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Two Cys or Not Two Cys? That Is the Question; Alternative Oxidase in the Thermogenic Plant Sacred Lotus

Abstract

Sacred lotus (*Nelumbo nucifera*) regulates temperature in its floral chamber to 32°C to 35°C across ambient temperatures of 8°C to 40°C with heating achieved through high alternative pathway fluxes. In most alternative oxidase (AOX) isoforms, two cysteine residues, Cys_1 and Cys_2, are highly conserved and play a role in posttranslational regulation of AOX. Further control occurs via interaction of reduced Cys_1 with α-keto acids, such as pyruvate. Here, we report on the in vitro regulation of AOX isolated from thermogenic receptacle tissues of sacred lotus. AOX protein was mostly present in the reduced form, and only a small fraction could be oxidized with diamide. Cyanide-resistant respiration in isolated mitochondria was stimulated 4-fold by succinate but not pyruvate or glyoxylate. Insensitivity of the alternative pathway of respiration to pyruvate and the inability of AOX protein to be oxidized by diamide suggested that AOX in these tissues may lack Cys_1. Subsequently, we isolated two novel cDNAs for AOX from thermogenic tissues of sacred lotus, designated as NnAOX1a and NnAOX1b. Deduced amino acid sequences of both confirmed that Cys_1 had been replaced by serine; however, Cys_2 was present. This contrasts with AOXs from thermogenic Aroids, which contain both Cys_1 and Cys_2. An additional cysteine was present at position 193 in NnAOX1b. The significance of the sequence data for regulation of the AOX protein in thermogenic sacred lotus is discussed and compared with AOXs from other thermogenic and nonthermogenic species.

Keywords

cyanide resistant respiration, AOX, plant respiration

Disciplines

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Two Cys or not two Cys, that is the question? Alternative oxidase in the thermogenic plant *Nelumbo nucifera*.

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ABSTRACT

Sacred lotus (*Nelumbo nucifera*) regulates temperature in its floral chamber to 32-35°C across ambient temperatures of 8-40°C with heating achieved through high alternative pathway fluxes. In most alternative oxidase (AOX) isoforms, two cysteine residues, Cys_1 and Cys_2, are highly conserved and play a role in post-translational regulation of AOX. Further control occurs via interaction of reduced Cys_1 with α-keto acids, such as pyruvate. Here, we report on the *in vitro* regulation of AOX isolated from thermogenic receptacle tissues of sacred lotus. AOX protein was mostly present in the reduced form, and only a small fraction could be oxidized with diamide. Cyanide resistant respiration in isolated mitochondria was stimulated 4-fold by succinate, but not pyruvate or glyoxylate. Insensitivity of the alternative pathway of respiration to pyruvate and the inability of AOX protein to be oxidized by diamide suggested that AOX in these tissues may lack Cys_1. Subsequently, we isolated 2 novel cDNAs for AOX from thermogenic tissues of *N. nucifera*, designated as *NnAOX1a* and *NnAOX1b*. Deduced amino acid sequences of both confirmed that Cys_1 had been replaced by serine, however Cys_2 was present. This contrasts with AOXs from thermogenic Aroids, which contain both Cys_1 and Cys_2. An additional Cys was present at position 193 in NnAOX1b. The significance of the sequence data for regulation of the AOX protein in thermogenic sacred lotus is discussed, and compared with AOXs from other thermogenic and non-thermogenic species.

INTRODUCTION

Thermogenesis in the sacred lotus

The sacred lotus (*Nelumbo nucifera*) is a thermogenic plant that regulates the temperature of its floral chamber between 32-35°C for up to 4 days (Seymour and Schultze-Motel, 1996). Heating of plant tissues has been described as an adaptation to: attract insect pollinators either by volatilization of scent compounds (Meeuse, 1975) or by providing a heat reward (Seymour et al., 1983), protect floral parts from low temperatures (Knutson, 1974), or provide the optimum temperature for floral development (Ervik and Barfod, 1999; Seymour et al., 2009). In the sacred lotus, heat is produced by high rates of alternative pathway respiration (Watling et al., 2006; Grant et al., 2008) however the mechanisms of heat regulation, which likely occur at a cellular level, remain unclear.

Alternative oxidase

Alternative pathway respiration is catalyzed by the alternative oxidase protein (AOX), which acts as a terminal oxidase in the electron transport chain but, unlike the energy conserving cytochrome pathway (COX), complexes III and IV are bypassed and energy is released as heat. Traditionally, AOX activity was measured using oxygen consumption of tissue, cells or isolated mitochondria in the presence or absence of AOX and COX inhibitors. However, this method does not accurately measure activity *in vivo* but does indicate the 'capacity' of the alternative pathway (Ribas-Carbo et al., 1995; Day et al., 1996). The only method to date to accurately determine AOX activity, that is flux of electrons through the AOX pathway *in vivo*, is to use oxygen isotope discrimination techniques (for review see Robinson et al., (1995)). Determining AOX activity *in vivo* is important because heat production in plants

could be due to activity of either the alternative oxidase (AOX), and/or plant uncoupling proteins. Using oxygen fractionation techniques we have shown that flux through the AOX pathway is responsible for heating in the sacred lotus (Watling et al., 2006; Grant et al., 2008). Furthermore, we were unable to detect any uncoupling protein in these tissues (Grant et al., 2008). AOX protein content within the sacred lotus receptacle increases markedly prior to thermogenesis, but it remains constant during heating (Grant et al., 2008), suggesting that regulation of heating occurs through post-translational modification of the protein.

Post-translational regulation of AOX protein

The plant alternative oxidase is a cyanide insensitive dimeric protein located in the inner mitochondrial membrane (Day and Wiskich, 1995). The dimer subunits (monomers) can be linked via a non covalent association (reduced protein) or covalently through the formation of a disulfide bridge (oxidized protein; Umbach and Siedow, 1993). The reduced protein when run on SDS-PAGE has a molecular mass of approximately 30-35 kDa and the oxidized protein 60-71 kDa; this holds true for AOX from a number of species including soybean roots and cotyledons (Umbach and Siedow, 1993), tobacco leaf (Day and Wiskich, 1995) and the thermogenic spadix of *Arum maculatum* (Hoefnagel and Wiskich, 1998).

Regulation of AOX has been well studied in non-thermogenic plant species and two mechanisms have been identified. Most AOX isoforms have two, highly conserved cysteine residues, Cys_1 and Cys_2 (defined by Berthold et al., 2000 and Holtzapffel et al., 2003), located near the N-terminal hydrophilic domain of the protein. In these isoforms, Cys_1 can either be reduced on both subunits of the AOX dimer or the Cys_1 sulfhydryl groups can be

oxidized to form a disulfide bridge (Rhoads et al., 1998). Reduction/oxidation modulation of AOX *in vitro* can be achieved using the sulfhydryl reductant dithiothreitol (DTT) to reduce the protein, or diamide to oxidize the cysteines. The reduced dimer can be further activated via the interaction of Cys_1 with α-keto acids, principally pyruvate (Rhoads et al., 1998; see McDonald (2008) for a model of post-translational regulation of AOX). In addition, Cys_2 may also be involved in regulating AOX activity through interaction with the α-keto acid glyoxlyate (which can also stimulate activity at Cys_1; Umbach et al., 2002).

Recently however, AOX proteins with different regulatory properties have been reported. Naturally occurring AOX proteins without the two regulatory cysteines have been identified and, along with site-directed mutagenesis studies, used to further elucidate the specific roles of Cys_1 and Cys_2. The LeAOX1b isoform from tomato which has serine residue at the position of Cys_1, and thus does not form disulfide linked dimers, it is also activated by succinate rather than pyruvate, when expressed in *S. cerevisiae* (Holtzapffel et al., 2003). In *Arabidopsis* uncharged or hydrophobic amino acid substitutions of either Cys result in an inactive enzyme, while positively charged substitutions produce an enzyme with higher than wild type basal activity but which is insensitive to pyruvate or succinate (Umbach et al., 2002). Single substitutions at Cys_1 or Cys_2 have revealed that glyoxylate can activate AOX via both cysteines, but only one is needed for glyoxylate stimulation (Umbach et al., 2002; Umbach et al., 2006). Double substitution mutants were not stimulated by either pyruvate or glyoxylate (Umbach et al., 2006).

Previously we have determined that thermogenesis via the AOX pathway in the sacred lotus

receptacle is precisely regulated through changes in AOX flux rather than changes to protein content (Grant et al., 2008). In this study we investigated the nature of this regulation in mitochondria isolated from heating receptacles. Our aim was to elucidate the reduction/oxidation behaviour of the AOX protein and the mechanisms of activation of cyanide resistant respiration in sacred lotus receptacles to provide insights into the mechanism(s) of heat regulation in this species. We further investigated AOX regulation, by determining the amino acid sequence of two novel AOX genes isolated from thermogenic receptacle tissue of sacred lotus.

RESULTS

Activity of sacred lotus AOX is stimulated by succinate but not pyruvate or glyoxylate

Residual mitochondrial respiration rates were quite low (<10 nmol O_2 min^{-1} mg^{-1} protein). Addition of NADH and KCN stimulated activity to an average of 50 nmol O_2 min^{-1} mg^{-1} protein but this stimulation was not statistically significant. No stimulation was observed with subsequent addition of 5 mM pyruvate (Fig. 1) nor with concentrations of pyruvate up to 20 mM. Addition of succinate, however, produced a 4-fold increase in activity to a mean of 196 ± 20 nmol O_2 min^{-1} mg^{-1} protein ($F_{2,20}$=48.70, p<0.0001; Fig. 1). Activation of respiration by succinate was similar in the presence or absence of malonate, which was used to inhibit complex II. Cyanide-resistant O_2 uptake was not stimulated by the addition of glyoxylate, either before or after succinate stimulation (Fig. 1C-F) and there was no increase in mitochondrial O_2 uptake with the possible substrates: citrate, fumarate, oxalate, α-ketoglutarate or malate (data not shown). Manipulation of AOX redox state by addition of the sulfhydryl redox reagents DTT (Fig. 1B) and diamide had no effect on O_2 uptake.

Cyanide-resistant oxygen uptake was almost completely inhibited by the AOX inhibitor *n*-PG (Fig. 1B, D &F).

The majority of AOX protein does not form disulfide-linked dimers in the presence of diamide

AOX protein isolated from thermogenic sacred lotus receptacles was predominantly in the reduced form (~32 kDa) with only 21% present in the oxidized state (~64 kDa; Fig. 2, lane 1). When treated with the reductant DTT (20 mM), almost all of the protein was present in the reduced state (Fig. 2, lane 2), although a small proportion (12%) remained oxidized. The reduced protein could be partially re-oxidized with 10 mM diamide (19%; Fig. 2, lane 3) however most of the protein was insensitive to diamide even at high concentrations (50-250 mM). In contrast, treatment with the Lys-Lys specific crosslinker EGS (1 mM) caused 76% dimerisation of the AOX protein (Fig. 2, lane 4).

Two novel AOX *isoforms lacking* Cys_1 *occur in thermogenic sacred lotus tissue*

RT-PCR based cloning of *AOX* transcripts was performed with total RNAs from thermogenic receptacles. Because two highly homologous partial fragments were detected during PCR analyses, full-length cDNAs of the corresponding transcripts were isolated and consequently named *NnAOX1a* and *NnAOX1b* (DDBJ accession numbers AB491175 and AB491176, respectively). The deduced amino acid sequences of the encoded proteins indicate that NnAOX1a and NnAOX1b encode proteins of 39.0 kDa and 39.3 kDa, respectively, 32.5 kDa and 32.6 kDa after cleavage of the mitochondrial targeting sequence. Both NnAOX1a and NnAOX1b contain some of the structural features typical of plant AOXs such as four α-helical bundles and ligands for the two iron atoms of the active center (Moore and Albury,

2008). However, both NnAOX1a and NnAOX1b were found to contain a Ser residue at the site of the highly conserved Cys_1 residue, which is necessary for the regulation of the plant AOX through both redox control and α-keto acid stimulation, although the second conserved cysteine, Cys_2, was present in both (Fig. 3). Additionally, in the case of NnAOX1b a leucine residue at position 193 was substituted by cysteine (Fig. 3).

NnAOX1a and NnAOX1b are similar to AOX isoforms from other dicots

Sequence alignment indicated that NnAOX1a and NnAOX1b are distinct from AOX isoforms reported from other thermogenic species in that they lack Cys_1, while *Dracunculus vulgaris, Philodendron bipinnafitidum, Sauromatum guttatum* and *Symplocarpus reinifolius* all contain both Cys_1 and Cys_2 (Fig. 3). Further analysis indicated that NnAOX1a and NnAOX1b from thermogenic sacred lotus were more similar to AOXs from other dicots than they were to AOXs from other thermogenic plants (Fig. 4, Supplemental Fig. S1). NnAOX1b also contains an extra Cys residue at position 193, this is similar to AtAOX1a, LeAOX1a, LeAOX1b and NtAOX1a, in which a leucine is replaced by cysteine at the same position (Supplemental Fig. S1). Based on the AOX model for *S. guttatum* (Andersson and Nordlund, 1999), this cysteine is located after the first α-helix, but we are unsure whether it sits within the membrane or matrix region of the protein.

DISCUSSION

In most plants studied to date the α-keto acid pyruvate stimulates AOX activity (Day et al., 1994) and the specific site of this regulation is reduced Cys_1 (Rhoads et al., 1998). Following pyruvate stimulation, glyoxylate can further increase AOX activity via Cys_2 (Umbach et al.,

2002) and can also initiate activity at either cysteine alone (Umbach et al., 2006). Recent studies on AOX isoforms without the regulatory cysteines have revealed stimulation by succinate, not pyruvate, when Cys_1 is not present (Djajanegara et al., 1999; Holtzapffel et al., 2003) and the glyoxylate effect is absent when both cysteines are missing (Umbach et al., 2006) . Here we report that AOX from thermogenic tissues of the sacred lotus is stimulated by succinate rather than pyruvate (Fig. 1A), that there is no glyoxylate effect (Fig 1 C-F), and that the majority of AOX could not be reversibly reduced and oxidized (Fig. 2). Our results thus suggested that the majority of AOX in these tissues lacked Cys_1, and that Cys_2 might also be missing. Subsequent sequencing of two cDNAs, *NnAOX1a* and *NnAOX1b*, isolated from thermogenic sacred lotus indicated that Cys_1 is replaced by serine, but that Cys_2 is present in both (Fig. 3). This confirmed our predictions, based on the *in vitro* studies of isolated mitochondria, that Cys_1 was missing from the majority of AOX protein in these tissues. The situation with Cys_2 is complicated, however, by the fact that glyoxylate stimulation of AOX containing this residue varies between naturally occurring and site-directed AOX substitutions. For example, similarly to our experiments, glyoxylate failed to stimulate tomato AOX (*Le*AOX1b) even though it contains Cys_2 (Holtzapffel et al., 2003). *Le*AOX1b was also activated by succinate in a similar fashion to the thermogenic lotus AOX. In contrast, site-directed mutation of both cysteines in *Arabidopsis* indicated that only one cysteine was needed for glyoxylate stimulation (Umbach et al., 2002; Umbach et al., 2006).

In the majority of plants, AOX can be reversibly reduced and oxidized (Umbach and Siedow, 1997). However, when extracted under non-reducing conditions, the sacred lotus receptacle AOX protein was predominantly in the reduced (i.e. non-linked) state and could not be

further oxidized with diamide across a range of concentrations up to 250 mM. This contrasts strongly with AOX proteins from soybean cotyledons and *Arabidopsis* leaves, where diamide concentrations of less than 5 mM were sufficient to oxidize AOX (Umbach and Siedow, 1993), whilst 200 mM diamide was able to oxidize AOX protein from chilled green tomato mitochondria (Holtzapffel et al., 2003). As formation of the oxidized dimer requires the presence of Cys_1 (Rhoads et al., 1998; Djajanegara et al., 1999; Umbach et al., 2006), our results are consistent with this regulatory cysteine being absent from the majority of AOX found in thermogenic sacred lotus. This results in an AOX that is permanently in the reduced state and ready for further activation by succinate. Thus, fine control of activity during heating may be modulated by succinate levels. Similarly, naturally occurring and mutated AOX proteins with serine substitutions at Cys_1 lack the ability to form oxidized dimers and, like the sacred lotus receptacle AOX, are poised for activation (Ito et al., 1997; Umbach et al., 2002; Holtzapffel et al., 2003; Umbach et al., 2006).

In contrast to the results with diamide, AOX from sacred lotus receptacle was able to form dimers when exposed to the Lys-Lys crosslinker, EGS. Monomeric AOX proteins such as those found in fungi (e.g. *Neurospora crassa* and *Pichia stipitis*) do not form dimers in the presence of EGS or diamide (Umbach and Siedow, 2000). Thus, while most of the thermogenic sacred lotus AOX protein is able to be covalently bound, only a small fraction (~20%) can form disulfide bonds in the presence of diamide (Fig. 2). This suggests that there may be an additional isoform, that unlike NnAOX1a and NnAOX1b contains Cys_1. Alternatively, there is the possibility that the additional Cys at position 193 in NnAOX1b, may be involved in disulfide bridge formation; although this Cys may not be close enough to

Cys_2 in the tertiary or quaternary structure of the protein to form disulfide bonds (Gilbert, 1990). Interestingly, a further isoform may be present in thermogenic sacred lotus, as we detected a small band around 60 kDa that could not be reduced in the presence of DTT (Fig 2, lane 2). This band represented around 12% of the total AOX protein present in our samples. Multiple AOX isoforms in the same tissue have been reported in a number of different species including thermogenic *Sauromatum guttatum*, in which a 37 kDa species is joined by a 35 kDa and a 36 kDa species during thermogenesis (Rhoads and McIntosh, 1992). It is possible that these different isoforms could form heterodimers. A mixture of homodimers and heterodimers have been proposed to occur in soybean (Finnegan et al., 1997), while in tomato it was suggested that heterodimeric associations between *Le*AOX1a and *Le*AOX1b could explain why full oxidation of tomato AOX dimers did not occur (Holtzapffel et al., 2003). Whether AOX heterodimers occur in thermogenic sacred lotus, and whether they have different catalytic properties from homodimers has yet to be investigated.

Crichton et al.,(2005) suggested that changes to amino acids other than the regulatory Cys_1 and Cys_2 may influence AOX activity in thermogenic species. This suggestion is based on a constitutively active SgAOX, with both conserved cysteines, which when expressed in yeast was insensitive to both pyruvate and succinate. However, the absence of Cys_1 in both NnAOX1a and NnAOX1b, and the fact that succinate was required for full alternative pathway activity in mitochondria isolated from thermogenic sacred lotus, makes it unlikely that these isoforms are regulated in a similar way to that hypothesized for *S. guttatum* (Crichton et al., 2005). Furthermore, AOX proteins that have been modified by amino acid substitutions or expressed in bacteria or yeasts may not reflect *in vivo* behavior, thus

comparisons with naturally occurring isoforms need to be approached with caution. Ours is the only study to date where naturally occurring AOX isoforms, without Cys_1, have been studied in plant mitochondria.

Regulation of heating via post-translational regulation of AOX

Sacred lotus is, to our knowledge, the only thermoregulating dicot so far described. Thus, it is perhaps not surprising that NnAOX1a and NnAOX1b were more closely aligned with AOXs from other dicots than with those from other thermogenic plants, all of which are monocots (Fig. 4). Based on our phylogenetic analysis, the two deduced sacred lotus AOX sequences were more similar to GhAOX1 from cotton than to any other AOX. It was also interesting that the only dicot AOX that fell within the same group as the thermogenic monocots was LeAOX1b from tomato. These results suggest that there is no specific AOX sequence associated with thermogenic activity in plants, rather it may be the amount of AOX synthesized that allows these plants to generate heat. This is further supported by the fact that there appear to be only a few mechanisms of post-translational regulation for AOX proteins from a wide variety of species, and that the same mechanism may be shared by both non-thermogenic and thergmogenic plants. For example, succinate activation of AOXs in which Cys_1 has been replaced by serine, is found in both thermogenic sacred lotus and non-thermogenic tomato (Holtzapffel et al., 2003). Similarly, pyruvate activation via reduced Cys_1, occurs in both thermogenic and non-thermogenic plants (Day et al., 1994; Onda et al., 2007). Modulation of AOX activity by either succinate or pyruvate could be important for those plants that thermoregulate, such as sacred lotus (Seymour and Schultze-Motel, 1996), *S. renifolius* (Knutson, 1974) and *P. bipinnafitidum* (Nagy et al., 1972). In contrast, *S.*

guttatum, the only thermogenic plant in which a constitutively active AOX has been found, does not thermoregulate. Rather this species has a single burst of heat production that lasts only a few hours (Meeuse, 1966).

Our observation that succinate stimulation of AOX occurs in thermogenic sacred lotus mitochondria even in the presence of malonate (a succinate dehydrogenase inhibitor), suggests a possible non-metabolic interaction of succinate with the AOX protein. As succinate is a common TCA cycle intermediate, it is possible that upstream substrate availability could be a signal for AOX activation. Other thermogenic species that are poised in the reduced state and that use lipids instead of carbohydrates to fuel thermogenesis, for example *P. bipinnafitidum* (N. Grant & R. Miller unpublished data) may use products from lipid metabolism to signal AOX activation. If substrate supply is the signal, succinate activation of sacred lotus AOX may play a larger role than previously thought, however this requires further investigation. Ubiquinol reduction status (Wagner et al., 2008) as well as regions in the AOX sequence located near the carboxy-terminus of the protein unique to thermogenic species (Crichton et al., 2005; Onda et al., 2008) could also be involved in controlled thermogenesis in these species.

Conclusion

Through a combination of biochemical and molecular techniques we have investigated the regulation of AOX activity in thermogenic tissues of the sacred lotus. This has enabled us to expand our understanding of how heating may be regulated in this and other thermoregulating species. The major isoforms of AOX found in lotus, NnAOX1a and

NnAOX1b lack Cys_1 and could therefore not form disulfide linked dimers. The lack of Cys_1 also explains the pyruvate insensitivity of alternative pathway respiration in thermogenic lotus, and also suggests that Cys-193, present in NnAOX1b, does not substitute for pyruvate activation via Cys_1. Our sequence data indicated that AOXs from thermogenic plants do not form a 'functional' grouping, and that heating in these plants may thus be a function of the amount of AOX protein present rather than the structure of the protein. Fine control of AOX activity in thermoregulating species is yet to be elucidated, but may involve modulation by the organic acids pyruvate or succinate, depending on which isoform of the protein is present.

MATERIALS AND METHODS

Plant material

Lotus flowers (*N. nucifera* Gaertn.) were collected from an outdoor pond in the Adelaide Botanic Gardens, South Australia, in January and February 2007 to 2009. Flowers for mitochondrial measurements were collected early during the thermoregulatory period classified as stage 1 by Grant et al., (2008). Stage 2 flowers were used for isolation of total RNA.

Isolation of mitochondria

Washed mitochondria were isolated from approximately 50 g of fresh sacred lotus receptacle tissue according to Day et al. (1985) with minor modifications (Grant et al., 2008). The mitochondria were purified using a three-step Percoll gradient (30 mL) made of equal amounts of 50% [v/v], 35% [v/v] and 20% [v/v] Percoll in a sucrose wash buffer (250 mM sucrose, 10 mM HEPES-KOH (pH 7.2), 0.2% [w/v] fatty acid free BSA). The gradients were centrifuged at 20 000 g for 1 h at 4°C and purified mitochondria were collected from the

20%-35% interface. Mitochondria were then washed (0.4 M mannitol, 10 mM MOPS/KOH (pH 7.2), 0.1% [w/v] fatty acid free BSA) twice by centrifugation at 10 000 g and the final pellet re-suspended in 1 mL wash buffer. Mitochondrial protein was determined according to the method of Bradford (1976).

Treatment of mitochondria with diamide and DTT

Percoll purified mitochondria were left untreated or treated with either DTT, diamide or ethylene glycol bis(succinimidylsuccinate) (EGS) to final concentrations of 20 mM, 10 mM and 5 mM respectively. Higher concentrations of EGS completely crosslinked the AOX protein however the AOX signal was greatly reduced. A high DMSO/protein ratio may have had a detrimental effect on the protein, therefore lower concentrations of EGS were used. Following the addition of DTT, mitochondria were incubated on ice for 30 min. Mitochondria treated with EGS or diamide (30 min at room temperature) were incubated with DTT first, to ensure the AOX protein was in the reduced form, and then washed before addition of the afore-mentioned reagents. Reactions were quenched by adding excess Tris-HCl (1 M pH 7.4). Stock solutions of diamide and EGS were prepared in DMSO. The DTT was prepared in purified water, however DMSO was added to both DTT treated and untreated mitochondria at the same final concentration as in the diamide treatment as a control. All solutions were prepared fresh on the day of use.

SDS-PAGE and immunoblotting

Mitochondrial protein samples were separated by non-reducing SDS-PAGE gels and immunoblotted as previously described (Grant et al., 2008). AOA antibody raised against

Sauromatum guttatum alternative oxidase (Elthon et al., 1989) was used to detect the AOX protein. The proteins were visualized using SuperSignal west femto maximum sensitivity substrate (Pierce, Rockford, USA). All buffers were reductant free.

Mitochondrial respiration measurements

Oxygen uptake by purified mitochondria was measured at 25°C using a Clark type oxygen electrode in 1.8 mL of reaction medium (0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 5 mM KH_2PO_4, 20 mM MOPS/KOH (pH 7.2), 0.1% [w/v] fatty acid free BSA). The O_2 concentration in air-saturated buffer at 25°C was estimated at 250 μM in each experiment. Mitochondrial O_2 uptake was initiated with 2 mM NADH and 20 mM succinate (final cuvette concentration). Approximately 100 μg of mitochondrial protein was used in each assay. KCN at a final concentration of 1 mM was used to inhibit the COX pathway and 100 μM *n*-propyl gallate (*n*-PG) was used to inhibit the AOX pathway. A steady state of O_2 uptake was reached before addition of subsequent constituents. Depending on the experiment, the following were added to the reaction mix (shown as final cuvette concentration): 20 mM pyruvate, 5 mM glyoxylate, 10 mM citrate, 10 mM fumarate, 10 mM oxalate, 10 mM α-ketoglutarate, 10 mM malate, 5 mM DTT and 5 mM diamide. To account for the effect of residual pyruvate, lactate dehydrogenase (LDH; 5 units/mL) was added to the reaction medium to scavenge residual pyruvate. Malonate (1-10mM) was used to determine whether succinate was acting as a substrate for succinate dehydrogenase (complex II) or an activator of AOX. Initial experiments showed no evidence of state 3 to state 4 transition following the addition of ADP and the succinate stimulated O_2 uptake was not inhibited by KCN suggesting that the bulk of respiration was occurring via the AOX pathway.

Isolation and sequencing of the full-length NnAOX1a and NnAOX1b

For the isolation of transcripts encoding AOX proteins by RT-PCR, total RNA was first extracted from thermogenic receptacles using Fruit-mate™ (Takara Bio Inc., Shiga, Japan) and FastPure™ RNA Kit (Takara Bio Inc.). Quality of the isolated RNAs was checked by using the FlashGel System (Lonza Inc., ME, USA). First strand cDNAs were generated with PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc.) using oligo (dT) primer. By aligning conserved cDNA sequences of *AOX* transcripts across several thermogenic plants (*Dracunculus vulgaris AOX* (Ito and Seymour, 2005), *Philodendron bipinnafitidum AOX* (Ito and Seymour, 2005) and *Sauromatum guttatum AOX* (Rhoads and McIntosh, 1991)), primers were designed to amplify partial fragments: NnAOXF1 (5'-ACA GCG GCG GGT GGA TCA AGG CCC TCC T-3') and NnAOXR1 (5'-TCG CGG TGG TGG GCC TCG TCG G-3'). The obtained fragments were cloned into pCR 2.1 with TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) then sequenced.

Based on the partial sequence data, 5'- and 3'-RACE reactions were performed using the SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) with the primers indicated below: NnRV1(5'-AAC TCG GTG TAG GAG TGG ATG GCC TCC T-3') and NnRV2 (5'-AAG GTC ATC AGG TGC ATC CGC TCG TTC T-3') for 5'-fragments of the *NnAOX1a* and *NnAOX1b*, NnFW1 (5'-AGA ACG AGC GGA TGC ACC TGA TGA CCT T-3') and NnFW2 (5'- AGG AGG CCA TCC ACT CCT ACA CCG AGT T-3') for 3'-fragment of the and *NnAOX1b.* RACE products were also cloned into pCR 2.1 and sequenced.

To obtain full length cDNAs of *NnAOX1a* and *NnAOX1b*, PCR amplification was performed using KOD -Plus- (TOYOBO Co., Ltd., Osaka, Japan). The final PCR products were subcloned into *Hinc*II site of pUC118 (TaKaRa Bio Inc.) and their sequences determined. Nucleotide sequence data were analyzed with GENETYX software (Genetyx Corp., Tokyo, Japan). Phylogenetic analyses of AOX sequence data were conducted using MEGA4 (Tamura et al., 2007). The phylogeny was deduced using the Neighbor-joining method for 29 molecular species of AOX proteins and tested by bootstrap analysis with 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset.

Statistical analysis

Changes in mitochondrial activity with respect to different substrates were compared using one-way analysis of variance (ANOVA; JMP 5.1 (SAS Institute Inc.)). Tukey HSD *post hoc* tests were used to identify significantly different means. Data sets were tested for normality and homogeneity of variances using Shapiro-Wilk W and Bartlett's tests, respectively. Significant differences between means were calculated at p=0.05.

SUPPLEMENTAL DATA

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignment of NnAOX1a, NnAOX1b and AOX1 proteins from other dicot species.

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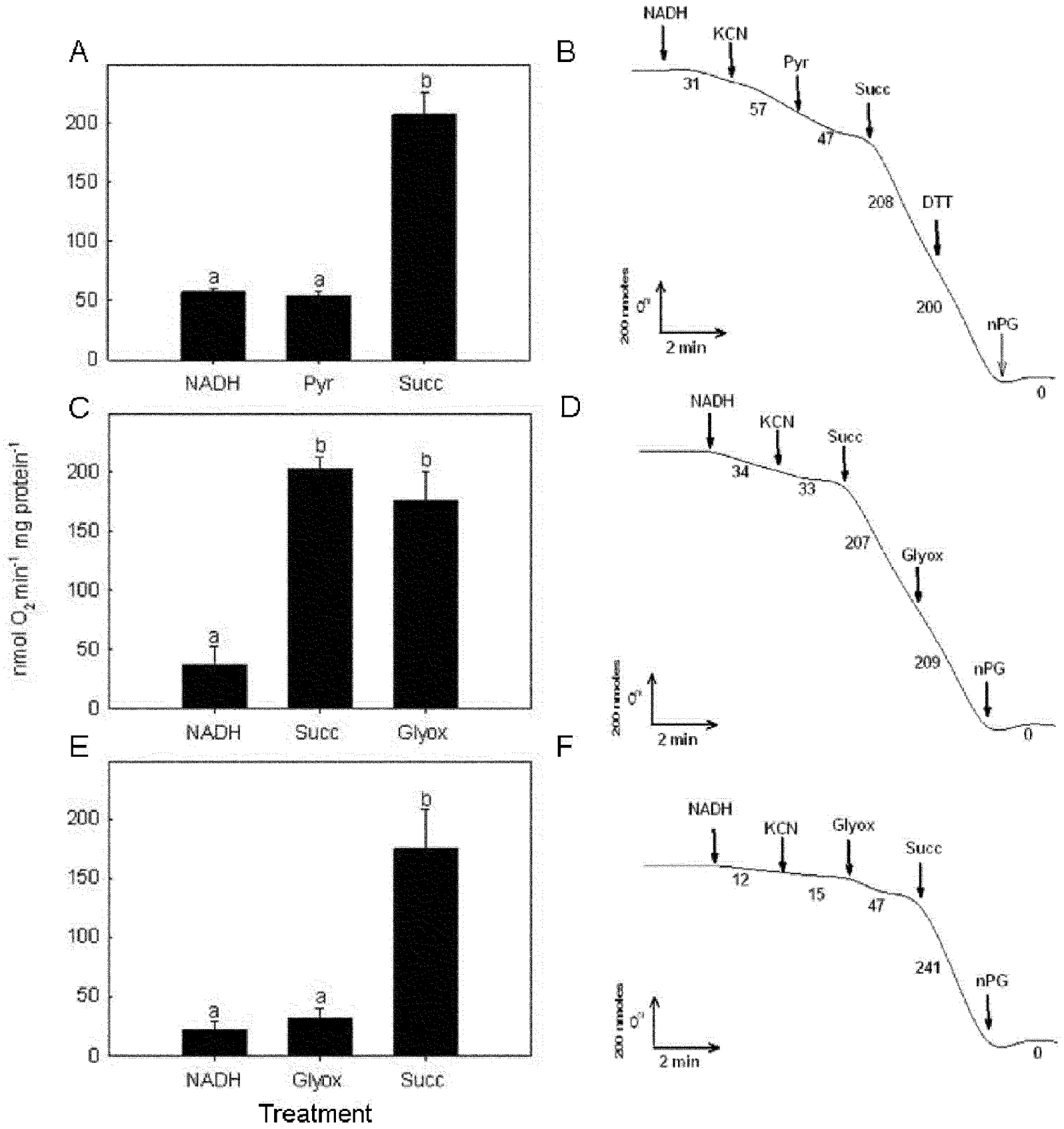
Figure 1. Rate of O_2 uptake in mitochondria from thermogenic *Nelumbo nucifera* receptacles. Each column (means ±SE, *n*=3-4) represents a subsequent addition to the O_2 electrode chamber of 2 mM NADH, 5 mM pyruvate (Pyr) or 20 mM succinate (Succ) and/or 5 mM glyoxylate (Glyox). These additions were made in the presence of 1mM KCN. Columns with different letters are significantly different. Typical O_2 uptake traces per mg of mitochondrial protein are shown to the right of the graphs. Numbers below the traces are the respiration rates (nmoles O_2 min^{-1} mg $protein^{-1}$).

Figure 2 Immunoblots of mitochondrial proteins from *Nelumbo nucifera* receptacle (as shown in Fig. 1) using antibodies raised against alternative oxidase proteins. Treatments are; lane 1 - untreated protein; lane 2 DTT (20 mM); lane 3 DTT (20mM), wash, diamide (10 mM) and lane 4 DTT (20mM), wash, EGS (5mM). There was no reductant used in the stock sample preparation. Numbers to the left of the blots are approximate positions of molecular weight markers, sizes in kDa. Numbers below the blots show the percentage of the protein oxidized/reduced or crosslinked/unlinked. Lane 4 has been added from another experiment.

Figure 3. Deduced amino acid sequences of NnAOX1a and NnAOX1b aligned with those of previously reported AOXs expressed in thermogenic tissues. Bold characters highlight residues conserved across all of the AOX sequences in the alignment. The putative structural features are indicated as follows: asterisks for two highly conserved cysteines, termed Cys_1 and Cys_2 (Berthold et al, 2000), double underline for ligands to iron atoms of the catalytic center, and grey underbars for four α-helices. Abbreviations and data sources: DvAOX, *Dracunculus vulgaris* AOX (BAD51465); PbAOX, *Philodendron bipinnatifidum* AOX (BAD51467); SgAOX, *Sauromatum guttatum* AOX (P22185); SrAOX, *Symplocarpus renifolius* AOX (BAD83866).

Figure 4. Unrooted dendrogram of a range of plant AOX proteins showing three distinct groups of AOX proteins: Monocot AOX1, Dicot AOX1 and Dicot AOX2. *Indicates LeAOX1b, which is unusual in that it is a dicot AOX1 that sits within the Monocot AOX1 grouping and Δ denotes thermogenic species. Abbreviations and data sources: as in Fig.3 and AtAOX1a, *Arabidopsis thaliana* AOX1a (NP_188876); AtAOX1b, *A. thaliana* AOX1b (NP_188875); AtAOX1c, *A. thaliana* AOX1c (NP_189399); AtAOX2, *A. thaliana* AOX2

(NP_201226); GhAOX1, *Gossypium hirsutum* AOX1 (ABJ98721); GmAOX1, *Glycine max* AOX1 (AAC35354); GmAOX2a, *G. max* AOX2a (AAB97285); GmAOX2b, *G. max* AOX2b (AAB97286); LeAOX1a, *Lycopersicon esculentum* AOX1a (AAK58482); LeAOX1b, *L. esculentum* AOX1b (AAK58483); NaAOX1, *Nicotiana attenuata* AOX1 (Q676U3); NtAOX1, *N. tabacum* AOX1 (AAC60576); OsAOX1a, *Oryza sativa* AOX1a (BAA28773); OsAOX1b, *O. sativa* AOX1b (BAA28771); OsAOX1c, *O. sativa* AOX1c (BAB71945); PtAOX1, *Populus tremula* x *P. tremuloides* AOX1 (Q9SC31); StAOX1a, *Solanum tuberosum* AOX1a (BAE92716); TaAOX1a, *Triticum aestivum* AOX1a (BAB88645); TaAOX1c, *T. aestivum* AOX1c (BAB88646); VuAOX1, *Vigna unguiculata* AOX1 (AAZ09196); VuAOX2a, *Vigna unguiculata* AOX2a (ABM66368); VuAOX2b, *V. unguiculata* AOX2b (AAZ09195); ZmAOX1a, *Zea mays* AOX1a (AAR36136).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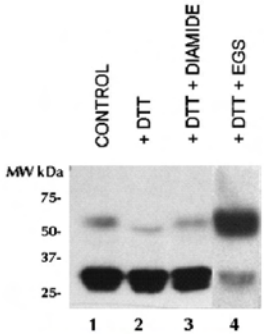

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% Reduced	78	87	80	23	% Unlinked

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DvAOX      1  MSS-RLAGTALCRQLSHVPVPH-LPVLRPTA--------GCSAATAQ-RA  39
PbAOX      1  MMSSRLTGTTVRQ-LGHALSATGLVV-RTTAEPASALRGGGAAA-PTPSH  47
SgAOX      1  MMSSRLVGTALCRQLSHVPVPQYLPALRPTADTASSLLHGCSAAAPAQRA  50
SrAOX      1  MMKSQLVGTALRH-LGQYLFSSSVPAARA-AEPVRTLLNEGLVQTPTG-P  47

NnAOX1a   47  GRRTWI-RFSCLGV--RNGSTSALNNKEKEEKGVRTSSTVGGANRPEDKM  93
NnAOX1b   51  VAS--I-RLPVLGV--RNGSTGALGGDEQTRNGLQTDSTGGTSDSPSDKP  95
DvAOX     40  G-LWP----PSWFSPPRRASTLSDPAQDGGKKKAGTA-GKVPPGEGGGGG  83
PbAOX     48  AHV-WMLRFPP-A---RGASTLSAPMTVDGQEEAAATKQTDAAKVAAAEQ  92
SgAOX     51  G-LWP----PSWFSPPRHASTLSAPAQDGGKEKAAGTAGKVPPGEDGGAE  95
SrAOX     48  VAV-WLLRLPG-AASLRSVSTLSAPLAVAGEEKEGKKAEVAAPKAGARVE  95

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DvAOX     84  EQKAVVSYWGVPPSRVSKEDGSEWRWTCFRPWDTYQADLSIDLQKHHAPT  133
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SrAOX     96  DKAVV-SHWGIPPSKATKEDGSEWRWSCFRPWETYEADLSIDLKKHHAPT  144
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PbAOX    141  TFLDKMAFRTVRALRWPTDIFFQRRYACRAMMLETVAAVPGMVGGMLLHL  190
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SrAOX    195  KSLRRFEHSGGWIKTLLNEAENERMHLMTFMEVSEPRWYERALVLAVQGV  244
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SrAOX    295  IAIDYWRLPQGSTLRDVVMVVRADEAHHRDVNHFASDIHYQGHELKKSPA  344
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NnAOX1b  344  PLGYH                                               348
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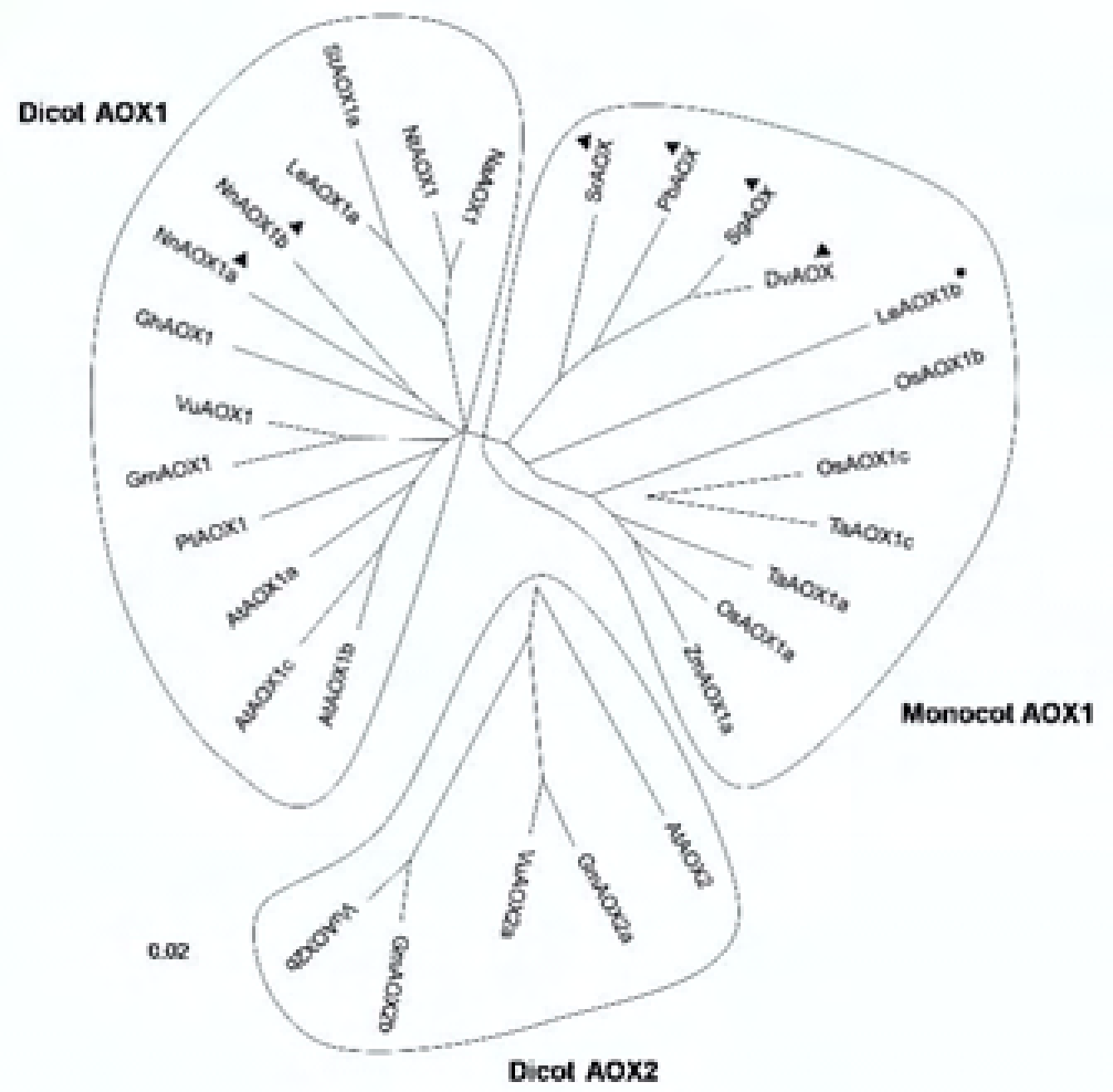
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