Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 137: 578–591. 2009

Differential expression and co-regulation of carrot *AOX* genes (*Daucus carota*)

Maria Doroteia Campos[a], Hélia Guerra Cardoso[a], Bettina Linke[b], José Hélio Costa[c], Dirce Fernandes de Melo[c], Lígia Justo[a], António Miguel Frederico[a] and Birgit Arnholdt-Schmitt[a,*]

[a]EU Marie Curie Chair, ICAAM, University of Évora, Apartado 94, 7002-554 Évora, Portugal
[b]Department of Biology, Humboldt University, Invalidenstr. 42, D-10115 Berlin, Germany
[c]Department of Biochemistry and Molecular Biology, Federal University of Ceará, PO Box 6029, 60455-900, Fortaleza, Ceará, Brazil

Correspondence
*Corresponding author,
e-mail: eu_chair@uevora.pt

Received 19 May 2009; revised 5 August 2009

doi:10.1111/j.1399-3054.2009.01282.x

Alternative oxidase (AOX) is a mitochondrial protein encoded by the nuclear genome. In higher plants *AOX* genes form a small multigene family mostly consisting of the two subfamilies *AOX1* and *AOX2*. *Daucus carota* L. is characterized by a unique extension pattern of *AOX* genes. Different from other plant species studied so far it contains two genes in both subfamilies. Therefore, carrot was recently highlighted as an important model in *AOX* stress research to understand the evolutionary importance of both *AOX* subfamilies. Here we report on the expression patterns of *DcAOX1a*, *DcAOX1b* and *DcAOX2a* and *DcAOX2b*. Our results demonstrate that all of the four carrot *AOX* genes are expressed. Differential expression was observed in organs, tissues and during de novo induction of secondary root phloem explants to growth and development. *DcAOX1a* and *DcAOX2a* indicated a differential transcript accumulation but a similar co-expression pattern. The genes of each carrot *AOX* sub-family revealed a differential regulation and responsiveness. *DcAOX2a* indicated high inducibility in contrast to *DcAOX2b,* which generally revealed low transcript abundance and rather weak responses. In search for within-gene sequence differences between both genes as a potential reason for the differential expression patterns, the structural organization of the two genes was compared. *DcAOX2a* and *DcAOX2b* showed high sequence similarity in their open reading frames (ORFs). However, length variability was observed in the N-terminal exon1 region. The predicted cleavage site of the mitochondrial targeting sequence in this locus is untypical small for both genes and consists of 35 amino acids for *DcAOX2a* and of 21 amino acids for *DcAOX2b*. The importance of structural gene organization and the relevancy of within-gene sequence variations are discussed. Our results strengthen the value of carrot as a model plant for future studies on the importance of AOX sub family evolution.

Abbreviations – AOX, alternative oxidase; EST, expressed sequence tag; ISH, in situ hybridization; ORF, open reading frame; UTR, un-translated region.

Introduction

Daucus carota L. was currently highlighted as an ideal model plant to progress alternative oxidase (AOX) stress research. This was deduced from its unique pattern of *AOX* genes and the easiness of cell reprogramming in this species upon stress, which led in 1958 to the first demonstration of totipotency in plants (Raghavan 2006, Costa et al. 2009a). Zottini et al. (2002) studied nitric oxide effects in carrot cells. Their data pointed for the first time to a role of AOX in carrot in response to stress-induced mitochondrial dysfunctioning and signaling. Plant AOX protein is encoded by a small nuclear multigene family. In higher plants, the *AOX* multigene family consists of two discrete subfamilies, *AOX1* and *AOX2* genes. The occurrence of two subfamilies is species-dependent. To date, the *AOX1* genes were found in monocots and eudicots, whereas *AOX2* genes were detected only in eudicot species (Considine et al. 2002) and in the gymnosperm *Pinus pinea* (see Frederico et al. 2009). In most of the species studied so far either the *AOX1* subgroup was expanded, as in the case of *Arabidopsis thaliana* (Saisho et al. 1997) or only *AOX2*, as in the case of *Glycine max* or *Vigna unguiculata* (Costa et al. 2004). However in *D. carota* a novel pattern of *AOX* sequences was discovered, showing a simultaneous occurrence of two gene sequences in both known *AOX* subfamilies.

The reason why two gene subfamilies evolved and the functional importance across species are not understood. Recently, conserved sequence differences between *AOX1* and *AOX2* genes were identified that covered also near-neighbor sequences of the CysI site (Costa et al. 2009a, Frederico et al. 2009). From in silico conservation analysis it is known that consideration of neighbor sequences can well-improve the prediction of conserved functional sites (Capra and Singh 2007) indicating the importance of such loci. Additional importance for the differential regulation of the two *AOX* gene subfamilies may come from different positions in the plant genomes related to the chromosomal territories. For example, it can be observed that in *A. thaliana AOX1a, AOX1b* and *AOX1c* are located at chromosome 3, *AOX1d* at chromosome 1, whereas *AOX2* is situated at chromosome 5. Costa et al. (2009b) confirmed the observation that *AOX2* is located separately now also for *Vitis vinifera* genes. Whereas, the two *VvAOX1* genes are located on chromosome 2 and *VvAOX2* is found at chromosome 12.

Expansion of a gene family can point to the evolution of pseudogenes or merely to a duplication of sequences related to the same function. Thus, to better understand the meaning of the specific pattern of *AOX* expansion in both *AOX* subfamilies in *D. carota*, it is important to study expression patterns and structural organization of the gene sequence. Here we report on the differential expression of *DcAOX1a*, *DcAOX1b*, *DcAOX2a* and *DcAOX2b* in various tissues or organs and during development and growth and highlight within-gene differences between *DcAOX2* sub-family member genes. The results revealed expression of all four carrot *AOX* genes. Independent regulation of both genes in each gene sub-family was accompanied by co-regulation of *DcAOX1a* and *DcAOX2a*. The role of within-gene differences is discussed.

Materials and methods

Plant material

Seeds of *D. carota* cv. Rotin were germinated and grown on MS solid medium (Murashige and Skoog 1962) under sterile and controlled-climate conditions (25 ± 1°C at 16 h photoperiod: 95–100 µmol m^{-2} s^{-1}, Philips). For partial *DcAOX2* genes identification, a mixture of several in vitro plants were taken, while for complete gene isolation, one single in vitro plant was used.

Tissue- or organ-specific expression studies in roots and leaves were performed in cv. Rotin in three individual eight-week-old in vitro plants, in the cambium and secondary phloem of carrot tap roots and during initiation of a primary culture. Expression in young and adult flowers was studied in cv. Marktgaertner. For the primary culture assays and the isolation of cambium and secondary phloem from carrot tap roots, plants of cv. Rotin were cultivated in pots under greenhouse conditions for 10 weeks and maintained in pots in a cooling chamber at 4°C until the experiment started.

For expression analyses and in situ hybridization (ISH) in flowers, plants of cv. Marktgaertner were cultivated under glasshouse conditions after vernalization for 10 weeks at 5°C. Floral traits were taken from male fertile plants and classified as umbels of first to fifth order. Florets of young (juvenile stages, S5–8) and adult (nearly mature stages, S11/12) flowers were collected according to stage definition by Linke et al. (1999 and 2003). Seeds were obtained from a collection of the Institute for Breeding Research on Horticultural and Fruits Crops (JKI), Quedlinburg, Germany.

Primary culture system

In order to study gene expression during de novo growth induction and differentiation, a primary culture was established. Slices from the third upper part of carrot tap roots of two individual plants (cv. Rotin) were cut and

five explants (2–4 mg) of the secondary phloem were inoculated in 100 ml Erlenmeyer flasks containing 20 ml of NL liquid medium (Neumann 1966) The cultures were incubated under continuous rotation (90 rpm) at continuous light (95–100 µmol m^{-2} s^{-1}, Philips) at 28°C. During culture *callus* formation is induced. After a lag-phase of 6–8 days exponential callus growth starts mainly as a result of cell division activity. At day 14, the linear phase of *callus* growth is running and a mixotrophic nutritional system is established (Arnholdt-Schmitt 1999). Samples for expression analysis were collected at inoculation (t0), and 1.5 (36 h), 3 (72 h), 6 and 14 days after inoculation. The selected sample collection times consider the induction of cell cycle activities in the system. Circadian rhythmic under the permanent light was not studied. Thirty-six hours after inoculation marks the termination of a first cell cycle round, which is initiated synchronously in some cells of the explant. Six days marks ending of the lag phase and 14 days relates to the linear phase of exponential growth ($r = 0.813$) (see in Gartenbach-Scharrer et al. 1990, Arnholdt-Schmitt et al. 1991, Arnholdt-Schmitt 1995, 1999).

In situ hybridization

Temporal and spatial transcript distribution was analyzed by ISH on tissue sections of young flower stages as described previously (Linke et al. 2003) using an *AOX1* probe of 650 bp in length. Signals were monitored with a stereomicroscope (Olympus, Hamburg, Germany). Images were taken with a digital camera (Colorview 12, Olympus, Hamburg, Germany).

RT-PCR analysis

Primer design for RT-PCR expression analyses were based on recently published carrot *AOX* gene sequences for *DcAOX1a* (*Acc. No* EU286573), *DcAOX1b* (*Acc. No* EU286574), *DcAOX2a (Acc. No* EU286575) and *DcAOX2b* (*Acc. No* EU286576) (see Costa et al. 2009a). For flower analyses, total RNA was extracted (RotiQuick-Kit, Roth, Karlsruhe, Germany), treated with DNase (Invitrogen, Karlsruhe, Germany) and recovered after phenolization and EtOH precipitation. Reverse transcription using SuperScript II (Invitrogen, Karlsruhe, Germany) and oligo d(T)-primers was performed according to manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Control reactions without reverse transcriptase were routinely conducted. Transcript abundance was analyzed by semi-quantitative RT-PCR using annealing temperatures between 58 and 61°C, different primer concentrations and varying cycle numbers (19–28 and 40 cycles). The *TubulinA* gene of *D. carota* (*Acc. No* AY007250) was used for normalization. Primers generating PCR products between 400 and 650 bp were *TuAfw1, TuArev1, AXfw1*, *AX1-rev2*, *AX2rev-1*, *AX1b-fw* and *AX1b-rev* (Table 1).

From plant organs (root and leaves), tap root tissues (cambium and secondary phloem), and primary cultures, total RNAs were extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with on-column digestion of DNA with the RNase-Free DNase Set (Qiagen, Hilden, Germany), according to manufacturer's instructions. For root tissues, the reverse transcription was performed using the RETROscript kit (Ambion, Austin, Texas, USA) with oligo d(T) primer and 2 µg of total RNA. For primary cultures and for plants a single strand cDNA was produced by RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Ontario, Canada) according to manufacturer's instructions with oligo d(T) primer and 5 µg of total RNA. Semi-quantitative RT-PCR was performed using Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) and the annealing temperature of 55°C with different cycle numbers (35 and/or 45 cycles). The following specific primers for each *DcAOX* gene were designed to generate amplicons between 250 and 350 bp: *DcAOX1aF* and *DcAOX1aR* for *DcAOX1a, DAOX1b_101F and DAOX1b_268R* for *DcAOX1b*, *DcAOX2aF* and *DcAOX2aR* for *DcAOX2a, DAOX2b_133F and DAOX2b_402Rev* for *DcAOX2b* (Table 1). In these assays RT-PCR for each gene was normalized by *D. carota Actin1* (*Acc. No* X17526) using the primers *DcA1F* and *DcA1R* (Table 1). In general, two to three biological repetitions were performed. For the primary culture assay and flower analyses, each biological sample consisted of bulked samples. The Ready-to-Go PCR Bead technology, which requires only addition of Aqua dest, primers and template cDNA, was applied to avoid technical non-reproducibility of PCR results and the necessity for technical repetitions. The reproducibility by this technique was monitored in former research and was validated even for sensitive RAPD studies (Arnholdt-Schmitt 2000, Schaffer and Arnholdt-Schmitt 2001). Nevertheless, the critical data from tap root tissues and primary cultures were confirmed by including at least one technical repetition.

Identification of *DcAOX2* genes

For partial gene identification, DNA extraction was performed from a mixture of several plants using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA quantification was made in comparison to defined concentrations of lambda DNA as a standard in 1% agarose gel by using GeneTools (Syngene, Cambridge,

UK). The degenerated primer pair P1/P2 designed in exon3 of *A. thaliana* was used for amplification according the conditions referred by Saisho et al. (1997). PCR was conducted with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) using 10 ng of DNA as a template.

Isolation of complete *DcAOX2* genes

To determine the 5′ and 3′ ends of both identified *DcAOX2* genes 5′ and 3′ RACE-PCRs were conducted. Total RNA from a selected in vitro plant was isolated using RNeasy Plant Mini Kit as described before. To isolate the 3′ ends a single strand cDNA was produced by RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) as described above.

For the 3′RACE-PCR of the *DcAOX2a* the reverse primer *VIAL 9* (Roche, Mannheim, Germany) and a gene-specific forward primer *DcAox2c1F* (Table 1) were used. RACE-PCR products were amplified as follows: denaturation at 94°C for 30 s, annealing at 52°C for 30 s and DNA synthesis at 72°C for 60 s (35 cycles). A fragment of 750 bp was amplified and isolated.

With the procedure previously described it was not possible to isolate the 3′UTR of the *DcAOX2b* gene. The FirstChoice RLM-RACE Kit (Ambion, Austin, Texas, USA) was applied according to manufacturer's instructions using the primers *DcAOX2bintF1* and the *3′ RACE Outer Primer* from the kit (PCR1) (Table 1). The parameters used were 30 s a 94°C, 30 s at 60°C and 1 min at 72°C for 35 cycles. 1 µl of PCR1 was used as template in PCR2 using the primers *DcAOX2b_404Fw* and the *3′RACE Inner Primer* from the kit (Table 1). The parameters used in this re-amplification reaction were: 1 min at 95°C, 2 min at 60°C and 2 min at 72°C for 35 cycles. PCR in both reactions were carried out with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England). Finally, a fragment of 522 bp was identified.

In order to isolate the 5′ end of both *DcAOX2* genes a cDNA library of *D. carota* cv. Marktgaertner M853 cloned into a Lambda gt22a phage vector

Table 1. List of primers used in this study.

Primer sequence	Application	Comments
TuAfw1: 5′-GTGCATTTGAGCCCTCTTCTATGATG-3′	RT	Designed from *DcTubulinA*
TuArev: 5′-CATAGAGAACAGCATAAAGAGTTGTTGC-3′	RT	Designed from *DcTubulinA*
AXfw1: 5′-GTAGCTGCTGTCCCTGGCATGG-3′	RT	Designed from *DcAOX1a*, *DcAOX1b*, *DcAOX2a* and *DcAOX2b*
AX1-rev: 5′-GAGAACCAGAGATTCCTCCACTTCAG-3′	RT	Designed from *DcAOX1a* and *DcAOX1b*
AX2rev-1: 5′-GATAACCAAGCGGAGCTGGGCATC-3′	RT	Designed from *DcAOX2a* and *DcAOX2b*
AX1b-fw: 5′-GGAAGAGGAGGCGATCCACTCG-3′	RT	Designed from *DcAOX1b*
AX1b-rev: 5′-CGCAAAGCGATTACTAGTCCATG.-3′	RT	Designed from *DcAOX1b*
DcA1F: 5′-ATGTTGCTATCCAGGCTGTGC-3′	RT	Designed from *DcActin1*
DcA1R: 5′-TCACGAACAATTTCCCGCTCG-3′	RT	Designed from *DcActin1*
DcAOX1aF: 5′-GCAAGTCACTCAGGCGCTTTG-3′	RT	Designed from *DcAOX1a*
DcAOX1aR: 5′-CATGGTTTGACGAGGGATTT -3′	RT	Designed from *DcAox1a*
DAOX1b_101F: 5′-TTTTCAATGCATACTTCTTGACC-3′	RT	Designed from *DcAOX1b*
DAOX1b_268R: 5′-AGTCGAGTTTGGTGGCATACG-3′	RT	Designed from *DcAOX1b*
DcAOX2aF: 5′-TGCTGCATCTGAGGTCTCTCC-3′	RT	Designed from *DcAOX2a*
DcAOX2aR: 5′-GGAGCAGGAACATTTTCAATTG-3′	RT	Designed from *DcAOX2a*
DAOX2b_133F: 5′-ACGGATATACTGTTCAAGAGACG-3′	RT	Designed from *DcAOX2b*
DAOX2b_402Rev: 5′-AGCTTTGGTGACAGTATGTATAGG-3′	RT	Designed from *DcAOX2b*
VIAL 9: 5′-GACCACGCGTATCGATGTCGAC-3′	RACE	oligo d(T) primer (Roche)
DcAox2c1F: 5′- AAGAAGCTGAGAATGAGAGG-3′	RACE	Designed from *DcAOX2a*
DcAOX2bintF1: 5′-TGAATAAACACCATAAACCTAAGG-3′	RACE	Designed from *DcAOX2b*
3′ RACE Out Primer: 5′-GCGAGCACAGAATTAATACGACT-3′	RACE	FirstChoice RLM-RACE Kit (Ambion)
3′RACE Inn Primer: 5′-CGCGGATCCGAATTAATACGACTCACTATAGG-3′	RACE	FirstChoice RLM-RACE Kit (Ambion)
DcAOX2b_404Fw: 5′-TCCTATACATACTGTCACCAAAGC-3′	RACE	Designed from *DcAOX2b*
P6 :5′-CGCGGAAGAAGGCACATGGCTGAATA-3′	RACE	Specific from the Lambda gt22a phage vector (Invitrogen)
DcAox2aR: 5′-GGAGCAGGAACATTTTCAATTG-3′	RACE	Designed from *DcAOX2a*
DAOX2b_402Rev: 5′-AGCTTTGGTGACAGTATGTATAGG-3′	RACE	Designed from *DcAOX2b*
DcAOX2a_30Fw: 5′-ATGAATCATCTGTTAGCCAAGTCTG-3′	Complete sequence	Designed from *DcAOX2a*
DcAOX2a_3UTRev: 5′-TTCAGAGATATATAGCTATGTGG-3′	Complete sequence	Designed from *DcAOX2a*
DAOX2b_40Fw: 5′-TGCATGCGTCCTTCCTTATTTTTC-3′	Complete sequence	Designed from *DcAOX2b*
DAOX2b_1188Rev: 5′-CGTCTGCTGTGATTTTCTGGAC-3′	Complete sequence	Designed from *DcAOX2b*

(Invitrogen, Karlsruhe, Germany) was generated as was previously described (Linke et al. 2003) and used for screening of full-length sequences. For amplification the vector-specific forward primer *P*6 (Table 1) was applied combined with the two gene-specific reverse primers: *DcAox2aR* for *DcAOX2a*, and *DAOX2b_402Rev* for *DcAOX2b* (Table 1). RACE-PCR products were amplified as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 2 min (35 cycles). Amplicons with 1100 bp for *DcAOX2a* gene and 950 bp for *DcAOX2b* gene were identified.

To isolate the complete gene sequences and the open reading frames (ORFs) of both *DcAOX2* genes, DNA and cDNA from an individual plant of cv. Rotin were used as templates. The cDNA was produced by RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) as described above. Two gene-specific primer sets (0.2 μM) were designed based on the 5′ and 3′ sequences isolated before (*DcAOX2a_30Fw* and *DcAOX2a_3UTRev* for the *DcAOX2a* and *DAOX2b_40Fw* and *DAOX2b_1188Rev* for the *DcAOX2b*) (Table 1). PCRs were performed using Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. PCR was carried out for 35 cycles each one consisting in 10 s at 98°C, 20 s at 52°C for *DcAOX2a* and 55°C for *DcAox2b* for primers annealing, and 2 min at 72°C.

Cloning and sequence analysis

PCR fragments were purified from agarose gels with GFX PCR DNA and Gel Band Purification Kit according to the manufacture protocol (GE Healthcare, Little Chalfont, England). They were separately cloned into a pGem® -T Easy vector (Promega, Madison, WI, USA). Plasmid DNA was extracted from putative recombinant clones (Birnboim and Doly 1979) and analyzed with the restriction enzymes *Eco*RI, *HpyF3I*, *AluI* and *Bsp143I* (Fermentas, Ontario, Canada). Clones showing different restriction patterns were completely sequenced (Macrogen company: www.macrogen.com) in the directions of sense and antisense strands using the primers T7 and SP6 (Promega, Madison, WI, USA). Sequence homology was explored in the NCBI data basis using BLAST algorithm (Karlin and Altschul 1993).

DNA and cDNA sequencing data were analyzed with SeqMan from LASERGENE 7 software (DNASTAR, Madison, WI, USA), in order to make the pairing of the 3′-end and 5′-end of each gene with each initial *AOX* partial sequence. The *DcAOX2a* and *DcAOX2b* sequences were translated to protein using the EditSeq from LASERGENE 7 software (DNASTAR, Madison, WI, USA). Phylogenetic studies included AOX sequences available in NCBI databases. The sequences were aligned with ClustalW Multiple alignment in BIOEDIT software (Hall 1999) and in MegAlign from LASERGENE 7. The alignments were bootstrapped with 1000 replicates by the Neighbor-Joining method using the MEGA 3.1 software. MITOPROT software (Claros and Vincens 1996) was used to predict the mitochondrial targeting sequence cleavage site.

Results

Expression of carrot *AOX* gene

Organ-specific expression

Differential expression between *DcAOX1* and *DcAOX2* genes was analyzed in total florets as well as in dissected stages consisting of juvenile stages during organ formation (S5–8) and of adult flowers shortly before anthesis (floral stages S11/12). Floral stages were assigned as previously described (Linke et al. 1999, 2003). Under stringent PCR conditions (19–22 cycle numbers) transcript abundance of *DcAOX1* genes in total flowers was generally higher than for *DcAOX2* (Fig. 1A). This is especially because of a rather low expression of *DcAOX2* genes in juvenile florets (Fig. 1B). Stable expression was observed between developmental stages of florets for *DcAOX1* genes, when a general primer pair for both *DcAOX1* genes has been used (Fig. 1B, left column). Abundant accumulation of *DcAOX1* transcripts at different stages of flower development was also shown by ISH (Fig. 1C). However, applying non-stringent PCR conditions (40 cycles) for the rather low expressed *DcAOX1b* gene, differential transcript accumulation became visible showing a clearly higher expression for this gene in juvenile florets (Fig. 1D). On the contrary, expression of *DcAOX2* genes was low in juvenile flowers and increased transcription was observed in nearly mature flower stages shortly before anthesis (Fig. 1B, right column). In the stalked bases of single umbelets only very low hybridization signals of *AOX* expression were detected and signals were nearly absent from leaf-like structures (involucels) that covered the compound carrot inflorescence (Fig. 1C). The hybridization assay was designed for *DcAOX1* expression, but because of the similarity of *DcAOX1* and *DcAOX2* sequences and the necessity of working with longer sequence information in ISH, an overlapping of *AOX1* and *AOX2* signals cannot be excluded.

Fig. 2 shows expression patterns of *DcAOX1a*, *DcAOX1b*, *DcAOX2a* and *DcAOX2b* in roots and leaves of carrot plants. In both organs, all *AOX* genes revealed expression signals. Differential expression was observed between paralogous genes in all three biological parallels. In general, the transcript abundance of *DcAOX1a*

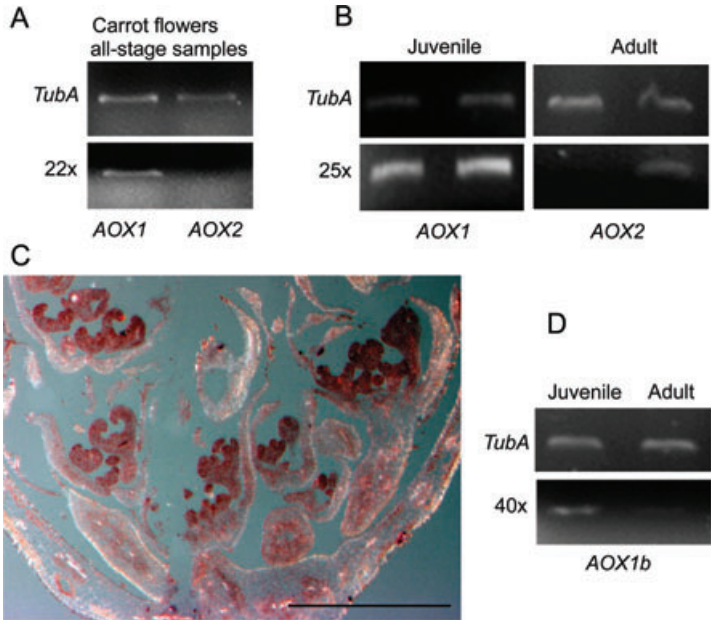

Fig. 1. Expression of *AOX* genes in carrot flowers. Transcript accumulation was analyzed by RT-PCR using the *Tubulin* gene (*TubA*) for normalization. (A) Discrimination between *DcAOX1* and *DcAOX2* expression in total florets. (B) Expression in juvenile and adult floral stages was analyzed by using primers discriminating between *DcAOX1* (left) and *DcAOX2* (right), respectively. (C) Tissue distribution in young carrot flowers during organ initiation shown by ISH of an *AOX1* probe indicates abundant expression in carrot inflorescences. Bar, 0.5 mm. (D) Expression in juvenile and adult floral stages was analyzed by using primers discriminating between *DcAOX1b*. Amplification products of *AOX* genes were studied after different cycle numbers (22, 25 (not shown) and 40× for *DcAOX1b*). Differential expression between *DcAOX1* and *DcAOX2* genes was observed in juvenile flowers that represented early stages of floral organ formation.

and *DcAOX2a* genes was higher in leaves than in roots, while *DcAOX2b* showed identical expression between both organs in two of the three biological repetitions.

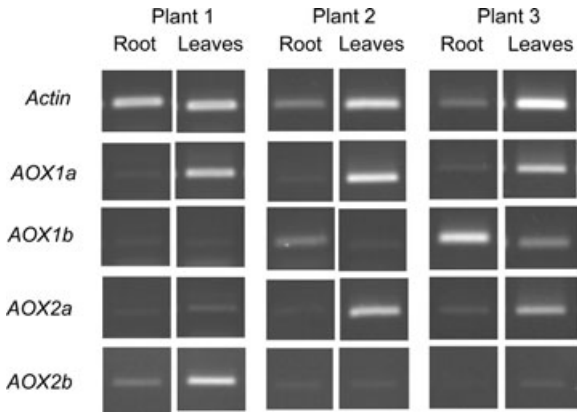

Fig. 2. Expression of *AOX* genes in carrot root and leaves. Transcript accumulation was analyzed by RT-PCR using the *Actin1* gene for normalization. Discrimination between *DcAOX1a, DcAOX1b, DcAOX2a* and *DcAOX2b* expression in three different in vitro plants was performed. Differential expression among genes and differences of gene expression between plants can be observed. The RT-PCR-products of root and leaves of all *AOX* genes were run together in the same electrophoresis.

Transcript abundance of *DcAOX1b* was higher in roots than in leaves in two biological repetitions.

De novo differentiation and tissue-specific expression

In order to focus on the dynamics of gene expression during development and growth, a primary culture experimental approach was chosen (Fig. 3A). *AOX* expression was studied after inoculation of differentiated secondary root phloem explants in a cytokinin-containing nutrient solution that induces tissue redifferentiation and *callus* growth.

An increase in the expression of *AOX* genes could be observed already after 36 h at the beginning of the lag-phase of growth induction. Typically, at this stage individual cells have been induced to enter into the cell cycle and first cycles are completed (Gartenbach-Scharrer et al. 1990, Arnholdt-Schmitt 1999). *DcAOX1a* was most responsive showing a clear up-regulation of expression with a peak after three days, still a high level of expression at the end of the lag-phase (day 6) and a decline at day 14. The same expression profile but at a lower abundance was obtained for *DcAOX2a*. After 14 days at linear cell division growth, the expression level of both, *DcAOX1a* and *DcAOX2a*,

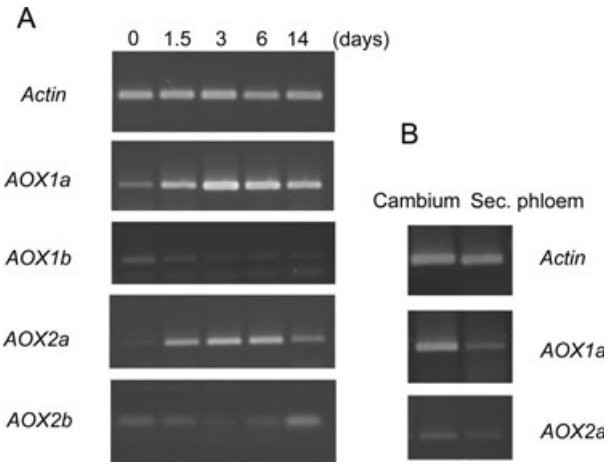

Fig. 3. Expression of *AOX* genes in carrot. Transcript accumulation was analyzed by RT-PCR using the *Actin1* gene for normalization. (A) Primary cultures from the secondary phloem of carrot roots. The explants were collected at the inoculation moment in the culture medium (0) and 1.5, 3, 6 and 14 days after inoculation. Discrimination between *DcAOX1a, DcAOX1b, DcAOX2a*, and *DcAOX2b* gene expression was performed. (B) Discrimination between *DcAOX1a* and *DcAOX2a* expression in the meristematic tissues cambium and secondary phloem.

was still higher than in the original quiescent tissue. Both gene subfamilies, *AOX1* and *AOX2* demonstrated differential expression of the extended genes. Expression of *DcAOX1b* and *DcAOX2b* remained overall low, and did not show specific patterns.

Because of the significance that *DcAOX1a* and *DcAOX2a* demonstrated during growth induction, the expression of these genes was analyzed in vivo in the meristem of tap roots, the cambium and for comparison in the adjacent secondary phloem, collected from root slices of individual carrot plants. Cambium cells showed clearly higher expression than the secondary phloem for both genes. *DcAOX1a* presented again the highest transcript accumulation (Fig. 3B).

Characterization of the full-length sequences of both *DcAOX2* genes

In search for differences between both extended genes of the *DcAOX2* sub family, two full-length cDNA sequences were isolated from an individual plant of *D. carota* cv. Rotin. Fig. 4 shows the nucleotide sequences and the two putative DcAOX2 proteins, *DcAOX2a* (*Acc. No* EU286575) and *DcAOX2b* (*Acc. No* EU286576).

The ORF length of both *DcAOX2* genes is similar. However, the *DcAOX2a* gene sequence is slightly longer containing a continuous ORF of 1014 bp that encodes a putative polypeptide consisting of 338 amino acid residues, whereas the *DcAOX2b* gene contains a continuous ORF of 957 bp encoding a polypeptide of 319 amino acids. The ATG in the beginning of the ORF of both genes is the correct start of translation, because it is the first start codon resulting in an open reading frame, and stop codons are present in all three reading frames of the transcript before this ATG. Fig. 4 demonstrates the conserved sites for intron positions. Both *DcAOX2* gene sequences indicate the expected genome organisation of four exons interrupted by three introns (see also Cardoso et al. 2009). The sizes of exons 2, 3 and 4 are the same in both *DcAOX2* genes with, respectively, 129 bp, 489 bp and 57 bp. However, the overall length difference observed for the whole gene sequences is exclusively because of exon1 in the N-terminal region. Exon1 has a size of 339 bp in *DcAOX2a* and of 282 bp in *DcAOX2b*.

As can be seen in the alignment of the deduced amino acid sequences of DcAOX2a and DcAOX2b and other AOX2 sequences available or identified through expressed sequence tags (ESTs) (Fig. 5) the N-terminal regions (that comprises the exon 1) between the AOX2 sequences analyzed are highly different. All sequences are complete, with exception of the sequences of *Centaurea maculosa* AOX2a and AOX2b where a small part from the N-terminal region is missing (Fig. 5). The predicted length of the cleavage site of the mitochondrial targeting sequence from the beginning of the protein contains 35 amino acids for DcAOX2a and 21 amino acids for DcAOX2b. All proteins contain the two conserved cysteines (boxed in Fig. 5).

In silico analysis allowed the identification of two additional AOX2 sequences for the asterids *C. maculosa* and *Triphysaria pussilla* in 'contigs' of different ESTs available in the database. A phylogenetic tree was constructed by the Neighbor-Joining method with the AOX2 protein sequences also used in the alignment, and two additional sequences used as outgroup (AOX from the fungus *Neurospora crassa* and a sequence of AOX1a from *A. thaliana*) (Fig. 6). For the construction of the phylogenetic tree, a small N-terminal part of 44 amino acids for DcAOX2a and 37 amino acids from DcAOX2b was removed after the alignment since it was missing in *C. maculosa* AOX2 sequences (Fig. 5).

In all three asterids two genes have been classified as belonging to sub family AOX2. The AOX2a and AOX2b sequences exhibited higher similarity between each other in each of these plant species, as can be seen in Fig. 6. *T. pussilla* demonstrated the highest homology (84.4%) for the deduced polypeptides of AOX2a and AOX2b. In contrast, AOX2a of the rosids (order fabales) *Glycine max, Vigna unguiculata* and *Lotus japonica* are closer to each other than to the corresponding AOX2b sequences, which form a distinguished group (Fig. 6). The plant species that show a single AOX2 sequence (*V. vinifera, Cucumis sativus* and *A. thaliana*) are included

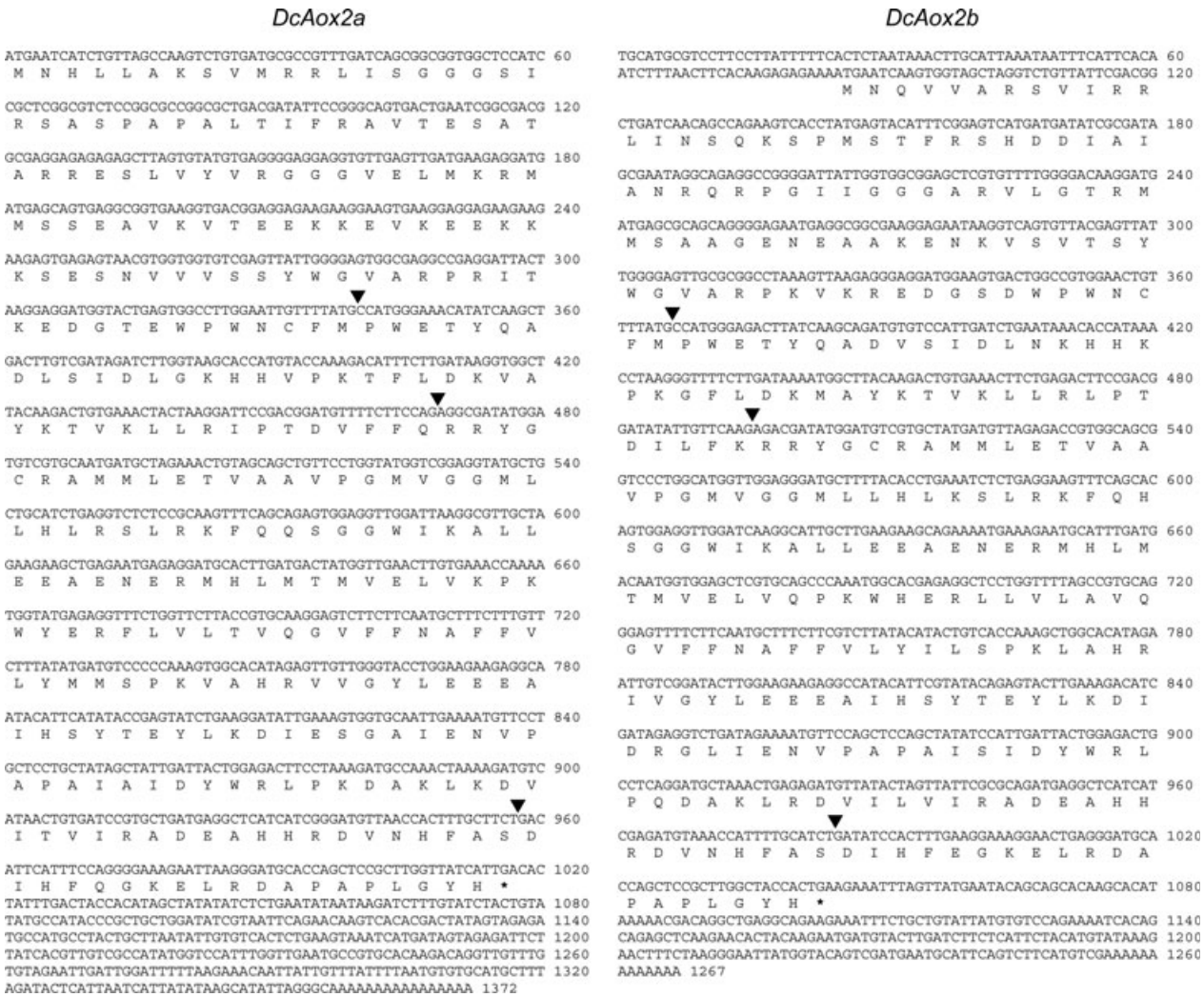

Fig. 4. Nucleotide and deduced amino acid sequences of two cDNAs encoding *D. carota* AOX, *DcAOX2a* (accession number EU286575) and *DcAOX2b* (accession number EU286576). The sites of introns are indicated by filled triangles, and * indicates stop codons.

in a group together with AOX2a and AOX2b from *D. carota, C. maculosa* and *T. pussilla* (Fig. 6).

Discussion

Our results present evidence for the expression of all of the four carrot *AOX* genes. They were expressed in all studied organs and tissues (roots, leaves, flowers, root cambium and secondary phloem, as well as in primary cultures induced to *callus* growth) and demonstrate differential expression in relation to organs, tissues, growth and development. *DcAOX1a* played the most important role at transcript level in various organs or tissues and responses on changing environmental conditions that need acclimation. On the contrary, *DcAOX1b* shows low expression and seems to confirm its specific significance in flowers, which is in agreement with observations in other plant species (Clifton et al. 2006; for review see Polidoros et al. 2009). However, the discussion of the results will not focus on similar responses described for genes from other species. Expression patterns of *AOX* genes must be considered in a species-specific manner, since transcript profiles of orthologous genes will not be a sufficient measure to group the functional importance of *AOX* genes (Thirkettle-Watts et al. 2003). Studies of gene orthology and gene ontology demand more exhaustive and systematic analyses. This view is getting confirmation through current knowledge on a high number of polymorphisms in *AOX* gene sequences in naturally growing plants and breeding lines. Individual genotypes and groups of genotypes can be distinguished by

polymorphic *AOX* gene sequences even within the same species (Holtzapffel et al. 2003, Cardoso et al. 2009, Costa et al. 2009b, Ferreira et al. 2009, Polidoros et al. 2009, Macedo et al. 2009). Thus, in future investigations the comparison of expression patterns and functionality should consider genetic differences within the *AOX* gene sequence under study. Further, it is known from a high number of studies with transgenic plants that the genomic background of plants can be expected to interfere strongly with gene expression patterns.

The expression data uncover two typical patterns for carrot *AOX* genes. At first, a close link between *DcAOX1a* and *DcAOX2a* expression was discovered. Both genes have been induced under the same conditions, although different transcript levels have been outlined. *DcAOX1a* shows typically a higher transcript accumulation. Secondly, both genes in each sub-family group, *DcAOX1a* and *DcAOX1b* and *DcAOX2a* and *DcAOX2b*, are differentially expressed among each other. Typically, *DcAOX1b* and *DcAOX2b* are less responsive.

AOX1 and *AOX2* genes can be discriminated by conserved nucleotide positions near important functional sites, such as the conserved cysteine in position CysI and the di-iron-binding sites. These sites have been recently highlighted by Costa et al. (2009a) and Frederico et al. (2009) (see arrows in Fig. 5). The meaning of these sites and their conservation is, though still obscure. However, all carrot genes contain both conserved cysteines, di-iron-binding sites and the currently highlighted conserved sites to distinguish *AOX1* from *AOX2* genes (Fig. 5). Notably, *Lotus japonicus* is exceptional in that it does not show the methionine (M) two positions downstream of CysII, but contains a serine (S) in this position. Differential or co-regulation of genes can be as a result of regulative elements outside gene sequences or because of within-gene sequence variations or similarities. Promoter sequences of *AOX* genes have been studied across species in soybean and *A. thaliana* (Thirkettle-Watts et al. 2003, Ho et al. 2007) and have been highlighted in a recent paper (Polidoros et al. 2009). It was concluded that promoter motifs will not be sufficient to explain common gene regulation. Clifton et al. (2005) suggested that the hierarchical order of common motifs in gene-upstream sequences can be important for similar responses. Recently, the importance of within-gene variability is better understood and target of intensive studies related to human diseases (e.g. Zacharova et al. 2005). Research on plant *AOX* gene sequences lacks systematic studies on sequence variations in paralogous genes of individuals and of different genotypes from the same species (see in Cardoso et al. 2009, Costa et al. 2009b, Ferreira et al. 2009, Frederico et al. 2009, Macedo et al. 2009). A comparison of complete *DcAOX1a* and *DcAOX2a* sequences can be expected to help in future approaches to reveal important within-gene motifs for co-regulation.

Contrarily, both *AOX2* sub-family member genes of *D. carota*, *DcAOX2a* and *DcAOX2b*, clearly indicated differential regulation patterns, but high similarity of their protein-coding sequences. Surprisingly, in the clade of asterids both of the deduced proteins of AOX2 show high similarity to each other in all three species (Fig. 6), whereas AOX2a from the solids *V. unguiculata*, *G. max* and *L. japonicus* form a distinguished group for AOX2b. The cause for high sequence similarities within the same species might originate from duplication events during evolution (Moore and Purugganan 2005). However, a tandem-linked duplication is unlikely, because of the differential regulation and obvious neofunctionalization (Lynch et al. 2001). Recently, we have mapped *DcAOX2a* and *DcAOX2b* and found that the two genes mapped to separate linkage groups (unpublished, see also Cardoso et al. 2009). Both observations point to independent regulation and function of both genes.

Sequence comparison of the complete protein-coding region confirmed the similarity in exon 2 and exon 3, but identified clear differences in exon 1 between both member genes of the *AOX2* sub family. The most pronounced difference was because of the deviating length of the cleavage sites of the mitochondrial targeting sequence between DcAOX2a and DcAOX2b. The meaning of this difference in the N-terminal region for the regulation of expression activities is unknown. Finnegan et al. (1997) refer that a lack of homology in mitochondrial targeting signals is common and typical of proteins requiring N-terminal signals for mitochondrial import. However, between phylogenetically very close species, the AOX orthologous proteins should be expected to present high identity in the N-terminus (Costa et al. 2004). Differences in the predicted length of the mitochondrial targeting peptide in AOX2 were reported for different plant species: for example 57 amino acids for *G. max* AOX2a, 51 amino acids for *G. max* AOX2b, 55 amino acids for *V. unguiculata* AOX2a and 50 amino acids for *V. unguiculata* AOX2b. In DcAOX2a and DcAOX2b proteins the predicted length of the mitochondrial targeting sequence cleavage site is smaller and displayed 35 and 21 amino acids, respectively.

Both carrot *AOX2* genes exhibited similar ORF lengths and the same exon–intron structure consisting of four exons and three introns. This confirms the conserved structure (Considine et al. 2002), typically found for *AOX* genes. This conserved structure was reported for the *AOX1a, AOX1c* and *AOX1d* genes of *Oryza sativa*

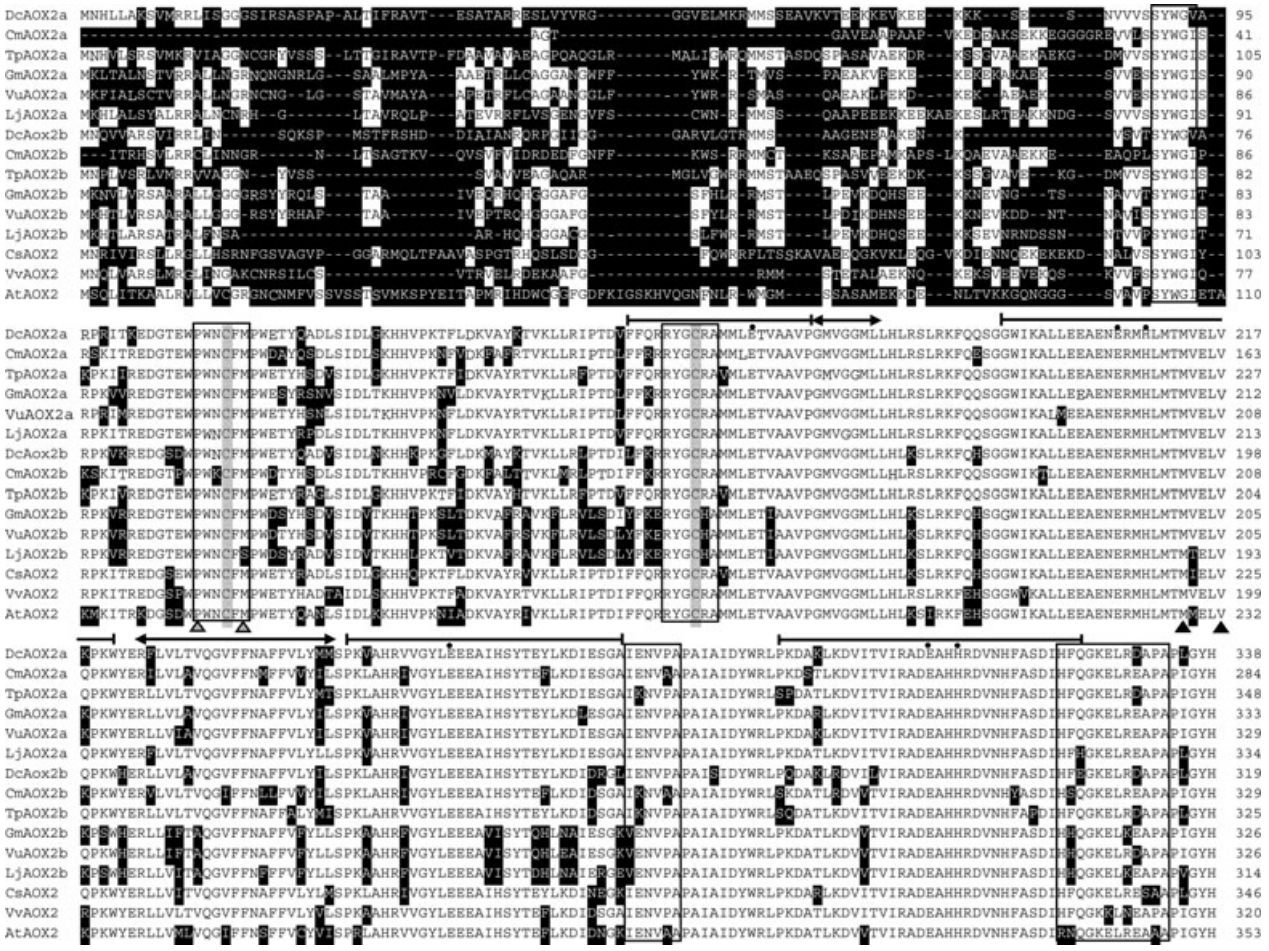

Fig. 5. Multiple alignment of the deduced amino acid complete sequences of *DcAOX2a* and *DcAOX2b* and 13 previously reported AOX2 proteins. The alignment was performed using the ClustalW method of LASERGENE 7 software. The sites of two conserved cysteins (CysI and CysII) that are involved in dimerization of the AOX protein by S–S bond formation (Umbach and Siedow 1993) are indicated in gray boxes. Gray arrows indicate the position of a conserved prolin (P) and a conserved methionine (M) in AOX2 sub family identified by Costa et al. (2009a). Black arrows indicate the positions of conserved methionine (M) and valine (V) in AOX2 sub family identified by Frederico et al. (2009). Helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown with overlines. E (glutamate) and H (histidine) amino acids residues involved in the iron-binding are indicated in filled circles. Possible membrane-binding domains (Andersson and Nordlund 1999, Berthold et al. 2000) are shown by two-headed arrows above the amino acid sequences. In black boxes are the structural elements proposed to influence AOX regulatory behavior by Crichton et al. (2005). Accession numbers to published sequences in the GenBank are as follows: DcAOX2a (EU286575), CmAOX2a (EH726462, EH727324, EH746720), TpAOX2a (EY182248, EY182249), GmAOX2a (U87906), VuAOX2a (AJ319899), LjAOX2a (AP007304), DcAOX2b (EU286576), CmAOX2b (EH723572, EH741730), TpAOX2b (EY177728, EY177727), GmAOX2b (U87907), VuAOX2b (AJ421015), LjAOX2b (AP007304), CsAOX2 (AAP33163), VvAOX2 (EU523224), AtAOX2 (AB003176).

(Ito et al. 1997, Saika et al. 2002), in *AOX1a, AOX1b* and *AOX1c* genes of *A. thaliana* (Saisho et al. 1997), in *AOX1, AOX2a* and *AOX2b* genes of *V. unguiculata* (*Acc. No* DQ100440, *Acc. No* EF187463 and *Acc. No* DQ100439) and in all three identified genes of *G. max* (*AOX1, AOX2a* and *AOX2b*) (Whelan et al. 1993, Finnegan et al. 1997, Thirkettle-Watts et al. 2003). Notable exceptions of this structure have been evolved by intron loss or gain. In *AOX2* from *A. thaliana* (Saisho et al. 2001) a gain of an intron occurred. The gene consists of five exons and four introns. *A. thaliana AOX1d* (Considine et al. 2002) and *O. sativa AOX1b* (Ito et al. 1997) are the two known examples that show a loss of intron 2. More information about intron loss or gain can be consulted in Polidoros et al. (2009). Interestingly, a pattern in the size of exons of the *AOX* genes can be observed. Exons 2, 3 and 4 of *AOX2a* and *AOX2b* genes of *D. carota* and other *AOX2* genes available in the NCBI database (*Cucumis sativus AOX2 Acc. No* AY258276; *V. unguiculata AOX2a Acc. No EF1874663; G. max AOX2a*

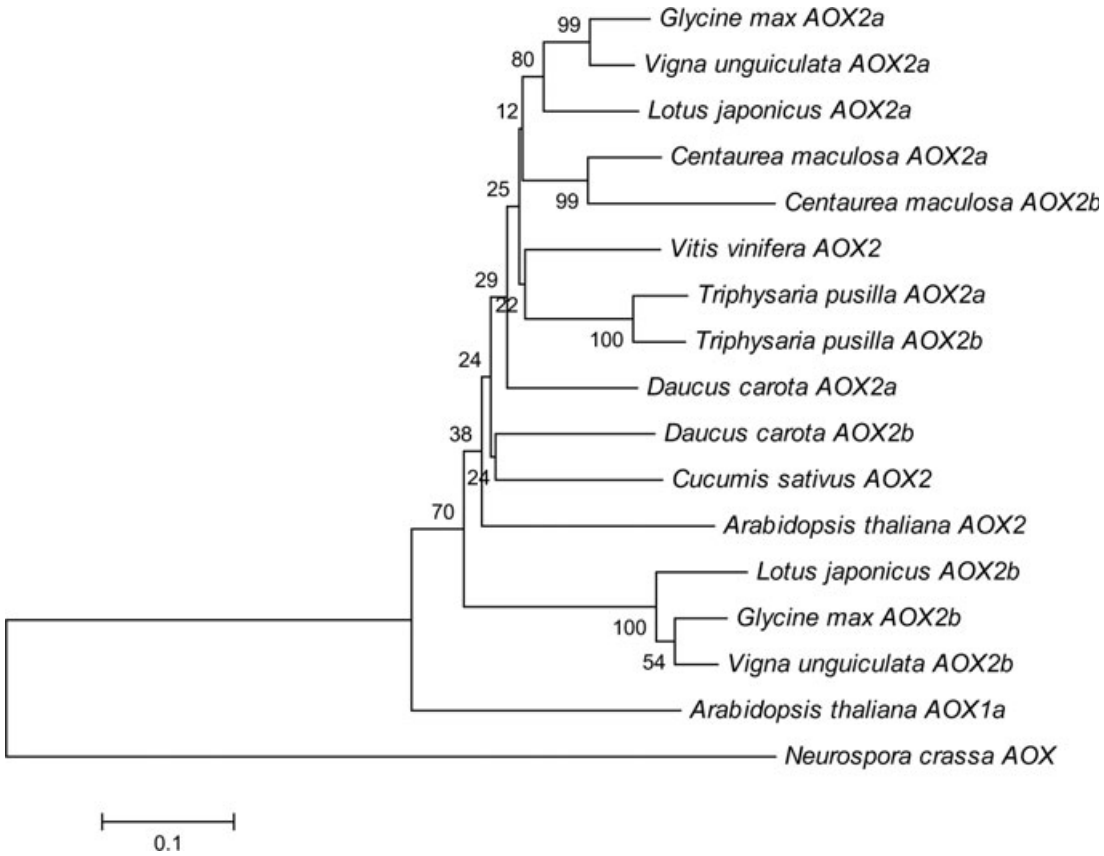

Fig. 6. Phylogenetic tree describing the relationship among AOX2 proteins from plants, including the two AOX2 sequences of *D. carota*. The alignments were bootstrapped with 1000 replicates by the Neigbor-Joining method using the MEGA 3.1 software. The fungus *Neurospora crassa* and *Arabidopsis thaliana* AOX1a were used as outgroup. The scale bar indicates the relative amount of change along branches. Accession numbers to published sequences in the GenBank are as follows: *Daucus carota* AOX2a (EU286575), *Daucus carota* AOX2b (EU286576), *Centaurea maculosa* AOX2a (EH726462, EH727324, EH746720), *Centaurea maculosa* AOX2b (EH723572, EH741730), *Triphysaria pusilla* AOX2a (EY182248, EY182249), *Triphysaria pusilla* AOX2b (EY177728, EY177727), *Glycine max* AOX2a (U87906), *Glycine max* AOX2b (U87907), *Vigna unguiculta* AOX2a (AJ319899), *Vigna unguiculata* AOX2b (AJ421015), *Lotus japonicus* AOX2a (AP007304), *Lotus japonicus* AOX2b (AP007304), *Cucumis sativus* AOX2 (AAP33163), *Vitis vinifera* AOX2 (EU523224), *Arabidopsis thaliana* AOX2 (AB003176).

and *AOX2b Acc. No AY303971)* count to, respectively, 129 bp, 489 bp and 57 bp. A newly described *AOX2* gene from olive shows the same size of 57 bp in exon 4 (Macedo et al. 2009). Exon 1 is the only exon sequence that presents variation in its size, in a typical four exon structure *AOX* gene: 363 bp in the *AOX2* gene of *C. sativus*, 312 bp in the *AOX2a* of *V. unguiculata*, 304 and 303 bp in the *AOX2a* and *AOX2b* of *G.m*ax and 339 and 366 bp in *AOX2a and AOX2b of D. carota.* In case of *AOX1d* of *A. thaliana* and *AOX1b* of *O. sativa,* where a loss of intron 2 took place, exon 2 has the size of 618 bp that corresponds to the sum of exon 2 and exon 3 of a typical four exon structure gene (129 and 489 bp). *AOX2* of *A. thaliana* (*Acc. No* AB003176) contains one extra intron in the upstream region (Saisho et al. 2001) that leads to the existence of two smaller exons (with 193 and 191 bp), but the other three downstream exons have the pattern sizes already referred (129, 489 and 57 bp).

Within-genomic DNA sequence differences can be suspected to affect diverse gene regulation mechanisms, such as alternative splicing, transcription binding sites, regulation through small RNAs or chromosomal organisation (see in Polidoros et al. 2009). Recent studies on polymorphisms in *AOX* genes of *Hypericum perforatum* L. (Ferreira et al. 2009), *Olea europaea* L. (Macedo et al. 2009) and *V. vinifera* L. (Costa et al. 2009b) are providing evidence of intron polymorphisms in *AOX* genes. Introns are generally known to provide a rich source for discriminatory SNPs or insertion/deletion polymorphisms between genotypes that can be useful in marker-assisted plant breeding (Arnholdt-Schmitt et al. 2006). Also, screening for polymorphic sequences in the carrot *AOX2a* gene between breeding lines and cultivars

revealed high variability in intron 3 of this gene (Cardoso et al. 2009). Variable 3′UTR-length induced differential polyadenylation and polymorphic single nucleotide sites in the 3′UTR related to miRNA target sites may also be a source for differential gene regulation. Currently, such types of polymorphisms have also been observed in *AOX* genes (Polidoros et al. 2005, Macedo et al. 2009). Thus, we speculate that exon 1-derived sequence variation between carrot *AOX2a* and *AOX2b* genes and differences in 5′ upstream sequences will not be sufficient to explain the observed clear differences in the expression regulation of both genes. We suggest that important regulative effects may also come from sequence variations within the three putative introns and the 3′UTR of these genes.

Conclusion

Results about transcript abundances in diverse carrot tissues, organs and a primary culture indicate a differential expression for all four identified *AOX* genes from *D. carota*. Similar expression profiles for *DcAOX1a* and *DcAOX2a* in the studied systems point to co-regulation of these two genes, although the extent of transcript accumulation differed between them. An independent and functional role of all four *AOX* genes can be suggested rather than merely the evolution of pseudogenes or non-functional gene duplication in each sub-family. The meaning of the length variation in the mitochondrial targeting sequence cleavage sites in exon 1 for differential regulation between *DcAOX2a* and *DcAOX2b* remains yet unclear and needs clarification. The high similarity between both ORFs despite a clearly differential regulation demands searching for important regulative cis-elements and/or within-gene differences at genomic DNA level.

As a forthcoming perspective, it can be concluded from the availability of two differentially regulated genes in each of the two *AOX* subfamilies that carrot has good potential as a model to advance current insights in the functionality and evolutionary importance of both subfamilies. A future transgenic approach for functional genomics and association studies can be expected to bring knowledge a significant step forward.

Acknowledgements – The authors are thankful to the European Commission for providing the Marie Curie Chair to Birgit Arnholdt-Schmitt. We are also grateful to Fundação para a Ciência e a Tecnologia for providing fellowships to Maria Doroteia Campos (GRICES/FCT:SFRH/BI/15991/2006), to Hélia Guerra Cardoso (FCT:SFRH/BPD/27016/2006) and to António Miguel Frederico (FCT:SFRH/BI/33048/2007). Collaboration with Germany was supported by a bilateral cooperation contract GRICES/FCT and DAAD.

References

Andersson ME, Nordlund P (1999) A revised model of the active site of alternative oxidase. FEBS Lett 449: 17–22

Arnholdt-Schmitt B (1995) Physiological aspects of genome variability in tissue culture. II. Growth phase-dependent quantitative variability of repetitive *Bst*NI fragments of primary cultures of *Daucus carota* L. Theor Appl Genet 91: 816–823

Arnholdt-Schmitt B (1999) On the physiology of yield production in carrots–Implications for breeding towards nutrient efficiency. Gartenbauwissenschaft 64: 26–32

Arnholdt-Schmitt B (2000) RAPD analysis: a method to investigate aspects of the reproductive biology of *Hypericum perforatum* L. Theor Appl Genet 100: 906–911

Arnholdt-Schmitt B, Costa JH, Fernandes de Melo D (2006) AOX–A functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11: 281–287

Arnholdt-Schmitt B, Holzapfel B, Schillinger A, Neumann KH (1991) Variable methylation and differential replication of genomic DNA in cultured carrot root explants during growth induction as influenced bt hormonal treatments. Theor Appl Genet 82: 283–288

Berthold DA, Andersson ME, Nordlund P (2000) New insight into the structure and function of the alternative oxidase. Biochim Biophys Acta 1460: 241–254

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513–1523

Capra JA, Singh M (2007) Predicting functionally important residues from sequence conservation. Bioinformatics 23: 1875–1882

Cardoso HG, Campos MD, Costa AR, Campos MC, Nothnagel T, Arnholdt-Schmitt B (2009) Carrot alternative oxidase gene *AOX2a* demonstrates allelic and genotypic polymorphisms in intron 3. Physiol Plant 137: 592–608

Claros MG, Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241: 779–786

Clifton R, Millar AH, Whelan J (2006) Alternative oxidases in Arabidopsis: a comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses. Biochim Biophys Acta 1757: 730–741

Clifton R, Lister R, Parker KL, Sappl PG, Elhafez D, Millar AH, Day DA, Whelan J (2005) Stress-induced co-expression of alternative respiratory chain

components in *Arabidopsis thaliana*. Plant Mol Biol 58: 193–212
Crichton PG, Affourtit C, Albury MS, Carré JE, Moore AL (2005) Constitutive activity of *Sauromatum guttatum* alternative oxidase in *Schizosaccharomyces pombe* implicates residues in addition to conserved cysteins in α-keto acid activation. FEBS Lett 579: 331–336
Considine M, Holtzapffel R, Day D, Whelan J, Millar A (2002) Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol 129: 949–953
Costa JH, Cardoso HC, Campos MD, Zavattieri A, Frederico AM, Fernandes de Melo D, Arnholdt-Schmitt B (2009a) *D. carota* L.–an old model for cell reprogramming gains new importance through a novel expansion pattern of *AOX* genes. Plant Physiol Biochem 47: 753–759
Costa JH, Fernandes de Melo D, Gouveia Z, Cardoso HG, Arnholdt-Schmitt B (2009b) The alternative oxidase family of *Vitis vinifera* reveals an attractive model to study the importance of genomic design. Physiol Plant 137: 553–565
Costa JH, Hasenfratz-Sauder MP, Pham-Thi AT, Lima MGS, Dizengremel P, Jolivet Y, Fernandes de Melo D (2004) Identification in *Vigna unguiculata* (L.) Walp. of two cDNAs encoding mitochondrial alternative oxidase orthologous to soybean alternative oxidase genes 2*a* and 2*b*. Plant Sci 167: 233–239
Ferreira A, Cardoso HG, Macedo ES, Breviario D, Arnholdt-Schmitt B (2009) Intron polymorphism pattern in *AOX1b* of wild St John's Wort (*Hypericum perforatum* L) allows discrimination between individual plants. Physiol Plant 137: 520–531
Finnegan PM, Whelan J, Millar AH, Zhang Q, Smith MK, Wiskish JT, Day DA (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. Plant Physiol 114: 455–466
Frederico AM, Zavattieri MA, Campos MD, Cardoso HG, McDonald AE, Arnholdt-Schmitt B (2009) The gymnosperm *Pinus pinea* contains both *AOX* gene subfamilies, *AOX1* and *AOX2*. Physiol Plant 137: 566–577
Gartenbach-Scharrer U, Habib S, Neumann KH (1990) Sequential synthesis of some proteins in cultured carrot explant (*Daucus carota*) cells during callus induction. Plant Cell Tissue Organ Cult 22: 27–35
Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98
Ho LHM, Giraud E, Lister R, Thirkettle-Watts D, Low J, Clifton R, Howell KA, Carrie C, Donald T, Whelan J (2007) Characterization of the regulatory and expression context of an alternative oxidase gene provides insights into cyanide-insensitive respiration during growth and development. Plant Physiol 143: 1519–1533
Holtzapffel RC, Castelli J, Finnegan PM, Millar AH, Whelan J, Day DA (2003) A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta 1606: 153–162
Ito Y, Saisho D, Nakazono M, Tsutsumi N, Hirai A (1997) Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature. Gene 203: 121–129
Karlin S, Altschul SF (1993). Applications and statistics for multiple high-scoring segments in molecular sequences. Proc Natl Acad Sci USA 90: 5873–5877
Linke B, Nothnagel T, Börner T (1999) Morphological characterization of modified flower morphology of three novel alloplasmic male sterile carrot sources. Plant Breed 118: 543–548
Linke B, Nothnagel T, Börner T (2003) Flower development in carrot CMS plants: mitochondria affect the expression of MADS box genes homologous to *GLOBOSA* and *DEFICIENS*. Plant J 34: 27–37
Lynch M, O'Hely M, Walsh B, Force A (2001) The probability of preservation of a newly arisen gene duplicate. Genetics 159: 1789–1804
Macedo ES, Cardoso HG, Hernández A, Peixe AA, Polidoros A, Ferreira A, Cordeiro A, Arnholdt-Schmitt B (2009) Physiological responses and gene diversity indicate olive alternative oxidase as a potential source for markers involved in efficient adventitious root induction. Physiol Plant 137: 532–552
Moore RC, Purugganan MD (2005) The evolutionary dynamics of plant duplicate genes. Curr Opin Plant Biol 8: 122–128
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissues cultures. Physiol Plant 15: 473–497
Neumann K (1966) Wurzelbildung und Nucleinsäuregehalt bei Phloem-Gewebekulturen der Karottenwurzel auf synthetischem Nährmedium verschiedener Hormonkombinationen. Phytohorm Organogen 38: 95–102
Polidoros AN, Mylona PV, Arnholdt-Schmitt B (2009) Aox gene structure, transcript variation and expression in plants. Physiol Plant 137: 342–354
Polidoros NA, Mylona PV, Pasentsis K, Scandalios JG, Tsaftaris AS (2005) The maize alternative oxidase 1a (*Aox1a*) gene is regulated by signals related to oxidase stress. Redox Rep 18: 71–78
Raghavan V (2006) Can carrot and Arabidopsis serve as model systems to study the molecular biology of somatic embryogenesis? Curr Sci 90: 1336–1343
Saika H, Ohtsu K, Hamanaka S, Nakazono M, Tsutsumi N, Hirai A (2002) *AOX1c*, a novel rice gene for alternative oxidase; comparison with rice *AOX1a* and *AOX1b*. Genes Genet Syst 77: 31–38
Saisho D, Nambara E, Naito S, Tsutsumi N, Hirai A, Nakazono M (1997) Characterization of the gene family

for alternative oxidase from *Arabidopsis thaliana*. Plant Mol Biol 35: 585–596
Saisho D, Nakazono M, Lee K-H, Tsutsumi N, Akita S, Hirai A (2001) The gene for alternative oxidase-2 (*AOX2*) from *Arabidopsis thaliana* consists of five exons unlike other *AOX* genes and is transcribed at an early stage during germination. Genes Genet Syst 76: 89–97
Schaffer S, Arnholdt-Schmitt B (2001) Characterization of genome variation in tissue cultures by RAPD fingerprinting–a methodological comment. Plant Biosyst 135: 115–120
Thirkettle-Watts D, McCabe TC, Clifton R, Moore C, Finnegan PM, Day D, Whelan J (2003) Analysis of the alternative oxidase promoters from soybean. Plant Physiol 133: 1158–1169
Umbach AL, Siedow JN (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol 103: 845–854
Whelan J, McIntosh L, Day DA (1993) Sequencing of a soybean alternative oxidase cDNA clone. Plant Physiol 103: 1481
Zacharova J, Chiasson J-L, Laakso M (2005) The common polymorphisms (single nucleotide polymorphism [SNP] + 45 and SNP + 276) of the adiponectin gene predict the conversion from impaired glucose tolerance to type 2 diabetes. Diabetes 54: 893–899
Zottini M, Formentin E, Scattolin M, Carimi F, Schiavo FL, Terzi M (2002) Nitric oxide affects plant mitochondrial functionality *in vivo*. FEBS Lett 515: 75–78

Edited by V. Hurry