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A single amino acid change in the plant alternative oxidase alters the specificity of organic acid activation

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Abstract The alternative oxidase is a quinol oxidase of the respiratory chain of plants and some fungi and protists. Its activity is regulated by redox-sensitive disulphide bond formation between neighbouring subunits and direct interaction with certain α-ketoacids. To investigate these regulatory mechanisms, we undertook site-directed mutagenesis of soybean and *Arabidopsis* alternative oxidase cDNAs, and expressed them in tobacco plants and *Escherichia coli*, respectively. The homologous C99 and C127 residues of *GmAOX3* and *AtAOX1a*, respectively, were changed to serine. In the plant system, this substitution prevented oxidative inactivation of alternative oxidase and rendered the protein insensitive to pyruvate activation, in agreement with the recent results from other laboratories [Rhoads et al. (1998) J. Biol. Chem. 273, 30750–30756; Vanlerberghe et al. (1998) Plant Cell 10, 1551–1560]. However, the mutated protein is instead activated specifically by succinate. Measurements of AtAOX1a activity in bacterial membranes lacking succinate dehydrogenase confirmed that the stimulation of the mutant protein's activity by succinate did not involve its metabolism. Examples of alternative oxidase proteins with the C to S substitution occur in nature and these oxidases are expected to be activated under most conditions in vivo, with implications for the efficiency of respiration in the tissues which express them.

Key words: Mitochondrion; Respiration; Alternative oxidase

1. Introduction

The plant respiratory chain contains two terminal oxidases which compete for electrons from the ubiquinone (Q) pool: the well known cytochrome oxidase (COX) and the cyanide-insensitive alternative oxidase (AOX). The latter enzyme catalyses the oxidation of ubiquinol and the reduction of molecular oxygen to water, but does not translocate protons and consequently is not linked to oxidative phosphorylation. Operation of AOX partially deregulates respiration and allows rapid respiration in thermogenic plant tissues and in climacteric fruits [1,2]. However, it is present in all plants so far examined and may play a more general role in cell metabolism by preventing over-reduction of the respiratory chain and production of reactive oxygen intermediates [3–5]. When activated, AOX competes with the cytochrome chain for electrons [6–8] but little energy is conserved in respiration via AOX [9]. It has, therefore, the potential to adversely affect the growth of plants if it is not carefully regulated.

In some plant tissues AOX is regulated at the transcript level and is only synthesised in large quantities when required, e.g. during thermogenesis or in response to environmental stress such as cold or infection [2]. Often AOX is encoded by a gene family and different genes are regulated in response to different signals [10]. Plants have also evolved post-translational regulatory mechanisms which control AOX activity. AOX activity is controlled by the redox status of the Q pool [11] and is directly stimulated by certain α-ketoacids (e.g. pyruvate [12]) which substantially increase the apparent V_{max} of the enzyme [13]. The oxidase can be inactivated by formation of disulphide bonds between neighbouring subunits [14]. The covalently linked AOX dimer is largely insensitive to pyruvate [15] but can be reduced and activated during oxidation of malate and isocitrate [16], presumably via matrix NAD(P)H and thioredoxin [17]. AOX synthesis can also be triggered by citrate accumulation and H_2O_2 presentation [18,19]. Taken together, these features could constitute a sophisticated feed-forward regulatory mechanism which ensures that AOX is active only when carbon substrates in the cell are plentiful or reactive oxygen intermediates are generated. However, these factors have not yet been placed in a physiological context.

Recently, site-directed mutagenesis has been used to identify a particular cysteine residue near the N-terminus of AOX[1] which is responsible for the disulphide linkage of subunits and also participates in the activation by α-ketoacids [20,21]. Expression in *Escherichia coli* of *Arabidopsis* AOX sequences in which the cysteine residue had been converted to alanine resulted in an enzyme which could not be covalently linked but was poorly active and did not respond to pyruvate [20]. Conversion of the same cysteine to glutamate resulted in a permanently active enzyme even in the absence of pyruvate, although the activity was somewhat less than that of the wild-type enzyme [20]. Homologous expression of the corresponding tobacco C to A mutant yielded plants whose mitochondria contained AOX which could not be oxidised but remained largely inactive in isolated organelles [21]. However, respiratory analysis of intact cells from these plants indicated that AOX was active in vivo and the authors suggested that

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Abbreviations: AOX, alternative oxidase; COX, cytochrome *c* oxidase; Q, ubiquinone; Qr, ubiquinol; pyr, pyruvate; succ, succinate

[1] C126 in NtAOX1 and C127 in AtAOX1a (but sometimes referred to as C78 in the truncated *Arabidopsis* protein first described), C99 in GmAOX3.

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another, as yet unknown, regulatory mechanism operated in plants.

We have expressed mutated soybean *AOX3*, in which C99 has been converted to serine, in tobacco plants, and have confirmed the results of [20,21]. However, while this mutated AOX protein cannot be activated by pyruvate, it is activated by succinate and cannot be dimerised by oxidation of sulphydryl groups. This implies that this enzyme will be permanently activated in vivo. Thus a single amino acid change in the AOX sequence can dramatically alter the regulation of the enzyme. Since natural examples of this change exist in rice and tomato AOX, it has important implications for respiratory regulation in some species.

2. Materials and methods

2.1. Materials

Percoll and DNA modifying enzymes were purchased from Pharmacia Biochemical Inc. (Uppsala, Sweden) and Folin and Ciocalteau's reagent from BDH Chemicals (Melbourne, Australia). All other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Site-directed mutagenesis and plasmid construction

Unless stated otherwise, all DNA manipulations were done according to Sambrook et al. [22]. Prior to introducing into tobacco, the C99 codon of the *GmAOX3* cDNA was converted to serine by in vitro site-directed mutagenesis using an Altered Sites kit (Promega, Madison, WI, USA). The mutagenic oligonucleotide was 5′-GAGTGGC-CGTGGAACTCCTTCATGCCATGGG-3′ where the new serine codon is underlined. A *Bsp*HI restriction site was added 1 bp upstream of the initiation codon and a *Bst*EII restriction site was added 1 bp downstream of the termination codon of the wild-type and mutagenised *GmAOX3* open reading frames by PCR amplification using the primers 5′-TCATGAAGAATGTTTTAGTAAGGTCAGCTGC-3′ and 5′-GGTCACCTCAGTGATAACCAATAGGAGC-3′. The products were cloned into pBluescript II KS(+) (Stratagene), sequenced to confirm their identities and then subcloned as *Bsp*HI-*Bst*EII fragments into the *Nco*I and *Bst*EII sites of pCAMBIA1301 (a kind gift of R. Jefferson, Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia), placing the open reading frames under the transcriptional control of the CaMV 35S promoter.

For *E. coli* transformation, the C127 codon of *AtAOX1a* was converted to serine using the method of recombination PCR [23]. The template DNA was constructed by cloning the 201-bp *Nhe*I-*Nco*I fragment of pAtAOmKX into pNK [24], which yielded the entire *Nhe*I-*Kpn*I fragment of *AtAOX1a* in a pUC118-derived vector. One PCR fragment was synthesised using the β-lactamase gene-specific primer 5′-GACTTGGTTGAATATTCACCAGTC-3′ and the *AtAOX1a*-specific primer 5′-TTCAGGCCGTGGGAAACGTAT-3′; the other fragment was synthesised using the β-lactamase gene-specific primer 5′-GACTGGTGAATATTCAACCAAGTC-3′ and the *AtAOX1a*-specific primer 5′-CCACGGCCTGAAAGAGTTCCACT-TCCA-3′. Co-transformation of the PCR mixtures into DH5-α *E. coli* resulted in recombination of the fragments to form pNNK-C127S, which contained the C127S mutation and lacked the nearby *Nco*I site, as confirmed by sequencing. The 233-bp *Nhe*I-*Bgl*II fragment of pNNK-C127S was then cloned into a similarly digested pAtAOmKX, to form the expression construct pKX-C127S.

2.3. Plant growth and transformation

The wild-type and C99S forms of *GmAOX3* were introduced into tobacco (*Nicotiana tabacum* cv. Petit havana) by *Agrobacterium tumefaciens* (strain LBA4404)-mediated transformation of leaf discs essentially as described [25]. Transformed material was selected on 100 μg/ml hygromycin in modified Murashige and Skoog medium (see below). Regenerated plantlets were moved from culture to soil and grown in a glass-house under natural light at ambient temperature. Genomic DNA was isolated from each transformant using a commercial kit (Promega). The presence and identity of the transgene was verified by PCR and subsequent subcloning and sequencing of the PCR products. Non-transformed tobacco was germinated on one-third strength Murashige and Skoog medium [26], modified to contain one-third strength salts, 1 mg/l nicotinic acid, 1 mg/l pyridoxine HCl, 1 mg/l thiamine-HCl, 1 mg/l glycine, 100 mg/l *myo*-inositol, 30 g/l sucrose and full-strength minor elements. Plantlets were moved to soil and grown alongside the transformants.

2.4. Bacterial growth and transformation

Plasmids pAtAOmKX and pKX-C127S were introduced into *E. coli* strain SASX41B, an 5-aminolevulinic acid auxotroph [27], and transformants grown on succinate-containing media as described [24].

2.5. Mitochondrial and membrane vesicle isolation

Mitochondria were isolated from mature leaves of tobacco and purified according to Day et al. [28]. Membrane vesicles were prepared from *E. coli* as described by Berthold [24] except that PMSF, DNase, and RNase were omitted from the breakage buffer, and pyruvate was omitted throughout.

2.6. Oxygen and ubiquinone analyses

O_2 consumption was measured at 25°C using a Rank-Bros (Cambridge, UK) electrode. A standard reaction medium (0.3 M sucrose, 10 mM TES buffer [pH 7.2], 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] bovine serum albumin) was used for mitochondria and assumed to contain an air-saturated O_2 concentration of 250 μM. Assays containing succinate also included 0.1 mM ATP to activate succinate dehydrogenase. For assay of *E. coli* membrane vesicles, the reaction buffer contained 100 mM NaCl, 50 mM potassium phosphate, 10 mM potassium chloride, 5 mM magnesium chloride, and 1 mM EDTA, pH 6.5.

The redox state of Q was measured voltammetrically with glassy carbon and platinum electrodes according to Moore et al. [29]. When NADH was used as a substrate with the Q electrode, a regeneration system comprising a small quantity of NAD^+, glucose 6-phosphate and glucose 6-phosphate dehydrogenase was used [30].

2.7. Protein analysis

Protein content of mitochondria was determined by the method of Lowry et al. [31] and that of *E. coli* vesicles by Petersen's modification [32]. Aliquots of mitochondria containing 40 μg of protein were separated by SDS-PAGE as described by Kearns et al. [33]. DTT and diamide (both 5 mM) were included in the sample buffer where indicated. A modified version of the method of Towbin et al. [34] was employed for immunoblotting with the AOA monoclonal antibody raised against AOX proteins from *Sauromatum guttatum* (generously supplied by T.E. Elthon, University of Nebraska and L. McIntosh, Michigan State University). Immunoreactive proteins were visualised using the BM Chemiluminescence system (Boehringer Mannheim, Mannheim, Germany).

3. Results

3.1. Expression of soybean AOX3 in tobacco

GmAOX3 was expressed at high levels in tobacco leaves (Fig. 1). Under the growth conditions used, wild-type tobacco produces little endogenous AOX protein, allowing ready detection of the soybean protein (Fig. 1). PCR analysis was used to confirm that the protein expressed was indeed that encoded by the soybean gene (results not shown; see Section 2).

Both the endogenous tobacco protein and GmAOX3 showed typical dimerisation when the mitochondria were treated with the oxidant diamide and this was reversed upon treatment with DTT (Fig. 1). The C99S mutant of GmAOX3, on the other hand, was not covalently linked upon oxidation with diamide (Fig. 1), as shown previously for C to A mutants of AOX [20,21]. We consistently observed a higher level of expression of the C99S mutant than of wild-type GmAOX3 (Fig. 1).

3.2. Activity of soybean AOX3 in tobacco mitochondria

Wild-type tobacco mitochondria showed only a modest

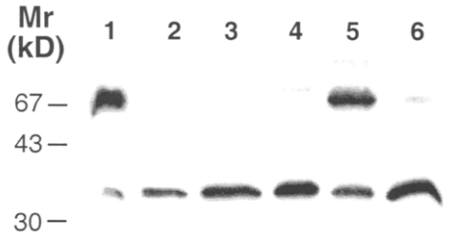

Fig. 1. Immunoblot of GmAOX3 proteins. Lane 1: wild-type GmAOX3 in tobacco mitochondria, treated with diamide; lane 2: wild-type GmAOX3 in tobacco mitochondria, treated with DTT; lane 3: C99S GmAOX3 in tobacco mitochondria, treated with DTT; lane 4: C99S GmAOX3 in tobacco mitochondria, treated with diamide; lane 5: soybean root mitochondria treated with diamide; lane 6: soybean root mitochondria treated with DTT. Diamide and DTT were added at 5 mM and 40 μg protein was loaded in each lane. Numbers on the left hand side of the figure are molecular weight markers in kDa.

AOX activity (O_2 uptake in the presence of the cytochrome chain inhibitor myxothiazol), which was dependent on the addition of both pyruvate and DTT (Fig. 2A). AOX activity was substantially greater in mitochondria from tobacco transformed with *GmAOX3* but maximal activity again required the presence of both pyruvate and reductant with NADH as substrate (Fig. 2B,C). When succinate was substrate, the dependence of AOX activity on added pyruvate and reductant was less pronounced (not shown), because of intramitochondrial malate and pyruvate production [16].

With NADH as substrate, mitochondria expressing the C99S mutant of *GmAOX3* showed low AOX activity and this changed little when pyruvate and DTT were added (Fig. 2F), as observed for C to A mutants of AOX in other systems [20,21]. The slow rate of O_2 uptake observed after addition of myxothiazol was unlikely to be due to the presence of endogenous tobacco AOX, since it was not stimulated by pyruvate (Fig. 2F, cf. Fig. 2A). When succinate was used as substrate, AOX activity was much greater (even though the state 3 rate prior to myxothiazol addition was slower with succinate than that with NADH), but, again, did not respond to either pyruvate or DTT (Fig. 2F). Addition of succinate to the C99S-containing mitochondria respiring NADH in the presence of myxothiazol stimulated O_2 uptake dramatically even when malonate was present to inhibit succinate dehydrogenase (Fig. 2D,F). This suggests that succinate is an activator of the C99S mutant AOX. The faster rate of AOX seen when succinate was added in the absence of malonate (Fig. 2F) reflects the increased donation of electrons into the Q pool when two substrates are oxidised simultaneously.

Voltammetric measurements of Q redox state confirmed the activation of the mutant AOX by succinate (Fig. 3). Malonate was added to mitochondria containing the C99S mutant GmAOX3 protein, to prevent succinate oxidation, and the concentration of NADH was kept low (via a regeneration system [30]; see legend to Fig. 3) to prevent a large reduction of the Q pool and maximise shifts due to changes in electron flow. Addition of NADH stimulated O_2 uptake and caused reduction of the Q pool. Subsequent addition of myxothiazol inhibited O_2 uptake severely which further reduced Q, indicating that most of the O_2 uptake in the presence of NADH was via COX. Addition of succinate at this point stimulated O_2 uptake and led to a substantial oxidation of the Q pool (Fig. 3), showing that succinate was not acting as a substrate but

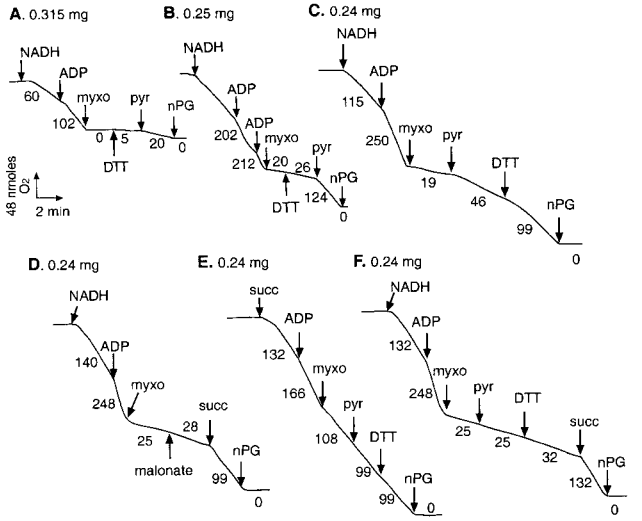

Fig. 2. Oxygen uptake by isolated tobacco mitochondria containing soybean AOX protein. A: Mitochondria from tobacco containing binary vector alone. B and C: Tobacco mitochondria containing wild-type GmAOX3. D–F: Tobacco mitochondria containing the C99S GmAOX3 mutant protein. Numbers on traces are nmol O_2 min^{-1} mg^{-1} protein. Where indicated, the following additions were made: 1 mM NADH, 0.1 mM ADP, 5 μM myxothiazol (myxo); 5 mM dithiothreitol (DTT); 5 mM pyruvate (pyr); 200 μM *n*-propyl gallate (nPG); 5 mM succinate (succ); 10 mM malonate.

rather as an activator of AOX. This effect of succinate on Qr and O_2 uptake was very similar to the effect of pyruvate on soybean root mitochondria [35,36] in which GmAOX3 is the sole AOX protein [10].

The relationship between Q and the different AOX proteins is analysed further in Fig. 4. Here the rate of O_2 uptake is plotted against the proportion of Q in the reduced state (Qr/Qt). The substrate was succinate in each case and the two parameters were varied by sequential additions of small quantities of malonate to inhibit succinate dehydrogenase [29]. In the mitochondria containing wild-type GmAOX3, a small quantity of isocitrate was included in the reaction medium to reduce and thereby activate AOX [16]. In these mitochondria, a typical AOX curve was observed, with AOX activity

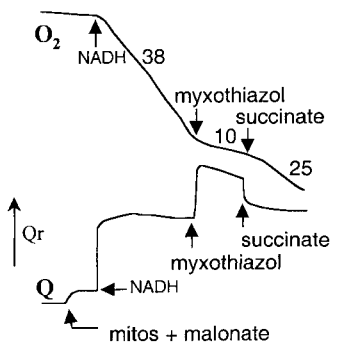

Fig. 3. NADH-dependent O_2 consumption and steady-state Q reduction in tobacco mitochondria containing the C99S mutant of GmAOX3. Where indicated the following additions were made to the reaction vessel: 12.5 μM NADH (generated by the addition of NAD^+, glucose 6-phosphate and glucose 6-phosphate dehydrogenase), 5 μM myxothiazol, 5 mM succinate, 0.56 mg mitochondrial protein and 5 mM malonate. The quantity of mitochondrial protein used in each assay is indicated at the top of each figure.

becoming observable only at a relatively high Qr/Qt ratio [11]. When pyruvate was included in the reaction medium to activate AOX, activity was detectable at a lower Qr/Qt as more AOX protein became engaged and apparent Vmax increased [30,35]. These results are very similar to those observed with mitochondria isolated from soybean roots and cotyledons [7,15,36], and show that the soybean AOX interacts in a 'normal' manner with the Q pool when produced in tobacco. In mitochondria containing the C99S mutant GmAOX3, on the other hand, pyruvate had virtually no effect on the response of AOX to Qr/Qt and appreciable AOX activity was observed at low Qr/Qt. That is, in the presence of succinate the mutant protein acted as if it was fully activated without treatment with pyruvate. The Qr response curve of AOX in these mitochondria was shifted slightly to the left compared to (activated) wild-type GmAOX3 activity, probably because of the higher expression of mutant protein (Fig. 1) which alters the relationship [35].

3.3. Expression of AOX in E. coli

Our attempts to express active *GmAOX3* in *E. coli* were unsuccessful. We therefore followed the lead of Rhoads et al. [20] and used the well characterised *Arabidopsis thaliana AOX1a* cDNA [24] to further analyse the effect of the converting the conserved cysteine residue (C127 in *AtAOX1a*) to serine. Membrane vesicles were prepared from haem$^-$ *E. coli* cultures expressing both wild-type and mutant *AtAOX1a*, in the absence of pyruvate, and AOX activity measured with an O_2 electrode (Fig. 5). Since haem is required for the functional assembly of succinate dehydrogenase [37], the electron transport chain of this strain presumably consisted of NADH dehydrogenase, Q and AOX.

In vesicles containing wild-type *AtAOX1a*, a low rate of O_2 uptake was seen upon addition of NADH and this was stimulated dramatically by subsequent addition of 0.5 mM pyruvate (Fig. 5A). This oxygen uptake was abolished by *n*-propyl gallate. With the C127S mutant, on the other hand, pyruvate had no significant effect on O_2 uptake even when added at high concentrations (Fig. 5B). However, addition of 0.5 mM succinate to vesicles containing the mutant protein stimulated O_2 uptake substantially and a further stimulation was seen upon addition of 5 mM succinate (Fig. 5C). Succinate had no significant effect on NADH-driven O_2 uptake by vesicles containing the wild-type oxidase (Fig. 5D). Since these membranes lack succinate dehydrogenase, no O_2 uptake was observed with succinate alone (Fig. 5E). These results show that the succinate stimulation of the mutant occurs with AOX proteins from different plants and confirm that the succinate effect does not involve its metabolism.

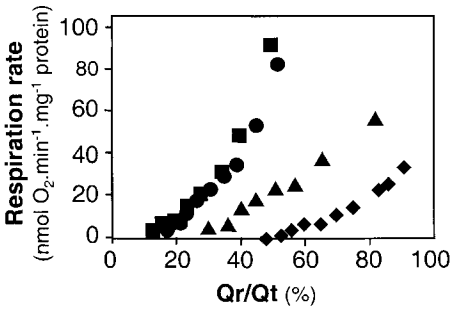

Fig. 4. Dependence of O_2 consumption rate on Q redox state in purified tobacco mitochondria containing GmAOX3. Qr refers to Q_1 reduced under steady-state conditions, and Qt to fully reduced Q_1 under anaerobic conditions. Succinate oxidation was inhibited by successive step-wise additions of malonate [29]. ADP (1 mM) was present in all cases and 0.1 mM ATP was added to activate succinate dehydrogenase. Isocitrate (0.1 mM) was included in the reaction medium to activate wild-type GmAOX3. ■: mitochondria containing the C99S mutant protein of GmAOX3, plus 5 mM pyruvate; ●: mitochondria containing the C99S mutant protein of GmAOX3, in the absence of pyruvate; ▲: mitochondria containing the wild-type GmAOX3 protein, plus 5 mM pyruvate; ◆: mitochondria containing wild-type GmAOX3 protein, in the absence of pyruvate.

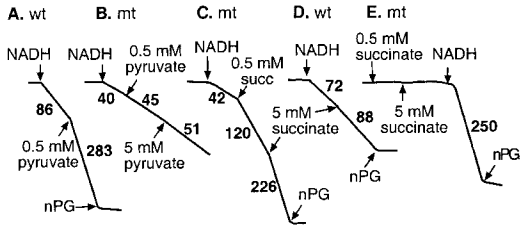

Fig. 5. AtAOX1a activity in membrane vesicles of *E. coli*. Expression of wild-type or mutated *Arabidopsis* AOX1a in SASX41B *E. coli* was induced by growing cells without the haem precursor 5-aminolevulinic acid. Membrane vesicles were prepared from these cells as described in Section 2. Typical recordings obtained with an O_2 electrode are shown. A and D: Wild-type AtAOX1a. B, C and E: C127S AtAOX1a mutant. NADH was added at 1 mM and *n*-propyl gallate (nPG) at 50 μM. Numbers on traces are nmol O_2 min^{-1} mg^{-1} protein succinate

4. Discussion

The results presented confirm recent evidence [20,21] that the highly conserved cysteine residue near the N-terminus of the AOX protein is responsible for both disulphide bond formation and organic acid activation of the oxidase. Clearly, any change in the amino acid at this position will have a dramatic effect on the regulation of AOX and has the potential to affect plant respiratory efficiency. We show here that replacing the cysteine in question with a serine residue results in an enzyme that cannot be inactivated by oxidation and which is stimulated by succinate. We anticipate that in vivo this enzyme will be active under most conditions.

The dramatic change in the specificity of organic activation in the C to S mutants was unexpected and has implications for the nature of the activation. Preliminary experiments (D. Berthold, G. Vanlerberghe and D. Day, unpublished results) indicate that the corresponding C to A mutant protein [20,21] also responds to succinate. This indicates that the formation of a thiohemiacetal [20,38] is not required per se for activation of the enzyme. The stimulation by succinate seems to be quite specific, since neither malonate (Fig. 2D) nor fumarate (not shown) stimulates the mutated enzyme. Half-maximal stimulation in intact mitochondria containing GmAOX3 was achieved at about 1 mM succinate while the *Arabidopsis* enzyme in membrane vesicles from *E. coli* required approximately 5 mM (not shown). These concentrations are likely to be physiologically relevant, especially as succinate is produced within the mitochondrial matrix where it may become concentrated. Importantly, two naturally occurring examples of AOX proteins with serine at this position have been identified recently, in rice [39] and tomato (R. Holtzapffel, P. Finnegan and D. Day, unpublished results). Nothing is

known concerning the expression or activity of the rice protein, but the tomato AOX is expressed in some tissues (R. Holtzapffel, P. Finnegan and D. Day, unpublished results). The regulation of these enzymes in vivo warrants extensive investigation and the transgenic tobacco plants described in this report will be valuable in this respect.

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