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Short Communication

Daucus carota L. – An old model for cell reprogramming gains new importance through a novel expansion pattern of alternative oxidase (*AOX*) genes

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ABSTRACT

The paper highlights *Daucus carota* L. as an ideal model to complement plant stress research on *Arabidopsis thaliana* L. Recently, alternative oxidase (AOX) is discussed as functional marker candidate for cell reprogramming upon stress. Carrot is the most studied species for cell reprogramming and our current research reveals that it is the only one that has expanded both AOX sub-family genes. We point to recently published, but not discussed results on conserved differences in the vicinity of the most active functional site of AOX1 and AOX2, which indicate the importance of studying AOX sequence polymorphism, structure and functionality. Thus, stress-inducible experimental systems of *D. carota* are especially appropriate to bring research on stress tolerance a significant step forward.

1. Introduction

Cell reprogramming is essential for the capacity of plants to adapt to changing conditions at their destined environment by physiological and/or morphological plasticity. In contrast to human beings and most animals, higher plants possess in their mitochondria besides a respiratory chain coupled to ATP synthesis, the alternative respiratory pathway as an energy dissipating system. The energy bypasses of the respiratory chain include the alternative oxidase (AOX) that appears as key regulatory enzyme and at least four biochemically discernible types of alternative dehydrogenases (DHs) that oxidize NADH or NADPH [1]. Independently from each other, Arnholdt-Schmitt et al. [2] and Clifton et al. [3] speculated about a crucial role of AOX in plant cell programming. Evidence emerges increasingly from plant research that all types of *in-vivo* and *in-vitro* cell reprogramming are related somehow to a change in environmental conditions, normally referred to as 'stress'. Adaptive cell reprogramming and morphological plasticity demand besides energy in terms of ATP and reduced equivalents a change in carbohydrate turnover. Mitochondria can play a crucial role in such kind of metabolic adjustment. Understanding the significance of the alternative pathway in respiration for stress adaptation is an important subject in current plant research [4–8]. For several years, the function of AOX in growth and development has been debated [6,9,10]. Currently, the functional role of individual genes of the multigene family is being considered more specifically [11]. The most prominent plant under study is the model Brassicacaea *Arabidopsis thaliana*. However, whereas *A. thaliana* is clearly advantageous for molecular and genetic studies (e.g. Reymond et al. [12]), *Daucus carota* has some important advantages over *A. thaliana* for investigating cell programming [13,14].

Eudicot species possess two distinct *AOX* gene families. Species studied so far have expanded either only *AOX1*, as in the case of *A. thaliana*, or only *AOX2*, as in the case of *Glycine max* or *Vigna unguiculata* [15]. For monocots, only genes related to the *AOX1* family have been detected. Phylogenetic studies show that *AOX*1 genes are more similar to genes from the *AOX1* family of different species than to *AOX*2 genes from the same specie. However, a specific role of the sub-family gene groups *AOX1* and *AOX2* has not been clearly identified. The potential importance of the differences

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between the two groups and the fact that monocots do not possess *AOX2* genes has not been previously highlighted. This may be a consequence of the observation that different *AOX* genes were shown to respond to the same regulatory metabolites [16] and furthermore that orthologous genes can demonstrate differences in the expression pattern related to stress or development, thus, suggesting discrimination between both groups could be less significant in terms of AOX function (see in Ho et al. [11]). To summarize, it is not understood, to date, why two *AOX* sub-family groups emerged during evolution. Work from our laboratory on *AOX* gene sequences of *D. carota* has demonstrated that carrot is unique in that it expanded both *AOX* families (GenBank accession: EU286573, EU286574, EU286575 and EU286576). We are highlighting this here for the first time. The carrot *AOX* multigene family is characterized by two *AOX1* and two *AOX2* genes. The high sensitivity and easy response of carrot tissues upon addition of cell-reprogramming modulators in *in-vitro* culture experiments are in contrast to the recalcitrant behavior of monocots to cell reprogramming. This observation together with the novel pattern of *AOX* genes in carrot lead to the hypothesis that these may be linked. In this short survey, we will highlight the importance of *D. carota* as a model system for further progress in understanding the general role of *AOX* genes of both groups in cell reprogramming under stress.

2. Two complementing model systems: Arabidopsis thaliana and Daucus carota

In life science, *D. carota* is most known for being the first successfully studied species in proving the integrity of living cells. Steward et al. [17,18] established a primary culture system by using the secondary phloem of carrot taproots, which finally demonstrated totipotency. Since the fifties, induction of somatic cells from carrot to embryogenesis has been of central importance to study cell reprogramming (e.g. [17–26]). Furthermore it was the experimental carrot system of Steward et al. [27] that provided hints for differential genome organization through varying DNA amounts per cell [28] being later standardized by Neumann [29]. Various biochemical and metabolic, as well as histo-chemical and electron microscopic studies, demonstrated the importance of plastid transformation during *de novo* differentiation (e.g. [30]). The highly reproducible system helped to elucidate interaction between growth regulators and genome organization and is still successfully in use for novel molecular biological studies (e.g. [31–35]). Cell reprogramming is particularly easy to achieve in *D. carota* from all levels of cell and tissue organization. The reproducibility of events is high, making it an ideal system for fundamental research. Recently, carrot obtained further novel importance from abiotic stress research [36–38]. Kikuchi et al. [26] established a carrot stress-induction system, which clearly showed an effect of abiotic stress on the acquisition of embryogenesis competence (see also [37]). The genome of carrot consists of nine chromosomes and is typically stably diploid ($2n = 18$). The genome is relatively small, covering around 2 pg of DNA from which around 50% consists of repetitive sequences [39].

A. thaliana has a smaller genome than carrot and a lower content of repetitive DNA, which facilitate genome sequencing. This species shows a very short reproduction cycle that facilitates rapid large-scale genetic studies in laboratories with segregating populations to correlate quantitative trait loci and underlying sequences. It also allows the production of complete knockout-mutant sets and subsequent molecular studies. For all these reasons, *A. thaliana* was selected as a representative plant genome to study genome organization, 'omics' and plant behavior. Ecotypes have also been collected from diverse environments to analyze gene drifts during evolution and the importance of distinct genes for plant stress adaptation. Such advantages have resulted in *A. thaliana* becoming the most widely chosen plant to study. Today, numerous research groups concentrate on establishing and characterizing mutagenic lines thereby discovering a vast amount of new genes involved in biochemical pathways related to development, growth and the interaction between plant and environment. However, despite significant success in translating data from *A. thaliana* to commercially relevant crops [12], the knowledge gained from *A. thaliana* is frequently of limited use, particularly when considering the importance of genes in relation to distinct morphological or agronomic traits in physiological studies and plant breeding of crops. Studies across species will be extremely helpful to improve our understanding of the general functionality of identified functional marker candidates for cell reprogramming under stress. However, to apply the knowledge with success to crop improvement, a more directed species-specific approach must be chosen to highlight and validate candidate markers (see e.g. [2,40,41]).

Recently, Raghavan [14] discussed the significance of carrot versus *A. thaliana* as model systems to study the molecular biology of somatic embryogenesis. Although the author stressed that both systems have so far not been able to reveal any key genes crucial for somatic embryogenesis induction, the author highlighted the complementing capacities of both models. In fact, Yazawa et al. [13] combined the advantages of both plant systems and used carrot for functional expression analysis of isolated *A. thaliana* genes (*lec*1) related to embryo morphogenesis.

Besides carrot being an ideal model plant in research, it has several additional advantages. It is of commercial importance not only in moderate and subtropical climate, but also under tropical climate conditions. It is produced at low cost in small and large farms and at the same time attracts emerging interest as an industrial crop related to secondary compounds. For consumers, the plant serves as low-cost vegetable and also helps to recover from nutritional disorders. Additionally, the importance of carrot as a model plant comes also by the fact that it serves as a highly adequate experimental system for gene transformation assays, e.g. related to the effect of biotic resistance factors [42] or the production of vaccines [43]. Synchronized carrot cells can be transformed with high efficiency by *Agrobacterium tumefaciens* and can easily be regenerated to functioning plants [23,43,44]. Unfortunately, however, Raghavan [14] only highlighted the disadvantages of both systems without suggesting any perspectives for future progress. Nevertheless Raghavan's approach makes clear again that the difficulty in identifying critical genes for somatic embryogenesis lies most probably in the non-existence of crucial genes for cell determination, which play at the same time a critical role for the expression of embryogenic competence. In carrot, he found the most important problem was in the overlapping importance of candidate genes, because they interfere not only with embryo formation, but also with callus growth. In *A. thaliana*, Raghavan stresses the problem of overlapping significance of genes involved in leaf-forming and in somatic embryogenesis. In our group, we have developed a new direction in thinking on cell reprogramming. We hypothesize that the metabolic environment of a cell can strongly limit the realization of a new cell program. Cell reprogramming is related with a rapid and massive initiation of *de novo* protein synthesis [23]. Thus, the availability of metabolized carbohydrates is a prerequisite for the initiation of a new program. We think that this can be the critical point, where targeted differential *AOX* expression has the potential to regulate cell reprogramming through a specific link to cell determination. To test this hypothesis, easy-to-handle experimental systems, such as those of *D. carota*, will be of particular importance.

3. Why is the carrot AOX gene pattern of special interest?

The biochemical function and cellular location of AOX isozymes is considered to be the same for all *AOX* genes. The multigene *AOX* family is encoded in the cell nucleus, whereas its activity occurs on the inner surface of the inner membrane of mitochondria. AOX isozymes belong to the non-haem, diiron carboxylate group of proteins [45]. The pattern of hydrophobic regions suggests an interfacial integral rather than a transmembrane nature of the membrane protein (see [46] for a review). The most active form is a non-covalently linked dimer. AOX activity short circuits the respiratory chain by transferring reducing equivalents from the ubiquinol pool directly to oxygen and, thus, bypasses cytochrome oxidase. As a result of being non-protonmotive [47], less ATP is synthesized and energy is dissipated as heat. Thus, cells can escape the feedback control on carbohydrate catabolism due to high cellular levels of ATP [48]. High AOX activity is often found in tissues with high metabolic turnover rates, such as meristems [49] and during fruit ripening [50]. However Millar et al. [51] and Azcón-Bieto et al. [52] referred that this has not been observed during development of roots and cotyledons. AOX activity can be regulated at transcription and/or protein levels. Additionally, post-translational control occurs via reducing the S–S bond of AOX dimer and metabolic activation. Metabolic regulation of AOX activity involves multiple organelle-to-nuclear communication, reactive oxygen species, organic acids, thioredoxin and presumably also pH changes [53]. AOX was shown to act in concert with NAD(P)H dehydrogenases at relatively highly reduced states of ubiquinone [5].

Despite these common characteristics of the *AOX* gene family related to cell location and general function, sequence alignment and phylogenetic analysis has resulted grouping *AOX* genes into two sub-families *AOX1* and *AOX2* [54] (Fig. 1). *AOX*2 from *A. thaliana* differs significantly at all levels from *AtAOX1* genes, i.e. at the level of protein, cDNA, gDNA and the intron sequences. Site-directed mutagenesis has provided insight in the regulation of selected sites in *AOX* and contributed to current modeling (e.g. [55,56]). All dicots studied have at least one member gene of both groups, each species showing typically between three and five *AOX* genes. However, to date no important superimposed differences in functional sites between both groups have been highlighted.

Differential regulation of plant AOX proteins at the post-translational level has been reported in several plants and it is generally accepted that this is due to the key role exerted by two highly conserved cysteine residues: Cys_I and Cys_{II}. Cys_I is located in the structurally undefined N-terminus, whereas Cys_{II} is located at the N-terminal end of the first diiron-binding helix of the AOX

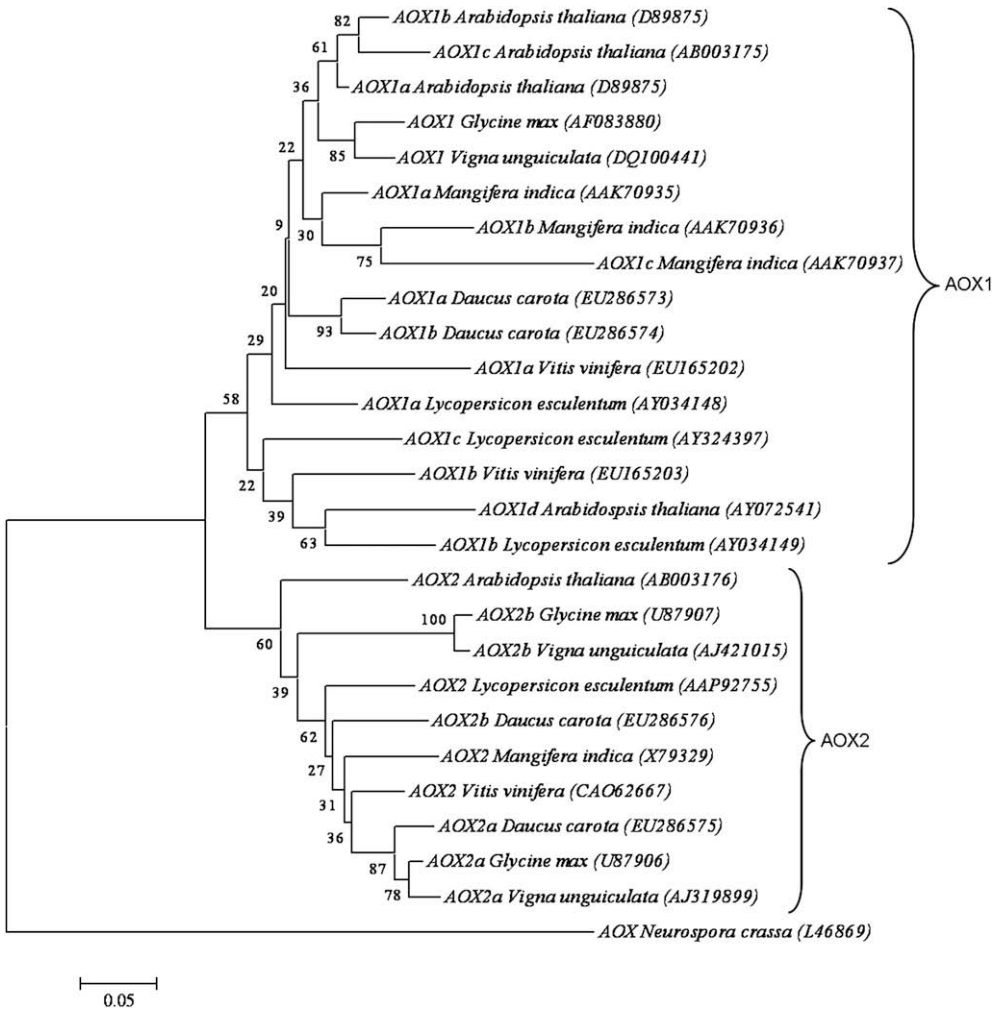

Fig. 1. Dendrogram describing the relationship among deduced AOX proteins from plants and fungus, including the four sequences of *Daucus carota* L. The sequences were aligned with ClustalW Multiple alignment in BioEdit software. The alignments were bootstrapped with 1000 replicates by the Neighbor-Joining method using the MEGA 3.1 software. The fungus *Neurospora crassa* was used as an outgroup. The scale bar indicates the relative amount of change along branches. Plant AOX proteins are divided into two groups (AOX1 and AOX2). Accession numbers are referred in brackets.

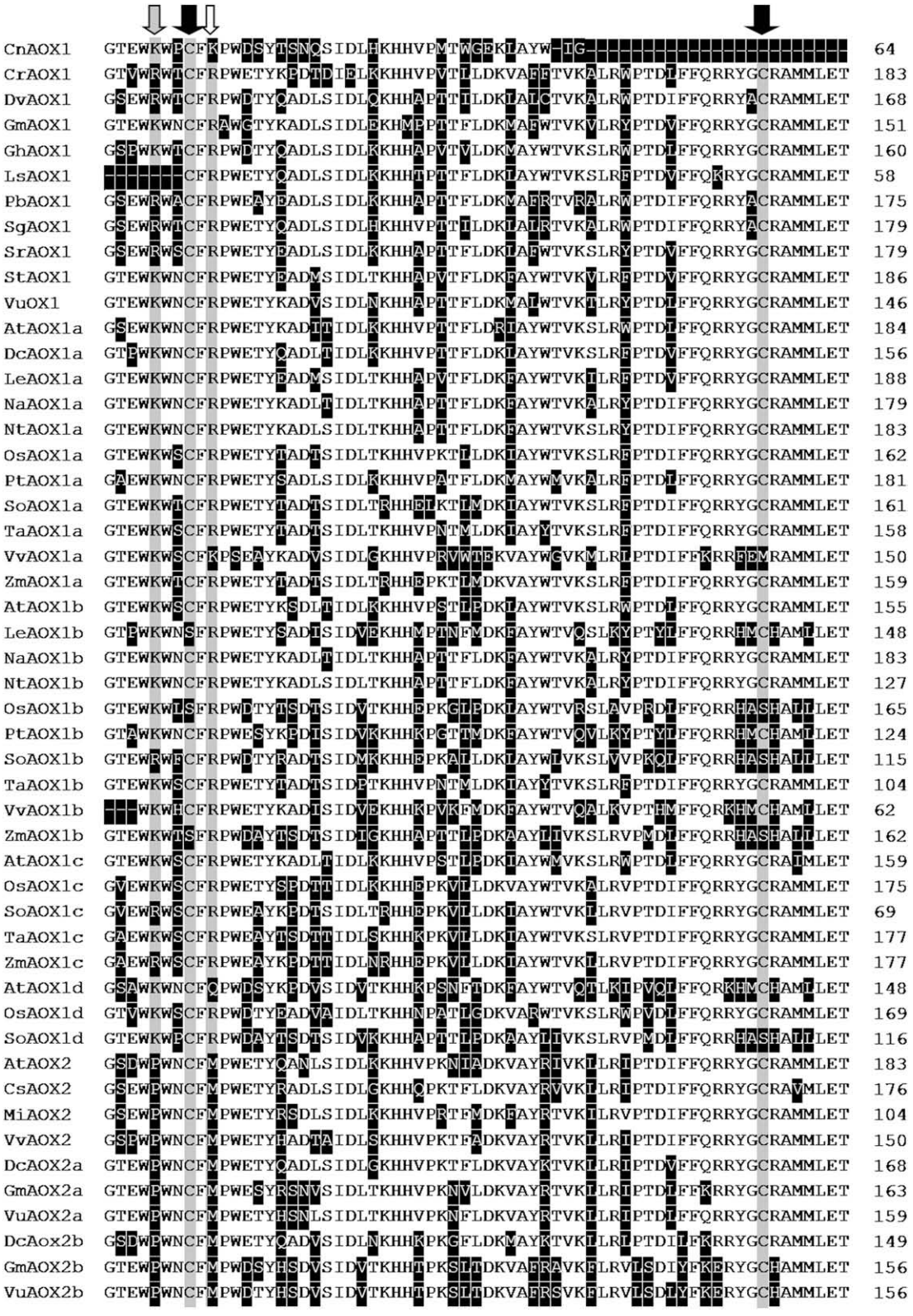

Fig. 2. Multiple alignment of the deduced amino acid sequences of 37 AOX genes from plants previously published in NCBI data bases. The alignment including AOX genes from both sub-families was performed using the ClustalW method of Lasergene 7 software. The accession numbers of the sequences are: CnAOX1 (AM167527), CrAOX1 (AB009395), DvAOX1 (AB189673), GmAOX1 (AF083880), GhAOX1 (DQ250028), LsAOX1 (AB268481), PbAOX1 (AB190213), SgAOX1 (M60330), SrAOX1 (AB183695), StAOX1 (AB176953), VuOX1 (DQ100441), AtAOX1a (D89875), DcAOX1a (EU286573), LeAOX1a (AY034148), NaAOX1a (AY422688), NtAOX1a (Q41224), OsAOX1a (O82807), PtAOX1a (AJ251511), SoAOX1a (AY644465), TaAOX1a (AB078882), VvAOX1a (EU165202), ZmAOX1a (AY059647), AtAOX1b (D89875), LeAOX1b (AY034149), NaAOX1b (AAR37365), NtAOX1b (Q40578), OsAOX1b (O82766), PtAOX1b (AJ271889), SoAOX1b (AAU11468), TaAOX1b (AF174004), VvAOX1b (EU165203), ZmAOX1b (AY059648), AtAOX1c (AB003175), OsAOX1c (Q8W855), SoAOX1c (AAU11469), TaAOX1c (AB078883), ZmAOX1c (AY059646), AtAOX1d (AY072541), OsAOX1d (AP004024), SoAOX1d (AAU11470), AtAOX2 (AB003176), CsAOX2 (AAP33163), MiAOX2 (X79329), VvAOX2 (EU523224), DcAOX2a (EU286575), GmAOX2a (U87906), VuAOX2a (AJ319899), DcAOX2b (EU286576), GmAOX2b (U87907), VuAOX2b (AJ421015). Amino acid residues differing are in black boxes and deletions are shown by minus signs (is due to the fact that partial sequences were used in this alignment). Grey arrow indicates the position of a conserved prolin in AOX2 sub-family and white arrow indicates the position of a conserved methionine in AOX2 sub-family. Black arrows indicate the sites of two conserved cysteines that are involved in dimerization of the AOX protein by S–S bond formation [65].

monomer [16,57]. All AOX2 proteins characterized to date contain both Cys_I and Cys_{II}, whilst in some AOX1 proteins there has been a substitution of Cys to serine (Ser) in one or in both positions [57]. These changes have been related to differential regulation of the protein activity. Crichton et al. [16] however stressed the effect of other residues, in addition to conserved cysteines, for metabolic activation of AOX, thus highlighting the importance of considering structural complexity of the isozymes.

Chemical and structural analysis of proteins is crucial to understand interaction of protein structure and function in active networks that rule cell behavior [58]. Although the AOX is considered to be a diiron carboxylate protein [59] its three-dimensional structure is yet to be elucidated. Furthermore, the reported size of AOX proteins varies between 29 and 37 kDa [11,60]. In rice OsAOX1a, a lysine residue was substituted in position 71 by asparagine through an artificially induced single nucleotide polymorphism (SNP). The corresponding proteins were isoforms of 32 and 34 kDa. The site of mutagenesis was tightly linked to a quantitative trait loci (QTL) for low temperature tolerance of anthers at the booting stage [61]. Thus, allelic variation in *AOX* through asynonymous SNP can be expected to change plant–environment interaction. The site of this SNP is linked to a region, which displays a higher number of conserved differences between *AOX1* and *AOX2* genes [62].

Conservation analysis is an important bioinformatics tool to predict functionally important residues in protein sequences. Current studies revealed that consideration of neighbor sequences of conserved sites can well improve the performance of methods for predicting conserved functional sites [63]. Reversely, we can expect that neighboring regions of conserved sites may have structural significance in relation to the regulatory function of conserved sites (see [16]). In this context, it is of interest to highlight recent results of Feenstra et al. [62] who developed a method to detect functional specificity from alignments and interestingly used AOX1 and AOX2 deduced protein sequences as an example to test their method. Analysis of the vicinity of both cysteine residues reveals a surprising result (Fig. 2) that will be highlighted here for the first time. All AOX1 and AOX2 sequences published so far can be clearly distinguished from one another from an examination of the residues on both sides of the first cysteine residue (Cys_I). While AOX2 sequences show a conserved proline three residues upstream and a conserved methionine in the second residue downstream, all AOX1 sequences displayed, instead, a lysine or an arginine three residues upstream and an arginine in the second position downstream. Only AtAOX1d contains a glutamine at this downstream position. This is the first time that AOX1 and AOX2 sequences were shown to have conserved differences in a functional region. In AOX1, Cys_I may be substituted by Ser. Nevertheless, the conserved differences in the vicinity of Cys_I are independent from such polymorphism (Fig. 2).

In contrast, the vicinity of Cys_{II} does not show any conserved differences in amino acid residues between *AOX1* and *AOX2*. This is of particular interest since Cys_I has been suggested to be the more important site for metabolic regulation in comparison to the conserved Cys_{II} [57]. From the available expression data, no clear distinction can be made between the mode and specificity of expression of AOX1 versus AOX2. For example, both sub-families can be stimulated by pyruvate [16]. An artificial substitution of Cys_I to Ser in *AtAOX1a* and *GmAOX2b* led in both cases to activation by succinate and not pyruvate [64]. Ho et al. [11] identified seven common active regulatory sites in the promoters of *AtAOX1c* and *GmAOX2b*. Both genes are related to development, tissue specificity and growth and do not respond to oxidative stress. However, all these studies compare orthologous gene expression or regulation of *AOX*1 and *AOX*2 from different species. To our knowledge, no systematic analyses are available from the metabolic regulation of all individual genes from one species. Until recently, it was believed that *AOX2* expression is typically constitutive and linked to tissues and development. However, Clifton et al. [5] showed that AOX2 also appears to play a role in stress responses related to plastid signaling. Interestingly, the only two species (*V. unguiculata* and *G. max*), which show expansion of AOX2, demonstrate similar tissue-specific expression of the orthologous *AOX2a* and *AOX2b* genes [15]. More recently, Costa et al. [8] showed that AOX2b is regulated by salt and drought stresses, thus, confirming the stress-inducibility of *AOX2* genes. The conserved differences in the functional site region around Cys_I between AOX1 and AOX2 sequences, described above, suggest that AOX1 and AOX2 can be distinguished by differential regulation at the Cys_I in a manner, which is not known today. For clarification as to the significance of these differences, we must await the outcome of further systematic expression studies and site-directed mutagenesis of AOX at species level.

In *A. thaliana*, *AOX2* is located at the end region of chromosome five, whereas all *AtAOX1* genes are found in the inner region of chromosome 3 (*AOX1a*, *b* and *c*) and chromosome 1 (*AOX1d*). Further, *AOX2* has less matrix attachment regions in the vicinity than *AOX1* genes (M. Xavier and B. Arnholdt-Schmitt, EU Marie Curie Chair, ICAM, University of Évora, Portugal, unpubl. res.). Both observations point to differences in global genome regulation of *AOX1* and *AOX2* genes related to genome looping and loop positioning within the cell nucleus and chromosome territories (see [35]).

4. Conclusion

D. carota is an interesting and important model system to advance our understanding on the role of individual *AOX* genes in cell reprogramming during stress. We suggest that experimentation with carrot can reveal the importance of defined functional sites in individual genes and the functional significance of *AOX1 versus AOX2* genes. Furthermore, we expect that studies on carrot polymorphisms in *AOX* genes will advance research on plant stress tolerance. This opinion is based on the following three observations, namely:

A. Tissues from *D. carota* show a high flexibility in cell reprogramming under diverse forms of stress. Carrot experimental systems are easy-to-handle in comparison with other species and are highly reproducible.
B. A functionally important region of *AOX1* and *AOX2* genes shows conserved differences.
C. The fact that *D. carota* genome expanded both sub-family groups of *AOX* makes carrot a unique experimental system.

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