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Regulation of Alternative Oxidase Activity in Higher Plants

David A. Day[1] and Joseph T. Wiskich[2]

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Plant mitochondria contain two terminal oxidases: cytochrome oxidase and the cyanide-insensitive alternative oxidase. Electron partioning between the two pathways is regulated by the redox poise of the ubiquinone pool and the activation state of the alternative oxidase. The alternative oxidase appears to exist as a dimer which is active in the reduced, noncovalently linked form and inactive when in the oxidized, covalently linked form. Reduction of the oxidase in isolated tobacco mitochondria occurs upon oxidation of isocitrate or malate and may be mediated by matrix NAD(P)H. The activity of the reduced oxidase is governed by certain other organic acids, notably pyruvate, which appear to interact directly with the enzyme. Pyruvate alters the interaction between the alternative oxidase and ubiquinol so that the oxidase becomes active at much lower levels of ubiquinol and competes with the cytochrome pathway for electrons. These requirements for activation of the alternative oxidase constitute a sophisticated feed-forward control mechanism which determines the extent to which electrons are directed away from the energy-conserving cytochrome pathway to the non-energy conserving alternative oxidase. Such a mechanism fits well with the proposed role of the alternative oxidase as a protective enzyme which prevents over-reduction of the cytochrome chain and fermentation of accumulated pyruvate.

KEY WORDS: Plant mitochondria; alternative oxidase; electron transport; protein disulfide bonds; enzyme activation.

INTRODUCTION

The alternative oxidase of the plant respiratory chain catalyzes cyanide-insensitive oxygen uptake. It is a quinol oxidase, branching from the main chain at ubiquinone and bypassing the proton-translocating cytochrome complexes. Apart from a role in thermogenic floral organs, where heat generation via the alternative pathway is a pollination aid (Meeuse, 1975), its function in plants is not clear. Recent evidence that its synthesis is induced by oxidative stress in plants (Purvis and Schewfelt, 1993) and by inhibition of the cytochrome chain in cell cultures (Minagawa *et al.*, 1992; Vanlerberghe and McIntosh 1992b), suggests that its function may be to prevent over-reduction of the respiratory chain and the consequent generation of harmful reactive oxygen species.

The plant alternative oxidase is encoded by nuclear gene(s) and consists of 1–3 proteins between 32 and 39 kDa in mass, depending on the species (McIntosh, 1994). It appears to operate as a dimer (either heterologous or homologous) (Umbach and Siedow, 1993) and probably contains Fe as a redox ligand (Minagawa *et al.*, 1990). In this short review, we focus on recent advances in our understanding of the regulation of alternative oxidase activity in plant tissues. For more detailed information, the reader is refered to reviews by Moore and Siedow (1991) and Day *et al.* (1995).

REGULATION BY PROTEIN SYNTHESIS

In all plants so far examined, cyanide-insensitive respiration is correlated with the presence of proteins

[1] Division of Biochemistry and Molecular Biology, and the Cooperative Research Centre in Plant Science, Australian National University, Canberra, ACT 0200.

[2] Botany Department, University of Adelaide, Adelaide, SA 5001, Australia.

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which react with monoclonal antibodies raised against alternative oxidase proteins (AOX)[3] from *Sauromatum guttatum* (Elthon and McIntosh, 1987; Elthon *et al.*, 1989). In general, the maximum rate of cyanide-insensitive oxygen uptake by isolated mitochondria varies roughly in proportion to the amount of AOX present. For example, in mitochondria from thermogenic Arum tissues, the quantity of AOX is about twentyfold that in soybean mitochondria and activity varies by approximately the same magnitude (Day *et al.*, 1995). In *Sauromatum guttatum,* induction of cyanide-insenstive respiration in the floral spadix during thermogenesis correlates with an increase in immunologically detectable AOX protein (Elthon *et al.*, 1989). In soybean cotyledons, the capacity of the alternative oxidase increases immediately after germination and during senescence, in concert with an increase in protein detected on western blots (T. McCabe and D. A. Day, unpublished results). Perhaps the best illustration of this coarse control of alternative oxidase activity by protein synthesis comes from recent studies with transgenic tobacco (Vanlerberghe *et al.*, 1994): in plants lacking AOX due to antisense transformation, cyanide-insensitive respiration is virtually undetectable, while in sense transformants which express more AOX than wild-type, alternative oxidase activity is much greater, providing the oxidase is activated (see below). However, while protein synthesis (and presumably gene transcription) may decide the potential for alternative oxidase activity in a tissue, several other factors determine how much of that potential is realized.

REGULATION BY AOX REDOX STATE

Cross-linking studies with soybean mitochondria indicate that the active alternative oxidase exists as a noncovalently linked dimer (Umbach and Siedow, 1993). The dimer can also be covalently linked by disulfide bonds upon addition of oxidants to isolated mitochondria (Umbach and Siedow, 1993; Umbach *et al.*, 1994). The oxidized dimer is readily detectable as a protein of higher molecular mass (about 70 kDa; Fig. 1) on SDS-PAGE in the absence of a reductant, and displays very little activity until it is re-reduced (Umbach and Siedow, 1993; Umbach *et al.*, 1994). The oxidized dimer is also quite unresponsive to the activator pyruvate (Umbach *et al.*, 1994; see below). Thus, for a given amount of AOX, the potential activity (capacity) of the oxidase will be determined by the ratio of oxidized to reduced protein. This ratio seems to vary considerably between species. For example, in soybean mitochondria, AOX is largely reduced and is active upon oganelle isolation (Umbach and Siedow, 1993; Day *et al.*, 1994); in mitochondria isolated from tobacco, it is largely oxidized and must be reduced before much activity is seen (Fig. 1). The AOX redox status in isolated mitochondria may not necessarily reflect that *in vivo,* at least in Arum spadices (A. L. Umbach and J. N. Siedow, personal communication). However, current evidence suggests that changes in AOX reduction occur relatively slowly once the mitochondria are isolated (following reduction in soybean and tobacco mitochondria, it is not altered by organelle re-isolation or lengthy assays: D. A. Day and A. H. Millar, unpublished results).

Reduction of AOX in isolated mitochondria can be achieved by addition of compunds such as DTT (Umbach and Siedow, 1993), but also occurs upon oxidation of certain TCA cycle intermediates (G. C. Vanlerberghe, D. A. Day, J. T. Wiskich, and L. McIntosh unpublished results). In tobacco leaf mitochondria, citrate/isocitrate are the most effective of these intermediates, even though they are poorly oxidized. Malate can also reduce AOX, providing care is taken to prevent oxaloacetate accumulation, but succinate, glycine, and 2-oxoglutarate are much less effective, even though the former two substrates are oxidized rapidly (G. C. Vanlerberghe, D. A. Day, and L. McIntosh, unpublished results). Of these substrates, isocitrate and malate are the only ones able to reduce $NADP^+$ directly in plant mitochondria (Rasmusson and Møller, 1990). This may, therefore, indicate that AOX reduction depends on NADPH generation in the matrix (the results also suggest that NADH and NADPH pools do not interact readily in tobacco mitochondria). It is likely that NADPH reduces AOX via an intermediate, but this has yet to be identified. A possible sequence of reactions leading to AOX reduction in plant mitochondria is shown in Fig. 2.

Two possible reductants ("X" in Fig. 2) for AOX have been suggested (Umbach and Siedow, 1993): thioredoxin and reduced glutathione. Reduction of both thioredoxin and glutathione usually involves NADPH as cofactor. A mitochondrial isoform of thioredoxin has been identified recently (Bodenstein-Lang *et al.*, 1989) and glutathione reductase also exists in plant

[3] Abbreviations: AOX, alternative oxidase protein; Q, ubiquinone; QH_2, ubiquinol; TCA, tricarboxylic acid.

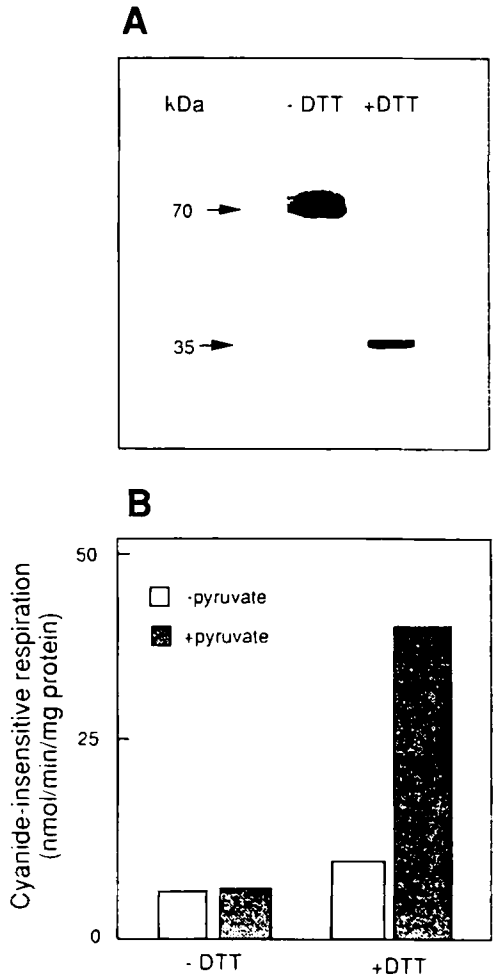

Fig. 1. Interaction between pyruvate and reductant in controlling alternative oxidase activity in isolated tobacco leaf mitochondria. (A) Immunoblot of tobacco mitochondrial proteins, probed with an antibody against AOX (Day *et al.*, 1994). In the presence of DTT, a single band at 35 kDa is seen; in the absence of DTT, all of AOX seems to be present as the oxidized dimer (70 kDa). (B) Effect of pyruvate on cyanide-insensitive oxygen uptake in the presence and absence of DTT. Isolated mitochondria were incubated with NADH (10 mM) as substrate, ADP (1 mM) and KCN (1 mM). Oxygen uptake was measured as described in Hoefnagel *et al.*, (1995). Where indicated, DTT (2.5 mM) and pyruvate (0.5 mM) were added. [M. Meijer and D. A. Day, unpublished results.]

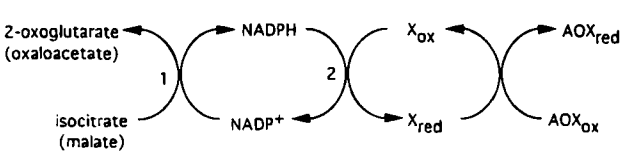

Fig. 2. Putative scheme for the reduction of the alternative oxidase in plant mitochondria. (1) NADP-isocitrate dehydrogenase or malate dehydrogenase; (2) either thioredoxin reductase or glutathione reductase; X: either thioredoxin or glutathione.

mitochondria (Rasmusson and Møller, 1990). Bacterial thioredoxin will reduce AOX in inside-out submitochondrial particles (Umbach and Siedow, 1994) and preliminary experiments suggest that exogenous reduced glutathione can also do this (M. H. N. Hoefnagel and J. T. Wiskich, unpublished results).

Although endogenous reduction of AOX is readily achieved, at least in tobacco mitochondria, reoxidation without use of artificial oxidants is more difficult to demonstrate. In chloroplasts, oxidation of cysteine residues on redox-sensitive stromal enzymes occurs in the dark via oxidized thioredoxin and this could also occur in mitochondria when the matrix pyridine nucleotide pools are oxidized.

REGULATION BY ORGANIC ACID ACTIVATORS

In mitochondria from thermogenic tissues such as Arum, cyanide-insensitive respiration is rapid with all substrates, including exogenous quinols (Elthon and McIntosh, 1987; Siedow and Moore, 1993). In fact, ubiquinol oxidation forms the basis of an assay for the solubilized oxidase (Rich, 1978). In mitochondria from nonthermogenic tissues, however, large differences are seen in alternative oxidase activity with different substrates (see Lance *et al.*, 1985). Maximum rates are usually seen with succinate as substrate; NAD-linked substrates often give slower rates but also reduce ubiquinone (Q) to a smaller extent (Day *et al.*, 1991). Exogenous NADH, on the other hand, is poorly oxidized via the alternative oxidase even though it maintains QH_2 at levels seen with succinate, and exogenous quinols are barely oxidized at all (Day *et al.*, 1991; Moore and Siedow, 1991).

It has subsequently been shown that certain organic acids can activate the alternative oxidase and eliminate the differences among substrates (Millar *et al.*, 1993). Glyoxylate and pyruvate are the most effective of these, but hydroxypyruvate and 2-oxoglutarate are also effective at higher concentrations (Day *et al.*, 1995). Activation by these organic acids does not involve their metabolism (Millar *et al.*, 1993). Intramitochondrial pyruvate generation during succinate and malate oxidation may explain why these compounds are better substrates for the alternative oxidase (Day *et al.*, 1994). In potato mitochondria, cyanide-insensitive exogenous NADH oxidation is stimulated by addition of D-malate or succinate, even when malonate is present to block succinate oxidation (Wagner *et al.*, 1989;

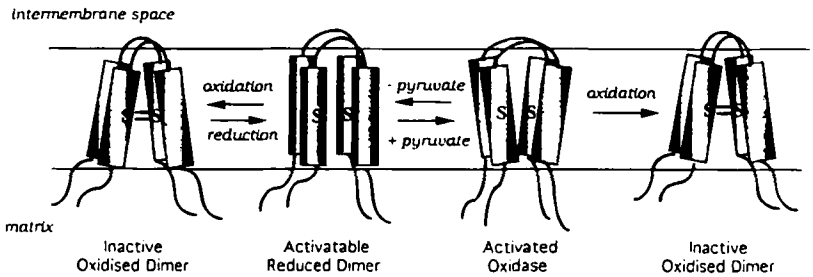

Fig. 3. Proposed sequence of alternative oxidase activation in plant mitochondria. The oxidized, covalently linked dimer shows little or no activity and is not stimulated by addition of pyruvate; formation of intermolecular disulfide bonds upon oxidation of the enzyme inactivates it and prevents its activation by pyruvate. The reduced form of the enzyme is activatable but shows very little activity unless pyruvate (or one of its analogues) is added. Pyruvate fully activates the oxidase: when activated, it not only shows full activity but does so at lower levels of ubiquinol. Oxidation of the enzyme, even in the presence of pyruvate, inactivates it.

Lidén and Ackerlund, 1993), but these compounds are needed at much higher concentrations and are not effective in soybean or tobacco mitochondria (Millar *et al.*, 1993; Day *et al.*, 1995). The fundamental structural motif required for stimulation seems to be an α-keto-carboxylic acid, as exemplified by glyoxylate. Modification of this fundamental structure by the addition of side groups decreases the effectiveness of the compound (Day *et al.*, 1995).

Pyruvate stimulates at low concentrations ($K_{0.5}$ = 0.1 mM when applied exernally) and appears to have its site of activation on the matrix side of the inner membrane (Day *et al.*, 1994). It is also effective in inside-out submitochondrial particles and with solubilized AOX (in fact, the solubilized enzyme from soybean is dependent on pyruvate Q. Zhang, M. H. N. Hoefnagel, and J. T. Wiskich, unpublished data), indicating that pyruvate interacts directly with AOX. The pyruvate stimulation is readily reversible, and pyruvate must be present continuously to maintain alternative oxidase activity (Day *et al.*, 1994). However, the oxidized form of AOX cannot be activated by pyruvate (Umbach and Siedow, 1993). In tobacco leaf mitochondria, where AOX is largely oxidized upon organelle isolation, alternative oxidase activity is very slow until both pyruvate and a reductant are added (Fig. 1B). Thus, the redox state of AOX sets alternative oxidase capacity, and pyruvate levels determine how much of that capacity is realized. The interaction between pyruvate and AOX redox state is summarized in Fig. 3.

REGULATION BY UBIQUINONE REDOX STATE

Early studies using inhibitors (Bahr and Bonner, 1973) indicated that the alternative pathway was only active when the cytochrome chain was either saturated with reducing equivalents or inhibited. For example, in some tissues the alternative oxidase was found to contribute to oxygen uptake in the absence of ADP (when the cytochrome chain is inhibited by the protonmotive force), but not in the presence of ADP. Additions of small quantities of cytochrome chain poisons, such as KCN and antimycin, also led to engagement of the alternative oxidase, but inhibition of alternative oxidase did not change the rate of electron flow throught the cytochromes (Bahr and Bonner, 1973; Day, 1992; but see Wilson, 1988). In other words, the activity of the alternative oxidase seemed to be governed largely by the activity of the cytochrome path, and electron switching from the alternative path to the cytochrome path was rarely seen. Bahr and Bonner (1973) suggested that an energetically unfavorable redox step existed in AOX so that high high levels of ubiquinol (QH_2) were required to elicit rapid turnover. From these observations, the notion of the alternative pathway being an overflow of the cytochrome path arose (Lambers, 1985).

Subsequent measurements with the so-called Q-electrode showed that the alternative oxidase only became active when the Q pool was 40–50% reduced, after which point activity increased dramatically and nonlinearly (Moore *et al.*, 1988; Dry *et al.*, 1989).

Cytochrome pathway activity, on the other hand, was more linearly related to the degree of Q reduction and saturated at a Q_r/Q_t ratio at which the alternative oxidase was inactive. Although not agreeing precisely with the electron transport model proposed by Bahr and Bonner (Dry *et al.*, 1989), these data explained the general observations outlined above and show that the redox poise of the Q pool is an important factor in determining alternative oxidase activity. Moore and Siedow (1991), Siedow and Moore (1993), and Ribas-Carbo *et al.* (1994) have developed a model which predicts this behavior (see Krab, this volume).

However, the presence of pyruvate alters the interaction between Q and AOX, so that the latter is active at much lower Q_r/Q_t ratios (Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995). Under these conditions, the alternative oxidase competes with the cytochrome chain in soybean cotyledon mitochondria and can contribute to respiration even in the presence of ADP (Hoefnagel *et al.*, 1995). Addition of pyruvate causes oxidation of the Q pool and redirection of electrons away from the cytochrome path to the alternative oxidase; conversely, addition of AOX inhibitors in the presence of pyruvate redirects electrons to the cytochrome path (Hoefnagel *et al.*, 1995). This makes the use of inhibitors to estimate the contribution of oxidases to respiration (Bahr and Bonner, 1973) very risky. The results also confirm those reported by Wilson (1988). It is probable that the differences observed between mung bean mitochondria (Wilson, 1988) and those from soybean (Day, 1992) were due to differences in intramitochondrial pyruvate levels in the two species under the assay conditions employed.

Although the presence of pyruvate allows alternative oxidase activity at lower Q_r/Q_t, the concentration of total ubiquinone in the membrane, and its redox state, remain important regulatory parameters, and these may differ between tissues (Ribas-Carbo *et al.*, 1995). Since the redox state of AOX and intramitochondrial pyruvate levels also vary between tissues and species (Day *et al.*, 1994, 1995; see above), care must be taken to fully activate the oxidase when estimating its capacity. In at least some isolated mitochondria, the capacity of the alternative oxidase is only realized when AOX is reduced, pyruvate is present, and a mixture of substrates is added (to ensure high levels of QH_2).

The mechanism by which pyruvate and analogues activate the alternative oxidase is not yet known. Since pyruvate needs to be present continuously, as if it were a cofactor, it is possible that it participates in the alternative oxidase reaction, although that is unlikely. Initial kinetic analyses (M. N. Hoefnagel and J. T. Wiskich, unpublished data) indicate that oxidized Q (Q_{OX}) may be an inhibitor of the alternative oxidase and that pyruvate relieves the inhibition. Pyruvate seems to increase the affinity of AOX for QH_2 (Umbach *et al.*, 1994) but V_{max} is also changed (M. N. Hoefnagel and J. T. Wiskich, unpublished data). Interaction of pyruvate with a Q_{OX}-binding site, preventing Q_{OX} from binding and causing a conformational change in the AOX dimer so that its affinity for QH_2 is increased, is possible, but much more work in this area is needed.

REGULATION *IN VIVO*

In a given tissue, or preparation of mitochondria, alternative oxidase activity will depend on:

(a) the amount of oxidase protein present;
(b) the redox status of that protein;
(c) the level of pyruvate, both intra- and extramitochondrial;
(d) the concentration of quinone (Qt) in the inner mitochondrial membrane; and
(e) the Qr/Qt ratio.

Parameters (b), (c), and (e) will all respond to perturbations in substrate and cofactor (such as ADP and NAD(P)H) concentration. The activity of the alternative oxidase *in vivo*, as determined by inhibitor studies (Lambers, 1985) or oxygen discrimination (Robinson *et al.*, 1994), varies dramatically between tissues, often being most active when respiratory substrates are plentiful. An increase in sugars in the cell would stimulate carbon flow through glycolysis, increasing cytosolic pyruvate concentration and consequently other TCA cycle intermediates, leading to an increase in NAD(P)H in the matrix. This would ensure activation of AOX.

Treatments that inhibit the cytochrome path will also affect alternative oxidase activity. If cytosolic ATP/ADP ratios rise to the point where electron flux through the cytochrome chain is inhibited, matrix pyridine nucleotide pools will become reduced leading to an inhibition of TCA cycle carbon flow. This could result in an increase in intramitochondrial pyruvate concentration. The increase in both NAD(P)H and pyruvate would then feed-forward to activate the alterna-

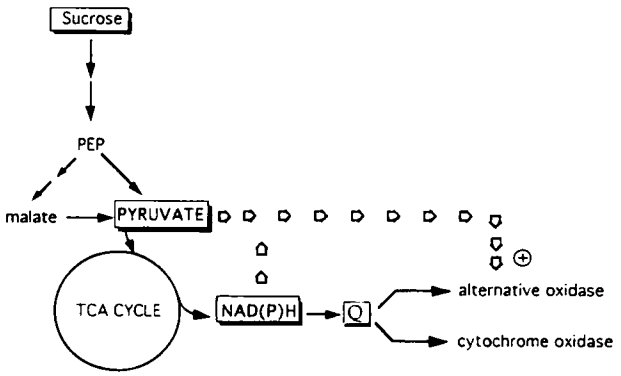

Fig. 4. Schematic of alternative oxidase control in plant cells. Sugar supply will affect pyruvate levels and NAD(P)H concentration in the matrix. Accumulation of pyruvate and NAD(P)H feedforward to stimulate the alternative oxidase (dotted arrow). Inhibition of the cytochrome chain will increase QH_2 levels and matrix NAD(P)H, also changing the activation state of the oxidase. See the text for details.

tive oxidase, increasing oxygen consumption and allowing TCA cycle turnover. Subsequent fluctuations in pyruvate concentration would provide short-term regulation of alternative oxidase operation.

This scheme has implications for the use of respiratory effectors in intact tissues. Uncoupling or poisoning of the cytochrome pathway in intact tissues may increase substrate flux and/or pyruvate levels (by decreasing cytosolic ATP and stimulating glycolysis), thereby changing alternative oxidase activity.

Pyruvate must be present continuously to maintain full alternative oxidase activity while the reduction state of AOX appears to be more stable. It is likely, therefore, that oxidation/reduction of the protein is a longer-term, coarse control mechanism, whereas fine tuning of activity occurs via fluctuations in pyruvate concentration. Activation of the alternative oxidase by this mechanism may prevent fermentation from accumulated pyruvate and may also prevent overreduction of the respiratory chain with its concomitant generation of harmful reactive oxygen species, such as superoxide (engagement of the alternative oxidase oxidizes ubiquinol and decreases electron flux through the cytochrome complexes; see Hoefnagel *et al.*, 1995). This is particularly important in the leaves of plants, where large fluctuations in carbon flux can occur.

Obviously more research on the regulation of respiration in intact cells and tissues is needed to confirm the control mechanisms described above. However, the few studies in which both pyruvate content and respiration have been measured generally support these ideas. For example, in a study of alternative oxidase activity in roots of various species, those species which showed highest activity also had highest levels of pyruvate (Day and Lambers, 1983). In CAM plants during de-acidification (Rustin and Queiroz-Claret, 1985; Robinson *et al.*, 1992), and in the bundle sheath certain C_4 plants (Gardeström and Edwards, 1983), pyruvate levels are also high and alternative oxidase is very active. It is also interesting to note that glyoxylate, another activator of the alternative oxidase, is a photorespiratory intermediate and that an increase in electron flow through the alternative oxidase occurs immediately following illumination under photorespiratory conditions in pea leaves (Azcón-Bieto *et al.*, 1983). The ability of oxoglutarate to stimulate alternative oxidase activity may be significant for ammonia assimilation, since oxoglutarate is needed for the GOGAT reaction.

Plants apparently possess a sophisticated feedforward control mechanism that ensures that the alternative oxidase is active when carbon substrates and reductant are plentiful, and this is summarized in Fig. 4.

SUMMARY

Our understanding of the regulation of electron partitioning in the plant respiratory chain has improved substantially over the last few years as mechanisms for the control of alternative oxidase activity have been identified. The challenge now is to describe these mechanisms in detail and to place them in a physiological context.

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