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REVIEW

Aox gene structure, transcript variation and expression in plants

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Alternative oxidase (*Aox*) has been proposed as a functional marker for breeding stress tolerant plant varieties. This requires presence of polymorphic *Aox* allele sequences in plants that affect plant phenotype in a recognizable way. In this review, we examine the hypothesis that organization of genomic *Aox* sequences and gene expression patterns are highly variable in relation to the possibility that such a variation may allow development of *Aox* functional markers in plants. Aox is encoded by a small multigene family, typically with four to five members in higher plants. The predominant structure of genomic *Aox* sequences is that of four exons interrupted by three introns at well conserved positions. Evolutionary intron loss and gain has resulted in the variation of intron numbers in some *Aox* members that may harbor two to four introns and three to five exons in their sequence. Accumulating evidence suggests that *Aox* gene structure is polymorphic enough to allow development of *Aox* markers in many plant species. However, the functional significance of *Aox* structural variation has not been examined exhaustively. *Aox* expression patterns display variability and typically *Aox* genes fall into two discrete subfamilies, *Aox1* and *Aox2*, the former being present in all plants and the latter restricted in eudicot species. Typically, although not exclusively, the *Aox1*-type genes are induced by many different kinds of stress, whereas *Aox2*-type genes are expressed in a constitutive or developmentally regulated way. Specific *Aox* alleles are among the first and most intensively stress-induced genes in several experimental systems involving oxidative stress. Differential response of *Aox* genes to stress may provide a flexible plan of plant defense where an energy-dissipating system in mitochondria is involved. Evidence to link structural variation and differential allele expression patterns is scarce. Much research is still required to understand the significance of polymorphisms within AOX gene sequences for gene regulation and its potential for breeding on important agronomic traits. Association studies and mapping approaches will be helpful to advance future perspectives for application more efficiently.

Introduction

Alternative oxidase (AOX) is a component of the mitochondrial respiration pathway present in all higher plants which diverts electrons from the energy conserving cytochrome pathway to catalyze the four-electron reduction of oxygen to water (Siedow and Umbach 2000). Although its presence is not restricted in plants (McDonald 2009, McDonald and Vanlerberghe 2006), much of the recent interest on this enzyme is because of

Abbreviations – AOX, Alternative oxidase; CS, cleavage site; FUE, far upstream element; MFA, monofluoroacetate; miRNA, microRNA; NUE, near upstream element; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; TCA, tricarboxylic acid; UTR, untranslated region.

its potential to serve as a functional marker in molecular plant breeding (Arnholdt-Schmitt et al. 2006).

Accumulating evidence suggests that AOX may play a significant role in cell adaptation under different types of stress. AOX is activated at high respiratory substrate availability and is induced by high levels of endogenous or exogenous reactive oxygen species (ROS) (Millar et al. 2001, Oliver et al. 2008, Vanlerberghe and McIntosh, 1997). AOX is able to prevent excessive reduction of the mitochondrial electron transfer chain, which could result in disruption of glycolysis and the tricarboxylic acid (TCA) cycle under conditions of high substrate availability (Lambers 1982). Thus, AOX activity enables high turnover rates of carbon and operation of TCA cycle under stress conditions, although with a cost in ATP synthesis. High induction of AOX with concomitant dramatic reduction in growth is also evident under nutrient limitation as, for example, was observed in tobacco cells, whereas the growth of transgenic tobacco cells lacking AOX was only moderately reduced by the nutrient deficiencies, and carbon use efficiency values remained the same as under nutrient-sufficient conditions (Sieger et al. 2005). These results indicate that AOX respiration provides an important general mechanism by which plant cells can modulate their growth in response to nutrient availability.

Usually, a specific *Aox* gene is highly induced by many types of abiotic stress in plants. The expression of over 60 genes encoding components of the classical, phosphorylating respiratory chain and TCA cycle remained largely constant when cells were subjected to a broad range of abiotic stresses, but expression of the alternative components responded differentially to the various treatments (Clifton et al. 2005). A common aspect of many types of abiotic and biotic stresses is the high production of ROS resulting in a cell damaging condition known as oxidative stress. As has been suggested long ago and shown by the results of many studies, AOX reduces the effects of oxidative stress by preventing accumulation of ROS in mitochondria (Amirsadeghi et al. 2006, Gray et al. 2004, Gupta et al. 2009, Maxwell et al. 1999, Møller 2001, Popov et al. 1997, Purvis and Shewfelt 1993).

Many attempts to elucidate the role of the AOX pathway have been based on the manipulation of *Aox* gene expression through overexpression and silencing experiments. Results of these reverse genetic strategies mainly regarding Arabidopsis and tobacco and manipulation of only one *Aox* gene at a time are reviewed in this issue (Vanlerberghe et al. 2009), and a major conclusion is that lack of an *Aox* allele (which is not compensated by induction of another counterpart allele) has little impact under normal growth conditions but has severe consequences for survival under stress. It is also evident that data from reverse genetic experiments provide support for most of the proposed physiological roles of AOX till date but do not allow the development of a simple model to explain all the observations. Thus, the authors make the hypothesis that AOX may control the mitochondrial stress signaling (perhaps ROS-based) pathway by defining the strength of the signal, that is determining cell death or survival. In doing so, AOX can either enhance or suppress ROS generation that is largely dependent upon a given set of metabolic conditions.

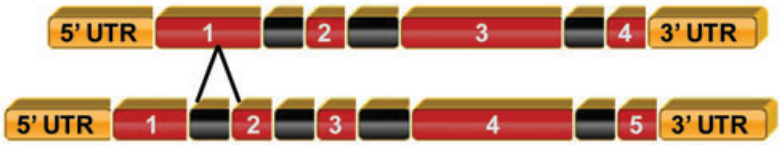
The above data from reverse genetics point to a significant role for AOX under adverse conditions in both modulation of plant growth and development and protection of cells from oxidative stress. More importantly, it is suggested that this role is not just housekeeping but can be regulatory, and evidence in support of this view is accumulating (Van Aken et al. 2009, Vanlerberghe et al. 2009). Thus, development of *Aox*-based functional markers for stress tolerance should be highly advantageous.

To be useful as a functional marker, *Aox* should fulfill several requirements: polymorphic sequences within species must be available; polymorphic, functional motifs affecting plant phenotype within *Aox* genes must be identifiable; and a strong relationship between DNA polymorphisms and variation of the trait of interest should be established. In this review, we provide evidence that organization of genomic *Aox* sequences and gene expression patterns are variable. This fulfills the first requirement in the path for development of functional markers based on *Aox* in plants. The second requirement, that is a strong correlation between *Aox* sequence polymorphism and trait variability, still needs to be broadly established.

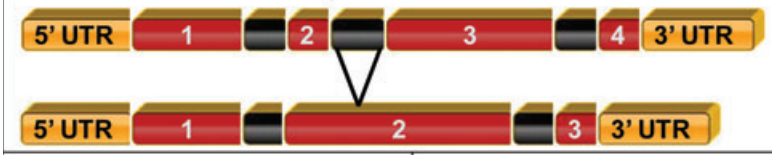
Variation of *Aox* gene organization

Plant AOXs are encoded by a small nuclear multigene family that is divided into two subfamilies, *Aox1* and *Aox2* in eudicots, whereas only *Aox1* members have been found in monocots. The number of *Aox* genes in different plant species is variable. For example, Arabidopsis has five genes: *Aox1a, Aox1b, Aox1c, Aox1d* and *Aox2* (Clifton et al. 2006, Saisho et al. 1997, 2001b), whereas rice has four: *Aox1a, Aox1b, Aox1c* and *Aox1d* lacking the *Aox2* as monocot, and *Vitis vinifera* has three genes: *Aox1a, Aox1b* and *Aox2* (Costa et al. 2009b). Variation has also been detected in copy numbers of each subfamily in different eudicot plants. Arabidopsis has four *Aox1*-type and one *Aox2*-type genes whereas soybean has one *Aox1*-type and two *Aox2*-type genes.

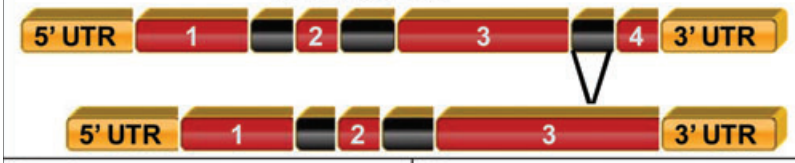
Conserved structure		
Species	**Gene**	**Reference**
Arabidopsis thaliana	*Aox1a, Aox1b, Aox1c*	(Considine et al., 2002)
Catharanthus roseus	*Aox*	*GenBank Acc. No.* AB055060
Citrus sinensis	*Aox1a*	*GenBank Acc. No.* EU723698
Glycine max	*Aox1, Aox2a, Aox2b*	(Thirkettle-Watts et al., 2003)
Gossypium hirsutum	*Aox1*	(Li et al., 2008)
Oryza sativa	*Aox1a, Aox1c, Aox1d*	(Considine et al., 2002)
Triticum aestivum	*Waox1a, Waox1c*	(Takumi et al., 2002)
Vigna unguiculata	*Aox1*	*GenBank Acc. No.* DQ100440
Vitis vinifera	*Aox1a, Aox2*	(Velasco et al., 2007)
Zea mays	*Aox1a*	(Polidoros et al., 2005)



Gain of intron in exon 1		
Species	**Gene**	**Reference**
Arabidopsis thaliana	*Aox2*	(Considine et al., 2002)



Loss of intron 2		
Species	**Gene**	**Reference**
Arabidopsis thaliana	*Aox1d*	(Considine et al., 2002)
Oryza sativa	*Aox1b*	(Considine et al., 2002)



Loss of intron 3		
Species	**Gene**	**Reference**
Solanum tuberosum	*Aox1a*	*GenBank Acc. No. DQ270421*

Fig. 1. Intron–exon organization of plant AOX genes. Most genes display the conserved structure of four exons interrupted by three introns. Intron gain and loss have resulted in variations in some species where Aox genes have five or three exons.

Thus, while *Aox1*-type genes have expanded in Arabidopsis, *Aox2*-type genes have expanded in legumes such as soybean and cowpea (Costa et al. 2004, McCabe et al. 1998). Usually, only one of the two *Aox* subfamilies has more than one member with carrot being the only reported exception where both subfamilies expanded having two members (Campos et al. 2009, Costa et al. 2009a). A tandem gene arrangement has been reported for Arabidopsis *Aox1b* and *Aox1a* (Saisho et al. 1997) as well as for soybean *Aox2b* and *Aox2a* (Thirkettle-Watts et al. 2003) and rice *Aox1b* and *Aox1a* (Ito et al. 1997), probably because of gene duplication. However, recently, it has been demonstrated that the two carrot AOX2 genes were linked to two linkage groups (unpublished, see Cardoso et al. 2009). *Aox* genes in both families present a conserved intron–exon structure that in many species consists of four exons interrupted by three introns at highly conserved splice site positions (Considine et al. 2002). Variations of this structure have been evolved by intron loss or gain and Fig. 1 shows *Aox* gene intron–exon structure in several species denoting the most prominent categories.

Sequence variation in *Aox* genes

Although *Aox* gene sequence information is available for several plant species at the genomic and even more at the transcript levels, evidence for variation in allelic sequences of *Aox* within species or in individual plants is limited. There are few reports for the presence of single nucleotide polymorphisms (SNPs) in *Aox* genes in rice and tomato, which may be related to differential gene expression and stress tolerance (Abe et al. 2002, Holtzapffel et al. 2003). Recently, an effort has been launched by Arnholdt–Schmitt and coworkers to gather more relevant information, and most of the results are presented in this issue. Variation in the intron length of *Aox1b* of *Hypericum perforatum* L. was observed when two fragments of 1408 and 1349 bps were identified for the partial *AOX1b* gene sequence. This intron length polymorphism was first identified in an individual plant, but was later verified through Exon-Primed Intron-Crossing (EPIC)-PCR in individual plants from six diverse regions in Portugal (Ferreira et al. 2009). In all plants, both fragments showed near identical sequences in all three exon regions. However, both complete intron regions revealed deletions that counted for the difference in the overall fragment sizes. Polymorphic sites were observed in several regions of *Aox2* cloned from the olive (*Olea europaea* L.) cultivars 'Galega vulgar,' 'Cobrançosa' and 'Picual.' Data revealed SNP polymorphisms in introns of the three varieties and variability in the 3′-untranslated region (3′-UTR) region among seven recombinant clones from 'Galega vulgar' (Macedo et al. 2009). Repetitive patterns of intron length variation have been observed in the carrot *DcAOX2a* gene. Polymorphic and identical PCR fragments revealed underlying high levels of sequence polymorphism encompassing insertion/deletion events, SNPs and polymorphism patterning (Cardoso et al. 2009). Variable transcript length of *Aox1a* and *Aox1b* was observed among two grape varieties, PN40024 and Pinot Noir. Also *Aox2* in PN40024 was found to harbor a retrotransposon rendering the gene sequence 5 kb longer than *Aox2* in Pinot Noir (Costa et al. 2009b). It is conceivable that evidence of variation among individual *Aox* genes in diverse plant species is accumulating and existing polymorphism may support the development of molecular markers for breeding purposes in these species.

Aox gene 3′-UTR microheterogeneity

Another aspect of polymorphism in *Aox* sequences is the observed 3′-UTR microheterogeneity as a result of alternative polyadenylation of *Aox* transcripts in maize (Polidoros et al. 2005) and olive (Macedo et al. 2009). Many processes of post-transcriptional control of gene expression involve the 3′-UTR and polyadenylation signal of the gene. In animals, the highly conserved AAUAAA signal, about 10–30 nt upstream of the cleavage site (CS), and a downstream U- or GU-rich element define the exact site where the poly(A) tail is added. In plants, unlike animals, the localization of the poly(A) tail addition depends on three major groups of poly(A) signals, namely the far upstream elements (FUEs), the near upstream elements (NUEs) – a functional equivalent to the vertebrate AAUAAA element – and the CS itself (Hunt 2007). It is a general phenomenon that plant transcripts are heterogeneous regarding the length of their 3′-UTR. This may affect as much as about 50% of the rice genes having at least two poly(A) sites that are 30 or more nucleotides apart (Shen et al. 2008). Also a total of 27.9% of 62 811 expressed sequence tags having an intact 3′ end of *V. vinifera* were found to contain alternative polyadenylation sites (Cai et al. 2008). Alternative poly(A) site selection may be accomplished by two different hypothetical mechanisms, the one involving a single polyadenylation signal defining multiple 3′ ends depending on changes in the relative efficiencies of the NUEs and/or CSs associated with the signal, and the other entailing different polyadenylation signals (complete combinations of FUE and associated NUEs and CSs) that might be selected differentially (Hunt 2007). Consequence of this phenomenon that may be widespread throughout the plant kingdom is that a high percentage of mRNAs transcribed from the same gene

are polymorphic at their 3′ ends. The nature of the 3′-UTR and the choice of polyadenylation site in genes with multiple sites may play a role in the expression of a gene, with important physiological consequences.

There are examples of several mechanisms capable of affecting the polyadenylation processing depending on signaling events as responses to environmental and developmental cues. Modification of various polyadenylation factors affect their activity and might contribute to alternative poly(A) site choice (Hunt 2007). For example, poly(A) synthesis in yeast is controlled by cycles of phosphorylation and dephosphorylation that require the action of the phosphatase Glc7 (He and Moore 2005). In Arabidopsis, signaling cascades stimulate alteration of the activity of the polyadenylation machinery components, as for instance the calcium dependent interaction of the cleavage and polyadenylation specificity factor *AtCPSF* with calmodulin, which inhibits the *AtCPSF* RNA-binding activity (Delaney et al. 2006). Finally, a relationship between polyadenylation and hormonal signaling was revealed by the abscisic acid (ABA) dependent inhibition of the alternative processing which downregulates the production of full-length FCA-encoding mRNAs that promote Arabidopsis development (Razem et al. 2006), providing a molecular link between ABA-mediated signaling, alternative polyadenylation and the regulation of flowering time.

These mechanisms could affect the polyadenylation process as a response to signaling and result in the production of transcripts with different 3′-UTR lengths. Such a case involving the maize *Aox1a* gene was provided by a study revealing alternative polyadenylation after treatment with H_2O_2, a well-known stress signaling molecule (Polidoros et al. 2005). Although the two major classes of 3′-UTRs in the transcript of maize *Aox1a* differing about 80 nt were also observed in control untreated tissue, the relative abundance of the two classes was different (unpublished observations). Interestingly, this difference could have a functional role if the deleted sequence in one class had a regulatory role(s).

There are several examples of regulatory elements residing in 3′-UTRs. A riboswitch (metabolite-sensing gene control element) function in the 3′-UTR of the thiamin biosynthetic gene *THIC* mediates feedback regulation of expression in response to changes in cellular thiamin pyrophosphate levels (Wachter et al. 2007). In this example, 3′-UTR length positively correlates with transcript accumulation, thereby establishing a basis for gene control by alternative 3′ end processing. In a more broad example, 3′-UTRs play an important role in post-transcriptional regulation that is mediated by microRNAs (miRNAs) in animals (Stark et al. 2005). Animal miRNA binding sites occur typically in the 3′-UTRs of the target genes, and a large set of genes involved in basic cellular processes avoid miRNA regulation because of short 3′-UTRs that are specifically depleted of miRNA binding sites (Stark et al. 2005). In plants, unlike animals, the complementary sites can exist anywhere along the target mRNA rather than exclusively at the 3′-UTR (Zhang et al. 2006). Regarding *Aox* genes, a repetitive deletion in intron 3 of the carrot *Aox2a* was found to affect a putative pri-miRNA site (Cardoso et al. 2009). Several examples exist where the 3′-UTR harbor miRNA targets in plants (Rhoades et al. 2002). Examining the maize *Aox1a* 3′-UTR for the presence of miRNA target sites, we identified a putative miR163 target motif (Fig. 2) having characteristics of a canonical site with good pairing to both 5′ and 3′ ends of the miRNA (Brennecke et al. 2005) and an overall pairing energy $\Delta G = -22.36$ kcal mol^{-1}. Although the functional significance of this motif in *Aox1a* is obscure and may be unlikely, as miR163 has been reported only in Arabidopsis and its putative target (S-adenosyl-L-methionine:carboxyl methyltransferase family members) is not related to *Aox*, the presence of this motif in the maize Aox 3′-UTR can be suggested as an example of how modulation of the 3′-UTR length can have significant effects of the regulation of *Aox* genes. miRNAs are regulatory RNAs with a mature length of about 21 nucleotides that are processed from hairpin precursors by Dicer-like enzymes and can negatively regulate gene expression by attenuating translation or by directing mRNA cleavage (Dugas and Bartel 2004). If the miRNA target site is between two CSs in the 3′-UTR, as is the case for maize *Aox1a* (Fig. 2), only transcripts with the long UTR will be affected by miRNA mediated silencing. Short UTR transcripts avoid this mechanism by not displaying the miRNA target site in their sequence. About half of the cloned maize *Aox1a* transcripts in H_2O_2 treated maize seedlings had short 3′-UTRs (Polidoros et al. 2005). Preferential expression of the *Aox1a* short UTR class points to a poly(A) site selection mechanism aiming to avoid

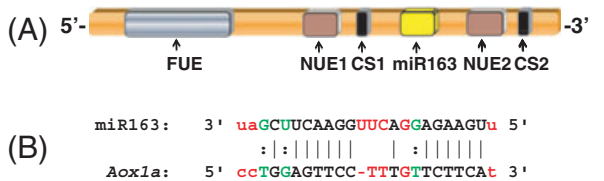


Fig. 2. (A) The structure of the maize *Aox1a* 3′-UTR showing the position of two different cleavage sites (CS1, CS2) with the neighboring near upstream elements (NUE1, NUE2) and the far upstream element (FUE). The position of the putative miRNA target site (miR163) between CS1 and CS2 is indicated. (B) Alignment of miR163 with its putative target site at the maize *Aox1a* 3′-UTR.

negative regulation through the miRNA or any other element that might reside in the long UTR transcript.

Aox 3′-UTR microheterogeneity may be not restricted to maize, as this phenomenon was also observed in olive *Aox2* that contained seven classes of short and long UTR variants (Macedo et al. 2009). A search for miRNA sites in the olive 3′-UTR revealed five putative miRNA targets that had an overall pairing energy of $\Delta G < -20$ kcal mol^{-1}. Three of these targets were present in all seven variants but the other two were absent in the shorter two variants. The functional significance of these sites remains to be examined. However, discovery of *Aox* 3′-UTR microheterogeneity in two phylogenetically distinct plant species strengthens the possibility that this phenomenon is widespread in plant *Aox* genes possibly because of a yet unknown regulatory function. Discovery of other *Aox* genes with variable 3′-UTR length of their transcripts will provide the tools to investigate the putative significance of these findings.

Regulatory element variation in *Aox* gene promoters

Different *Aox* gene family members display differential expression patterns depending on the plant species, tissues, growth, development and environment. This can be an effect of within-gene polymorphism and subsequent differences in gene regulation as discussed above, or as a consequence of differences in the structure of the promoter regions of these genes. Extensive promoter characterization has been conducted in Arabidopsis as well as in soybean (Ho et al. 2007, Thirkettle-Watts et al. 2003). These studies have revealed positive as well as negative regulatory regions that function in a species-specific manner and sequence motifs that are common between the two species. It was also found that the expression patterns observed between Arabidopsis and soybean were not conserved with gene orthology. Comparisons made between promoters driving the expression of genes with similar expression profiles revealed that the promoter region of soybean *GmAOX2b* contained seven sequence elements in common with the promoter region of *AtAOX1c*, pointing to the putative significance of these elements in co-regulation of the two genes. It should be noted that these genes are not induced by oxidative stress. Nevertheless, both genes share three out of the seven elements with *ZmAox1a*, which is highly responsive to oxidative stress (Polidoros et al. 2005). Thus, identification of common elements in the promoter region is not enough to potentiate common gene regulation. Identification of common sequence elements directing the co-expression of *Aox1a* and *NDB2* observed in Arabidopsis under a number of treatments suggested that common motifs arranged hierarchically in the upstream promoter regions of these genes may be related to similar responses (Clifton et al. 2005). It is then possible that multiple common regulatory elements with similar organization may be important to support the necessary specificity and selectivity for common gene regulatory context. Such a similarity was found between the maize *ZmAox1a* and the rice *OsAox1a* promoters (Polidoros et al. 2005). Particularly, a 90 bp upstream the TATA-box region was 73.4% homologous and a TGACG motif (as inverse repeat) within this region was conserved between the two promoters. The TGACG motif is conserved in the promoters of soybean *GmAox2b* and Arabidopsis *AtAox1a* and *AtAox1b* genes, and represents the binding motif of the TGA1 protein known to interact with the redox-activated NPR1 protein (Despres et al. 2003) important in plant responses to pathogens. In conclusion, there are many similarities in regulatory motifs among different *Aox* promoters, although their relative significance in common regulation of the harboring genes is dependent upon the presence of other motifs and the local hierarchical organization of the regulatory elements in these promoters.

Aox gene expression patterns

Aox gene expression in plants was investigated after cloning the first *Aox* cDNA in 1991 (Rhoads and McIntosh 1991). In the following years, a wealth of information has been accumulated regarding *Aox* expression patterns in various species. Data indicated that *Aox* genes are expressed either constitutively at specific stages, organs and tissues during development or are highly induced under stress. Thus, it is conceivable that AOX isozymes could be involved in two major functions: maintenance of mitochondrial respiratory capacity during plant development and preservation of mitochondrial functionality as a consequence of the high induction of *Aox* expression under various stresses. *Aox* genes are distributed in two subfamilies in eudicots in which the *Aox1* subfamily is regarded as the inducible one whereas *Aox2* is usually thought as constitutively or developmentally regulated (Considine et al. 2002, Juszczuk and Rychter 2003). Differential tissue, developmental and stress regulation of *Aox* genes that fits to the above model has been reported in Arabidopsis (Clifton et al. 2006, Saisho et al. 2001a, 2001b) and soybean (Considine et al. 2002, Djajanegara et al. 2002, Finnegan et al. 1997, McCabe et al. 1998). Orthologous genes tend to have a similar regulation as, for instance, the soybean and *Vigna unguiculata Aox2a* and *Aox2b* genes (Costa et al. 2004) but there are examples that are not compatible to this

model in other species (Considine et al. 2002). Developmentally regulated *Aox* genes although belonging to the *Aox1* subfamily have been reported in monocots, for example rice (Saika et al. 2002), wheat (Takumi et al. 2002) and maize (Karpova et al. 2002). *Aox* genes in various species also respond differentially to biotic and abiotic stresses (Maxwell et al. 1999, 2002, McIntosh et al. 1998, Mizuno et al. 2008, Simons et al. 1999, Vanlerberghe and McIntosh 1997), mitochondrial mutations (Karpova et al. 2002), treatments that interrupt mitochondrial functions, hormones and signaling molecules, particularly ROS, salicylic acid and nitric oxide (Djajanegara et al. 2002, Fung et al. 2006, Huang et al. 2002, Li et al. 2008, Maxwell et al. 1999, McIntosh et al. 1998, Millar and Day 1996, Polidoros et al. 2005, Rhoads and McIntosh 1992, Wagner 1995). In Arabidopsis, several studies comparing *Aox1a* expression with other stress defense genes reported *Aox1a* to be the most stress responsive (Clifton et al. 2005, Huang et al. 2002, Saisho et al. 1997). It should be noted, however, that in experiments with different species conflicting results have been reported (Clifton et al. 2006, Frederico et al. 2009)

There are multiple signaling pathways leading to *Aox* induction that can be either ROS dependent or ROS independent (Gray et al. 2004). ROS dependent pathways are probably very significant in stress related studies, as many types of biotic and abiotic stresses induce ROS production and accumulation at much higher than normal levels (Mittler 2002). In Arabidopsis, a network of at least 152 genes is involved in managing the level of ROS. In a comparative analysis of microarray expression data for the different genes of this network in three different knockout or antisense lines (with reduced or lacking ascorbate peroxidase 1, Cu/Zn superoxide dismutase 2 and catalase 2) that were overproducing ROS and in plants subjected to five different abiotic stress conditions (heat, drought, salt, cold or high light), the Arabidopsis *Aox1a* expression was induced to all but one condition, exposure to high light, where it was actually slightly reduced (Mittler et al. 2004). No other gene of this network displayed such a wide range of induction and responses were more specific. The regulation of the transcriptional induction of *Aox1a* was studied by the analysis of the *Aox1a* promoter using deletion and mutagenesis, and a common region especially important for strong induction by both the mitochondrial electron transport chain inhibitor antimycin antimycin A (AA) and the TCA cycle inhibitor monofluoroacetate (MFA) was identified (Dojcinovic et al. 2005). Although ROS production by AA is well documented and could be possibly responsible for the induction of the gene, the ROS related induction by the concentration of MFA used in these experiments looked unlikely. The authors concluded 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. The above data suggest that *Aox* may play a significant role not only in preventing ROS buildup, but also in sensing metabolic perturbations and in the coordination of stress responses. This is furthermore supported by expression studies using *Aox* overexpression, suppression or knockout lines mainly in Arabidopsis where altered *Aox* expression results in an altered cellular metabolic state (Umbach et al. 2005), pointing also to the significance of *Aox* function outside the mitochondria, in the organism level, because altered *Aox* expression could inflict more significant extramitochondrial metabolic or antioxidant defense effects than mitochondrial ones (Clifton et al. 2006, Fiorani et al. 2005, Giraud et al. 2008, Smith et al. 2009, Van Aken et al. 2009).

Aox alleles are regulated independently of each other (Clifton et al. 2006) but studies in knockout Arabidopsis plants lacking *Aox1a* showed increased induction of *Aox1d*, although not enough to fully compensate for the lack of AOX1a under restricting cytochrome pathway conditions (Strodtkotter et al. 2009). Overall, the above studies suggest that differential regulation of *Aox* genes is correlated with specific roles each gene has to fulfill in normal development or under stress and multiple stress defense, and metabolic pathway signals are integrated in a 'regulatory apparatus' responsible to effect specificity in *Aox* expression.

Given the well-documented *Aox* induction at the transcriptional level under various stress conditions, there are two important questions regarding the significance and the role of this response. The first is whether there is a direct correlation between *Aox* expression, protein abundance and its activity or engagement in respiration. This is an issue that will be covered thoroughly elsewhere in this special issue (Florez-Sarasa et al. 2009, Rasmusson et al. 2009). The second, in relation to the main theme of this review, that is assessment of *Aox* polymorphisms and use as a tool for plant improvement, is whether *Aox* expression and induction under stress is variable in different genotypes and related with phenotypic differences and adaptation to stress. In other words, are there any data pointing to a significant differential *Aox* expression between a tolerant and a sensitive variety under a certain stress and is there any relationship of variation in *Aox* expression with differential response to stress? This is a fundamental question (Arnholdt-Schmitt et al. 2006), although almost totally unexplored up to now.

There is only one published study that has undertaken the task to examine variation of *Aox* expression in contrasting genotypes regarding their response to stress (Costa et al. 2007). In that study the *VuAox1*, *VuAox2a* and *VuAox2b* gene expression and AOX protein level and capacity were examined in roots of a sensitive and a tolerant *V. unguiculata* variety after hydroponic exposure to high salt concentration or osmotic stress. The results indicated differential *VuAox2b* expression among the two varieties under both conditions whereas *VuAox1* and *VuAox2a* remained unchanged. In the tolerant cultivar (Vita 3), the expression of *VuAox2b* gene was induced by an osmotic stress but it was underexpressed in salt stress conditions. In the sensitive cultivar (Vita 5), the transcript level of the *VuAox2b* was unchanged in response to Polyethylene glycol (PEG) treatment, and upon salt stress, it was overexpressed.

Arnholdt-Schmitt et al. (2006) have pointed to the importance of considering species- and genotype-specific AOX expression and its kinetics. Additionally, a theoretical strategy was developed that highlighted the significance of designing molecular functional markers for agronomic traits from important target tissues in a top-down approach starting at whole plant level (Arnholdt-Schmitt 2005a, 2005b). Recent data confirm this view concerning genotype dependent AOX expression on salt treatments (Mhadhby, Fotopoulos, Mylona, Aouani and Polidoros unpublished results). Examining the differential response of a set of antioxidant genes with real-time RT-PCR in *Medicago truncatula* leaves and roots to high salt concentration in one sensitive and two tolerant varieties, a different picture regarding *Aox* expression was recorded than in *V. unguiculata*. The response of an *Aox1a* ortholog in this species was dependent on salt concentration and showed differences depending on the duration of the treatment (24 or 48 h) and the organs studied in interaction with the genotype.

Conclusions

Many lines of evidence suggest that AOX may act as a central regulator of plant growth and development as well as an integrator of stress signals for defense deployment under stress. Therefore, it has been proposed that this gene could be a promising candidate for functional marker-assisted breeding strategies for stress tolerance. The use of *Aox* as a marker should be based on the presence of polymorphic sequences and the identification of polymorphic motifs related to altered gene expression, and ultimately modified AOX engagement and capacity, leading to phenotypic variation for the trait under investigation. In this review, we presented evidence documenting that *Aox* genes are highly polymorphic regarding the gene family organization and gene sequence, and display variable expression patterns in normal plant development and as a response to stress. However, limited data are available and still do not allow an unequivocal link of sequence polymorphism with phenotypic variation regarding stress tolerance. The possibility to establish the missing link exists as sequence variation has been observed at all levels and gene regions encompassing SNPs, indels and 3′-UTR microheterogeneity. This variation can have immense effects on both *Aox* expression and AOX function. Initial reports show that different genotypes displaying various degrees of tolerance under a certain stress condition have also differences in *Aox* expression responses to the stress. Many factors seem to affect the course and magnitude of the response. Further studies are needed to establish which of the different parameters affecting *Aox* gene expression will be critical for a strong correlation between *Aox* gene expression and the stress response phenotype. Understanding the potential of using *Aox* as a functional marker for stress breeding will be largely dependent upon our ability to recognize these factors and integrate their effects into a system correlating *Aox* sequence polymorphisms, the kinetics and variability of gene expression at transcript level, and AOX activity and capacity to the stress response. However, from the perspective of a breeder, it will be sufficient to closely associate or link identified AOX polymorphic sequences to target agronomic traits. This would allow initiating marker-assisted breeding strategies but broad establishment of this association or link is still not documented by the limited available results and is a question of increasing interest for plant breeders and plant biologists.

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