



Role of sugars and organic acids in regulating the concentration and activity of the alternative oxidase in *Poa annua* roots

Frank F. Millenaar^{1,6}, Miquel A. Gonzalez-Meler^{2,3}, James N. Siedow², Anneke M. Wagner⁴ and Hans Lambers^{1,5}

¹Plant Ecophysiology, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands

²Botany Department-DCMB Group, Duke University, Durham, North Carolina, USA

³Department of Biological Sciences, University of Illinois, 845 West Taylor Street, Chicago, IL 60607, USA

⁴Department of Molecular Cell Physiology, Vrije Universiteit, Amsterdam, The Netherlands

⁵School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley WA 6009 Australia.

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Abstract

Detached roots of *Poa annua* were used to study alternative oxidase protein expression upon the addition of sucrose, glucose, fructose, inositol, mannitol, citrate or malate, at a concentration of 1 or 10 mM for 24 h. After 24 h the capacity of cytochrome c oxidase was decreased equally in all treatments. Only citrate induced the expression of the alternative oxidase, especially at a concentration of 1 mM (15-fold). The activity of the alternative pathway (measured with the ¹⁸O-fractionation technique) was not affected by the addition of sucrose for 24 h as compared with time zero. However, after the addition of citrate or mannitol the activity of the alternative pathway decreased to almost zero. The discrepancy between the large increase in alternative oxidase protein concentration when citrate was applied and the concomitant decrease in alternative pathway activity is discussed.

Key words: Alternative oxidase, citrate, organic acid, *Poa annua*, sugar.

Introduction

The cytochrome and alternative pathways constitute the respiratory electron-transport pathways of all higher

plant mitochondria. In contrast to the cytochrome pathway, beyond the branch point (ubiquinone), the alternative pathway does not contribute to the generation of a proton-motive force (Moore and Siedow, 1991). The AOX protein is found in every plant species examined and in almost every plant organ, and the genes encoding AOX have regions that are very conserved (Vanlerberghe and McIntosh, 1997), suggesting that the alternative pathway plays a vital role in plant functioning. However, a clear function has not yet been identified.

In the recent past, an understanding of the mechanisms that account for activation of the alternative pathway in isolated mitochondria has increased substantially. It is now known that the alternative pathway becomes more activated when the AOX protein is reduced and when specific α -keto acids, for example, pyruvate, are present in sufficiently high concentration (Millar *et al.*, 1993, 1996; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995).

It has previously been shown that the AOX protein invariably occurs in its reduced form during the day in roots of *Poa annua* (Millenaar *et al.*, 1998). Similarly, both in control leaves of *Arabidopsis thaliana* and in leaves infected with *Pseudomonas syringae* there was no oxidized form of the AOX protein (Simons *et al.*, 1999). Also in roots of several *Poa* species no oxidized form of AOX was present (Millenaar *et al.*, 2001). In roots of soybean seedlings the AOX protein was largely in the

⁶To whom correspondence should be addressed. Fax: +31 3025 18366. E-mail: F.F.Millenaar@bio.uu.nl

Abbreviations: AOX, alternative oxidase; Cyt, cytochrome; FM, fresh mass; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; SHAM, salicylhydroxamic acid.

reduced form at day 7 and day 17, but was partially oxidized at day 4 (Millar *et al.*, 1998). There is also no oxidized form of the AOX protein in roots of *Poa annua* after an exposure of the plants to 4 d low light or complete darkness (Millenaar *et al.*, 2000). During the low-light experiment both the sugar concentration and total respiration decreased; however, the activity, protein concentration and reduction (activation) state of the alternative oxidase did not change. Addition of sucrose for 45–60 min affected the cytochrome pathway, but not the alternative pathway. Thus the relative contribution of the alternative pathway increased with decreasing sugar concentration and decreased upon addition of sucrose (Millenaar *et al.*, 2000). The previous experiments concern short-term treatment with sugars. Equally interesting is the question whether long-term sugar addition affects the activity, protein concentration or reduction state of the alternative oxidase.

The effects of the addition of 1 or 10 mM sucrose, glucose, fructose, inositol, citrate, and malate, for 24 h on the levels of the alternative oxidase have been investigated. Citrate addition increases the alternative oxidase protein concentration in tobacco cell suspension cultures (Vanlerberghe and McIntosh, 1996) and the question is whether it also induces the alternative oxidase in other cells of other species, for example, roots of *Poa annua*. It has also been investigated whether other organic acids (e.g. malate) can induce the alternative oxidase and whether cytochrome *c* oxidase is expressed to a different extent after the treatments than the alternative oxidase is. To address these questions, cytochrome *c* oxidase was also measured.

Materials and methods

Plant material and growth conditions

Poa annua L. roots of 5–6-week-old plants were used for all measurements. Seeds were germinated on moistened filter paper for 1 week and seedlings were transferred to sand for 1 week. Plants were then placed in 30 l containers (24 plants per container) and grown on an aerated hydroponic nutrient solution (Poorter and Remkes, 1990; with the exception that Fe concentration was doubled). The nutrient solution was replaced every week and the pH was adjusted every other day to a value of 5.8. Plants grew at 20 °C, 60% RH, with a photoperiod of 14 h at an irradiance of 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR).

Sugar and organic acid addition to Poa annua roots

To study the expression and reduction (activation) state of the alternative oxidase, detached roots of *Poa annua* were exposed to sucrose, glucose, fructose, inositol, citrate, and malate for 24 h. Mannitol was used as a control, to compensate for possible differences in osmolarity, since mannitol is not metabolized by most plants. The compounds, at a concentration of 1 or 10 mM, were added to a nutrient solution with a pH 5.8. About 1 g of root material was added to 50 ml solution in a 100 ml Erlenmeyer flask. The Erlenmeyer flasks were shaken gently

during the entire 24 h period to avoid oxygen depletion in the solution. After 24 h the respiration, AOX concentration, and cytochrome *c* oxidase concentrations were measured.

Unless stated otherwise, experiments were repeated twice and for each concentration 3–4 replicates were used.

Respiration of intact roots

Roots of *Poa annua* (1.0 g fresh mass (FM)) were transferred to an air-tight cuvette containing nutrient solution without Fe, and respiration was measured as a decrease of the oxygen concentration 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 0.5 mM (from a 0.5 M stock solution in 20 mM HEPES, pH 8). The rate of respiration measured at 10–15 min after addition of the inhibitors was used to calculate the percentage inhibition from control respiration rates. Short-term effects of glucose and citrate were studied at a concentration of 1 mM from a 1 M stock at pH 7.0.

AOX protein

The total protein content of the extracts was determined (Lowry *et al.*, 1951). Root extracts were prepared from 100 mg (FM) 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% SDS, 10% glycerol, and 0.001% bromophenol blue (v/v)). After boiling for 5 min the samples were centrifuged for 10 min at 16 000 *g* in an Eppendorf centrifuge to precipitate cell debris, the proteins were separated by SDS/PAGE according to well-established procedures (Laemmli, 1970), and subsequently electro-transferred onto nitrocellulose filters using blot transfer buffer (25 mM TRIS, 192 mM glycine, 20% (v/v) methanol). Immunodetection of the AOX protein was carried out according to the product protocol of the AOX monoclonal antibody (GTMA, Lincoln, NE, USA). Antibodies were obtained from Dr TE Elthon (Elthon *et al.*, 1989) and used as a primary antibody (1:100). Antimouse 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 manufacturer's instructions (Pierce, Rockford, IL, USA). An IBAS image-analysis system (Kontron/Zeiss, Eching, Germany) was used to quantify the bands from the autoradiograms. Films were scanned with a Panasonic b/w CCD camera (WC-CD50), digitized four times and averaged to improve the signal to noise ratio (frame size 640 \times 512 pixels; 256 grey levels). Band intensity was corrected for the background.

Cytochrome c oxidase capacity

Root extracts were prepared from 300 mg (FM) 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. Cyt *c* oxidase was measured at 550 nm in the presence of 12 μM reduced Cyt *c* (5 μl) and 0.3 ml extract in the cuvette with 1 ml KH_2PO_4 buffer. Cyt *c* (in KH_2PO_4 buffer) was reduced with sodium dithionite. Excess dithionite was removed by a gentle flow of normal air in the solution for a few minutes. The assay was measured at 25 °C and the first-order rate constant was

calculated ($\text{g}^{-1} \text{FM s}^{-1}$) (Smith, 1961). The final extinction was measured by adding $\text{K}_3\text{Fe}(\text{CN})_6$ ($3 \mu\text{l}$ of a 0.1 mM solution) in a final concentration of $0.23 \mu\text{M}$ (whereby the volume changes only by 0.2%), which completely oxidized the reduced cyt *c*. Addition of 0.5 mM KCN or bubbling with CO inhibited the reaction to $6 \pm 1\%$ and $16 \pm 4\%$, respectively (average and standard error). The measured activity should represent the maximal activity of cytochrome *c* oxidase in the extract, and is, therefore, related to the concentration of cytochrome *c* oxidase present.

Oxygen fractionation and gas-phase respiration measurements

Root samples ($0.5\text{--}1.2 \text{ g FM}$) were kept in the dark for 25 min before gas-phase respiratory measurements were taken 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 (González-Meler *et al.*, 1996). Oxygen extraction and isotope analysis were carried out as described earlier (Robinson *et al.*, 1995) with modifications (González-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 (Ribas-Carbo *et al.*, 1995, 1997; Lennon *et al.*, 1997; González-Meler *et al.*, 1999). 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 tissue respiratory recovery from the inhibitor. Calculations of oxygen-isotope fractionation were made as described previously (Guy *et al.*, 1989) with modifications (González-Meler *et al.*, 1999). Electron partitioning between the two pathways in the absence of inhibitors was calculated as described earlier (Guy *et al.*, 1989). Preliminary results show that there is no difference in respiration rate between the two methods.

Mitochondria and SMP preparation

Mitochondria and inside-out submitochondrial particles (SMP) from cold-stored (4°C) cauliflower inflorescences (*Brassica oleracea*, a commercial cultivar from a local store in the Netherlands was used) were isolated (Van den Bergen, 1994).

Statistics

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

Results

To study the expression, reduction (activation) state, and activity of the alternative oxidase, detached roots of *Poa annua* were exposed to sucrose, glucose, fructose,

inositol, citrate, malate, mannitol or only the nutrient solution (NS) for 24 h. Mannitol was used as a control, to correct for possible differences in osmolarity. The blots from the extracts treated with 1 mM glucose failed in succession; this was most likely not caused by the treatment but by the blotting and visualization procedure.

There was no difference in the alternative oxidase protein concentration 24 h after the addition of sucrose, glucose, fructose, inositol, malate or mannitol, at 1 and 10 mM (Fig. 1). At 1 mM citrate, there was a large increase in the alternative oxidase concentration, up to 15 times, as compared with the addition of mannitol (Fig. 2). At 10 mM citrate, there was also more ($5\times$) alternative oxidase as compared with mannitol; however, this further increase was not significant, compared with the other treatments. The alternative oxidase was invariably in its active (reduced) form after all treatments (Fig. 2; data not shown).

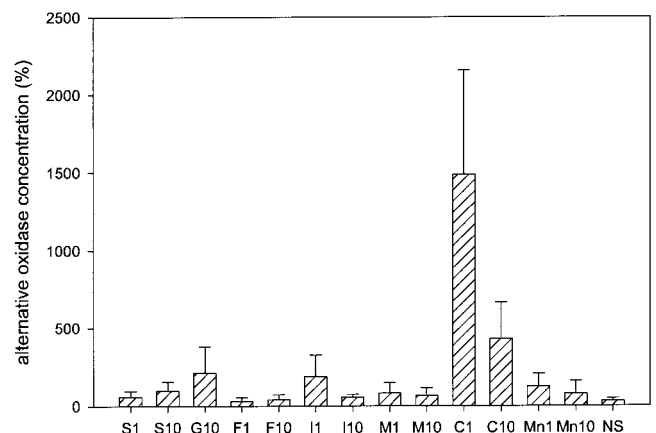


Fig. 1. Concentration of the alternative oxidase in *Poa annua* after 24 h of incubation with sucrose (S), glucose (G), fructose (F), inositol (I), malate (M), citrate (C), or mannitol (Mn), or the nutrient solution (NS) only as the control. Two concentrations were applied, 1 and 10 mM . Western blots were detected with monoclonal antibodies and the intensity of the bands was measured. The average AOX concentration with mannitol was set at 100% ; error bars represent standard error, number of replicates was at least three. Only the AOX concentration after 1 mM citrate was different from the other values ($P_{\text{ANOVA}} < 0.1\%$).

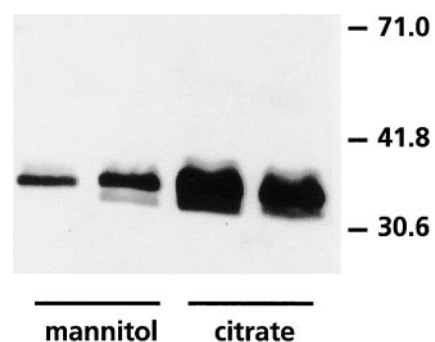


Fig. 2. Immunoblots of alternative oxidase (detected with monoclonal antibodies) directly isolated from roots of *Poa annua* 24 h after addition of 1 mM mannitol (lanes 1, 2) or citrate (lanes 3, 4).

Cytochrome *c* oxidase capacity was measured only after the treatments with 1 mM concentrations and decreased about 4-fold in 24 h, independent of the treatment. There were no significant differences between the treatments (Fig. 3).

The activity (assessed using ¹⁸O fractionation) of the alternative pathway decreased after 24 h of exposure to citrate or mannitol; there was no decrease in activity with sucrose compared with the control at zero h (Table 1). There was no significant change in the cytochrome pathway activity after 24 h of treatment; this lack of significance is most likely due to the low number of replicates. At time zero and after sucrose addition relative activity of the alternative pathway was 33–35%; after 24 h of exposure to mannitol or citrate the relative activity of the alternative pathway was 6 ± 5% (Table 1).

The total respiration (assessed with the oxygen electrode) was 4.6 ± 0.8 and 4.2 ± 0.8 nmol O₂ g⁻¹ FM s⁻¹ at time zero and after 24 h incubation with citrate, respectively (average and standard deviation, *n* ≥ 7). After 24 h of exposure to 10 mM mannitol the respiration

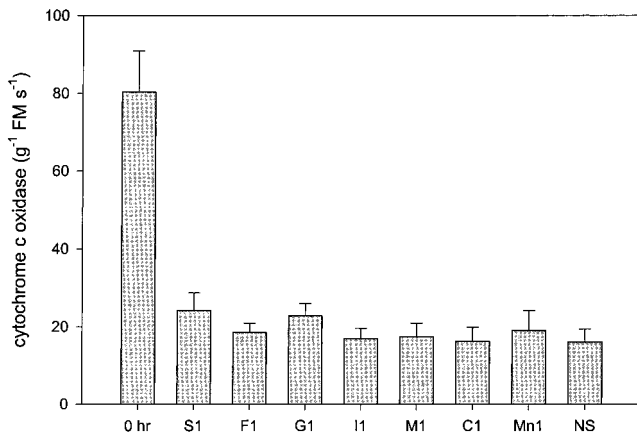


Fig. 3. Cytochrome *c* oxidase capacity (g⁻¹ FM s⁻¹) in roots of *Poa annua* at time 0 and after 24 h in a 1 mM sucrose (S), fructose (F), glucose (G), inositol (I), malate (M), citrate (C), or mannitol (Mn), or the nutrient solution (NS) only as the control. Bars represent standard error; number of replicates was at least four. Only the value at 0 h was significant different from the other values (*P*_{ANOVA} = 2.7%).

Table 1. Activity of the alternative and cytochrome pathway, absolute (*v*_{alt}, *v*_{cyt} in nmol O₂ g⁻¹ FM s⁻¹) and relative (% alt path, % cyt path in %) and the fractionation (Δ in ‰) as already defined (Farquhar and Richards, 1984) after exposure for 24 h to 10 mM sucrose, mannitol or citrate, and at time zero (control) in *Poa annua* roots

Mean and standard error; values with a different letter are significantly different (Tukey B, *P* < 0.05); number of replicates is three. Fractionation of the alternative pathway (with KCN) was 26.55 ± 0.10‰ and for the cytochrome pathway (with SHAM) 19.51 ± 0.32‰.

	<i>v</i> _{alt}	<i>v</i> _{cyt}	Δ (‰)	% alt path	% cyt path
Control (0 h)	1.33 ± 0.24 b	2.67 ± 0.39 a	21.88 ± 0.43 a	33 ± 6 a	66 ± 6 a
Sucrose	1.93 ± 0.39 b	3.43 ± 0.13 a	22.00 ± 0.33 a	35 ± 5 a	65 ± 5 a
Mannitol	0.20 ± 0.17 a	3.51 ± 0.32 a	19.90 ± 0.34 b	6 ± 5 b	94 ± 5 b
Citrate	0.23 ± 0.20 a	4.30 ± 0.72 a	19.90 ± 0.32 b	6 ± 5 b	94 ± 5 b

was significantly decreased to 2.9 ± 0.1 nmol O₂ g⁻¹ FM s⁻¹ (Fig. 4). The KCN-insensitive respiration decreased after 24 h exposure to citrate or mannitol as compared with the control at zero h (Fig. 4). The percentage respiration that was insensitive to KCN decreased (from 80% to 50%) after 24 h exposure to 10 mM citrate.

None of the treatments had any effect on the residual rate of respiration (Fig. 4). Short-term glucose addition (15 min) did not have an effect on the rate of respiration at either time zero nor after 24 h of incubation with citrate or mannitol. At time zero citrate did not have an effect on the rate of respiration either (Fig. 4).

To test the short-term effect of citrate on the alternative oxidase, inside-out submitochondrial particles were used to avoid citrate uptake problems. For the isolation of inside-out submitochondrial particles a high yield of mitochondria is necessary; therefore, the inflorescences

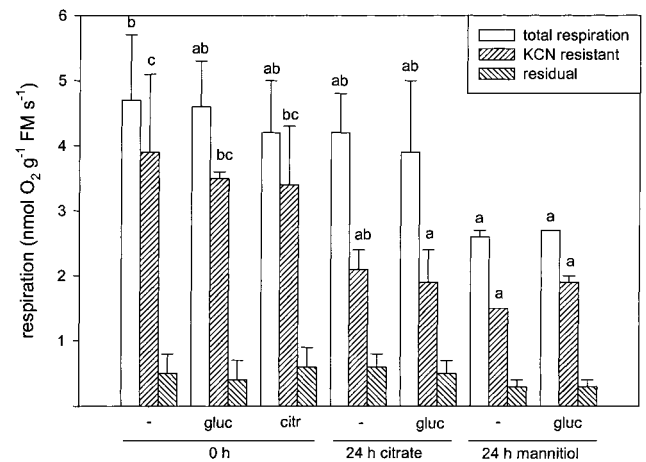


Fig. 4. Total respiration (1st bars, *P*_{ANOVA} = 1.4%), CN-resistant respiration (2nd bars, *P*_{ANOVA} < 0.1%) and residual respiration (3rd bars) in roots of *Poa annua* at 0 h (in the absence or presence of citrate or in the absence or presence of glucose for 15 min), after 24 h of exposure to citrate and mannitol (in the absence or presence of glucose for 15 min). Lines on top of the bars represent standard deviation, number of replicates was 3–7; bars with a different letter are significantly different (total respiration and CN-resistant respiration are separated). There are no significant differences between residual respiration (with KCN and SHAM) (*P*_{ANOVA} = n.s.).

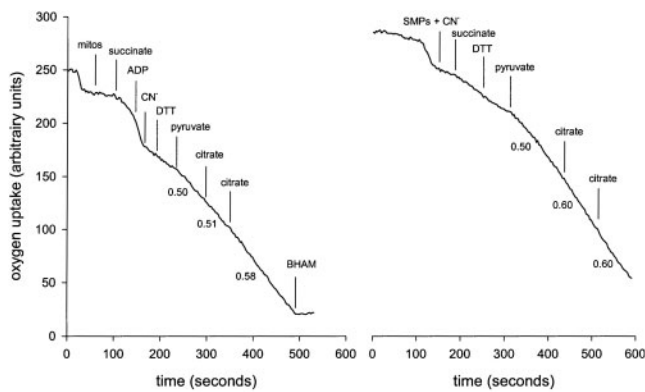


Fig. 5. Oxygen uptake traces of mitochondria and inside-out sub-mitochondrial particles (SMPs) from cauliflower. The following concentrations are used: 50 μl mitochondria, 100 μl SMPs, 10 mM succinate, 0.1 mM ADP, 10 mM CN^- , 10 mM DTT, 10 mM pyruvate, and 10 mM citrate. The numbers at the left of the traces are respiration rates in $\text{nmol O}_2 \text{ cuvette}^{-1} \text{ s}^{-1}$.

of cauliflower were used. To have reasonable concentrations of AOX, the cauliflowers were pre-treated with 1 week of cold storage of 4 °C (Vanlerberghe and McIntosh, 1992; Gonzalez-Meler *et al.*, 1999). Short-term exposure of mitochondria and inside-out sub-mitochondrial particles of cauliflower to citrate increased the respiration rate slightly in the presence of CN^- (Fig. 5).

Discussion

Total respiration

The total rate of respiration in detached roots of *Poa annua* decreased by 40% during a 24 h treatment with mannitol. Mannitol cannot be used as a respiratory substrate in most plants, and during the 24 h of mannitol treatment respiratory substrates were probably exhausted. There is no proof that mannitol cannot be metabolized in *Poa annua* roots; however, mannitol addition for 24 h decreased the rate of root respiration as compared with the effect of addition of citrate, suggesting a relative slow metabolism. Short-term (15 min) exposure to glucose, however, did not increase the rate of respiration after 24 h exposure to mannitol. An explanation may be that carbohydrates are not capable of restoring the respiration because of a low capacity of cytochrome *c* oxidase or other components of respiratory pathways. The capacity of cytochrome *c* oxidase decreased to the same extent in all the treatments. The total respiration did not decrease when, for example, citrate was added for 24 h compared with time zero. Therefore, the concentration of cytochrome *c* oxidase is not a major controlling step for the respiratory rates of the present *Poa annua* roots.

In conclusion, the decrease in respiration after 24 h of exposure to mannitol is not caused by the low concentration of cytochrome *c* oxidase and cannot be restored by glucose addition. Apparently other steps in the respiratory chain are limiting the respiration. This agrees with earlier results (Bingham and Farrar, 1988), which concluded that the respiration of roots from control, and leaf- or root-pruned plants was not limited by carbohydrates but rather by the turnover rates of ATP, since short-term sucrose feeding did not stimulate respiration.

AOX concentration and activation state

In the recent past, understanding of the mechanisms that account for activation of the alternative oxidase in isolated mitochondria has increased substantially. Post-translational features that control the activation state of the alternative pathway are the redox state of the AOX protein, being more active in its reduced form, and the presence of α -keto acids (e.g. pyruvate) that further activate the reduced form of the enzyme at sufficiently high concentrations (Millar *et al.*, 1993; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996). An increase in alternative oxidase expression was also found after citrate addition in a cell suspension of tobacco (Vanlerberghe and McIntosh, 1996).

Previously, it has been shown that the AOX protein occurs invariably in its reduced form during the day in roots of *Poa annua* (Millenaar *et al.*, 1998). Similarly, both, in control leaves of *Arabidopsis thaliana* and in leaves infected with *Pseudomonas syringae*, there was no oxidized form of the AOX protein (Simons *et al.*, 1999). In roots of soybean seedlings the AOX protein was largely in the reduced form at day 7 and day 17, but was partially oxidized at day 4 (Millar *et al.*, 1998). After 24 h of root incubation with a variety of sugars and organic acids, no oxidized form of alternative oxidase (less active, around 66 kDa) was seen on immunoblots (Fig. 2). This observation is especially striking, because the activity of the alternative pathway decreased to almost zero after mannitol or citrate incubations, despite the fact that the incubation treatment with 1 mM citrate caused an increase in alternative oxidase protein concentration by about 15-fold as compared with the mannitol treatment.

These results strongly suggest that an additional regulatory mechanism is required to modulate alternative pathway activity in intact tissues, since it is difficult to explain this study's observations based on the currently known regulatory factors only.

AOX activity and KCN sensitivity

The citrate treatment caused a 15-fold difference in the alternative oxidase concentration as compared to

mannitol (Fig. 1). All the protein was in the reduced, more active, form. The pyruvate concentration found in the roots is probably sufficiently high to activate the reduced alternative oxidase protein fully (Millar *et al.*, 1998; Millenaar *et al.*, 1998). Yet, citrate incubation not only decreased the activity of the alternative pathway, but roots also became less resistant to KCN, which implies less AOX protein or more inactive AOX. The present results are in contrast with those of others (Vanlerberghe and McIntosh, 1996) who found an increase in cyanide-resistant activity and AOX protein concentration with an increase in the exogenous citrate concentration. Their experiments were done on a different species (tobacco) grown in heterotrophic cell cultures and citrate was applied for a shorter time period (8 h), which may account for some of the differences.

Data are not available on the reduction state of the ubiquinone pool or the ubiquinone concentration. It is unlikely that the low activity of the alternative pathway during the citrate treatment was solely due to differences in the amount of substrate, because of the large differences in alternative oxidase protein concentration. Also, changes observed in the reduction state of the ubiquinone pool and ubiquinone concentration are, in general, relatively small, including roots of *Poa annua* (Wagner and Wagner, 1995; Millar *et al.*, 1998). However, the Q_r/Q_t can play an important role modulating the AOX activity (Millenaar *et al.*, 1998).

The large increase of alternative oxidase protein induced by the citrate treatment was not followed by an increase in alternative pathway activity. The activity of the alternative pathway was very similar in roots incubated with either citrate or mannitol, despite the fact that the mannitol treatment did not induce an increase in alternative oxidase protein levels. These results are also in contrast with those obtained after sucrose incubation where the alternative pathway activity remained constant with no change in alternative oxidase protein level.

In the case of mannitol, detached roots were probably substrate-starved (carbohydrates) because there was no carbon source during the 24 h incubation time, limiting the rate of respiration. Decrease in root respiration rates with mannitol was probably because of down-regulation of some enzymes in the respiratory chain, although as discussed previously respiration can also be limited by rates of ATP turnover (see total respiration paragraph). In the presence of citrate, however, detached roots do not appear to become substrate-starved, since rates of respiration did not decrease. However, the concentration of the alternative oxidase protein is higher in the presence of citrate as compared to mannitol. These results indicate that most of the alternative oxidase present in roots treated with citrate seems to be inactive by an unknown mechanism, because the alternative oxidase protein was in its reduced form.

Inactivation of the AOX after the citrate incubation

After citrate addition the concentration of the alternative oxidase increases with no concomitant increase in either the activity of the alternative pathway or the KCN-resistant respiration. In fact the two activities actually decreased after citrate incubation treatment. The following observations may shed some light for the explanation of these results. (1) The alternative oxidase contains a Fe in its active centre (Siedow *et al.*, 1995; Andersson and Nordlund, 1999). (2) Organic acids chelate metal cations depending on the pH. At a pH below 8 citrate is capable of binding some Fe. At a pH below 6.0 100% of the free Fe is chelated by citrate (Jones, 1998). (3) The pH in the mitochondrial matrix ranges from 7–8 units. Therefore, it is very likely that, citrate chelates a substantial amount of free Fe, and it is possibly that iron can be withdrawn directly or indirectly from enzyme complexes, such as AOX.

One potential result of the binding of Fe by citrate (directly or after AOX protein turnover) is an alternative oxidase protein with no Fe in its active centre, with the potential consequence that the enzyme will be inactivated. Does the plant synthesize more alternative oxidase protein as a response to the (partly) inactive alternative oxidase after citrate addition? Interestingly, malate binds Fe only at pH below 4 (Jones, 1998). Therefore it is unlikely that a significant amount of Fe is chelated by malate inside the mitochondria. In this case, there is little potential for inactivation of the alternative oxidase due to a lack of iron. The fact that alternative oxidase protein levels do not increase upon incubation with malate supports this explanation (Fig. 1). Interestingly, exposure of yeast (*Hansenula anomala*) to either antimycin, alone or in combination with *o*-phenanthroline (Fe²⁺ chelator) increased AOX expression, but cyanide-resistant respiration only increased if *o*-phenanthroline was omitted (Minagawa *et al.*, 1990).

Short-term effects of citrate exposure to isolated mitochondria and inside-out SMPs does not inhibit the alternative oxidase oxygen uptake in the presence of cyanide; there is even a slight stimulation (Fig. 5). There are a few differences in the experimental design compared with the *in vivo* situation that should be noticed. The isolated mitochondria and the SMPs are from a different plant species, and, more importantly, the exposure of citrate to the alternative oxidase was only for a few min. Exposure may have to be much longer for citrate to bind the Fe that is in the AOX protein, and AOX protein turnover may be necessary for citrate to have an effect.

Induction of the alternative oxidase via inactivation of the protein is hypothetical and only parts of the puzzle have been discovered. Further research is required to test the hypothesis in more detail in the future.

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