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Review

Alternative oxidases in Arabidopsis: A comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses

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Abstract

The emergence of Arabidopsis as a model plant provides an opportunity to gain insights into the role of the alternative oxidase that cannot be as readily achieved in other plant species. The analysis of extensive mRNA expression data indicates that all five *Aox* genes (*Aox1a, 1b, 1c, 1d* and *2*) are expressed, but organ and developmental regulation are evident, suggesting regulatory specialisation of *Aox* gene members. The stress-induced nature of the alternative pathway in a variety of plants is further supported in Arabidopsis as *Aox1a* and *Aox1d* are amongst the most stress responsive genes amongst the hundreds of known genes encoding mitochondrial proteins. Analysis of genes co-expressed with *Aox*s from studies of responses to various treatments altering mitochondrial functions and/or from plants with altered Aox levels reveals that: (i) this gene set encodes more functions outside the mitochondrion than functions in mitochondria, (ii) several pathways for induction exist and there is a difference in the magnitude of the induction in each pathway, (iii) the magnitude of induction may depend on the endogenous levels of Aox, and (iv) induction of Aox can be oxidative stress-dependent or -independent depending on the gene member and the tissue analysed. An overall role for Aox in re-programming cellular metabolism in response to the ever changing environment encountered by plants is proposed.

Keywords: Alternative oxidase; *Arabidopsis thaliana*; Multi-gene family; Co-expression; Function; Stress

1. Introduction

The alternative oxidase (Aox), which catalyses cyanide insensitive respiration, has been studied in a wide variety of plant species over the last 40 years. In voodoo lily (*Sauromatum guttatum*), Aox is expressed at high levels during flowering and was used as a model to initially clone *Aox* [1]. Studies in potato, tobacco, maize and soybean have elucidated various aspects of the control of Aox expression and activity [2–4]. Physiological investigations have used *Poa*, *Hakea* and *Vicia* to determine the effect of Aox on growth under various conditions [5–9]. In recent years, Arabidopsis has become the pre-eminent plant model with the availability of the complete genome sequence allowing experimental approaches which are only possible with extensive, high quality sequence data [10]. In this review, we will focus on the knowledge and insights that have been gained regarding Aox from studies in Arabidopsis. More specifically, we will utilise the huge repository of expression data from hundreds of array experiments to analyse all five *Aox* genes and other genes that are co-expressed with *Aox*, to provide insights into control of expression. Finally, various genetic screening approaches have been used to examine regulatory pathways. This review focuses on the analysis of these data in Arabidopsis, other aspects of Aox structure and function that are more adequately covered elsewhere [2,4,11].

2. Alternative oxidase gene structure in Arabidopsis

The first *Aox* gene was fortuitously cloned from Arabidopsis by complementation of a heme deficient mutant of *E. coli* [12].

Abbreviations: Aox, alternative oxidase; CAREs, *cis*-acting regulatory elements; ND, alternative NAD(P)H dehydrogenase; JA, Jasmonic acid; MPSS, Massively Parallel Signature Sequencing; ROS, reactive oxygen species; SA, salicylic acid

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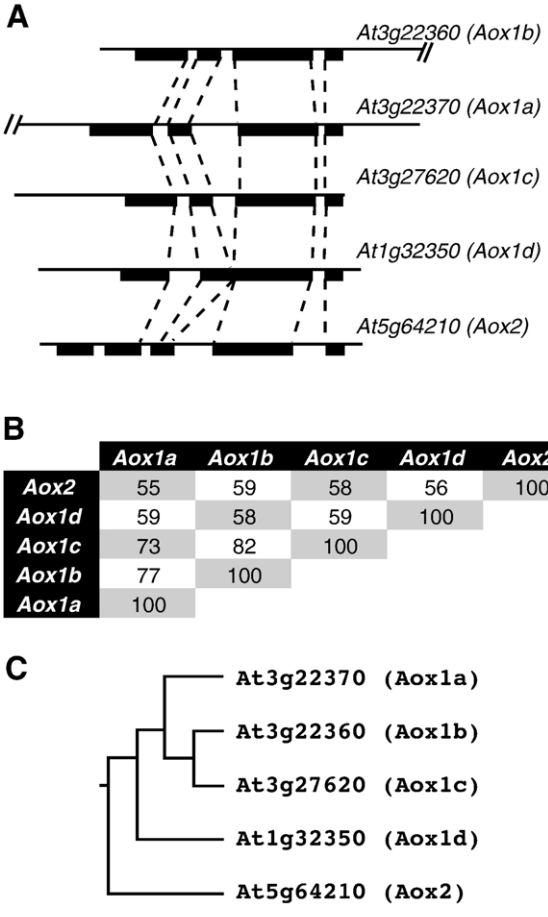
Complete genome sequencing revealed five genes, classified as four *Aox1* type and one *Aox2* type (Fig. 1) [13]. In contrast to the four exon structure of most *Aox* genes cloned from a variety of organisms, *Aox1d* contains three exons and *Aox2* contains five exons (Fig. 1A). Protein sequence identity varies from 55% to 82% between the various members [14] (Fig. 1B). Analysis of the chromosomal position reveals that *Aox1b* and *Aox1a* are in tandem on chromosome 3, but notably *Aox1b* and *Aox1c* display the highest protein sequence identity of 82% (Fig. 1B and C). All five proteins are predicted to be located in mitochondria by a variety of targeting prediction programs. Examination of the targeting signals indicates that *Aox1a*, *b* and *c* contain a putative -2 arginine processing signal, but no clear prediction for the mature N-terminus for *Aox1d* and *Aox2* is evident [15]. Overall, the protein sequences of all five Arabidopsis Aoxs are typical of the plant Aox family and do not give any novel insights into Aox structure and function compared to Aox protein sequences known from a variety of other plants [2].

In comparison to current data from other plants, Arabidopsis contains more *Aox* genes, but this is likely a reflection of the completeness of the genome [13] than a biological difference. *Aox* genes are classified into two families, *Aox1* and *Aox2*. Comparison of *Aox1* members indicates that they display higher sequence similarity to each other between species than they do to *Aox2* members within a species [14]. However it is clear that by the process of duplication and subsequent divergence that the number of members of specific gene types have expanded in some species. Thus, while *Aox1* type genes have expanded in Arabidopsis, *Aox2* type genes have expanded in legumes such as soybean and cowpea [16,17]. In tobacco, another widely used model to study Aox [18], it appears that there is one of each type of gene, but two allelic copies of each exist due to the amphidiploid nature of tobacco. In monocots only *Aox1* type genes have been characterised to date [19,20]. Given this diversity in the number and type of *Aox* genes in any species, knowing the sequence of all genes, as in Arabidopsis, is a considerable advantage for expression analysis if the role and regulation of Aox is to be comprehensively understood.



	Aox1a	Aox1b	Aox1c	Aox1d	Aox2
Aox2	55	59	58	56	100
Aox1d	59	58	59	100	
Aox1c	73	82	100		
Aox1b	77	100			
Aox1a	100				

Fig. 1. Comparison of the five Arabidopsis *Aox* genes. (A) Scale diagram illustrating the intron/exon structure of Arabidopsis *Aox* genes. (B) A ClustalW multiple sequence alignment was performed using the predicted protein sequences encoded by the five *Aox* genes. The number in each box represents the percentage sequence identity between the corresponding proteins indicated on each axis. (C) Dendrogram describing the relationship between the five Arabidopsis Aox protein sequences.

3. Alternative oxidase gene expression

Expression analysis using Northern blots indicated that *Aox1a*, *1b*, *1c* and *2* displayed overlapping but distinct profiles [21,22]. Two whole genome tiling array studies [23,24], indicated that all five *Aox* genes were expressed over a range of developmental stages and in a range of tissues, with seedlings, anthers, flowers and roots examined as well as two different suspension cell cultures.

3.1. Tissues and development

The Genevestigator database allows a comprehensive overview of the expression profile of *Aox* genes in Arabidopsis from over 2000 arrays [25]. The Gene Chronologer tool was used to examine the expression patterns of the *Aox* gene family over Arabidopsis development, from 1 day to 50 days of age (Fig. 2A). *Aox1a* transcript expression is abundant throughout Arabidopsis development peaking at 14–17 days and again at 25–28 days as flowers emerge. The *Aox1d* transcript is predominately found during early rosette development and during flowering, whereas *Aox2* expression is limited to developmental stages associated with the presence of seed—during early germination and the latter stages of silique maturation (Fig. 2A). *Aox1b* expression appears limited to stages involving early flower development whereas *Aox1c* appears ubiquitously expressed albeit at a very low level. The Genevestigator Gene Atlas tool is designed to address the question: how strongly is my gene of interest expressed in different organs or tissues? (Fig. 2B) In callus tissue and cell culture *Aox1a* and *Aox1c* are the predominant transcripts. In seedlings and roots, *Aox1a*, *Aox1c*, *Aox1d* and *Aox2* transcripts are detected. *Aox1a*, *Aox1c* and *Aox1d* transcripts are found in rosettes with *Aox1d* expression peaking in the senescent leaf. All five *Aox* genes are detected in the inflorescence; further dissection of this organ reveals that expression of each *Aox* gene is limited to a number of organs within the inflorescence. For example, *Aox2* expression is restricted to the seed whereas

Aox1b expression is found only in stamen tissue where *Aox1c* expression also peaks (Fig. 2B). Whilst *Aox1a*, *Aox1c* and *Aox1d* transcripts are detected in several inflorescence organs, expression of *Aox1a* and *Aox1d* is highest in sepal tissue. In addition to the microarray data described above, studies on the expression profiles of the various *Aox* genes have been carried out using quantitative RT-PCR [26]. These data are in general agreement with the results from array analysis and indicate that *Aox1a* is the prominently expressed gene in a variety of tissues, followed by *Aox1c*, *Aox1b*, *Aox2* and *Aox1d*, with some of the latter displaying more restricted organ-specific expression.

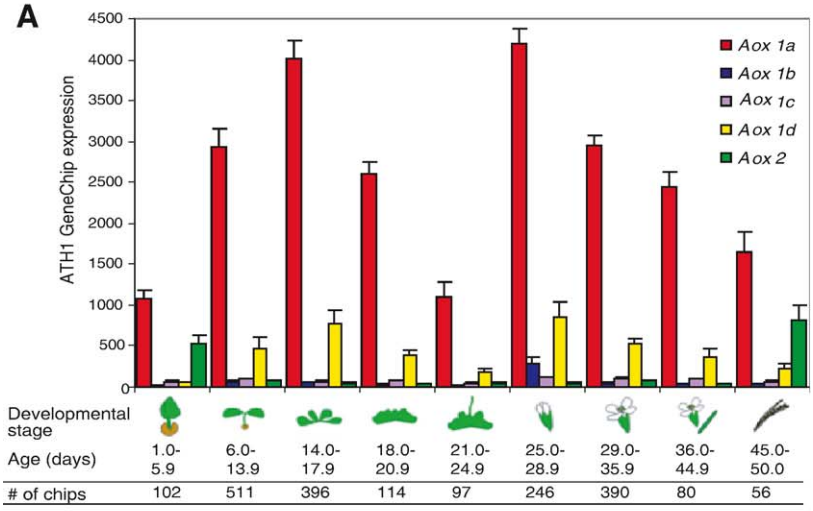
Multi-gene families are especially prevalent in plant genomes, arising from gene duplication events resulting from illegitimate recombination or segmental duplication following a polyploidization event [13,27]. The paralogous genes arising from such duplications may retain different subsets of the ancestral gene function (sub-functionalization), they may evolve new functions (neo-functionalization), or they may maintain overlapping functions [28]. Functional divergence of the members of gene families may occur via mutations both within the coding sequence and within the regulatory regions—resulting in either altered biochemical properties or altered spatial, temporal or stimuli responsive transcript expression [29]. A number of models have been proposed to explain the processes leading to functional divergence. The DDC model (duplication, degeneration, complementation) proposes that after duplication the two gene copies acquire complementary loss-of-function mutations in independent sub-functions such that both copies are required to produce the full complement of functions of the single ancestral gene [28,30]. This model requires sub-functions to be independent, such that mutations affecting one will not affect another. Several properties of transcriptional regulatory regions make them excellent targets to drive the sub-functionalization process, including the modular structure of regulatory regions and the short length of *C*AREs whose function can be disrupted by point mutations. Reflecting these properties, many researches have emphasized that evolutionarily important changes might occur primarily at the level of gene regulation rather than protein function [28,30,31].

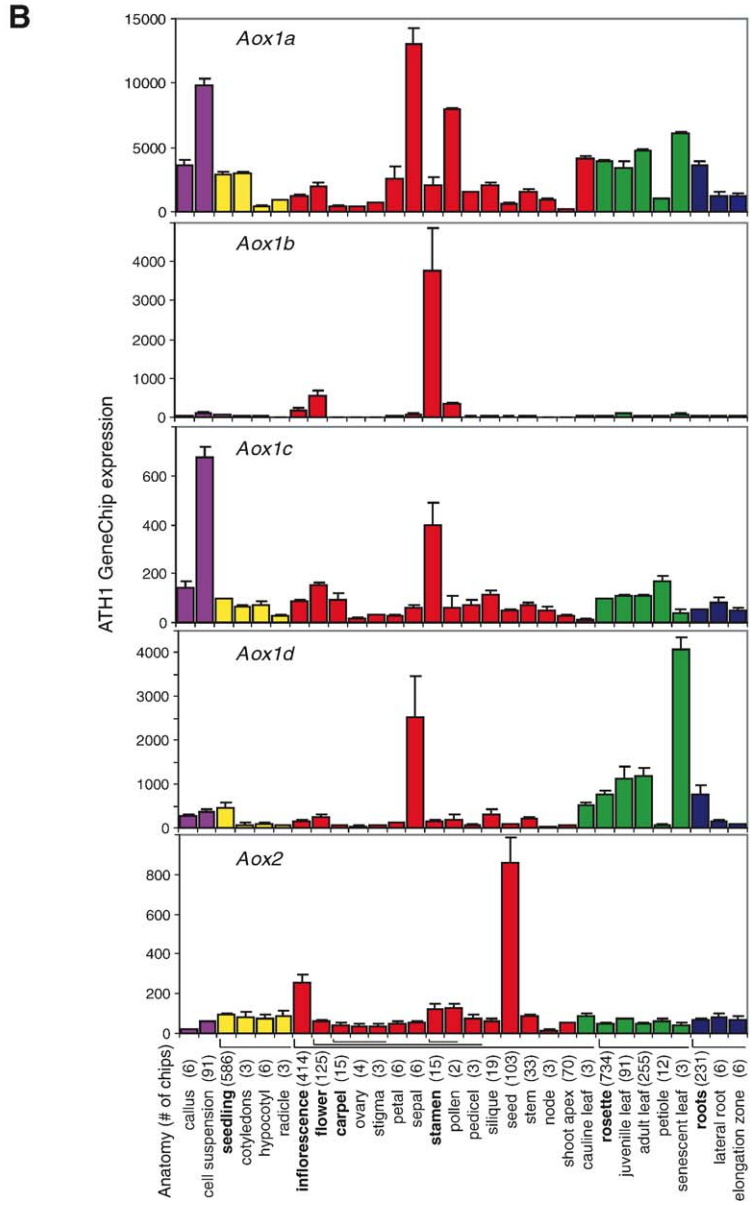
The distribution of the *Aox* genes across three of the five chromosomes suggests ancient polyploidization events may have contributed to the evolution of at least four members of this gene family. Organ-specific expression changes commonly arise with the onset of polyploidy [32], supporting the organ-specific expression patterns observed for *Aox2*, *Aox1b* and *Aox1d*. Thus, the dynamic nature of the transcript abundance of the members of the *Aox* gene family represents a trend observed across eukaryotic multigene families, making the *Aox* gene family an interesting model to investigate the regulatory mechanisms controlling and coordinating the expression of a mitochondrial targeted nuclear gene family.

It is unknown whether this divergence has yielded *Aox* genes encoding proteins with significantly different regulatory or catalytic properties. However examination of ^{18}O discrimination data for various species shows a range of values from 23.5 to 31.2, indicating that access of larger oxygen isotopes to the active site of different Aox isoforms may vary [2]. Aox can be regulated by its oxidation/reduction state and the presence of α-keto acids [33,34], but apart from several rare natural variants lacking a conserved cysteine that are important for this mechanism [19,35,36], there is no clear evidence for differences in regulation between conserved cys containing isoforms in the Aox family. It is also unclear if these in vitro regulatory mechanisms play a substantial role in control of Aox function in vivo based on transgenic studies [2].

Plant growth and development are tightly regulated processes with each growth stage having specific cellular demands. Certain growth stages require a substantial energy commitment, such as the transition to flowering and seed production. Interestingly, transcripts of the three *Aox* genes that display organ- or developmental-specific expression, *Aox1b*, *Aox1d* and *Aox2*, are predominately found in organs associated with high metabolic rate, such as *Aox2* in the seed (ready for rapid metabolism during germination) and *Aox1b* in young inflorescences (ready for rapid metabolism to drive pollen formation). Multiple genes encoding proteins of the same function resulting in increased gene dosage may be advantageous under particular circumstances. The up-regulation of additional *Aox* genes to complement the universal expression of *Aox1a* may reflect increased demand for the alternative pathway under certain conditions. It is well documented that there is an increase in mitochondrial gene expression during flowering [37–39], development and organ identity [40], in meristem maintenance [41], and programmed cell death [42–45]. As Aox plays an integral role in mitochondrial metabolism it is thus not surprising that expression displays cellular and developmental variations. The role(s) that Aox plays in these various circumstances is as yet unclear, but precisely defining expression may give functional insights. At face value induction of Aox in tissues with high metabolic and energy demands may appear paradoxical given Aox operation makes respiration less

Fig. 2. Analysis of Alternative oxidase gene expression overdevelopment and organ distribution. (A) Genevestigator gene chronologer expression map describing the transcript expression patterns of the five members of the *Aox* gene family over Arabidopsis development. Growth stages from seed germination to senescence are arbitrarily grouped into subcategories based on Boyes standard [89]. Each category contains averaged raw signal data and standard error derived from all ATH1 chips hydridized with RNA from the corresponding growth stages in the Genevestigator database, including all organs available at that stage. Each of the Arabidopsis life cycle stages examined is shown and age of the plant in each category is indicated, as is the number of chips contributing to each category. Error bars represent standard error. (B) Genevestigator atlas of transcript expression of the *Aox* gene family in the wild type Arabidopsis plant. Organs in bold represent data from all chips hybridized with RNA from that whole tissue in addition to all chips with RNA from individual or specific tissues within that organ, as described by the underline. For example, the 'roots' category contains all chips hybridized with RNA extracted from whole roots as well as from specific root tissues such as 'lateral roots' and the 'elongation zone'. In contrast organs not in bold contain data from chips hybridized with RNA extracted from those subcategories only, such as 'petal'. Error bars represent standard error. Data were sourced from https://www.genevestigator.ethz.ch/.


A
ATH1 GeneChip expression
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Aox 1a
Aox 1b
Aox 1c
Aox 1d
Aox 2
Developmental stage
Age (days)
1.0-5.9
6.0-13.9
14.0-17.9
18.0-20.9
21.0-24.9
25.0-28.9
29.0-35.9
36.0-44.9
45.0-50.0
# of chips
102
511
396
114
97
246
390
80
56


B
ATH1 GeneChip expression
Aox1a
Aox1b
Aox1c
Aox1d
Aox2
Anatomy (# of chips)
callus (6)
cell suspension (91)
seedling (586)
cotyledons (3)
hypocotyl (6)
radicle (3)
inflorescence (414)
flower (125)
carpel (15)
ovary (4)
stigma (3)
petal (6)
sepal (6)
stamen (15)
pollen (2)
pedicel (3)
silique (19)
seed (103)
stem (33)
node (3)
shoot apex (70)
cauline leaf (3)
rosette (734)
juvenile leaf (91)
adult leaf (255)
petiole (12)
senescent leaf (3)
roots (231)
lateral root (6)
elongation zone (6)

efficient in terms of ATP synthesis. However, when the cytochrome pathway is saturated, Aox operation would provide a method for both enhanced respiratory pathway flux and ATP production albeit the latter at a lower efficiency above the threshold of the cytochrome pathway. Additionally, high metabolic demands are not only met by ATP, so non-phosphorylating Aox operation will enhance TCA cycle provision of organic acids as building blocks for amino acid and nucleic acid synthesis in metabolically demanding tissues.

3.1.1. Aox1b and the flower

The transcript expression of *Aox1b* suggests this gene may play a floral-specific role. In Arabidopsis the transcriptional programs during floral induction [46], during early flower development [47], in floral organs [48] and during the reproductive stages [49] have all been investigated and general support for the floral-specific expression of *Aox1b* is found. For example, by comparing the expression patterns of floral organ identity mutants with wild type flowers, *Aox1b* is described as displaying a stamen-specific expression pattern [48]. Another study that profiled the transcriptome at three stages of flower and fruit development found that *Aox1b* transcript expression was specific to the floral bud [49].

3.1.2. Aox1d and senescence

The expression patterns observed suggest the function of *Aox1d* may be associated with senescence. *Aox1d* transcript levels are induced in leaves following salicylic acid (SA) treatment, in leaves following a dark treatment, in aging suspension cells grown in the light, and in senescing leaves, with each of these conditions used to model senescence [50–54]. The link between *Aox1d* and senescence is further substantiated by the presence of this gene in a list of ESTs associated with leaf senescence [52], and in the list of 827 senescence up-regulated genes identified by whole transcriptome analysis [50]. Whilst both *Aox1a* and *Aox1d* are found in this second set, the raw data from these experiments reveals senescence results in a more dramatic induction of *Aox1d* (23-fold vs. 3.5 fold for *Aox1a*, [50]).

The initiation and progression of plant senescence involves a complex combination of signalling pathways with considerable cross talk between other plant responses. The plant growth regulators, ethylene, jasmonic acid (JA) and SA, are thought to participate in interconnected signalling pathways that control senescence as they all increase during senescence and induce the expression of specific genes [52]. Of the characterised set of 827 senescence induced genes, 19% show reduced expression in *NahG* transgenic plants defective in SA signalling, 12% show reduced expression in the *coi1* mutant defective in the JA signalling pathway and 9% show reduced expression in the ethylene signalling mutant *ein2* [50]. The same study found *Aox1d* expression significantly down-regulated in the *ein2* mutant and to a lesser extent in the *coi1* and *NahG* mutants. Whole genome transcript profiling studies have observed that the majority of genes that depend on JA and ethylene signalling pathways show increased abundance during both dark-induced and cell suspension senescence [50,53,54]. Whilst mutant studies revealed a role for JA and ethylene in regulating the expression of *Aox1d,* MPSS (massively parallel signature sequencing) data suggest that SA may also participate in regulating *Aox1d* expression [55].

3.1.3. Aox2 and the seed

The gene expression map of Arabidopsis development revealed a diminished transcriptome in the seed compared to other vegetative tissues, with two opposing expression trends observed from early to late stages of seed development. Roughly 800 transcripts were induced during the transition from silique to mature seed including *Aox2*, whereas 1500 were repressed [56]. *Aox2* expression appears to be specific to the mature seed, remaining in the dry seed and decreasing following imbibition [57]. The specific expression of *Aox2* in this organ suggests a role for the alternative oxidase in seed maturation and in the early stages of germination, although this requires further investigation.

All the above analysis are at a transcript level and some caution needs to be exercised to presume that these increases lead to a corresponding increase in protein and activity. It is well documented that Aox is affected or regulated at a protein level by oxidation/reduction of the protein and the presence of pyruvate or other α-keto acids [58]. Thus, increases in transcript cannot be directly related to increases in Aox activity. However studies examining *Aox* transcript and protein abundance often show a correlation; the numerous studies in tobacco with induction, sense and antisense plants [4], and induction studies in maize by genetic or chemical means all show changes in protein corresponding to the presence or absence of transcript [19]. In soybean, all three *Aox* genes have been cloned, and changes in protein abundance of each isoform of Aox can be assessed as each isoform has a different apparent molecular mass distinguishable by SDS-PAGE [59]. Studies in soybean inducing Aox by chemical means [60], and analysis in various tissues overdevelopment indicate that the relative abundance of the different Aox isoforms reflects differences in transcript abundance [17,61].

Numerous studies have been carried out in a variety of plant species examining the expression of *Aox* in various plant organs and during development [2, 3]. While the lack of gene-specific data from the bulk of these studies limits direct comparison across species it is clear that orthologous genes do not display the same expression profiles. This can be illustrated by comparing Arabidopsis and soybean; both expression analysis at an mRNA level and using promoter and reporter gene constructs indicate that *Aox* is expressed in the inflorescence, but in a gene-specific manner [26]. In soybean, *Aox1* is expressed in carpels and filaments, *Aox2a* is expressed in tapetal cells of anthers and *Aox2b* is expressed in filaments supporting anthers, the tip of the style and stigma. As outlined above, Arabidopsis *Aox* genes also display gene-specific expression patterns in the inflorescence. It is clear that the orthologous genes do not display similar expression patterns and thus, care must be taken when making comparisons between species. Comparison between maize and Arabidopsis *Aox* expression patterns in response to mitochondrial inhibition also indicate response patterns should not be assumed to be consistent between orthologous genes in different species [19,62].

3.2. Stress

A large number of studies in a variety of species have indicated that Aox is induced at a gene, protein and activity level by a variety of treatments, generally labelled as stresses [2]. Notably, comparison between species often leads to conflicting results. For example, treating potato leaves with antimycin A had no effect on Aox, while in tobacco suspension cells antimycin A induced Aox strongly [63,64]. Also, cold or chilling treatment lead to no change in Aox in soybean, but a large increase in Aox in *Vigna*, [65]. Arabidopsis is an extensively used model to determine plant molecular responses to various stresses. Several Arabidopsis studies have described *Aox1a* as the most stress responsive *Aox* gene [22,62]. Expression analysis using an Arabidopsis suspension cell cultures reveals *Aox1c* as unresponsive to most treatments, suggesting a house-keeping role, and based on dramatic inductions in response to specific treatments it was proposed that *Aox2* may play a role in mitochondrial-plastid communication [62].

Detailed studies using quantitative RT-PCR examining the response of Arabidopsis *Aox* genes to various treatments indicated that the timing and magnitude of the response varies depending on the treatment [62]. Furthermore, in vivo studies with transgenic Arabidopsis suggest that the kinetics and magnitude of *Aox1a* transcript induction are tissue and treatment specific [66]. At least four distinct but overlapping pathways that lead to the induction of specific *Aox* genes have been proposed: a ROS dependent pathway, a redox pathway linked to plastid dysfunction, a pathway triggered by ATP depletion and a pathway triggered by altered metabolic conditions [62,66]. An analysis of the response of 670 genes encoding mitochondrial proteins to 219 stress conditions, revealed *Aox* genes are highly stress responsive, particularly *Aox1a* and to a lesser extent *Aox1d* (Table 1, Supplementary Figure 1 and Table 1). This confirms the stress responsive nature of *Aox* genes shown from other studies in a variety of plants but also provides a unique opportunity to elucidate which other genes respond in a similar manner to understand the context in which *Aox* gene expression is changing.

3.2.1. Mitochondrial context

An analysis of the genes that displayed similar expression profiles with *Aox1a* indicated that *NDB2*, an external NAD(P)H dehydrogenase, displayed the strongest co-expression pattern when both quantitative RT-PCR and array data were analysed (Supplementary Fig. 1, [62,67]). Interestingly, these gene products can form a functional respiratory pathway oxidising external NAD(P)H. *NDA2*, encoding an internal NAD(P)H dehydrogenase also displays co-expression with *Aox1a* and *NDB2* under many conditions. Similarly, *Aox1c* and *NDA1* transcripts appear to be co-expressed under a number of conditions (Supplementary Table 1, [62]).

Analysis of the expression patterns of genes encoding mitochondrial proteins performed in response to 8 conditions generates a mitochondrial context to Aox and alternative respiratory pathway induction [68]. The treatments all affect mitochondrial function, primarily by inhibiting electron transport directly by chemical inhibition or indirectly by affecting organelle gene expression [68]. This study identified genes encoding mitochondrial proteins that are co-expressed with the induction of the alternative transport chain components. These include substrate dehydrogenases, providing alternative avenues for NADH generation [68] and several mitochondrial metabolite carrier proteins [68]; Supplementary Table 1). The substrates for these alternative NADH generating pathways are likely to include TCA cycle intermediates and reducing

Table 1
A list of the twenty responsive genes encoding mitochondrial proteins, detected by microarray analysis (data taken from Supplementary Table 1)

Gene locus	Name	# down-regulated	# up-regulated	# 2-fold change
AT3G50930	BCS1 protein-like	1	57	58
AT4G24570	mitochondrial carrier protein family	17	38	55
AT5G51440	mitochondrial heat shock 22 kd protein-like	8	36	44
AT3G01290	expressed protein	11	31	42
AT2G22500	mitochondrial carrier protein family	5	34	39
AT4G05020	rotenone insensitive NADH-dehydrogenase (external) (NDB2)	2	36	38
AT4G30270	xyloglucan endotransglycosylase (meri5B)	0	34	34
AT2G21640	unknown protein	13	21	34
AT1G74360	leucine-rich protein kinase	1	32	33
AT3G22370	alternative oxidase (Aox1a)	0	33	33
AT3G48850	mitochondrial phosphate transporter	0	31	31
AT5G07440	glutamate dehydrogenase 2	7	24	31
AT3G28580	hypothetical protein	4	26	30
AT1G06570	4-hydroxyphenylpyruvate dioxygenase (HPD)	8	22	30
AT2G18700	putative trehalose-6-phosphate synthase	4	26	30
AT2G41380	putative embryo-abundant protein	0	29	29
AT4G25200	Arabidopsis mitochondrion-localized small heat shock protein (AtHSP23.6-mito)	4	22	26
AT3G30775	hypothetical protein	12	13	25
AT1G32350	alternative oxidase 1d (Aox1d)	1	23	24
AT1G73260	trypsin inhibitor (protease inhibitor)	0	24	24

The number of up-regulated or down-regulated indicates the number of treatments to which a response greater or less than 2-fold was detected. The list is constructed from analysis of 219 publicly available microarrays downloaded from NASC. The mitochondrial protein set consists of 670 proteins defined as mitochondrial by proteomic analysis [77], by homology to mitochondrial genes from other organisms and targeting predictions [78–80].

equivalents generated by catabolism of amino acids. Indeed, genes involved in catabolism of valine, isoleucine, cysteine, tyrosine, alanine and glutamate are also induced [68] (Supplementary Table 1). The up-regulation of several mitochondrial metabolite carrier proteins of unknown function, as observed in yeast with mitochondrial perturbation [69], likely facilitate this altered metabolic state.

3.2.2. Global context

An analysis of the wider cellular context of genes that are induced in a similar manner to *Aox*, indicates a variety of UDP-glycosyl transferases, glutathione S-transferases, ubiquitination-related function and stress related transcription factors [70], all widely associated with responses to various stresses in plants suggesting that induction of *Aox1a* is part of a more general stress response [71–74]. The induction of *Aox1a* as part of a general stress response is further supported by array analysis of Arabidopsis treated with the respiratory inhibitor antimycin A, which is widely used to induce *Aox* in a variety of systems [75]. This study indicated similarities in response profiles caused by inhibition of mitochondrial electron transport with metal toxicity and hydrogen peroxide treatment.

Examination of the wider context under which *Aox* is induced, reveals that *Aox* is part of a larger stress response at a mitochondrial and cellular level [68,70]. Plants engineered with altered Aox levels display subtle but pervasive transcript adjustments, supporting the notion that altered Aox expression results in an altered cellular metabolic state [76].

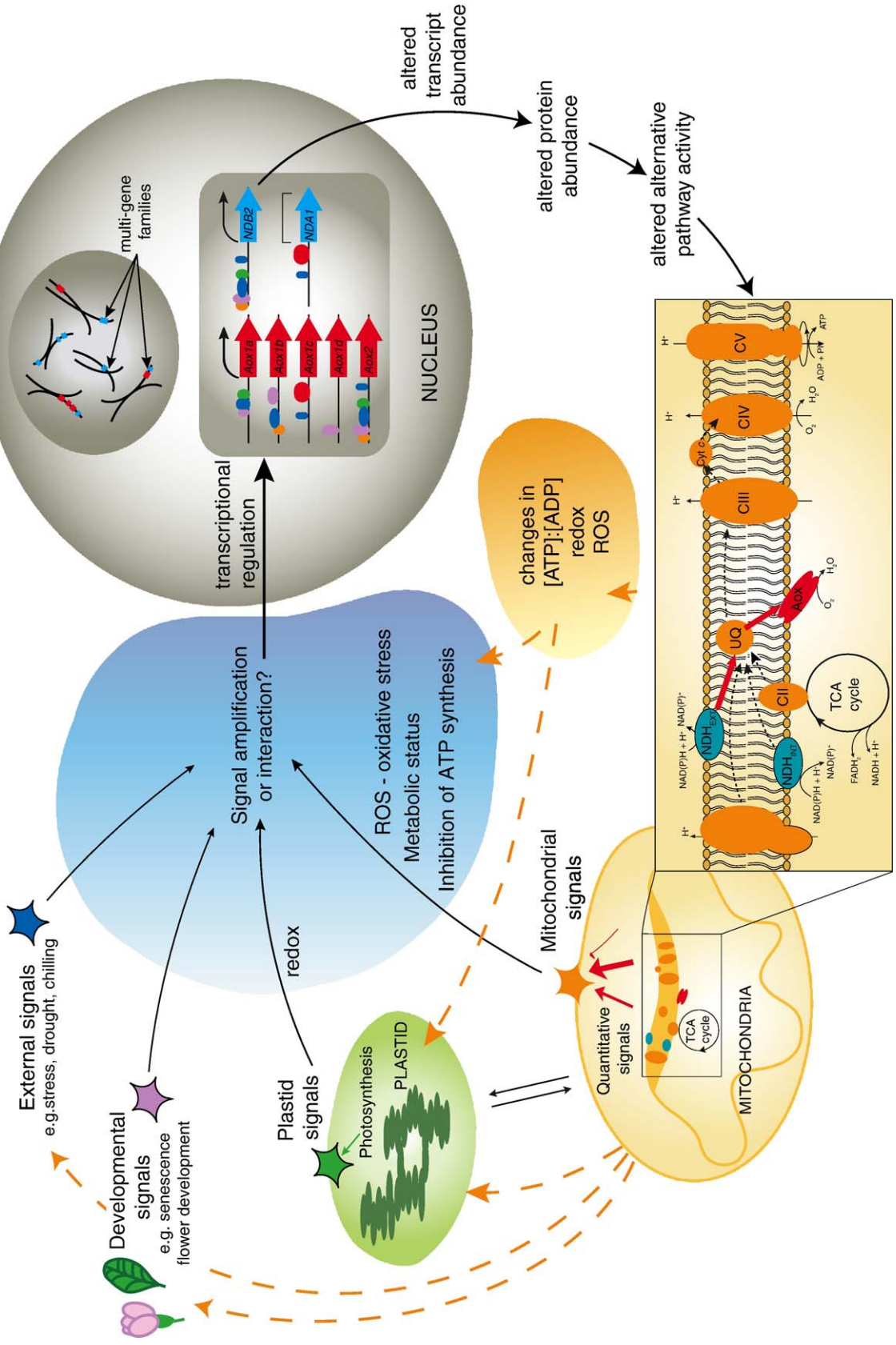
3.2.3. Altered Aox expression

Modification of *Aox* expression by genetic means provides a powerful approach to elucidate function and role in a cellular and plant context. Although such studies have been previously carried out in potato and tobacco [77–82], Arabidopsis allow greater examination of these modifications due to the analysis tools available for Arabidopsis research. Two reports modifying *Aox1a* in Arabidopsis resulting in over- and under-expression have been analysed in considerable detail [76,83]. In response to cold stress, a vegetative phenotype was observed for plants altered in expression of Aox1a; anti-sense lines had ~25% reduction in leaf area and rosette size while over-expressors had 30% or greater increase in these parameters [83]. These studies revealed that under normal conditions there was no difference in the oxidative stress of wild type plants compared to plants engineered for altered levels of Aox1a, and that the cold induced phenotype observed only partially correlated with the oxidative stress status, suggesting that Aox1a does not function exclusively to prevent formation of reactive oxygen species (ROS), a focus of much Aox related research. These reports highlight the need to consider the role and function of Aox in a whole cellular and plant context as changed levels of Aox resulted in alterations of extramitochondrial metabolism that were more significant than mitochondrial effects per se [76,83]. A general lack of response in the transcript abundance of cytochrome and TCA cycle components to genetic Aox modification supports this point, similar to the lack of responses seen in studies when mitochondrial function was perturbed in Arabidopsis wild type plants and suspension cell cultures [62,75]. However, in these same transgenic plants, changes in transcripts associated with other aspects of plant carbon metabolism, including chloroplast-related transcripts, were reported [76]. The interaction of Aox levels with extramitochondrial metabolism is further illustrated by the correlation of anthocyanin levels and the transcription of flavonoid pathway genes with Aox levels in cold stressed plants [83].

3.2.4. Regulation of alternative oxidase gene expression

A reporter gene system, in which the *Aox1a* promoter region drives the expression of the firefly luciferase gene, has been established in Arabidopsis to characterise by genetic means the factors that bind and regulate expression of *Aox1a* in Arabidopsis [66]. Mutagenesis of this line has generated mutants with altered luciferase expression driven by the *Aox1a* promoter thus identifying mutants with components involved in the *Aox* regulatory network. Whilst the identity of any such factors has yet to be reported, this is a potentially powerful system for elucidating elements of the Aox retrograde regulatory pathways. Promoter analysis has begun to elucidate elements upstream of the transcriptional start site that may play a role in aspects of Aox expression overdevelopment and in response to stress in dicot species [26,84]. Analysis of the *Aox1a* promoter region using promoter deletion and mutagenesis studies has identified a 93 bp region containing regulatory elements necessary for induction of *Aox1a* expression in response to specific inhibition of both the TCA cycle and the mitochondrial electron transport chain by monoflouroacetate and antimycin A respectively [84]. Preliminary reports indicate proteins interact with this region which may play a role in regulating *Aox1a* expression, however, identification of any such protein has yet to be described [84]. In this study analysis of transient and stably transformed Arabidopsis coupled with in vitro and in vivo assays lead to the conclusion that induction of *Aox1a* in response to perturbation of mitochondrial function relies on a complex set of interactions at the level of promoter, rather than simple transcription factor-transcription factor binding site interaction. Both the constructs and transgenic lines used in these studies will provide an excellent resource for identifying promoter regions and elements in the regulatory

Fig. 3. Overview of the signalling pathways that regulate expression of the five *Aox* genes in Arabidopsis. This diagram depicts the potential signalling pathways that influence the expression of *Aox* in a gene-specific manner. Signals originating within the cell from mitochondrial or plastidic function interact with external signals from organ, developmental or environmental stimuli to activate expression of *Aox*. These inputs are processed via the promoters of genes where transcription factors involved in these signal transduction pathways converge to activate gene expression. The components involved in transducing the signals which ultimately result in altered expression are unknown. These pathways may interact to affect the magnitude and/or *Aox* gene induced. Following induction and expression of Aox the potential for its activity to alter or modulate gene expression programs is indicated (dashed arrows—orange). Arrows link the steps involved, illustrating the pathway by which changes in *Aox* expression can affect cellular expression programs. Abbreviations: CI = complex I, CII = complex II, CIII = complex III, CIV = Complex IV, CV = Complex V, Aox = alternative oxidase, NDH = alternative type II NAD(P)H dehydrogenase, INT = internal, EXT = external, ROS = reactive oxygen species.


altered transcript abundance
altered protein abundance
altered alternative pathway activity
multi-gene families
NUCLEUS
NDB2
NDA1
Aox1a
Aox1b
Aox1c
Aox1d
Aox2
transcriptional regulation
changes in [ATP]:[ADP] redox ROS
Signal amplification or interaction?
ROS - oxidative stress
Metabolic status
Inhibition of ATP synthesis
External signals
e.g.stress, drought, chilling
Developmental signals
e.g. senescence flower development
Plastid signals
redox
Photosynthesis
PLASTID
Mitochondrial signals
Quantitative signals
TCA cycle
MITOCHONDRIA
CV
CIV
CIII
CII
UQ
AOX
NDH
TCA cycle

pathway that are important for induction of *Aox1a* following stress or specific mitochondrial inhibition and for developmental expression.

3.2.5. Conclusions and perspectives

So what have we learned about Aox from Arabidopsis and how does this help us understand the role of Aox in plant metabolism? The general data sets available from Arabidopsis combined with specific studies have revealed that:

(A) There are clear gene-specific roles for *Aox* in specific organs, overdevelopment and under stress, that likely relates to regulatory diversification of the genes. Notably, orthologous genes do not display the same regulation of expression properties and thus care needs to be taken when extrapolating from one species to another.
(B) The induction of the expression of *Aox* needs to be examined and interpreted in light of correlated changes in the mitochondrial and cellular context. The point above (A) is also of importance here as the co-expressed set will vary depending on the *Aox* gene expressed.
(C) Aox plays a role in both stress and normal growth conditions. In Arabidopsis, expression of *Aox1a* and to a lesser extent *Aox1c*, takes place under normal growth and development. Careful analysis reveals that altering Aox1a levels affects normal plant growth and thus, Aox is likely to play a number of roles in the cell.
(D) There are several pathways leading to the induction of Aox that go beyond the two way split into ROS dependent and ROS independent pathways. These pathways are likely to interact to affect the magnitude and timing of the response.

Our current understanding of the factors that influence the expression of *Aox* in Arabidopsis are summarised in Fig. 3. A combination of organ, developmental, external and internal signals act via a number of pathways to induce expression, which is notably gene specific. To date, it is not known how these pathways interact or cross over.

All these points indicate that the variety of functions previously proposed for Aox in the literature; a specialised role in thermogenesis, electron overflow, ROS metabolism under a variety of conditions, a role in nutrient (Pi) limitation, inter-organelle communication with plastids and regulation of cellular pH, are all likely to occur in different situations [2]. Certain genes appear to provide a basal level of the enzyme, *Aox1a* and to a lesser extent *Aox1c*, providing housekeeping functions and ensuring the presence of the pathway should conditions alter. Additional genes appear to have been recruited to participate under certain developmental situations and in response to stress and cellular disturbances. Consequently, these data provide support for both the traditional 'overflow' hypothesis [85] and the more recent homeostatic regulator model [79,86]. Given the predominance of *Aox1a* expression under basal and induced conditions, and assuming that translational efficiency of *Aox* genes are the same, the regulation of *Aox* gene expression in Arabidopsis may represent an intermediate state where the "minor" expressed genes have yet to be fully activated. In other species such as soybean and maize the same predominance of expression of a single isoform is not observed and the multi-gene family members more evenly 'share' the role [17,19,61].

Is there any overarching function of Aox that the above descriptions could fall under, i.e. are they each a consequence of a primary or master function of Aox activity? The dramatic induction of *Aox*, primarily *Aox1a* but also *Aox2* with a subset of treatments that affect plastid function, upon various treatments is a characteristic of many of the proposed functions. We have observed that even minor disturbances of cell cultures or plants can cause a rapid and dramatic induction of *Aox*, even in comparison to many characterised stress responsive genes. However, under short-term conditions of cytochrome pathway impairment or saturation, studies suggest the degree to which Aox can prevent ROS formation may be dependent upon how much excess alternative pathway capacity is present before the imposition of the stress [76]. Should altered Aox expression be considered: (a) as a general stress response, (b) as a modulator of existing programs, (c) as an initiator of novel expression programs, or d) as a combination of the above? Perhaps Aox has the potential to be an initiator of cellular reprogramming, acting to trigger novel expression programs, in addition to its activity as a terminal oxidase. A potential scheme for how Aox may function as an initiator of expression programs is illustrated in Fig. 3. The induction of Aox activity itself may act as one of the early responses in a general stress response through which alteration of redox, adenylate balance and ROS may in fact be the trigger or signal for subsequent responses. An emerging theme in regulatory systems is the abundance of dual function proteins. Several examples exist where regulatory roles have recently been assigned to well characterised mitochondrial enzymes. Both aconitase, an enzyme of the TCA cycle, and acetohydroxy acid reductoisomerase, which catalyses a step in branched chain amino acid metabolism, have been associated with the maintenance of mitochondrial DNA in yeast [87,88]. Cytochrome c also plays a dual role, in electron transport and signalling of programmed cell death [43]. Thus Aox may play a central role as a mediator of a cellular response to changing conditions. The rapid induction of Aox and subsequent changes that result from its activity may act as the signals to induce the changes in expression of a variety of other components to achieve an overall cellular response to changing conditions.

Accessing the wealth of Arabidopsis mutants will provide further insights into the role and regulation of Aox. The reporter gene system recently described by Zarkovic et al. [66] and plants that have altered *Aox1a* expression need to be extensively utilised [76,83] as well as comprehensive molecular profiling of stress responses to test the hypothesis and conclusions outlined above. If *Aox*, and in particular *Aox1a*, is the initiator of a wider response to stress, then plants unable to upregulate its expression should have altered responses beyond alternative respiratory chain activity. The observed co-expression of *Aox* with other genes need to be pursued to determine if this is due to co-regulation, and if so how this is altered in the genetic backgrounds of the regulatory or functional mutants. These

approaches are complicated by the fact that Aox is encoded in a small gene family, but a combination of knock-outs and subsequent studies will provide an in-depth analysis that is not possible in any other plant. With more studies combining and utilising the resources that are now available, an understanding of the response and regulation of Aox will become more comprehensive (and perhaps more complex), providing a platform to understand the role of Aox in a variety of situations in all plant species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbabio.2006.03.009.

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