The alternative oxidase in roots of Poa species: *in vivo* regulation and function

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Alternatieve oxydase in de wortels van verschillende *Poa* soorten: *in vivo* regulatie en functie

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

Introduction

General background

In green plant leaves, light energy is captured and driving the photosynthetic reduction of CO_2 with H_2O to carbohydrates. This energy can become available again as ATP at a different time and/or place, by oxidizing these carbohydrates to CO_2 and H_2O in the respiratory process. The rate of respiration in non photosynthesizing tissues is determined by the metabolic activity of the tissue, and is a measure of the tissue's energy (i.e. ATP) consumption. Respiration can be measured as the production of CO_2 or as O_2 consumption.

Depending on the species and growth conditions of a plant, 30 to 70% of all the carbohydrates fixed in photosynthesis are respired in the same day (van der Werf *et al.*, 1992). The terminal part of the respiratory path, starting with the degradation of carbohydrates via glycolysis, consists of the mitochondrial electron-transport pathway, in which, among other components, two terminal oxidases participate, cytochrome *c* oxidase and the alternative oxidase (Fig. 1). The alternative oxidase branches from the main electron-transport pathway at the ubiquinone pool and, beyond the branch-point, does not contribute to ATP production. The energy conservation is less than maximal if a part of the respiration proceeds via this nonphosphorylating (alternative) pathway. Because of its energy-wasting nature, it is most interesting (scientifically as well as economically) to investigate under which conditions and to what extent the alternative respiration is used and how its activity is regulated.

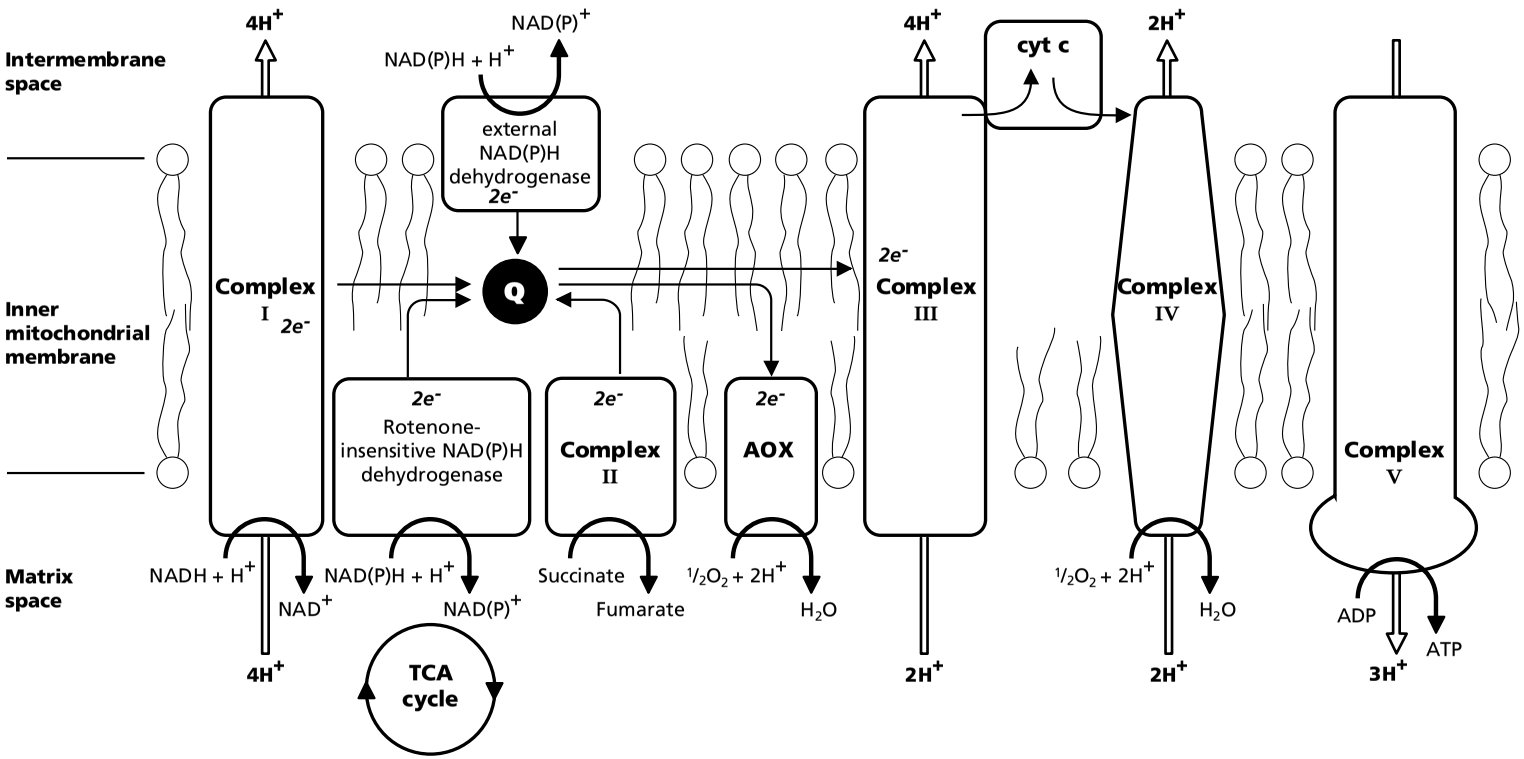
Mitochondrial electron-transport pathway

The mitochondrial electron-transport pathway consists of several dehydrogenases and the cytochrome and alternative oxidases, which are linked via the ubiquinone pool, and are all located in the inner mitochondrial membrane (Fig. 1). NADH and succinate are produced inside the mitochondria in the citric acid cycle and donate their electrons to their respective dehydrogenases, complex I and II. In plant mitochondria there are more dehydrogenases than in their animal counterparts; there are non-phosphorylating NAD(P)H dehydrogenases on the outside as well as on the inside of the inner mitochondrial membrane. All these dehydrogenases are linked to the ubiquinone (Q) pool, which is reduced by input of electrons via the various dehydrogenases and oxidized by the cytochrome and/or the alternative pathways. As a consequence, the ubiquinone pool consists of an oxidized (ubiquinone) and reduced (ubiquinol) part, of which the ratio varies with the conditions (substrate, energy state of the mitochondria) (Fig. 2). The cytochrome pathway consists of a series of 3 enzymes; complex III, cytochrome *c* and complex IV (cytochrome *c* oxidase), respectively. Cytochrome *c* oxidase is sensitive to cyanide, in contrast to the alternative oxidase. Also the composition of the respiratory pathway beyond ubiquinone is more complex in plants as compared with animals, since it not only contains the cytochrome pathway, but also the alternative pathway. The alternative pathway consists of only one protein, the quinol-oxidizing alternative oxidase (AOX). A proton gradient across the inner mitochondrial membrane is necessary to generate ATP from ADP and Pi by ATP-synthase (complex V). Only complex I, III and IV contribute to the extrusion of protons out of the matrix space of the mitochondria. When the electrons from reduced ubiquinone are donated to the alternative oxidase, proton pumping of the cytochrome pathway (complexes III and IV) is bypassed and, therefore, there is no energy conservation via this part of the respiratory pathway.

Dehydrogenase and cytochrome pathway activity

The activity of the cytochrome pathway and the activity of complex I depend on the proton gradient (or internal ADP concentration) across the inner mitochondrial membrane (Fig. 3). A low ADP concentration results in a high proton gradient and occurs during state 4 respiration, as opposed to state 3 respiration. ATP-synthase dissipates the proton gradient to produce ATP

► **Figure 1: The organization of the electron-transport pathway in the inner membrane of plant mitochondria. Some of the components are membrane-spanning, others face the mitochondrial matrix or the space between the inner and the outer mitochondrial membrane. Q, ubiquinone, a pool of quinone and quinol molecules; AOX, alternative oxidase; TCA, tricarboxylic acid cycle.**


Intermembrane space
Inner mitochondrial membrane
Matrix space
4H+
NAD(P)+
NAD(P)H + H+
external NAD(P)H dehydrogenase
2e-
Complex I
2e-
Q
Rotenone-insensitive NAD(P)H dehydrogenase
2e-
Complex II
2e-
AOX
2e-
4H+
Complex III
2e-
cyt c
2H+
Complex IV
Complex V
NADH + H+
NAD+
4H+
NAD(P)H + H+
NAD(P)+
TCA cycle
Succinate
Fumarate
1/2O2 + 2H+
H2O
2H+
1/2O2 + 2H+
H2O
2H+
ADP
ATP
3H+

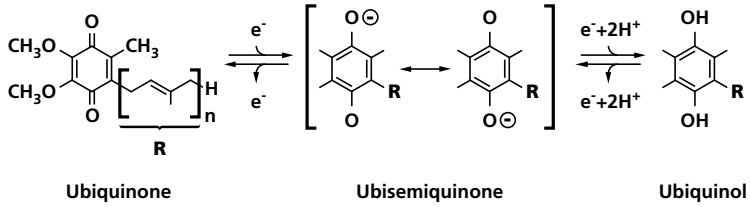


Figure 2: The structure and redox states of ubiquinone (Q). Ubiquinone is a substituted 1,4-benzoquinone, the side chain (R) is composed of isoprenyl units, the number after the Q (e.g., Q_{10}) is equal to the number of isoprenyl units. Reduction of ubiquinone requires two electrons and two protons. Reduction with only one electron gives ubisemiquinone.

from ADP and P_i; therefore, in the absence of ADP there will be a steep proton gradient across the inner mitochondrial membrane.

Internal NADH and succinate from the citric acid cycle, localized in the mitochondrial matrix, are the major substrates for the respiratory chain. The production of NADH and succinate is linked via the activity of the citric acid cycle if no export or import of NADH and succinate takes place. Three NADH and 1 succinate are produced in one cycle of the citric acid cycle.

Function of the alternative oxidase

In the reproductive structures of aroids (Meeuse, 1975) and also in a number of other species, including water lilies (Skubatz *et al.*, 1990) and cycads (Skubatz *et al.*, 1993), the alternative path plays a role in thermogenesis. During its "respiratory crisis" the temperature of the short-lived inflorescence of aroids may rise to 10°C above ambient. Similar increases in temperature have been found for water lilies, whereas in cycads the temperature rises only a few degrees. Because of the increase in temperature of the tissue, chemicals are volatilized and pollinators are attracted. During the respiratory crisis, respiration is largely cyanide-resistant. Apart from these thermogenic floral organs, there is no evidence that heat production plays a role. Breidenbach *et al.* (1997) calculated that the alternative oxidase cannot increase the temperature more than a few hundredths of a degree, except in thermogenic flowers that are bulky and have very high respiration rates. Tobacco sprayed with salicylic acid (induces AOX gene expression) increased their temperature, and also the total respiration and the cyanide-resistant respiration increased (Van der Straeten *et al.*, 1995). This rise in temperature, however, is not caused by the alternative oxidase activity, but is due to stomatal closure upon salicylic acid addition (Chaerle *et al.*, 1999). The presence of the alternative oxidase has been demonstrated in every examined plant species and plant part (except freshly harvested potato tubers) and, therefore, it is likely that it has another function in addition to heat production.

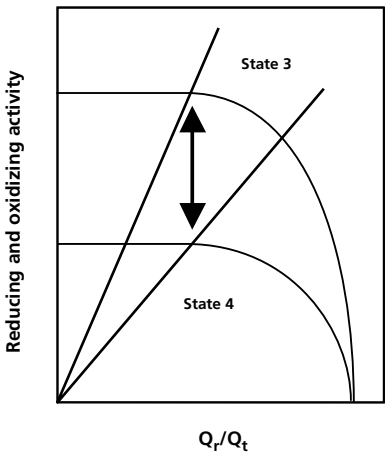


Figure 3: The relationship between the cytochrome pathway (straight lines) and the dehydrogenases (curved lines) with the Q_r/Q_t. A higher proton gradient (state 4 = in the virtual absence of ADP) lowers the activity of both the cytochrome pathway and the dehydrogenases, and ample ADP increases the activity (state 3).

It has been proposed that a more generally important function for the alternative oxidase is the prevention of the formation of oxygen free radicals. Oxygen free radicals may lead to severe metabolic disturbances and a wide range of environmentally induced plant disorders, including chilling damage, are mediated by reactive oxygen species (Scandalios, 1993). It has been suggested that a high reduction state of the ubiquinone pool (Q_r/Q_t) promotes oxygen free radical formation when the cytochrome pathway is inhibited or restricted; respiration via the alternative pathway might help to maintain Q_r/Q_t at a low level. Purvis and Shewfelt (1993) and Wagner and Wagner (1995) speculated that the alternative pathway helps to stabilize the reduction state of the mitochondrial ubiquinone pool (Q_r/Q_t). The use of the alternative pathway results in a mild form of uncoupling, so there is no build-up of a proton gradient (after the Q pool), especially in combination with the use of the external or internal rotenone-insensitive dehydrogenases (Skulachev, 1996).

Alternative oxidase genes

The alternative oxidase is present in all angiosperms; many algae and some fungi also contain the genetic capacity to express this pathway (Ordentlich *et al.*, 1991; Vanlerberghe and McIntosh 1997). In soybean, rice and *Arabidopsis* (and probably also other plant species) the alternative oxidase consists of a multigene family whose individual products can be separated on a protein gel (Whelan *et al.*, 1996, Finnegan *et al.*, 1997; Ito *et al.*, 1997). The sequenced genes in the different species contain highly conserved regions (Vanlerberghe and McIntosh, 1997).

Alternative oxidase structure

Both the C terminus and the N terminus face the matrix (Fig. 4). The C terminus contains the postulated binuclear iron center; the N terminus

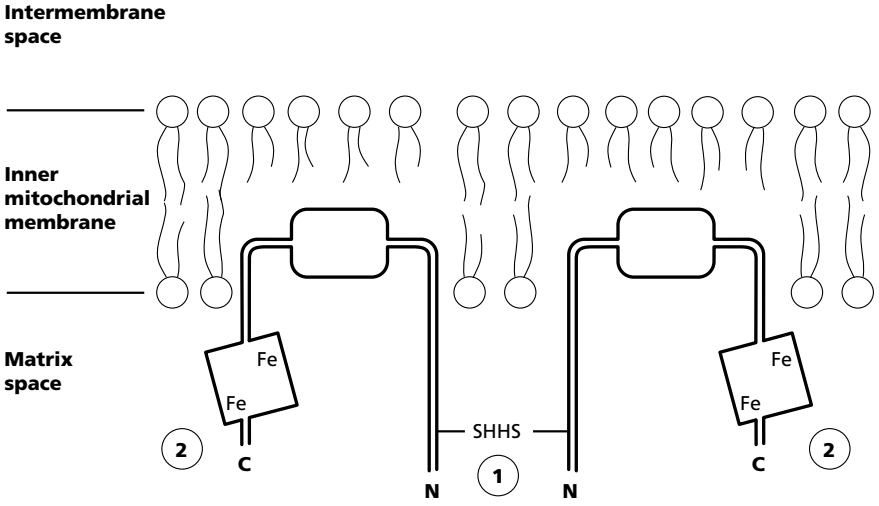


Figure 4: Schematic representation of the alternative oxidase dimer (AOX) and its proposed conformation in the inner mitochondrial membrane. The AOX is embedded in the inner mitochondrial membrane via an inter-membrane helix region. 1, Sulfydryls that are involved in redox regulation and pyruvate activation; 2, proposed iron-active site of AOX.

contains, in most species, a conserved cysteine residue (Siedow and Umbach, 1995; Andersson and Nordlund, 1999). The cysteine residue is involved in the dimerization of two alternative oxidase subunits.

The two ends are connected via an inter-membrane helix region, where the potential ubiquinone-binding site is located (Andersson and Nordlund, 1999).

Alternative oxidase activation

The alternative oxidase can occur as a noncovalently linked dimer (reduced) or as a covalently linked dimer (oxidized), when the sulfhydryl groups are oxidized to form sulfur bridges that link the dimer. In the reduced configuration, the alternative oxidase is more active compared with the oxidized dimer (Fig. 5) (Umbach and Siedow, 1993; Umbach *et al.*, 1994). Some α-keto acids, like pyruvate, influence the activity of the alternative oxidase (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996); in the presence of pyruvate, the alternative oxidase becomes active at low substrate (Q_r/Q_t) concentrations (Fig. 5). The α-keto acids appear to bind to the same cysteine residue that is used for dimerisation (Rhoads *et al.*, 1998; Vanlerberghe *et al.*, 1998; Djajanegara *et al.*, 1999). In transgenic plants that have an alternative oxidase that lacks the cysteine residue, the oxidase is insensitive to pyruvate and the alternative oxidase does not occur

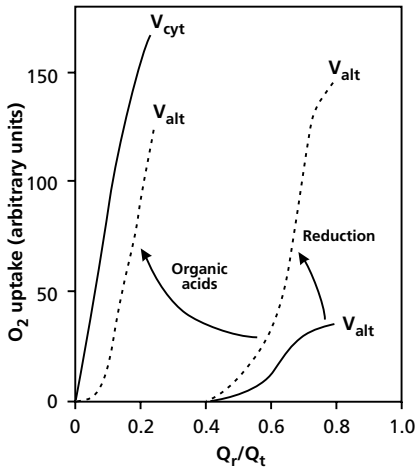


Figure 5: Dependence of the activity of the cytochrome and of the alternative path on the fraction of ubiquinone that is in its reduced state (Q_r/Q_t). When the alternative oxidase is in its reduced (higher-activity) configuration, it has a greater capacity to accept electrons. In its reduced state, the alternative oxidase can be affected by α-keto acids like pyruvate, which enhance its activity at low levels of Q_r/Q_t.

in the oxidized (less active) form (Djajanegara *et al.*, 1999). Up to now, rice and tomato are the only plant species that have been found to have AOX genes that lack the conserved cysteine residue (Djajanegara *et al.*, 1999). Also in amoeba cells there is no oxidized, less-active form of alternative oxidase, not even after addition of oxidizing reagent like diamide (Jarmuszkiewicz, 1997).

Alternative oxidase activity

Many reports have appeared, in which the activity of the alternative oxidase was assessed with the use of specific inhibitors of the cytochrome (e.g., CN^-, azide, antimycin) and alternative (e.g., SHAM, BHAM, propyl gallate) pathway (Bahr and Bonner, 1973; Lambers 1997). In these studies it was assumed that the cytochrome pathway was saturated, which appeared to be supported by reports. It was also assumed that the alternative pathway became active at very high reduction levels of the Q pool. However, since the discovery of the activation by pyruvate and the influence of the reduction state of the protein on AOX activity, which is accompanied by a lowering of the redox state of the Q pool at which AOX becomes active, it became clear that the alternative pathway is not a simple overflow for the cytochrome pathway, and inhibitors cannot be used to estimate the activities (Day *et al.*, 1996). The activity of both pathways can be measured with the ^{18}O-fractionation technique (Guy *et al.*, 1989; Gonzàlez-Meler *et al.*, 1999). In this thesis the advanced ^{18}O-fractionation technique has been used to measure the partitioning of electrons to the alternative and cytochrome pathway. Up to now, very few data were available on AOX activity *in vivo* as measured with the ^{18}O-fractionation technique.

Outline of the thesis

Extensive research has been done on isolated mitochondria, and new regulatory mechanism were discovered (pyruvate, AOX reduction state); however, little is known about the importance of these mechanisms *in vivo*. Also little is known about the extent of *in vivo* AOX activity. In this thesis I combine the study of the *in vivo* AOX regulatory mechanisms with the *in vivo* ^{18}O-fractionation technique to measure AOX activity.

One of the postulated functions of the alternative pathway is to stabilize the ubiquinone pool, to avoid the production of extra oxygen free radicals. It is known from isolated mitochondria that the alternative oxidase can stabilize the reduction state of the ubiquinone pool. Can the alternative oxidase also stabilize the reduction state of the ubiquinone pool *in vivo*? Chapter 2 describes experiments to test this hypothesis.

What is the influence of pyruvate and the reduction state of the alternative oxidase *in vivo*? From studies on isolated mitochondria, it is known that pyruvate and the reduction state of the protein can influence the activity of the alternative oxidase. What is the activation state of the alternative oxidase *in vivo*? In chapter 2, I examine the role of pyruvate and the AOX reduction state in *Poa annua* roots during the light period.

Does the reduction state and activity of the alternative pathway change if the sugar concentration decreases? In chapter 3, I examine the activation state and activity of the alternative oxidase in roots of *Poa annua* after the plants have been exposed to a long night (16 hr instead of the normal 10 hr) and to a low light intensity (70 instead of 450 μmol m^{-2} s^{-1} PAR).

Can the AOX activity be explained by the known regulatory mechanisms? What is the influence of different concentrations of alternative oxidase on the *in vivo* activity and is there a relation between whole plant relative growth rate and alternative oxidase activity? In chapter 4 I address these questions using 5 closely related monocotyledonous species.

What is the influence of exposure of roots to sugars and organic acids on the alternative pathway? In chapter 5, I examine the activation state, activity and concentration of the alternative oxidase in detached roots of *Poa annua* after 24 hours exposure to sugars or organic acids.

To study the influence of the AOX concentration in one species, antisense plants are needed. In chapter 6, I describe the process of making transgenic alternative oxidase plants, together with some preliminary results.

The last chapter 7 is a general discussion of the key results of the preceding chapters.

References

Andersson ME, Nordlund P (1999) A revised model of the active site of alternative oxidase. FEBS Lett. 449: 17-22

Bahr JT, Bonner WD (1973) Cyanide-insensitive respiration; II control of the alternate pathway. J. Biol. Chem. 248: 3446-3450

Breidenbach RW, Saxton MJ, Hanson LD, Criddle RS (1997) Heat generation and dissipation in plants: can the alternative oxidative phosphorylation pathway serve a thermoregulatory role in plant tissues other than specialized organs? Plant Physiol. 114: 1137-1140

Chaerle L, Van Caeneghem W, Messens E, Lambers H, Van Montagu M, Van der Straeten D (1999) Presymptomatic visualisation of plant-virus interactions by thermography. Nature Biotechnol. 17: 813-816

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, Wiskich JT (1996) The cyanide-resistant oxidase: To inhibit or not to inhibit, that is the question. Plant Physiol. 110: 1-2

Djajanegara I, Holtzapffel R, Finnegan PM, Hoefnagel MHN, Berthold DA, Wiskich JT, Day DA (1999) A single amino acid change in the plant alternative oxidase alters the specificity of organic acid activation. FEBS Lett. 454: 220-224

Finnegan PM, Whelan J, Millar AH, Zhang Q, Smith MK, Wiskich JT, Day DA (1997) Differential expression of the multigene family encoding the Soybean mitochondrial alternative oxidase. Plant Physiol. 114: 455-466

Gonzalez-Meler MA, Ribas-Carbo M, Giles L, Siedow JN (1999) The effect of growth and measurement temperature on the activity of the alternative respiratory pathway. Plant Physiol. 120: 765-772

Guy RD, Berry JA, Fogel ML, Hoering TC (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. Planta 177: 483-491

Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400

Ito Y, Saisho D, Nakazone M, Tsutsumi N, Hirai A (1997) Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature. Gene 203: 121-129

Jarmuszkiewicz W, Wagner AM, Wagner MJ, Hryniewiecka L (1997) Immunological identification of the alternative oxidase of *Acanthamoeba castellanii* mitochondria. FEBS Lett. 411: 110-114

Lambers H (1997) Oxidation of mitochondrial NADH and the synthesis of ATP. In: Plant Metabolism (eds) Dennis DT, Layzell DB, Lefebvre DD and Turpin DH. Longman Singapore, pp:200-219

Meeuse BJD (1975) Thermogenic respiration in aroids. Annu. Rev. Plant Physiol. 26: 117-126

Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618

Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Let. 329: 259-262

Ordentlich A, Linzer RA, Raskin I (1991) Alternative respiration and heat evolution in plants. Plant Physiol. 97: 1545-1550

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol. Plant. 88: 712-718

Rhoads DM, Umbach AL, Sweet CR, Lennon AM, Rauch GS, Siedow JN (1998) Regulation of the cyanide-resistant alternative oxidase of plant mitochondria. J. Biol. Chem. 273: 30750-30756

Scandalios JG (1993) Oxygen stress and superoxide dismutase. Plant Physiol. 101: 7-12

Siedow JN, Umbach AL (1995) Plant mitochondrial electron transfer and molecular biology. Plant Cell 7: 821-831

Skubatz H, Williamson PS, Schneider EL, Meeuse BJD (1990) Cyanide-insensitive respiration in thermogenic flowers of *Victoria* and *Nelumbo*. J. Exp. Bot. 41: 1335-1339

Skubatz H, Tang W, Meeuse BJD (1993) Oscillatory, heat-production in the male cones of cycads. J. Exp. Bot. 44: 489-492

Skulachev VP (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. Q. Rev. Biophys. 29: 169-202

Umbach AL, Siedow JN (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett. 348: 181-184

Van der Straeten D, Chaerle L, Sharkov G, Lambers H, Van Montagu M (1995) Salicylic acid enhances the activity of the alternative pathway of respiration in tobacco leaves and induces thermogenicity. Planta 196: 412-419

Van der Werf W, Welschen R, Lambers H (1992). Respiratory losses increase with decreasing inherent growth rate of a species and with decreasing nitrate supply: a search for explanations for these observations. In: Molecular, Biochemical and Physiological aspects of plant respiration eds: Lambers, H. van der Plas, L.H.W. Academic Publishing, The Hague. Pp 421-432

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: From gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Vanlerberghe GC, McIntosh L, Yip JYH (1998) Molecular localization of a redox-modulated process regulating plant mitochondrial electron transport. Plant Cell 10: 1551-1560

Wagner AM, Wagner MJ (1995) Measurements of *in vivo* ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283

Whelan J, Millar AH, Day DA (1996) The alternative oxidase is encoded in a multigene family in soybean. Planta 198: 197-201

Chapter 2

The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool and the activation state of the alternative oxidase

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Abbreviations:
AOX, alternative oxidase; FM, fresh mass; Q_r, reduced ubiquinone; Q_r/Q_t, reduction state of the ubiquinone pool; Q_t, total ubiquinone; SHAM, salicylhydroxamic acid.

Summary

A possible function for the alternative (nonphosphorylating) pathway is to stabilize the reduction state of the ubiquinone pool (Q_r/Q_t), thereby avoiding an increase in oxygen free radical production. If the Q_r/Q_t were stabilized by the alternative pathway, then Q_r/Q_t should be less stable when the alternative pathway is blocked. Q_r/Q_t increased when we exposed roots of *Poa annua* (L.) to increasing concentrations of KCN (an inhibitor of the cytochrome pathway). However, when salicylhydroxamic acid, an inhibitor of the alternative pathway, was added at the same time, Q_r/Q_t increased significantly more. Therefore, we conclude that the alternative pathway stabilizes Q_r/Q_t. Salicylhydroxamic acid increasingly inhibited respiration with increasing concentrations of KCN. In the experiments described here the alternative oxidase protein was invariably in its reduced (high-activity) state. Therefore, changes in the reduction state of the alternative oxidase cannot account for an increase in activity of the alternative pathway upon titration with KCN. The pyruvate concentration in intact roots increased only after the alternative pathway was blocked or the cytochrome pathway was severely inhibited. The significance of the pyruvate concentration and Q_r/Q_t on the activity of the alternative pathway in intact roots is discussed.

Introduction

The cytochrome pathway and the alternative pathway constitute the respiratory electron-transport pathways of 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. The AOX protein is found in every examined plant species and in 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 clearly identified function for the alternative pathway has been documented only once to our knowledge (in thermogenic flowers; Meeuse, 1975). Purvis and Shewfelt (1993) and Wagner and Wagner (1995) speculated that the alternative pathway helps to stabilize Q_r/Q_t. Q_r is a common substrate for both respiratory pathways. It has been suggested that high Q_r/Q_t levels promote oxygen free radical formation when the cytochrome pathway is inhibited or restricted; respiration via the alternative pathway might then help to maintain Q_r/Q_t at a low level. Although there is a linear relationship between the rate of mitochondrial respiration and the rate of radical formation (Puntelarulo *et al.*, 1991; Leprince *et al.*, 1994), radical formation is not directly connected to O_2 consumption, because uncouplers increase radical formation only to a minor extent (Chance *et al.*, 1977; Leprince *et al.*, 1994) and may even decrease it (Liu and Huang, 1996). Rather, radical formation is linked to the relative reduction state of the respiratory chain (Forman and Boveris, 1982). The addition of uncoupler enhances respiration but not Q_r/Q_t (Wagner and Wagner, 1995). Radical formation increases if the appropriate inhibitors (Purvis *et al.*, 1995) are used to block one or more respiratory pathways (Chance *et al.*, 1977; Forman and

Boveris, 1982, and refs. therein; Rich and Bonner, 1987). However, when the transmembrane potential increases, the production of radicals and H_2O_2 increase as well (Liu and Huang, 1996), so it is reasonable to assume that the formation of radicals increases with an increase in Q_r/Q_t. If Q_r/Q_t is stabilized by the alternative pathway, then the Q_r/Q_t should be less stable if the alternative pathway is blocked (with SHAM) than when it is not blocked. To determine if Q_r/Q_t is stabilized by the alternative pathway *in vivo*, we titrated root respiration of *Poa annua* (L.) with KCN (an inhibitor of the cytochrome pathway) in the absence or presence of SHAM. We used a range of KCN concentrations to achieve no inhibition, a small inhibition, or full inhibition of the cytochrome pathway. On the basis of data on isolated mitochondria and kinetic modeling (Wagner and Krab, 1995) it can be expected that the alternative pathway stabilizes Q_r/Q_t *in vivo*; however, this hypothesis remains to be proven. In the recent past our understanding of the mechanisms that account for activation of the alternative pathway in isolated mitochondria has increased dramatically. We now know that the alternative pathway is more active when the AOX protein becomes reduced or when specific organic acids, e.g. pyruvate, are present in sufficiently high concentrations (Millar *et al.*, 1993; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996). If and how the activity of the alternative pathway is controlled *in vivo* is still entirely unknown. To determine the activation state of the alternative pathway in intact roots, we measured the concentration of the activator pyruvate and the reduction state of the AOX protein in the roots that were used in the titration experiments.

Materials and methods

Plant Material and Growth Conditions

Roots of 6- to 7-week-old *Poa annua* (L.) plants were used for the measurements. Seeds were germinated on moistened filter paper for 1 week and then transferred to sand for 1 week, after which time they were placed in 30L containers (24 plants per container) and grown on an aerated nutrient solution (as described by 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 2nd day to 5.8. The growth conditions were 20°C, 60% RH, 14hr day length, and 450 µmol m^{-2} s^{-1} PAR.

Respiration of Intact Roots

Roots (1.5-2.0 g FM) were severed and transferred to an airtight cuvette containing nutrient solution without Fe, and respiration was measured as the

decrease in the O_2 concentration using a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The alternative pathway was inhibited with 3 mM SHAM (1 M stock solution in methoxyethanol). To inhibit the cytochrome pathway, KCN was used in a wide range of concentrations (0–400 μM; stock solutions were made in 20 mM Hepes, pH 8.0). The respiration 10 to 15 minutes after addition of the inhibitors was used to calculate the percent inhibition.

Measurements of Pyruvate, Ethanol, and Lactate in Intact Roots

Pyruvate (lactate dehydrogenase), ethanol (alcohol dehydrogenase), and lactate (lactate dehydrogenase) concentrations in intact roots were measured enzymatically according to the product protocol of Boehringer Mannheim (Almere, The Netherlands). About 1 g of fresh root material was used for every measurement. To reduce the background extinction, an extra purification step was included by mixing active carbon (approximately 30 mg per 1.5 mL sample) to the sample mixture, followed by filtration. The recovery was 101% ± 4.5% (n=3), 81% ± 1.5%, and 99% ± 6.6% (n=3) for pyruvate, ethanol, and lactate, respectively.

AOX Protein

Root extracts were prepared from 100 mg FM of frozen root material that was ground in liquid N_2 using a mortar and pestle and then suspended in a total volume of 400 mL of protein sample mix (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromophenol blue), and boiled for 5 min. After centrifugation for 10 min at 16,000g in an Eppendorf centrifuge to precipitate cell debris, the proteins were separated by SDS-PAGE according to the method of Laemmli (1970), and then electrotransferred to nitrocellulose filters using blot-transfer buffer (25 mM Tris, 192 mM Gly, 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). Antibodies were kindly provided by Dr. T.E. Elthon (Elthon *et al.*, 1989) and used as a primary antibody (1:50). Anti-mouse IgG Fab fragments conjugated to peroxidase (Boehringer Mannheim) were used as a secondary anti-body (1:25,000), using a chemiluminescent substrate (SuperSignal Ultra, Pierce) according to the product-usage protocol supplied by the manufacturer. To quantify the bands in the autoradiograms, an image-analysis system (IBAS, Kontron/Zeiss) was used. Scanning was performed with a black-and-white CCD camera (WC-CD50, Panasonic), digitized four times, and averaged to improve the signal-to-noise ratio (frame size, 640 × 512 pixels; 256 gray levels). The bands were corrected for the background.

Measurement of Ubiquinone Reduction Levels in Intact Roots

The ubiquinone assays were based on the method of Wagner and Wagner (1995). Root extracts were prepared from 1 g of fresh root material that was ground in liquid N_2 using a mortar and pestle, and then suspended in a total volume of 15 mL of methanol and 15 mL of petroleum ether (boiling point, 40-60°C) and vortexed for 30 s. The mixture was centrifuged at 1,500*g* for 1 minute and the upper petroleum ether phase was removed, transferred to a test tube, and evaporated to dryness under a flow of N_2. Another 15 µL 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 N_2-purged ethanol and analyzed 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 minutes in 20% ethanol, and then through a gradient to 70% ethanol at 40 minutes as the mobile phase at 1 mL min^{-1}) was used. Detection was performed at 290 and 275 nm for Q_r and oxidized ubiquinone, respectively. Commercially obtained Q_{10} and Q_9 were used as standards (Sigma and Fluka). The extinction of Q_r measured at 290 nm was multiplied by 3.56 according to the method of Crane (1963) because of the lower extinction coefficient for Q_r compared with oxidized ubiquinone. The ubiquinone measurements were made with a recovery for Q_{10} of 93% (n= 4); the Q_{10} was added to the sample just after grinding.

Isolation of Mitochondria from Roots

One gram FM of roots was used for a fast isolation procedure to obtain mitochondria. Roots were ground using an ice-cold mortar and pestle with sand, and suspended in a total volume of 5 mL of buffer (0.05 M Mops, pH 7.4, 0.4 M mannitol, 0.25% BSA [m/v]). After centrifugation at 4,000*g* for 3 min at 2°C, the supernatant was centrifuged at 19,100*g* for 7 minutes at 2°C. The pellet was suspended in 5 mL of buffer and centrifuged again at 19,100*g* for 7 minutes at 2°C. The pellet was suspended in 200 µL of protein sample mix.

Results

To study the effects of a (partial) inhibition of the cytochrome pathway on the levels of Q_r/Q_t with or without the alternative pathway operating, we first examined the KCN concentrations at which the cytochrome pathway is inhibited and electrons are diverted to the alternative pathway.

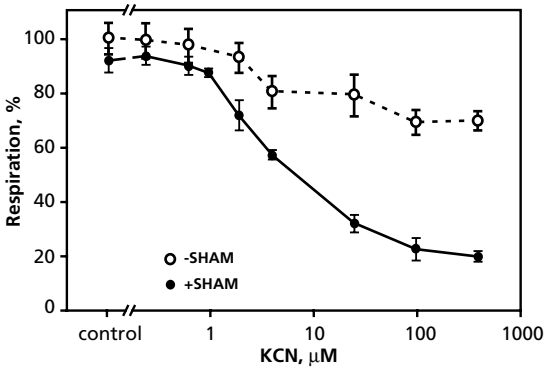

Figure 1: O_2 consumption (percent of control) by intact roots (detached from the plant) of *P. annua* plotted against the KCN concentration (note the logarithmic scale). The following KCN concentrations were used: 0 (control), 0.25, 0.63, 1, 2, 4, 25, 40, and 400 μM. The error bars indicate the SD. ○, Measurements made in the absence of SHAM; ●, measurements made in the presence of SHAM (3 mM). The number of replicates was at least three and these were from different plants and plant batches. The control respiration was 4.4 nmol O_2 g^{-1} FM s^{-1}.

O_2 uptake in intact roots was measured at a range of KCN concentrations in the absence or presence of SHAM. The rate of root respiration in the absence of SHAM was unaffected by KCN concentrations lower than 2 μM and was 4.4 nmol O_2 g^{-1} FM s^{-1} (Fig. 1). The respiration decreased by 35% in the presence of 100 to 400 μM KCN. SHAM (3 mM) alone significantly decreased the rate of respiration by 11% ($p = 0.013$). In the presence of SHAM, KCN concentrations between 0.6 and 25 μM inhibited respiration significantly more, up to 75%. Therefore, in the absence of SHAM, the alternative pathway apparently takes over an increasing number of electrons from the increasingly inhibited cytochrome pathway. At KCN concentrations exceeding 25 μM, the difference in inhibition of respiration with and without SHAM was constant, with an average of 49%.

To determine if the presence of an alternative pathway acting as an overflow for an inhibited cytochrome pathway can stabilize Q_r/Q_t, we determined Q_r/Q_t at different KCN concentrations in the absence and presence of SHAM. In intact roots of *P. annua* we found mainly Q_9 (67%), as has been found for many other species (Threlfall and Whistance, 1970), some Q_8 (23%), and almost no Q_{10} (10%). The present results for Q_r/Q_t are for Q_9 only, which had an average concentration of 4.4 ± 0.6 nmol g^{-1} FM. Ribas-Carbo *et al.* (1995) showed that different ubiquinones have the same redox behavior in isolated mitochondria.

To determine if the alternative pathway stabilizes Q_r/Q_t, we looked for an extra increase in Q_r/Q_t , which would be expected when the alternative pathway is inhibited by SHAM. By measuring Q_r/Q_t at different KCN concentrations, we determined if an increase in Q_r/Q_t coincided with an increase in activity of the alternative pathway. Q_r/Q_t was constant (44% ± 3%) up to a KCN concentration of 0.63 μM with or without SHAM (Fig. 2). Between 0.63 and 2 μM KCN, Q_r/Q_t increased much more in the presence

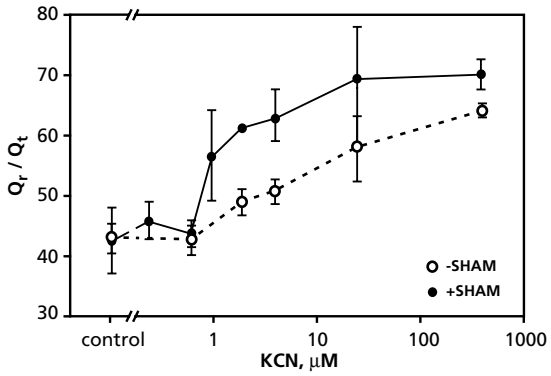

Figure 2: Q_r/Q_t (percent) in intact roots (detached from the plant) of *P. annua* plotted against the KCN concentration (note the logarithmic scale). The following KCN concentrations were used: 0 (control), 0.25, 0.63, 1, 2, 4, 25, 40, and 400 μM. The error bars indicate the SD. ○, Measurements made in the absence of SHAM; ●, measurements made in the presence of SHAM (3 mM). The number of replicates was at least three and these were from different plants and plant batches.

of SHAM than in its absence. Between 2 and 25 μM KCN, the increase in Q_r/Q_t was the same (12%) in the absence and presence of SHAM. With increasing inhibition of the cytochrome pathway, the alternative pathway became increasingly engaged in respiration. An increase in the activity of the alternative pathway might be the result of an increased substrate concentration (Q_r). However, AOX might also be activated when the cytochrome pathway is inhibited. Such an activation could be the result of an increase in pyruvate concentration and/or in the reduction state of the protein.

The pyruvate concentration in the roots was constant (58 ± 7 nmol g^{-1} FM) up to a KCN concentration of 0.63 μM with or without SHAM (Fig. 3). Up to a KCN concentration of 4 μM (without SHAM), the pyruvate concentration did not change, whereas after the addition of SHAM, it increased significantly ($p = 0.05$) to 206 nmol g^{-1} FM. At the two highest KCN concentrations there was no further effect of SHAM on the pyruvate concentration.

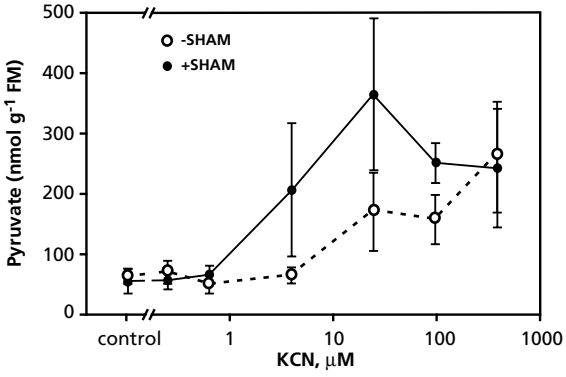

Figure 3: Pyruvate concentration (nanomoles per gram FM) in intact roots (detached from the plant) of P. annua 10 to 15 min after the inhibitors were applied plotted against the KCN concentration (note the logarithmic scale). The following KCN concentrations were used: 0 (control), 0.25, 0.63, 1, 2, 4, 25, 40, and 400 μM. The error bars indicate the SD. ○, Measurements made in the absence of SHAM; ●, measurements made in the presence of SHAM (3 mM). The number of replicates was at least three and these were from different plants and plant batches.

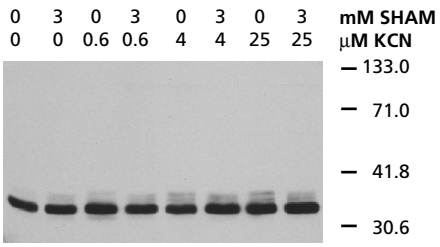

Figure 4: Western blot of AOX detected with monoclonal antibodies of whole-root extracts of *P. annua* at different concentrations of KCN in the presence or absence of SHAM (3 mM). Lane 1, 0 μM KCN without SHAM; lane 2, 0 μM KCN + SHAM; lane 3, 0.63 μM KCN without SHAM; lane 4, 0.63 μM KCN + SHAM; lane 5, 4 μM KCN without SHAM; lane 6, 4 μM KCN +1 SHAM; lane 7, 25 μM KCN without SHAM; lane 8, 25 μM KCN + SHAM.

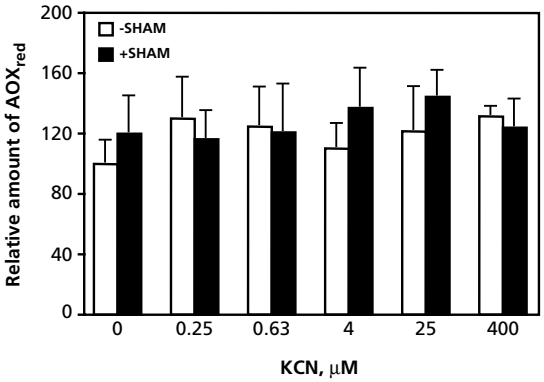

Figure 5: Relative levels of reduced AOX protein from whole-root extracts of *P. annua* at different concentrations of KCN in the presence (cross-hatched columns) or absence (open columns) of SHAM (3 mM), with the control (0 KCN without SHAM) as 100%. The error bars indicate the SD. The number of replicates was at least three and these were from different plants and plant batches.

We also determined the reduction state of the AOX protein *in vivo* in a tissue extract without the intermediate step of isolating mitochondria. No changes in the reduction state of the protein upon addition of KCN or SHAM were observed. The protein was invariably almost completely in its reduced (higher-activity) state (Figs 4 and 5). The reduced AOX protein gave one prominent and three minor bands around 35 kD. The oxidized dimer gave two bands around 66 kD. The difference between the intensity of the reduced (monomer) and oxidized (dimer) bands was at least 50-fold. However, when we first isolated mitochondria from the roots and then assayed AOX, the oxidized bands were more abundant than the reduced forms. In lanes with a mixture of isolated mitochondria and whole-root extract the oxidized bands were clearly detectable (data not shown). We conclude that the absence of visible oxidized bands of AOX on our gels was not an artifact, but was caused by the virtual absence of oxidized protein in intact *P. annua* roots.

Figure 6 shows the root respiration in the absence and presence of SHAM (Fig. 6A) and pyruvate concentrations (Fig. 6B) plotted against Q_r/Q_t, calculated from Figures 1 to 3. Both O_2 uptake and pyruvate concentration were dependent on Q_r/Q_t and these relationships were generally unaffected by the presence of SHAM. The increase in Q_r/Q_t was obtained because the respiration was increasingly blocked by inhibitors. Because at every point a

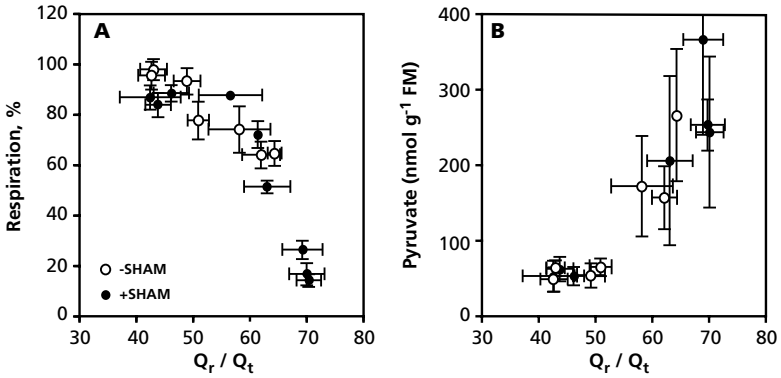

Figure 6: Respiration (A; percent of control) and pyruvate concentration (B; nanomoles per gram FM) plotted against Q_r/Q_t in roots of *P. annua* . ○, Measurements made in the absence of SHAM (3 mM); ●, measurements made in the presence of SHAM. The error bars indicate the SD. The number of replicates was at least three. The data were extracted from Figures 1 to 3.

steady state was reached at which the rate of ubiquinone oxidation by definition equaled the rate of ubiquinone reduction, the curve shown in Figure 6B represents the kinetics of the combined actions of the several dehydrogenases operating *in vivo*: when Q_r/Q_t increases, the dehydrogenases are less able to donate electrons to the Q_r/Q_t pool. With the dehydrogenases becoming less active, the concentration of respiratory substrates (pyruvate) increases (compare Fig. 6, A and B).

There was no significant change in the concentration of lactate with SHAM (235 ± 44, 240 ± 48, and 189 ± 87 nmol g^{-1} FM for 0, 4, and 100 mM KCN, respectively; mean values and sd; n=3) or ethanol (9.9 and 9.0 nmol g^{-1} FM for 0 and 100 μM KCN, respectively) 15 minutes after the addition of inhibitors.

Discussion

A Role for the Alternative Pathway in the Stabilization of Q_r/Q_t in Intact Roots

Our data supports a hypothesized physiological role for the alternative pathway to stabilize Q_r/Q_t, as proposed by Purvis and Shewfelt (1993) and Wagner and Wagner (1995). If the alternative pathway stabilizes Q_r/Q_t, then it should be expected that when respiration via the cytochrome pathway is inhibited by KCN, Q_r/Q_t increases to a greater extent in the presence of SHAM than in its absence.

We conclude from the data presented in Figures 1 and 2 that, especially at low KCN concentrations (up to 4 μM), when respiration is inhibited by

20%, Q_r/Q_t increases by almost 20% when SHAM is present, but only by approximately 8% when the alternative pathway is able to accept electrons. In addition, O_2 uptake proceeds at a faster rate when the alternative pathway participates in respiration. Because a high Q_r/Q_t favors the formation of oxygen free radicals, it is feasible that engaging the AOX when the cytochrome pathway is inhibited is an important mechanism to prevent such a potentially harmful situation. With increasing KCN concentrations, Q_r/Q_t further increases, also in the absence of SHAM, suggesting that the AOX is not able to fully buffer a considerable inhibition of the cytochrome pathway.

Activity of the Alternative Pathway in Intact Roots

At KCN concentrations between 0.63 and 25 μM there was an increasing effect of SHAM on the inhibition of respiration. That part of the cytochrome pathway that was not inhibited by KCN most likely increased in activity as a result of an increase in Q_r/Q_t. SHAM caused an extra increase in Q_r/Q_t compared with that in roots that were not exposed to SHAM. Therefore, SHAM inhibition of root respiration was an underestimation of the activity of the alternative pathway.

At KCN concentrations greater than 25 μM, SHAM inhibited O_2 uptake, with an average inhibition of 48%, whereas Q_r/Q_t was similar in the absence and presence of SHAM. This is explained by the relationship between O_2 uptake and Q_r/Q_t as plotted in Figure 6B. At a high Q_r/Q_t, a considerable decrease in respiration no longer results in a further increase in Q_r/Q_t (Fig. 7).

When the cytochrome pathway is partially inhibited by low KCN concentrations (<0.60 μM), a small (11%) but significant inhibition of O_2 uptake by SHAM was observed in intact roots of *P. annua*. If this inhibition

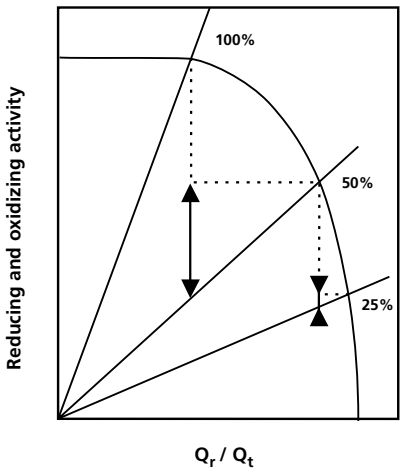

Figure 7: Hypothetical reducing (curved line) and oxidizing (straight lines) pathway activities against Q_r/Q_t. In a steady state, the reducing activity (combined dehydrogenases) is by definition equal to the oxidizing activity (cytochrome and alternative pathway). If 50% of the oxidizing pathways is inhibited, the other 50% will become more active (long arrow) because of the increase in Q_r/Q_t, and the inhibition will be underestimated. If subsequently 75% of the oxidizing pathways is inhibited, the 25% that is left becomes only slightly more active (short arrow) because of the steep slope of the reducing pathways and therefore the estimation of the inhibition becomes more accurate.

represented the participation of the alternative pathway, it is expected that at any of those low KCN concentrations, Q_r/Q_t would be higher in the presence of SHAM than in its absence (Wagner and Krab, 1995). However, no difference was observed in Q_r/Q_t with or without SHAM, suggesting that the alternative pathway does not contribute to respiration under these conditions. If, on the other hand, the inhibition were caused by a nonspecific effect on the reducing side of ubiquinone, a change in Q_r/Q_t would be expected: a shift to a more oxidized situation. However, Q_r/Q_t remained unchanged, so SHAM probably inhibited a nonmitochondrial component of O_2 uptake.

The Mechanisms Accounting for Increased AOX Activity

The data presented in Figures 1 and 2 clearly show that at KCN concentrations greater than 1 μM, the alternative pathway contributes to respiration (without SHAM) to an increasing extent, although the exact rates cannot be established.

In vitro the AOX can become more active by increasing the concentration of its substrate (Q_r), by reduction of the AOX protein to its higher-activity configuration (Umbach and Siedow, 1993; Umbach *et al.*, 1994), or by increasing the concentration of some α-keto acids, e.g., pyruvate (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996). It is unknown if and to what extent these mechanisms occur in intact tissues. *In vivo* the amount of AOX protein might also change over time; however, because our experiments lasted only 10 to 15 minutes, we can exclude such an effect of protein synthesis.

Activation of the AOX by the Reduction State of AOX

The slope of the kinetic curve of AOX activity (O_2 uptake against Q_r/Q_t) is much steeper when the AOX protein is reduced (Umbach and Siedow, 1993; Umbach *et al.*, 1994). If the reduction state of the AOX protein functions as a mechanism to increase respiration via the alternative pathway when the cytochrome pathway in intact grass roots is inhibited, then the AOX protein should become more reduced at KCN concentrations greater than 0.6 μM. However, at all KCN concentrations and independent of the presence of SHAM, the AOX protein was mainly in its reduced state (a factor of 50 difference in intensity between the oxidized and reduced bands). The oxidized form appears around 66 kD. We conclude, therefore, that the reduction state of the AOX protein has no regulatory function in the experiments described here. This does not necessarily mean that the reduction state of the AOX protein never changes to the oxidized (less-active) form *in vivo* under other circumstances, e.g., different developmental stages, different growth conditions, but this remains to be confirmed.

The AOX protein of *P. annua* roots was much more oxidized in the same experiments when mitochondria were isolated before the AOX protein measurements (data not shown). The oxidized form of the protein is clearly detectable in isolated mitochondria and in samples with a mixture of isolated mitochondria with whole-root extracts.

This indicates that in our experiments, the primary antibodies recognized both the oxidized and the reduced forms of the protein, as has been found previously in comparable experiments (e.g., Umbach and Siedow, 1993). The only difference between the AOX protein measurements in intact plant material and those in isolated mitochondria was the isolation of the mitochondria before immunodetection. Therefore, our results show that the AOX protein becomes more oxidized during isolation of the mitochondria material, as observed previously by Umbach and Siedow (1997). Estimations of the reduction state of the AOX protein *in vivo* using isolated mitochondria are therefore not suitable for obtaining information on the reduction state of the protein in intact plants.

Activation of the AOX by Pyruvate

The kinetic curve of the AOX (O_2 uptake against Q_r/Q_t) shifts to the left upon the addition of pyruvate or other α-keto acids in isolated mitochondria, so α-keto acids increase the activity of the AOX (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Hoefnagel and Wiskich, 1996; Millar *et al.*, 1996). If pyruvate activated the alternative pathway in the present experiments, the pyruvate concentration would be expected to increase at KCN concentrations greater than 1 μM. However, assuming that our results on concentrations in whole tissue reflect the intramitochondrial pyruvate concentration, then these results do not indicate that the alternative pathway is activated by an increase in the pyruvate concentration. At those KCN concentrations at which the activity of the alternative pathway increased (without SHAM) the pyruvate concentration did not increase.

If the alternative pathway were activated by increased concentrations of pyruvate, the concentrations inside the mitochondria (Umbach and Siedow, 1996) should change in a range around the half-maximum pyruvate stimulation. Values for the half-maximum pyruvate stimulation vary between different studies, from 128 μM in mitochondria isolated from potato tubers (Wagner *et al.*, 1995) to 500 μM in mitochondria isolated from tobacco leaves (Vanlerberghe *et al.*, 1995). Values for half-maximum pyruvate stimulation were determined by adding pyruvate to intact mitochondria. The binding site of pyruvate on the AOX is at the matrix side of the mitochondria (Umbach and Siedow, 1996). The pyruvate concentration outside the mitochondria may not be representative of the concentration inside the mitochondria at the

activation site because of a low capacity of the mitochondrial pyruvate transporter and the production of pyruvate by malic enzyme from malate inside the mitochondria, especially at pH values around 6.5 (Millar *et al.*, 1996).

Zang *et al.* (1996) found a half-maximum pyruvate stimulation of 400 μM for purified AOX protein from arum lily and soybean. However, in these studies the protein was isolated from the mitochondrial membranes, which does not necessarily represent the situation when the protein is still in a membrane, e.g., due to conformational changes.

Millar *et al.* (1996) used inside-out mitochondrial particles to measure the half-maximum pyruvate stimulation and found a value of less then 4 μM (in sweet potato and soybean). Finnegan *et al.* (1997) found comparable half-maximum pyruvate stimulation: 4.5 μM in cotyledons and 51 μM in roots of soybean inside-out mitochondrial particles. This is presumably the most appropriate method for determining the half-maximum pyruvate stimulation, because it gives the pyruvate concentration at the site where it reacts with the AOX protein to stimulate its activity.

The measured pyruvate concentrations are values pertaining to the whole tissue and were 58 ± 7 nmol g^{-1} FM for the control plants and increased to 250 nmol g^{-1} FM at the highest KCN concentrations (Fig. 3). These concentrations are similar to those found for other plants (e.g., carrot roots, 39 nmol g^{-1} FM [Kato-Noguchi, 1996]; petunia cell suspensions, 100 nmol g^{-1} FM [Wagner and Wagner, 1997]; and 60, 32, and 67 nmol g^{-1} FM for roots of spinach, bean, and wheat, respectively [Day and Lambers, 1983]). During hypoxia, the pyruvate concentration in barley roots increases from 60 to 120 nmol g^{-1} FM (Good and Muench, 1993). In tobacco cell suspensions, the pyruvate concentration increases upon the addition of antimycin from 100 to 550 nmol g^{-1} FM (assuming a dry matter percentage of 10%) in less than 1 h (Vanlerberghe *et al.*, 1997).

If we estimate the concentration of pyruvate in the cytoplasm we might be able to conclude if it is in the range of the half-maximum pyruvate stimulation. The mitochondrial volume is about 0.2% to 0.7% of the total cell volume (Douce, 1985). The volume of the mitochondria is small in comparison with that of the whole cell; therefore, it is impossible to separate the pyruvate concentration inside the mitochondria from whole-tissue data. If pyruvate is equally distributed over the mitochondria, cytosol, and vacuole, then the concentration is 60 μM, which is 1.2 to 15 times as high as the half-maximum pyruvate stimulation of 50 and 4 μM (Millar *et al.*, 1996; Finnegan *et al.* (1997). If the pyruvate concentration in the vacuole is 0 (which it probably is), then the concentration in the cytoplasm (10% of cell volume) will be about 10 times as high, i.e. 600 μM (12–150 times as high as the half-maximum pyruvate stimulation of 50 and 4 μM). If the pyruvate concentration in the

mitochondria is at least equal to that in the cytosol, then the AOX is always fully activated by pyruvate. The end product of glycolysis in plants is not only pyruvate but also a substantial amount of malate (Day and Hanson, 1977). Malate by itself cannot be the substrate for the citric acid cycle, but needs to be converted into pyruvate via malic enzyme. The pyruvate concentration inside the mitochondria may be even higher than that in the cytosol.

Increase in AOX Activity Caused by an Increase in Q_r/Q_t

The alternative pathway becomes more active if there is more of its substrate, Q_r. The slope of the activity of the alternative pathway (respiration against Q_r/Q_t) determines the change in activity of the alternative pathway for a given change in Q_r/Q_t. If the slope is steep, then a small change in Q_r/Q_t has a major influence on the activity of the alternative pathway. In the present experiments Q_r/Q_t increased by 8% between 0.6 and 25 mM KCN (in the absence of SHAM), and under these conditions the activity of the alternative pathway increased (indicated by an inhibition of SHAM).

If we consider data from the literature that describe the relationship between Q_r/Q_t and O_2 uptake via the alternative pathway, a nonlinear relationship is often found when the oxidized form is measured in the absence of pyruvate. If the AOX protein is reduced, the relationship often approaches linearity, except for the first approximately 10% of the O_2 uptake (nanomoles of O_2 per milligram of protein per minute). If a straight line is fitted through these points, an average slope of 143% ± 39% (with pyruvate) and 208% ± 96% (without pyruvate) AOX activity $(\%Q_r/Q_t)^{-1}$ is found (Umbach *et al.*, 1994; Day *et al.*, 1995; Hoefnagel *et al.*, 1995; Hoefnagel and Wiskich, 1996; Millar *et al.*, 1996, 1997). The first nonlinear part was ignored if necessary, and the values were recalculated to percentages to compare the different respiration rates. Millar *et al.* (1996) found even steeper slopes using inside-out submitochondrial particles, 476% AOX activity $(\%Q_r/Q_t)^{-1}$ in the presence of pyruvate.

If the Q_r/Q_t increases by 10% (on a 0%–100% scale), then the activity of the alternative pathway increases by 14%, and if the active ubiquinone pool is 50% of the Q_t pool, then a change in Q_r/Q_t of 10% results in an increase of 28% in alternative pathway activity. The alternative pathway can stabilize Q_r/Q_t because of its steep kinetics, even though Q_r/Q_t cannot be kept absolutely constant; if Q_r/Q_t increases marginally, the alternative pathway rapidly becomes more active and thereby prevents a further increase in Q_r/Q_t.

Kinetic Properties of Mitochondrial Dehydrogenases

All of the mitochondrial dehydrogenases have unique kinetic properties that may differ between the different types (van den Bergen *et al.*, 1994).

Because at steady state the Q_r/Q_t activity of ubiquinone-reducing pathways by definition equals the activity of ubiquinone-oxidizing pathways, the obtained rate at equilibrium also represents the rate of the combined dehydrogenase activity. By manipulating the oxidizing pathways with inhibitors, a relationship between activity of dehydrogenases and ubiquinone reduction can be obtained (van den Bergen *et al.*, 1994; Millar *et al.*, 1995; Wagner and Krab, 1995). Figure 6A shows the combined activity of all of the dehydrogenase acting in *P. annua* roots *in vivo* as a function of Q_r/Q_t. The resulting curve suggests that there is no large or sudden change in the use of different types of dehydrogenases either at the various levels of Q_r/Q_t or in the absence or presence of SHAM. If the activity of the combined dehydrogenases decreases at higher Q_r/Q_t values, and if glycolysis is not decreased to the same extent, the concentration of the end products of glycolysis will increase. Figure 6A shows an increase in the concentration of pyruvate when the combined dehydrogenases become less active as a result of the increase in Q_r/Q_t.

Vanlerberghe et al. (1997) showed that if there is an imbalance between oxidation of organic acids (production of NADH) and the activity of the electron-transport pathway (production of NAD^+), pyruvate will accumulate, which may result in fermentation under aerobic conditions. In roots of *P. annua* there was no accumulation of ethanol or lactate, and therefore fermentation did not occur, probably because the respiration measurements lasted only 10 to 15 minutes. In roots of maize (Wignarajah and Greenway, 1976), barley, and rice (Wignarajah *et al.*, 1976), the maximum activity of alcohol dehydrogenase and especially pyruvate decarboxylase is low and increases upon anoxia. These enzymes probably have to be synthesized first in the roots of *P. annua* before lactate/ethanol accumulates, and that takes longer than 15 minutes.

The alternative pathway can avoid fermentation because it can prevent an increase in Q_r/Q_t and therefore prevent the dehydrogenases from becoming less active, so pyruvate accumulates.

Concluding remarks

Our results show that the alternative pathway can stabilize Q_r/Q_t in roots of *P. annua* (Figs 1 and 2) when the cytochrome pathway is restricted by KCN. By stabilizing Q_r/Q_t, an increase in the production of radicals and fermentation products can be prevented. In this way potential cell damage is avoided. The increased activity of the alternative pathway as a result of KCN inhibition of the cytochrome pathway is not caused by a further reduction of the AOX

protein (Fig. 5); almost all of the AOX is already in its reduced state in the intact *P. annua* roots in the absence of inhibitors. A small change in Q_r/Q_t has a large effect on the activity of the alternative pathway. Therefore, the alternative pathway stabilizes Q_r/Q_t. The role of pyruvate in the increased activity of the alternative pathway is not entirely clear from our results, but the pyruvate concentration always seemed to be higher than the half-maximum pyruvate stimulation.

References

Atkin OK, Villar R, Lambers H (1995) Partitioning of electrons between the cytochrome and alternative pathways in intact roots. Plant Physiol. 108: 1179-1183

Chance B, Boveris A, Oshino N (1977) Alcohol and Aldehyde Metabolizing Systems. Academic Press, New York, pp 261-274

Crane FL (1963) The coenzyme Q group (ubiquinones). Prog. Chem. Fats Other Lipids. 7: 267-289

Day DA, Hanson JB (1977) Pyruvate and malate transport and oxidation in corn mitochondria. Plant Physiol. 59: 630-635

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, Wiskich JT (1996) The cyanide-resistant oxidase. To inhibit or not to inhibit, that is the question. Plant Physiol. 110: 1-2

Day DA, Lambers H (1983) The regulation of glycolysis and electron transport in roots. Plant Physiol. 58: 155-160

Day DA, Whelan J, Millar AH, Siedow JN, Wiskich JT (1995) Regulation of the alternative oxidase in plants and fungi. Aust. J. Plant Physiol. 22: 497-509

Douce R (1985) Mitochondria in Higher Plants: Structure, Function, and Biogenesis. Academic Press, New York, pp 4-7

Elthon TE, Nickels RL, McIntosh L (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89: 1311-1317

Finnegan PM, Whelan J, Millar AH, Zhang Q, Smith MK, Wiskich JT, Day DA (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. Plant Physiol. 114: 455-466

Forman HJ, Boveris A (1982) Superoxide radical and hydrogen peroxide in mitochondria. In Free Radicals in Biology, Vol V. Academic Press, New York, pp 65-90

Good AG, Muench DG (1993) Long-term anaerobic metabolism in root tissue. Plant Physiol. 101: 1163-1168

Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400

Hoefnagel MHN, Wiskich JT (1996) Alternative oxidase activity and the ubiquinone redox level in soybean cotyledon and *Arum* spadix mitochondria during NADH and succinate oxidation. Plant Physiol. 110: 1329-1335

Kato-Noguchi H (1996) Effects of citrate on respiratory gas exchange and metabolism in carrot root tissues. Phytochemistry. 45: 225-227

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685

Leprince O, Atherton NM, Deltour R, Hendry GAF (1994) The involvement of respiration in free radical processes during loss of desiccation tolerance in germinating *Zea mays* L. Plant Physiol. 104: 1333-1339

Liu SS, Huang JP (1996) Coexistence of a "reactive oxygen cycle" with the "Q-cycle" in the respiratory chain: a hypothesis for generating, partitioning, and function of superoxide in mitochondria. In Proceedings of International Symposium on Natural Antioxidants: Molecular Mechanisms and Health Effects. AOCS Press, Champaign, IL, pp 513-529

Meeuse BJD (1975) Thermogenic respiration in aroids. Annu. Rev. Plant Physiol. 26: 117-126

Millar AH, Atkin OK, Lambers H, Wiskich JT, Day DA (1995) A critique of the use of inhibitors to estimate partitioning of electrons between mitochondrial respiratory pathways in plants. Physiol. Plant. 95: 523-532

Millar AH, Finnegan PM, Whelan J, Drevon JJ, Day DA (1997) Expression and kinetics of the mitochondrial alternative oxidase in nitrogen-fixing nodules of soybean roots. Plant Cell Environ. 20: 1273-1282

Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation on the alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618

Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329: 259-262

Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia. 83: 553-559

Puntelarulo S, Galleano M, Sanchez RA, Boveris A (1991) Super-oxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. Biochim. Biophys. Acta. 1074: 277-283

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissue? Physiol. Plant. 88: 712-718

Purvis AC, Shewfelt RL, Gegogeine JW (1995) Superoxide production by mitochondria isolated from green bell pepper fruit. Physiol. Plant. 94: 743-749

Ribas-Carbo M, Wiskich JT, Berry JA, Siedow JN (1995) Ubiquinone redox behavior in plant mitochondria during electron transport. Arch. Biochem. Biophys. 317: 156-160

Rich PR, Bonner WD (1987) The sites of superoxide anion generation in higher plant mitochondria. Arch. Biochem. Biophys. 188: 206-213

Threlfall DR, Whistance GR (1970) Biosynthesis of ubiquinone: a search for polyprenyl phenol and quinone precursors. Phytochemistry. 9: 355-359

Umbach AL, Siedow JN (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Siedow JN (1996) The reaction of the soybean cotyledon mitochondrial cyanide-resistant oxides with sulfhydryl reagents suggests that α-keto acid activation involves the formation of a thiohemiacetal. J. Biol. Chem. 271: 25019-25026

Umbach AL, Siedow JN (1997) Changes in the redox state of the alternative oxidase regulator sulfhydryl/disulfide system during mitochondrial isolation: implications for inferences of activity *in vivo*. Plant Sci. 123: 19-28

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett. 348: 181-184

Van den Bergen CWM, Wagner AM, Krab K, Moore AL (1994) The relationship between electron flux and the redox poise of the quinone pool in plant mitochondria: interplay between quinol-oxidizing and quinone-reducing pathways. Eur. J. Biochem. 226: 1071-1078

Vanlerberghe GC, Day DA, Wiskich JT, Vanlerberghe AE, McIntosh L (1995) Alternative oxidase activity in tobacco leaf mitochondria. Plant Physiol. 109: 353-361

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: from gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1997) Molecular genetic evidence of the ability of alternative oxidase to support respiratory carbon metabolism. Plant Physiol. 113: 657-661

Wagner AM, Bergen van CWM, Wincencjusz H (1995) Stimulation of the alternative pathway by succinate and malate. Plant Physiol. 108: 1035-1042

Wagner AM, Krab K (1995) The alternative respiration pathway in plants. Role and regulation. Plant Physiol. 95: 318-325

Wagner AM, Wagner MJ (1995) Measurements of in vivo ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283

Wagner AM, Wagner MJ (1997) Changes in mitochondrial respiratory chain components of petunia cells during culture in the presence of antimycin A. Plant Physiol. 115: 617-622

Wignarajah K, Greenway H (1976) Effect of anaerobiosis on activities of alcohol dehydrogenase and pyruvate decarboxylase in roots of *Zea mays*. New Phytol. 77: 575-584

Wignarajah K, Greenway H, John CD (1976) Effect of waterlogging on growth and activity of alcohol dehydrogenase in barley and rice. New Phytol. 77: 585-592

Zhang Q, Hoefnagel MHN, Wiskich JT (1996) Alternative oxidase from *Arum* and soybean: its stabilization during purification. Physiol. Plant. 96: 551-558

Chapter 3

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

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Abbreviations:
AOX, alternative oxidase; Cyt, cytochrome; FM, fresh mass; SHAM, salicylhydroxamic acid; Q_r/Q_t, reduction state of the ubiquinone pool; Q_{ox}, oxidized ubiquinone; Q_r, (reduced) ubiquinol, Q_t, total ubiquinone pool, Q_r-max, maximum obtainable Q_r.

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 (AOX) 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 with 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 AOX 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.

Introduction

The respiratory electron-transport pathways of plant mitochondria comprise the cytochrome pathway and the alternative pathway; beyond the branchpoint (ubiquinone), the alternative pathway does not contribute to the generation of a proton-motive force, in contrast to the cytochrome pathway (recent review: Vanlerberghe and McIntosh, 1997). The alternative pathway consists of only one protein, the alternative oxidase. 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 disulfide bond and by α–keto acids, such as pyruvate (Millar *et al.*, 1993; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996). 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 roots of *Poa annua*, the alternative oxidase is invariably in the reduced form during the light period (Millenaar *et al.*, 1998, Chapter 2). 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, Chapter 2) concluded that alternative pathway activity can stabilize 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, Chapter 2).

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 what will happen with 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 SHAM sensitivity. In roots of *Cucumis sativus*, a diurnal fluctuation in the sugar concentration is correlated 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 for absence of alternative path activity (Hoefnagel *et al.*, 1995; Ribas-Carbo *et al.*, 1995; Wagner and Krab, 1995; Day *et al.*, 1996).

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 hr instead of the normal 10 hr) and to a low light intensity (70 instead of 450 μmol m^{-2} s^{-1} 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 pathway (Robinson *et al.* 1995; Lennon *et al.* 1997). During the low-light treatment, sugar concentrations and cytochrome *c* oxidase activity 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.

Materials and methods

Plant material and growth conditions

Roots of 5- to 6-weeks-old *Poa annua* (L) plants were used for all measurements. Seeds were germinated on moistened filter paper for one week and the seedlings were transferred to sand for one 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% RH, with a photoperiod of 14 hr at 450 μmol m^{-2} s^{-1} (PAR). The low-light treatment consisted of transferring 5- to 6-weeks-old plants to an 8 hr daylength at 60-70 μmol m^{-2} s^{-1} (PAR) for 4 days, or to complete darkness for 4 to 7 days

Respiration of intact roots

Roots (1.5-2.0 g fresh mass (FM)) 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, USA) (Lambers *et al.*, 1993). The alternative pathway was inhibited with 3 mM SHAM (1 M stock solution in methoxy-ethanol). 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 to 15 minutes 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 Sweely *et al.* (1963). Root extracts were prepared from 400 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 5 mL ethanol/water (38% v/v) with mannitol as an internal standard, and subsequently vortexed (2 × 30 sec) and centrifuged at 1,500 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 1,500 g for 4 min. The supernatant was mixed with approx. 0.1 g of Dowex (BDH Chemicals Ltd., Poole, UK) to remove amino acids. This extract was vortexed 4 times for 30 sec with 15 minutes 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 stay for at least 45 minutes, after which 2 μL of the sililated extract was analyzed on a gas chromatograph (GC, HP 5890 A, Hewlett Packard, Amstelveen, the Netherlands), with a 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 minutes to 280°C with a rate of 5°C min^{-1}. Individual compounds were identified by their retention times as compared with commercially obtained standard compounds; also the response values were obtained from these standards.

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 minutes, 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. 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} FM s^{-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 cyt c. Addition of 0.5 mM KCN or bubbling with CO inhibited the reaction to 6 ± 1 and 16 ± 4%, respectively (average and 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 (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% (v/v) SDS, 10% (v/v) glycerol, 0.001% (v/v) bromophenol blue]. After centrifugation for 10 minutes 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, Ne, USA) were used as primary antibodies (Elthon *et al.*, 1989) with a dilution factor of 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 product usage protocol (Pierce, Rockford, 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 gray levels). The intensities of the bands were corrected for the background.

Measurement of ubiquinone reduction levels in intact roots

The ubiquinone assays were done 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 40-60ºC) and vortexed for 30 sec. The mixture was centrifuged (1,500*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 analyzed by HPLC (HP 1050 series, 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 minutes 20% (v/v) ethanol, and from 10 minutes a gradient to 70% (v/v) ethanol at 40 minutes) as the mobile phase (1 mL min^{-1}) was used. Detection was performed at 290 and 275 nm for Q_r and Q_{ox}, respectively. Commercially obtained Q_{10} and Q_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 done 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 to 1.2 g fresh mass) were kept in the dark for 25 minutes 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 (Gonzàlez-Meler *et al.*, 1996). Oxygen extraction and isotope analysis were carried out as described in Robinson *et al.* (1995) with modifications of 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 5 data points) were greater than the value of 0.995 considered minimally acceptable (Ribas-Carbo

et al., 1995; 1997; Lennon *et al.*, 1997; Gonzàlez-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 sulfoxide) were applied by sandwiching the roots between medical wipes soaked with the corresponding inhibitor and incubating in the dark for at least 25 minutes (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 made as described in 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.

Results

Carbohydrates

To change the endogenous sugar concentration in roots of *Poa annua*, we transferred plants from a 10 hr to a 16 hr night period and at the same time to a lower light intensity during the day (70 instead of 450 µmol m^{-2} s^{-1}, PAR). During the following four days, the concentrations of glucose, fructose, sucrose, inositol, malate, and citrate were measured (Fig. 1). The sugar concentrations in the roots were not significantly changed after the normal 10 hr period in the dark, and recovered when no low-light treatment was given (data from time 0 are an average of data from several days with normal light conditions). After transfer to a light regime with 16 hr 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 hr (6.2, 10.5, and 1.6 µmol g^{-1} FM, respectively, Fig. 1A-C). After two long nights, glucose and fructose concentrations were only 14% of their initial concentration at time 0, while the sucrose concentration 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 (Fig. 1D, E). The citrate concentration (1.0 $\pm$ 0.3 µmol g^{-1} FM) did not change significantly during the low-light treatment (Fig. 1F).

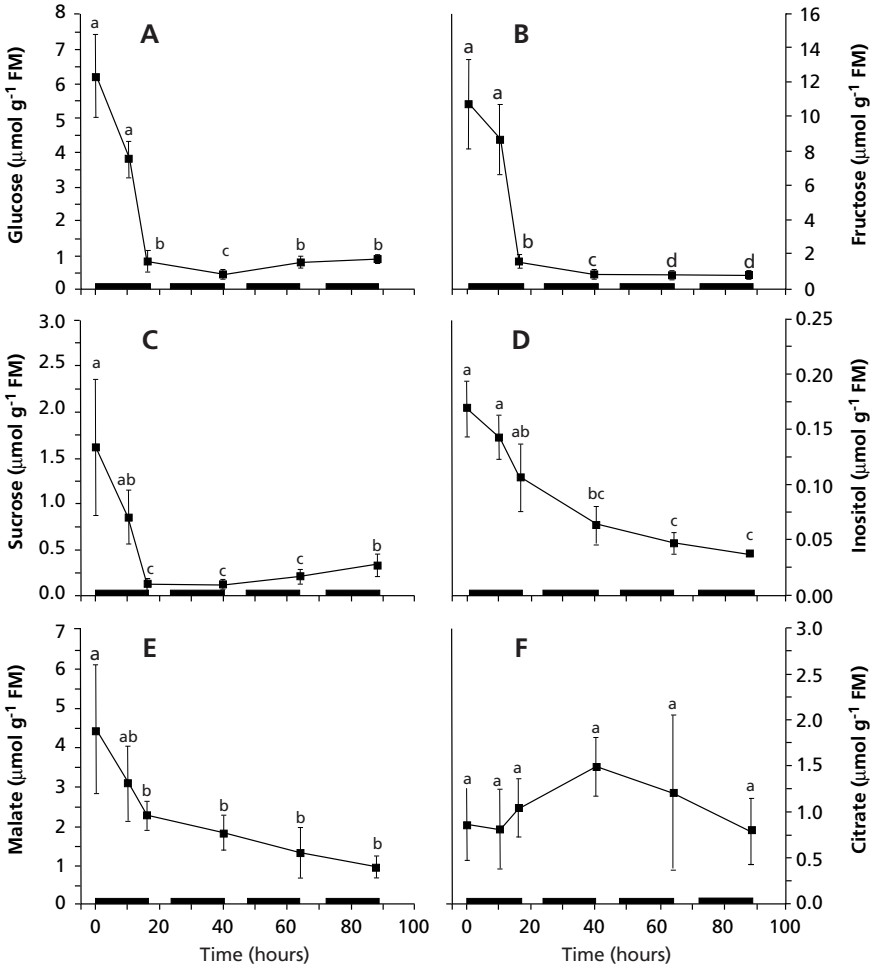

Figure 1: Roots of *Poa annua* plants during four days exposure to low-light conditions. Error bars represent standard deviation and the number of replicates was at least three, based on different plants and plant batches; points with a different letter are significantly different. All concentrations are in μmol g^{-1} FM. A) Glucose; B) Fructose; C) Sucrose; D) Inositol; E) Malate, and F) Citrate.

Respiration

Root respiration did not significantly decrease during the 10 hr of normal darkness compared with the initial respiration rate in the light period (5.3 ± 0.6 nmol O_2 g^{-1} FM s^{-1}, data from time 0 are an average from several days with normal light conditions). However, when the night period was extended to 16 hr, root respiration decreased by 45%. During the following 3 days, respiration decreased further, reaching a value of 27% of the initial control respiration (Fig. 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

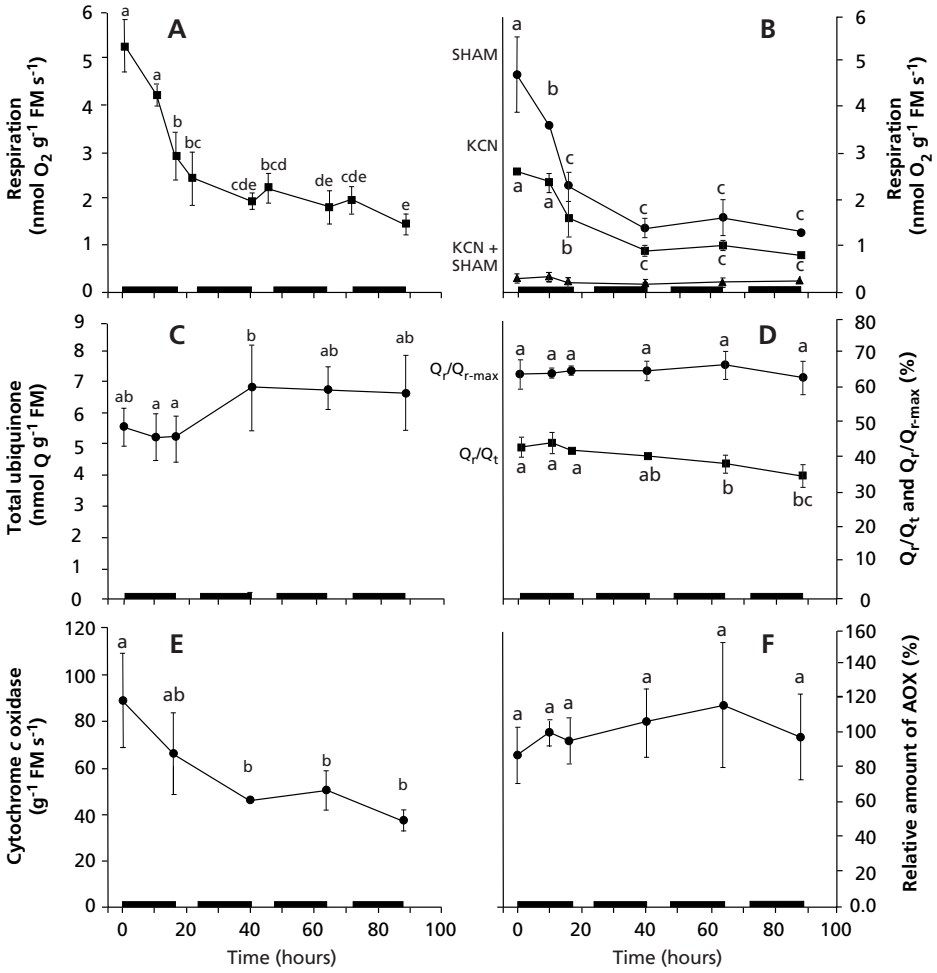

Figure 2: Roots of *Poa annua* plants during four days exposure to low-light conditions. Error bars represent standard deviation and the number of replicates was at least three, based on different plants and plant batches; points with a different letter are significantly different. A) Respiration (nmol O_2 g^{-1} FM s^{-1}); B) SHAM (circles), KCN (squares) and SHAM plus KCN (triangle) resistant respiration; C) Total ubiquinone pool (nmol g^{-1} FM); D) Q_r/Q_t (squares) and $Q_r/Q_{r\text{-}max}$ (circles); E) Cytochrome *c* oxidase activity (g^{-1} FM s^{-1}); F) Relative alternative oxidase protein (%), detected with monoclonal antibodies.

respiration decreased during the low-light treatment (Fig. 2B), when compared with plants grown under initial conditions. The residual respiration did not change with time.

The alternative pathway activity was measured with 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 ± 0.10‰, and that for cytochrome *c* oxidase was 19.5 ± 0.32‰ (with SHAM); for the residual respiration it was 21.4 ± 0.24‰ (+KCN and SHAM, mean and

Table 1: Fractionation (Δ, ‰; as defined by Farquhar and Richards, 1984), the percentage alternative respiration (% alt path), the percentage total respiration (% tot resp) and absolute total respiration (nmol O_2 g^{-1} FM s^{-1}), versus time (hr) of the low-light experiment, with or without sucrose addition just before the measurement. Mean and standard error; *n* = number of replicates. The fractionation was 19.5±0.32 for the cytochrome path and 26.6±0.10 for the alternative path.

hr	sucrose	*n*	Δ (‰)	% alt path	% tot resp	tot resp
0	-	5	21.6 ± 0.44	29.9 ± 6.3	100.0 ± 6.7	4.0 ± 0.3
10	-	2	22.2 ± 0.81	38.4 ± 11.6	96.7 ± 13.8	3.6 ± 0.7
16	-	4	23.6 ± 0.43	57.9 ± 6.2	57.5 ± 3.1	2.2 ± 0.2
64	-	3	22.9 ± 0.29	48.1 ± 4.1	40.3 ± 2.5	1.5 ± 0.2
0	+	4	20.9 ± 1.49	19.8 ± 10.6	118.5 ± 5.4	4.4 ± 0.4
64	+	3	22.1 ± 0.72	36.8 ± 5.9	52.2 ± 3.0	2.0 ± 0.2

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 hr of the first long night 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 (Fig. 3). In contrast, the activity of the cytochrome pathway decreased in response to the low-light treatment. When roots of *Poa annua* were fed sucrose at times 0 and 64 hr, 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 (Fig. 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 were measured. The total concentration of ubiquinone (mainly Q_9 + Q_8 Millenaar *et al.*, 1998, Chapter 2) before transfer to the long-night treatment was 5.5 ± 0.6 nmol g^{-1} FM, and this increased during the second long night to 131% of the control values. No further changes occurred during the last 3 days (Fig. 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 time 0, 16, 40, 64, and 88 hr, 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 time 0, 10, 16, 40, 64, and 88 hr, respectively (average and standard deviation;*n*=3), suggesting

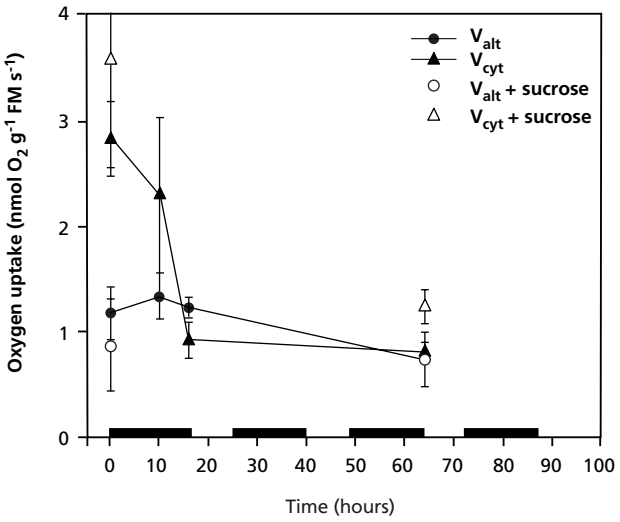

Figure 3: Activity (nmol O_2 g^{-1} FM s^{-1}) of the alternative (circles) and cytochrome pathways (squares) with sucrose (open symbols) or without sucrose (closed symbols), measured using the ^{18}O isotope fractionation technique. Error bars represent standard error, *n*=5, 2, 4, 3, 4, 3 for 0, 10,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

that a gradually increasing part of the Q pool could not be reduced and seemed to be inactive. A significant correlation was found between $Q_{r\text{-}max}$ and Q_r/Q_t. The reduction state of the Q pool, corrected for the maximal obtainable reduction state ($Q_r/Q_{r\text{-}max}$) had a constant value of 65% during the treatment (Fig. 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} FM at time 0, 10, 16, 40, 64, and 88 hr, respectively (average and 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 (Fig. 1F) and all of the AOX was in the reduced form (Fig. 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 (Fig. 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.

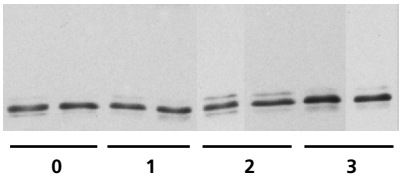

Figure 4: Immunoblot of alternative oxidase in whole-root extracts of *Poa annua* at different times during the low-light experiment detected with monoclonal antibodies. Lane 1 & 2 day 0, with the normal light conditions; Lane 3 & 4 after one long night, Lane 5 & 6 after 2 long nights; Lane 7 & 8 after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

Cytochrome *c* oxidase

The cytochrome *c* oxidase capacity (control 89 ± 20.6 g^{-1} FM s^{-1}, first order rate constant) decreased 26% during the first night, and 49% during the following night; it did not change any further during the last two days (Fig. 2E). The cytochrome *c* oxidase capacity after 4 and 7 days of darkness was 48 ± 13.6 and 38 ± 2.6 g^{-1} FM s^{-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 hr) and, at the same time, from high to low light intensity (from 450 to 70 µmol m^{-2} s^{-1}), showed a dramatic decrease in the 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, indicating greater engagement of the alternative oxidase. Possible regulatory mechanisms that account for this are discussed below.

The total respiration measured with the GC during the activity measurements (^{18}O fractionation) at Duke University was 22% lower as 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 as 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

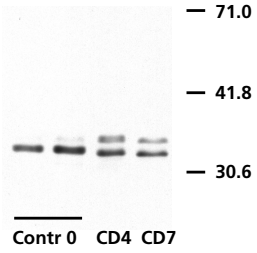

Figure 5: Immunoblot of alternative oxidase in whole-root extracts of *Poa annua* at different times in complete darkness detected with monoclonal antibodies. Lane 1 & 2, day 0, with the normal light conditions; Lane 3, after 4 days of darkness, Lane 4, after 7 days of darkness.

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 four days of low-light treatment, the *in vitro* capacity of cytochrome *c* oxidase decreased by 73%. McDonnell and 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 hr, 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 antioxidant (Nohl *et al.*, 1998), which raises the question if 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. That is, 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 (Van den Bergen *et al.*, 1994; Ribas-Carbo *et al.*, 1995). 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 Q-pool that cannot be fully oxidized, however, it is unknown if 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 not only stable during the low-light treatment, but also 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 as 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 also under these conditions the alternative pathway has an important function.

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 is unknown. The amount of AOX increased three-fold in leaves of *Pisum sativum* when they were transferred from 19 to 28°C. After transfer from 28 to 19°C, the concentration was back to normal levels in about 24 hr; the half-life time is, therefore, estimated at 18 hr (MA Gonzàlez-Mehler and JN 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 appears on the blot (Fig. 5). Therefore, the roots are capable of synthesizing new AOX protein, even under extreme low-light conditions. We do not know, however, if 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 (Fig. 4). Plants that are exposed for 4 or 7 days to complete darkness (Fig. 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 (Umbach and Siedow, 1997; Millenaar *et al.*, 1998, Chapter 2). 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 (Chapter 4). 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 (Fig. 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 entirely caused 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 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.

The rate of root respiration after 64 hr cannot be completely restored to the level of the high-light plants by the addition of sucrose. Therefore, the cytochrome path is only limited to a minor extent by the immediate sugar supply, although sucrose readily entered the roots, since the respiration increased.

Why do roots maintain the alternative pathway? This pathway appears to have a crucial role in stabilizing Q_r/Q_t *in vivo* (Millenaar *et al.*, 1998, Chapter 2) in preventing the formation of harmful reactive oxygen species. Especially when respiration proceeds closer to state 4 (low ADP concentration), when the proton gradient across the inner mitochondrial membrane is relatively high, the production of harmful oxygen free radicals tends to be enhanced (Skulachev, 1996). In addition, engagement of the alternative pathway can also prevent fermentation and maintain a continuing citric acid cycle when the cytochrome pathway is restricted (Vanlerberghe *et al.*, 1997; Millenaar *et al.*, 1998, Chapter 2).

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 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.

Acknowledgements

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References

Azcón-Bieto J, Lambers H, Day DA (1983) Effect of photosynthesis and carbohydrate status on respiratory rates and the involvement of the alternative pathway in leaf respiration. Plant Physiol. 72: 598-603

Crane FL (1963) The coenzyme Q group (ubiquinones). Progress in the chemistry of fats and other lipids. 7(II): 267-289.

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, Wiskich JT (1996) The cyanide-resistant oxidase: To inhibit or not to inhibit, that is the question. Plant Physiol. 110: 1-2

Elthon TE, Nickels RL, McIntosh L (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89: 1311-1317

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust. J. Plant Physiol. 11: 539-552

Gonzàlez-Meler MA, Ribas-Carbo M, Siedow JN, Drake BG (1996) Direct inhibition of plant mitochondrial respiration by elevated CO_2. Plant Physiol. 112: 1349-1355

Gonzàlez-Meler MA, Ribas-Carbo M, Giles L, Siedow JN (1999) The effect of growth and measurement temperature on the activity of the alternative respiratory pathway. Plant Physiol. 120: 765-772

Guy RD, Berry JA, Fogel ML, Hoering TC (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. Planta. 177: 483-491

Hoefnagel MHN, Millar AH, Wiskich T, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685

Lambers H, Scheurwater I, Atkin OK (1996) Respiration patterns in roots in relation to their functioning. In Plant roots. The hidden half (Waisel Y, Eshel A and Kafkafi U eds.) New York, Marcel Dekker Inc. pp 323-362

Lambers H, Van der Werf A, Bergkotte M (1993) Respiration: the alternative pathway. In Methods in comparative plant ecology (Hendry, G.F and Grime, J.P., eds.). pp 140-144. Chapman & Hall, London.

Lennon AM, Neuenschwander UH, Ribas-Carbo M, Giles L, Ryals JA, Siedow JN (1997) The effects of salicylic acid, tobacco mosaic virus infection on the alternative oxidase of tobacco. Plant Physiol. 115: 783-791

Lowry OH, Rosenbrough NJ, Farr AL, Randall R (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275

McDonnell E, Farrar JF (1992) Substrate supply and its effect on mitochondrial and whole tissue respiration in barley roots. In: Molecular, biochemical and physiological aspects of plant respiration (Lambers H and Van der Plas LHW, eds.). The Hague, SPB Academic Publishing, pp. 455-462.

Millar AH, Atkin OK, Menz RI, Henry B, Farquhar G, Day DA (1998) Analysis of respiratory chain regulation in roots of soybean seedlings. Plant Physiol. 117: 1083-1093

Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618

Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329: 259-262

Millenaar FF, Benschop JJ, Wagner AM, Lambers H (1998) The role of the alternative oxidase in stabilizing the in vivo reduction state of the ubiquinone pool; and the activation state of the alternative oxidase. Plant Physiol. 118: 599-607

Nohl H, Gille L, Kozlov AV (1998) Antioxidant-derived prooxidant formation from ubiquinol. Free Radical Biol. Medicine. 25: 666-675

Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia 83: 553-559

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol. Plant. 88: 712-718

Ribas-Carbo M, Lennon AM, Robinson SA, Giles L, Berry JA, Siedow JN (1997) The regulation of electron partitioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. Plant Physiol. 113: 903-911

Ribas-Carbo M, Wiskich J, Berry JA, Siedow JN (1995) Ubiquinone redox behavior in plant mitochondria during electron transport. Arch. Biochem. Biophys. 317: 156-160

Robinson SA, Ribas-Carbo M, Yakir D, Giles L, Reuveni Y, Berry JA (1995) Beyond SHAM and cyanide: Opportunities for studying the alternative oxidase in plant respiration using oxygen isotope fractionation. Aust. J. Plant Physiol. 22: 487-496

Simons BH, Millenaar FF, Mulder L, Van Loon LC, Lambers H (1999) Enhanced expression and activation of the alternative oxidase during infection of *Arabidopsis* with *Pseudomonas syringae* pv tomato. Plant Physiol. 120: 529-538

Smith L (1955) Spectrophotometric assay of cytochrome c oxidase. In: Methods of Biochem. Anal. (Glick, D.M., eds.). Interscience, New York, 2: 427-434

Skulachev VP (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. Q. Rev. Biophys. 29: 169-202

Sweeley CC, Bentley R, Makita M, Wells WW (1963) Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. 85: 2497-2507

Umbach AL, Siedow JN (1993) Covalent and Noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett. 348: 181-184

Umbach AL, Siedow JN (1997) Changes in the redox state of the alternative oxidase regulator sylfhydryl/disulfide system during mitochondrial isolation: Implications for inferences of activity *in vivo*. Plant Sci. 123: 19-28

Van den Bergen CWM, Wagner AM, Krab K, Moore AL (1994) The relationship between electron flux and the redox poise of the quinone pool in plant mitochondria; Interplay between quinol-oxidizing and quinone-reducing pathways. FEBS Lett. 226: 1071-1078

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: From gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1997) Molecular genetic evidence of the ability of alternative oxidase to support respiratory carbon metabolism. Plant Physiol. 113: 657-661

Wagner AM, Krab K (1995) The alternative respiration pathway in plants: Role and regulation. Physiol. Plant. 95: 318-325

Wagner AM, Purvis AC (1998) Production of reactive oxygen species in plant mitochondria. A dual role for ubiquinone? In: Plant mitochondria, from gene to function. (Møller IM, Gardeström P, Glimelius K and Glaser E eds). Leiden, Backhuys Publishers, pp 537-541.

Wagner AM, Wagner MJ (1995) Measurements of *in vivo* ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283

Chapter 4

Regulation of alternative oxidase activity in six wild monocotyledonous species; an *in vivo* study at the whole root level

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Abbreviations:
AOX, alternative oxidase; CCCP, carbonyl cyanide-m-chlorophenyl hydrazone; Cyt, cytochrome; FM, fresh mass; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SHAM, salicylhydroxamic acid; Q_r/Q_t, reduction state of the ubiquinone pool; Q_r, reduced ubiquinone, Q_t, total ubiquinone pool; TES, 2-{[tris(hydroxymethyl)-methyl]amino}-1-ethane-sulfonic acid.

Summary

The activity of the alternative pathway is affected by a number of factors, including the level and reduction state of the alternative oxidase (AOX) protein, and the reduction state of the ubiquinone pool. To investigate the significance of these factors for the rate of alternative respiration *in vivo*, we studied root respiration of six wild monocotyledonous grass species that were grown under identical controlled conditions. The activity of the alternative pathway was determined using the oxygen isotope-fractionation technique. In all species the AOX protein was invariably in its reduced (high-activity) state. There was no correlation between alternative oxidase activity and alternative oxidase protein concentration, ubiquinone (total, reduced or oxidized) concentration, or the reduction state of the ubiquinone pool. However, when some of these factors are combined in a linear regression model, a good fit to alternative oxidase activity is obtained. The function of the alternative oxidase is still not fully understood. Interestingly, we found a positive correlation between the activity of the alternative pathway and relative growth rate; a possible explanation for this correlation is discussed. Inhibition of the alternative oxidase (with SHAM) decreases respiration rates less than the activity present before inhibition (i.e., measured with the ^{18}O-fractionation technique).

Introduction

When herbaceous plants are grown with free access to nutrients, they exhibit inherent differences in relative growth rate (RGR) and rates of nutrient uptake (Poorter and Remkes 1990, Poorter *et al.*, 1991, Garnier 1991, Van der Werf *et al.*, 1992). For example, fast-growing species exhibit RGR values that are more than three-fold higher than those of slow-growing species (Poorter and Remkes 1990). Similarly, the rate of net NO_3^- uptake is four to six-fold higher in fast-growing species than in slow-growing ones (Poorter *et al.*, 1991). Rates of root respiration are expected to be higher, since more respiratory energy is needed for growth and ion uptake. Although the measured rates of root respiration are, indeed, (approximately 1.7-fold) higher in fast-growing species than in slow-growing ones, they are not as high as predicted from their high rates of growth and ion uptake. Calculations about the specific respiratory costs for energy-requiring processes (Poorter *et al.*, 1991) suggested that fast-growing species should exhibit three-fold higher rates of respiration than their slow-growing counterparts. Scheurwater *et al.* (1998) concluded that the major cause of the relatively low rates of root respiration in fast-growing grasses is the lower specific costs for nitrate uptake in fast-growing grasses compared with their slow-growing counterparts.

Another possible explanation for the relatively low rate of root respiration in fast-growing species might be the occurrence of lower relative activities of the nonphosphorylating alternative pathway, compared with that in slow-growing species. The cytochrome pathway and the alternative pathway constitute the respiratory electron-transport pathways of plant mitochondria. In contrast to the cytochrome pathway the alternative pathway does not contribute to the generation of a proton-motive force beyond the branch point

(ubiquinone) (Vanlerberghe and McIntosh 1997). The alternative oxidase protein is found in every examined plant species and in almost every plant organ. The alternative oxidase gene is encoded by a small gene family which exhibits highly conserved regions (Whelan *et al.*, 1996; Ito *et al.*, 1997; Vanlerberghe and McIntosh, 1997). Taken together, these findings suggest that the alternative pathway plays a vital role in plant functioning, but a clear function for the alternative pathway has yet to be established.

If the relative activity of the alternative pathway in fast-growing species were lower than that in slow-growing species, the production of respiratory energy (ATP) per unit oxygen consumption would be higher. To test if fast-growing species have a lower relative alternative pathway activity, we studied six monocotyledonous grass species, all grown under the same controlled conditions. The six wild grass species differ in their relative growth rate (RGR, mg g^{-1} day^{-1}): *Poa annua* (272), *P. alpina* (166), *P. compressa* (188), *P. pratensis* (182), *P. trivialis* (255) and *Holcus lanatus* (268) (Van Arendonk and Poorter, 1994; Atkin *et al.*, 1996). There are only small differences in the RGR reported by different authors for the same species (Scheurwater, 1999 chapter 5). There are three fast-growing and three slower-growing species. The RGR of a whole plant is closely related to the separate RGR of the roots or that of the shoot in vegetative herbaceous species (Hunt and Lloyd, 1987; Hunt and Cornelissen, 1997). *Poa annua* is an annual species while the others are all perennials. All the species occur naturally in lowland regions, except *Poa alpina*, which is a sub-alpine species (Van Arendonk and Poorter, 1994; Atkin *et al.*, 1996).

A determination of the activity of the alternative pathway in six wild grass species also raises questions about the main factors that control this activity. The total concentration of the protein is certainly important, but no correlation between activity and concentration can be expected in the six species if there were large differences in substrate concentration or AOX reduction state. Therefore, the reduction state of the AOX protein and the substrate concentration (Q_r/Q_t) were also determined. In addition, we explored the relationships between the root alternative oxidase protein concentration, alternative pathway activity, and other respiratory parameters (e.g., cytochrome *c* oxidase, ubiquinone concentration).

Materials and methods

Plant material and growth conditions

Roots of 5- to 6-weeks-old plants were used for all measurements. Seeds were germinated on moistened filter paper for one week. The seedlings were then transferred to sand for one 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 second day to 5.8. Plants grew at 20ºC, 60% RH, with a photoperiod of 14 h at 450 µmol m^{-1} s^{-1} (PAR).

Respiration of intact roots

Roots (1.5-2.0 g fresh mass (FM)) were severed and transferred to an airtight 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 2 mM (*P. alpina*), 3 mM (*P. compressa*, *P. annua*, *P. trivialis*, *H. lanatus*) or 10 mM (*P. pratensis*) SHAM (1 M stock solution in methoxyethanol). To inhibit the cytochrome pathway, KCN was used at a concentration of 0.5 mM (0.5 M stock solution in 20 mM Hepes, pH 8). To uncouple respiration, 0.5 µM CCCP and/or 1.5 µM valinomycin (both 1 mM stock in methanol) were used. The rate of respiration in the time interval between 10 to 15 minutes after addition of the inhibitors was used to calculate the percentage inhibition. Because the control respiration rate decreased somewhat during the measurement period (3-15%), the rates of respiration after addition of SHAM, CCCP or valinomycin were corrected for this decline. The KCN-resistant respiration (AOX 'capacity') was corrected for a residual component of respiration (measured in the presence of both KCN and SHAM). The true maximum capacity cannot easily be reached *in vivo*, since the capacity should then be measured with a Q_r/Q_t of 1.0, which cannot be reached under normal conditions *in vivo* because the dehydrogenases decrease in activity at high Q_r/Q_t. Therefore, the term 'capacity' is placed between quotes.

AOX protein

The total protein content of root extracts was determined according to Lowry *et al.* (1951). Mitochondria were isolated according to Umbach and Siedow (1997). For AOX protein detection, root extracts were prepared from 100 mg (FM) of frozen root material that were homogenized 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, 0.001% bromophenol blue (v/v)). After centrifugation for 10 minutes 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). Immunodetections of the AOX protein were

carried out according to the product protocol of the AOX monoclonal antibody (GTMA, Lincoln, NE, USA). Antibodies from Dr T.E. Elthon (Elthon *et al.*, 1989) were used as the primary antibody (1:100). Anti-mouse IgG Fab fragments conjugated to peroxidase (Boehringer Mannheim, Germany) were used as the secondary antibody (1:25000), using SuperSignal ULTRA Chemiluminescent Substrate according to the protocol supplied by Pierce (Rockford, IL, USA). There was no difference in the total protein concentration in the samples between the species; therefore, the same amount of protein per gram fresh mass was loaded onto the gels.

To quantify the intensity of the bands in the autoradiograms an IBAS 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 gray levels). The band intensities were corrected for the background.

Cytochrome *c* oxidase capacity

Root extracts were prepared from 300 mg (FM) of frozen root material that was homogenized 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 minutes, 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 performed at 25°C and the first-order rate constant was calculated (g^{-1} FM s^{-1}) (Smith, 1961). The extinction coefficient of Cyt *c* was measured by adding $K_3Fe(CN)_6$ (3 µL of a 0.1 mM solution) at a final concentration of 0.23 µM (whereby the volume changes only by 0.2%) which completely oxidizes the reduced Cyt *c*. Addition of 0.5 mM KCN or bubbling with CO inhibited the reaction to 6 ± 1 and 16 ± 4%, respectively (average and standard error). The activity measured, should represent the maximal activity in the extract, and is, therefore, related to the concentration of cytochrome *c* oxidase present.

Measurement of ubiquinone reduction levels in intact roots

The ubiquinone assays were done 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, suspended in 15

mL methanol and 15 mL petroleum ether (boiling point 40-60°C), and vortexed for 30 sec. The mixture was centrifuged (1,500*g*, 1 minute) 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 obtained previously. The extracted ubiquinones were resuspended with a glass rod in 75 µL of nitrogen-purged ethanol and analyzed by HPLC (HP 1050 series, Amstelveen, the Netherlands). A reversed phase Lichrosorb 5 RP 18 column (Chrompack, Bergen op Zoom, the Netherlands) with an ethanol-methanol mixture as the mobile phase (1 mL min^{-1}) was used (starting with 10 minutes 20% (v/v) ethanol, followed by a gradient to 70 % (v/v) ethanol at 40 minutes). Detection was performed at 290 and 275 nm for Q_r and Q_{ox}, respectively. Commercially obtained Q_{10} and Q_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}.

Oxygen Fractionation and Gas-Phase Respiration Measurements

Root samples (0.5 to 1.2 g fresh mass) were kept in the dark for 25 minutes 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 (Gonzàlez-Meler *et al.*, 1996). Oxygen extraction and isotope analysis were carried out as described in Robinson *et al.*, (1995) with modifications of 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 5 data points) were greater than the value of 0.995 considered minimally acceptable (Ribas-Carbo *et al.*, 1995; 1997; Lennon *et al.*, 1997; Gonzàlez-Meler *et al.*, 1999). During inhibitor treatments, either 0.5 mM KCN (in 1 mM TES, pH 8.0) and 2 mM (*P. alpina*), 3 mM (*P. compressa*, *P. annua*, *P. trivialis*, *H. lanatus*) or 10 mM (*P. pratensis*) SHAM (in water from a 1 M stock in dimethyl-d_6 sulfoxide) were applied by sandwiching the roots between medical wipes soaked with the corresponding inhibitor and incubating in the dark for at least 25 minutes (Lennon *et al.*, 1997). All stock solutions 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 made as described in 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 Tukey B *post-hoc* test was used for the statistical analysis. The correlations were calculated with the Pearson correlation test.

Results

Total respiration rates differed for the six species (between 5.1 and 7.2 nmol O_2 g^{-1} FM s^{-1}); *P. pratensis* and *P. trivialis* had a significantly faster rate of root respiration than the other four species (Fig. 1A). Root respiration was either stimulated (6%) or inhibited (up to 21%) by SHAM, and the KCN-resistant SHAM-sensitive respiration was between 25 and 40% (Table 1).

The ^{18}O-fractionation technique was used to measure the activity of the alternative pathway. Fractionation values for the alternative pathway (with KCN) and for the cytochrome pathway (with SHAM) in all five *Poa* species were similar to values reported previously for mitochondria isolated from non-green tissue (Ribas-Carbo *et al.*, 1997) (Table 2).

We were not able to measure the activity of the alternative pathway in *H. lanatus*, because the fractionation by the alternative oxidase (in the presence of KCN) did not give consistent results.

There was a positive trend (Pearson one-tailed correlation coefficient 0.78, $p=0.061$) between the relative growth rate (measured under the same conditions as used before in our laboratory; Van Arendonk and Poorter, 1994; Atkin *et al.*, 1996) and alternative pathway activity (Fig. 2). There was no correlation between the life-history trait (annual/perennial) or the natural habitat (lowland/sub-alpine) and alternative or cytochrome pathway activity.

AOX activation state and activity

In an effort to understand the reason for the observed differences in alternative pathway activity in the various species, several parameters associated with alternative oxidase and respiratory biochemistry were assayed. There were large differences (almost 10-fold) in alternative oxidase protein concentration

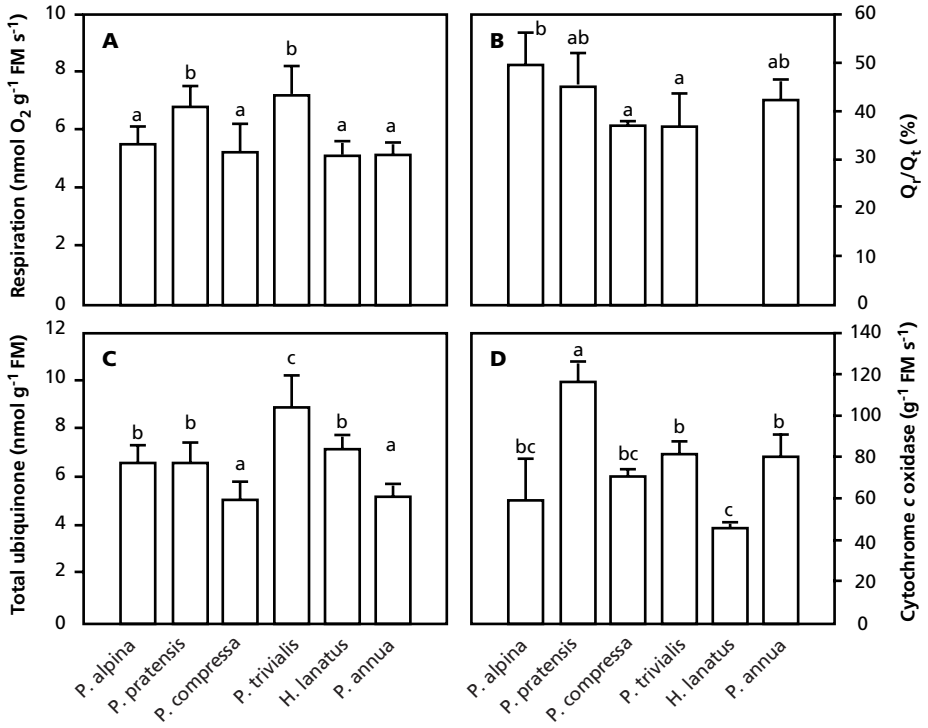


Figure 1: Respiration (A), reduction state of the ubiquinone pool (B), ubiquinone concentration (C) and cytochrome *c* oxidase capacity (D) in *P. alpina*, *P. pratensis*, *P. compressa*, *P. trivialis*, *Holcus lanatus*, and *Poa annua*. Error bars represent standard deviation. The number of replicates was at least four; columns with a different letter are significantly different (per measured parameter).

Table 1: Relative growth rate (RGR, mg g^{-1} day^{-1}, Van Arendonk and Poorter, 1994; Atkin *et al.*, 1996). Inhibition of respiration (percentage) after a treatment in six monocotyledonous species in the presence of SHAM or KCN. The SHAM-sensitive respiration is corrected with the control and the AOX 'capacity' is the KCN-insensitive, SHAM-sensitive respiration (see 'Material and methods'). Mean and standard deviation are presented. Means with the same character per treatment are not significantly different (p=0.05), n >= 4. See Figure 1A for the respiration rate.

	RGR	SHAM sensitive	AOX 'capacity'
P. alpina	166	5.3 ± 5.2 b	30.6 ± 5.0 ab
P. pratensis	182	18.2 ± 2.5 c	40.8 ± 7.2 b
P. compressa	188	-5.9 ± 7.8 a	30.3 ± 3.0 ab
P. trivialis	255	8.7 ± 0.4 bc	26.8 ± 7.4 ab
H. lanatus	268	20.6 ± 1.8 c	25.1 ± 9.7 a
P. annua	272	11.2 ± 4.6 b	38.6 ± 5.9 ab

Table 2: Fractionation (Δ in ‰), as defined by Farquhar and Richards (1984), of the alternative oxidase (Δ_{alt}; with KCN), cytochrome oxidase (Δ_{cyt}; with SHAM), and control (no additions) measurements (Δ). The values are given both as percentage alternative path (% alt path) and absolute activity (alt path in nmol O_2 g^{-1} FM s^{-1}). Mean and standard error, number in brackets is the number of replicates.

	Δ_{alt}, ‰	Δ_{cyt}, ‰	Δ, ‰	% alt path	alt path
P. alpina	25.34 ± 0.15 (3)	19.16 ± 0.28 (3)	20.49 ± 0.23 (4)	22 ± 4	0.65 ± 0.10
P. pratensis	26.33 ± 0.73 (2)	20.10 ± 0.06 (2)	20.93 ± 0.16 (4)	13 ± 3	0.63 ± 0.17
P. compressa	25.17 ± 0.38 (3)	19.60 ± 0.22 (3)	20.06 ± 0.50 (3)	11 ± 7	0.36 ± 0.24
P. trivialis	26.06 ± 0.64 (2)	18.69 ± 0.19 (2)	22.29 ± 0.48 (4)	49 ± 7	2.02 ± 0.48
H. lanatus	19.84 ± 0.27 (2)	-	-	-	-
P. annua	26.55 ± 0.10 (3)	19.51 ± 0.32 (3)	21.61 ± 0.44 (6)	30 ± 6	1.18 ± 0.25

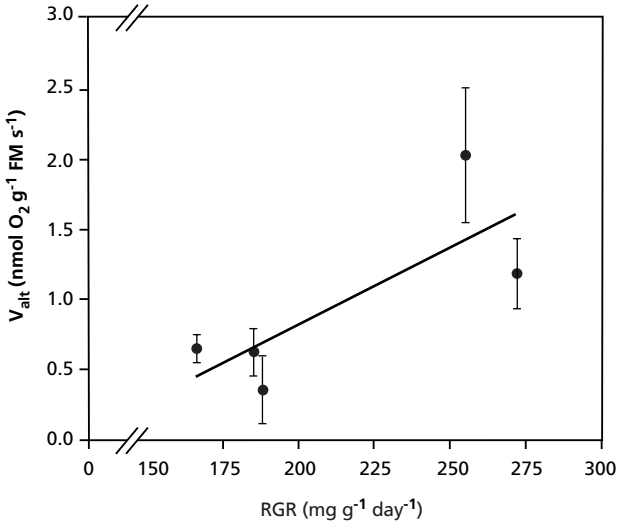
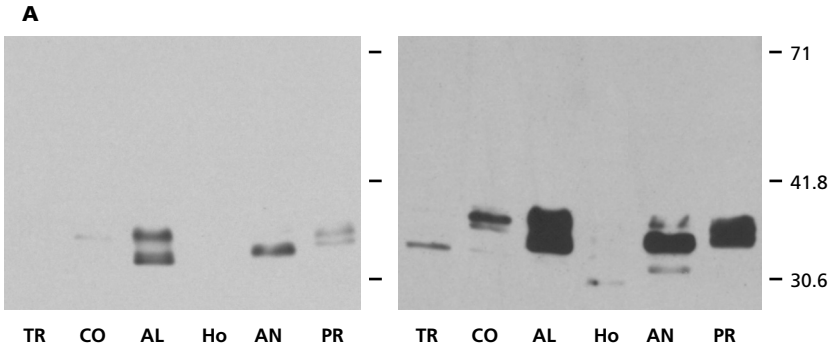


Figure 2: Activity of the alternative oxidase versus relative growth rate (RGR, mg g^{-1} day^{-1}). Relative growth rate was measured previously in our laboratory under the same conditions used to grow plants for the alternative pathway measurements (Van Arendonk and Poorter, 1994). From left to right *Poa alpina*, *P. pratensis*, *P. compressa*, *P. trivialis*, and *P. annua*. Pearson one-tailed correlation coefficient 0.78, p=0.061.

between the species (Fig. 3). Not one of the six species had an oxidized (covalently bound) form of the alternative oxidase, which should appear around 66 kD (Siedow and Umbach, 1995). When mitochondrial extracts (with oxidized alternative oxidase) were added to tissue just before the extraction, there was no change between the oxidized and reduced form of the AOX protein from the whole tissue or from the isolated mitochondria (Fig. 4).

The total ubiquinone concentration was between 5.1 and 8.8 nmol g^{-1} FM (Fig. 1C). Substrate concentration (Q_r/Q_t) for both oxidizing pathways was different (between 37 and 49%) for the species used (Fig. 1B). It was not possible to obtain reliable Q_r/Q_t measurements in *Holcus lanatus*; the measured values varied and were much more oxidized compared with the



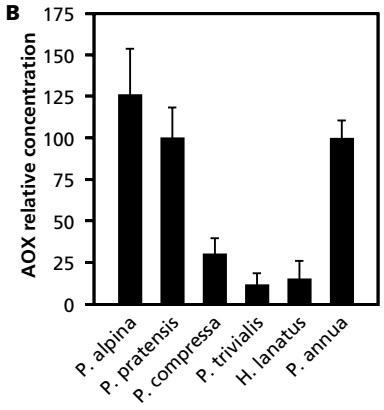


Figure 3: A) Immunoblots of alternative oxidase (detected with monoclonal antibodies) in whole root tissue extracts from *Poa trivialis* (TR), *Poa compressa* (CO), *Poa alpina* (AL), *Holcus lanatus* (HO), *Poa annua* (AN), and *Poa pratensis* (PR) roots. One example of a blot with a short (left panel) and a long (right panel) exposure. B) Relative alternative oxidase concentration (intensity of the bands) for *Poa annua* is set as 100%. Average concentration (%) and standard error; samples are from two different batches, n ≥ 5.

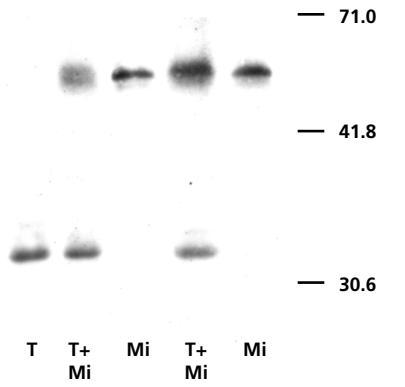


Figure 4: Immunoblots of alternative oxidase (detected with monoclonal antibodies) isolated from whole tissue (T) or isolated mitochondria (Mi, 2x) and from whole tissue where a mitochondrial extract was added (T+Mi, 2x) to the tissue just before the extraction (*Poa annua* roots).

other five species. The Q_r/Q_t increased after addition of KCN and KCN with SHAM, but SHAM alone did not increase Q_r/Q_t. In *P. annua* and *P. pratensis* there was even a decrease of the Q_r/Q_t after SHAM addition (Fig. 5). CCCP alone (18 ± 4.6%) and in combination with valinomycin (22 ± 3.6%) stimulated respiration, but valinomycin alone (7 ± 3.2%) did not significantly stimulate respiration rate (n>6, averages with standard errors). After addition

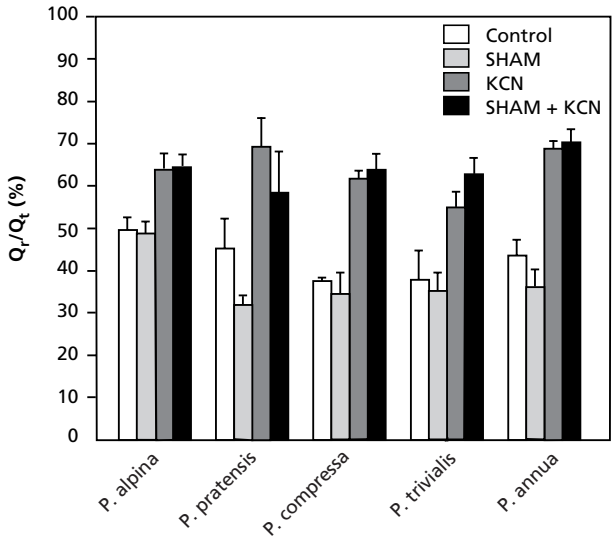


Figure 5: Relative reduction state of the ubiquinone pool (Q_r/Q_t), without inhibitors (control) or in the presence of SHAM, KCN, or SHAM + KCN in *P. alpina*, *P. pratensis*, *P. compressa*, *P. trivialis*, *Holcus lanatus*, and *Poa annua*. Error bars represent standard deviation and the number of replicates was at least three.

Table 3: Reduction state of the ubiquinone pool (Q_r/Q_t) after treatment with CCCP, valinomycin, SHAM, and KCN in roots of *Poa annua*. Average values and standard deviations are shown. Averages with a different letter are significantly different (Tukey B, $p<0.05$), n is 4 to 8.

treatment	Q_r/Q_t
Control	45.0 ± 5.0 c
SHAM	36.2 ± 3.8 ab
KCN + SHAM	67.0 ± 6.7 d
CCCP	43.5 ± 2.7 bc
CCCP + SHAM	37.8 ± 1.0 a
Valinomycin	42.6 ± 4.6 bc
Valinomycin + SHAM	37.1 ± 4.0 abc
Valinomycin + CCCP	38.8 ± 1.6 abc
Valinomycin + CCCP + SHAM	39.7 ± 4.8 bc

of uncouplers (CCCP and/or valinomycin) the Q_r/Q_t did not change compared with the control in *P. annua* roots (Table 3). Q_r/Q_t decreased or did not change after addition of SHAM and uncouplers together.

There was no relation between the activity of the alternative oxidase or KCN resistance and the concentration of alternative oxidase protein, Q_r/Q_t, or total ubiquinone concentration (Fig. 6). There was no relation between the activity of the AOX and the cytochrome *c* oxidase concentration; however, there was a correlation between KCN-resistant respiration and cytochrome *c* oxidase.

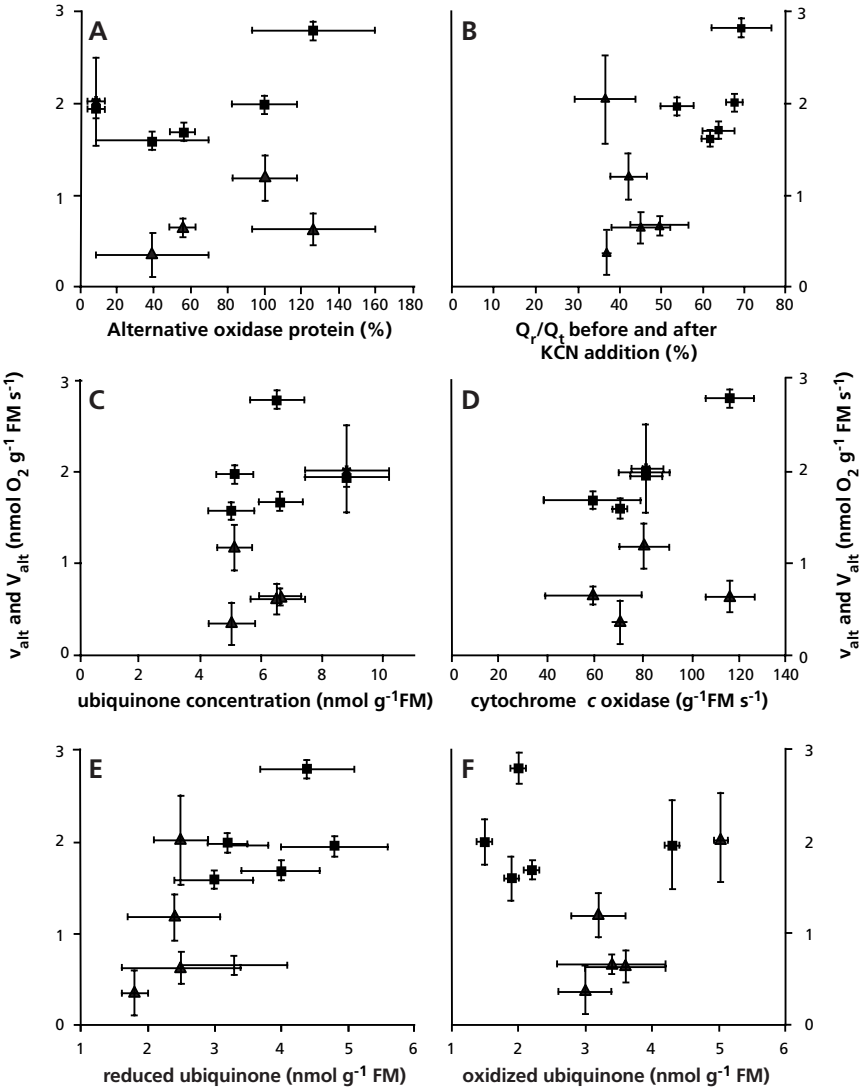


Figure 6: Relation between activity (▲ v_{alt}) and 'capacity' (■ V_{alt}, KCN-resistant, SHAM-sensitive respiration) with (A) alternative oxidase concentration, (B) reduction state of the ubiquinone pool before (activity) or after ('capacity') addition of KCN, (C) total ubiquinone concentration, (D) cytochrome *c* oxidase capacity, (E) reduced - and (F) oxidized ubiquinone concentration. Error bars represent standard deviation.

Discussion

Relative growth rate and alternative oxidase activity

Fast-growing species have a relatively low rate of root respiration when compared with slow-growing ones, considering their high rates of growth and

ion uptake. A possible explanation might be that fast-growing species have a lower relative activity of the nonphosphorylating alternative pathway (Scheurwater *et al.*, 1998).

Total respiration rate measured with the GC during the activity measurements (^{18}O fractionation) at Duke University was 22-44% lower as compared with the respiration rates 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 measurements 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; the GC measurements are 11-26 % lower as compared with the oxygen electrode. Any decrease in total respiration does not influence the partitioning between the two 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.

There was no difference in respiration rate between the species used. One reason is the smaller range in RGR among the grasses used here, compared with those by Poorter *et al.*, (1991). However, on the basis of the theoretically calculated respiration rates differences in respiration rate were expected (Poorter *et al.*, 1991).

There was no positive correlation, but actually a positive trend (Pearson one-tailed correlation coefficient 0.78, $p=0.061$), between the relative growth rate and the activity of the alternative pathway (Fig. 2). Therefore, contrary to what might be expected, fast-growing species have a relatively higher alternative pathway activity compared with slow-growing ones. Consequently, the low root respiration rate of the fast-growing species compared with that of the slow-growing ones is probably not related to more efficient ATP production.

The flux of carbohydrates to the roots of a fast-growing species is about 3-fold higher than that in a slow-growing one (Poorter *et al.*, 1990). This could result in those plants having a higher carbohydrate production also having a higher alternative pathway activity. However, 1 or 24 hours after sugar addition and after 4 days of sugar starvation, no effect on the activity of the alternative pathway activity was observed (Millenaar *et al.*, 2000, Chapters 3 and 4). Therefore, it is unlikely that plants with a higher carbohydrate level have a higher alternative pathway activity.

The question still remains whether the relatively greater contribution of respiration via the alternative pathway is a prerequisite for fast growth, or whether plants grow faster *despite* the apparent larger waste of energy (and

carbohydrates) via the alternative oxidase. In several papers, a role of the alternative oxidase in the protection against oxidative stress has been suggested (Purvis and Shewfelt, 1993; Wagner and Moore, 1997). The alternative oxidase can stabilize the reduction state of the ubiquinone pool *in vivo* (Millenaar *et al.*, 1998, Chapter 2), which may prevent damage by reactive oxygen species, since radical formation is linked to the relative reduction state of the respiratory chain (Forman and Bovaris, 1982). Recently, it was shown that plant cells which have a genetically low concentration of alternative oxidase show an increase in radical production, and cells with higher amounts of alternative oxidase protein have less oxygen free radical production (Maxwell *et al.*, 1999). Similarly, maize plants which overproduce iron superoxide dismutase from *Arabidopsis thaliana* cope better with oxidative stress, and their relative growth rate was also faster as compared with the control plants (Van Breusegem *et al.*, 1999). Combining these results with the present findings, it is tempting to suggest that a high alternative pathway activity leads to less oxygen free radical production, and hence allows faster growth.

AOX activity and KCN resistance

There were large differences in alternative oxidase activity (measured with the ^{18}O-fractionation technique) between the investigated species. In the recent past, our understanding of the mechanisms that account for activity of the alternative pathway in isolated mitochondria has increased substantially. We now know that the alternative oxidase can become more activated when the AOX protein is reduced or when α-keto acids, e.g., pyruvate, are present in sufficiently high concentration (Millar *et al.*, 1993; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.* , 1996).

During the isolation of mitochondria the reduction state of the alternative oxidase changes to a more oxidized (less-active) form (Umbach and Siedow, 1997; Millenaar *et al.*, 1998, Chapter 2). However, when mitochondrial extracts (with oxidized alternative oxidase) were added to tissue just before the extraction, there was no change between the oxidized and reduced form of the AOX protein from the whole tissue or from the isolated mitochondria (Fig. 4). Therefore, the procedure that we used for whole tissue extracts does not change the reduction state of the protein.

We have previously shown that the AOX protein invariably occurs in its reduced form during the light period in roots of *Poa annua* (Millenaar *et al.*, 1998, Chapter 2). Similarly, both in control leaves of *Arabidopsis thaliana* and in leaves infected with *Pseudomonas syringae* no oxidized form of the AOX protein was observed (Simons *et al.*, 1999). There is also no oxidized form of the AOX protein in roots of *Poa annua* after an exposure of the plants to 4 days of low light or complete darkness (Millenaar *et al.*, 1999, Chapter 3).

Similarly, none of the six species used in this study had an oxidized form (less active, covalently bound dimer) of alternative oxidase (Fig. 3).

The present species were also chosen on the basis of their different SHAM sensitivity. Several studies have revealed a correlation between the rate of respiration that is sensitive to inhibition by salicylhydroxamic acid (SHAM) in the presence of cyanide and the amount of the alternative oxidase (AOX). Hilal *et al.* (1997) found a correlation between the immunolocalisation of the AOX in soybean roots and hypocotyls and SHAM-sensitive O_2 uptake in the presence of KCN. It was also shown that the SHAM-sensitive O_2 uptake in the presence of KCN depends on AOX levels in transgenic plants (Vanlerberghe *et al.*, 1994; Hiser *et al.*, 1996). However, no data are available on the occurrence of this relationship in different species and *in vivo*. We expected that the chosen species also differed in the concentration of alternative oxidase protein. There were, indeed, large differences (almost 10-fold) in the signal observed in immunoblots (Fig. 3). The alternative oxidase antibody binds to a highly conserved region of the protein (Finnegan *et al.*, 1999); therefore, the signal obtained on immunoblots is probably a reflection of the alternative oxidase concentration.

In all of the present wild monocotyledonous species the alternative oxidase is in the active form, and also the pyruvate concentration is probably sufficiently high to fully activate AOX (Millenaar *et al.*, 1998, Chapter 2). Therefore, one might expect a correlation between the concentration of the alternative oxidase protein and its activity; such a correlation, however, was not found (Fig. 6A). In the literature there is also no clear relationship between the alternative oxidase concentration and the alternative oxidase activity (^{18}O fractionation). During a variety of stresses the concentration of the alternative oxidase increases. Infection of tobacco leaves with tobacco mosaic virus, resulted in an increased concentration of alternative oxidase; however, no change in the activity of the alternative pathway was observed (Lennon *et al.*, 1997). In mung bean grown at 19°C the concentration of the alternative oxidase increased over two-fold in both hypocotyls and leaves compared with plants grown at 28°C. The plants grown at 19°C maintained a higher activity of the alternative pathway compared with the ones grown at 28°C. This response, however, was not observed in soybean cotyledons, despite the increased concentration of alternative oxidase (Gonzàlez-Meler *et al.*, 1999). There is no clear relation between the alternative oxidase concentration and activity, despite the lack of differences in the activation state (reduction state of the AOX protein and pyruvate concentration).

The substrate concentration (Q_r/Q_t) also influences the activity of the alternative oxidase. The different species had different reduction states of the ubiquinone pool (Q_r/Q_t) as well as different total ubiquinone concentrations

Table 4: The coefficients of several models (multiple linear regression) to explain the activity and 'capacity' (KCN-resistant respiration, corrected for residual respiration) with the alternative oxidase concentration, reduction state of the ubiquinone pool before or after addition of KCN, and the total ubiquinone concentration; or with the reduced and oxidized ubiquinone pool instead of the Q_r/Q_t and Q_t. E.g., Activity model 1 = [AOX] × a1 + Q_r/Q_t × a2 + Q_t × a3. Capacity model 1 = [AOX] × a1 + Q_r × a4 + Q_{ox} × a5. The a1 to a5 coefficients correspond to the respective coefficients listed in the table. Note that some models do not contain the AOX concentration and all models go through the origin. The fit of the linear model is represented by the r^2 and the p value shows the significance of the model; the significance of the individual coefficients is also shown, + = p < 0.1, * = p < 0.05, ** = p < 0.01.

	Coefficients					Statistics	
Model	AOX (a1)	Q_r/Q_t (a2)	Q_t (a3)	Q_r (a4)	Q_{ox} (a5)	r^2	p
Activity 1	0.003	-0.039	0.372			0.91	n.s.
Activity 2		-0.027	0.328+			0.90	*
Activity 3	-0.001			-0.287	0.493	0.90	n.s.
Activity 4				-0.369	0.528	0.90	*
Capacity 1	0.001	0.023	0.079			0.97	*
Capacity 2		0.024	0.072			0.97	**
Capacity 3	-0.009			1.325*	-1.061+	1.00	**
Capacity 4				0.807*	-0.488	0.99	**

(Q_t). It should be noted that it is not valid to compare Q_r/Q_t values if the total ubiquinone pool is different; however, there was no relation between the total ubiquinone concentration or Q_r/Q_t and the activity of the alternative oxidase (Fig. 6B,C). Moreover, we found no relation between the concentration of reduced or oxidized ubiquinone and the activity of the alternative oxidase (Fig. 6E,F). Ribas-Carbo *et al.* (1997) came to the conclusion that the AOX concentration is limiting the AOX activity in etiolated soybean cotyledons and the ubiquinone concentration is limiting the AOX activity in soybean roots.

The different species have different activities of the alternative oxidase, and the two changing factors are concentration of alternative oxidase and the substrate concentration (Q_r/Q_t and Q_t or Q_r and Q_{ox}); however, none of these factors alone can explain the activity in these species. Apparently, no one of the known factors has a crucial role in determining the activity, since there is no correlation between any of the known factors and the activity (Fig. 6). Assuming that these factors are important, and hence can counteract each other, e.g., when large amounts of substrate are present, but concentration of AOX is low, the activity will still not be very high. Therefore it is reasonable to suggest that a combination of two or more factors are determining the AOX activity.

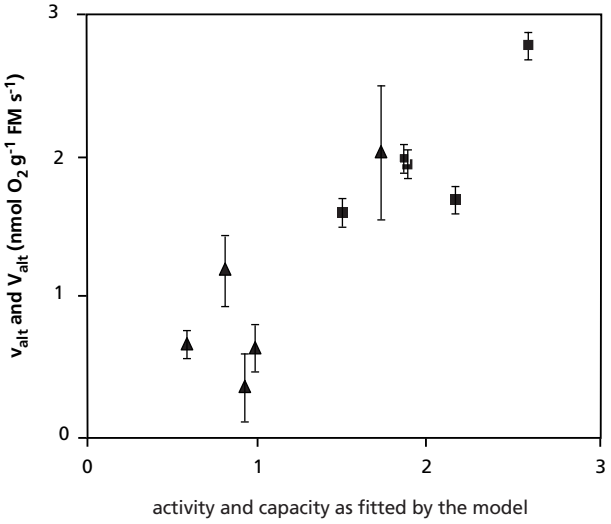


Figure 7: A model (multiple linear regression) to explain the activity (▲ v_{alt}) and 'capacity' (■ V_{alt}, KCN-resistant, SHAM-sensitive respiration) with the alternative oxidase concentration, reduction state of the ubiquinone pool before (activity) or after ('capacity') addition of KCN and the total ubiquinone concentration. On the x-axis the predicted values and on the y-axes the measured values are shown. Activity or 'capacity' in the model = Qr × a1 + Q_{ox} × a2, (a1 and a2 correspond to the respective coefficients). For activity a1=-0.369 and a2=0.528 and for 'capacity' a1=0.807 and a2=-0.488.

An attempt to model the AOX activity and capacity

We analyzed if more factors can be combined in a multiple linear regression model to explain the AOX activity, e.g., activity or capacity = [AOX] × a1 + Q_r/Q_t × a2 + Q_{tot} × a3 or [AOX] × a1 + Q_r × a4 + Q_{ox} × a5. The coefficients (a1..a5) are estimated via the least-square method (SPSS). The outcome of the model predicts activities. However, since it is an additive experimental model, it is only possible to use values between the minimum and maximum values that are used to calculate the coefficients. For instance, calculating the activity with an AOX concentration of 0% will obviously not result in a proper activity.

If alternative oxidase concentration and Q_r/Q_t and Q_t are combined in a multiple linear regression, the activity can be explained with a r^2 of 0.91 (Table 4). If the alternative oxidase concentration, Q_r and Q_{ox} are used as parameters, then the activity is explained with a r^2 of 0.90. The alternative oxidase concentration has little influence, because if the AOX concentration is taken out in the activity model, then the regression becomes significant (Table 4, Fig. 7).

Adding cytochrome *c* oxidase or cytochrome pathway activity hardly improves the model, and replacing it with one of the three factors decreases

the accuracy of the model. Similarly, there is no relation between KCN resistance (corrected for residual respiration) and the alternative oxidase concentration, Q_r/Q_t and Q_t (Fig. 6). If all these factors are combined, then, again, the KCN-resistant respiration can be fitted with a r^2 of 0.97 (Table 4, Fig. 7). If alternative oxidase concentration, Q_r and Q_{ox} are used as parameters, then the KCN-resistant respiration is explained with a r^2 of 1.00.

When combining alternative oxidase concentration, Q_r/Q_t and Q_t (or Q_r and Q_{ox}) it is possible to explain both the activity and the KCN-resistant respiration ('capacity'). The individual coefficients are not discussed, since most of the coefficients are not significant, although the regressions are, which is due to the low number of replicates.

Future experiments where one or more factors (concentration of the alternative oxidase, cytochrome *c* oxidase, and ubiquinone, and Q_r/Q_t) can be modified should resolve if our results reflect causal relations or not. However, we conclude that there does not seem to be one single most important factor that explains the alternative oxidase activity, but a combination of factors. It will be interesting to examine more plants whereby the several factors can be changed more independently from each other.

SHAM inhibition and the effect of uncouplers

Addition of SHAM alone to the roots of the grass species had different effects on their root respiration, from a slight stimulation (5%) to inhibition (20%) of respiration. The activity of the alternative oxidase was 10 to 50% of the respiration (measured with ^{18}O fractionation). With the exception of *P. pratensis* (possibly caused by peroxidases), the SHAM inhibition of respiration (absolute or in percentage) is less than the activity as estimated with the ^{18}O fractionation. Therefore, the cytochrome pathway has become more active after SHAM addition compared with the situation before SHAM addition. The cytochrome pathway can become more active if Q_r/Q_t increases (Van den Bergen *et al.*, 1994; Wagner and Krab, 1995) or if there is a shift from closer to state 4 to closer to state 3. Addition of SHAM did not cause any increase in Q_r/Q_t; there was even a decrease in some species. Q_r/Q_t increased or did not further change when KCN was applied first, before SHAM was added. Therefore, the cytochrome pathway became more active without an increase in Q_r/Q_t after SHAM addition. This suggests a shift from nearer to state 4 toward state 3 after SHAM addition. The flux through the cytochrome pathway is apparently not only affected by the reduction state of the ubiquinone pool.

In order to establish whether state 4-state 3 transitions are an issue here, we measured the reaction upon addition of SHAM in the presence of uncoupler. The proton gradient across the inner mitochondrial membrane can be removed by adding an uncoupler, and hence the control of ADP/ATP ratios

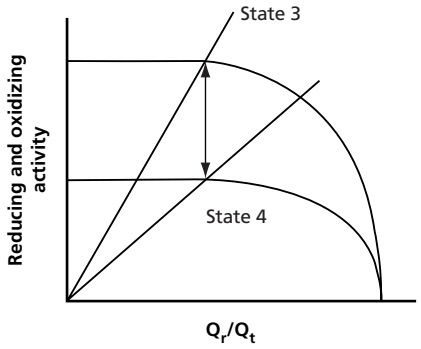


Figure 8: The relationship between the cytochrome pathway (straight lines) and the dehydrogenases (curved lines). A higher proton gradient (state 4) lowers the activity of both the cytochrome pathway and the dehydrogenases, and vica versa (state 3). If both the cytochrome pathway and the dehydrogenases are changing equally in activity (according to the arrow), the respiration can change with no change in the reduction state of the ubiquinone pool (Q_r/Q_t).

will be impaired. The cytochrome pathway cannot change from state 4 to state 3 if there is no proton gradient. Addition of CCCP and/or valinomycin did not change Q_r/Q_t compared with the control (Table 3). Addition of SHAM after CCCP and/or valinomycin resulted in no change or a decrease in Q_r/Q_t. If we assume that the activity of the alternative oxidase is not decreased after addition of uncouplers, then the activity of the cytochrome pathway appears to increase without a change from closer to state 4 towards state 3 and without an increase in Q_r/Q_t. Also Wagner and Wagner (1995) found no change in Q_r/Q_t after BHAM addition, independent of the presence of an uncoupler in a *Petunia* cell suspension. At present the only possible explanation is that, when the rate of respiration changes with no change in Q_r/Q_t, the dehydrogenase activity changes as much as the oxidase activity (Fig. 8).

Acknowledgements

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Reference

Atkin OK, Botman B, Lambers H (1996) The causes of inherently slow growth in alpine plants: an analysis based on the underlying carbon economies of alpine and lowland species. Functional Ecology 10: 698-707

Crane FL (1963) The coenzyme Q group (ubiquinones). Progress in the chemistry of fats and other lipids 7(II): 267-289

Elthon TE, Nickels RL, McIntosh L (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89: 1311-1317

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust. J. Plant Physiol. 11: 539-552

Finnegan PM, Wooding AR, Day DA (1999) An alternative oxidase monoclonal antibody recognises a highly conserved sequence among alternative oxidases subunits. FEBS Lett. 447: 21-24

Forman HJ, Boveris A (1982) Superoxide radical and hydrogen peroxide in mitochondria. In Free Radical in Biology, Vol V. Academic Press, New York, pp 65-90
Garnier E (1991) Resource capture, biomass allocation and growth in herbaceous plants. Tree 6: 126-131
Gonzàlez-Meler MA, Ribas-Carbo M, Giles L, Siedow JN (1999) The effect of growth and measurement temperature on the activity of the alternative respiratory pathway. Plant Physiol. 120: 765-772
Gonzàlez-Meler MA, Ribas-Carbo M, Siedow JN, Drake BG (1996) Direct inhibition of plant mitochondrial respiration by elevated CO2. Plant Physiol. 112: 1349-1355
Guy RD, Berry JA, Fogel ML, Hoering TC (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. Planta 177: 483-491
Hilal M, Castagnaro A, Moreno H, Massa EM (1997) Specific localization of the respiratory alternative oxidase in meristematic and xylematic tissues from developing soybean roots and hypocotyls. Plant Physiol. 115: 1499-1503
Hiser C, Kapranov P, McIntosh L (1996) Genetic modification of respiratory capacity in potato. Plant Physiol. 110: 277-286
Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400
Hunt R, Lloyd PS (1987) Growth and partitioning. New Phytol. 106: 235-249
Hunt R, Cornelissen JHC (1997) Components of relative growth rate and their interrelations in 59 temperate plant species. New Phytol. 135: 395-417
Ito Y, Saisho D, Nakazone M, Tsutsumi N, Hirai A (1997) Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature. Gene 203: 121-129
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685
Lambers H, Van der Werf A, Bergkotte M (1993) Respiration: the alternative pathway. In: Methods in comparative plant ecology (eds: Hendry, G.F. and Grime, J.P.) pp 140-144. Chapman & Hall, London
Lennon AM, Neuenschwander UH, Ribas-Carbo M, Giles L, Ryals JA, Siedow JN (1997) The effects of salicylic acid and tobacco mosaic virus infection on the alternative oxidase of tobacco. Plant Physiol. 115: 783-791
Lowry OH, Rosenbrough NJ, Farr AL, Randall R (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275
Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc. Natl. Acad. Sci. USA 96: 8271-8276
Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618
Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329: 259-262
Millenaar FF, Benschop JJ, Wagner AM, Lambers H (1998) The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool; and the activation state of the alternative oxidase. Plant Physiol. 118: 599-607
Millenaar FF, Roelofs R, Gonzàlez-Meler MA, Siedow JN, Wagner AM, Lambers H (2000). The alternative oxidase in roots of *Poa annua* after transfer from high-light to low-light conditions. Plant J., accepted
Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia 83: 553-559
Poorter H, Remkes C, Lambers H (1990) Carbon and nitrogen economy of 24 wild species differing in relative growth rate. Plant Physiol. 94: 621-627
Poorter H, van der Werf A, Atkin OK, Lambers H (1991) Respiratory energy requirement of roots vary with the potential growth rate of a plant species. Physiol. Plant. 83: 469-475

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol. Plant. 88: 712-718

Ribas-Carbo M, Berry JA, Yakir D, Giles L, Robinson SA, Lennon AM, Siedow JN (1995) Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. Plant Physiol. 109: 829-837

Ribas-Carbo M, Lennon AM, Robinson SA, Giles L, Berry JA, Siedow JN (1997) The regulation of electron partitioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. Plant Physiol. 113: 903-911

Robinson SA, Ribas-Carbo M, Yakir D, Giles L, Reuveni Y, Berry JA (1995) Beyond SHAM and cyanide: Opportunities for studying the alternative oxidase in plant respiration using oxygen isotope discrimination. Aust. J. Plant Physiol. 22: 487-496

Scheurwater I, Koren M, Lambers H, Atkin OK (1999) The contribution of roots and shoots to whole plant nitrate reduction in fast- and slow-growing grass species. In: Variation in specific respiratory costs in the roots of fast- and slow-growing grass species. Scheurwater I. Thesis, pp 77-96

Scheurwater I, Cornelissen C, Dictus F, Welschen R, Lambers H (1998) Why do fast- and slow-growing grass species differ so little in their rate of root respiration, considering the large differences in rate of growth and ion uptake? Plant, Cell Environ. 21: 995-1005

Simons BH, Millenaar FF, Mulder L, Van Loon LC, Lambers H (1999) Enhanced expression and activation of the alternative oxidase during infection of *Arabidopsis* with *Pseudomonas syringae* pv tomato. Plant Physiol. 120: 529-538

Smith L (1961) Spectrophotometric assay of cytochrome *c* oxidase. Methods of Biochemical Analysis 2: 427-434

Umbach AL, Siedow JN (1993) Covalent and Noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Siedow JN (1997) Changes in the redox state of the alternative oxidase regulatory sulfhydryl/disulfide system during mitochondrial isolation: implication for inferences of activity *in vivo*. Plant Sci. 123: 19-28

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett. 348: 181-184

Van Arendonk JJCM, Poorter H (1994) The chemical composition and anatomical structure of leaves of grass species differing in relative growth rate. Plant, Cell Environ. 17: 963-970

Van Breusegem F, Sloote L, Stassart J-M, Moens T, Van Botterman J, Van Montagu M, Inzé D (1999) Overproduction of *Arabidopsis thaliana* FeSOD confers oxidative stress tolerance to transgenic maize. Plant Cell Physiol. 40: 515-523

Van den Bergen CWM, Wagner AM, Krab K, Moore AL (1994) The relationship between electron flux and the redox poise of the quinone pool in plant mitochondria; Interplay between quinol-oxidizing and quinone-reducing pathways. FEBS Lett. 226: 1071-1078

Van der Werf W, Welschen R, Lambers H (1992) Respiratory losses increase with decreasing inherent growth rate of a species and with decreasing nitrate supply: a search for explanations for these observations. In Molecular, Biochemical and Physiological aspects of plant respiration eds: Lambers, H. van der Plas, L.H.W. Academic Publishing, The Hague, pp 421-432

Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1994) Molecular genetic alteration of plant respiration. Silencing and overexpression of alternative oxidase in transgenic tobacco. Plant Physiol. 106: 1503-1510

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: From gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Wagner AM, Krab K (1995) The alternative respiration pathway in plants: Role and regulation. Physiol. Plant. 95: 318-325

Wagner AM, Moore AL (1997) Structure and function of the plant alternative oxidase: Its putative role in the oxygen defence mechanism. Bioscience reports 17: 319-333
Wagner AM, Wagner MJ (1995) Measurements of *in vivo* ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283
Whelan J, Millar AH, Day DA (1996) The alternative oxidase is encoded in a multigene family in soybean. Planta 198: 197-201

Chapter 5

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

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Abbreviations:
AOX, alternative oxidase; Cyt, cytochrome; FM, fresh mass; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SHAM, salicylhydroxamic acid; SMP, submitochondrial particles.

Summary

Detached roots of *Poa annua* were used to study alternative oxidase protein expression upon addition of sucrose, glucose, fructose, inositol, mannitol, citrate, and malate, at a concentration of 1 or 10 mM for 24 hr. 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 ^{18}O-fractionation technique) was not affected upon addition of sucrose for 24 hr as compared with time zero. After addition of citrate or mannitol the activity of the alternative pathway decreased to almost zero. The discrepancy between the large increase in alternative oxidase concentration when citrate was applied and the concomitant decrease in its activity is discussed.

Introduction

The cytochrome pathway and the alternative pathway constitute the respiratory electron-transport pathways of 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. The AOX protein is found in every examined plant species 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 our understanding of the mechanisms that account for activation of the alternative pathway in isolated mitochondria has increased substantially. We now know that the alternative pathway becomes more activated when the AOX protein is reduced or when specific α-keto acids, e.g., pyruvate, are present in sufficiently high concentration (Millar *et al.*, 1993; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996).

We have previously shown that the AOX protein invariably occurs in its reduced form during the day in roots of *Poa annua* (Millenaar et al., 1998, Chapter 2). 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 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 days of low light or complete darkness (Millenaar *et al.*, 2000; Chapter 3). During the low-light experiment the sugar concentration and total respiration decreased; however, the activity, concentration and reduction (activation) state of the alternative oxidase did not change. Addition of sucrose for 45-60 minutes only 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; Chapter 3). The previous experiments concern short-term treatment with sugars. Equally interesting is the question whether long-term sugar addition affects the activity, concentration and reduction state of the alternative oxidase.

We investigated alternative oxidase expression upon addition of sucrose, glucose, fructose, inositol, citrate, and malate, at a concentration of 1 or 10 mM for 24 hr. Citrate addition increases the alternative oxidase concentration in tobacco cell suspension (Vanlerberghe and McIntosh, 1996) and the question is whether it also induces the alternative oxidase in other cells of other species, e.g., roots of *Poa annua*. We also 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, we also measured cytochrome *c* oxidase.

Materials and methods

Plant material and growth conditions

Poa annua L. roots of 5- to 6-weeks old plants were used for all measurements. Seeds were germinated on moistened filter paper for one week and the seedlings were transferred to sand for one 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% RH, with a photoperiod of 14 h at 450 μmol m^{-1} 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 hr. Mannitol was used as a control, to compensate for possible differences in osmolarity, since mannitol is not metabolised by most plants. The compounds, at a concentration of 1 or 10 mM, were added to a nutrient solution with a pH of 5.8. About 1 gram 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 hr to avoid oxygen depletion in the solution. After 24 hr the respiration, AOX concentration, and cytochrome *c* oxidase concentration were measured.

Respiration of intact roots

Roots of *Poa annua* (1.0 g fresh mass (FM)) were transferred to an airtight 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 (0.5 M stock solution in 20 mM Hepes, pH 8). The rate of respiration at 10 to 15 minutes after addition of the inhibitors was used to calculate the percentage inhibition. Short-term effects of glucose and citrate were studied at a concentration of 1 mM from a 1 M stock with a pH of 7.

AOX protein

The total protein content of the extracts was determined according to 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, 0.001% bromophenol blue (v/v)). After centrifugation for 10 minutes 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). Immunodetections of the AOX protein were carried out according to the product protocol of the AOX monoclonal antibody (GTMA, Lincoln, NE, USA). Antibodies were obtained from Dr T.E. 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 product usage protocol supplied by Pierce (Rockford, IL, USA). To quantify the bands in the autoradiograms, an IBAS image-analysis system (Kontron/Zeiss, Eching, Germany) was used. Scanning was performed with a Panasonic b/w CCD camera (WC-CD50), digitized 4 times and averaged to improve the signal to noise ratio (frame size 640 × 512 pixels; 256 gray levels). The 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

minutes, 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. The excess of dithionite was removed by a gentle flow of normal air in the solution for a few minutes. The assay was measured at 25ºC and the first-order rate constant was calculated (g^{-1} FM s^{-1}) (Smith, 1961). The final extinction was measured by adding $K_3Fe(CN)_6$ (3 µL of a 0.1 mM solution) in a final concentration of 0.38 µM (whereby the volume changes only by 0.2%), which completely oxidizes the reduced cyt *c*. Addition of 0.5 mM KCN or bubbling with CO inhibited the reaction to 6 ± 1 and 16 ± 4%, respectively (average and standard error). The activity measured, should represent the maximal activity in the extract, and is, therefore, related to the concentration of cytochrome *c* oxidase.

Oxygen Fractionation and Gas-Phase Respiration Measurements

Root samples (0.5 to 1.2 g fresh mass) were kept in the dark for 25 minutes 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 (Gonzàlez-Meler *et al*, 1996). Oxygen extraction and isotope analysis were carried out as described in Robinson *et al*. (1995) with modifications of 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 5 data points) were greater than the value of 0.995 considered minimally acceptable (Ribas-Carbo *et al.*, 1995; 1997; Lennon *et al.*, 1997; Gonzàlez-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 sulfoxide) were applied by sandwiching the roots between medical wipes soaked with the corresponding inhibitor and incubating in the dark for at least 25 minutes (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 made as described in 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).

Mitochondria and SMP preparation

Mitochondria and inside-out submitochondrial particles (SMP) from cold-stored cauliflower (a commercial cultivar from local store was used) were isolated according to 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, we exposed detached roots of *Poa annua* to sucrose, glucose, fructose, inositol, citrate, malate, mannitol or only the nutrient solution (NS) for 24 hr. Mannitol was used as a control, to correct for possible differences in osmolarity. The experiment was repeated twice and for each concentration 3 to 4 replicates were used. 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 visualisation procedure.

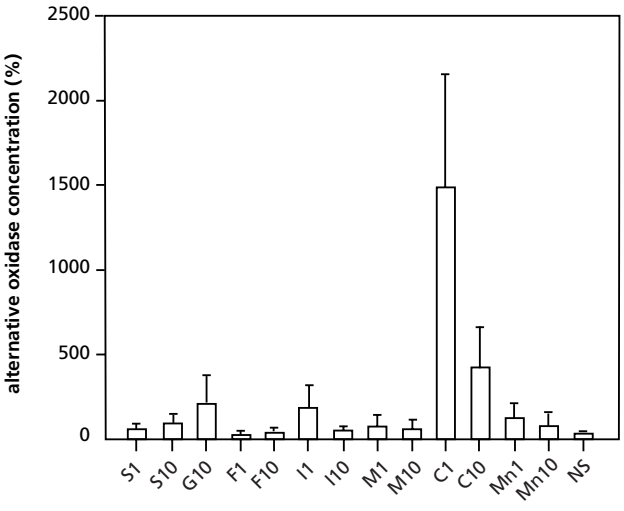

Figure 1: Concentration of the alternative oxidase in *Poa annua* after 24 hr of incubation with sucrose (S), fructose (F), malate (M), inositol (I), citrate (C), glucose (G), or mannitol (Mn), or only the nutrient solution (NS) as controls. Two concentrations were applied, 1 and 10 mM. Western blots were detected with monoclonal antibodies and the intensity of the bands was measured. The average AOX concentration with mannitol was set as 100%; error bars represent standard error, number of replicates was at least 3. Only the AOX concentration after 1 mM citrate was significantly different from the other values.

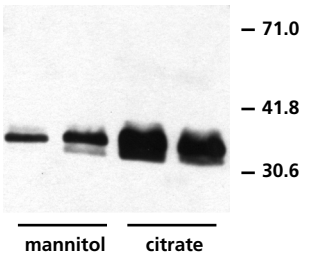

Figure 2: Immunoblots of alternative oxidase (detected with monoclonal antibodies) directly isolated from roots of *Poa annua* 24 hr after addition of 1 mM mannitol (lane 1 & 2) or citrate (lane 3 & 4).

Neither sucrose, nor glucose, fructose, inositol, and malate at 1 and 10 mM for 24 hr increased the alternative oxidase concentration (Fig. 1). At 1 mM, citrate strongly increased the alternative oxidase concentration, up to 15 times (Fig. 2). Also at 10 mM citrate the alternative oxidase was induced (5x increased); however, this was not significant, compared with the other treatments. The alternative oxidase was invariably in its active (reduced) form after all treatments (Fig. 2 and data not shown).

Cytochrome *c* oxidase capacity was measured only at the treatments with 1 mM concentrations and decreased about four-fold in 24 hr, independent of the treatment, and there were no differences between the treatments (Fig. 3).

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

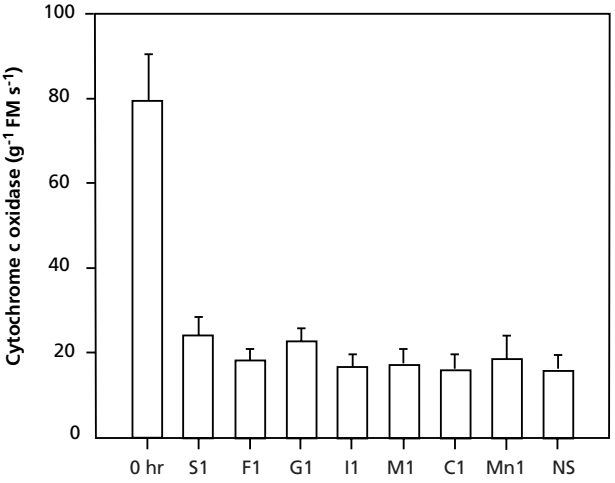

Figure 3: Cytochrome *c* oxidase capacity (g^{-1} FM s^{-1}) in roots of *Poa annua* at time 0 and after 24 hour in a 1 mM sucrose (S), fructose (F), malate (M), inositol (I), citrate (C), glucose (G), or mannitol (Mn), or only the nutrient solution (NS) as controls. Bars represent standard error; number of replicates was at least 4. Only the value at zero hr was significantly different from the other values.

Table 1: Activity of the alternative and cytochrome pathway, absolute (v_{alt}, V_{alt} in nmol O_2 g^{-1} FM s^{-1}) and relative (% alt path, % cyt path in % of total respiration) and the fractionation (Δ in ‰) as defined by Farquhar and Richards (1984) after exposure for 24 hr 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.

	v_{alt}	V_{alt}	Δ, ‰	% alt path	% cyt path
Control (0 hr)	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

The total respiration (assessed with an oxygen electrode) was 4.6 ± 0.8 and 4.2 ± 0.8 nmol O_2 g^{-1} FM s^{-1} at time zero and after 24 hr citrate, respectively (average and standard deviation, $n \geq 7$). After 24 hr of exposure to mannitol the respiration was significantly decreased to 2.9 ± 0.1 nmol O_2 g^{-1} FM s^{-1} (Fig. 4). The KCN-insensitive respiration decreased after 24 hr exposure to citrate or mannitol as compared with the control at zero hr (Fig. 4). The percentage respiration insensitive to KCN especially decreased (from 80 to 50%) after 24 hr exposure to citrate.

None of the treatments had any effect on the residual respiration (Fig. 4). Short-term glucose addition (15 minutes) did not have an effect on the rate

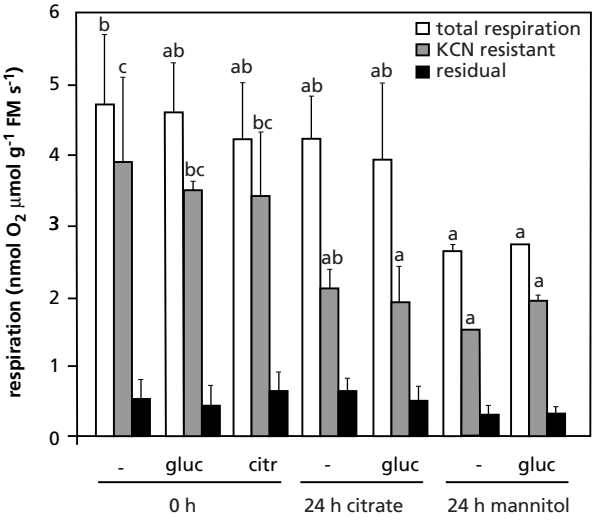

Figure 4: Total respiration (1st bars), CN-resistant respiration (2nd bars) and residual respiration (3rd bars) in roots of *Poa annua* at zero hour (in the absence or presence of citrate or in the absence or presence of glucose for 15 minutes), after 24 hr of exposure to 1 M citrate and mannitol (in the absence or presence of glucose for 15 minutes). Lines on top of the bars represent standard deviation, number of replicates was 3 to 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).

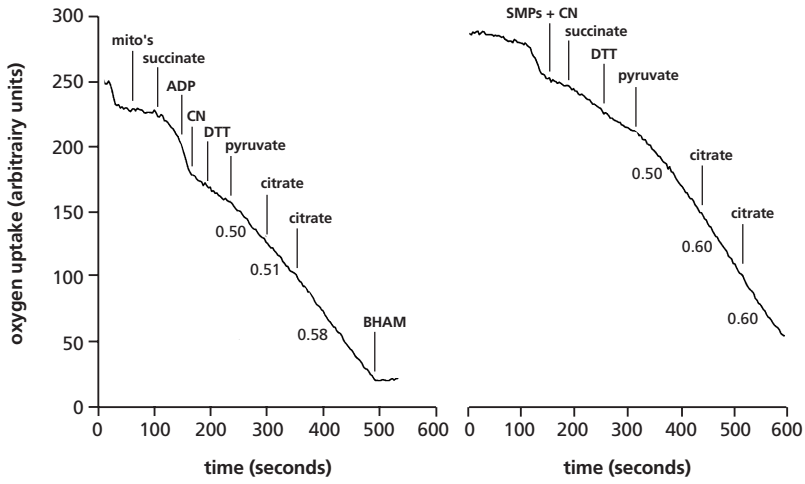

Figure 5: Oxygen uptake traces of mitochondria and inside-out submitochondrial particles (SMPs) from cauliflower. The following concentrations are used: 50 µL mitochondria, 100 µL SMPs, 10 mM succinate, 0.1 mM ADP, 10 mM CN^-, 10 mM DTT, 10 mM pyruvate, 10 mM citrate. The numbers at the left of the traces are respiration rates in nmol O_2 $cuvette^{-1}$ s^{-1}.

of respiration, neither at time zero nor after 24 hr 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 of cauliflower were used. To have a reasonable concentration of AOX, the cauliflowers were pre-treated with one week of cold storage (4°C). Short-term exposure of mitochondria and inside-out submitochondrial particles of cauliflower to citrate increased the respiration rate slightly in the presence of KCN (Fig. 5).

Discussion

Total respiration

The total respiration in detached roots of *Poa annua* decreased by 40% during a 24 hr treatment with mannitol. Mannitol cannot be used as a respiratory substrate in most plants, and during the 24 hr of mannitol treatment respiratory substrates were probably exhausted. Short-term (15 minutes) exposure to glucose, however, did not increase the rate of respiration after 24 hr 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, e.g., citrate was added for 24 hr compared with time zero. Therefore, the concentration of cytochrome *c* oxidase is not the major controlling step for the respiratory rates of the present *Poa annua* roots.

In conclusion, the decrease in respiration after 24 hr 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 results of Bingham and Farrar (1988), who concluded that the respiration of roots from control, and leaf- or root-pruned plants was not limited by carbohydrates, since short-term sucrose feeding did not stimulate respiration, but via the turnover of ATP.

AOX concentration and activation state

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

Vanlerberghe and McIntosh (1996) also found an increase in alternative oxidase expression after citrate addition in a cell suspension of tobacco.

Previously we have shown that the AOX protein occurs invariably in the reduced form during the day in roots of *Poa annua* (Millenaar *et al.*, 1998, Chapter 2). 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 17, but was partially oxidized at day 4 (Millar *et al.*, 1998). Also 24 hr after the addition of the various sugars or organic acids used in the experiments described here, no oxidised (less active, around 66 kD) form of alternative oxidase is present on the western blot. This is especially striking since the activity of the alternative pathway decreased to almost zero after mannitol or citrate addition, while treatment with the latter increased the alternative oxidase concentration about 15-fold.

An additional regulatory mechanism may be required to explain these observations, since it is difficult to explain our observations on AOX activity

by the known factors only. A new model, incorporating changes in the oxygen reaction center, is discussed below.

AOX activity and KCN sensitivity

The citrate treatment caused a 15-fold increase in alternative oxidase concentration (Fig. 1). All the protein was in the reduced (active) form. The pyruvate concentration is probably sufficiently high to fully activate AOX (Millar *et al.*, 1998; Millenaar *et al.*, 1998, Chapter 2). Yet, not only does the activity of the alternative respiration decrease after citrate addition, but also the roots become less resistant to KCN, which implies less AOX or more inactive AOX. This is in contrast with Vanlerberghe and McIntosh (1996) who found an increase in cyanide resistance and AOX concentration with an increase in citrate concentration. They used a different species in a cell culture and applied citrate for a shorter time period (8 hr), which might account for some of the differences.

We do not have data on the reduction state of the ubiquinone pool or the ubiquinone concentration. The low activity in the citrate treatment can (most likely) not be explained by differences in the amount of substrate, because of large differences in alternative oxidase concentration. Also the differences in reduction state of the ubiquinone pool and ubiquinone concentration are in general relatively small (Wagner and Wager, 1995; Millar *et al.*, 1998; Chapter 3 and 4). However the Q_r/Q_t can play an important role in changing the AOX activity (Millenaar *et al.*, 1998, Chapter 2; Chapter 4).

How can this large pool of alternative oxidase, induced with the citrate treatment have such a low activity, which is in fact about equal to the activity after a mannitol treatment, during which no extra AOX is induced? And why does the activity remain high after a sucrose treatment?

The detached roots in mannitol were probably substrate-starved (carbohydrates) because there is no carbon source for 24 hr and the rate of respiration decreased, probably because of down-regulation of some enzymes in the respiratory chain, or ATP turnover (see total respiration paragraph). In the presence of citrate, however, detached roots do not appear to become substrate-starved, since the respiration did not decrease, yet, the concentration of the alternative oxidase increased. Most of the induced alternative oxidase after citrate addition seems to be inactive. In the following section, we propose a hypothesis to explain these results.

Inactive AOX after citrate addition

After citrate addition the concentration of the alternative oxidase increases; however, the activity (almost zero) and the KCN-resistant respiration decreases. The following observations may help to explain our results.

(1) The alternative oxidase most likely contains a Fe in its oxygen reaction centre (Siedow *et al.*, 1995; Anderson and Nordlund, 1999).
(2) Organic acids can bind metal cations depending on the pH and the type of organic acid. At a pH below 8 citrate is capable of binding Fe, below a pH of 6, 100% of the Fe is bound (Jones, 1998).
(3) The pH inside the mitochondria is between 7 and 8; therefore, it is very likely that, depending on the concentration of Fe and citrate, a substantial amount of Fe is bound to citrate, and Fe is possibly even withdrawn directly or indirectly from enzyme complexes, like AOX.

The result of a binding of Fe with citrate (directly or indirectly) is an alternative oxidase with no Fe in its oxygen reaction centre and, the enzyme will, therefore, be inactive. Does the plant make more alternative oxidase as a response to the (partly) inactive alternative oxidase after citrate addition? Interestingly, malate binds Fe only below a pH of 4 (Jones, 1998). Therefore, malate does not bind Fe inside the mitochondria, and there is no inactivation of alternative oxidase and no reaction to make more alternative oxidase to compensate, which agrees with our data (Fig. 1). Interestingly, exposure to either antimycin, alone or in combination with o-phenanthroline (Fe^{2+} chelator) increased the AOX expression, but the cyanide resistance 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 mitochondria and SMPs are from a different plant species, and, more importantly, the exposure of citrate to the alternative oxidase was only a few minutes. Exposure may have to be much longer for citrate to bind 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.

Acknowledgements

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References

Andersson ME, Nordlund P (1999) A revised model of the active site of alternative oxidase. FEBS Lett. 449: 17-22

Bingham IJ, Farrar JF (1988) Regulation of respiration in roots of barley. Physiol. Plant. 73: 278-285

Elthon TE, Nickels RL, McIntosh L (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89: 1311-1317

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust. J. Plant Physiol. 11: 539-552

Gonzàlez-Meler MA, Ribas-Carbo M, Giles L, Siedow JN (1999) The effect of growth and measurement temperature on the activity of the alternative respiratory pathway. Plant Physiol. 120: 765-772

Gonzàlez-Meler MA, Ribas-Carbo M, Siedow JN, Drake BG (1996) Direct inhibition of plant mitochondrial respiration by elevated CO2. Plant Physiol. 112: 1349-1355

Guy RD, Berry JA, Fogel ML, Hoering TC (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. Planta. 177: 483-491

Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400

Jones DL (1998) Organic acids in the rhizosphere - a critical review. Plant and Soil. 205: 25-44

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685

Lambers H, van der Werf A, Bergkotte M (1993) Respiration: the alternative pathway. In: Methods in comparative plant ecology (eds: Hendry, G.F. and Grime, J.P.) pp 140-144. Chapman & Hall, London

Lennon AM, Neuenschwander UH, Ribas-Carbo M, Giles L, Ryals JA, Siedow JN (1997) The effects of salicylic acid and tobacco mosaic virus infection on the alternative oxidase of tobacco. Plant Physiol. 115: 783-791

Lowry OH, Rosenbrough NJ, Farr AL, Randall R (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275

Millar AH, Atkin OK, Menz RI, Henry B, Farquhar G, Day DA (1998) Analysis of respiratory chain regulation in roots of soybean seedlings. Plant Physiol. 117: 1083-1093

Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618

Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329: 259-262

Millenaar FF, Benschop JJ, Wagner AM, Lambers H (1998) The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool; and the activation state of the alternative oxidase. Plant Physiol. 118: 599-607

Millenaar FF, Roelofs R, Gonzàlez-Meler MA, Siedow JN, Wagner AM, Lambers H (2000). The alternative oxidase in roots of *Poa annua* after transfer from high-light to low-light conditions. Plant J., accepted.

Minagawa N, Sakajo S, Komiyama T, Yoshimoto A (1990) Essential role of ferrous iron in cyanide-resistant respiration in *Hansenula anomala*. FEBS Lett. 267: 114-116

Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia. 83: 553-559

Ribas-Carbo M, Berry JA, Yakir D, Giles L, Robinson SA, Lennon AM, Siedow JN (1995) Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. Plant Physiol. 109: 829-837

Ribas-Carbo M, Lennon AM, Robinson SA, Giles L, Berry JA, Siedow JN (1997) The regulation of electron partitioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. Plant Physiol. 113: 903-911

Robinson SA, Ribas-Carbo M, Yakir D, Giles L, Reuveni Y, Berry JA (1995) Beyond SHAM and cyanide: Opportunities for studying the alternative oxidase in plant respiration using oxygen isotope discrimination. Aust. J. Plant Physiol. 22: 487-496

Siedow JN, Umbach AL, Moore AL (1995) The active site for the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett. 362: 10-14

Simons BH, Millenaar FF, Mulder L, Van Loon LC, Lambers H (1999) Enhanced expression and activation of the alternative oxidase during infection of *Arabidopsis* with *Pseudomonas syringae* pv tomato. Plant Physiol. 120: 529-538

Smith L (1961) Spectrophotometric assay of cytochrome c oxidase. Methods of Biochemical Analysis. 2: 427-434

Umbach AL, Siedow JN (1993) Covalent and Noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett. 348: 181-184

Van den Bergen C (1994) Preparation and characteristics of inside-out submitochondrial particles (SMPs) from potato tuber mitochondria. In: Regulation of respiration in plant mitochondria. Van den Bergen C, Thesis, pp 63-67

Vanlerberghe GC, McIntosh L (1996) Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. Plant Physiol. 111: 589-595

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: From gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Wagner AM, Wagner MJ (1995) Measurements of *in vivo* ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283

Chapter 6

Transgenic alternative oxidase tobacco plants

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Abbreviations:
AOX, alternative oxidase; Cyt, cytochrome; FM, fresh mass; MES, 4-morpholineethane-sulfonic acid; SHAM, salicylhydroxamic acid; Q_r/Q_t, reduction state of the ubiquinone pool; X-Gluc,5-bromo-4-chloro-3-indolyl-B-D-glucuronide.

Summary

Tobacco plants were transformed with a sense and anti-sense alternative oxidase construct. We selected two overexpressor lines, two underexpressor lines and two that do not differ (much) in the alternative oxidase concentration. None of the lines showed an oxidized (less active) form of the alternative oxidase on the Western blots. Plants that were altered in the concentration of the alternative oxidase did not show a different phenotype in the primary tranformants, F1 and F2 (self fertilized) compared with the wild-type.

Introduction

The cytochrome pathway and the alternative pathway constitute the respiratory electron-transport pathways of 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. The alternative oxidase (AOX) protein is found in every examined plant species and in almost every plant organs, 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.

One of the possible functions of the alternative pathway is to prevent oxygen free radical formation (Purvis and Shewfelt, 1993; Wagner and Wagner, 1995). Experiments to test this by culturing plants in the presence of inhibitors of AOX (e.g., SHAM) are troublesome, because a characteristic of these inhibitors, although capable of penetrating the plant tissue, is that they also act as radical scavengers. Therefore, it is necessary to have plants with an altered expression of this protein, since inhibitors cannot be used to examine the role of the alternative oxidase. Although AOX anti-sense and sense tobacco plants have been described in the literature since 1994 (Vanlerberghe and McIntosh, 1994), little progress has been made to assess the physiological significance of AOX. In phosphate-deficient tobacco cell cultures, oxygen free radical production, growth and morphology are changed in antisense AOX cells (Parson, *et al.*, 1999). These tobacco cells with a lowered concentration of AOX produce more oxygen free radicals than control cells (Maxwell *et al.*, 1999). Cultured cells, however, are not necessarily comparable with the situation in intact plants. Therefore, we decided to make our own sense and anti-sense plants.

A prerequisite for studying the effect of over- or under-expression of AOX is that this indeed affects the activity of the protein *in vivo*. There is no correlation between the alternative oxidase concentration and the activity (measured with ^{18}O fractionation) and the KCN-insensitive respiration in 5 monocotyledonous grass species (*Poa annua*, *P. alpina*, *P. compressa*, *P.*

pratensis, *P. trivialis*) (Chapter 4). Although the species used are closely related, they differ more than just in AOX concentration. In sense and anti-sense AOX plants from one and the same species, the relationship between the alternative oxidase concentration and the activity and the KCN-insensitive respiration can be studied more precisely. In this chapter, we describe how the sense and anti-sense tobacco plants have been made and present some preliminary results.

Materials and methods

Plasmid constructs and plant transformation

Standard techniques were used to construct recombinant DNA plasmids in *Escherichia coli* strain XL1Blue (Sambrook *et al.*, 1989). An AOX1 cDNA clone from tobacco was kindly provided by Dr Jim Whelan (The University of Western Australia, Perth, Australia). The cDNA clone (*Eco*R1 fragment) is 1161 bp long and contains the coding region. This cDNA was ligated in the EcoR1 site of the expression vector pMON999 (Monsanto, London, UK) in both sense and anti-sense orientation to the P-e35S promoter (enhancer of 35S CaMV) and a NOS (nopaline synthase) 3' tail. The presence of AOX1 and its orientation in *E. coli* was tested with Bgl II and Pst I digestion. The promoter, AOX1 coding region and tail (Sma I, Hind III) was ligated in the multiple cloning site (pUC18 MCS) of the binary expression vector pCAMBIA2301 (Hajdukiewicz *et al.*, 1994). pCAMBIA2301 contains a gusA reporter gene and a kanamycin selection gene both with a CaMV 35S promoter (Fig. 1). The number of insertions in pCAMBIA2301 was tested with Sma I and Pst I digestion. Transformation of *Agrobacterium* strain LBA4404 was done by a direct DNA uptake method (An *et al.*, 1988). Tobacco (*Nicotiana tabacum* cv SR1 Petit Havanna) was transformed by the leaf disc method and kanamycin-resistant plants were regenerated as described by Horsch *et al.* (1988).

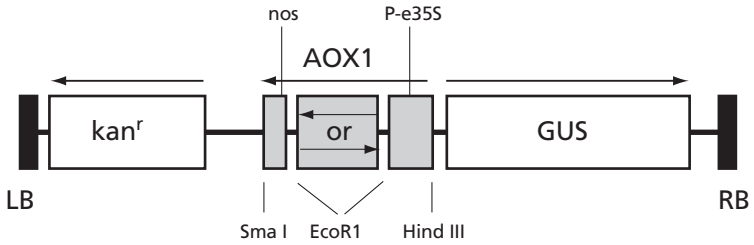

Figure 1: Sense and anti-sense constructs used to generate transgenic tobacco. The AOX-coding region with 35S promoter and tail are ligated with Sma I, Hind III in the binary expression vector pCAMBIA2301. The orientation was tested with Bgl II and Pst I. LB, left border; RB, right border.

GUS assay

Leaf or root section were fixed in 0.3% formaldehyde in 10 mM MES, pH 5.6, 0.3 M mannitol for 45 min at room temperature, followed by several washes in 50 mM NaH_2PO_4, pH 7.0. Histochemical reactions with the indigogenic substrate, X-Gluc were performed with 1 mM substrate in 50 mM NaH_2PO_4, pH 7.0 at 37°C for 2 hours. After staining, sections were rinsed in 90% ethanol at 30°C until the chlorophyll was extracted.

Plant material and growth conditions

Seeds were germinated in petri dishes on a modified Murashige and Skoog (MSO) medium with kanamycin (Horsch *et al.*, 1988). The seedlings were transferred after they had 2 to 4 leaves and were placed in 30 L containers (8 plants per container) and grown on an aerated nutrient solution (Poorter and Remkes, 1990). The nutrient solution was replaced every week and the pH was adjusted every other day to 5.8. Plants grew at 20°C, 60% RH, with a photoperiod of 14 hr at 450 µmol m^{-2} s^{-1} (PAR).

AOX protein

The total protein content of root extracts was determined according to Lowry *et al.* (1951). For AOX protein detection, root extracts were prepared from 100 mg (FM) of frozen root material that were homogenized 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, 0.001% bromophenol blue (v/v)). After centrifugation for 10 minutes 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). Immunodetections of the AOX protein were carried out according to the product protocol of the AOX monoclonal antibody (GTMA, Lincoln, NE, USA). Antibodies from Dr T.E. Elthon (Elthon *et al.*, 1989) were used as a primary antibody (1:100). Antimouse IgG Fab fragments conjugated to peroxidase (Boehringer Mannheim, Germany) were used as a secondary antibody (1:25000), using SuperSignal ULTRA Chemiluminescent Substrate according to the product usage protocol supplied by Pierce (Rockford, IL, USA). There was no difference in the total protein concentration in the samples between the species; therefore, on gel the same amount of total protein is loaded either expressed per gram fresh mass or per total protein concentration.

To quantify the intensity of the bands in the autoradiograms an IBAS image analysis system (Kontron/Zeiss, Eching, Germany) was used. Scanning was performed with a Panasonic b/w CCD camera (WC-CD50), digitized 4

times and averaged to improve the signal to noise ratio (frame size 640 × 512 pixels; 256 gray levels). The band intensities were corrected for the background.

Results

Tobacco plants were transformed with a sense and anti-sense AOX construct. About 40 sense and anti-sense kanamycin-resistant lines were regenerated; eventually in total 51 lines survived after the transfer to the greenhouse. All the primary transformants were screened for GUS activity and alternative oxidase concentration in the leaves. Remarkably, there was no relation between GUS activity and alterations in AOX concentration in the primary transformants. Thirteen plants with an altered concentration of AOX were selected. After sowing the F1 lines on a kanamycin medium, it became clear that three lines were not transformed and one line did not germinate. The alternative oxidase concentration in the roots was determined for the nine remaining lines (Table 1). Six lines were selected to make homozygous lines. There were two overexpressor lines, two underexpressor lines and two that did not differ (much) in the alternative oxidase concentration. We did not find an oxidized (less active) form of the alternative oxidase on the Western blots in any of the lines, in either roots or leaves (data not shown). Plants from lines that were altered in the concentration of alternative oxidase did not show a phenotype in the primary tranformants, when either F1 or F2 were compared with the wild-type. Seedlings from the 6 selected F2 lines did not have GUS activity, except line S44.

Table 1: Relative concentration of alternative oxidase in nine F1 lines that are altered in the expression of alternative oxidase. Six lines were selected for the F2. Averages and standard errors, n = 1-3.

Line	AOX Concentration	Over/under-expressor	Selected For F2
Wild-type	100		
S7	638 ± 386	++	F2
S10	23		
S12	306 ± 316	+	F2
S17	250 ± 140		
S25	16	-	F2
S44	211 ± 297	=/+	F2
S46	166 ± 190		
AS44	119 ± 93	=	F2
AS51	41 ± 17	-	F2

Discussion

AOX concentration and activation state

Tobacco was transformed with AOX1 cDNA in the sense and anti-sense orientation. Two overexpressor and underexpressor lines were selected, and two with no alteration in AOX concentration and a wild-type as a control. Two lines in which the alternative oxidase was inserted in the sense direction were not overexpressors, but underexpressors (S10 and S25). Apparently there is co-suppression in those two lines. Remarkably, there was no relation in the F2 seedlings between the GUS activity and a change in gene expression; only in S44 was there a detectable GUS expression, while this line did not differ much in AOX concentration. It is unknown why there was no relation between GUS activity and AOX concentration.

In the recent past our understanding of the mechanisms that account for activation of the alternative pathway in isolated mitochondria has increased substantially. We now know that the alternative pathway becomes more activated when the AOX protein is reduced or when specific α-keto acids, e.g., pyruvate, are present in sufficiently high concentration (Millar *et al.*, 1993; Umbach and Siedow, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996). Previously, we have shown that the AOX protein invariably occurs in the reduced form in roots of *Poa annua* (Millenaar *et al.*, 1998, Chapter 2). 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 the present tobacco leaves and roots there was no oxidized (less active) form of the alternative oxidase, which should be present around 66 kD on the Western blot. Up to now there are hardly any data available that show an oxidized form of the alternative oxidase *in vivo*. Vanlerberghe *et al.* (1999) showed a faint oxidized band in cells treated with antimycin A and H_2O_2. They compared the oxidized band with the highest reduced band, claiming a major shift in reduction state after the treatment; however, they ignored the much more abundant reduced bands with a slightly lower molecular weight. There was an oxidized band in roots of 4 days old soybean seedlings, which was not visible at day 7 and 17 (Millar *et al.*, 1998).

Phenotype

The plants with a higher or lower alternative oxidase concentration did not show an obvious phenotype as compared with the wild-type. Why are plants with less alternative oxidase not different?

No data are available on the AOX activity in antisense AOX plants. The

question is how much the activity has changed, since a change in the concentration of AOX does not necessarily lead to a change in activity of the alternative pathway (Chapter 4, 5).

Purvis and Shewfelt (1993) and Wagner and Wagner (1995) speculated that the alternative pathway helps to stabilize the reduction state of the ubiquinone pool (Q_r/Q_t), which would be important because oxygen free radical production is linked to the relative reduction state of the respiratory chain (Forman and Boveris, 1982). Indeed, the alternative pathway is capable of stabilizing Q_r/Q_t *in vivo* in roots of *Poa annua* (Millenaar *et al.*, 1998, Chapter 2). In accordance, tobacco cells with a lowered concentration of alternative oxidase produce more oxygen free radicals than control cells (Maxwell *et al.*, 1999), which is an indication of a lower activity of the alternative pathway. It is, therefore, expected that plants with less alternative oxidase also have to cope with a higher production of oxygen free radicals. Plants may compensate for a decrease in alternative oxidase with, e.g., more superoxide dismutase (SOD). Maxwell *et al.* (1999) showed that tobacco cells that underexpress AOX did not increase the concentration of Mn and Fe SOD RNA. The concentration of RNA does not necessary reflect the concentration of the protein. Apparently the plants can cope with a slight increase in oxygen free radical production without an effect on the phenotype.

The relation between free oxygen radical and H_2O_2 production with the membrane potential is nonlinear. Small changes at relatively high membrane potentials have a large effect, compared with changes at a lower membrane potential (Liu and Huang, 1996). As long as the membrane potential is in the lower range, the need for an alternative oxidase may not be high. If, during certain conditions (decreased cytochrome pathway activity, decreased ADP concentration) the membrane potential increases, however, then the alternative pathway can be necessary to prevent a large increase in membrane potential, thereby preventing a large increase the oxygen free radical production. There might only be a phenotype under conditions when AOX is normally very active, like in cells with a low-P treatment (Parsons *et al.*, 1999). In those tobacco cell cultures where phosphate is deficient, oxygen free radical production, growth and morphology are changed in antisense AOX cells.

After transfer of *Poa annua* plants from high light (450 µmol m^{-2} s^{-1}) and long days (14 hr light) to low light (80 µmol m^{-2} s^{-1}) and short days (8 hr light), the alternative pathway activity on a percentage basis increased from 30 to 55% (Millenaar *et al.*, 2000, Chapter 3). These are conditions where the need for an alternative oxidase can be tested. Our tobacco plants do not show an obvious phenotype under normal conditions; therefore, our transformants can cope with a different concentration of alternative oxidase. However, we plan to continue this research by testing the plants under different conditions.

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References

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. In: Plant molecular biology manual. Eds. Gelvin SB, Schilperoort RA. Kluwer Academic Publishers, Dordrecht, pp A3, 1-19

Elthon TE, Nickels RL, McIntosh L (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89: 1311-1317

Forman HJ, Boveris A (1982) Superoxide radical and hydrogen peroxide in mitochondria. Free radicals in biology. Vol V, Academic Press, Inc., New York, pp. 65-90

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile Ppzp family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. 25: 989-994

Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1988) Leaf disc transformation. In: Plant molecular biology manual. Eds. Gelvin SB, Schilperoort RA. Kluwer Academic Publishers, Dordrecht, pp A5, 1-9

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Liu S-S, Huang J-P (1996) Coexistence of a "reactive oxygen cycle" with the "Q-cycle" in the respiratory chain; a hypothesis for generating, partitioning, and functioning of superoxide in mitochondria. In: Proc. of Intern. Symp. on natural antioxidants: Molecular mechanisms and health effects. Eds. D. Moores. AOCS Press, Champaign (IL) pp. 513-529

Lowry OH, Rosenbrough NJ, Farr AL, Randall R (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275

Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc. Natl. Acad. Sci. USA 96: 8271-8276

Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618

Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329: 259-262

Millenaar FF, Benschop JJ, Wagner AM, Lambers H (1998) The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool; and the activation state of the alternative oxidase. Plant Physiol. 118: 599-607

Millenaar FF, Roelofs R, Gonzàlez-Meler MA, Siedow JN, Wagner AM, Lambers H (2000). The alternative oxidase in roots of *Poa annua* after transfer from high-light to low-light conditions. Plant J., accepted.

Parson HL, Yip JYH, Vanlerberghe GC (1999) Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. Plant Physiol. 121: 1309-1320

Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia 83: 553-559

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol. Plant. 88: 712-718

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Simons BH, Millenaar FF, Mulder L, Van Loon LC, Lambers H (1999) Enhanced expression and activation of the alternative oxidase during infection of *Arabidopsis* with *Pseudomonas syringae* pv tomato. Plant Physiol. 120: 529-538

Umbach AL, Siedow JN (1993) Covalent and Noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS lett. 348: 181-184

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: From gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1994) Molecular genetic alteration of plant respiration. Silencing and overexpression of alternative oxidase in transgenic tobacco. Plant Physiol. 106: 1503-1510

Vanlerberghe GC, Yip JYH, Parsons HL (1999) In organello and *in vivo* evidence of the importance of the regulatory sulfydryl/disulfide system and pyruvate for alternative oxidase activity in tobacco. Plant Physiol. 121: 793-803

Wagner AM, Wagner MJ (1995) Measurements of *in vivo* ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283

Chapter 7

Summary of results and general discussion

The terminal part of the respiratory path, starting with the degradation of carbohydrates via glycolysis, consists of the mitochondrial electron-transport pathway, in which, among others components, two terminal oxidases participate, cytochrome *c* oxidase and the alternative oxidase (Fig. 1, Introduction). The alternative oxidase branches from the main electron-transport pathway at the ubiquinone pool and, beyond the branch-point, does not contribute to ATP production. The energy conservation is less than maximal if a part of the respiration proceeds via this nonphosphorylating (alternative) pathway. Because of its energy-wasting nature, it is most interesting (scientifically as well as economically) to investigate under which conditions and to what extent the alternative respiratory path is used, and how its activity is regulated.

Regulation of AOX activity

In the last few years a number of factors have been discovered, which influence the AOX activity (Chapter 1). All these AOX-regulating factors have been studied in isolated mitochondria, and until recently hardly anything was known about the importance of these factors *in vivo*.

The alternative oxidase activity can be influenced by a number of factors:

1 The alternative oxidase can occur as a noncovalently linked dimer (reduced) or as a covalently linked dimer (oxidized). In the reduced configuration the alternative oxidase is more active compared with the oxidized dimer (Figs 4 and 5, Introduction) (Umbach and Siedow, 1993; Umbach *et al.*, 1994).
2 Some α-keto acids like pyruvate, influence the activity of the alternative oxidase (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996); the alternative oxidase becomes active at lower substrate concentrations if α-keto acids are present (Fig. 5, Introduction).
3 More reduced ubiquinone (substrate) will result in a higher activity (Fig. 5, Introduction).
4 A higher concentration of AOX protein can result potentially in a higher AOX activity.
5 The activity of the cytochrome pathway will influence the AOX activity since the two pathways compete for electrons from the reduced ubiquinone pool (Millar *et al.*, 1995; Ribas-Carbo *et al.*, 1995; Wagner and Krab, 1995; Day *et al.*, 1996).

1 Reduction state of the alternative oxidase

The alternative oxidase can occur as a noncovalently linked dimer (reduced) or as a covalently linked dimer (oxidized), when the sulfur bridges that link the subunits of the dimer are oxidized. In the reduced configuration the alternative oxidase is more active compared with the oxidized dimer (Figs 4 and 5, Introduction) (Umbach and Siedow, 1993; Umbach *et al.*, 1994). The oxidized form has a molecular mass of around 66 kD, and the reduced form around 30 to 36 kD; therefore, the two forms can readily be separated on a protein gel. The isolation procedure of mitochondria may cause a change in the reduction state of the AOX protein (Umbach and Siedow, 1997; Millenaar *et al.*, 1998, Chapter 2). In whole tissue extracts the alternative oxidase is mainly in the reduced form (see below). Is the protein mainly in the reduced state because of the extraction from whole tissue? 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 (Chapter 4). Therefore, the procedure that we used for whole tissue extracts does not change the reduction state of the protein.

There was no inactive form of AOX in *Sauromatum guttatum* floral appendix tissue and in the roots and cotyledons of *Glycine max* (Umbach and Siedow, 1997). In roots of *Poa annua*, the alternative oxidase is invariably in the reduced form during the light period (Millenaar *et al.*, 1998, Chapter 2). In roots of soybean seedlings the AOX protein was largely in the reduced form at day 7 and 17, but was partially oxidized at day 4 (Millar *et al.*, 1998). 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 after infection with *Pseudomonas syringae*. Vanlerberghe *et al.* (1999) showed a faint oxidized band in cells treated with antimycin A and H_2O_2. They compared the oxidized form with the reduced form, but only the reduced form with the largest molecular weight, claiming a major shift in reduction state after the treatment; however, they ignored the much more abundant reduced bands with a slightly lower molecular weight.

During the low-light experiments there is no shift to a more oxidized (less active) form of the protein (Fig. 4, Chapter 3). Plants that are exposed for 4 or 7 days to complete darkness (Fig. 5, Chapter 3) do not show an oxidized band with whole tissue extracts. In six closely related wild grass species that differ in their relative growth rate [(RGR, mg g^{-1} day^{-1}): *Poa annua* (272), *P. alpina* (166), *P. compressa* (188), *P. pratensis* (182), *P. trivialis* (255), and *Holcus lanatus* (268)] there was no oxidized band with whole tissue extracts (Fig. 3, Chapter 4). Detached roots of *Poa annua* were exposed to sucrose, glucose, fructose, inositol, citrate, malate, mannitol, or only the nutrient solution for 24 hours. Again, the alternative oxidase was invariably in its active (reduced) form after all treatments (Fig. 2 and data not shown, Chapter 5). In either roots or leaves of tobacco, there was no oxidized (less active) form of the alternative oxidase on the Western blots in any of the transgenic lines or wild-type plants (Chapter 6).

In roots of the *Poa* species measured the alternative oxidase was always in the active form, independent of the species, the carbohydrate status, respiration rate, AOX concentration, and activity. Also in leaves of *Arabidopsis* and tobacco (and in leaves of *Poa annua*, preliminary results) there was no inactive form of AOX. In almost all organisms the cysteine residue region that is responsible for the reduction state of the AOX protein is very conserved (Chapter 1); however, up to now in two organisms (rice and tomato) the conserved cysteine residue is missing (Djajanegara *et al.*, 1999). Therefore, these organisms are not capable of forming an oxidized AOX form. Amoeba

cells are not capable of forming an oxidized AOX form and also pyruvate does not have a stimulatory effect (Jarmuszkiewicz *et al.*, 1997).

In conclusion, up to know there is very little evidence that the inactive form of the AOX protein does exists *in vivo* (except Millar *et al.*, 1998; Vanlerberghe *et al.*, 1999).

2 Pyruvate

The curve that describes the kinetics of the alternative oxidase (oxygen uptake against Q_r/Q_t) shifts to the left upon addition of pyruvate or other α-keto acids in isolated mitochondria (Fig. 4, Introduction). It has been demonstrated that α-keto acids increase the activity of the alternative oxidase (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Millar *et al.*, 1996). Pyruvate reacts with the part of the AOX protein (cysteine residue) on the matrix side of the inner mitochondrial membrane to stimulate its activity (Rhoads *et al.*, 1998; Vanlerberghe *et al.*, 1998; Djajanegara *et al.*, 1999). The pyruvate concentration for half-maximum stimulation found with inside-out mitochondrial particles from *Ipomoea batatas* and *Glycine max* is less then 4 μM (Millar *et al.* 1996). Finnegan *et al.* (1997) found a half-maximum pyruvate stimulation of 4.5 μM in cotyledons and 51 μM in roots of *Glycine max*. The concentration of pyruvate in several tissues of several species does not vary much, and is mostly between 40 to 100 nmol g^{-1} (FM) (Table 1, and Chapter 2).

Given the concentration for half-maximum pyruvate stimulation, the pyruvate concentration and the mitochondrial volume, it is possible to estimate the concentration of pyruvate inside the mitochondria. This concentration is most likely to be 12 to 150 times higher than the concentration for half-maximum pyruvate stimulation (Millenaar *et al.*, 1998, Chapter 2). The end-product of glycolysis in plants is not only pyruvate, but, for a substantial part, malate (Day and Hanson, 1977). Malate by itself cannot be the substrate for the citric acid cycle, but needs to be converted into pyruvate via malic enzyme. Therefore, pyruvate is also produced inside the mitochondria. The malic enzyme activity in isolated mitochondria did not change in 4 to 17 days old soybean roots; however, the *in vivo* AOX activity changed from virtually zero to rather high levels. Moreover, pyruvate was invariably necessary to get a reasonable AOX activity in isolated mitochondria at physiological Q_r/Q_t values. Therefore, Millar *et al.* (1998) concluded that pyruvate is not involved in the regulation of the AOX activity in soybean roots, although they do not know the *in vivo* activity of malic enzyme.

In conclusion, there is no evidence, so far, that pyruvate stimulation of AOX has a regulatory function *in vivo*.

Table 1: Pyruvate concentration (nmol g^{-1} FM) in several species, organs and treatments.

species	organ and treatment	conc.	reference
Arabidopsis thaliana	leaves	110	Simons *et al.*, 1999
Arabidopsis thaliana	leaves, infected	440	Simons *et al.*, 1999
Daucus carota	roots	39	Kato-Noguchi, 1996
Glycine max	roots	60	Miller *et al.*, 1998
Hordeum vulgare	roots	60	Good and Muench, 1993
Hordeum vulgare	roots, hypoxia	120	Good and Muench, 1993
Nicotiana tabacum	cell suspension	100[1]	Vanlerberghe *et al.*, 1997
Nicotiana tabacum	cell suspension + antimycin	550[1]	Vanlerberghe *et al.*, 1997
Petunia hybrida	cell suspension	100	Wagner and Wagner, 1995
Phaseolus vulgaris	roots	32	Day and Lambers, 1983 and H. Lambers, pers. comm.
Poa annua	roots	60	Millenaar *et al.*, 1998
Spinacea oleracea	roots	60	Day and Lambers, 1983
Triticum aestivum	roots	67	Day and Lambers, 1983

[1] = assuming % DM = 10%.

3 Reduction state of the ubiquinone pool

The substrate concentration of the alternative oxidase is ubiquinol; more substrate will result in a higher activity (Fig. 4, Introduction). The slope of the kinetic curve of the cytochrome and alternative pathway will determine the extent to which a particular pathway is stimulated by an increase in the reduction state of the ubiquinone pool (Q_r/Q_t). During the titration of the cytochrome pathway with KCN, the alternative pathway will become more active. It was concluded that small changes in Q_r/Q_t are the cause of this increase in AOX activity (Millenaar *et al.*, 1998, Chapter 2), since the other regulatory mechanisms did not change. Also, from literature it is known that the kinetic curve of AOX is in general steep, if pyruvate is present and the AOX protein is in its reduced (active) form (Millenaar *et al.*, 1998, Chapter 2). The alternative pathway activity differs in different *Poa* species; in Chapter 4 it was concluded that the substrate availability is one of the factors necessary to explain the activity.

In a batch culture of *Petunia hybrida* cells the respiration and sugar availability changed dramatically; however, Q_r/Q_t hardly changed (Wagner and

Wagner, 1995). Millar *et al.* (1998) also found constant Q_r/Q_t values in roots of 4 to 17-days-old soybean seedlings, while respiration decreased. During the low-light treatment of *Poa annua* the respiration and sugar concentrations decreased; however, the Q_r/Q_t did not change, after correcting for the non-active ubiquinone pool (Millenaar *et al.*, 2000, Chapter 3).

In the literature there are very few data about *in vivo* Q_r/Q_t values; however, the values appear to be independent of the respiration rate and carbohydrate concentration. Although small changes in Q_r/Q_t can have relatively large effects on the activity of the alternative pathway, they do not appear to be a major controlling factor, since the Q_r/Q_t is very stable.

4 Alternative oxidase concentration

The concentration of alternative oxidase potentially influences the alternative pathway activity. Transgenic tobacco plants with an altered concentration of AOX are changed in the 'capacity' of the alternative pathway (CN-insensitive respiration, Vanlerberghe *et al.*, 1994). However, no data have been published about the AOX activity in plants which are altered in the AOX concentration, despite the fact that the first publications of AOX transgenic plants date from 1994 (Vanlerberghe *et al.*). Chapter 4 describes an experiment where 5 closely related wild grass species have been compared. The species differed in the AOX concentration and in the AOX activity (^{18}O-fractionation technique); however, there was no positive correlation between the AOX concentration and activity. No relation is expected if the species differ in the reduction state of the AOX protein, pyruvate activation, or Q_r/Q_t. Since these factors did not differ between the species, except for some variation in the Q_r/Q_t, it is surprising how small the influence of the concentration of AOX protein is on its activity. Chapter 4 discusses an additive linear model with the AOX and substrate (Q_r/Q_t and Q_{tot} or Q_r and Q_{ox}) concentration, as variables to explain the AOX activity. This model could explain the AOX activity in a satisfactory manner (Table 4, Fig. 7, Chapter 4). The species used differed in more characteristics than AOX concentration alone; therefore, transgenically changed plants that differ in AOX concentration are more suitable to test the influence of the AOX concentration on the AOX activity. In Chapter 6 it is described how transgenic tobacco plants have been made with a sense and antisense construct with AOX. However, further research is needed to analyze those plants.

Another way of measuring the influence of the AOX concentration is stimulating gene expression; e.g., citrate induces AOX gene expression (Vanlerberghe and McIntosh, 1996). Detached roots of *Poa annua* were used to study alternative oxidase protein expression upon addition of sucrose, glucose, fructose, inositol, mannitol, citrate, and malate, at a concentration of

1 or 10 mM for 24 hours. Only citrate induced the expression of the alternative oxidase, especially at a concentration of 1 mM (15-fold). The AOX activity (measured with the ^{18}O-fractionation technique) decreased to almost zero, compared with the activity before the experiment or with exposure for 24 hours to sucrose (Table 1, Chapter 5). Chapter 5 speculates about possible side effects of citrate, which might cause a decrease in AOX activity.

During a variety of stresses, the concentration of the alternative oxidase increases. Infection of tobacco leaves with tobacco mosaic virus resulted in an increased concentration of alternative oxidase; however, no change in the activity of the alternative pathway was observed (Lennon et al., 1997). In mung bean grown at 19ºC the concentration of the alternative oxidase increased over two-fold in both hypocotyls and leaves compared with plants grown at 28ºC. The plants grown at 19ºC maintained a higher activity of the alternative pathway compared with the ones grown at 28ºC. This response, however, was not observed in soybean cotyledons, despite the increased concentration of alternative oxidase (Gonzàlez-Meler *et al.*, 1999).

There is no clear positive correlation between the alternative oxidase concentration and its activity *in vivo*, despite the lack of differences in the activation state (reduction state of the AOX protein and pyruvate concentration).

5 Cytochrome pathway

The alternative and cytochrome pathways compete for electrons from ubiquinol (Millar *et al.*, 1995; Ribas-Carbo *et al.*, 1995; Wagner and Krab, 1995; Day *et al.*, 1996). The activity of the cytochrome pathway, among others, depends on the ADP concentration (state3/state4) (Fig. 3, Introduction). Depending on the energy (ATP) production and the energy demand, the activity of the cytochrome pathway changes. The relative importance of the alternative pathway in the total respiration can potentially easily be changed by changes in the cytochrome pathway activity. However, no changes in the absolute alternative pathway activity are expected if there are no changes in the AOX reduction state, AOX concentration, pyruvate action, or substrate concentration.

During the development of soybean roots the absolute AOX activity was relatively stable from day 7 to 17; however, due to a decrease in the cytochrome pathway activity the relative AOX activity increased in this period (Millar *et al.*, 1998). The cytochrome pathway activity decreased in roots from plants that were transferred to low-light conditions (Fig. 3, Chapter 3). The activity of the alternative pathway did not change in those roots; however, the relative importance in the total respiration increased.

Concluding: the *in vivo* regulation of AOX activity

The reduction state of the AOX protein and the pyruvate action were thought to be very important factors in regulating the AOX activity (e.g., Vanlerberghe and McIntosh, 1997). However, as discussed in this Chapter, these two factors do not seem to change to a major extent *in vivo*: the AOX protein is mostly in the active (reduced) from and the pyruvate concentration is probably always much higher than the concentration required for half-maximum stimulation. The substrate concentration and the AOX concentration have some effect on the AOX activity, but are by themselves not very important. However, there is no need to postulate an as yet unknown activation mechanism, because, when the two known factors are combined, the activity can be fitted with great accuracy (Chapter 4).

The function of the alternative pathway

Although the phenomenon of cyanide-resistant plant respiration has been known for a long time (Van Herk, 1937; Marsh and Goddard, 1939), it was not until the beginning of the 1970^s that a lot of research was done on the alternative pathway. However, a clear general function for the alternative path has not yet been found or proven. In this section several possible functions of the alternative pathway are discussed.

Thermogenesis

In the reproductive structures of aroids (Meeuse, 1975) and also of a number of other species, including water lilies (Skubatz *et al.*, 1990) and cycads (Skubatz *et al.*, 1993), the alternative path plays a role in thermogenesis. The function of the alternative pathway in these flowers can be regarded as a special one, but not as the general function of AOX, since there is no temperature increase in other organs due to the alternative pathway (Chapter 1). AOX is also present in some Protista and hence evolved long before higher plants appeared. The alternative oxidase, which occurs in every examined plant species and in almost every plant organ, must have another function in addition to heat production.

Energy overflow

Until 1995, the alternative pathway was thought to function as an overflow for the cytochrome pathway. On this basis a energy overflow hypothesis was formulated (Lambers, 1982). Experiments with inhibitors of the two pathways showed that if the carbohydrate concentration or supply is

higher than required (growth, ion uptake and maintenance), the alternative pathway will oxidize the excess of carbohydrates and so prevent a build-up of a large pool of carbohydrates in the tissue. These inhibitor studies now have to be reevaluated (with ^{18}O-fractionation measurements), since we now know that the alternative pathway competes with the cytochrome pathway (Day *et al.*, 1996).

After the transfer of plants from high-light to low-light conditions the sugar concentration in the roots decreased dramatically (Fig. 1, Chapter 3). At the same time the absolute AOX activity in the roots did not change (Fig. 3, Chapter 3). Also sucrose addition after 3 days of low light did not change the AOX activity; however, both with sugar depletion and with sugar addition the cytochrome pathway responded with a decrease or increase in activity, respectively (Fig. 3, Chapter 3). In these experiments the alternative pathway did not act as an energy overflow when the carbohydrate availability changed. However, more experiments need to be done to accept or rule out the energy overflow hypothesis.

Reductant overflow

In principle, the flux though glycolysis, citric acid cycle, and the electron-transport chain is tightly regulated. The rate of respiration is regulated via ADP concentration (state3/state4). At low ADP concentrations the activity of the electron-transport chain (complexes 1, 3 and 4) slows down. Also glycolysis (via phosphofructokinase) slows down. However, in plants there are several bypasses to overcome the decreased activity of glycolysis and the electron-transport chain at low ADP concentrations. The regulating phosphofructo-kinase step in glycolysis can be bypassed by the pentose phosphate pathway (e.g., Dennis *et al.*, 1997; Simons *et al.*, 1999) and also directly by fructose-6-phosphate phosphotransferase with the use of pyrophosphate (Stitt, 1998).

Also the electron-transport chain has bypasses. The bypass for complex 1 is another internal (and external) NADH dehydrogenase (rotenone insensitive, Fig. 1, Chapter 1). The bypass for complexes 3 and 4 (the cytochrome pathway) is the alternative oxidase. The bypass for complex 1 is only used if the concentration of NADH is relatively high, since the K_m value for NADH oxidation for complex 1 is much lower (10x) than that of the rotenone-insensitive bypass (Møller and Palmer, 1982; Palmer *et al.*, 1982; Møller and Lin, 1986; Agius *et al.*, 1998). Therefore, the significance of the alternative pathway may be to oxidize the NADH pool. This can be necessary for the continuation of the citric acid cycle and / or to prevent over-reduction of the electron-transport chain (see below).

Continuation of the citric acid cycle

The function of the citric acid cycle in plants is not only to generate reducing molecules for the electron-transport chain to produce ATP, but also for the production and breakdown of amino acids and for nitrate assimilation.

The citric acid cycle can only continue if the produced NADH will be oxidized again to NAD^+. Under state 4 respiration (low ADP concentration) this can be difficult, since complex 1 and the cytochrome pathway are less active. Therefore, the rotenone-insensitive bypass and the alternative pathway are necessary for the continuation of the citric acid cycle, when the cytochrome pathway is restricted.

The cytochrome pathway operates at a slower rate under state 4 conditions; however, there are also situations were the cytochrome pathway is inhibited directly, e.g., with 1: CN^-, 2: CO, 3: NO, 4: low oxygen concentration, 5: H_2S and 6: low temperature.

1 Cyanide resistance

Seeds of some species contain cyanogenic compounds (Lambers *et al.*, 1998 p. 111). These compounds liberate HCN after hydrolysis during imbibition. However, those seeds also contain a mitochondrial β-cyanoalanine synthase (β-CAS) to detoxifies HCN (Hagesawa *et al.*, 1995; Maruyama *et al.*, 1998). Also in other species and organs the concentration of cyanogenic compounds can be high, e.g., in *Manihot esculenta* and *Sorghum bicolor* (Nartey, 1981). In seedlings of *Glycine max* and *Hordeum vulgare* the cyanide concentration is 0.44 and 3.9 nmol g^{-1} FM, respectively; in both species β-CAS is present (Tittle *et al.*, 1990). However, considering their method these authors also measured the CN^- bound to organic compounds; therefore, the actual CN^- concentration is probably much lower.

The β-CAS enzyme is the most important enzyme in plants to detoxify CN^- and occurs in seeds, berries, roots, leaves, and whole seedlings and is widely distributed in higher plants species (Miller and Conn, 1980; Wurtle *et al.*, 1985). The maximum β-CAS activity is at least 0.5 mmol H_2S g^{-1} FM hr^{-1}; however, in most species it is much higher, up to 60 mmol H_2S g^{-1} FM hr^{-1}.

In the ethylene biosynthetic pathway, ACC oxidase produces CN^-, probably in the cytosol (ACC + $^1/_2O_2$ → ethylene + CO_2 + HCN) (Kende, 1993). Table 2 lists the rate of ethylene release in several species, organs and treatments. Per mole of ethylene one mole of CN^- is produced. Concentrations in excess of 0.63 nmol CN^- g^{-1} FM will inhibit the cytochrome pathway in *Poa annua* roots (Figs 2 and 3, Chapter 2). If one of the highest values for ethylene release is taken (1 nmol g^{-1} FM hr^{-1}), then it will take about 40

Table 2: Ethylene release (nmol g^{-1} FM hr^{-1}) in several species, organs and treatments.

Species	Additional information	Ethylene release	Reference
Leaves or shoots			
Arabidopsis thaliana, etr		0.250	Bleecker *et al.*, 1988
Arabidopsis thaliana, wt		0.190	Bleecker *et al.*, 1988
Arabidopsis thaliana, wt		0.163	Romano *et al.*, 1993
Arabidopsis thaliana, wt		0.152	Knoester *et al.*, 1999
Lycopersicon esculentum cv Pearson		0.408	Lund *et al.*, 1998
Lycopersicon esculentum cv Pearson	Infection	1.102	Lund *et al.*, 1998
Nicotiana tabacum Samsun NN wt	Leaf disks	0.120	Knoester *et al.*, 1998
Nicotiana tabacum Samsun NN wt	Infection	1.062	Knoester *et al.*, 1998
Nicotiana tabacum Samsun wt		0.037	Romano, *et al.*, 1993
Nicotiana tabacum tetr18		0.191	Knoester *et al.*, 1998
Poa alpina		0.022[1]	Fiorani *et al.*[2]
Poa annua		0.028[1]	Fiorani *et al.*[2]
Poa compressa		0.014[1]	Fiorani *et al.*[2]
Poa trivialis		0.030[1]	Fiorani *et al.*[2]
Rumex palustris		0.262	Vriezen *et al.*, 1999
Rumex palustris	Flooding	0.700	Vriezen *et al.*, 1999
Roots			
Arabidopsis thaliana Samsun NN wt		0.105	Knoester *et al.*, 1999
Helianthus annuus		0.230	Finlayson and Reid, 1996
Lycopersicon esculentum cv Ailsa Craig		0.163	Konings and Jackson, 1979
Lycopersicon esculentum diageotropica		0.102	Konings and Jackson, 1979
Lycopersicon esculentum cv Moneymaker		0.143	Konings and Jackson, 1979
Oryza sativa IR 20		0.061	Konings and Jackson, 1979
Sinapis alba		0.273	Konings and Jackson, 1979

[1] =assuming a steady release over 24 hours.
[2] = unpublished data: Fiorani F, Bögeman GM, Visser EJW, Voesenek LACJ and Lambers H.

minutes to reach a concentration where CN^- starts to inhibit the cytochrome pathway, if no CN^- detoxification takes place. The cyanide produced cannot only be detoxified but will also be volatilized to the air because at a pH of up to 7, a substantial part of the cyanide is in the HCN form, which is a gas.

Therefore, the CN^- produced during synthesis of ethylene might inhibit the cytochrome path during an extreme ethylene burst, if the rate of CN^- detoxification and the HCN diffusion is slow; however, in most cases the CN^- production will be too low.

2 CO_2 resistance

The respiration can be reduced by elevated CO_2 concentrations in some species (Drake *et al.*, 1999), but not in all species (e.g., Scheurwater *et al.*, 1998). It is also known that CO_2 can directly inhibit cytochrome *c* oxidase and succinate dehydrogenase; however, these enzymes are normally not rate limiting (Gonzàlez-Meler *et al.*, 1996). Therefore, it is questionable if the decrease in respiration is caused by direct effects of CO_2 on enzymes of the respiratory pathway (Drake *et al.*, 1999). Also the alternative pathway cannot overcome a possible effect of an inhibited cytochrome pathway if succinate dehydrogenase is inhibited more than cytochrome *c* oxidase.

3 Nitric oxide resistance

In the past decade nitric oxide has been studied elaborately in biological systems (Hausladen and Stamler, 1998; Durner and Klessig, 1999). Nitric oxide (NO) reversibly inhibits cytochrome *c* oxidase (Brown and Cooper, 1994; Brown 1995; Cleeter *et al.*, 1994, Borutaite and Brown, 1996). NO inhibits cytochrome *c* oxidase at lower concentrations if the oxygen concentration is decreased (Brown and Cooper, 1994). The K_i is 270 nM NO at 145 µM O_2 (half O_2-saturated water) and 60 nM NO at 30 µM O_2, in synaptosome cells from the brain of rats. NO is highly lipophilic and can diffuse through cell membranes without the aid of specific membrane transporters (Leshem, 1996). Nitric oxide inhibits the cytochrome pathway to a much greater extent than the alternative pathway (Millar and Day, 1996).

NO is produced by nitric oxide synthase (NOS; arginine + O_2 + NADPH $\rightarrow$ citrulline + NO + $NADP^+$) and is present in mitochondria of mammalian heart, skeletal muscle, and kidney cells (Bates *et al.*, 1996). Immuno-fluorescence showed that the maize NOS protein is present in the cytosol of cells in the division zone and is translocated into the nucleus in the elongation zone of maize root tips (Ribeiro *et al.*, 1999). NO can also be produced by nitrate reductase NR (Dean and Harper, 1988). The normal reaction of NR is $NAD(P)H + H^+ + NO_3^- \rightarrow NO_2^- + H_2O + NAD(P)^+$, but NR can also react with NO_2^- to form mainly NO.

Interestingly, NO can react with reduced molecules including ubiquinol to form NO^- (Poderoso *et al.*, 1999), which suggests, the more the cytochrome pathway is inhibited by NO the more ubiquinol is available to neutralize the NO radicals.

There are very few articles about NO production in plants. Table 3 lists NO release rates of several species, organs, and treatments. The release of NO is in most cases too low to inhibit the cytochrome pathway; however, after NO_3^- addition (or rewatering after a period of drought) the NO release increased to such a high level that inhibition of the cytochrome pathway is

Table 3: Nitric oxide release (nmol NO kg^{-1} FM s^{-1}) in several species, organs and treatments.

Species	Additional information	NO release	Reference
Actinidia sinensis Hayward	Fruits, green	0.10	Leshem *et al.*, 1998
Actinidia sinensis Hayward	Fruits, mature	0.07	Leshem *et al.*, 1998
Brassica napus c.v. Bronowski	Shoot, day	0.90	Wildt *et al.*, 1997
Brassica napus c.v. Bronowski	Shoot, night	0.03	Wildt *et al.*, 1997
Diospyros kaki Triumph	Fruits, green	0.20	Leshem *et al.*, 1998
Diospyros kaki Triumph	Fruits, mature	0.07	Leshem *et al.*, 1998
Glycine max Merr	Shoot, day	0.30	Wildt *et al.*, 1997
Glycine max Merr	Shoot, night	0.03	Wildt *et al.*, 1997
Glycine max Merr	Shoot, NO_3^- addition	3-117	Wildt *et al.*, 1997
Helianthus annuus cv. Giganteus	Shoot, day	0.60	Wildt *et al.*, 1997
Helianthus annuus cv. Giganteus	Shoot, day, soil	0.90	Wildt *et al.*, 1997
Helianthus annuus cv. Giganteus	Shoot, night	0.03	Wildt *et al.*, 1997
Helianthus annuus cv. Giganteus	Shoot, NO_3^- addition	0.6-66	Wildt *et al.*, 1997
Helianthus annuus cv. Giganteus	Shoot, NO_3^- addition, soil	9-12	Wildt *et al.* , 1997
Lycopersicon esculentum			
Bet Dagan 139 (cherry)	Fruits, green	1.17	Leshem *et al.*, 1998
Lycopersicon esculentum			
Bet Dagan 139 (cherry)	Mature fruits	0.60	Leshem *et al.*, 1998
Musa sapientum Cavendich	Fruits, green	0.23	Leshem *et al.*, 1998
Musa sapientum Cavendich	Fruits, mature	0.07	Leshem *et al.*, 1998
Persea americana Ettinger	Fruits, green	0.23	Leshem *et al.*, 1998
Persea americana Ettinger	Fruits, mature	0.13	Leshem *et al.*, 1998
Saccharum officinarum	Shoot, NO_3^- addition	12-57	Wildt *et al.*, 1997
Zea mays cv. Dento	Shoot, NO_3^- addition	0.3-0.9	Wildt *et al.*, 1997

possible. The NO release is measured on whole organs and represents average organ production rates; it is unknown if the NO production is equally distributed in an organ. If the oxygen concentration in a tissue is decreased, then the cytochrome *c* oxidase becomes also more sensitive to NO. In conclusion, further research is required to find out whether NO is an important factor in influencing the activity of the cytochrome pathway.

4 Low oxygen resistance

Both the alternative and the cytochrome pathway oxidize O_2 to H_2O. The K_m value for O_2 is different for the two pathways. Under normal condition the K_m values are not important since the K_m values for both pathways are in

the low micromolar range. The K_m for the alternative pathway determined in isolated mitochondria of *Glycine max* and *Vigna radiata* varied between the organs and was between 9.9 and 25.2 μM (Ribas-Carbo *et al.*, 1994). In isolated mitochondria from *Glycine max nodules the* K_m was 2.5 μM (Millar *et al.*, 1997). The K_m for cytochrome *c* oxidase was 0.08 to 0.4 μM in mitochondria from passive mammal organs and 0.35 to 0.8 μM in active mitochondria (Gnaiger *et al.*, 1998), and in mitochondria from *Glycine max* nodules the K_m was 0.05 μM (Millar *et al.*, 1997). In *Glycine max* root and cotyledon mitochondria the K_m for the cytochrome pathway was 0.125 to 0.147, and for the alternative pathway it was 1.6 to 1.8 μM (Millar *et al.*, 1994).

In conclusion the alternative pathway is inhibited more at low oxygen concentrations compared with the cytochrome pathway. Therefore, the alternative pathway does not have a function at low oxygen concentrations.

5 Sulfide resistance

Sulfide (H_2S) is a very effective inhibitor (K_i = 1 μM = 1 nmol H_2S g^{-1} FM) of cytochrome *c* oxidase (Nicholls, 1975). There is a lot of animal literature that reports about the toxic effects of sulfide in animals (e.g., Julian *et al.*, 1998). Also animals have several ways of coping with sulfide, including oxidizing sulfide to less toxic forms (Vetter *et al.*, 1987; Arp *et al.*, 1995). Sulfide can be oxidized to sulfite (SO_3^{2-}) and then to sulfate (SO_4^{2-}) by plants (Rennenberg *et al.*, 1982). Sulfide is a gas that some plants emit in rather high amounts. Leaf disks of *Cucurbita pepo* and *Cucumis sativus* emit 0.5 to 7.5 nmol H_2S g^{-1} FM min^{-1} after addition of sulfate or L-cysteine. The sulfide emission depended on the leaf position and leaf age (Rennenberg and Filner, 1983). Reybould *et al.* (1977) concluded that a wheat canopy lost about 50% of its sulfur in the postanthesis period, most likely via emission to the air of an unknown sulfur form.

It will take less than two minutes to reach a sulfide concentration equal to the K_i if no emission and oxidation of sulfide takes place. However, this is only based on one article and on a situation where also the production of sulfide was stimulated. In the detoxification of CN^- via β-CAS H_2S is produced (see above). Remarkably, not one of the articles on the detoxification of CN^- via β-CAS in plants mentioned the toxic effect of sulfide! More research is required to clarify the influence of sulfide in plants *in vivo*.

6 Low-temperature resistance

The concentration of AOX protein often increases after transfer to lower temperatures (Vanlerberghe and McIntosh 1992; Gonzàlez-Meler *et al.*, 1999). Also the alternative pathway capacity (KCN-insensitive, SHAM-sensitive

respiration) increases as found frequently (Vanlerberghe and McIntosh, 1992, and references therein). However, in a chilling-sensitive maize cultivar the activity of the alternative pathway was higher during the recovery period than in a less chilling-sensitive cultivar (Ribas-Carbo *et al.*, 2000).

Cold-grown *Vigna radiata* plants increased their concentration of AOX protein compared with the ones grown at higher temperatures. The AOX activity measured at lower temperatures was also higher compared with the ones grown at higher temperatures. This response of increase in both AOX concentration and activity was not found in *Glycine max* cotyledons (Gonzàlez-Meler *et al.*, 1999). From inhibitor studies it was concluded that the cytochrome pathway has a higher Q_{10} compared with that of the alternative pathway. In *Vigna radiata* leaves the alternative pathway was more temperature sensitive (higher Q_{10}) (Gonzàlez-Meler *et al.*, 1999). Up to now there are not enough data to generalize which pathway is more sensitive to temperature. However, some species respond to a lower growth temperature by increasing their AOX concentration and activity.

Prevention of radical formation

Another proposed function for the alternative oxidase is the prevention of the formation of oxygen free radicals. Oxygen free radicals may lead to severe metabolic disturbances and a wide range of environmentally induced plant disorders, including chilling damage, are mediated by reactive oxygen species (Scandalios, 1993). Ubiquinol is a common substrate for both the cytochrome and alternative pathway. It has been suggested that a high reduction state of the ubiquinone pool (Q_r/Q_t), occurring when the cytochrome pathway is inhibited or restricted, promotes oxygen free radical formation; respiration via the alternative pathway might then help to maintain Q_r/Q_t at a low level. Purvis and Shewfelt (1993) and Wagner and Wagner (1995) speculated that the alternative pathway helps to stabilize the reduction state of the mitochondrial ubiquinone pool. Also a high membrane potential stimulates oxygen free radical formation, and respiration via AOX in combination with a rotenone-insensitive NADH dehydrogenase can result in uncoupled respiration, lowering the membrane potential (Skulachev, 1996).

Titration of *Poa annua* roots with cyanide caused an increase in Q_r/Q_t; however, in the presence of SHAM (an inhibitor of the alternative pathway) the Q_r/Q_t increased even more (Fig. 1, Chapter 2). Therefore, the alternative pathway is indeed capable of stabilizing the Q_r/Q_t *in vivo*. In accordance, tobacco cells with a lowered concentration of alternative oxidase produce more oxygen free radicals than control cells do (Maxwell *et al.*, 1999). The same antisense AOX cell lines produce also more radicals if a low-P treatment is given (Parsons *et al.*, 1999).

Concluding - the function of the alternative pathway

Heat production by the alternative oxidase is functional only in some flowers. With the limited data available, the energy (carbohydrate) overflow theory is not supported. The cytochrome pathway can be inhibited by the naturally occurring cyanide, nitric oxide, sulfide, and high concentrations of CO_2, or low temperatures. However, it is not clear how important these inhibitory effectors are in plants. The alternative oxidase can prevent the production of extra oxygen free radicals via stabilizing the Q_r/Q_t. This can be important if the NADH concentration is too high (reductant overflow), or the cytochrome pathway is inhibited. At the same time the citric acid cycle can continue to operate under those conditions.

References

Agius SC, Bykova NV, Igamberdiev AU, Møller IM (1998) The internal rotenone-insensitive NADPH dehydrogenase contributes to malate oxidation by potato tuber and pea leaf mitochondria. Physiol. Plant. 104: 329-336

Arp AJ, Menon JG, Julian D (1995) Multiple mechanisms provide tolerance to environmental sulfide in *Urechis caupo*. Amer. Zool. 35: 132-144

Bates TE, Loesch A, Burnstock G, Clark JB (1996) Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation? Biochem. Biophys. Res. Comm. 218: 40-44

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086-1089

Borutaite V, Brown GC (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem. J. 315: 295-299

Brown GC (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett. 369: 136-139

Brown GC, Cooper CE (1994) Nanomolar concentrations of nitric oxide reversible inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. 356: 295-298

Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncadea S, Schapira AHV (1994) Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. FEBS Lett. 345: 50-54

Day DA, Hanson JB (1977) Pyruvate and malate transport and oxidation in corn mitochondria. Plant Physiol. 59: 630-635

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, Wiskich JT (1996) The cyanide-resistant oxidase: To inhibit or not to inhibit, that is the question. Plant Physiol. 110: 1-2

Day DA, Lambers H (1983) The regulation of glycolysis and electron transport in roots. Physiol. Plant. 58: 155-160

Dennis DT, Huang, Y, Negm FB (1997) Glycolysis, the pentose phosphate pathway and anaerobic respiration. In: Plant metabolism. Eds: Dennis DT, Turpin DH, Lefebvre DD, Layzell DB. Longman Singapore pp:103-123

Djajanegara I, Holtzapffel R, Finnegan PM, Hoefnagel MHN, Berthold DA, Wiskich JT, Day DA (1999) A single amino acid change in the plant alternative oxidase alters the specificity of organic acid activation. FEBS Lett. 454: 220-224

Drake BG, Azcon-Bieto J, Berry J, Bunce J, Dijkstra P, Farrar J, Gifford RM, Gonzàlez-Meler MA, Koch G, Lambers H, Siedow J, Wullschleger S (1999) Does elevated atmospheric CO_2 concentration inhibits mitochondrial respiration in green plants? Plant, Cell Environ. 22: 649-657

Durner J, Klessing DF (1999) Nitric oxide as a signal in plants. Curr. Opin. Plant Biol. 2: 369-374

Finlayson SA, Reid DM (1996) The effect of CO2 on ethylene evolution and elongation rate in roots of sunflower (*Helianthus annuus*) seedlings. Physiol. Plant. 98: 875-881

Finnegan PM, Whelan J, Millar AH, Zhang Q, Smith MK, Wiskich JT, Day DA (1997) Differential expression of the multigene family encoding the Soybean mitochondrial alternative oxidase. Plant Physiol. 114: 455-466

Gonzàlez-Meler MA, Ribas-Carbo M, Siedow JN, Drake BG (1996) Direct inhibition of plant mitochondrial respiration by elevated CO_2. Plant Physiol. 112: 1349-1355

Good AG, Muench DG (1993) Long-term anaerobic metabolism in root tissue. Plant Physiol. 101: 1163-1168

Hagesawa R, Muruyama A, Esashi Y (1995) The presence of two types of ß-cyanoalanine synthase in germinating seeds and their response to ethylene. Physiol. Plant. 93: 713-718

Hausladen A, Stamler JS (1998) Nitric oxide in plant immunity. Proc. Natl. Acad. Sci. USA 95: 10345-10347

Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch. Biochem. Biophys. 318: 394-400

Jarmuszkiewicz W, Wagner AM, Wagner MJ, Hryniewiecka L (1997) Immunological identification of the alternative oxidase of *Acanthamoeba castellanii* mitochondria. FEBS Lett. 411: 110-114

Kato-Noguchi H (1997) Effects of citrate on respiratory gas exchange and metabolism in carrot root tissues. Phytochemistry 45: 225-227

Kende H (1993) Ethylene biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44: 283-307

Knoester M, Pieterse CMJ, Bol JF, van Loon LC (1999) Systemic resistance in *Arabidopsis* induced by rhizobacteria requires ethylene-dependent signaling at the site of application. Mol. Plant-Microbe Interact. 12: 720-727

Knoester M, van Loon LC, van den Heuvel J, Henning J, Bol JF (1998) Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proc. Natl. Acad. Sci. USA 95: 1933-1937

Konings H, Jackson MB (1979) A relationship between rates of ethylene production by roots and the promoting or inhibiting effects of exogenous ethylene and water on root elongation. Z. Pflanzenphysiol. 92: 385-397

Lambers H (1982) Cyanide-resistant respiration: A non-phosphorylating electron transport pathway acting as an energy overflow. Physiol. Plant. 55: 478-485

Lambers H, Chapin FS III, Pons TL (1998) Plant physiological ecology. Springer-Verlag, New York

Leshem YY (1996) Nitric oxide in biological systems. Plant Growth Regul. 18: 155-159

Leshem YY, Wills RBH, Ku VV-V (1998) Evidence for the function of the free radical gas - nitric oxide ($NO^{\cdot}$)- as endogenous maturation and senescence regulating factor in higher plants. Plant Physiol. Biochem. 36: 825-833

Lund ST, Stall RE, Klee HJ (1998) Ethylene regulates the susceptible response to pathogen infection in tomato. Plant Cell 10: 371-382

Marsh PB, Goddard DR (1939) Respiration and fermentation in the carrot, *Daucus carota*; I Respiration. Amer. J. Bot. 26: 724-728

Maruyama A, Ishizawa K, Takagi T, Esashi Y (1998) Cytosolic ß-cyanoalanine synthase activity attributed to cysteine synthase in cocklebur seeds. Purification and characterization of cytosolic cysteine synthases. Plant Cell Physiol. 39: 671-680

Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc. Natl. Acad. Sci. USA 96: 8271-8276
Meeuse BJD (1975) Thermogenic respiration in aroids. Annu. Rev. Plant Physiol. 26: 117-126
Millar AH, Atkin OK, Lambers H, Wiskich JT, Day DA (1995) A critique of the use of inhibitors to estimate partitioning of electrons between mitochondrial respiratory pathways in plants. Physiol. Plant. 95: 523-532
Millar AH, Atkin OK, Menz RI, Henry B, Farquhar G, Day DA (1998) Analysis of respiratory chain regulation in roots of soybean seedlings. Plant Physiol. 117: 1083-1093
Millar AH, Day DA (1996) Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. FEBS Lett. 398: 155-158
Millar HA, Bergersen FJ, Day DA (1994) Oxygen affinity of terminal oxidases in soybean mitochondria. Plant Physiol. Biochem. 32: 847-852
Millar AH, Hoefnagel MHN, Day DA, Wiskich JT (1996) Specificity of the organic acid activation of alternative oxidase in plant mitochondria. Plant Physiol. 111: 613-618
Millar AH, Finnegan PM, Whelan J, Drevon JJ, Day DA (1997) Expression and kinetics of the mitochondrial alternative oxidase in nitrogen-fixing nodules of soybean roots. Plant, Cell Environ. 20: 1273-1282
Millar AH, Wiskich JT, Whelan J, Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329: 259-262
Millenaar FF, Benschop JJ, Wagner AM, Lambers H (1998) The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool; and the activation state of the alternative oxidase. Plant Physiol. 118: 599-607
Millenaar FF, Roelofs R, Gonzàlez-Meler MA, Siedow JN, Wagner AM, Lambers H (2000). The alternative oxidase in roots of *Poa annua* after transfer from high-light to low-light conditions. Plant J., accepted.
Miller JM, Conn EE (1980) Metabolism of hydrogen cyanide by higher plants. Plant Physiol. 65: 1199-1202
Møller IM, Palmer JM (1982) Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria. Physiol. Plant. 54: 267-274
Møller IM, Roberts TH, Rasmusson AG (1996) Ubiquinone-1 Induces external deamino-NADH oxidation in potato tuber mitochondria. Plant Physiol. 112: 75-78
Nartey F (1981) Cyanogenesis in tropical feeds and foodstuffs. In: Cyanide in biology (eds) B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing. Academic Press, London pp:115-132
Palmer JM, Schwitzguebel J-P, Møller IM (1982) Regulation of malate oxidation in plant mitochondria; Response to rotenone and exogenous NAD^+. Biochem. J. 208: 703-711
Parsons HL, Yip JYH, Vanlerberghe GC (1999) Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. Plant Physiol. 121: 1309-1320
Poderoso JJ, Carreras MC, Schöpfer F, Lisdero CL, Riobo NA, Giulivi C, Boveris AD, Boveris A, Cadenas E (1999). The reaction of nitric oxide with ubiquinol: kinetic properties and biological significance. Free Radic. Biol. Med. 26: 925-935
Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol. Plant. 88: 712-718
Raybould C, Unsworth MH, Gregory PJ (1977) Sources of sulfur in rain collected below a wheat canopy. Nature 267: 146-147
Rennenberg H, Filner P (1983) Developmental changes in the potential for H_2S emission in cucurbit plants. Plant Physiol. 71: 269-275
Rennenberg H, Sekija J, Wilson LG, Filner P (1982) Evidence for an intracellular sulfur cycle in cucumber leaves. Planta 154: 516-524

Rhoads DM, Umbach AL, Sweet CR, Lennon AM, Rauch GS, Siedow JN (1998) Regulation of the cyanide-resistant alternative oxidase of plant mitochondria. J. Biol. Chem. 273: 30750-30756

Ribas-Carbo M, Aroca R, Gonzàlez-Meler MA, Irigoyen JJ, Sanchez-Diaz M (2000) The electron partitioning between the cytochrome and alternative respiratory pathways during chilling recovery in two cultivars of maize differing in chilling sensitivity. Plant Physiol. 122: 199-204

Ribas-Carbo M, Berry JA, Azcon-Bieto J, Siedow JN (1994) The reaction of the plant mitochondrial cyanide-resistant alternative oxidase with oxygen. Biochim. Biophys. Acta 1188: 205-212

Ribas-Carbo M, Berry JA, Yakir D, Giles L, Robinson SA, Lennon AM, Siedow JN (1995) Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. Plant Physiol. 109: 829-837

Ribeiro EA, Cunha FQ, Tamashiro WMSC, Martins IS (1999) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Lett. 445: 283-286

Romano CP, Cooper ML, Klee HJ (1993) Uncoupling auxin and ethylene effects in transgenic tobacco and *Arabidopsis* plants. Plant Cell 5: 181-189

Scandalios JG (1993) Oxygen stress and superoxide dismutase. Plant Physiol. 101: 7-12

Scheurwater I, Cornelissen C, Dictus F, Welschen R, Lambers H (1998) Why do fast- and slow-growing grass species differ so little in their rate of root respiration, considering the large differences in rate of growth and ion uptake. Plant, Cell Environ. 21: 995-1005

Simons BH, Millenaar FF, Mulder L, Van Loon LC, Lambers H (1999) Enhanced expression and activation of the alternative oxidase during infection of arabidopsis with *Pseudomonas syringae* pv tomato. Plant Physiol. 120: 529-538

Skubatz H, Tang W, Meeuse BJD (1993) Oscillatory, heat-production in the male cones of cycads. J. Exp. Bot. 44: 489-492

Skubatz H, Williamson PS, Schneider EL, Meeuse BJD (1990) Cyanide-insensitive respiration in thermogenic flowers of *Victoria* and *Nelumbo*. J. Exp. Bot. 41: 1335-1339

Skulachev VP (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. Q. Rev. Biophys. 29: 169-202

Stitt M (1998) Pyrophosphate as an energy donor in the cytosol of plant cells: an enigmatic alternative to ATP. Bot. Acta 111: 167-175

Tittle FL, Goudey JS, Spencer MS (1990) Effect of 2,4-dichlorophenoxyacetic acid on endogenous cyanide, ß-cyanoalanine synthase activity, and ethylene evolution in seedlings of soybean and barley. Plant Physiol. 94: 1143-1148

Umbach AL, Siedow JN (1993) Covalent and Noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol. 103: 845-854

Umbach AL, Siedow JN (1997) Changes in the redox state of the alternative oxidase regulatory sulfhydryl/disulfide system during mitochondrial isolation: implication for inferences of activity *in vivo*. Plant Sci. 123: 19-28

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett. 348: 181-184

Van Herk AWH (1937) Die chemischen Vorgänge im Sauromatum-Kolben. Rec. Trav. Bot. Neerl. 34: 69-156

Vanlerberghe GC, McIntosh L (1992) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. Plant Physiol. 100: 115-119

Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1994) Molecular genetic alteration of plant respiration. Silencing and overexpression of alternative oxidase in transgenic tobacco. Plant Physiol. 106: 1503-1510

Vanlerberghe GC, McIntosh L (1996) Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. Plant Physiol. 111: 589-595

Vanlerberghe GC, McIntosch L (1997) Alternative oxidase: From gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 703-734

Vanlerberghe GC, McIntosh L, Yip JYH (1998) Molecular localization of a redox-modulated process regulating plant mitochondrial electron transport. Plant Cell 10: 1551-1560

Vanlerberghe GC, Vanlerberghe AE, McIntosch L (1997) Molecular genetic evidence of the ability of alternative oxidase to support respiratory carbon metabolism. Plant Physiol. 113: 657-661

Vetter RD, Wells ME, Kurtsman AL, Somero GN (1987) Sulfide detoxification by the hydrothermal vent crab *Bythograea thermydron* and other decapod crustaceans. Physiol. Zool. 60: 121-137

Vriezen WH, Hulzink R, Mariani C, Voesenek LACJ (1999) 1-Aminocyclopropane-1-Carboxylate oxidase activity limits ethylene biosynthesis in *Rumex palustris* during submergence. Plant Physiol. 121: 189-195

Wagner AM, Krab K (1995) The alternative respiration pathway in plants: Role and regulation. Physiol. Plant. 95: 318-325

Wagner AM, Wagner MJ (1995) Measurements of *in vivo* ubiquinone reduction levels in plant cells. Plant Physiol. 108: 277-283

Wildt J, Kley D, Rockel A, Rockel P, Segschneider HJ (1997) Emission of NO from several higher plant species. J. Geophys. Res. 102: 5919-5927

Wurtele ES, Nikolau BJ, Conn EE (1985) Subcellular and developmental distribution of ß-cyanoalanine synthase in barley leaves. Plant Physiol. 78: 285-290

Samenvatting

De titel van dit proefschrift is "Alternative oxidase in roots of *Poa* species: *in vivo* regulation and function" ofwel, "Alternatieve oxidase in de wortels van verschillende *Poa* soorten: *in vivo* regulatie en functie". De functie, maar vooral ook de regulatie in intacte weefsels, staan centraal in dit proefschrift.

De alternatieve ademhaling is een energieverspillend biochemisch proces. Het doel van de ademhaling is het verbranden (oxyderen) van voornamelijk suikers om ATP te produceren. ATP is een energierijke stof die nodig is voor talrijke biochemische reacties. De ademhaling bestaat uit drie onderdelen; de glycolyse, de citroenzuurcyclus en de elektronentransportketen (deze laatste is in de mitochondriële binnenmembraan gelokaliseerd). Aan het einde van de laatste stap wordt ATP gegenereerd. Producten (NADH en succinaat) van vooral de citroenzuurcyclus, maar ook van de glycolyse (NADH), zijn het substraat voor de elektronentransportketen. De elektronentransportketen bestaat uit de dehydrogenases, de ubichinon pool, de cytochroomketen en de alternatieve keten (Fig. 1, Introduction). NADH en succinaat worden geoxydeerd door de dehydrogenases die de elektronen overdragen aan de ubichinon pool. Deze dragen op hun beurt de elektronen over aan de cytochroomketen of de alternatieve keten. Het gereduceerde ubichinon (ubichinol, Fig. 2 Introduction) is dus het substraat voor beide ketens. Een van de dehydrogenases en de cytochroomketen pompen protonen over de binnen membraan van de mitochondria. De protonengradiënt wordt gebruikt om ATP te genereren. De alternatieve keten bestaat uit één eiwit, de alternatieve oxydase (AOX); dit eiwit pompt geen protonen en draagt dus niet bij aan de

produktie van ATP. Als suikers worden geoxydeerd tot H_2O en CO_2 en de elektronen stromen via de alternatieve keten in plaats van de cytochroomketen naar O_2 dan wordt energie verspild.

De regulatie van de activiteit van de alternatieve keten

In geïsoleerde mitochondria zijn diverse regulatiemechanismen ontdekt die de activiteit van de alternatieve keten beïnvloeden. Het alternatieve oxydase (AOX) eiwit bestaat als dimeer in de mitochondriële binnen membraan; als deze dimeer niet covalent gebonden is via een zwavelbrug is het AOX meer actief (Fig. 4, Introduction). De twee AOX vormen worden gescheiden op een Western blot omdat ze verschillen in grootte. Als er voldoende α-ketozuren zoals pyruvaat aanwezig zijn wordt AOX al actief bij lage substraatconcentraties (Fig. 5, Introduction). Van deze regulatiemechanismen is niet bekend hoe belangrijk ze zijn in intacte weefsels. Een belangrijk deel van dit proefschrift gaat over deze regulatiemechanismen in intacte weefsels. Er is gekozen om vooral in wortels te meten, omdat het meten van de reductietoestand van de ubichinon pool in bladeren niet mogelijk is.

Het AOX eiwit komt vooral voor in de actieve (niet covalent gebonden) vorm gedurende de lichtperiode in de wortels van straatgras, *Poa annua* (Hoofdstuk 2). Nadat planten werden overgezet van hoog licht en lange dagen naar laag licht en korte dagen (450 - 80 μmol m^{-2} s^{-1} PAR en 16 - 10 uur licht per dag) daalde in 4 dagen de totale ademhaling (met 45%) en de suiker concentratie (met 90%) in de wortels sterk (Hoofdstuk 3), maar de absolute AOX activiteit en aktivatietoestand veranderden niet. In de wortels van verschillende *Poa* soorten (*P. annua*, *P. alpina*, *P. compressa*, *P. pratensis*, *P. trivialis*) die verschilden in ademhalingssnelheid, relatieve groeisnelheid, AOX concentratie (10 keer) en AOX activiteit (10-50%) komt het AOX eiwit alleen in de actieve vorm voor (Hoofdstuk 4). Na het toedienen van sucrose, mannitol of citraat aan afgesneden wortels van *P. annua* gedurende 24 uur komt het AOX eiwit alleen in de actieve vorm voor ondanks de grote verschillen in AOX concentratie (15 keer) en activiteit (5-30%) (Hoofdstuk 5). Ook in bladeren en wortels van tabak komt het AOX eiwit alleen in de actieve vorm voor. In geïsoleerde mitochondria komt een aanzienlijk deel van het eiwit voor in de inactieve vorm. Dit komt omdat de activatie toestand van het eiwit verandert op het moment dat de mitochondria worden geïsoleerd uit weefsels, en dit gebeurt niet tijdens de AOX isolatie direct uit weefsels (Hoofdstuk 2, 4).

De pyruvaatconcentratie beïnvloedt de AOX activiteit. Als de pyruvaatconcentratie wordt geschat in de mitochondria en wordt vergeleken met de pyruvaatconcentratie die nodig is om het AOX eiwit te activeren is het zeer waarschijnlijk dat in intacte weefsels altijd voldoende pyruvaat aanwezig is voor een volledige stimulatie (Hoofstuk 2).

De hoeveelheid AOX eiwit kan de AOX activiteit mogelijk beïnvloeden. In de vijf *Poa* soorten die verschillen in AOX concentratie (10 keer) en activiteit (10-50%) was geen correlatie tussen deze twee parameters (Hoofdstuk 4). Een betere manier om verschillen in AOX concentratie te bestuderen is deze te veranderen via sense en antisense genetisch getransformeerde planten binnen een soort (Hoofdstuk 6). Er waren verschillen in de AOX concentratie bij een aantal getransformeerde tabakslijnen, maar deze zagen er uiterlijk niet anders uit. Er was echter geen tijd meer om deze planten nader te analyseren.

De activiteit van de alternatieve keten wordt ook beïnvloed door de hoeveelheid beschikbaar substraat (ubichinol). De reductietoestand van de ubichinon pool wordt gewoonlijk uitgedrukt als percentage ubichinol ten opzichte van de totale pool, afgekort Q_r/Q_t. De Q_r/Q_t verandert niet veel in weefsels, ook niet als de ademhaling verandert (Hoofdstuk 2, 3, 4). Kleine veranderingen in Q_r/Q_t kunnen echter wel een relatief grote invloed hebben op de activiteit van de alternatieve keten, omdat de kinetische curve stijl is (Hoofdstuk 2).

Aangezien de cytochroomketen en de alternatieve keten in competitie zijn voor de elektronen afkomstig van de gereduceerde ubichinon pool beïnvloedt de activiteit van de ene keten de activiteit van de andere keten. In het experiment waarbij planten zijn overgeplaatst van hoog licht en lange dagen naar laag licht en korte dagen veranderde de absolute activiteit van de alternatieve keten niet. Maar omdat de cytochroomketen in activiteit daalde steeg het aandeel van de alternatieve keten sterk (van 30 naar 60%, Hoofdstuk 3).

De activatietoestand van het AOX eiwit en de pyruvaat concentratie spelen geen grote rol in de beïnvloeding van de AOX activiteit in de bestudeerde weefsels (Hoofdstuk 4, 7). Verschillen in activiteit van de alternatieve keten tussen soorten kan niet worden verklaard door een van de bekende factoren (Q_r/Q_t, AOX concentratie, etc.) die invloed hebben op de activiteit. Als echter de substraat- en de AOX-concentraties worden gebruikt in een lineair regressiemodel is een goed passende regressie mogelijk (Hoofstuk 4). Dit verkleint de noodzaak om te zoeken naar een nieuwe onbekende factor die de AOX activiteit beïnvloedt.

De functie van de alternatieve keten

In sommige bloemen van de aronskelk-familie wordt de alternatieve keten gebruikt om warmte te produceren zodat de bloemen vele graden warmer worden in vergelijking met hun omgevingstemperatuur. Hierdoor worden stoffen vluchtig die insecten lokken die nodig zijn voor de bestuiving. Het AOX eiwit is in alle plantensoorten en in vrijwel alle organen gevonden die onderzocht zijn. Behalve in bloemen van soorten zoals van de aronskelk-familie is er geen temperatuursverhoging gemeten en dat is theoretisch ook

niet mogelijk. De alternatieve ademhaling moet dus een andere functie hebben in deze planten.

Als de cytochroomketen geremd is of de aanvoer van substraat (NADH) niet gelijk loopt met de afvoer via de cytochroomketen wordt de elektronentransportketen meer gereduceerd (onder andere de Q_r/Q_t). Dit heeft tot gevolg dat schadelijke vrije zuurstofradicalen worden gevormd. Als onder deze omstandigheden de alternatieve keten er voor kan zorgen dat de Q_r/Q_t niet te gereduceerd wordt, wordt voorkomen dat extra vrije zuurstofradicalen ontstaan. Door de cytochroomketen te blokkeren met toenemende concentraties cyanide neemt de Q_r/Q_t ook steeds meer toe. Wordt echter tegelijkertijd ook SHAM (remmer van AOX) toegediend dan neemt de Q_r/Q_t nog sneller toe (Hoofstuk 2). De alternatieve keten is dus in staat om in weefsels de Q_r/Q_t enigszins te stabiliseren.

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Publications

Reviewed publications in journals and conference proceedings

Frank F. Millenaar, Roeland Roelofs, Miquel A. Gonzàlez-Meler, James N. Siedow, Anneke M. Wagner, Hans Lambers (2000) The alternative oxidase in roots of *Poa annua* after transfer from high-light to low-light conditions. Plant Journal, accepted.

Frank F. Millenaar, Miquel A. Gonzàlez-Meler, Fabio Fiorani, Rob Welschen, Miquel Ribas-Carbo, James N. Siedow, Anneke M. Wagner and Hans Lambers (200?) Regulation of alternative oxidase activity in six wild monocotyledonous species; an *in vivo* study at the whole root level. Plant Physiology, submitted.

Frank F. Millenaar, Joris J. Benschop, Anneke M. Wagner and Hans Lambers (1998) The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool; and the activation state of the alternative oxidase. Plant Physiology 118: 599-607.

Frank F. Millenaar, Roeland Roelofs and Hans Lambers (1998) Induction and reduction state of the alternative oxidase during an extra-long night. I. M. Møller, P. Gardeström, K. Glimelius and E. Glaser (Eds.) Plant mitochondria; from gene to function, pp. 499-502. Backhuys, Leiden

Fabio Fiorani, Frank F. Millenaar and Hans Lambers (1998) Relationship between KCN-resistant respiration and alternative oxidase amount in four *Poa* species. I. M. Møller, P. Gardeström, K. Glimelius and E. Glaser (Eds.) Plant mitochondria; from gene to function, pp. 455-458. Backhuys, Leiden

Bert H. Simons, Frank F. Millenaar, Lonneke Mulder, Leendert C. Van Loon and Hans Lambers (**1999**) Enhanced expression and activation of the alternative oxidase during infection of arabidopsis with *Pseudomonas syringae* pv. tomato. Plant Physiology 120: 529-538.

Van Hellemond JJ, Simons B, Millenaar FF and Tielens AGM (**1998**) *Leismania* spp. lack the gene encoding the plant-like alternative oxidase. Journal of Eukaryotic Microbiology 45: 426-430.

Contributions to edited books

Hans Lambers, Owen K. Atkin, and Frank F. Millenaar (**2000**) Respiratory Patterns in Roots in Relation to Their Functioning. Y. Waisel, A. Eshel, and K. Kafkaki, (Eds.) Plant Roots; The Hidden Half. Marcel Dekker, Inc. New York, submitted

Lambers H, Scheurwater I and Millenaar F (**1997**) Variation in carbon utilization in root respiration and exudation as dependent on a species potential growth rate and nutrient supply. H. E. Flores, J. P. Lynch and D. Eissenstat (Eds.) Radical Biology: Advances and perspectives on the function of plant roots, pp. 116-130

Curriculum Vitae

Frank Millenaar was born on 26 December 1968 in Rheydt, the Netherlands. He passed the 'Lagere tuinbouwschool' College for horticultural studies (1985), the 'Middelbare tuinbouwschool' College for horticultural studies (1988) and the 'Hogeschool Delft' Institute for higher professional education in nature, agriculture, food and management, horticultural division (degree of 'ing', 1992). In the same year he started to study horticulture at Wageningen Agricultural University. For his Masters of Science (MSc or 'Ir.') which he obtained in 1995, two projects were carried out:

1) Horticultural production systems and product quality group, Wageningen Agricultural University. "Water relations during aging of cut and non-cut Freesia flowers". Supervisors: Dr. Ir. U. van Meeteren and Prof. Dr. J. Tromp.
2) Plant physiology, Wageningen Agricultural University. "Changes of citric acid cycle intermediates and of ICDH activity during tuber and shoot formation of *in vitro* potatoes". Supervisors: Dr. D. Vreugenhil and Prof. Dr. L.H.W. van der Plas.

In September 1995 he was appointed as a Ph.D. student at Utrecht University to work in the Plant Ecophysiology group. The research, of which the results are presented in this thesis, was supervised by Professor Dr. Hans Lambers and Dr. Anneke Wagner and took place in the Graduate School Experimental Plant Sciences. From September 2000 onwards, he will be working as a post-doc in the laboratory of Professor Dr. Rens Voesenek in the Plant Ecophysiology group of Utrecht University.