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Review

Alternative oxidase in higher plants✪

Izabela M. Juszczuk and Anna M. Rychter✉

Department of Plant Bioenergetics, Institute of Experimental Plant Biology, Warsaw University, Miecznikowa 1, 02-096 Warszawa, Poland

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Plant respiratory chain branches at the level of ubiquinone from where the electrons flow through the cytochrome pathway or to alternative oxidase. Transfer of electrons from ubiquinone to oxygen by alternative oxidase has a non-protonmotive character and, by bypassing two sites of H^+ pumping in complexes III and IV, lowers the energy efficiency of respiration. In this paper we review theoretical and experimental studies about the structure and possible function of alternative oxidase. The evidence for specific gene expression dependent on the physiological, developmental and environmental conditions is also described. We underline the physiological role of alternative oxidase as a "survival" protein that allows plants to cope with the stressful environment.

Cyanide-resistant respiration was discovered at the beginning of the 20th century as a curiosity in thermogenic plants during anthesis and was later found to be a typical feature of plant respiration. The phenomenon of respiration resistant to cyanide is connected with the presence in the respiratory chain of an additional terminal oxidase – alternative oxidase (AOX). The presence of AOX in higher plant mitochondria poses an intriguing question regarding its regulation *in vivo* and the role in plant metabolism. The progress in AOX research was achieved by biochemical studies but also in a great part by

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✉Address correspondence to Anna M. Rychter; tel.: (48 22) 554 3005; fax: (48 22) 554 2022; e-mail: anna@rychter.com

Abbreviations: AOX, alternative oxidase; HR, hypersensitive response; PCR, polymerase chain reaction; PCD, programmed cell death; Q, ubiquinone; Qr, reduced ubiquinone, ubiquinol; ROS, reactive oxygen species; SA, salicylic acid.

the identification of AOX gene families and the utilization of transgenic plants with altered levels of AOX to study the regulation of its expression. The progress included an increased understanding of the biochemical/metabolic mechanisms regulating AOX activity (McDonald *et al.,* 2002; Millar *et al.,* 1993; Umbach & Siedow, 1993; Vanlerberghe & McIntosh, 1997). Several excellent reviews in the last few years have covered the subject of structure, regulation and the possible role of AOX in plant mitochondria (Vanlerberghe & McIntosh, 1997; Berthold *et al.,* 2000; Siedow & Umbach, 2000; Affourtit *et al.,* 2002; Considine *et al.,* 2002; McDonald *et al.,* 2002; Berthold & Stenmark, 2003).

For most species examined under particular stress influence and/or growth conditions AOX presence is indicated by combination of biochemical (oxygen isotope discrimination and maximum activity/capacity) and molecular assays (AOX transcript level, protein level) (McDonald *et al.,* 2002, and references therein). More often AOX is studied in details using transgenic plants or *in vitro* cell cultures. Most information on *AOX* genes and AOX regulation comes from experiments on *Sauromatum guttatum, Arabidopsis thaliana, Glycine max, Pisum sativum* and *Nicotiana tabacum*.

Although we have learnt a lot about the molecular nature of the alternative oxidase its role in plant respiratory metabolism is still a matter of debate. The present review summarises our present knowledge and understanding of the regulation and role of alternative oxidase in higher plant respiration.

STRUCTURE AND REGULATION OF ACTIVITY

AOX branches from the main respiratory chain at the level of ubiquinone and catalyses the four-electron reduction of oxygen to water (Fig. 1). In contrast to electron transfer by the

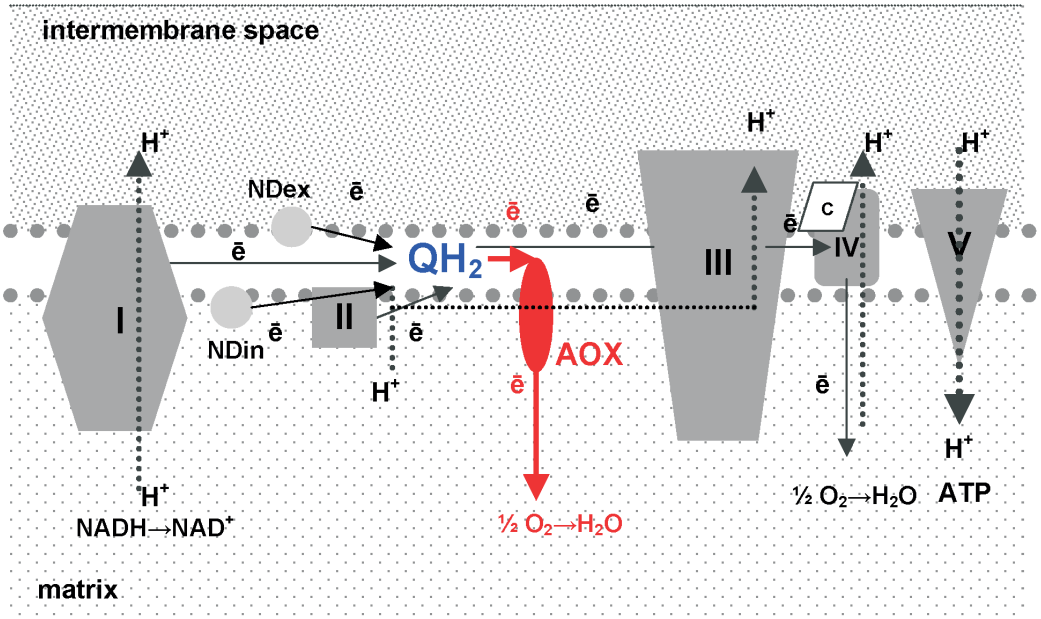

Figure 1. Alternative oxidase in plant mitochondrial respiratory chain.

Alternative oxidase catalyzes cyanide-resistant reduction of oxygen to water without translocation of protons across the inner mitochondrial membrane, and thus functions as a non-energy-conserving component of the respiratory electron transfer chain. I, NADH – ubiquinone oxidoreductase; II, succinate – ubiquinone oxidoreductase; III, ubiquinol – cytochrome *c* oxidoreductase; IV, cytochrome *c* oxidase; V, ATP synthase; AOX, alternative oxidase; c, cytochrome *c*; QH_2, ubiquinol (reduced ubiquinone); ND ex/in, NAD(P)H dehydrogenases, respectively external/internal.

cytochrome chain (complex III and IV), AOX does not pump H^+, so the transfer of electrons by AOX does not generate a transmembrane potential and the drop in free energy between ubiquinol and oxygen is dissipated, or 'wasted', as heat (Vanlerberghe & McIntosh, 1997).

The enzyme remains difficult to purify to homogeneity in a stable, active form (Berthold & Siedow, 1993; Bonner *et al.,* 1986; Elthon & McIntosh, 1987; Huq & Palmer, 1978; Zhang *et al.,* 1996). However, a few models of AOX structure delivered from complete cDNA sequences encoding the alternative oxidase protein have been presented during the last decade. It was proposed (Umbach & Siedow, 1993; Siedow *et al.,* 1995) that AOX is an approximately 32-kDa homodimeric integral mitochondrial inner membrane protein with a non-haem di-iron centre and two membrane-spanning helices connected by a single α-helix at the inter-membrane space. Subsequently, in 1999, it was pointed out by Andersson and Nordlund that this model was not in agreement with the structure of other well-characterized di-iron proteins. The revised model considered AOX not as a trans-membrane protein but rather an interfacial protein peripherally associated with the matrix side of the inner membrane (Andersson & Nordlund, 1999; Siedow & Umbach, 2000). Such a location is generally supported by site-directed mutagenesis experiments (Albury *et al.,* 2002). However, the question is, to what extent is the structure of AOX as a di-iron centre interfacial mitochondrial protein conserved? (Affourtit *et al.,* 2002; Moore *et al.,* 2002; Berthold and Stenmark, 2003).

Studies *in vitro* showed that the activity of AOX increased considerably upon reduction of the intersubunit disulfide bridge, yielding a non-covalently linked homodimeric protein (Umbach & Siedow, 1993; Umbach *et al.,* 1994). The reduced enzyme is further activated by α-keto acids, particularly pyruvate, which forms a thiohemiacetal with a protein-derived sulfhydryl moiety (Umbach & Siedow, 1996; Rhoads *et al.,* 1998; Umbach *et al.,* 2002). The suggested catalytic site of AOX activation by α-keto acids involves at least one cysteine residue, highly, but not universally, conserved in the known plant AOX sequences (Affourtit *et al.,* 2002; Umbach *et al.,* 2002).

The structure of the ubiquinone (Q) binding site(s) of AOX was proposed by Berthold *et al.* (1998; 2000). It is based on the similarity in structure and chemical catalysis between AOX and the R2-type di-iron proteins (Berthold & Stenmark, 2003). The two electrons needed to reduce the diferric centre of AOX would be delivered from reduced ubiquinone (Berthold *et al.,* 2000). A similar model of the AOX catalytic cycle was presented by Affourtit *et al.* (2002) and contained the analogues of the hydroxylase component of methane monooxygenase (MMOH) and AOX binuclear centre. Analysis of site-directed mutant proteins in combination with the recent development of more efficient bacterial over-expression systems should help test the structural and functional hypotheses.

GENE EXPRESSION

AOX in higher plants is encoded by the nuclear genome (Elthon *et al.,* 1989). The first AOX antibody was raised against the protein isolated from *Sauromatum guttatum* and gene (*AOX1*) was cloned (Elthon *et al.,* 1989; Rhoads & McIntosh, 1991). By the use of techniques such as cDNA library screening, PCR (polymerase chain reaction) with primers designed from highly conserved regions or complementation methods, AOX was found to be encoded by a small family of nuclear genes (subfamilies *AOX1, AOX2a* and *AOX2b,* formerly named *AOX3*) among a wide variety of nonthermogenic monocotyledon and eudicotyledon plants (Considine *et al.,* 2002, and references therein). Differential expression of the soybean (*G. max*) genes in response to developmental cues, environmental perturba-

tions or site-directed mutagenesis was studied (Millar & Day, 1997; Finnegan *et al.,* 1997; 1998; McCabe *et al.,* 1998; Karpova *et al.,* 2002). During soybean development two AOX2-type isoenzymes, AOX2-a and AOX2-b, have been described (Finnegan *et al.,* 1997; McCabe *et al.,* 1998).

The *AOX1* gene is most widely known for its expression by stress stimuli in many tissues and is present in both monocotyledon and eudicotyledon plant species. On the other hand, the *AOX2* gene is usually constitutively or developmentally expressed in eudicotyledon species but is absent from the genomes of all monocotyledon species examined to date (Considine *et al.,* 2002, and references therein). There is a need to investigate the apparent absence of the *AOX2* gene from monocotyledon plants. It has been proposed that *AOX1* gene expression constitutes the plant's adaptation to stress factors whereas *AOX2* expression depends on the tissue and developmental stage and is required for a "housekeeping" function in respiratory metabolism but does not respond to stress (Vanlerberghe & McIntosh, 1997; Considine *et al.,* 2002). Among other species the soybean and *A. thaliana* gene expression is best characterised and fits this model. In these plants *AOX1* gene expression is limited to conditions of extreme environmental stress, whereas *AOX2* is not induced by stress but is expressed during normal seed development (Finnegan *et al.,* 1997; McCabe *et al.,* 1998; Millar *et al.,* 1997; Saisho *et al.,* 1997; 2001a; 2001b; Simons *et al.,* 1999; Tanudji *et al.,* 1998; 1999).

The background of the studies of *AOX* genes and their expression were recently summarised in details (Vanlerberghe & McIntosh, 1997; Simons & Lambers, 1999; McDonald *et al.,* 2002). Alternative oxidase gene expression under a variety of biotic and/or abiotic stress conditions indicates that AOX belongs to the stress-induced plant proteins but its constitutive presence in some tissues determines its potential role in integrated plant metabolism. Several environmental as well as developmental conditions involve changes in *AOX* mRNA, protein, and/or cyanide-resistant respiration. These are, for example, pathogen infections (Simons *et al.,* 1999; Maxwell *et al.,* 2002; Ordog *et al.,* 2002), freezing injury, cold (Vanlerberghe & McIntosh, 1992; Purvis & Shewfelt, 1993), phosphate deficiency (Parsons *et al.,* 1999; Juszczuk *et al.,* 2001), changes in oxygen concentration (Szal *et al.,* 2003) and also flowering (Raskin *et al.,* 1987), ageing (Hisher & McIntosh, 1990; Maxwell *et al.,* 2002), and fruit ripening (Cruz-Hernandez & Gomez-Lim, 1995; Considine *et al.,* 2001). However, in some stresses like cold, it has been reported that AOX is not always induced (Gonzales-Meler *et al.,* 1999; Svensson *et al.,* 2002).

Based on a sophisticated molecular approach it has been shown how *AOX* gene expression may change in response to an experimental treatment in a developmental-specific, tissue-specific, and stress-specific manner. In *S. guttatum,* mRNA for AOX1 increased before thermogenesis and after application of salicylic acid to the immature appendix (Rhoads & McIntosh, 1992). In *Arabidopsis* the concentration of mRNA for AOX changed in response to inhibitors of electron transport or ATP synthesis (Saisho *et al.,* 1997; 2001a; 2001b). In soybean (Finnegan *et al.,* 1997; McCabe *et al.,* 1998) the expression of three *AOX* genes in the roots and cotyledons differs in the amount of particular gene transcripts and protein levels. In soybean cotyledons the relative amount of both *AOX2* transcripts and protein increase upon greening of dark-grown seedlings (McCabe *et al.,* 1998). In potato, *AOX* mRNA and AOX protein accumulate during ageing (Hisher & McIntosh, 1990). Under phosphate deficiency in bean roots the level of AOX protein increased (Juszczuk *et al.,* 2001). In contrast to the common reaction to stress factors of inducing AOX (see the references above), low oxygen conditions caused no change in *AOX* transcript but a large decrease in AOX protein

level and AOX activity in barley roots (Szal *et al.,* 2003).

During the last decade, alternative oxidase gene(s) expression has been specifically altered through genetic transformations in both "sense" and "antisense" orientations. The most popular model system is the sense and antisense tobacco lines (Vanlerberghe *et al.,* 1994; 1998; 2002; Parsons *et al.,* 1999; Yip & Vanlerberghe, 2001; Robson & Vanlerberghe, 2002). Other transgenic plant systems have been also constructed with potato mutants (Hisher *et al.,* 1996) and *Arabidopsis* mutants (Johnson-Potter *et al.,* 2001). Comparison of plant phenotypes and changes in the respiratory metabolism between wild-type and *AOX* gene-mutated plants would be useful to provide insight into the physiological role of AOX.

SIGNAL TRANSDUCTION FROM MITOCHONDRIA TO THE NUCLEUS

AOX is induced not only in response to different environmental stress factors but also as a result of inhibition of the respiratory chain *in vivo* or *in vitro* (Vanlerberghe & McIntosh, 1997, and references therein). Experiments with respiratory-deficient mutants combined with inhibitors treatment indicate that the *AOX* gene expression pattern is specific for the type of mitochondrial respiratory chain modifications (Karpova *et al.,* 2002). The NADH dehydrogenase-defective maize NCS2 mutant has high expression of *AOX2a*, whereas the cytochrome oxidase-defective NCS6 mutant predominantly expresses *AOX2b* (formerly named *AOX3*). Similarly, *AOX2a* and *AOX2b* (*AOX3*) can be induced differentially in normal maize seedlings by rotenone or KCN, specific inhibitors of these complexes (Karpova *et al.,* 2002).

There is increasing evidence suggesting signal transduction from stressed mitochondria to the nucleus for transcription of genes whose products (among them AOX) are needed to cope with altered metabolic conditions. Partition of electrons between the cytochrome chain and alternative oxidase is highly regulated and influenced by stress conditions. This implies that the signal inducing the expression of *AOX* gene(s) is perceived in the mitochondrion and transmitted to the nucleus (McIntosh *et al.,* 1998). While AOX protein concentration and AOX activity are generally low in unstressed plants, both increase when plants are subjected to suboptimal conditions such as, for example, chilling (Purvis & Shewfelt, 1993), pathogen attack (Simons *et al.,* 1999), or phosphate deficiency (Parsons *et al.,* 1999; Juszczuk *et al.,* 2001).

Most if not all stress conditions induce oxidative stress resulting in an increased formation of reactive oxygen species (ROS) by the mitochondrial respiratory chain. In non-photosynthetic tissues ROS are thought to arise mainly from mitochondria and function as second messengers (McIntosh *et al.,* 1998; Maxwell *et al.,* 1999; Møller, 2001; Maxwell *et al.,* 2002). Several experimental observations suggest a role of ROS in the increase of the alternative oxidase protein. Addition of 1 mM H_2O_2 to cultured *Petunia hybrida* cells resulted in a strong induction of the AOX protein (Wagner & Krab, 1995; Wagner & Wagner, 1995). In tobacco suspension cells addition of 5 mM H_2O_2 caused a considerable increase in *AOX1* mRNA levels and AOX capacity (defined as salicylhydroxamic acid (SHAM)-sensitive O_2 uptake in the presence of KCN) (Vanlerberghe & McIntosh, 1996). As shown earlier genetic alteration of the level of AOX protein in transgenic tobacco was sufficient to alter the capacity for AOX respiration (Vanlerberghe *et al.,* 1994).

It was demonstrated on intact plant cells that restriction of the cytochrome chain exacerbated mitochondrial ROS levels and that over-expression of AOX decreased ROS formation (Maxwell *et al.,* 1999). Treatment of tobacco cells with mitochondrial electron transport inhibitor antimycin A (AA) caused a rapid *AOX* gene expression (Vanlerberghe *et al.,*

1994; 2002) probably mediated by increased ROS formation (Maxwell *et al.,* 2002; Vanlerberghe *et al.,* 2002). When cultured tobacco cells were treated with AA, or H_2O_2 or salicylic acid (SA) *AOX1* gene was found to be induced. Antimycin A caused the fastest induction of *AOX1* while H_2O_2 the slowest (Maxwell *et al.,* 2002). There was no clear correlation between the intensity of ROS formation and accumulation of *AOX1* transcript. However, addition of antioxidants lowered the intracellular ROS level and inhibited *AOX1* gene expression (Maxwell *et al.,* 2002). Since ROS generation is a by-product of aerobic metabolism and its increased production is a common feature in most stresses that induce *AOX1*, an increase in ROS alone may not be sufficient to activate the signal transduction pathway leading to changes in gene expression and the signal transduction pathway of *AOX1* induction may be more complex.

Maxwell *et al.* (2002) suggested that neither H_2O_2 nor SA alter the gene expression directly but lead to a rapid rise in the intracellular ROS concentration, which causes a non-specific disruption of mitochondrial function (similarly as AA treatment). Their findings support the notion that mitochondria may serve as an intermediary in intracellular stress signalling, the nature of which remains to be established. The respiratory-deficient as well as direct *AOX*-gene mutants could serve as model systems for the analysis of the mitochondrial-nuclear signalling pathways.

It would be interesting to determine whether the communication between the mitochondria and nucleus is a common response to mitochondrial dysfunction (for example a decrease in the activity of the cytochrome pathway caused by stressful conditions) and to other conditions not involving any obvious mitochondrial dysfunction (Robson & Vanlerberghe, 2002; Vanlerberghe *et al.,* 2002). Biochemical and genetic studies confirm that hydrogen peroxide is a signalling molecule in plants that mediates responses to biotic and/or abiotic stresses (Neill *et al.,* 2002a). H_2O_2 modulates the expression of various genes, mainly encoding antioxidant and programmed cell death-involved enzymes (Desikan *et al.,* 2001; Baxter-Burrell *et al.,* 2002; Neill *et al.,* 2002b). H_2O_2 specific-regulatory DNA sequences and their transcription factors have not yet been isolated and characterised (Neill *et al.,* 2002a) but it is proposed that H_2O_2 may directly oxidise transcription factors or modulate phosphorylation processes (Neill *et al.,* 2002a). The synthesis and action of hydrogen peroxide and other ROS appear to be linked to those of nitric oxide (NO) (Millar & Day, 1997; Neill *et al.,* 2002b). Treatment of *Arabidopsis* cell cultures with NO strongly induced *AOX1a* transcription (Huang *et al.,* 2002). NO-induced *AOX* expression might be an important part of the cell death mechanism (Huang *et al.,* 2002).

Besides the hypothesis that H_2O_2 and/or other ROS regulate *AOX* gene expression, there are some studies indicating that the carbon flux through the TCA cycle can effectively control *AOX* gene expression. The almost forgotten hypothesis of citrate-induced *AOX1* expression (Vanlerberghe & McIntosh, 1996) may rise again as a result of considerations that the signals influencing *AOX1* gene expression are connected with the carbon and redox status of mitochondria (Vanlerberghe *et al.,* 2002).

In spite of different experiments with wild-type and *AOX*-mutated plants or with *in vitro*-cultured cells, it still needs to be established whether ROS are indeed involved in the induction of AOX as a messenger in a specific signalling pathway, or alternatively, cause cell damage involving a more general change in the cellular metabolism.

POSTTRANSLATIONAL CONTROL OF ACTIVITY

In a given species or tissue under specific growth conditions the abundance of AOX pro-

tein is one of the obvious factors. However, there is no direct correlation between AOX protein abundance and its activity or "engagement" in respiration (McDonald *et al.,* 2002). This observation suggests that partitioning of electrons to AOX is primarily determined by posttranslational mechanisms. *In vitro* substrate level, ubiquinone (Q) concentration and its redox poise, the redox state of AOX itself and α-keto acids, (mainly pyruvate) are factors regulating AOX activity (Millar *et al.,* 1996; Vanlerberghe & McIntosh, 1997; Siedow & Umbach, 2000; Affourtit *et al.,* 2002; McDonald *et al.,* 2002; Umbach *et al.,* 2002). Since reduced ubiquinone (Qr, ubiquinol) is the common substrate of the cytochrome chain and alternative oxidase, the absolute concentration of Qr and/or the redox state of total Q are thought to be important factors regulating AOX activity. However, in the presence of pyruvate AOX becomes active at a much lower level of ubiquinone reduction than in the absence of pyruvate (Hoefnagel *et al.,* 1995). Voltametric assays (Moore *et al.,* 1988) as well as organic extraction and HPLC analysis (Ribas-Carbo *et al.,* 1995; Wagner & Wagner, 1995; Millar *et al.,* 1998; Millenaar *et al.,* 2001) were performed to study the redox state of Q pool in isolated mitochondria and intact cells or tissues. The results indicate in general that Q redox state remains remarkably constant over a wide range of AOX engagement in respiration.

A second biochemical regulatory mechanism of AOX activity concerns the fact that AOX exists as a homodimer, where the subunits are linked in a reversible manner by a disulphide bond (Umbach & Siedow, 1993; Umbach *et al.,* 1994; Vanlerberghe *et al.,* 1995). The relative amount of oxidized dimer appears to depend on the redox state of the mitochondrial pyridine nucleotide pool and may change quickly in response to mitochondrial metabolism (Vanlerberghe *et al.,* 1995). The Cys-residue responsible for this redox regulation was determined using site-directed mutagenesis (Rhoads *et al.,* 1997; 1998; Vanlerberghe *et al.,* 1998). The regulatory cysteine corresponds to the more N-terminal of the two highly, but not universally, conserved cysteines found among plant alternative oxidase sequences (Siedow & Umbach, 2000). When alternative oxidase is reduced, the enzyme can be activated by pyruvate (Millar *et al.,* 1993; Day *et al.,* 1994; Umbach *et al.,* 1994; 2002; Vanlerberghe *et al.,* 1999; Siedow & Umbach, 2000). The amino-acid residue at which this regulatory feature operates is that forming the covalent association of the two AOX subunits (Umbach & Siedow, 1996; Rhoads *et al.,* 1997; 1998; Berthold *et al.,* 2000; Affourtit *et al.,* 2002). Pyruvate forms thiohemiacetal with cysteine sulfydryl. This results in the introduction of a negatively charged carboxylate (Umbach & Siedow, 1996; Umbach *et al.,* 2002). The regulatory mechanisms *in vitro* suggest that pyruvate concentration in the mitochondrial matrix as well as matrix redox state control AOX engagement in respiration (Umbach & Siedow, 1996; Juszczuk *et al.,* 1998; Djajanegara *et al.,* 1999; Vanlerberghe *et al.,* 1999; Umbach *et al.,* 2002). However, the importance of this type of regulation for the functioning of alternative oxidase *in vivo* is still unclear (McDonald *et al.,* 2002; Millar *et al.,* 1998; Umbach *et al.,* 2002). No experimental approaches to readily manipulate and measure these matrix parameters (and their effects on AOX engagement) *in vivo* have been reported (Millenaar *et al.,* 2001; McDonald *et al.,* 2002; Umbach *et al.,* 2002).

Future experiments to investigate the importance of the regulatory mechanisms *in vivo* must utilize transgenic plants expressing mutated AOX proteins with altered *in organello* regulatory properties (McDonald *et al.,* 2002; Umbach *et al.,* 2002). Obviously, the study of AOX posttranslational regulation would be aided by purification of the active enzyme as well as by improvements in the sophisticated systems for heterologous expression of plant AOX in bacteria and yeast systems (McDonald *et al.,* 2002).

PHYSIOLOGICAL ROLE IN PLANT OXIDATIVE METABOLISM

AOX has been recognized to act as an overflow mechanism (Lambers, 1985), also as a enzyme limiting mitochondrial ROS production (Møller, 2001; Purvis & Shewfeld, 1993) and more recently the role AOX was described as part of a plant's ability to regulate energy balance in response to a changing environment (Hansen *et al.,* 2002; Moore *et al.,* 2002).

The only confirmed function for respiration with AOX engagement is the thermogenesis in *Araceae* species where the heat produced during anthesis mobilises aromatic compounds to attract pollinators (Meeuse, 1975; Skubatz *et al.,* 1993). In spite of very intensive studies on the structure and regulation of alternative oxidase (at the gene and protein level) its function *in vivo* in non-thermogenic tissues remains unclear. Considering the possible role of AOX in plant metabolism it is important to take into account the nonphosphorylating nature of AOX and its operation during periods of high rates of substrate oxidation (Simons & Lambers, 1999). Moreover, the results of a wide range of experiments indicate that AOX is a "stress-induced protein" because of its expression under several stressful treatments, such as chilling, wounding, drought, osmotic and nutrient stress and pathogen attack. It is also clear that any restriction (inhibition and/or decrease) of cytochrome *c* oxidase activity induces AOX (Moore *et al.,* 2002; Vanlerberghe & McIntosh, 1997).

The 20-year-old hypothesis of AOX as an "energy overflow" (Lambers, 1985) is still generally accepted with the one modification that AOX can compete for and share electrons with cytochrome *c* oxidase (Hoefnagel *et al.,* 1995; Day *et al.,* 1996; Simons & Lambers, 1999). Given the biochemical controls of the alternative oxidase enzyme, any metabolic condition that leads to accumulation of either reduced ubiquinone, mitochondrial pyridine nucleotides, or pyruvate, citrate and other organic acids has the potential to increase electron flow to the alternative pathway. The function of AOX as an "overflow" may therefore be to balance carbon metabolism and electron transport (co-ordinated regulation between the upstream respiratory carbon metabolism and the downstream electron transport) (Vanlerberghe & McIntosh, 1997; Simons & Lambers, 1999; Moore *et al.,* 2002, and references therein). Tightly connected with the "energy overflow" is the "energy overcharge" hypothesis (De Visser *et al.,* 1986). The ability of the mitochondrial electron transport to adjust its capacity rapidly *via* activation of alternative pathway may have at least two important consequences for plant metabolism. First, if cells require a large amount of carbon skeletons, but do not have a high demand for ATP, AOX allows operation of tricarboxylic acid cycle with minimal ATP synthesis (Simons & Lambers, 1999). Second, AOX may prevent over-reduction of the respiratory chain components that might otherwise result in the formation of harmful ROS (Møller, 2001; Vanlerberghe & McIntosh, 1997).

It has been proposed that AOX plays a role in avoidance of damage to the cell by ROS (Maxwell *et al.,* 1999; Møller, 2001; Parsons *et al.,* 1999; Purvis & Shewfelt, 1993; Robson & Vanlerberghe, 2002; Yip & Vanlerberghe, 2001). In the mitochondrial respiratory chain AOX effectively allows the interaction of molecular oxygen with reduced ubiquinone (ubiquinol). Such a mechanism of the prevention of "long life" of ubiquinol is one way to minimize ROS production (Møller, 2001). *In vitro* experiments with tobacco AOX mutants showed that cells in which AOX had been overexpressed contained half as much ROS as control cells (Maxwell *et al.,* 1999). In contrast, cells in which the expression of AOX had been reduced by antisensing contained five times more ROS than control cells. The AS8 antisense tobacco *AOX* mutant generates about twice as much ROS as wild

wide-type cells during phosphate starvation as well as under control conditions (Parsons *et al.,* 1999; Yip & Vanlerberghe, 2001). The authors suggest that induction of AOX respiration in cell culture is an important metabolic adaptation to phosphate limitation. Evidence of AOX involvement together with symptoms of oxidative stress during acclimation to phosphate deficiency were also observed for bean plants *in vivo* (Juszczuk *et al.,* 2001).

Generation of ROS and oxidative stress in plants have been implicated in biotic and abiotic stresses (Møller, 2001; Tiwari *et al.,* 2002). Very often the increased ROS formation results in the induction of programmed death of individual cells or groups of the cells in a tissue. The potential role of mitochondria in the integration of the cellular stress and regulation of programmed cell death (PCD) has been suggested (Sun *et al.,* 1999; Jones, 2000; Lam *et al.,* 2001). A few studies have implicated the role of AOX in plant PCD (Lacomme & Roby, 1999; Maxwell *et al.,* 2002; Ordog *et al.,* 2002; Robson & Vanlerberghe, 2002; Vanlerberghe *et al.,* 2002). In *Arabidopsis AOX* genes are induced early in hypersensitive response (HR) (Lacomme & Roby, 1999). Transgenic tobacco lacking AOX showed increased susceptibility to three different death-inducing compounds (H_2O_2, salicylic acid and the protein phosphatase inhibitor cantharidin) in comparison with wild-type cells (Robson & Vanlerberghe, 2002). Ordog *et al.* (2002) found that overexpression of AOX in S24 tobacco mutants reduced the size of HR lesions. But, in the same tobacco cells with enhanced susceptibility to tobacco mosaic virus infection, the overexpression of AOX did not significantly increase viral resistance. Vanlerberghe *et al.* (2002) showed that loss of the cytochrome pathway induced by cysteine or antimycin treatment in AS8 cells unable to induce AOX (due to the presence of an antisense transgene) causes a complete loss of respiratory capacity and results in massive cell death. Higher levels of AOX protein after anoxic treatment of soybean cells correlated with increased resistance to H_2O_2-induced PCD (Amor *et al.,* 2000). It has been suggested that the ability of AOX to attenuate cell death might relate to its ability to generate small amounts of ATP (during NADH oxidation by complex I) (Vanlerberghe *et al.,* 2002). Moreover, AOX may dampen the excessive mitochondrial generation of ROS by preventing over-reduction of the respiratory chain complexes (Møller 2001, Vanlerberghe *et al.,* 2002).

The hypothesis of the role of AOX in oxidative stress defence has an evolutionary background with the postulated early function of AOX in di-oxygen scavenging (Gomes *et al.,* 2001). The primary structure of AOX suggests that it belongs to the di-iron carboxylate family and an evolutionary relationship with primitive members of that family (Moore *et al.,* 2002). However, plant cell have a more efficient ROS-scavenging system consisting of haem-copper enzymes (catalases, peroxidases, dismutases). Moore *et al.* (2002) ask the question: "if the scavenging of di-oxygen is not the principle function of the alternative oxidase, what is its role?" Hansen *et al.* (2002) suggest that the present-day function of alternative oxidase is to allow flexible control of ATP synthesis to maintain growth rate homeostasis. This is in agreement with the hypothesis that AOX takes part in the plant stress-acclimation process as a "survival protein" (Robson & Vanlerberghe, 2002). AOX protein is induced during plant response to nutritional phosphate deprivation (Juszczuk *et al.,* 2001), and in green pepper under cold stress (Purvis & Shewfelt, 1993), as a result of PCD occurring during plant development and in response to biotic stress (Beers & McDowell, 2001), for example the HR response (Alvarez, 2000). It appears that the non-protonmotive nature of AOX allows a flexible and rapid adaptation of mitochondria to maintain relatively stable energy charge and, consequently, to maintain the growth rate under variable environmental conditions (Moore *et al.,* 2002).

The potential role of AOX is obviously even more complicated in green tissues because of the evident interaction between mitochondria and chloroplasts in ATP and reducing power utilisation. Igamberdiev *et al.* (1997) proposed that AOX could participate in photorespiratory glycine oxidation helping to maintain the ATP/ADP ratio and oxidizing NADH. In potato AOX showed induction in mature leaves and light dependence indicating its role in photosynthetically associated mitochondrial metabolism (Svensson & Rasmusson, 2001).

Most hypotheses for the role of AOX in plant metabolism are based on *in vitro* experiments of using cell cultures. Future studies should verify whether the mechanisms of AOX regulation *in vivo* are the same. Transgenic plants with altered AOX expression and *in vivo* examination of the interactions between the organelles will help to understand the role of AOX in plant metabolism. Moreover, in addition to alternative oxidase, plant mitochondria contain uncoupling proteins (Vercesi *et al.,* 1995) which also dissipate energy. The functioning of the two systems of modification of plant cell energy status also remains to be studied.

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