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Oxygen concentration regulates alternative oxidase expression in barley roots during hypoxia and post-hypoxia

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During flooding, when roots are submerged in oxygen-free water, root tissue becomes hypoxic and its metabolism is characterized by fermentation processes and limited respiratory activity. After returning to aerobic conditions (post-hypoxic period) high respiration rates together with symptoms of oxidative stress are observed. Plant mitochondria have two terminal oxidases: a cytochrome oxidase with high affinity to oxygen and an alternative (cyanide resistant) oxidase (AOX) with a relatively low oxygen affinity. We compared mitochondrial respiration and AOX expression immediately after hypoxic and during post-hypoxic period. Four-day-old barley (*Hordeum vulgare* L. cv Gregor) seedlings were transferred for 5 days to Knop nutrient medium flushed with air (control) or nitrogen (hypoxia) and after returned for 24 h to aerated nutrient medium (post-hypoxia). $NADH/NAD^+ + NADH$ and UQH_2/UQ_{total} ratios increased in hypoxia-treated roots. After the hypoxic roots were returned to aerated medium, increases in respiration rate and ATP concentration were observed. Mitochondria isolated from barley roots at the end of hypoxic period exhibited high respiratory rates as compared to mitochondria from control and post-hypoxic roots. Control root mitochondria expressed high AOX capacity in the presence of pyruvate and DTT. Mitochondria isolated at the end of the hypoxic period were highly cyanide sensitive under AOX-activating conditions, however, after 24 h of post-hypoxia, AOX capacity was comparable to that observed in the control. The capacity of AOX was correlated with the amount of AOX protein determined by Western blotting. Faint or no bands were observed immediately after hypoxia with the AOX protein appearing again during the post-hypoxic period. Examination of AOX transcript levels showed no significant differences between control, hypoxic and post-hypoxic roots indicating that the regulation of AOX expression by oxygen availability in barley roots may take place at the translation level.

Introduction

Plants are subjected to variations in oxygen concentration in their environment. Such changes may occur during flooding when roots or whole plants are submerged in water. The oxygen concentration and the rate of oxygen diffusion in water are much lower than in air and, due to O_2 consumption by respiring roots and micro-organisms, roots may be in a hypoxic (oxygen concentration between 2 and 4% v/v, equivalent to 2–4 kPa oxygen partial pressure) or an anoxic (no oxygen) environment. When roots are submerged in oxygen-free water (anoxia), root tissue do not become fully anoxic since some oxygen can be delivered from shoots exposed to air (Erdmann and Wiedenroth 1988). Hypoxic conditions are more common in nature than anoxic conditions and can have adverse effects on growth (Drew 1997). During hypoxia, plant metabolism is characterized by fermentation processes and limited respiratory activity (Ricard et al. 1994). All plants are able to survive for some periods of low oxygen availability. The degree to which plants can recover after a period of anaerobiosis depends on its duration and on the changes in metabolism resulting in acclimation of plant tissues to a low oxygen environment (Andreev et al. 1991, Drew 1997). Pea root mitochondria, after a limited period of anoxia, restored their function despite the observed changes in their ultrastructure (Andreev et al. 1991).

Abbreviations – ADH, alcohol dehydrogenase; AOX, alternative oxidase; DTT, dithiothreitol; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; UQ, ubiquinone; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.

Plant mitochondria possess two pathways of electron transport branched at the ubiquinone level – a cyanide-sensitive cytochrome pathway and a cyanide-resistant alternative pathway, the latter accounted for by a single enzyme, the alternative oxidase (AOX). Electron transfer through the cytochrome pathway is coupled to the synthesis of ATP while the alternative oxidase catalyses the oxidation of reduced ubiquinone without formation of an electrochemical gradient and consequently without any ATP production (Vanlerberghe and McIntosh 1997, McDonald et al. 2002). Cyanide-resistant and SHAM-sensitive respiration were reported in all higher plant species studied so far and in numerous species of algae, fungi and yeast (Vanlerberghe and McIntosh 1997).

AOX is encoded by two nuclear gene subfamilies and consists of one to three proteins (32–39 kDa) depending on the plant species (Considine et al. 2002). *aox1* is induced by a large number of stress conditions (Vanlerberghe and McIntosh 1997, Considine et al. 2002) and is present in both monocotyledons and eudicotyledons whereas *aox2* is constitutively or developmentally expressed in eudicotyledons but is considered to be absent in monocotyledons (Considine et al. 2002). An increase in AOX activity usually corresponds to an increase in the amount of AOX protein (Vanlerberghe and McIntosh 1997). Besides that, AOX activity is determined by the redox poise of ubiquinone, the redox status of the AOX disulphide bond and the presence of pyruvate which is an allosteric activator of AOX (Vanlerberghe and McIntosh 1997). Alternative oxidase has a relatively low affinity for oxygen compared to cytochrome oxidase, with K_m (O_2) values exceeding 30 μ*M* (Affourtit et al. 2001).

Plant mitochondria may differ in structure, activity and protein composition depending on their metabolic activity and function (Thompson et al. 1998). Low oxygen concentrations in root tissue resulting from an anaerobic environment allow for limited cytochrome oxidase activity (Ricard et al. 1994), while the behaviour of AOX is unknown. When, after anaerobiosis, plants were exposed to aerobic conditions, a rapid increase in root respiration, as the result of glycolytic product accumulation and high energy demand, was observed in wheat seedlings (Albrecht and Wiedenroth 1994). This indicates that mitochondria, after several days of hypoxia, were able to quickly restore their oxidative activity. Also during the post-hypoxic period, symptoms of oxidative stress manifested by activation of antioxidative defence systems were observed (Biemelt et al. 1998).

The aim of our study was to characterize mitochondrial activity and alternative oxidase expression under hypoxic and post-hypoxic conditions. We compared the activities of mitochondria isolated from barley roots grown on an aerated culture medium with mitochondria isolated from roots after 5 days of growth in a culture medium devoid of oxygen (hypoxic roots) and also with mitochondria isolated from roots subjected to 24 h of aerobic treatment following a hypoxic period (post-hypoxia). AOX levels were assessed by a combination of assays including AOX transcript level, protein level and capacity of the enzyme as suggested by McDonald et al. (2002).

Materials and methods

Plant material and growth conditions

Barley caryopses (*Hordeum vulgare* L. cv. Gregor) were germinated on moistened filter paper and 2-day-old seedlings were transferred to 3.5-l boxes with complete Knop medium (Rychter and Mikulska 1990) flushed with air. After 1 day, one part of the seedlings was left for 6 or 7 days on aerated medium (control). A second part was transferred to 3.5-l boxes with Knop medium in which the oxygen concentration was approximately 0.62 μ*M* (measured using oxygen meter PO-551; Elmetron, Zabrz, Poland) and was kept for 5 days with continuous flushing of pure nitrogen to the growth medium and shoots exposed to air. Such treatment created the hypoxic conditions in root tissue since some oxygen was transported from the shoots (Erdmann and Wiedenroth 1988). After 5 days of hypoxia, part of the seedlings were transferred to Knop medium and flushed for 24 h with air (post-hypoxic treatment).

The nutrient solution was supplemented every day. Plants were grown under a 16-h photoperiod at 150 $\mu mol\, m^{-2}\, s^{-1}$ PAR (daylight and warm white 1 : 1, LF-40 W; Pilá, Poland) day/night temperature of 25/20°C and 60/70% relative humidity.

Enzyme assays and metabolite concentration

Alcohol dehydrogenase (EC 1.1.1.1) and lactate dehydrogenase (EC 1.1.1.27) used as markers of anaerobiosis were assayed according to Russell et al. (1990) and Hanson and Jacobsen (1984), respectively. Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured according to Wigge and Gardeström (1987) in a medium consisting of 0.45 *M* mannitol, 10 m*M* phosphate buffer pH 7.2 and 100 m*M* KCl, 50 μ*M* cytochrome *c* (reduced with a few crystals of sodium dithionite) in the presence of 0.04% (w/v) Triton X-100. PEP carboxylase was assayed according to Fontaine et al. (1999). Pyruvate concentration was measured according to Lamprecht and Heinz (1985) and malate concentration as described by Möllering (1985).

Isolation of mitochondria

Mitochondria were isolated from 60 g of roots ground with a cold mortar and pestle in 300 ml of homogenization medium: 0.45 *M* mannitol, 30 m*M* MOPS, pH 7.4, 5 m*M* EDTA, 5 m*M* DTT, 0.6% (w/v) PVP-40, 10 m*M* ascorbate and 1% (w/v) BSA. The homogenate was filtered through miracloth and the mitochondria were isolated by differential centrifugations: 1500 *g* for 10 min, 9600 *g* for 20 min. The pellet was suspended in 20 ml of homogenization medium and centrifuged again at 1000 *g* for 10 min and 9600 *g* for 20 min. The pellet was re-suspended in 4 ml of homogenization medium and the mitochondria were purified on a discontinuous Percoll gradient using a modified method of Nishimura et al. (1982). The discontinuous gradient was composed of: 4.5 ml 60% (v/v) Percoll, 6 ml 45% (v/v) Percoll, 6 ml 28% (v/v) Percoll and 6 ml 5% (v/v) Percoll all in 0.45 *M* mannitol, 20 m*M* MOPS, pH 7.2, 0.1% (w/v) BSA and 1% (w/v) PVP-25. The gradient was centrifuged at 7000 *g* for 40 min. The mitochondrial fraction appeared at the interface between 45 and 28% (v/v) Percoll layer. The mitochondrial fraction was collected and washed in a medium containing: 0.45 *M* mannitol, 10 m*M* MOPS, pH 7.2, 1 m*M* EDTA and 0.5% (w/v) BSA. The pellet was suspended in about 150 µl of washing medium.

Mitochondrial integrity was calculated by comparison of cytochrome *c* oxidase (EC 1.9.3.1) activity measured in isotonic medium first in the absence, then in the presence of 0.04% (w/v) Triton X-100 to disrupt mitochondrial membranes.

Respiration measurements

Roots, approximately 0.1–0.2 g FW, were rinsed and placed in 5 ml of air-saturated 0.1 *M* MOPS-KOH, pH 7.2 in a Clark-type electrode (Yellow Springs, Ohio, USA). The oxygen uptake was measured at 25°C for a period of approximately 10 min. The roots were then blotted and weighed.

Oxygen uptake by isolated mitochondria was measured using a Clark-type electrode (Hansatech, King's Lyn, UK) at 25°C in 0.5 ml of incubation medium containing: 0.45 *M* mannitol, 10 m*M* phosphate, pH 7.2, 5 m*M* $MgCl_2$, 10 m*M* KCl, 0.1% (w/v) BSA and 60–100 µg of mitochondrial protein. Substrates were used at the following concentrations: 10 m*M* malate (with 2 m*M* glutamate), 20 m*M* succinate, 1 m*M* NADH (with 1 µ*M* $CaCl_2$) and 80 µ*M* ADP. The cytochrome pathway was inhibited with 800 µ*M* KCN in the presence of 1 m*M* pyruvate and 10 m*M* DTT to activate the alternative pathway. The alternative pathway was inhibited with 750 µ*M* SHAM.

Ubiquinone extraction and determination

Root extracts were prepared from 1 g of fresh root tissue. Roots were cut and immediately dropped in liquid nitrogen, ground to a powder with mortar and pestle and homogenized in the presence of 10 ml 0.2 *M* $HClO_4$ in methanol (0°C). The ubiquinone (UQ) assays were performed according to the method described by Wagner and Wagner (1995). After evaporation, the extracts were stored at −20°C for a maximum of 2 days. Immediately before use, the UQ extracts were re-suspended with a glass rod in 50 µl of N_2-purged ethanol and analysed by HPLC with a pump system and detector (Pharmacia LKB, Uppsala, Sweden). A reversed-phase column with ethanol: methanol (1 : 1, v/v) was used. Detection of Q was performed at 290 nm and the amounts of UQ_{10} and $UQ_{10}H_2$ were calculated from peak areas using CHROMA FOR WINDOWS version 1.96 program. Commercially obtained ubiquinone-10 (Sigma, Steinhem, Germany) was used as a standard and ubiquinone was reduced to ubiquinol by addition of sodium borohydride.

Pyridine nucleotide extraction and determination

NAD^+ and NADH were extracted according to Carrier and Neve (1979) and determined by the method of Lechevallier et al. (1977) as described by Juszczuk and Rychter (1997).

ATP extraction and determination

One gram of fresh roots were blotted and rapidly homogenized in a pre-cooled mortar with liquid nitrogen essentially as described by Rychter et al. (1992). Briefly, 1 ml 17% perchloric acid were added, the homogenate was centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant was neutralized by mixing with equal volumes of 1,1,2-trichlorotrifluoroethane (Sigma) and tri-*n*-octylamine (Sigma). After centrifugation, the top layer which contained the neutralized extract was decanted and stored at −20°C. The ATP concentration was assayed by the luciferin-luciferase reaction according to Pradet (1967).

SDS-PAGE and immunoblotting

Extracts were prepared from 300 mg of frozen roots. Tissue was crushed to a fine powder with a mortar and pestle in the presence of liquid nitrogen and a small amount of sand. The sample was suspended in 1 ml of buffer (2% (w/v) SDS, 65.5 m*M* Tris-HCl, pH 6.8, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue) centrifuged at 14 000 *g* for 10 min and the supernatant was boiled for 5 min. The boiled supernatant, 40 µg of protein per lane, were separated by SDS polyacrylamide electrophoresis and electotransferred to nitrocellulose membrane (PROTRAN® Nitrocellulose Transfer Membrane, Order no. 4401196; Schleicher & Schuell GmbH, Dassel, Germany) using a semi-dry blotting apparatus. AOX monoclonal antibodies (gift of Dr T. Elthon to Y.J) (Elthon et al. 1989) were used as a primary antibody at a dilution factor of 1/500. Anti-mouse IgG-HRP were used as a secondary antibody at a dilution of 1/25000 (Catalogue no. 170–6516, Bio-Rad, Hercules, CA, USA.). Visualization was performed with a chemiluminescent reagent system (Western blot Chemiluminescence Reagent *Plus*, NEL 103, NEN® Life Science Products, Inc, Boston, MA, USA). Blots were quantified by densitometry (Bio-Rad Model GS 690 Imaging densitometer). The signal intensities were estimated using MULTI ANALYST software after the bands were corrected for background.

Relative quantitative RT-PCR

Total RNA was extracted from barley roots according to the NucleoSpin® RNA Plant Kit Macherey-Nagel manual (Macherey–Nagel, Düren, Germany). One microgram

Table 1. Effect of 5-day hypoxic conditions on growth of barley plants, alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities in root extracts. Mean value ± SD.

	Control	5-days hypoxia	Post-hypoxia
Length of roots (cm)	10.0 ± 0.7	6.7 ± 0.4	–
Length of shoots (cm)	19.0 ± 0.6	16.8 ± 0.5	–
ADH (nmol NADH mg^{-1} protein min^{-1})	29 ± 10	160 ± 22	58 ± 9
LDH (nmol NADH mg^{-1} protein min^{-1})	31 ± 2	72 ± 4	35 ± 5

of total RNA was converted to the first strand of cDNA with a random primer mixture (Random Decamers; Ambion, Huntingdon, UK) at 42°C for 40 min, using AMV reverse transcriptase (F-570S; Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The cDNA was then amplified by PCR with two degenerated primers, AOX1 (5′-TGGACCTGCTTCAGGCCATGG-GAGACGTA) as sense primer and AOX6 (5′-TGR-TTIACRTCICKRTGRTGIGCYTCRTCIGC) as anti-sense primer. One microlitre of the reverse transcription reaction was used directly for PCR amplification under the following conditions: 1 × buffer supplemented to 2 m*M* $MgCl_2$, 100 µ*M* deoxynucleotides; 5 pmol of each primer AOX1 and AOX6 and one unit of Taq polymerase (REDTaq® DNA Polymerase, D-5684; Sigma) in a final volume of 25 µl. PCR amplification consisted of a first cycle of 10 min at 94°C followed by 35 cycles each consisting of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C, followed by a final polymerization step at 72°C for 10 min. An endogenous standard was amplified simultaneously as a control (QuantumRNA® Universal 18S Internal Standards; Ambion). The number of cycles was established empirically to amplify the target DNA and the control in a linear range.

Protein determination

The protein concentration was determined with the Bio-Rad DC Protein Assay for Western blotting or the method of Bradford (1976) for enzymatic analysis and mitochondrial protein estimation.

Results

Hypoxic treatment of barley roots led to increased activities of marker enzymes of anaerobiosis: lactate dehydrogenase and alcohol dehydrogenase, indicating induction of fermentation processes (Table 1). Oxygen deprivation also influenced barley seedlings growth, since shoot and root length was about 10 and 30% smaller after hypoxia than in control plants, respectively (Table 1).

Hypoxic conditions increased the total NAD(H) pool in root tissue but the most pronounced was the increase of NADH concentration, leading to a slightly but significantly higher ratio NADH/NAD$(H)_{total}$ (Table 2). After 24 h of post-hypoxia, both NADH and NAD^+ concentrations returned to levels slightly above those observed in control roots but the ratio NADH/NAD$(H)_{total}$ remained higher than under control conditions (Table 2).

The highly reduced state of hypoxic root tissue was also indicated by the level of reduction of the ubiquinone pool (Table 2). Oxygen deprivation led to a rise in the size and reduction level of the total ubiquinone pool. The reduction level was approximately 30% in control roots and 66% in hypoxic roots. After 24 h of post-hypoxia the UQ pool remained highly reduced and the amount was even higher than in control roots (Table 2).

The concentration of pyruvate in hypoxic roots was similar to that in control plants (Fig. 1A) whereas the malate concentration increased (Fig. 1B) together with an increase in PEP carboxylase activity (Fig. 1C). This tendency continued during post-hypoxia (Fig. 1B and C).

After 5 days of hypoxia, the respiratory activity of whole roots, measured under aerobic conditions, was higher than that of control plants and remained at elevated levels after 24 h of re-aeration (Table 3). The increase in respiration immediately after the hypoxic period was accompanied by a higher tissue ATP concentration, which became less pronounced after 24 h of post-hypoxia (Table 3).

Purified mitochondria, isolated from the control, hypoxic and post-hypoxic barley roots, showed high

Table 2. Effect of hypoxic and post-hypoxic conditions on pyridine nucleotide and ubiquinone concentration in barley roots. Data are given as mean value ± SD from three to five independent preparations.

	Control	5-days hypoxia	Post-hypoxia 24 h
Pyridine nucleotides (nmol g^{-1} FW)			
NAD^+	3.1 ± 0.1	7.5 ± 0.9	3.2 ± 0.1
NADH	0.6 ± 0.0	1.9 ± 0.1	0.8 ± 0.0
NAD^+ + NADH	3.7 ± 0.1	9.4 ± 1.1	4.0 ± 0.1
NADH/NADH + NAD^+	0.16	0.20	0.20
Ubiquinone (nmol g^{-1} FW)			
UQ	0.43 ± 0.11	0.34 ± 0.07	0.61 ± 0.08
UQH_2	0.18 ± 0.08	0.74 ± 0.12	0.99 ± 0.11
UQ + UQH_2	0.61 ± 0.12	1.08 ± 0.12	1.60 ± 0.17
UQH_2/UQ + UQH_2	0.34	0.66	0.63

integrity, usually between 80 and 90% (Table 4). Respiratory rates were the highest with exogenous NADH as substrate (Table 4). After hypoxic treatment, barley root mitochondria had higher respiratory rates than mitochondria from control roots with all substrates

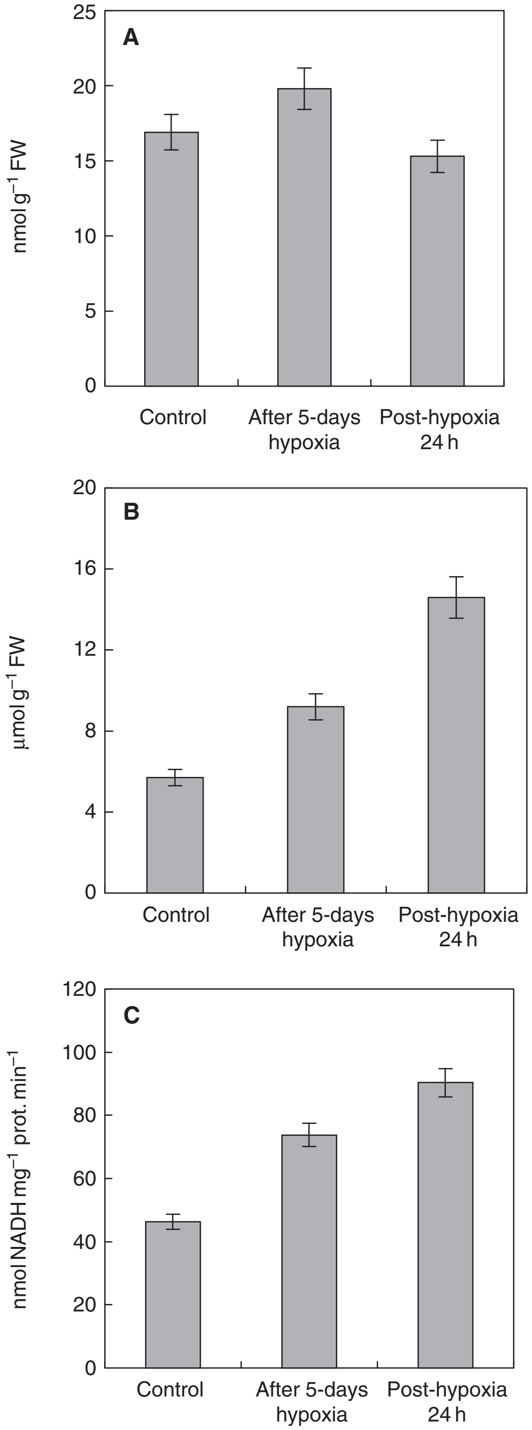

Fig. 1. Concentration of pyruvate (A), malate (B) and phosphoenolopyruvate carboxylase activity (C) in control, hypoxic and post-hypoxic barley root tissues.

tested, the most pronounced increase was with NADH as substrate, about 60% higher than control (Table 4). In contrast, after 24 h of post-hypoxia, the respiratory rates in isolated mitochondria were similar to that of the control (Table 4). Whatever the treatment, the levels of respiratory control and ADP/O ratio were unchanged for all substrates tested, similarly as cytochrome *c* oxidase activity (Table 4).

Respiration of mitochondria from control barley roots was inhibited by cyanide, but a cyanide-insensitive oxygen uptake was observed after addition of pyruvate and DTT with all the substrates tested (Table 4). AOX capacity in control root mitochondria was about 30–40% of the state 3 respiration rate and was completely inhibited by SHAM. In mitochondria isolated from hypoxic roots no AOX capacity was detected in the presence of cyanide, pyruvate and DTT, except some low capacity observed with malate. In post-hypoxia, respiration after the addition of cyanide and in AOX-activated conditions was 25–40% of the state 3 respiration rate and was also completely inhibited by SHAM (Table 4). Thus, AOX capacity in post-hypoxia was similar to that observed in mitochondria isolated from control roots.

AOX capacity depends primarily on AOX protein levels (Vanlerberghe and McIntosh 1997). The amount of AOX protein was determined as the immunoreactive signal with AOX antibody in control, hypoxic and post-hypoxic roots. We found that most of the AOX protein was present in its oxidized homodimeric form in mitochondria isolated from control roots. In whole-root extracts, strong cross reacting bands at 32–36 kDa, corresponding to the reduced form of AOX, were detected in control plants (Fig. 2). After 5 days of hypoxia (H), a faint band (about 10–14% relative to control) was observed on Western blots and after 24 h of post-hypoxia (PH), AOX protein bands appeared again and the amount of AOX protein detected was about 50% of that observed in control roots (Fig. 2). We also observed a protein band of apparent molecular mass of 50 kDa on immunoblots. Although the identity of this band is not known, we speculate that it could be a degraded product of the oxidized form (which has a mass of about 65–70 kDa) or a precursor form of AOX as proposed by Albury et al. (1996) who observed similar AOX band in *S. pombe*.

To examine AOX transcript levels, we designed two oligonucleotides (AOX1 and AOX6) corresponding to highly conserved regions among plant alternative oxidase sequences. The PCR amplification of cDNA using these primers generated the expected 620-bp fragment. Comparing the RT-PCR products obtained from the same amount of total RNA, there was no significant difference in AOX transcript levels between control (C), hypoxic (H) and post-hypoxic (PH) roots (Fig. 3).

Discussion

Among several environmental conditions controlling AOX abundance *in vivo*, oxygen availability is usually

Table 3. Effect of hypoxic and post-hypoxic conditions on respiration of whole roots measured in air-saturated buffer (nmol O_2 min^{-1} g^{-1} FW) and ATP concentration (nmol g^{-1} FW) of barley plants. Data are presented as mean values ± SD.

	Control	5-days hypoxia	Post-hypoxia 24 h
Respiration of whole roots	108.9 ± 15.5	183.3 ± 11.8	157.6 ± 11.2
ATP concentration	34.7 ± 6.9	67.0 ± 6.1	46.8 ± 2.0

neglected and competition for oxygen as electron acceptor is seldom considered as a factor controlling the flow of electrons between the cytochrome and alternative pathways. When root tissue is exposed to air after hypoxic treatment, oxygen concentration in the tissue rapidly increases resulting in higher respiration and activation of antioxidative defence systems (Biemelt et al. 1998). Increased respiratory activity of barley roots after hypoxia compared to control corresponded to high ATP concentrations (Table 3). Similar results of increased respiration rate after hypoxia were obtained in wheat roots by Albrecht and Wiedenroth (1994). We observed that more reduced substrates accumulated during the hypoxic period, for example NADH (Table 2) or malate (Fig. 1B) and the decrease of NADH in the post-hypoxic period (Table 2). Immediately after hypoxia the higher respiratory rates were observed in isolated mitochondria with all tested substrates, especially with external NADH, but the respiration was highly cyanide sensitive even under AOX-activating conditions (Table 4) indicating mainly the participation of the cytochrome pathway. After 24 h of post-hypoxia the respiratory rates and AOX capacity were similar to the control (Table 4).

During hypoxia, low AOX activity in the intact tissue may result from decreasing concentrations of oxygen at the mitochondrial level and low AOX affinity for oxygen compared to cytochrome oxidase (Affourtit et al. 2001). AOX capacity found in isolated mitochondria from barley roots grown on aerated medium, oxygen-free medium (hypoxia) and on 24 h-aerated medium (post-hypoxia) corresponded to AOX protein levels determined by Western blot as commonly observed in other plants and tissues (Vanlerberghe and McIntosh 1997). Apparently, in barley root tissues exposed to 5 days of hypoxia, oxygen availability limited AOX protein synthesis (or increased the rate of breakdown) resulting in a low level of AOX capacity. We found only a faint band of AOX protein present at the end of the hypoxic period (Fig. 2). The amount of AOX protein started to increase after the exposure of hypoxic roots to an oxygen environment for 4 h (results not shown) and after 24 h of aerobic treatment this amount increased together with AOX capacity to a level comparable to that of the control (Table 4, Fig. 2). The expression of AOX due to low oxygen concentration treatment may also depend on other experimental conditions and plant material. In soybean cell culture, the amount of AOX protein was higher after 16 h incubation in an anoxic atmosphere in comparison with that in aerated cell culture (Amor et al. 2000). It has been proposed that AOX is one of the mechanisms for minimizing reactive oxygen species (ROS) production (Purvis 1997, Maxwell et al. 1999, Møller 2001). Low expression of AOX in barley roots after hypoxia may explain the observed symptoms of oxidative stress (Albrecht and Wiedenroth 1994, Biemelt et al. 1998).

Regulation of gene expression under anaerobic conditions may be both at the transcriptional and translational

Table 4. Respiratory activity of mitochondria isolated from barley roots grown on aerated medium (control), after 5 days of hypoxia and additional 24 h post-hypoxia. Substrate concentrations were: 10 m*M* malate-2 m*M* glutamate, 20 m*M* succinate, 1 m*M* NADH and 80 μ*M* ADP. The term 'AOX capacity' refers to SHAM-sensitive oxygen consumption in the presence CN^-, pyruvate and DTT. Data are presented as mean values ± SD. ND, not detected. Data are averages from three to six mitochondrial isolations.

	Control	Hypoxia	Post-hypoxia
Oxygen consumption (state 3) (nmol O_2 consumed mg^{-1} protein min^{-1})			
Malate	74 ± 10	99 ± 12	51 ± 19
Succinate	135 ± 42	190 ± 27	116 ± 6
NADH	152 ± 41	239 ± 50	162 ± 30
Respiratory control			
Malate	2.1 ± 0.6	1.8 ± 0.2	1.9 ± 0.5
Succinate	1.5 ± 0.2	1.7 ± 0.2	2.1 ± 0.4
NADH	1.4 ± 0.2	1.9 ± 0.2	2.2 ± 0.2
ADP/O			
Malate	2.4 ± 0.5	2.1 ± 0.4	2.6 ± 0.3
Succinate	1.7 ± 0.4	1.4 ± 0.2	1.7 ± 0.2
NADH	1.6 ± 0.6	1.0 ± 0.2	1.6 ± 0.2
AOX capacity			
Malate	35 ± 7	7 ± 3	24 ± 5
Succinate	44 ± 5	ND	39 ± 9
NADH	44 ± 10	ND	45 ± 7
Integrity of mitochondrial membranes (%)	87 ± 3	86 ± 4	84 ± 6
COX activity (nmol cyt *c* mg^{-1} protein min^{-1})	935 ± 58	890 ± 27	947 ± 51

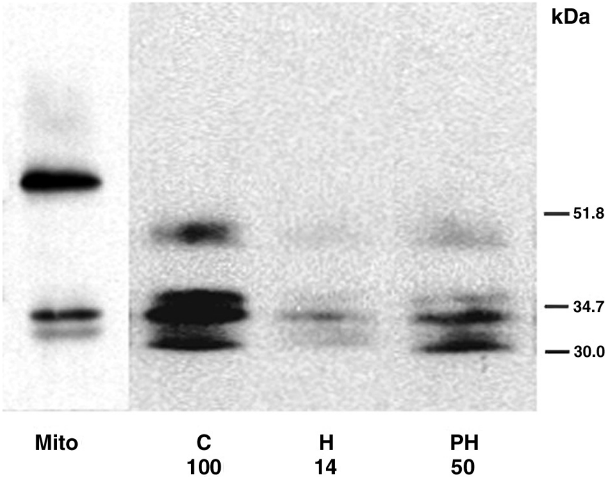

Fig. 2. Immunoblotting of AOX protein from isolated mitochondria and barley whole-root extracts. Mitochondria (Mito) were isolated from roots of control plants. The extracts were prepared from barley roots grown on aerated medium (C), after 5 days of growth in culture medium devoid of oxygen (H) and from roots subjected to 24 h of aerobic treatment after the hypoxic period (PH). Mitochondrial protein (10 μg) and whole-root extracts (40 μg protein per lane) were separated by SDS-PAGE, immunoblotted with AOX monoclonal antibody and visualized by chemiluminescence. The signal intensities were estimated using MULTI-ANALYST software after the bands were corrected for the background, results are expressed relative to control. Arrows indicate molecular masses (kDa) of standard protein markers. Representative results are shown.

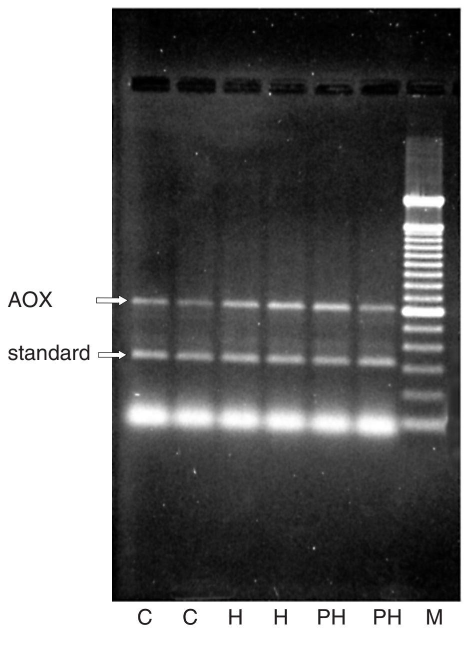

Fig. 3. Detection of barley alternative oxidase mRNA by PCR amplification. Total RNA was isolated from barley roots grown on aerated medium (C), after 5 days of growth in culture medium devoid of oxygen (H) and from roots subjected to 24 h of aerobic treatment after the hypoxic period (PH). M, Marker DNA. One microgram of each sample was reverse-transcribed and PCR-amplified using specific primers as described in Materials and methods. Representative results are shown.

level (Huq and Hodges 1999 and references therein). In rice, an anoxia-tolerant plant, hypoxic treatment decreased the transcript level of AOX and it was demonstrated that Ca^{2+} is a physiological transducer of a low-oxygen signalling pathway for expression of the *AOX* 1a gene (Tsuji et al. 2000). It was observed previously that the amount of AOX transcripts was positively correlated to the amount of AOX protein detected by immunoblot (Cruz-Hernandez and Gomez-Lim 1995, Aubert et al. 1997). However, in barley roots, the examination of AOX mRNA by a semi-quantitative RT-PCR assay, indicated that the AOX transcript level was comparable in control, hypoxic and post-hypoxic roots (Fig. 3). Since we also used primers for 18S RNA as an internal standard in the PCR-experiments, these data reflect the actual amounts of AOX transcripts. The presence of AOX transcripts after 5 days of hypoxia but with almost no AOX protein and AOX capacity indicate that the regulation of AOX expression by oxygen availability in barley roots may take place at the translation level. Translational regulation of nuclear-encoded genes has been shown to occur in response to environmental conditions (Cohen and Mayfield 1997). It was observed that in potato tubers during hypoxia translation activity was inhibited and previously translated mRNAs were not degraded but remained associated with polysomes for up to 24 h (Crosby and Vayda 1991). A similar situation may be found in hypoxic barley roots.

The changes in the redox state of barley root tissue under hypoxic conditions were determined by the NAD reduction level. During hypoxia, glycolysis was stimulated with an activation of the fermentative pathways indicated by increased activities of lactate and alcohol dehydrogenases (Fig. 1). NADH accumulation during hypoxia, could result from an excess of supply over its oxidation through fermentative processes. We also observed a transient increase in the total nucleotide pool during hypoxia (Table 2). These results are hard to explain, similar observation was made by Reggiani et al. (1985).

A higher reduction state of hypoxic root tissue was accompanied also by an increased malate concentration (Fig. 1). Malate accumulation could be the result of increased PEP carboxylase activity (Fig. 1). Contrary to the results obtained by Good and Muench (1993), prolonged hypoxic treatment of barley roots did not increase pyruvate concentration (Fig. 1). This could be explained by unchanged rates of malic enzyme activities (both cytosolic and mitochondrial) and an increase in alanine concentration in hypoxic as compared to control roots (results not shown), similarly to results reported by Good and Muench (1993).

Low oxygen concentration also resulted in an increase in the total amount of UQ in barley roots (Table 2). The reason for an increased amount of UQ during hypoxia is not clear but a similar increase was found in cauliflower plants treated with a high oxygen concentration (Popov et al. 2001), cold-stored bell pepper (Wagner and Purvis 1998, Popov et al. 2001) and antimycin A-treated petunia cells (Wagner and Wagner 1997). It is known that a considerable part of UQ in plant mitochondria often remains reduced in the absence of respiratory substrates (Wagner and Purvis 1998). In barley roots after hypoxia, a great increase in reduced ubiquinone was observed (Table 2). It has been proposed that the redox-inactive ubiquinone could be localized in the outer mitochondrial membrane and might not be linked to the electron transport chain in the inner membrane (Ribas-Carbo et al. 1995). The increase in UQ concentration was connected with an increasing amount of the inoxidizable part of UQ pool during cold storage of green pepper (Wagner and Purvis 1998) and antimycin A treatment of petunia cell culture (Wagner and Wagner 1997). In animal tissue, where ubiquinol is known as a very effective antioxidant, reduced ubiquinone in the inner mitochondrial membrane has similar antioxidant efficiency to tocopherol but the molar concentration of UQ is 10-fold greater than tocopherol (Frei et al. 1990 and references therein).

Finally, in post-hypoxia, the increase of respiration together with severely reduced energy balance could promote free radical production. Positive correlation of free radical content and oxygen consumption was found in germinating maize during desiccation stress (Leprince et al. 1994). Oxidative stress during post-hypoxia was indicated by Biemelt et al. (1998) and Albrecht and Wiedenroth (1994). Consequently, an increase in the expression of AOX protein during post-hypoxia in barley roots may help to minimize ROS production and the higher pool of ubiquinol may act as a scavenger of ROS to diminish the oxidative stress after hypoxia. Our results indicate that besides the amount of AOX protein and the reduction state of mitochondria, the concentration of oxygen in the tissue may be one of the control mechanisms of AOX activity in barley roots.

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