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Biochemical and genetic controls exerted by plant mitochondria

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Abstract

Higher plant mitochondria contain two terminal oxidases, cytochrome *c* oxidase and a cyanide-resistant 'alternative' oxidase. Electron flux through these two respiratory pathways is controlled by environmental conditions, stimuli received by mitochondria. In general, stresses such as cold, wounding, pathogen attack and others favor electron flow through the alternative oxidase. One of the proposed functions of the alternative pathway is to relieve the tricarboxylic acid (TCA) cycle of inhibition from cytochrome pathway products and allow the cycle to furnish carbon skeletons for anabolic requirements. We are currently investigating, with an NADP-linked isocitrate dehydrogenase in plant mitochondria, a possible link between respiratory control and carbon flux from the TCA cycle. Regulation of the nuclear gene encoding the alternative oxidase, *Aox*1, is also being employed as a model for perception of the many stresses by the mitochondria and transfer of these signals to the nucleus. Our initial results indicate that hydrogen peroxide is an intermediate in this signalling process.

Keywords: Alternative oxidase; Respiration; Hydrogen peroxide; NADP-isocitrate dehydrogenase; Mitochondrion

1. Introduction

Among the unique qualities of higher plant mitochondria [1–4] is a striking functional difference between plant and animal mitochondria, i.e. the presence of two terminal oxidases in higher plants. Higher plants contain, as other eukaryotes do, a cytochrome *c* oxidase [5], however, they also possess an 'alternative' oxidase. This oxidase was first identified by its resistance to cyanide [6]. The electrons flowing along this alternative pathway are not linked to the production of a transmembrane potential and thus are lost, or 'wasted', for the production of ATP. Because this pathway branches at the ubiquinone pool and does not pump protons, its contribution to energy balance is one third that of the cytochrome pathway. Mitochondrial energy conservation in plants is thus a balancing act between two competing pathways.

The electron flow, division of energy between the two terminal oxidases, is regulated by the environment, especially during stress. This implies a function for the alternative pathway of energy flow and indicates that the signal(s) regulating its expression is (are) perceived in the mitochondrion and transmitted to the nucleus. One current challenge is to link the function of the bifurcated plant mitochondrial electron flow with plastid function and ultimately with carbon balance within the intact plant. More immediate questions are (1) to understand the carbon balance

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'function' of alternative pathway respiration, especially as a response to stress, and (2) to unravel the sensory transduction pathway from the mitochondrion back to the nucleus.

2. Mitochondrial expression of the two plant respiratory pathways

Electron (energy) flow in plant mitochondria is a Y-shaped pathway (Fig. 1). Alternative pathway respiration branches from the cytochrome pathway at ubiquinone and donates electrons directly to oxygen to form water [7]. An important feature of this pathway is that it does not contribute to a transmembrane potential and energy flowing down this pathway loses two of the three potential coupling sites for proton transport and, thus, ATP production. One path leads to coupled ATP production and the other 'wastes' this electron energy.

The only confirmed function for alternative pathway respiration, until now, is to supply heat in thermogenic blooms of such plants as *Sauromatum guttatum*, of the Araceae, and some other inflorescences [8]. In *S. guttatum* (the 'voodoo lily'), heat is produced in ephemeral inflorescences, to better volatilize scents to attract their insect pollinators, in this case, flies.

Environmental stimuli are probably more significant and also serve to induce this apparently 'wasteful' respiratory pathway in plants. These stimuli include low temperature, wounding, pathogen attack, elevated carbohydrate status, cell culture stage, addition of ethylene, fruit ripening and elevation of salicylic acid levels [3,4,8–10]. It is easier to understand the function of the alternative respiratory pathway if it is viewed as a regulatory phenomenon allowing a metabolic response. In the past, it has been described as serving a regulatory function as an 'overflow' for excess electrons when the cytochrome pathway is saturated or limited [10,11]. It would thus act by allowing the turnover of the tricarboxylic acid (TCA) cycle (due to lowered ATP and NADH levels) to continue, supplying carbon skeletons for biosynthetic demands. It has also been proposed that alternate pathway respiration is induced under many different environmental conditions as a mechanism to allow the plant to respond to, and survive, these stresses [3], specifically oxidative stress [12,13].

3. Mitochondrial regulation of nuclear gene expression and plant responses to stress

3.1. Mitochondrial/nuclear interaction: Genetic paradigms

Organelle/nuclear interaction proceeds by (1) synthesis and the contribution of essential polypeptides and components facilitating biosynthetic processes, transport and assembly, and (2) a bidirectional information flow, allowing growth and response to the

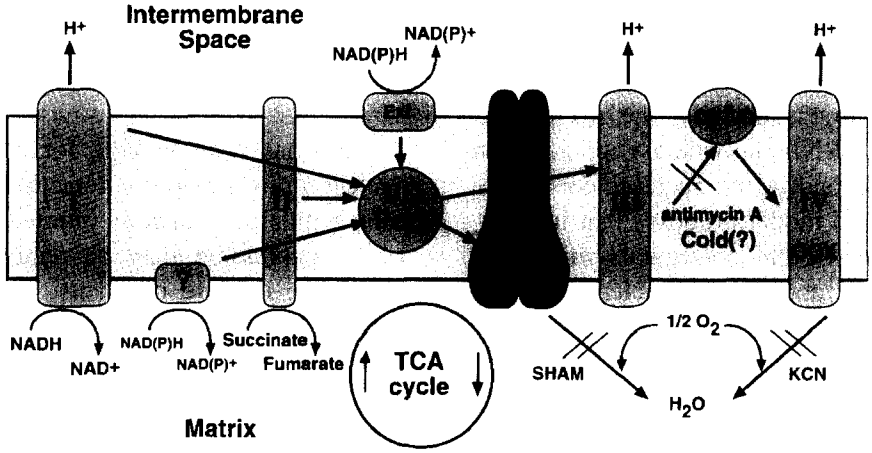

Fig. 1. Plant mitochondrial electron transport complexes shown in schematic fashion: Complex I, NADH dehydrogenase; Complex II, succinate dehydrogenase; Complex III, cytochrome bc_1; Complex IV, cytochrome oxidase (COX); AOX, alternative oxidase; UQ, ubiquinone; Ext., an external plant NADH dehydrogenase; ?, another proposed NADH dehydrogenase for plants; A, antimycin A; SHAM, salicylhydroxamic acid; TCA, tricarboxylic acid.

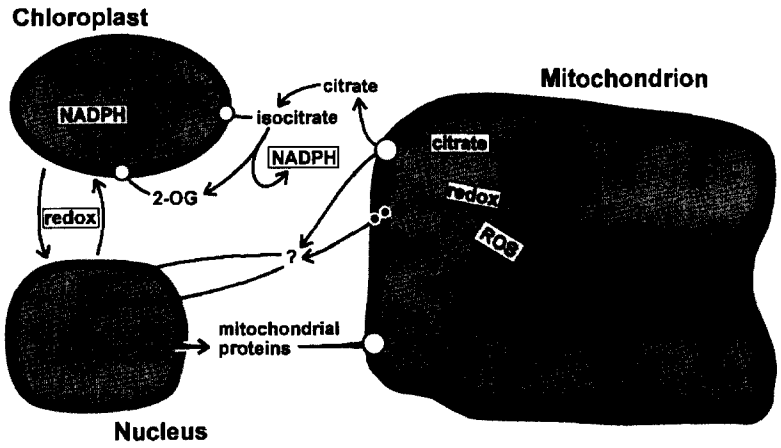

Fig. 2. Three organelles involved in the regulation of energy transduction: the nucleus, plastid and mitochondrion. Plant mitochondria contain two terminal oxidases: cytochrome oxidase (COX) and alternative oxidase (AOX). The alternative oxidase is nuclear-encoded, by *Aox*1, and appears to be regulated by mitochondrial redox status, reactive oxygen species (ROS) and the metabolite citrate. Citrate, a major export metabolite of plant mitochondria [14,15], may contribute to the production of redox NAD(P)H equivalents, through its oxidation to isocitrate and 2-oxyglutarate (2-OG), in the cytoplasm and organelles.

environment (Fig. 2). Regulatory models from bacterial energy transduction, ones involving two-component regulation, have been extended to photosynthesis in plastids [16–18]. For example, transcription of the nuclear-encoded chlorophyll *a*/*b* binding protein appears to be regulated by the 'redox' state of the plastoquinone pool [19]. Redox regulation is presented as being dependent upon a redox-sensing kinase and specific DNA-binding proteins [20]. It has also been shown that translation of the chloroplast-encoded Photosystem II *psbA* gene product in *Chlamydomonas reinhardtii* is light-regulated through changes in chloroplast redox poise, which affect an RNA binding protein [21].

Yeast serves as the best model for nuclear/mitochondrial interaction. Two general models have been proposed [22]. The first states that the nuclear-encoded mitochondrial RNA polymerase operates in parallel in both mitochondria and nuclei to coordinate transcription. A second model proposes that nuclear-encoded transcription factors co-regulate mitochondrial RNA polymerase and control nuclear structure genes for mitochondrial components. Recent work casts considerable doubt on the first model, while supporting the second [22–28]. The key to the yeast paradigm is that carbon and heme concentrations serve to mediate expression of mitochondrial components, with oxygen serving as the main regulator of heme biosynthesis. These factors influence the positive and negative regulators affecting transcription of nuclear-encoded mitochondrial genes.

3.2. Expression of the alternative pathway of respiration as a model for mitochondrial/nuclear interaction

Nuclear/mitochondrial interaction is much less studied in plants. The best examples are (1) cytoplasmic male sterility (CMS), where the interaction of nuclear and mitochondrial genomes gives rise to male sterility [29] and (2) maize nonchromosomal stripe mutants (NCS), which are involved with deletions in a number of different mitochondrial genes [30]. These systems are not very experimentally amenable to developmental studies in the laboratory.

What has been lacking for plants is a model of nuclear/mitochondrial interaction with (1) a dominant regulatory function unique to plants and (2) whose expression may be regulated precisely. The alternative oxidase of plants seems to offer such a model, as its gene expression and activity are induced in plants and suspension cells by a number of different stimuli, e.g. cold, oxidative stress, pathogen attack and chemical additions such as salicylic acid and antimycin A, an inhibitor of the cytochrome pathway [4]. Upon the addition of antimycin A to cultured tobacco cells or plants, accumulation of

*Aox*1 mRNA is induced within 60 min, alternative oxidase protein within 3–5 h and activity within 5 h [31]. The same pattern is found following the addition of citrate or H_2O_2 [32].

Antimycin A is thought to induce alternative oxidase due to inhibition of the cytochrome pathway [3] and this inhibition may be recognized through production of reactive oxygen species (ROS) (Fig. 2) [12,13,32,33]. ROS are thought to arise from mitochondrial electron transport (especially in the presence of cyanide or antimycin A [34]), and function as second messengers [35], potentiated through TNF (tumor necrosis factor) stimulation of the mammalian transcription factor NFκB [36]. It has also been shown that the alternative oxidase of yeast *Hansenula anomola* is induced by ROS [37], such as H_2O_2, and it has been postulated that cold-induction of alternative oxidase occurs in plants to ameliorate superoxide production by mitochondria [12]. In their recent review on nuclear/mitochondrial 'crosstalk', Poyton and McEwen [22] suggested that 'a potentially useful experimental paradigm for the identification of genes regulated by mitochondrially produced ROS is to search for genes that are differentially expressed in response to antimycin A'.

4. Reactive oxygen species: Messengers for plant mitochondria?

It has been demonstrated that alternative activity increases in response to H_2O_2 as well as transcription of *Aox*1 [4,32], thus, for plant mitochondria, we appear to have a valuable plant paradigm for expression mediated by ROS. Is this response, i.e. of increased alternative pathway activity, a functional one for plants, to protect them from ROS and, if so, does the H_2O_2 signal originate in mitochondria and is it transported? In recent experiments, we have employed the fluorescent dye, dichlorofluorescein diacetate (DCF-DA), to follow H_2O_2 production in plant cells. DCF-DA is non-polar, non-fluorescent and easily enters cells. Resident esterases convert it to DCF, a polar compound that is sensitive to H_2O_2, which gives rise to an oxidized form of DCF that is fluorescent [38]. Using this approach, a recent publication claimed that the addition of antimycin A elevated H_2O_2 levels in soybean and pea mitochondria [39]. To investigate the importance of the alternative oxidase to this phenomenon, we have employed a transgenic plant line that was either lacking most alternative oxidase activity, AS8, or one engineered to overexpress the alternative oxidase, S11 [40,41]. In this experiment (Table 1), 2 μM antimycin A was added to suspension cell cultures of wild-type, AS8 (antisense) and S11 (sense) cells at time zero. Cells lacking the alternative pathway show a dramatic increase in H_2O_2 production when compared with wild-type, while cells overexpressing the oxidase show repressed amounts of H_2O_2. These results indicate that the alternative pathway could play a protective function in oxidative stress in plant cells.

4.1.1. Could H_2O_2 serve as a signal on the pathway from mitochondrion to the nucleus, and the expression of the alternative oxidase?

In mammalian fibrosarcoma cells, ROS produced from mitochondrial electron transport appear to be signal transducers for TNF-induced gene expression [36]. Interestingly, additions of antimycin A to these cells appear to initiate this process through expression of the transcription factor NFκB. To determine if H_2O_2 may be part of the signal transduction pathway in plants, we have assayed the mRNA expression of a set of enzymes that are known to function in response to oxidative stress and whose cellular location is recognized (Fig. 3). The genes encoding these enzymes are catalase 2 (CAT2), localized to the cytosol/peroxisome fraction(s) and catalase 3, localized to the mitochondrion [42–44]. As shown in Fig. 3, control and transgenic tobacco lines (with both repressed and overabundant alternative oxidase, AS8

Table 1
Formation of reactive oxygen species as a function of time after the addition of antimycin A (2 μM) to tobacco suspension cells

Cell line	0 h	2 h	4 h
WT	9.7±2.5	76.9±4.5	142.8±8.5
AS8	21.4±2.2	165±18.5	290.6±25.2
S11	1.4±0.8	9.5±1.5	18.4±5.8

Reactive oxygen species were assayed by measuring dichlorofluorescein fluorescence.
Cells were incubated for 30 min in dichlorofluorescein diacetate (5 μM), starting at each time of the time-points indicated.
Excitation, 488 nm; emission, 525 nm.
Data are presented per mg dry weight. Mean±SD, $n=3$.

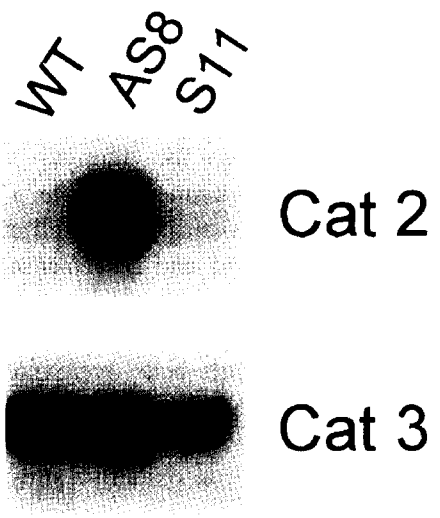

Fig. 3. Northern blots of total RNA extracted from tobacco suspension cells from wild-type (WT), an 'antisense' line to alternative oxidase gene, *Aox*1 (AS8), and two lines that overexpress the *Aox*1 gene (S11). The blot was probed with a gene encoding the cytosolic form of catalase, CAT2, and the gene encoding the catalase localized to mitochondria, CAT3.

and S11) show similar amounts of RNA accumulated for the mitochondrial CAT3. It is interesting to note that the catalase isozyme specific to the cytosol/peroxisomal fractions, CAT2, is only elevated in the cell line with repressed alternative oxidase expression. These results may indicate that cells lacking alternative oxidase activity have increased H_2O_2 concentrations and that it has diffused to the cytosol where its presence has induced the CAT2 isozyme. Our future work is aimed at further elucidating the mitochondrial signal transduction pathway and isolating possible transcription factors involved, or influenced, by ROS in plants.

5. TCA cycle metabolite regulation of respiratory carbon balance

Our previous work implicated the mitochondrial NADPH-dependent isocitrate dehydrogenase (mtICDH) as part of a biochemical regulatory reduction step to 'reductively' activate alternative oxidase, specifically at one of two highly conserved cysteine residues (Fig. 4) [4,45,46]. In addition, we have proposed that mtICDH may link carbon outflow from the TCA cycle to respiration under stress conditions. It is interesting that mtICDH in *Escherichia coli* is a controlling enzyme in the distribution of carbon flux at the branch point of the TCA cycle and the glyoxylate shunt [47]. Also, in animal mitochondria, a proton-translocating transhydrogenase and mtICDH have also been proposed to operate in the fine control of the TCA cycle [48].

5.1. Carbon balance: Mitochondrial NADP-isocitrate dehydrogenase (mtICDH)

If mtICDH is involved in the reductive activation of alternative oxidase, through production of NADPH, then how is it regulated and is it involved in a carbon 'shuttle' out of the TCA cycle? To answer this question, we have purified this enzyme from mitochondrial matrix fractions (Table 2), obtained

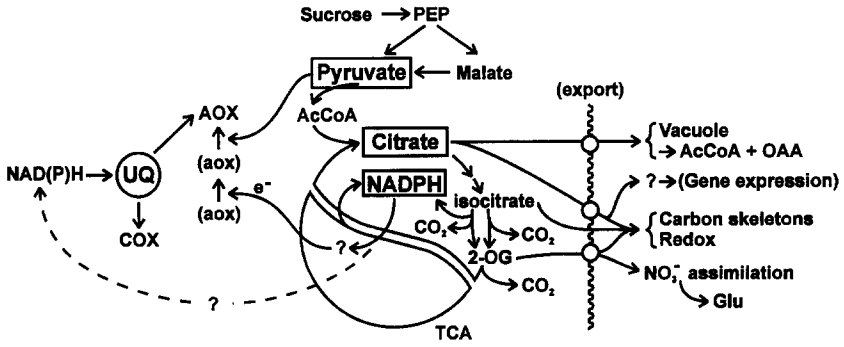

Fig. 4. Integration of metabolite and respiratory effectors in plant mitochondria. Under many different environmental conditions, the TCA cycle may act more as a carbon 'shuttle' than as a cycle [49]. Pyruvate, the main carbon entry into mitochondria, acts to allosterically activate alternative oxidase [50]. NADPH reducing power from NADP-isocitrate dehydrogenase (a non-TCA cycle matrix enzyme) and/or malate dehydrogenase may reduce alternative oxidase (to increased activity) and be mediated by mitochondrial thioredoxin or glutathione, both of which require NADPH [51,52]. Citrate, the major TCA cycle intermediate exported from mitochondria [15], also contributes to carbon export and regulation of *Aox*1 expression [32].

Table 2
Specific activities for mitochondrial $NADP^+$-dependent isocitrate dehydrogenase in cell-suspension cultures of transgenic tobacco (*Nicotiana tabacum* L. cv Petit Havana) containing *Aox*1 in the sense (S11 or S24) or antisense (AS8) orientations

Tobacco line	Specific activity (mU mg^{-1})
WT SR1	102
AS8	185
S11	63
S24	72

One mU of activity is defined as the production of 1 nmol of NADPH min^{-1}.
Specific activity is expressed per mg of soluble matrix protein.

partial protein sequence data, and will clone the gene encoding this isoenzyme.

We proposed that the reducing equivalents generated by the (non-TCA cycle) NADP-isocitrate dehydrogenase in the matrix act through a thioredoxin or glutathione system [51] to mediate cysteine reduction and activation of the alternative oxidase [4]. Further biochemical evidence demonstrates that changes in mtICDH activity correlate with altered alternative oxidase activities in transgenic tobacco lines. We have assayed mtICDH activities (Table 2) in control lines and lines of tobacco where alternative oxidase activity has been increased (S11, S24) or repressed (AS8). In lines that overexpress alternative oxidase, the activity of mtICDH was lowered, while in a single line with little or no alternative oxidase activity, mtICDH activity was almost doubled (compared to control). This may indicate that a positive feedback from the amount of alternative oxidase protein in the cell affects mtICDH activity and/or expression.

Cytosolic and chloroplastic NADP-isocitrate dehydrogenase has been purified previously from plant sources [53–56] and some their cDNAs have been cloned [57]. Plant mtICDHs have been, to some extent, purified [49,54,58], but, as yet, no authentic NADP-mtICDH cDNA clone has been identified. In initial experiments, we have isolated, from large-batch tobacco cell cultures, mitochondrial matrix fractions and have assayed NAD-dependent and NADP-dependent isocitrate dehydrogenase activity [49]. We then employed HPLC (high-performance liquid chromatography), following the method of Hayes et al. [59], in conjunction with a HiTrap Blue affinity column (Pharmacia). Sodium dodecyl sulfate–polyacrylamide gels were used to isolate a single protein band correlating to mitochondrial matrix MtICDH. Initial protein sequencing of this band confirmed its identity as MtICDH. The protein sequence will be employed to identify the cDNA clone specific for the mitochondrial isozyme.

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