrogenases decreases in a similar manner to that of the combined oxidases, to maintain the homeostatic mitochondrial metabolism.

### *Alternative oxidase concentration and reduction state*

The respiration decreased by 59% during the low-light treatment while the absolute activity of the alternative oxidase did not change. The alternative pathway is active, even under conditions where the carbohydrate concentration is lowered, suggesting that the alternative pathway has an important function even under these conditions.

Why does the plant not decrease the concentration of AOX? One reason might be that AOX proteins have slow turnover rates. The turnover rates of the AOX protein in *Poa annua* roots under the present treatment conditions are unknown. The amount of AOX increased threefold in leaves of *Pisum sativum* when they were transferred from 19 to 28°C. After transfer from 28 to 19°C, the concentration returned to normal levels in about 24 h; the half life is therefore estimated at 18 h (M.A. Gonzàlez-Mehler and J.N. Siedow, unpublished data). If these turnover rates are valid for *Poa annua*, then the roots must make new AOX protein to maintain a constant concentration during the low-light treatment. Plants that are kept in the dark for 4 or 7 days do make new AOX proteins, and even isozymes that are different from those synthesized in the light, since a new band appeared on the blot (Figure 5). Therefore, the roots are capable of synthesizing new AOX protein, even under extreme low-light conditions. We do not know, however, whether this new AOX protein is expressed in the same cells as the existing protein.

During the low-light experiment, the activity of the alternative pathway was constant while the fractionation increased. There is no shift to a more oxidized (less active) form of the protein (Figure 4). Plants that are exposed for 4 or 7 days to complete darkness (Figure 5) do not show an oxidized band with whole-tissue extracts. Failure to show an oxidized AOX band in our whole-root extracts is not due to experimental error, since we were able to show both oxidized and reduced bands in extracts of isolated mitochondria of our plants. The difference between mitochondrial blots and whole-tissue blots is caused by the isolation procedures that are used for mitochondria, during which the alternative oxidase becomes more oxidized (Millenaar *et al.*, 1998; Umbach and Siedow, 1997). When mitochondrial extracts of *Poa annua* roots are added to whole tissue just before the extraction of the proteins, there is no alteration between the oxidized and reduced form of the AOX protein (data not shown). Therefore, the procedure that we used for whole-tissue extracts does not change the reduction state of the protein.

### *Activity of the alternative pathway*

To measure the activity *in vivo* in the absence of inhibitors of the respiratory chain, we used the $^{18}O$-isotope fractionation method. The partitioning of electrons to the alternative pathway increases from 35 to 60% during the low-light treatment, but there is no change in the absolute activity (Figure 3, Table 1). In contrast, the relative and

absolute cytochrome pathway activity decreases during the low-light treatment. The relative increase in partitioning of electrons to the alternative pathway is caused entirely by the decrease in the activity of the cytochrome pathway. Millar *et al*. (1998) reached a similar conclusion, stating that the cytochrome pathway is regulated more tightly during the development of soybean roots than the alternative pathway is. Therefore, the relative contribution of the alternative pathway is not increased by changes in the activation state of the protein or in the substrate ($Q_r$), but by changes in the activity of the cytochrome pathway.

The activity of the alternative oxidase remained constant; apparently the alternative oxidase is also important for the root cell homeostasis when the carbohydrate concentration is low.

## Conclusions

Even after a decrease in the rate of root respiration, endogenous sugar concentration, and *in vitro* cytochrome *c* oxidase capacity during the low-light treatment, there is no change in the amount and in the reduction state of alternative oxidase in the roots of *Poa annua*. There is also no change in $Q_r/Q_t$ after correction for the maximum obtainable $Q_r/Q_t$, which is defined as $Q_r/Q_{r\text{-}max}$. The absolute activity of the alternative pathway does not change during the low-light treatment or upon the addition of sugars. Only the cytochrome pathway responds to these treatments. Even under severe light deprivation, the concentration and activity of the alternative oxidase were not changed.

## Experimental procedures

### *Plant material and growth conditions*

Roots of 5–6-week-old *Poa annua* (L) plants were used for all measurements. Seeds were germinated on moistened filter paper for 1 week and the seedlings were transferred to sand for 1 week, after which they were placed in 30 l containers (24 plants per container) and grown on an aerated nutrient solution (Poorter and Remkes, 1990), with the exception that the Fe concentration was doubled. The nutrient solution was replaced every week and the pH was adjusted every other day to 5.8. Plants grew at 20°C, 60% relative humidity, with a photoperiod of 14 h at 450 μmol $m^{-2}$ $sec^{-1}$ (PAR). The low-light treatment consisted of transferring 5–6-week-old plants to an 8 h day length at 60–70 μmol $m^{-2}$ $s^{-1}$ (PAR) for 4 days, or to complete darkness for 4–7 days.

### *Respiration of intact roots*

Roots (1.5–2.0 g fresh weight (FW)) were severed and transferred to an airtight cuvette containing nutrient solution without Fe, and respiration was measured polarographically, using a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH, USA) (Lambers *et al*., 1993). The alternative pathway was inhibited with 3 mM SHAM (1 M stock solution in methoxyethanol). To inhibit the cytochrome pathway, KCN was used at a concentration of 0.4 mM (0.5 M stock solution in 20 mM HEPES, pH 8). The rate of respiration at 10–15 min after addition of the inhibitors was used to calculate the percentage inhibition.

### *Measurements of carbohydrates in intact roots*

Individual soluble sugars were measured according to Sweeley *et al*. (1963). Root extracts were prepared from 400 mg (FW) of frozen root material that was ground in liquid nitrogen using a mortar and pestle and then suspended in a total volume of 5 ml ethanol/water (38% v/v) with mannitol as an internal standard, and subsequently vortexed (2 × 30 sec) and centrifuged at 1500 *g* for 7 min. The supernatant was transferred to a second test tube with 1 ml chloroform to remove lipids, vortexed (2 × 30 sec) and centrifuged at 1500 *g* for 4 min. The supernatant was mixed with approximately 0.1 g of Dowex (BDH Chemicals Ltd, Poole, UK) to remove amino acids. This extract was vortexed four times for 30 sec with 15 min intervals. Then 100 μl of extract was dried under a nitrogen flow. To the dried sample, 50 μl of TMS reagent (pyridine:hexamethyldisilazane:trimethylchloorsilane, 5:1:1, v/v/v) was added for sililation of the sugars and organic acids. The samples were allowed to stand for at least 45 min, after which 2 μl of the sililated extract was analysed on a gas chromatograph (GC, HP 5890A, Hewlett Packard, Amstelveen, The Netherlands), with an FID detector (Hewlett Packard) and a CP Sil 5b column of 25 m (Chrompack, Bergen op Zoom, The Netherlands), with an injection and detection temperature of 285°C. The temperature of the column was 150°C and was increased after 2 min to 280°C at a rate of 5°C $min^{-1}$. Individual compounds were identified by their retention times as compared with commercially obtained standard compounds; the response values were also obtained from these standards.

### *Cytochrome c oxidase capacity*

Root extracts were prepared from 300 mg (FW) of frozen root material that was ground in liquid nitrogen using a mortar and pestle and then suspended in a total volume of 1.2 ml with 0.1 M $KH_2PO_4$ (pH 7.5) and 0.1% (w/v) Triton X-100. The extract was centrifuged at 13 000 *g* for 5 min, and the supernatant was used for a spectrophotometric assay. Cytochrome *c* oxidase was measured at 550 nm in the presence of 12 μM reduced cytochrome *c* (5 μl) and 0.3 ml extract in the cuvette containing 1 ml $KH_2PO_4$ buffer. Cytochrome *c* (in $KH_2PO_4$ buffer) was reduced with sodium dithionite. The excess of dithionite was removed by a gentle flow of normal air in the solution for a few min. The assay was measured at 25°C and the first-order rate constant was calculated ($g^{-1}$ FW $sec^{-1}$) (Smith, 1955). The final extinction was measured by adding $K_3Fe(CN)_6$ (3 μl of a 0.1 mM solution) in a final concentration of 0.23 μM (whereby the volume changes only by 0.2%), which completely oxidizes the reduced cytochrome *c*. Addition of 0.5 mM KCN or bubbling with CO inhibited the reaction to 6 ± 1 and 16 ± 4%, respectively (mean ± standard error). The activity measured should represent the maximal activity in the extract, and is therefore related to the concentration of cytochrome *c* oxidase.

### *AOX protein*

The total protein content of extracts was determined according to Lowry *et al*. (1951). Root extracts were prepared from 100 mg (FW)

of frozen root material that was ground in liquid nitrogen using a mortar and pestle, and then suspended in a total volume of 400 µl of protein sample mix (62.5 mM Tris–HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.001% (v/v) bromophenol blue). After centrifugation for 10 min at 16 000 *g* in an Eppendorf centrifuge to precipitate cell debris, the proteins were separated by SDS–PAGE according to Laemmli (1970), and subsequently electro-transferred to nitrocellulose filters using blot transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). AOX monoclonal antibodies (GT Monoclonal Antibodies, Lincoln, NB, USA) were used as primary antibodies (Elthon *et al.*, 1989) with a dilution factor of 1:100. Anti-mouse IgG Fab fragments conjugated to peroxidase (Boehringer Mannheim, Germany) were used as a secondary antibody (1:25 000), using SuperSignal ULTRA Chemiluminescent Substrate according to the product usage protocol (Pierce, Rockford, MD, USA). To quantify the bands in the autoradiograms, an image analysis system (Kontron/Zeiss, Eching, Germany) was used. Scanning was performed with a Panasonic b/w CCD camera (WC-CD50), digitized four times and averaged to improve the signal to noise ratio (frame size 640 × 512 pixels; 256 grey levels). The intensities of the bands were corrected for the background.

### *Measurement of ubiquinone reduction levels in intact roots*

The ubiquinone assays were performed according to Wagner and Wagner (1995). Root systems were vertically split in two, and treated with or without KCN + SHAM. Root extracts were prepared from 0.8 g of fresh root material that was ground in liquid nitrogen, using a mortar and pestle, and then suspended in a total volume of 15 ml methanol and 15 ml petroleum ether (boiling point 40–60°C) and vortexed for 30 sec. The mixture was centrifuged (1500 *g*, 1 min) and the upper petroleum ether phase was removed, transferred to a test tube, and evaporated to dryness under a flow of nitrogen. Another 15 ml of petroleum ether was added to the lower phase, and the vortex and centrifugation steps were repeated. The upper phase was added to the one previously obtained. The extracted ubiquinones were resuspended with a glass rod in 75 µl of nitrogen-purged ethanol and analysed by HPLC (HP 1050 series, Hewlett Packard, Amstelveen, The Netherlands). A reversed-phase Lichrosorb 5 RP 18 column (Chrompack, Bergen op Zoom, The Netherlands) with an ethanol–methanol mixture (starting with 10 min 20% (v/v) ethanol, and from 10 min a gradient to 70% (v/v) ethanol at 40 min as the mobile phase (1 ml $min^{-1}$) was used. Detection was performed at 290 and 275 nm for $Q_r$ and oxidized unbiquinone ($Q_{ox}$), respectively. Commercially obtained ubiquinone-10 and ubiquinone-9 were used as standards (Sigma, Zwijndrecht, The Netherlands, and Fluka, Zwijndrecht, The Netherlands). The extinction of $Q_r$ measured at 290 nm was multiplied by 3.56 according to Crane (1963), because of the lower extinction coefficient for $Q_r$ as compared with that of $Q_{ox}$. The ubiquinone measurements were performed with a recovery for $Q_{10}$ of 93% ($n = 4$); the $Q_{10}$ was added to the sample just after grinding.

### *Oxygen fractionation and gas-phase respiration measurements*

Root samples (0.5–1.2 g fresh mass) were kept in the dark for 25 min before gas-phase respiratory measurements were performed in a 4.96 ml stainless-steel closed cuvette at 20°C. A $CO_2$ absorber (ascarite II) was present during measurements to avoid inhibition of respiration as a consequence of build-up of $CO_2$ in the closed cuvette during the course of the experiment (Gonzàlez-Meler *et al.*, 1996). Oxygen extraction and isotope analysis were carried out as described by Robinson *et al.* (1995) with modifications by Gonzàlez-Meler *et al.* (1999). Roots were carefully surface-dried prior to measurements to minimize diffusion resistance to tissue gas exchange. Over the course of the experiment, each sample consumed at least 30% but no more than 50% of the initial oxygen. The $r^2$ values for all unconstrained linear regressions of the fractionation values (with a minimum of five data points) were greater than the value of 0.995 considered minimally acceptable (Gonzàlez-Meler *et al.*, 1999; Lennon *et al.*, 1997; Ribas-Carbo *et al.*, 1995; Ribas-Carbo *et al.*, 1997). During inhibitor treatments, either 0.5 mM KCN (in 1 mM TES, pH 8.0) or 3 mM SHAM (in water from a 1 M stock in dimethyl sulphoxide) were applied by sandwiching the roots between medical wipes soaked with the corresponding inhibitor and incubating in the dark for at least 25 min (Lennon *et al.*, 1997). All stocks were freshly prepared before use. The $CO_2$ absorber was not present in experiments requiring KCN to avoid recovery from the inhibitor. Calculations of oxygen-isotope fractionation were performed as described by Guy *et al.* (1989) with modifications (Gonzàlez-Meler *et al.*, 1999). Electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy *et al.* (1989).

### *Statistics*

SPSS (Chicago, IL, USA) for Windows 8.0 was used for statistical analysis. One-way analysis of variance with a Bonferroni *post hoc* test was used for the statistical analysis. The correlations (two-tailed) were calculated with the Pearson correlation test.

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