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Alternative Oxidase Isoforms Are Differentially Activated by Tricarboxylic Acid Cycle Intermediates[1[OPEN]]

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The cyanide-insensitive alternative oxidase (AOX) is a non-proton-pumping ubiquinol oxidase that catalyzes the reduction of oxygen to water and is posttranslationally regulated by redox mechanisms and 2-oxo acids. Arabidopsis (*Arabidopsis thaliana*) possesses five AOX isoforms (AOX1A–AOX1D and AOX2). AOX1D expression is increased in *aox1a* knockout mutants from Arabidopsis (especially after restriction of the cytochrome *c* pathway) but cannot compensate for the lack of AOX1A, suggesting a difference in the regulation of these isoforms. Therefore, we analyzed the different AOX isoenzymes with the aim to identify differences in their posttranslational regulation. Seven tricarboxylic acid cycle intermediates (citrate, isocitrate, 2-oxoglutarate, succinate, fumarate, malate, and oxaloacetate) were tested for their influence on AOX1A, AOX1C, and AOX1D wild-type protein activity using a refined in vitro system. AOX1C is insensitive to all seven organic acids, AOX1A and AOX1D are both activated by 2-oxoglutarate, but only AOX1A is additionally activated by oxaloacetate. Furthermore, AOX isoforms cannot be transformed to mimic one another by substituting the variable cysteine residues at position III in the protein. In summary, we show that AOX isoforms from Arabidopsis are differentially fine-regulated by tricarboxylic acid cycle metabolites (most likely depending on the amino-terminal region around the highly conserved cysteine residues known to be involved in regulation by the 2-oxo acids pyruvate and glyoxylate) and propose that this is the main reason why they cannot functionally compensate for each other.

Higher plant mitochondria possess two distinct pathways for the transfer of electrons from ubiquinol to molecular oxygen: the cytochrome *c* oxidase (COX) pathway and the alternative oxidase (AOX) pathway. In the COX pathway, electron transport is coupled to proton translocation and concomitant ATP formation. Electron transport through the AOX pathway occurs without proton translocation and, consequently, is not coupled to ATP synthesis or energy conservation (for review, see Millar et al., 2011). In this case, most of the energy is dissipated as heat (Sluse and Jarmuszkiewicz, 1998; Affourtit et al., 2002). The dimeric AOX mediates the terminal step of the alternative pathway and is localized to the inner mitochondrial membrane, with its catalytic centers oriented toward the matrix (Juszczuk and Rychter, 2003).

In different plant species, the number of nuclear genes encoding AOX isoforms varies between two (e.g. *Nelumbo nucifera*) and seven (e.g. *Arum maculatum*). Studies on various transgenic plants indicate that these isoforms are not redundant and cannot compensate for each other under stress or adverse growth conditions (Table I). These include studies on Arabidopsis (*Arabidopsis thaliana*), which has five nuclear AOX genes: four AOX1 type (A–D) and one AOX2 type (Polidoros et al., 2009). In Arabidopsis, an *aox1a* knockout cannot be compensated by the expression of other isogenes (Table I; Strodtkötter et al., 2009; Kühn et al., 2015). Although the expression of AOX1D is increased in *aox1a* mutants, it cannot functionally replace the lack of AOX1A, since, in contrast to the wild type, *Ataox1a* knockout plants do not survive treatment with antimycin A, an inhibitor of the cytochrome *c* pathway acting at the site of cytochrome bc_1 in complex III (Alexandre and Lehninger, 1984; Campo et al., 1992; Maguire et al., 1992; Xia et al., 1997; Pham et al., 2000; Strodtkötter et al., 2009; Kühn et al., 2015). Moreover, a double mutant impaired in both the COX and AOX pathways (*aox1a:rpoTmp*) shows more severe growth impairment, even though AOX1D is highly expressed at the transcript and protein levels (Kühn et al., 2015). These results suggest that differences in posttranslational activation of AOX isoforms are more likely to occur than differences in transcriptional regulation in the *aox1a:rpoTmp* mutants (Strodtkötter et al., 2009; Kühn et al., 2015).

Besides transcriptional regulation, AOX activity has been shown to be posttranslationally regulated at the two highly conserved Cys residues (CysI and CysII)

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present in the N-terminal domain of the protein (Millar et al., 1993, 1996; Umbach and Siedow, 1993, 1996; Day and Wiskich, 1995; Day et al., 1995; Rhoads et al., 1998; Siedow and Umbach, 2000; Umbach et al., 2006; Selinski et al., 2016, 2017). Two interrelated mechanisms were identified regulating the activation/inactivation of AOX: (1) oxidation/reduction of the disulfide bridge formed between the two CysI residues in the AOX dime; and (2) further activation of the reduced form via allosteric regulation by 2-oxo acids, also involving the conserved Cys residues (Millar et al., 1993, 1996; Umbach and Siedow, 1993; Rhoads et al., 1998; Umbach et al., 2006; Moore and Albury, 2008; Selinski et al., 2016, 2017).

Although recombinant AOX1A, AOX1C, and AOX1D from Arabidopsis are posttranslationally activated by pyruvate and glyoxylate (Rhoads et al., 1998; Umbach et al., 2002, 2006; Selinski et al., 2016, 2017), the influence of tricarboxylic acid cycle (TCAC) intermediates on AOX activity has not been studied in detail. In soybean (*Glycine max*) mitochondria and submitochondrial particles, the AOX pathway also is activated by oxaloacetate (OAA) and 2-oxoglutarate (2-OG), but at higher concentrations than pyruvate (Day et al., 1995; Millar et al., 1996). However, these effectors have not been studied in detail, and their effects have not been confirmed directly with AOX protein. Furthermore, these studies used mitochondria isolated from soybean cotyledons, where the only expressed AOX isoform is AOX2a (Finnegan et al., 1997), and tell us nothing about AOX1 isoforms, which are predominant in most other plants. In this work, a detailed comparison of posttranslational activation by TCAC intermediates of the three Arabidopsis isoforms AOX1A, AOX1C, and AOX1D was performed using a sensitive experimental setup with prolonged linear time intervals, based on *Escherichia coli* membranes enriched in individual AOX isoforms after heterologous expression, as described by Selinski et al. (2016, 2017).

[1] This work was supported by the Collaborative Research Center 944, Physiology and Dynamics of Cellular Microcompartments (R.S. and G.D.-H.), the government of Lower Saxonia (Lichtenberg Fellowship to J.S.), the Frauenförderpool of Osnabrück University (J.S.), La Trobe University School of Life Science Publication Booster Award, and the Alexander von Humboldt Foundation (Feodor Lynen Research Fellowship to J.S.). J.W. was supported by the Australian Research Council Centre of Excellence Program in Plant Energy Biology (CE140100008).

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J.S. conceived the study, participated in its design and coordination, performed activity measurements and statistical analyses, and wrote the article; A.H. carried out membrane vesicle isolation, immunoassays, and activity measurements; G.D.-H. participated in the design of the study as well as in writing the article; J.W. and D.A.D. helped to draft the article and participated in discussions and data analysis; D.A.D. also contributed to the design of the study; R.S. contributed to the design and helped to draft the article; all authors read and approved the final article.

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www.plantphysiol.org/cgi/doi/10.1104/pp.17.01331

RESULTS

AOX Isoforms Are Differentially Activated by Organic Acids of the TCAC

To analyze the isoform-specific sensitivities of Arabidopsis AOX1A, AOX1C, and AOX1D to TCAC intermediates, each isoform was recombinantly expressed in *E. coli* BHH8, and membrane vesicles enriched in individual AOX proteins were isolated. A one-letter code for amino acids was used to describe the composition at Cys sites I, II, and III occurring in native and mutant forms combined with a three-letter code, resulting in CCC for the AOX1A wild type (AOX1A-WT), CCF for AOX1C-WT, and CCL for AOX1D-WT, respectively (Selinski et al., 2017). Due to the fact that AOX proteins were heterologously expressed under reducing conditions (these conditions are present in the *E. coli* cytosol) and that a reductant (DTT) was present during membrane vesicle isolation and activity measurements, AOX proteins are present in their reduced, and therefore activatable, state. Isoform-specific oxygen consumption was measured by linking the NADH dehydrogenases and ubiquinol pool of the *E. coli* respiratory chain to the heterologously expressed AOX isoform using a Clark-type oxygen electrode (Selinski et al., 2016, 2017).

Based on the observations that monocarboxylic 2-oxo acids such as pyruvate and glyoxylate stimulate AOX1A, AOX1C, and AOX1D activity (Rhoads et al., 1998; Umbach et al., 2002, 2006; Selinski et al., 2016, 2017) and that the dicarboxylic acid succinate activates AOX1A from Arabidopsis after substitution of CysI by Ser or Ala (Djajanegara et al., 1999; Umbach et al., 2002; Selinski et al., 2017), a variety of other organic acids (dicarboxylic acids: fumarate, malate, OAA, 2-OG [and succinate]; tricarboxylic acids: citrate and isocitrate) were tested for their effects on the activity of the three AOX wild-type proteins (Fig. 1A). Neither of the tested tricarboxylic acids (citrate nor isocitrate) influenced the activity of any isoform (Fig. 1A). This also was the case for the dicarboxylic acids fumarate, malate, and succinate. However, OAA and 2-OG (the only TCAC intermediates belonging to the group of 2-oxo acids) significantly stimulated the activity of AOX1A-CCC (WT) (Fig. 1A) but not AOX1A-SCC derivatives (Supplemental Fig. S1). While the AOX1A protein exhibited a 7-fold increase in activity after treatment with OAA compared with its basal activity (no effector), the addition of 2-OG led to a 3-fold increase only. This indicates that AOX1A is more prone to be

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Table I. *Overview of studies on* aox *mutants*

AS, Antisense; ir, RNA interference construct harboring a fragment of a gene in an inverted repeat orientation; OE, overexpression; RNAi, RNA interference; rpoTmp, T3/T7 bacteriophage-type RNA polymerase, mitochondrial and plastidial. So far, in no case could compensation by another isoform be observed.

Plant Species	AOX Isoforms	Mutants	References
Arabidopsis thaliana	AOX1A to AOX1D, AOX2	*aox1a*	Giraud et al. (2008); Watanabe et al. (2008, 2010); Strodtkötter et al. (2009); Yoshida et al. (2010, 2011a, 2011b, 2011c); Gandin et al. (2012, 2014); Vishwakarma et al. (2014, 2015); Keunen et al. (2015)
		aox1a/rpoTmp	Kühn et al. (2015)
		AOX1A AS	Fiorani et al. (2005); Umbach et al. (2005); Florez-Sarasa et al. (2011)
Chlamydomonas reinhardtii	AOX1, AOX2	*AOX1 RNAi*	Mathy et al. (2010)
Glycine max	AOX1A, AOX2a, AOX2b	*GmAOX2b AS*	Chai et al. (2010)
Nicotiana tabacum	AOX1A, AOX1B, AOX2	*AOX1a RNAi*	Wang et al. (2011); Cvetkovska and Vanlerberghe (2012, 2013); Wang and Vanlerberghe (2013); Cvetkovska et al. (2014); Dahal et al. (2014)
		AOX1A AS	Vanlerberghe et al. (1997); Maxwell et al. (1999); Parsons et al. (1999); Robson and Vanlerberghe (2002); Guy and Vanlerberghe (2005); Sieger et al. (2005); Zhang et al. (2009); Dahal et al. (2017); Dahal and Vanlerberghe (2017)
		AOX1A OE	Guy and Vanlerberghe (2005); Dahal et al. (2017); Dahal and Vanlerberghe (2017)
Nicotiana attenuata	AOX1A, AOX1B, AOX2	*irAOX*	Zhang et al. (2012)
Solanum lycopersicum	AOX1A to AOX1C, AOX2	*AOX1A RNAi*	Xu et al. (2012)

activated by OAA than by 2-OG. Due to the fact that OAA and 2-OG belong to the group of 2-oxo acids, it is likely that the activation of AOX1A by OAA and 2-OG is based on the same mechanism as is the case for pyruvate and glyoxylate, namely the proposed formation of a thiohemiacetal. AOX1D was activated by 2-OG (2-fold increase) but was insensitive to OAA (Fig. 1A), while the AOX1C-WT protein was insensitive to all tested dicarboxylic and tricarboxylic acids (Fig. 1A). That is, AOX1A responds differentially to metabolites compared with AOX1C and AOX1D, suggesting that isoform-specific functions depend on environmental and/or tissue-specific conditions.

The influence of metabolites on AOX activity also was investigated in isolated mitochondria from Arabidopsis (Fig. 2). The AOX pathway in these mitochondria was stimulated by the 2-oxo acids 2-OG, pyruvate, and glyoxylate in a similar manner to that in isolated *E. coli* membrane vesicles (Fig. 2). Likewise, the AOX pathway was insensitive to the addition of citrate, isocitrate, fumarate, and malate compared with its activity under reducing conditions solely (no effector; Fig. 2). However, isolated mitochondria pose some problems when testing different metabolites. (1) Isoform-specific responses to the tested metabolites cannot be observed because the isoforms cannot be analyzed separately and, in any case, AOX1A is the predominant form. (2) The effect of OAA on the activity of the AOX pathway cannot be analyzed due to the presence of free malate dehydrogenase (MDH) in isolated mitochondria. Although the intactness of mitochondria was determined to be 90% to 91%, the few broken mitochondria release MDH that then will catalyze NADH oxidation directly in competition with the electron transport chain. (3) The influence of succinate on AOX activity cannot be analyzed accurately because it is an electron transport substrate (Jacoby et al., 2015).

AOX Isoforms Cannot Be Transformed into One Another by Amino Acid Substitutions at Position CysIII

Given that the AOX1A-WT protein is differentially regulated by metabolites compared with AOX1C and AOX1D (Fig. 1) and that AOX1A possesses a third Cys residue (CysIII) near the diiron center, while AOX1C and AOX1D contain a Phe or Leu residue at this position (Selinski et al., 2017), derivatives containing Phe or Leu at the position of CysIII in AOX1A were generated to mimic AOX1C or AOX1D and vice versa.

Conversion of AOX1A into the CCF form (i.e. like AOX1C-WT) did not change its activation by 2-oxo acids or its basal activity (Fig. 3, top); in this respect, AOX1A-CCF resembled AOX1C. However, AOX1A-CCF was much more sensitive to pyruvate and glyoxylate (25- to 18-fold increase) compared with AOX1A-CCC#(WT) (7-fold increase) and AOX1C (Fig. 3).

Conversion of AOX1C to the CCC form (like AOX1A-WT), on the other hand, had little effect on its response to organic acids: it remained stimulated by

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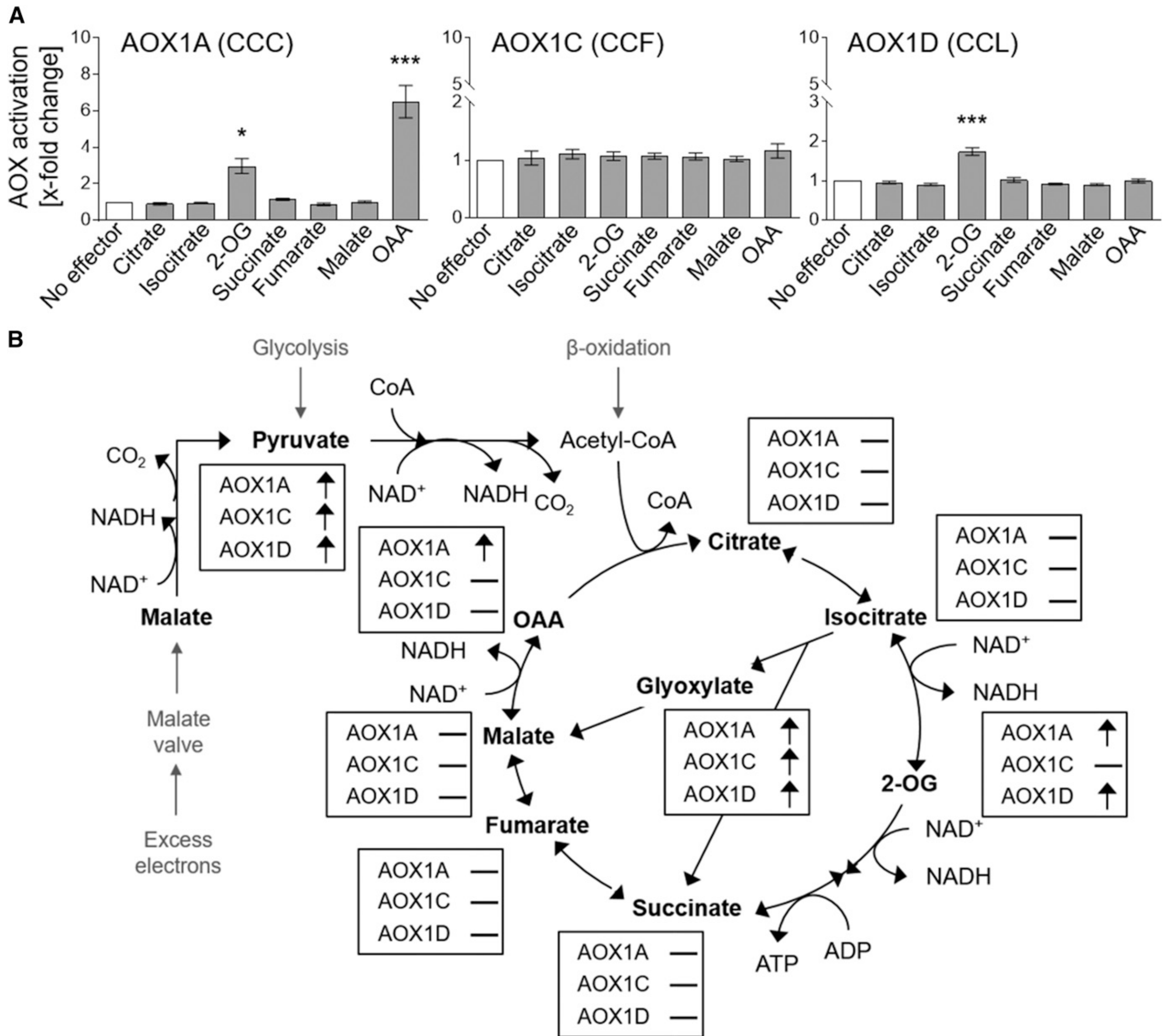

Figure 1. Influence of organic acids on the activity of AOX wild-type proteins. A, The effect of different organic acids on the activity of AOX1A-, AOX1C-, and AOX1D-WT proteins was analyzed. AOX activity was determined as described by Selinski et al. (2016) using 5 mM citrate, isocitrate, 2-OG, succinate, fumarate, malate, or OAA as effectors. Measurements were carried out as three independent biological replicates. Each biological replicate was measured twice, leading to a total of six values per column. Basal activities (no effector) were 5.7 ± 0.21 nmol oxygen min^{-1} density units $(DU)^{-1}$ for AOX1A-WT, 39.28 ± 3.94 nmol oxygen min^{-1} DU^{-1} for AOX1C-WT, and 15.26 ± 0.67 nmol oxygen min^{-1} DU^{-1} for AOX1D-WT. Asterisks indicate that the differences (*, $P < 0.05$ and ***, $P < 0.001$) between the basal activity (no effector) and activities in the presence of the effectors are statistically significant as determined by two-way ANOVA with posthoc Tukey's honestly significant difference (HSD) test. B, Schematic overview of AOX activation by TCAC intermediates.

pyruvate and glyoxylate and insensitive to the other, larger 2-oxo acids tested (Figs. 1 and 3, middle). Obviously, the transformation of AOX1A into AOX1C and vice versa is not possible simply by substituting CysIII in AOX1A by Phe or PheIII in AOX1C by Cys. Likewise, neither isoform can be transformed into AOX1D simply by substitution of CysIII in AOX1A or PheIII in AOX1C by Leu. In both cases, the basal activity was increased dramatically (20-fold for AOX1A-CCL and 7-fold for AOX1C-CCL) over the activity of the WT proteins. In addition, AOX1A-CCL activity was insensitive to 2-oxo acids (Fig. 3), in contrast to the AOX1D-WT protein, which is stimulated by 2-OG (Fig. 1).

AOX1D-CCL#(WT) and AOX1D-CCF (like AOX1C) exhibited nearly identical basal activities and were activated by pyruvate and glyoxylate (Fig. 3, bottom). The

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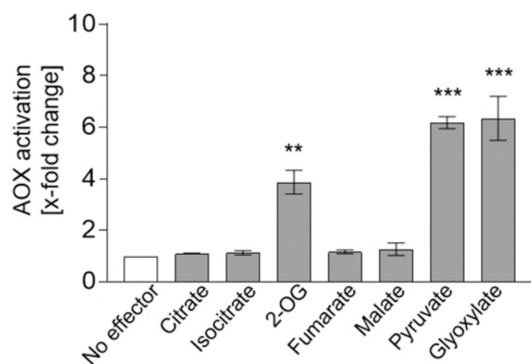

Figure 2. Influence of organic acids on the activity of the AOX pathway in plant mitochondria. The effect of different organic acids on the activity of the AOX pathway in isolated mitochondria from Arabidopsis was analyzed. AOX activity was determined as described by Jacoby et al. (2015) using 5 mM citrate, isocitrate, 2-OG, fumarate, malate, pyruvate, or glyoxylate as effectors. Measurements were carried out as three independent biological replicates. Each biological replicate was measured twice, leading to a total of six values per column. The basal activity (no effector) of the AOX pathway was 3.96 ± 0.6 nmol oxygen min^{-1} mg^{-1} protein. Asterisks indicate that the differences (**, $P < 0.01$ and ***, $P < 0.001$) between the basal activity (no effector) and activities in the presence of the effectors are statistically significant as determined by two-way ANOVA with posthoc Tukey's HSD test.

substitution of LeuIII in AOX1D by Cys (as in the AOX1A-WT protein), on the other hand, resulted in a very low overall activity, but this form was nonetheless stimulated by the 2-oxo acids pyruvate and glyoxylate. However, as found with AOX1C-CCC, AOX1D-CCC was insensitive to the 2-oxo acids OAA and 2-OG (Fig. 3), indicating that substitution of LeuIII by Cys or Phe does not, in itself, confer similar responsiveness to 2-OG to that seen with AOX1A-WT or AOX1C-WT.

Overall, these results demonstrate that the differential regulation by metabolites is not dependent solely on the amino acid present at position CysIII in the AOX protein.

DISCUSSION AND CONCLUSION

About 25 years ago, the posttranslational regulation of plant AOX activity by redox mechanisms and 2-oxo acids like pyruvate and glyoxylate was discovered (Millar et al., 1993; Umbach and Siedow, 1993). Succinate also was shown to activate specific AOX isoforms that contain a Ser residue instead of a Cys residue at position I (Djajanegara et al., 1999; Holtzapffel et al., 2003). It also has been shown that AOX1A, AOX1C, and AOX1D are activated by pyruvate and glyoxylate, although AOX1A is activated to a greater extent (Selinski et al., 2017). Consequently, we used isolated membrane vesicles of *E. coli* BHH8 containing heterologously expressed AOX protein and have confirmed that AOX-WT proteins are insensitive to succinate but are all activated by pyruvate and glyoxylate, consistent with the previous studies. It has been shown that AOX2 in soybean is activated by OAA and 2-OG (Day et al., 1995; Millar et al., 1996), but these studies were carried out with mitochondria that contain multiple isoforms of AOX; thus, isoform-specific activation could not be determined. Using a bacterial expression system expressing individual AOX isoforms (Selinski et al., 2016, 2017), differential activation by metabolites is possible to detect. In this study, we used this system to demonstrate several important differences between the different isoforms in their responsiveness to the 2-oxo acids OAA and 2-OG. AOX1A was stimulated by both OAA and 2-OG, AOX1D was activated by 2-OG solely, while AOX1C was insensitive to all tested TCAC intermediates (Fig. 1).

Citrate does not directly influence AOX activity, but its accumulation, triggered by the inhibition of aconitase, was shown to induce AOX gene expression, especially *AOX1A* and *AOX1D*, and therefore leads to an increased capacity of the AOX pathway under stress conditions in plants (Vanlerberghe and McIntosh, 1997; Gray et al., 2004; Gupta et al., 2012; Konert et al., 2015). Malate itself also does not influence AOX activity directly (Fig. 1). However, under various stress conditions, malate [e.g. generated in the chloroplast via NAD(P)-dependent MDH] can be transported into mitochondria, where its conversion to pyruvate (via malic enzyme) or OAA (via MDH) occurs (Fig. 1B). The 2-oxo acids pyruvate and OAA both stimulate AOX activity (the latter only AOX1A) and, therefore, could prevent an overreduction of the mitochondrial electron transport chain under various stress conditions when excess reducing equivalents are exported indirectly from the chloroplast via the malate valve (Scheibe, 2004). This is likely to be particularly important under photoinhibitory conditions in leaf cells. The accumulation of OAA also could occur when citrate levels increase and inhibit citrate synthase (Wiegand and Remington, 1986). In isolated mitochondria, OAA accumulates substantially during malate oxidation under conditions where malic enzyme is not activated and the cytochrome *c* pathway is restricted by adenylates, curtailing NADH oxidation and causing a severe inhibition of electron transport (Tobin et al., 1980; Day et al., 1984). This is a consequence of the equilibrium conditions of the MDH reaction. If this occurs in vivo, for example under conditions where respiration is limited by the energy status of the cell, then OAA activation of AOX would help to alleviate this problem by providing an additional avenue for NADH oxidation.

While pyruvate and OAA possibly represent the most important regulatory metabolites for AOX(1A) activity under stress conditions, 2-OG and glyoxylate also can accumulate in mitochondria, especially during photorespiration (Bari et al., 2004). Glyoxylate, 2-OG, and pyruvate also are substrates of the γ-aminobutyric acid (GABA) shunt, which is functionally linked to the TCAC (Narayan and Nair, 1990; Bouché and Fromm, 2004; Studart-Guimarães et al., 2007). The interconversion of Glu to GABA takes place in the cytosol. Subsequently, GABA is transported into mitochondria,

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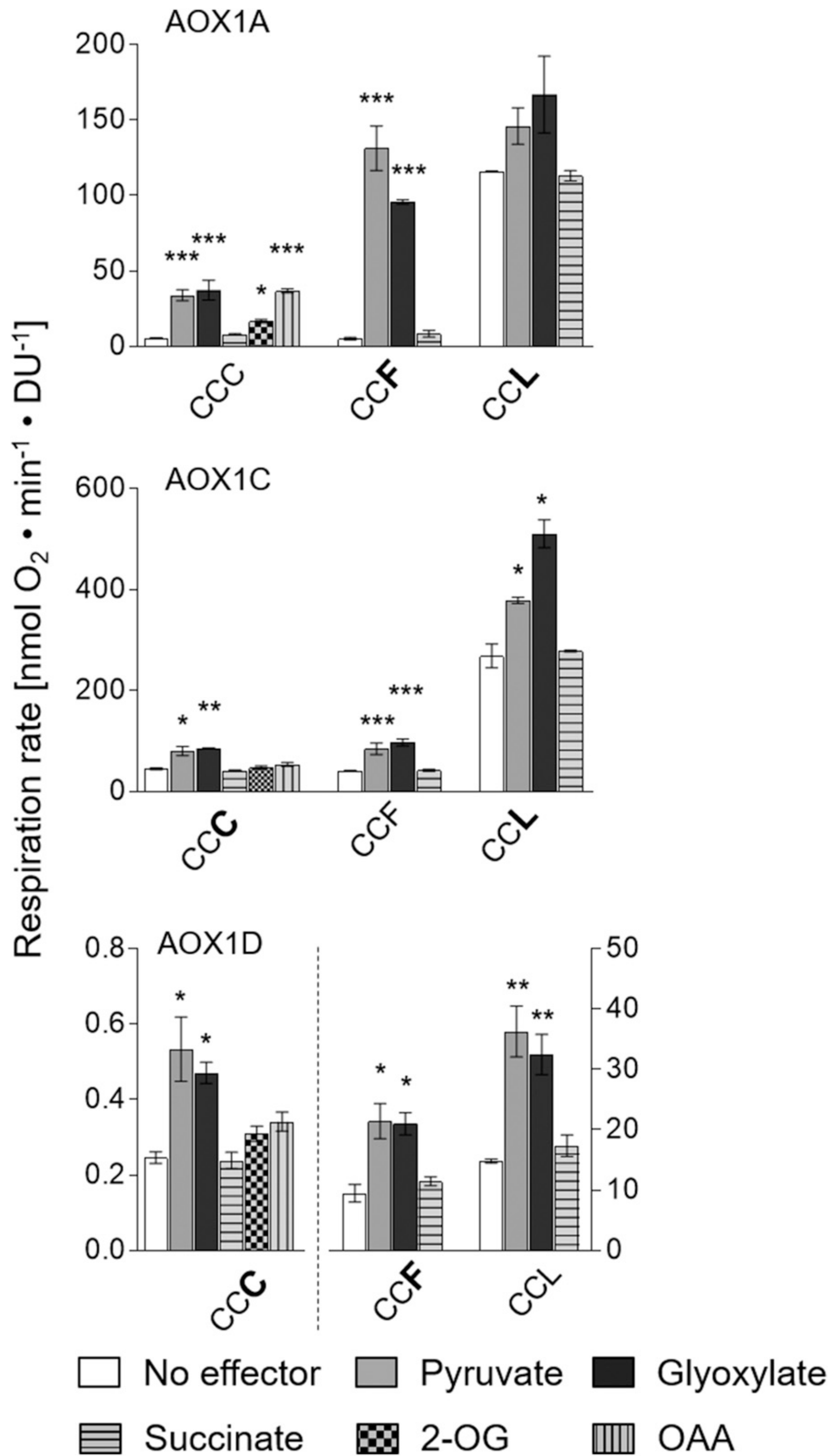

Figure 3. Convertibility of AOX isoforms into one another by substitutions of amino acid residues at position CysIII. Oxygen consumption measurements and calculations of specific respiration rates (nmol oxygen min^{-1} density units $[DU]^{-1}$) were performed as described by Selinski et al. (2016). Measurements were carried out as three independent biological replicates. Each biological replicate was measured twice, leading to a total of six values per column. Basal activities (no effector) were 5.71 ± 0.15 nmol oxygen min^{-1} DU^{-1} for AOX1A-WT (CCC), 40.89 ± 0.87 nmol oxygen min^{-1} DU^{-1} for AOX1C-WT (CCF), and 14.81 ± 0.34 nmol oxygen min^{-1} DU^{-1} for AOX1D-WT (CCL). Asterisks indicate that the differences (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$) between the basal activity (no effector) and activities in the presence of the effectors are statistically significant as determined by two-way ANOVA with posthoc Tukey's HSD test. Wild types are as follows: AOX1A, CCC; AOX1C, CCF; and AOX1D, CCL. Substitutions are presented in the one-letter code for amino acids in enlarged boldface letters. Note the difference in scale for AOX1D proteins: the left *y* axis belongs to AOX1D-CCC, and the right *y* axis belongs to AOX1D-CCF and AOX1D-CCL.

where it is converted to succinic-semialdehyde catalyzed by GABA-transaminase (GABA-T). Depending on the enzyme's substrate specificity, the GABA-T enzyme can be divided into two types: the 2-OG-dependent GABA-T (GABA-TK) and the pyruvate-dependent GABA-T (GABA-TP). While GABA-TK uses 2-OG to generate Glu, the bispecific GABA-TP uses pyruvate to generate Ala or glyoxylate to generate Gly (Clark et al., 2009a, 2009b; Shimajiri et al., 2013; Trobacher et al., 2013). Succinic-semialdehyde

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generated by GABA-T is further converted to succinate, which can be used in the TCAC and as an electron donor of the mitochondrial electron transport chain. In both cases, increased activity of AOX will be important to avoid an overreduction of the mitochondrial electron transport chain. Studies in vivo with a variety of plant species show that AOX activities are correlated with GABA and 2-OG (Florez-Sarasa et al., 2016), and the correlation of increases in AOX activity with 2-OG is consistent with the stimulatory effect of 2-OG observed in the study outlined here.

In Arabidopsis, AOX1A and AOX1D are highly stress responsive at the transcriptional level, in contrast to the other AOX genes, which do not respond to various stress conditions (Clifton et al., 2006). It is interesting, therefore, that these two isoforms are activated by 2-OG (both), OAA (only AOX1A), while AOX1C is insensitive to all tested TCAC intermediates. This may ensure that electron flux through the AOX pathway is optimized, helping to minimize oxidative stress under various environmental conditions.

Differential posttranslational activation of AOX1 isoforms also may explain why these proteins are unable to functionally substitute for each other in planta. While this is clearly the case for plants under stress conditions, cyanide-resistant respiration has been reported in vivo in *aox1a* T-DNA lines under nonstress conditions but was proposed to be due to a variety of other oxidases, as it was inhibited by salicylhydroxamic acid, an inhibitor of AOX (Watanabe et al., 2008, 2010). In our hands in two different laboratories, the *Ataox1a* T-DNA lines do not show expression of any of the other AOX genes under normal conditions, as measured by real-time quantitative reverse transcription-PCR, and mitochondria isolated from these lines do not contain detectable AOX protein or activity (J. Selinski, D.A. Day, and J. Whelan, unpublished data).

The differential activation by metabolites may be an example of a neofunctionalization of AOX isoforms, where, due to activation by different metabolites, various isoforms are only active under certain conditions. This proposal is consistent with the variation in the number of AOX genes that are observed in plants, which is not linked to their phylogenetic history. Expression of a number of isoproteins that are differentially activated by metabolites may allow fine-tuning of their activity in different tissues and under different circumstances. Differential activation is likely to occur at the N-terminal end of the AOX protein that displays more variation in sequence identity (amino acid identity of 19%–43%, calculated for the first 50 amino acids in the N-terminal region of the mature proteins) compared with the C-terminal end (amino acid identity of 82%–94%, calculated for the last 50 amino acids in the C-terminal region). The fact that the stimulation of AOX isoforms does not depend just on the size of the binding pockets (otherwise, OAA also would stimulate AOX1D, because this molecule is smaller than 2-OG, which is activating; Fig. 1) further supports the importance of the N-terminal region. Especially the position of the additional negative charge in the 2-oxo acids OAA and 2-OG in relation to the 2-oxo group appears to be essential for their interaction with the amino acid residues in close proximity to the conserved Cys residues.

In this study, we used a concentration of 5 mM of added metabolites to ensure maximal activation, as the mitochondrial concentration of these metabolites in vivo is unknown. The concentration of OAA in vivo has not been measured even in whole cells, presumably due to rapid conversion to citrate or the spontaneous decarboxylation to pyruvate. While it is recognized that the TCAC exists in plants in many situations, it does not always operate as a complete cycle, with intermediates used in various biosynthetic reactions. For example, 2-OG is used in the assimilation of nitrogen and GABA synthesis (Sweetlove et al., 2010). Again, the extent of these pathways differs between species and tissues, and in mature illuminated leaves, noncyclic flux is proposed (Sweetlove et al., 2010). Added onto this is the occurrence of metabolic channeling of citrate and fumarate, but not 2-OG, in plant mitochondria (Zhang et al., 2017), and the fact that most steps in the TCAC can be bypassed by similar nonmitochondrial activities in other cellular locations means that the matrix subcompartment concentrations are unknown. Finally, the existence of alternative respirasomes, where AOX isoforms are associated with alternative NAD(P)H dehydrogenases, suggests subcompartmentalizations of respiration and that local concentrations of substrates may differ significantly from the concentrations in a given compartment (Senkler et al., 2017).

While our results offer a possible explanation for the finding that different AOX isoforms cannot completely substitute for each other, the situation in planta needs to be considered under normal and stress conditions. Inactivation of *AOX1* in Arabidopsis and tobacco (*Nicotiana tabacum*) has no apparent physiological or growth consequences under normal, optimal growth conditions. This can be interpreted as either that AOX is not required for growth under normal conditions or that other isoforms can compensate for each other. However, an alternative explanation is that, under normal conditions, changes in the underlying transcriptional program compensate for a lack of AOX. In Arabidopsis, it has been shown that the transcriptome of *aox1a* plants is greatly altered compared with that in wild-type plants under conditions where no changes in growth or physiology are apparent (Giraud et al., 2008). In these and other studies, no other AOX isoforms are expressed or detected in *aox1a* T-DNA lines (Giraud et al., 2008; Watanabe et al., 2008, 2010). This differs from antisense lines, where significant, albeit reduced, AOX can be detected (Guy and Vanlerberghe, 2005; Florez-Sarasa et al., 2011), and in Arabidopsis, the remaining AOX capacity in antisense lines appears to be fully engaged (Florez-Sarasa et al., 2011). Thus, under normal growth conditions, while the other isoforms are not expressed, changes in the complete underlying transcriptional program in T-DNA lines suggest that compensation occurs via other mechanisms.

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In contrast, under stress conditions, other isoforms are induced in Arabidopsis, in particular AOX1D (Table I). Despite this induction, the altered phenotypes of *aox1a* mutants indicate that they are not able to compensate for the absence of AOX1a. This may be due to the fact that there is not sufficient AOX1D protein, but in several studies, it took several days for the altered phenotypes to emerge and significant AOX1D transcripts and protein could be detected (Giraud et al., 2008; Strodtkötter et al., 2009; Kühn et al., 2015). This suggests that the biochemical properties of isoforms may differ, as shown in this study. Supportive for this suggestion is that, in investigations of the in vivo activities in different plant species under high light, the activity of AOX was independent of AOX protein abundance, and differential posttranslational regulation of AOX was proposed (Florez-Sarasa et al., 2016). Our findings are consistent with this in vivo study highlighting that differences may occur between species.

Some further questions remain to be answered in future studies. (1) Homodimerization/heterodimerization of AOX isoforms. AOX has been proposed to be present in plant mitochondria as covalently and noncovalently linked homodimers (Umbach and Siedow, 1993). However, it is not possible to distinguish AOX homodimers and heterodimers based on SDS-PAGE, because monomers of different isoforms are of nearly identical size (e.g. for Arabidopsis, molecular masses of mature monomeric proteins vary between 32 kD [AOX1B and AOX1D] and 33 kD [AOX1A and AOX1C]). AOX2 is larger (38 kD) but is poorly expressed in Arabidopsis. It is possible that heterodimers possess different properties from homodimers, which would allow further fine-tuning of their activities. (2) Amount of total AOX protein and capacity in plant cells. Although the expression of AOX1D is increased when AOX1A is lacking (Strodtkötter et al., 2009), the total amount of AOX protein is lower compared with wild-type plants (data not shown). Therefore, the total AOX protein present in *aox1a* mutants might not be sufficient to compensate for the lack of AOX1A, depending on the AOX pathway capacity. As shown by Selinski et al. (2017), fully activated AOX1A and AOX1D (after the addition of pyruvate or glyoxylate) exhibit similar activities, indicating that AOX1D should be able to compensate for the lack of AOX1A in *aox1a* knockout mutants. Since this is not the case, other regulatory mechanisms must play a role in this context. (3) Cell-specific expression patterns of AOX isoforms. It is also conceivable that AOX isoforms cannot compensate for each other because of differences in their cell-specific localizations. This has to be investigated further.

MATERIALS AND METHODS

Plasmids Used in This Study

Plasmids p536 (AOX1A in pET-22b), p537 (AOX1D in pET-22b), and p583 (AOX1C in pET-22b) were obtained from former studies by Selinski et al. (2016, 2017). For mutagenesis, plasmids p536, p537, and p583 were amplified via PCR using PfuUltra II Fusion HS DNA-Polymerase (Agilent Technologies) and specific mutagenesis primers for amplification (Supplemental Table S1). Following PCR, products were treated with *Dpn*I for 1 h at 37°C to eliminate the maternal DNA template. Constructs were verified by sequencing.

Isolation and Oxygen Consumption Measurements of Plant Mitochondria

Intact plant mitochondria were isolated from Arabidopsis (*Arabidopsis thaliana* Columbia-0) water cultures, and subsequent respiratory measurements were carried out as described by Murcha and Whelan (2015) with some modifications. To analyze the effect of metabolites on the activity of the AOX pathway, citrate, isocitrate, 2-OG, fumarate, malate, pyruvate, or glyoxylate was added to the reaction chamber. The final concentration of each effector used in this study was 5 mM solubilized in respiration medium (for details, see Jacoby et al., 2015) with a pH adjusted to 7.

Activity Measurements of Recombinantly Expressed AOXs

Cell growth, protein expression, *Escherichia coli* membrane vesicle isolation, and AOX activity measurements with concomitant immunoblot analysis and calculations were carried out according to Selinski et al. (2016). The final concentration of each effector (citrate, isocitrate, 2-OG, succinate, fumarate, malate, or OAA) used in this study was 5 mM. The pH of all effectors was adjusted to 7 using NaOH before use.

Statistical Analysis

Statistical evaluations were conducted by means of two-way ANOVA with posthoc Tukey's HSD test integrated in GraphPad Prism 7 (GraphPad Software). Differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered as significant and indicated as *, **, and ***, respectively.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Influence of organic acids on the activity of AOX derivatives after substitution of CysI by Ser.

Supplemental Table S1. Primers used in this study.

Received September 18, 2017; accepted November 30, 2017; published December 5, 2017.

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