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Identification of duplicated and stress-inducible *Aox2b* gene co-expressed with *Aox1* in species of the *Medicago* genus reveals a regulation linked to gene rearrangement in leguminous genomes

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ABSTRACT

In flowering plants, alternative oxidase (Aox) is encoded by 3–5 genes distributed in 2 subfamilies (*Aox1* and *Aox2*). In several species only *Aox1* is reported as a stress-responsive gene, but in the leguminous *Vigna unguiculata Aox2b* is also induced by stress. In this work we investigated the *Aox* genes from two leguminous species of the *Medicago* genus (*Medicago sativa* and *Medicago truncatula*) which present one *Aox1*, one *Aox2a* and an *Aox2b* duplication (named here *Aox2b1* and *Aox2b2*). Expression analyses by semi-quantitative RT-PCR in *M. sativa* revealed that *Aox1*, *Aox2b1* and *Aox2b2* transcripts increased during seed germination. Similar analyses in leaves and roots under different treatments (SA, PEG, H_2O_2 and cysteine) revealed that these genes are also induced by stress, but with peculiar spatio-temporal differences. *Aox1* and *Aox2b1* showed basal levels of expression under control conditions and were induced by stress in leaves and roots. *Aox2b2* presented a dual behavior, *i.e.*, it was expressed only under stress conditions in leaves, and showed basal expression levels in roots that were induced by stress. Moreover, *Aox2a* was expressed at higher levels in leaves and during seed germination than in roots and appeared to be not responsive to stress. The *Aox* expression profiles obtained from a *M. truncatula* microarray dataset also revealed a stress-induced co-expression of *Aox1*, *Aox2b1* and *Aox2b2* in leaves and roots. These results reinforce the stress-inducible co-expression of *Aox1*/*Aox2b* in some leguminous plants. Comparative genomic analysis indicates that this regulation is linked to *Aox1*/*Aox2b* proximity in the genome as a result of the gene rearrangement that occurred in some leguminous plants during evolution. The differential expression of *Aox2b1*/*2b2* suggests that a second gene has been originated by recent gene duplication with neofunctionalization.

Introduction

Mitochondria possess a cytochrome pathway that culminates in oxygen reduction and in ATP synthesis. However, plant mitochondria have an alternative route mediated by alternative oxidase (Aox) that branches from the main respiratory chain at the ubiquinone level. As a consequence, ATP generation is reduced and most of the resulting energy is released as heat (Chai et al., 2012). Many studies have been focused on elucidating Aox function in plants. So far, the prominent role of Aox in thermogenic floral tissues in plants from Araceae family is well established (Ito-Inaba et al., 2009). However, in non-thermogenic plants, it is suggested that Aox plays an important role on reactive oxygen species (ROS) prevention, cell reprogramming under stress conditions and maintenance of the TCA cycle in situations of high cellular ATP concentration (Maxwell et al., 1999; Arnholdt-Schmitt et al., 2006; Clifton et al., 2006). Although progress has been made in the last several years, the exact physiological and molecular details of the metabolic regulation mediated by Aox remain largely unknown and represent an area of considerable research interest (Sircar et al., 2012).

In plants, Aox is encoded by a small nuclear family of 3–5 members distributed in 2 subfamilies (*Aox1* and *Aox2*) (Whelan et al.,

Abbreviations: Aox, alternative oxidase; cDNA, DNA complementary to RNA; Chr, chromosome; LEA proteins, Late Embryogenesis Abundant proteins; NAA, 1-naphthaleneacetic acid; PEG, polyethylene glycol; ROS, reactive oxygen species; SA, salicylic acid; TCA, tricarboxylic acid; TSS, transcriptional start site.

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1996; Considine et al., 2002; Borecky et al., 2006). *Aox1* is found in all studied angiosperms while *Aox2* is present only in eudicots species (Considine et al., 2002). The majority of the investigated plant species have duplicated *Aox1* genes, as is the case of *Arabidopsis thaliana*, while other species, particularly those belonging to the Fabales order such as soybean and *Vigna unguiculata*, have duplicated *Aox2* (*Aox2a* and *Aox2b*) (Considine et al., 2002; Costa et al., 2004, 2009a). Up to now, duplication of both subfamilies has been described only in *Daucus carota* (*DcAox1a*, *DcAox1b*, *DcAox2a* and *DcAox2b*) (Costa et al., 2009a; Campos et al., 2009). Gene duplication in plants occurs mainly due to polyploidy events in their genome, and evolutionary pressures can lead to neofunctionalization of paraloguos genes (Clifton et al., 2006; Moore and Purugganan, 2005). In this context, the expression of *Aox* gene members found in a species is under tissue-specific and developmental regulation and could be differentially affected by stress conditions (Considine et al., 2002; Clifton et al., 2005, 2006; Campos et al., 2009; Costa et al., 2010). Generally, *Aox1* is reported as a stress-responsive gene while *Aox2* is a housekeeping gene and/or related to developmental events (Considine et al., 2002). In *Arabidopsis*, *Aox2* has been also linked to plastid-dependent signaling (Clifton et al., 2005). However, more recently it was demonstrated that both *Aox2* (specifically *Aox2b*) and *Aox1* of *V. unguiculata* are responsive to several stress conditions (Costa et al., 2010). Conflicting results were reported for soybean. In the Stevens cultivar, only *Aox1* was shown to be responsive to stress (Millar et al., 1997; Djajanegara et al., 2002; Thirkettle-Watts et al., 2003), while in the Cresir cultivar both *Aox1* and *Aox2b* were induced in response to salicylic acid (Matos et al., 2009), a direct ROS inducer.

Insight into signaling pathways that regulate *Aox* gene expression have been obtained from large scale *Arabidopsis* expression data (Clifton et al., 2005, 2006). In general, there are several pathways leading to Aox induction that go beyond the two way split of ROS-dependent and ROS-independent pathways. It was suggested that these pathways interact to affect the magnitude and timing of gene response (Clifton et al., 2006). Indeed, co-expression of stress-induced alternative respiratory chain components suggested a co-regulation directed by common sequence elements in the upstream promoter regions of responsive genes (Clifton et al., 2005; Costa et al., 2010). These findings have been obtained submitting plants to specific stresses from a broad range of treatments such as H_2O_2 (oxidative stress inducer), NaCl (osmotic, ionic and oxidative stress inducer), PEG (water deficit inducer) (Clifton et al., 2005, 2006; Costa et al., 2007, 2010), and to salicylic acid, which is known to be involved in biotic stress signaling. Insights in Aox regulation indicate Aox involvement in cell reprogramming processes during somatic embryogenesis (Frederico et al., 2009; Macedo et al., 2009). In this context, it is known that auxin such as 1-naphthaleneacetic acid (NAA) is able to induce cell division and somatic embryogenesis (Mohajer et al., 2012), an event that occurs with the concomitant induction of several genes responsive to stress conditions that increase ROS production (El-Gaied et al., 2013).

Promoter analysis combined with *Aox* gene expression data between *Arabidopsis* and soybean indicated that the expression pattern is not conserved with gene orthology (Thirkettle-Watts et al., 2003; Ho et al., 2007). In fact, both species have opposite types of Aox families: while *Arabidopsis* has duplicated *Aox1*, soybean has duplicated *Aox2*, and thus it is difficult to find an exact correlation between orthologous genes. In this context, to advance in the study of orthologous genes, it appears more reliable to compare *Aox* genes of closely related species that generally present similar Aox families. Therefore, we investigated here the *Aox* gene family in leguminous species of the *Medicago* genus that belong to the same subfamily of well studied species such as *V. unguiculata* and *G. max* but differ at the tribe level. Although *V. unguiculata* and soybean have similar Aox gene families, contrasting expression profiles of orthologous *Aox1*/*Aox2b* genes under stress conditions were found (Costa et al., 2010). Thus, studies focusing other close related species are necessary to clarify the function of the different *Aox* gene members in legumes.

In this report we characterized the multigene family of Aox in *Medicago sativa* and *Medicago truncatula* revealing an *Aox2b* duplication. Expression analyses were carried out under different stress conditions and in different organ/tissues of *M. sativa* in order to understand the functionality of *Aox* gene members in this species. Stress-induced expression of *Aox* genes was also evaluated in *M. truncatula* using publicly available Affymetrix GeneChip expression data (http://mtgea.noble.org/v2). Moreover, bioinformatic analyses of available genomes of the Fabales order and other eudicot species were performed with the purpose of gaining insight into the regulation and evolution of *Aox* genes.

Materials and methods

Identification of Aox genes in Medicago truncatula and other Fabales order species

The *Aox* genes of *Medicago truncatula* Gaertn were retrieved from genomic and EST databases using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) searches. Briefly, one *Aox1* and two *Aox2b* were obtained from *M. truncatula* genome in the phytozome database (http://www.phytozome.net/medicago.php) (Goodstein et al., 2012), while one *Aox2a* sequence was found at the *M. truncatula* Genome Project database (http://www.jcvi.org/cgi-bin/medicago/overview.cgi). Full-length cDNA sequences were obtained using CAP3 software (Huang and Madan, 1999) to generate contigs from expressed sequence tag (EST) data retrieved from GenBank (http://www.ncbi.nlm.nih.gov). DNA and cDNA sequences were translated into putative proteins sequence using the translation tool of EXPASY web server (http://web.expasy.org/translate).

Aox genes were also retrieved from *Glycine max* (L.) Merrill and *Phaseolus vulgaris* L. genomes available in phytozome as well as from *Cajanus cajan* (L.) Millsp. and *Lotus japonicus* (Regel) K. Larsen draft genomes available in WGS (Whole Genome Shotgun) database from GenBank (NCBI – National Center for Biotechnology Information) using BLAST searches with the purpose of detecting family composition and/or gene arrangement in these genomes.

Cloning and sequencing of Aox cDNAs from Medicago sativa

A pool of total RNA extracted from leaves of 30 day-old seedlings of *Medicago sativa* L. submitted to different treatments [control, polyethylene glycol (PEG), H_2O_2 and sodium salicylate] was used to amplify the *M. sativa Aox* cDNAs by RT-PCR using specific primers designed from orthologous *M. truncatula Aox* genes (Table 1).

The synthesis of first cDNA strand was carried out using 2 μg of total RNA and the Improm II kit (Promega, USA) following instructions of the manufacturer. RT-PCR reactions were performed using specific primer pairs (see Table 1) placed in the 5′- and 3′-ends of each *M. truncatula Aox* gene. The PCR amplification was executed following a pre-denaturation step at 94 °C for 3 min, employing 40 cycles of amplification at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, followed by a final extension step at 72 °C for 5 min. The PCR products were purified using the QIAEX kit (QIAGEN, Germany), cloned into pGEM T-easy vector (Promega, USA) using the Jm109 strain of *Escherichia coli*. Inserts of positive clones were sequenced at the Universidade Estadual Paulista Julio de Mesquita Filho (UNESP/Botucatu – Brazil).