UAlg FCT
UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DE ÉVORA

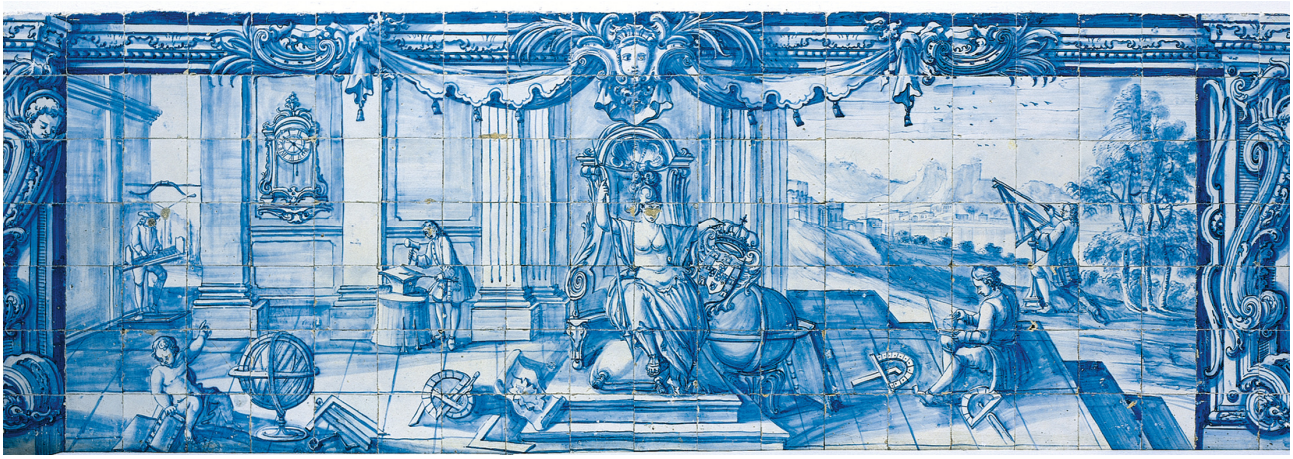
TRANSCRIPTION OF ALTERNATIVE OXIDASE (*AOX*) AND PLASTID TERMINAL OXIDASE (*PTOX*) DURING STRESS-REGULATED ROOT TISSUE GROWTH IN *Daucus carota* L. – AN APPROACH TO IDENTIFY FUNCTIONAL MARKER CANDIDATES FOR BREEDING ON CARROT YIELD STABILITY

Maria Doroteia Murteira Rico da Costa Campos

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Agrárias
Especialidade: Biotecnologia

ORIENTADORA: *Professora Doutora Birgit Arnholdt-Schmitt*
COORIENTADORA: *Doutora Hélia Guerra Cardoso*

ÉVORA, JULHO DE 2016



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

Thesis publications

This thesis includes manuscripts originating from part of results obtained in the experimental work referenced as:

Campos MD, Cardoso H, Linke B, Costa JH, Fernandes de Melo D, Justo L, Frederico AM, Arnholdt-Schmitt B (2009) Differential expression and co-regulation of carrot *AOX* genes (*Daucus carota*). Physiol Plant 137: 578–591

Campos MD, Frederico AM, Nothnagel T, Arnholdt-Schmitt B, Cardoso H (2015) Selection of suitable reference genes for reverse transcription quantitative real-time pcr studies on different experimental systems from carrot (*Daucus carota* L.). Sci Hortic 186: 115-123

Campos MD, Nogales A, Cardoso HG, Rajeev Kumar S, Nobre T, Sathishkumar R, Arnholdt-Schmitt B (2016) Stress-induced accumulation of *DcAOX1* and *DcAOX2a* transcripts coincides with critical time point for structural biomass prediction in carrot primary cultures (*Daucus carota* L.). Front Genet 7:1

Campos MD, Campos C, Cardoso HG, Simon PW, Oliveira M, Nogales A, Arnholdt-Schmitt B (2016) Isolation and characterization of plastid terminal oxidase gene from carrot and its relation to carotenoid accumulation. Plant Gene 5: 13–21

Campos MD, Cardoso HG, Nogales A, Campos C, Arnholdt-Schmitt B. Characterization of the plastid terminal oxidase gene in carrot - involvement in carotenoids accumulation during storage root development. Acta Hortic *in press*

Other publications were achieved in the frame of the work developed in this thesis, namely:

Cardoso H, Campos MD, Nothnagel T, Arnholdt-Schmitt B (2011) Polymorphisms in intron 1 of carrot *AOX2b* – an useful tool to develop a functional marker? Plant Genet Resour, 9:177-180

Nogales A, Noceda C, Ragonezi C, Cardoso HG, Campos MD, Frederico AM, Sircar D, Iyer R, Polidoros A, Peixe A, Arnholdt-Schmitt B (2015) Functional marker development from *AOX* genes requires deep phenotyping and individualized diagnosis. In: Gupta KJ, M.L., Neelwarne B (eds) Alternative respiratory pathways in higher plants. John Wiley & Sons, Inc, Oxford, pp 275-280

Nogales A, Nobre T, Cardoso HG, Muñoz-Sanhueza L, Valadas V, Campos MD, Arnholdt-Schmitt B (2016) Allelic variation on *DcAOX1* gene in carrot (*Daucus carota* L.): an interesting simple sequence repeat in a highly variable intron. Plant Gene 5: 49–55

Table of contents

Acknowledgements

During these last years of work, many people contributed to the successful completion of this thesis. Here, I would like to demonstrate my appreciation for their invaluable contribution.

First of all, I would like to thank my supervisors Professor Birgit Arnholdt-Schmitt and Doutora Hélia Cardoso. Professor Birgit gave me the opportunity of working with her research group, and the necessary impulse to pursue this doctoral project. For all support, knowledge and enthusiastic motivation through all this years, I am deeply grateful. Working with Doutora Hélia Cardoso was invaluable through all this time. My knowledge on molecular biology is completely attributed to her, and her contribution in the preparation and running of all the experimental work was huge. I am extremely grateful for her unreserved support during all the development of my work, and for her friendship.

I want to thank Fundação para a Ciência e a Tecnologia (FCT) for financial support (SFRH/BD/65354/2009).

I am very grateful to my good friends Elisete Macedo, Alexandre Ferreira, Carla Ragonezi and Rajeev Kumar for their help, share of knowledge and also for the funniest times that we spent together in the Lab.

Also, I deeply acknowledge my current colleagues in the Lab Tânia Nobre, Vera Valadas, Amaia Nogales and Isabel Velada for their great support and friendship. Tânia and Amaia collaboration in the manuscripts reviewing was also extremely helpful.

I want to thank very much to my mom Adelaide, my sister Catarina, my brother-in-law Gonçalo, my in-laws Domingas and Manuel, and also my dearest friends Susana, Cláudia and Ana Elisa for their support and encouragement during all this time.

And finally, I want to thank the love, patience, encouragement and unconditional support from my husband Carlos and from our children António, Teresa and Luísa.

In memoriam of my father

Estudos de transcrição da oxidase alternativa (*AOX*) e da oxidase terminal da plastoquinona (*PTOX*) na regulação do crescimento dos tecidos da raíz de cenoura (*Daucus carota* L.) sob condições de stresse - uma abordagem para identificação de candidatos a marcadores funcionais para o melhoramento da estabilidade de produção

Resumo

A presente tese explora a hipótese de utilização dos genes da oxidase alternativa (*AOX*) e da oxidase terminal da plastoquinona (*PTOX*) como genes-alvo para o desenvolvimento de marcadores funcionais (MF) para avaliar a performance do crescimento em cenoura, fator determinante da produtividade. Para avaliar se os referidos genes estão associados com o crescimento da cenoura procedeu-se ao seu isolamento e posterior análise dos seus perfis de transcrição em diversos sistemas biológicos. O sistema *in vitro* selecionado, denominado sistema de culturas primárias, permitiu avaliar alterações na quantidade de transcritos desses genes durante os processos de reprogramação celular e crescimento. Ao nível da planta foi também estudado o efeito do frio na expressão precoce dos genes *AOX.* Ambos os genes *DcAOX1* e *DcAOX2a* revelaram uma resposta rápida e um padrão semelhante após stresse (inoculação *in vitro* e resposta ao frio). Foi Igualmente verificado um incremento na expressão do gene *DcPTOX* durante a fase inicial do processo de reprogramação celular. Estudos de expressão dos genes *AOX* durante o desenvolvimento da raíz da cenoura revelaram que o gene *DcAOX2a* será potencialmente o gene mais envolvido neste processo. De modo a avaliar a hipótese de envolvimento do gene *DcPTOX* no crescimento da raíz, procederam-se a estudos de expressão ao nível do tecido meristemático. Todavia, para um mais completo entendimento da ligação entre *DcPTOX* e o crescimento secundário e/ou acumulação de carotenos, a expressão do gene *DcPTOX* foi também avaliada em raízes de cenoura durante o desenvolvimento, utilizando cultivares caracterizadas por distintos conteúdos de carotenos. Os resultados obtidos demonstraram a associação do gene *DcPTOX* a ambos os processos. O envolvimento da PTOX no crescimento adaptativo da raiz foi analisado com um ensaio que permitiu identificar, no tecido meristemático, uma resposta precoce do gene *DcPTOX* face a uma diminuição da temperatura. Adicionalmente, foi efetuada a seleção de genes de referência para uma análise precisa da expressão génica por RT-qPCR em diversos sistemas biológicos de cenoura, e a importância do seu estudo ao nível do sistema biológico foi realçada.

Os resultados desta tese são encorajadores para prosseguir os estudos de utilização dos genes *AOX* e *PTOX* como MF no melhoramento da performance do crescimento adaptativo em cenoura, fator determinante para a produtividade.

Palavras-chave: cenoura; oxidase alternativa; oxidase terminal da plastoquinona; marcador funcional; crescimento

Abstract

This thesis explores the hypothesis of using the alternative oxidase (*AOX*) and the plastid terminal oxidase (*PTOX*) as target genes for functional marker (FM) development for yield-determining growth performance in carrot. To understand if these genes are associated to growth, different *AOX* gene family members and the single *PTOX* gene were isolated, and their expression patterns evaluated in diverse carrot plant systems. An *in-vitro* primary culture system was selected to study *AOX* and *PTOX* transcript changes during cell reprogramming and growth performance. At plant level, a putative early response of *AOX* to chilling was also evaluated. In fact, both *DcAOX1* and *DcAOX2a* were early responsive and showed similar patterns under stress conditions (*in vitro* inoculation and chilling). A role for *DcPTOX* during earliest events of cell reprogramming was also suggested. Next, the expression profiles of *AOX* gene family members during carrot tap root development were investigated. *DcAOX2a* was identified as the most responsive gene to root development. In order to evaluate if DcPTOX is associated with carrot tap root growth performance, *DcPTOX* transcript levels were measured in the central root meristem. To further understand whether DcPTOX is associated with secondary growth and/or carotenoids accumulation, *DcPTOX* expression was also studied in developing carrot tap roots in cultivars with different carotenoids contents. The results indicated that *DcPTOX* associates to both carotenoid biosynthesis and secondary growth during storage root development. To obtain further insights into the involvement of PTOX on adaptive growth, the early effects of temperature decrease were explored in the root meristem, where a short-term early response in *DcPTOX* was found, probably associated with adaptive growth. Furthermore, a selection of the most suitable reference genes for accurate RT-qPCR analysis in several carrot experimental systems was performed and discussed.

The present research provides the necessary toolbox for continuing studies in carrot *AOX* and *PTOX* genes as promising resources for FM candidates in order to assist breeding on yield-determining adaptive growth performance.

Keywords: carrot; alternative oxidase; plastid terminal oxidase; functional marker; growth

List of abbreviations

ACT	Actin
AFP	Anti-freezing protein
AOX	Alternative oxidase
cDNA	Complementary DNA
CE	Chiling exposure
Cq	Quantification cycle
Ct	Cycle threshold
dpi	Days post inoculation
EF1α or *EF-1A*	Elongation factor 1 alpha
EST	Expressed sequence tag
ETC	Electron transport chain
FM	Functional marker
FW	Fresh weight
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Growth chamber
gDNA	Genomic DNA
GGPP	geranylgeranyl diphosphate
hpi	Hours post inoculation
hsp70	Heat shock protein 70
InDels	Insertion/deletions
LCYB	Lycopene β-cyclase
LCYE	Lycopene ε-cyclase
M	Expression stability
miRNA	Micro RNA
NCBI	National Center for Biotechnology Information
NF	Normalization factor
ORF	Open reading frame
PCS	Primary culture system
PDS	Phytoene desaturase
PQ	Plastoquinone
PSI, PSII	Photosystem I and II
PSY	Phytoene synthase
PTOX	Plastid terminal oxidase
QTL	Quantitative trait loci
ROS	Reactive oxygen species
rpL2	Ribosomal protein L2 encoding gene
RT-qPCR	Reverse transcription quantitative real time polymerase chain reaction
RT-sqPCR	Reverse transcription semi-quantitative polymerase chain reaction
SNPs	Single-nucleotide polymorphisms
SV	Stability value
TBA	Alpha-tubulin
TIF1	Transcriptional initiation factor
UBQ	Ubiquitin
UTR	Untranslated region
V	Pairwise variation
ZDS	ζ-carotene desaturase
18S, 25S, 5.8S rRNA	Ribosomal RNAs
ß-TUB	Beta-tubulin

CHAPTER 1 - INTRODUCTION

1.1. General aspects of carrot (*Daucus carota* L.) biology and production

Carrot (*Daucus carota* var. *sativus* L.) is the most widely cultivated member of the Apiaceae family, which comprises up to 3700 species across 450 genera distributed worldwide (Constance 1971; Pimenov and Leonov 1993). There are approximately 20 members in the genus *Daucus*, where cultivated carrot is included.

D. carota var. *sativus* L. is an outcrossing, insect-pollinated diploid (2n = 2x = 18) plant, with a genome size of approximately 480 Mb (Iorizzo et al. 2011). Carrot is a biennial plant, not flowering during the vegetative phase of its life cycle, when the storage root forms and grows (going from 60 to 150 days or more, depending on the environment and genotype). The vegetative phase of carrot life cycle is essential to successful crop production. Cool temperature is the primary stimulus that initiates carrot flowering, so plants exposed to cold weather in the field will therefore make the transition from vegetative phase and initiate flowering.

Carrot storage root is rich in pro-healthy antioxidants, both of lipophylic (carotenoids) and hydrophilic (phenolic compounds) characters. Root pigmentation depends on the relative proportion of different carotenoids in combination with anthocyanins (Surles et al. 2004). Orange and red-rooted carrots accumulate large amounts of carotenoids, mainly α- and β-carotene for the orange type and lycopene and β-carotene for the red type. Yellow-rooted carrots present low amounts of carotenoids, especially lutein and β-carotene. White-rooted carrots contain negligible amounts of carotenoids. Polyphenol substances, mostly anthocyanins, are typical for purple roots (Sun et al. 2009; Arscott and Tanumihardjo 2010).

Nowadays, carrot production represents, together with turnips, more than 37 million tons a year worldwide, according to 2013 data from the Food and Agriculture Organization of the United Nations (http://faostat3.fao.org). Since 1961 (http://faostat3.fao.org), carrot world production has been increasing and this is in part, attributed to an increased awareness of health benefits associated to carrot human consumption (Arscott and Tanumihardjo 2010).

1.2. Carrot genetics and breeding

Cultivated carrots have their origin in the Afghanistan region before the 900s and were initially yellow and purple (Mackevic 1929). When the domestication of carrot began, carrots were selected for storage root size and smoothness. From this center of domestication, carrots were grown as a root crop to the East and West with the incorporation of several characteristics which led to the development of contrasting phenotypes between those two

geographic regions. The Eastern carrot spread to central and north Asia and then to Japan. Red coloured carrots are typical of India and were also introduced to Japan (Laufer 1919; Shinohara 1984). In contrast, Western carrot type is characterized by its initial yellow and later orange root colour. The orange carrots were not commonly found until the 16th and early 17th centuries (Banga 1963) and its origin is unknown. Although it was suggested that the outcrossing of cultivated yellow and purple carrot with wild carrot may have contributed to the development of the now common orange carrot, this hypothesis has not been substantiated (Simon and Goldman 2007). Since the 19th century, orange-rooted carrots have spread from Europe to other continents and have become commercially attractive vegetable (Rubatzky et al. 1999). Genetic analysis to assess genetic diversity among cultivated carrot cultivars recently revealed a genetic subdivision between Western (European and American) and Eastern (Asian) accessions (Clotault et al. 2010; Baranski et al. 2011).

The development of a highly reliable cytoplasmic male sterility system was developed between 1950s and 70s and provided the foundation for carrots' hybrid development (Simon and Goldman 2007). With diverse germplasms available across the world, breeding programs have made great strides in carrot improvement. Carrot root size has been selected and improved for a variety of consumer markets (Simon and Goldman 2007). Another important breeding goal for carrot is reducing the tendency for early flowering or bolting. Under cold conditions, bolting results in changes in the composition of the storage root, making it tough, woody and unmarketable (Peterson and Simon 1986).

Carrot genetics has traditionally focused on traits important to growers and consumers. Disease resistance is a focus for modern carrot breeding and genetics programs. For instance, *Alternaria* leaf blight, caused by the fungus *Alternaria dauci* has been extensively investigated, and a recent program has included wild relatives to try to trace back the resistance to that disease (see http://www.bioversityinternational.org/uploads/tx_news/Report_of_a_ Working _Group_on_Umbellifer_Crops_Second_Meeting4.pdf). The root knot nematodes *Meloidogyne javanica* and *Meloidogyne incognita* can also have a high impact on carrot production, and for this reason carrot resistance to these pathogens has been extensively studied, and a single gene has been identified conferring resistance to both nematodes (Boiteux et al. 2004). Moreover, two genes have been identified for carrots' resistance to the nematode *Meloidogyne hapla* (Wang and Goldman 1996). In addition to breeding programs focused on pathogens resistance and temperature tolerance, seed companies are also trying to fulfill market demands for new and different products, including various carrot sizes, shapes, thickness and flavors.

As previously referred, the content of antioxidants in carrot storage root is a trait that has gained importance due to the link with human health. Important loci regarding carotenoid accumulation have been identified in carrot, which regulate the accumulation of carotenoid pigments throughout the carrot root (Santos and Simon 2002; Fernandes Santos et al. 2005; Just et al. 2009). In the carotenoid biosynthetic pathway (Fig. 1), the coloured carotenoids are synthesized within plastids from phytoene, a non-coloured precursor that results from two geranylgeranyl diphosphate (GGPP) molecules, catalysed by phytoene synthase (PSY). Desaturation of phytoene by the sequential activity of the enzymes phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) (Carol and Kuntz 2001; Simkin et al. 2008) results in the production of lycopene, a substrate for the formation of both *α*- and *β*-carotene (Fig. 1).

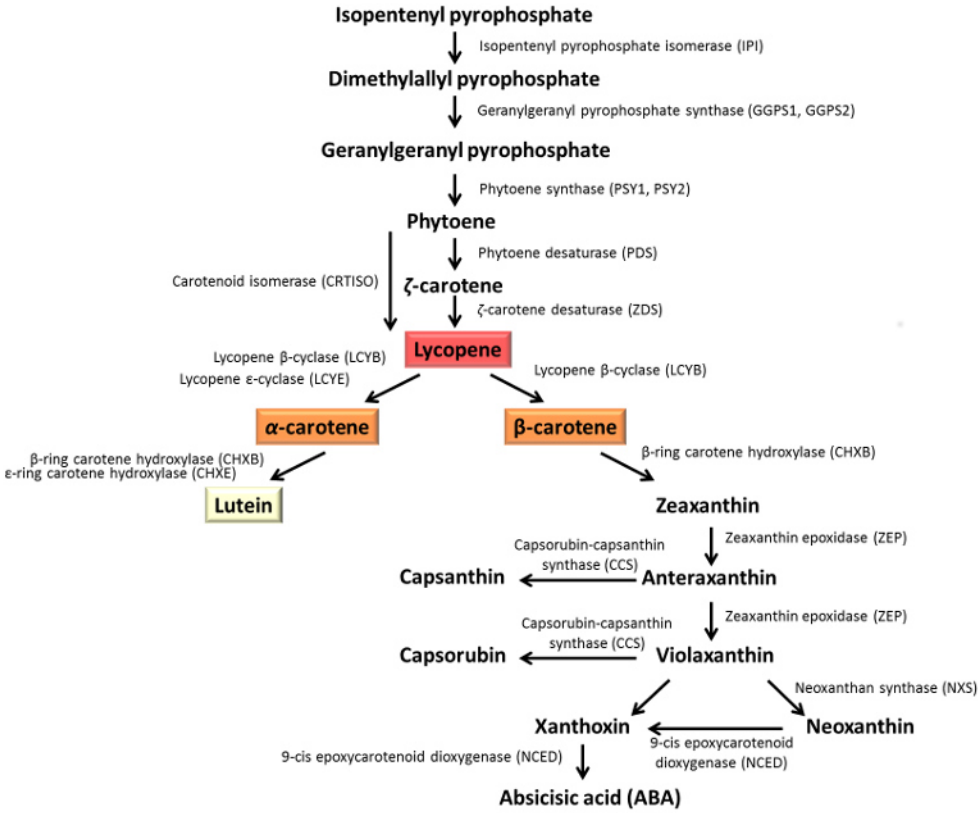


Fig. 1. A simplified diagram of the enzymes and major products in the carotenoid pathway (adapted from Just et al. 2007). Boxes indicate main carotenoids found in carrot (Surles et al. 2004).

Carotenoid biosynthesis pathway analysis suggests that PSY is the major driver for accumulation of carotenoids in carrot (Maass et al. 2009). Fernandes Santos et al. (2005) also refers an important role for *PSY* in regulating carotenoid accumulation in a carrot population segregating for white, yellow and orange root colour. Several quantitative trait loci (QTL) have

been also associated with products of the carotenoid pathway (Santos and Simon 2002; Just et al. 2009).

1.3. Aspects of carrot physiology on yield production

The storage organ derives mainly from root tissues, but in the mature state, the hypocotyl comprises about 2.5 cm of the upper part of the storage organ (Esau 1940). The secondary growth resulting in the swollen tap root begins with the initiation of the secondary cambium between primary xylem and primary phloem. The cambium is formed simultaneously to the first leaves (Esau 1940). Hole et al. (1984) observed initiation of the cambium at 11 days after sowing and completion of the cambial ring after 20 days in a controlled environment. Under field conditions, initiation and completion of the cambium occurred ten days later (Hole et al. 1987b). Esau (1940) described the development of the secondary growth of carrot root as the division of cambial cells to form xylem on the inside and phloem on the outside. Most of the secondary tissues consist of parenchyma cells, which embed the vessels in xylem and sieve tubes and companion cells in the phloem. As a consequence of enlargement of the circumference of the root, cells of the cortex and endodermis disrupt, simultaneously to the appearance of the colour, in the coloured varieties. Periderm, arising from meristematic activity in the pericycle, forms the new protective layer. Cell division continues throughout the development of the storage root together with cell expansion (Hole et al. 1987a).

To understand the physiology of yield production in carrots and to improve nutrient efficiency mechanisms, it is crucial to analyse the factors that contribute to control cambial activity (Arnholdt-Schmitt 1999). A first approach to be considered is the importance of regulation by source or sink strength. Hole et al. (1987c) reported the existence of young carrot plants with genotypic differences in the shoot to root ratio, already observable at 34 to 48 days after sowing. Nevertheless, the distribution of assimilates to shoot or root and its loss by respiration were not cultivar specific (Hole and Dearman 1991). Arnholdt-Schmitt et al. (1993) found that positive correlation between carrot shoot and root fresh weight became significant at the main phase of secondary root growth, suggesting that shoot may become a limiting factor during the increase of yield production. Arnholdt-Schmitt (1999 and references therein) pointed for a basic role of cytokinins as key regulating factors for carrot yield production, thus seeming to be directly involved in the control of cell cycle activity in the root meristem.

Isolated cambial tissue of carrot roots was shown able to synthesize cytokinins, whereas in non-cambial regions this synthesis was not observed (Chen et al. 1985). During secondary root growth an increase of cytokinin activity was also observed (Palussek and

Neumann 1982). Arnholdt-Schmitt (1993) referred to differences of carrot root cells in their ability to transduce a given cytokinin signal into cell division growth. This potential termed cytokinin sensitivity was related to the growth potential of carrot root cells in tissue culture experiments and was found to be genotype dependent (Arnholdt-Schmitt 1999).

1.4. Characterization of alternative oxidase and plastid terminal oxidase

1.4.1. Alternative oxidase (AOX)

Plants possess a so-called alternative respiration pathway as part of the total respiration process. A key enzyme in this pathway is the multigenic alternative oxidase (AOX). AOX is a nucleus encoded terminal ubiquinol oxidase, and in eukaryotes is located at the substrate side of the Cytochrome bc_1 complex, forming an integral part of the electron transport chain in the mitochondria (Siedow et al. 1995). It catalyses the oxidation of ubiquinol and provides an alternative route for electrons to reduce oxygen to water prior to proton translocation by complexes III and IV, in turn hindering ATP formation and thus energy is dissipated as heat (Siedow et al. 1995; Moore et al. 2013) (Fig. 2). AOX bypasses adenylate and local P_i (inorganic phosphorus) control and, under a high-energy charge, AOX helps to avoid incomplete reduction of oxygen to water as a source for reactive oxygen species. Many publications point to the protective role of AOX against oxidative stress in plants (see Vanlerberghe 2013 and references therein). However, it was in the thermogenic floral tissues of Araceae that the alternative respiration was first recognized, and its involvement with pollination was first related. The high rate of electron flux that occurs in thermogenic plants generates heat that consequently volatilizes important compounds to attract insect pollinators (Meeuse 1975).

AOX proteins belong to the membrane-bound di-iron carboxylate proteins (Berthold and Stenmark 2003). The location of the hydrophobic regions suggests an interfacial rather than a transmembrane nature of the protein (Andersson and Nordlund 1999; Berthold et al. 2000; Albury et al. 2009; Moore et al. 2013). AOX occurrence is ubiquitous in the plant kingdom but is also found in many fungi, algae, protists and nematodes (Siedow and Umbach 2000; Mercy et al. 2015; Valadas et al. 2015). In higher plants, *AOX* is nuclear encoded by a small multigene family, comprising one to six gene members (Cardoso et al. 2015) distributed by two subfamilies, the *AOX1* and *AOX2*. In monocots, only genes belonging to the *AOX1* subfamily have been identified, while in dicots, genes from both subfamilies are present (Cardoso et al. 2015). The number of genes belonging to each subfamily varies considerably with the plant species (Cardoso et al. 2015). In carrot, one gene of the *AOX1* subfamily (*DcAOX1,* Costa et al. 2014a) and two genes belonging to the *AOX2* subfamily (*DcAOX2a* and

DcAOX2b) were identified (Campos et al. 2009; Costa et al. 2009). Recently, Costa et al. (2014b) proposed a classification scheme, based on protein phylogenies and sequence harmony methods, to clarify the taxonomic distribution and evolutionary history of AOX in angiosperms. The predominant structure of genomic *AOX* sequences consists of four exons interrupted by three introns at well conserved positions (Saisho et al. 1997; Considine et al. 2002; Saika et al. 2002; Polidoros et al. 2009; Cardoso et al. 2015). This typical structure was previously identified in both carrot *AOX2* genes (Campos et al. 2009). Evolutionary intron loss and gain has resulted in the variation of intron number in some *AOX* members (Cardoso et al. 2014; Considine et al. 2002; Ito et al. 1997; Polidoros et al. 2009; Saisho et al. 2001). Campos et al. (submitted, see in CHAPTER 3) described the structure of carrot *AOX1* gene as composed by only three exons interrupted by two introns due to the loss of intron 3. High variability at intron size has been also reported within members of *AOX* genes from a single species, as well across plant species (Cardoso et al. 2009; Cardoso et al. 2015). Nevertheless, high conservation in the protein coding sequence leads to peptides around 300 amino acid residues with highly conserved regions. Two highly conserved cysteine residues located towards the N-terminal hydrophilic domain mark the target site of redox and α-keto acid regulation (Rhoads et al. 1998; Holtzapffel et al. 2003). AOX proteins contain a four-helix bundle coordinating the di-iron center (Berthold et al. 2002; Berthold and Stenmark 2003; Moore et al. 2008; Moore et al. 2013). The presence of iron-binding motifs within the four helical regions, rich in histidine and glutamate (4 glutamates and 2 histidines), that coordinate the diiron center, and those that interact with the AOX inhibitor salicylhydroxamic acid (SHAM) are identified in AOX across kingdoms (Moore et al. 2013).

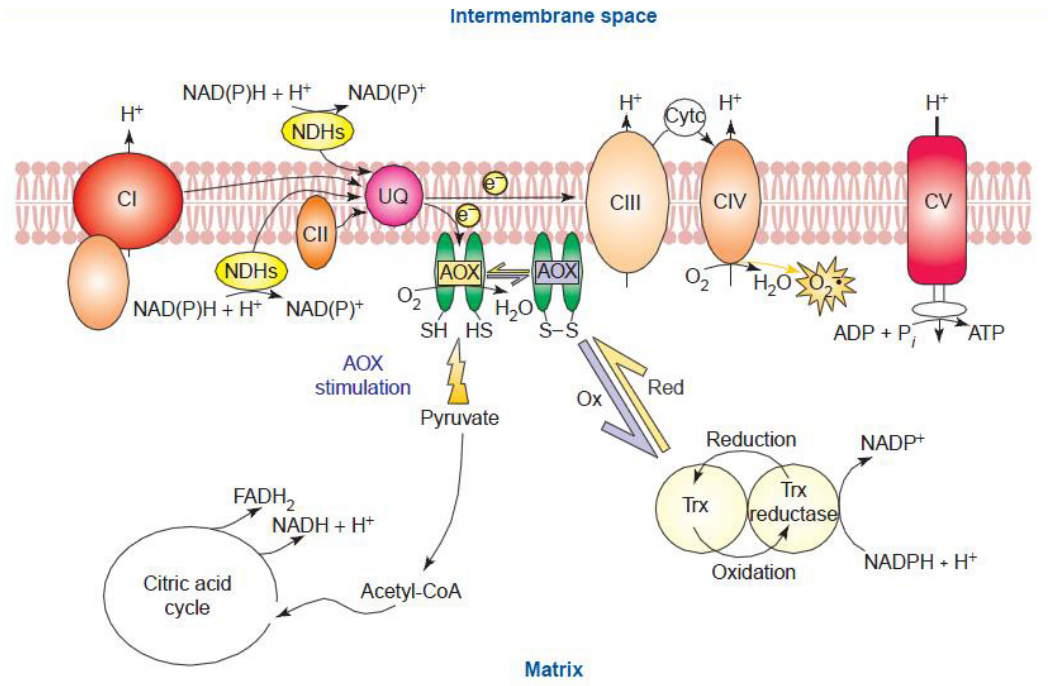


Fig. 2. The mitochondrial electron transport chain showing the position of the alternative oxidase. AOX, alternative oxidase; CI, NADH: ubiquinone oxidoreductase; CII, succinate: ubiquinone oxidoreductase; CIII, Cytochrome bc1 complex; CIV, cytochrome c oxidase; CV, ATP synthase; Cytc, cytochrome c; NDHs, rotenone-insensitive NAD(P)H dehydrogenases; Pi, inorganic phosphorus; Trx, thioredoxin; UQ, ubiquinone pool (Arnholdt-Schmitt et al. 2006).

AOX1 genes are frequently induced by many different kinds of stress (Thirkettle-Watts et al. 2003; Clifton et al. 2005; Umbach et al. 2005; Costa et al. 2010; Cavalcanti et al. 2013; Belozerova et al. 2014; Tang et al. 2014; Vishwakarma et al. 2014). Perhaps, the most studied abiotic condition in relation to alternative respiration is temperature, particularly low temperature, with studies in many species showing a sharp increase in *AOX* transcript and/or protein after changing to, or growing at a low temperature (Vanlerberghe and McIntosh 1992; Ito et al. 1997; Fiorani et al. 2005; Watanabe et al. 2008; Wang et al. 2009; Tang et al. 2014; Velada et al. 2014). Several reports have also demonstrated a link between AOX capacity/activity and response to other abiotic factors such as drought, nutrient limitation, salinity or low oxygen (Amor et al. 2000; Bartoli et al. 2005; Vassileva et al. 2009; Vijayraghavan V 2010; Martí et al. 2011). Stress-inducible cell signaling molecules for growth and development, such as reactive oxygen species (ROS) and nitric oxide, have also been shown to induce *AOX* genes (Huang et al. 2002; Gray et al. 2004; Clifton et al. 2005; Liao et al. 2012; Zidenga et al. 2012). Other compounds, known to activate various stress signaling pathways (e.g. methyl jasmonate and salicylic acid) demonstrated also an association with the increase in *AOX* transcript levels (Matos et al. 2009; Sircar et al. 2012; Belozerova et al. 2014).

Regarding plant growth and development, there are several research groups that report a relationship with AOX (see in Arnholdt-Schmitt et al. 2006; Vanlerberghe 2013). Differently to *AOX1*, *AOX2*-subfamily gene members are usually reported as constitutively or developmentally expressed (Considine et al. 2002; Costa et al. 2014b). Nevertheless, several studies have shown that *AOX2* genes are also able to respond to stress conditions (Clifton et al. 2005; Costa et al. 2010; Cavalcanti et al. 2013; Mróz et al. 2015).

1.4.2. Plastid terminal oxidase (PTOX)

Major reactions of oxygenic photosynthesis consist in a vectorial electron transfer from water to $NADP^+$ involving protein complexes present in thylakoid membranes, namely photosystem II (PSII), the cytochrome *b6/f* complex, photosystem I (PSI) and ferredoxin $NADP^+$ reductase, connected with soluble carriers such as plastoquinones (PQs), plastocyanin and ferredoxin (Fig. 2). Besides major photosynthetic complexes of oxygenic photosynthesis, new electron carriers have been identified in thylakoid membranes of higher plant chloroplasts (Rumeau et al. 2007). In fact, there is a chlororespiratory pathway (Bennoun 1982; Peltier and Cournac 2002), mediated by a chloroplastic dehydrogenase (NDH), that uses stromal NAD(P)H for the non-photochemical reduction of PQ to PQH_2, which in turn, is oxidized by a chloroplast targeted plastid terminal oxidase (PTOX) (Fig. 2). PTOX is a nucleus encoded plastid-located plastoquinone (PQ)-O_2 oxidoreductase (plastoquinol oxidase), localized in stromal thylakoid membranes of chloroplast. PTOX has been suggested to be the chloroplasts' functional analogous to AOX in mitochondria (Aluru and Rodermel 2004; Kuntz 2004). Both enzymes are non-heme diiron-carboxylate proteins linked to the adjustment of the cell redox state and have been modeled as interfacial membrane proteins with an active site (DOX) domain exposed to the matrix (for AOX) or stroma (for PTOX) (Fu et al. 2012).

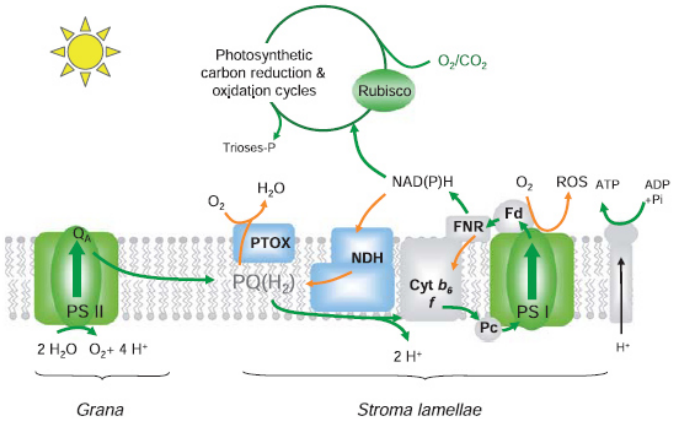


Fig. 3. Electron transfer reactions during oxygenic photosynthesis, showing the position of plastid terminal oxidase (PTOX). Granal thylakoids contain photosystem II (PSII) complexes and the cytochrome b559 complex (not shown on the figure), whereas stroma lamellae contain photosystem I (PSI) complexes, ATPases, the cytochrome *b*6/*f* complex, the NDH complex and PTOX. Rubisco, ribulose 1·5-bisphosphate carboxylase/oxygenase (Rumeau et al. 2007).

In the same way as AOX, PTOX proteins have several conserved domains, such as iron-binding residues (4 glutamates and 2 histidines) (McDonald et al. 2011). Both enzymes exhibit, at their C-terminus, the iron-binding motifs typical of Type II di-iron carboxylate proteins (Carol and Kuntz 2001). Protein sequences' analysis showed that PTOX shares sequence similarity with AOX in a number of plant species (Carol et al. 1999; Wu et al. 1999; Berthold and Stenmark 2003), reason why there are frequently misunderstandings in the annotation of both genes (Nobre et al. in preparation). Interestingly, recent findings in *A. thaliana* indicate that AtAOX2 was imported into the chloroplasts using its own transpeptide (Fu et al. 2012). These authors proposed that AtAOX2 is able to function in chloroplasts to supplement PTOX activity during early events of chloroplast biogenesis. Similar results were obtained when AtAOX1a was reengineered to target the plastid. The ability of AtAOX1a and AtAOX2 to substitute PTOX in the correct physiological and developmental contexts is a striking example of the capacity of a mitochondrial protein to replace the function of a chloroplast protein and illustrates the plasticity of the photosynthetic apparatus (Fu et al. 2012).

Chlororespiration was suggested as an effective electron safety valve preventing over-reduction of the PQ pool and protecting PSII reaction centers from photo-damage under excessive light conditions (Niyogi 2000). PTOX is the terminal oxidase of chlororespiration and regulates the redox state of the PQ pool (Peltier and Cournac 2002; Aluru and Rodermel 2004) by transferring excess electrons to O_2, in order to maintain the relative redox balance in the photosynthetic electron transport chain and reducing the possibility for oxidative damage by

ROS under environmental stresses (McDonald et al. 2011). Sun and Wen (2011) suggested a protective function for PTOX when stress-caused inhibition of photosynthetic electron transport chain occurs. For instance, In high alpine plants acclimated to a low temperature, PTOX was shown to play an important role in photoprotection of PSII (Streb et al. 2005). Increased expression levels were also detected in plants exposed to various stress conditions such as heat, cold, high light, drought, and salinity (reviewed by Sun and Wen 2011), indicating a important metabolic role for PTOX upon adverse conditions. According to Sun and Wen (2011) PTOX is likely helpful under stress conditions in certain plants, in which additional PTOX-related physiological regulation may exist in response to stresses, but probably, does not act as a universal or essential safety valve in the whole plant kingdom. In fact, other studies argue that PTOX more likely plays important roles in plant development (Rosso et al. 2006; Shahbazi et al. 2007; Busch et al. 2008; Okegawa et al. 2010) or other physiological processes.

Recent reports further indicate PTOX as a key enzyme of the carotenoid biosynthesis pathway. Using a transgenic approach, Carol and Kuntz (2001) showed that the lack of PTOX blocks carotenoid synthesis. PTOX absence gives rise to the *immutans* phenotype in *Arabidopsis* and to the *ghost* phenotype in *Solanum lycopersicum* (Carol et al. 1999; Josse et al. 2000; Carol and Kuntz 2001; Rodermel 2001; Aluru et al. 2006). These phenotypes are characterised by variegated leaves with green and bleached sectors and in *S. lycopersicum*, by a yellow-orange ripe fruit. In *immutans*, the variegated phenotype might thus be due to a block in the desaturation of phytoene in the carotenoid biosynthetic pathway, as a result of insufficient oxidized PQ, which is needed as an electron acceptor for this reaction (Wu et al. 1999; Carol and Kuntz 2001), leading to photobleaching of green tissues. PTOX has also a preponderant role in carotenoid biosynthesis in fruit chromoplasts (Josse et al. 2000), as observed in the yellow-orange *S. lycopersicum* fruit, which is characterised by a reduced carotenoid content (Barr et al. 2004). In *S. lycopersicum*, a dual role for PTOX in efficient carotenoid desaturation as well as in chlororespiration in green tissues is referred by Shahbazi et al. (2007). Furthermore, PTOX is the most likely oxidase involved in the barely studied respiratory process that occurs in chromoplast, called chromorespiration (Carol et al. 1999; Wu et al. 1999; Renato et al. 2014). Agreeing to its role in chromorespiration and carotenoid biosynthesis, the expression of *PTOX* increases during the ripening process of *S. lycopersicum* and *Capsicum annuum* fruits, in parallel with chromoplast differentiation (Josse et al. 2000).

PTOX exists widely in photosynthetic species, including algae and higher plants (Cournac et al. 2000; Carol and Kuntz 2001; Archibald et al. 2003; Kuntz 2004). In higher plants *PTOX* has been described as a single gene (Wang et al. 2009; Houille-Vernes et al. 2011). In some eukaryotic algae genomes (at least in *Chlamydomonas*, *Haematococcus*, *Ostreococcus*

and *Cyanidioschyzon*) *PTOX* is present as a small multigene family, composed by two gene members (*PTOX1* and *PTOX2*) (Wang et al. 2009). Bioinformatic analyses revealed that so far, PTOX is known to be present in a total of 15 cyanobacterial strains found in marine, terrestrial, and freshwater environments (McDonald et al. 2011). From those, the marine cyanobacterium *Acaryochloris marina* is unique in that it possesses two *PTOX* genes, while all other cyanobacteria only encode a single *PTOX* gene.

1.5. AOX potential role for functional marker (FM) development

This sub-chapter is adapted from the book chapter:

Nogales A, Noceda C, Ragonezi C, Cardoso HG, Campos MD, Frederico AM, Sircar D, Iyer R, Polidoros A, Peixe A, Arnholdt-Schmitt B (2015) Functional marker development from *AOX* genes requires deep phenotyping and individualized diagnosis. In: Gupta KJ, M.L., Neelwarne B (eds) Alternative respiratory pathways in higher plants. John Wiley & Sons, Inc, Oxford, pp 275-280

Marker assisted selection is commonly used in plant breeding programs to select traits with agronomic interest (e.g. productivity, disease resistance, stress tolerance, quality) using molecular markers closely associated to that trait. FMs can be used to detect the presence of allelic or copy number variation for genes underlying a desired characteristic, thus increasing the efficiency and precision of plant breeding programs. For this reason, FM development has become an area of considerable research interest during the last years (Andersen and Lübberstedt 2003; Neale and Savolainen 2004; Arnholdt-Schmitt 2005; Lübberstedt and Varshney 2013).

Development of FMs can be laborious and time-consuming, and depending on the nature of the selected agronomic trait, the strategies to follow may differ. The first critical step for FM development is the identification of candidate genes and sequence polymorphisms that affect protein (enzyme) activity and consequently induce phenotypic variations (functional polymorphisms). Candidate genes for FM development can be identified by high-throughput differential gene expression (expression quantitative trait loci, eQTL), association mapping, and QTL analysis followed by fine mapping, (bulk) segregation for a trait or by hypothesis-driven research. Hypothesis driven selection of candidate genes is a targeted approach and is thus a highly promising strategy in molecular plant breeding (Arnholdt-Schmitt 2005; Collins et al. 2008).

Plant abiotic stress tolerance is one of the most important and complex traits considered in breeding programs. Adaptive plasticity upon environmental changes influences

the stability of plant biomass and consequently yield production. Plant stress tolerance, as a quantitative multigenic trait, involves the effect of a large set of genes belonging to different signalling and metabolic pathways, hampering the selection of the most appropriate gene(s) for FM development. Good candidate genes are genes that are involved in global cell coordination and decision making of cell fate in plant responses to environment. Candidate gene-based association studies are commonly used to establish a link between genotypes and phenotypes. *AOX* genes have been proposed and adopted as candidate genes for FM development related to multi-stress tolerance and phenotype plasticity (Arnholdt-Schmitt et al. 2006; Polidoros et al. 2009; Cardoso and Arnholdt-Schmitt 2013). However, although *AOX* genes could be general candidate markers related to diverse types of abiotic and biotic stress reactions, the role of AOX can differ between species and needs to be validated at species as well as at target tissue or cell level, depending on the crop and breeding goals (Arnholdt-Schmitt 2005; Arnholdt-Schmitt et al. 2006; Vanlerberghe 2013).

Phenotypic changes related to adaptation to environmental changes might be coordinated by AOX, due to its upstream role in biotic and abiotic stress responses (McDonald and Vanlerberghe 2006; Plaxton and Podestá 2006; Cardoso and Arnholdt-Schmitt 2013; Vanlerberghe 2013) and these changes can include morphogenic responses (Fiorani et al. 2005; Ho et al. 2007; Campos et al. 2009; Frederico et al. 2009; Santos Macedo et al. 2009; Santos Macedo et al. 2012). Differential expression of *AOX* genes in genotypes from the same species but with contrasting stress responses likely provides supporting evidence for a functional role of this gene in stress adaptation (Mhadhbi et al. 2013).

After selecting a suitable candidate gene, the next step for FM development consists, as referred above, on the identification of polymorphisms within the candidate gene sequence that are likely functional and associated with phenotypic variation. This includes characterisation of alleles and/or copy number variation in genotypes with different degree of stress tolerance or responses, affecting plant phenotypes. The subsequent validation of such polymorphisms as markers is then needed. Characterisation of FMs from candidate gene sequences will be less time consuming and will require a lower amount of samples when phenotyping of the polymorphic genotypes is done in a focused way, i.e. by performing deep phenotyping. By identifying the relevant biochemical and/or physiological processes in target tissues/cells, i.e. “deep traits”, the association between them and polymorphic sequences is easier to explore, due to the requirement of less samples, as the studied process is more directly influenced by the candidate gene (i.e., targeted and thus with less factors masking the gene effect). Functional polymorphisms that can be used as FM will be much more easily

identified than just measuring the final trait which is influenced by many other factors, thus reducing the degree of robustness of the putative FM.

While this approach might be valid for many candidate genes, it is especially relevant in the case of *AOX*. The central and upstream role that AOX has in adaptive metabolism and several biological processes makes its regulation too complex to easily obtain a link with a specific desirable trait. Consequently, identifying a link between the *AOX* gene sequence and a biochemical or metabolic 'deep trait' which highly determines the agronomical trait of interest will make FM development more efficient.

This strategy is being applied recently in several studies related to FM development for *AOX* genes. For example, Santos Macedo et al. (2009; 2012) and Hedayati et al. (2015) investigated the involvement of AOX in olive adventitious rooting for FM development related with the efficiency of this process. Adventitious root formation can be considered a morphological response upon stressful treatments which involves cell reprogramming and *de novo* differentiation. That fact leads to the selection of *AOX* as a candidate gene for FM development. In the studies performed by Santos Macedo et al. (2009; 2012), the ring from the basal portion of olive semi-hardwood shoots was taken, which is the place where cells are reprogrammed to perform adventitious rooting, a process that is important for efficient and commercially relevant propagation of the trees. Metabolic analyses were performed in the target tissues and demonstrated that phenylpropanoid and/or lignin content could be suitable 'deep traits' for association studies with *AOX* polymorphisms (Santos Macedo et al. 2012).

The appropriateness of *in vitro* – culture systems for studying the linkage of AOX to a morphological process could recently be confirmed by comparing *AOX* transcript levels during adventitious root induction in semi-hardwood olive shoots and *in vitro* microshoots. A similar *AOX* gene expression pattern could be found in both systems (C. Noceda and E.S. Macedo, personal communications), which makes future studies on the functionality of *AOX* gene polymorphisms for efficient adventitious rooting reasonable. Applying the *in vitro* – system will make screening much more efficient. Different genotypes can be checked under *in vitro* – culture conditions at the same time in a reasonably small space compared to the necessary space for greenhouse plant trials. Additionally, genetic stability and robustness of the polymorphic sites and their effects can easily be screened under these conditions.

Another example of *in vitro*- culture application as a strategy for 'deep phenotyping' for FM development is the use of a primary culture system (PCS) for *Daucus carota* (Campos et al. 2009, see CHAPTER 2 and 3). This system was first established by Steward and Caplin (1952) and consists of inducing a cell program change in differentiated secondary phloem explants from tap roots in a nutrient media containing cytokinin and auxin which initiates *callus* growth.

The primary culture system has been applied at diverse temperatures and was adopted as a test system for the genetic potential for carrot yield production and to distinguish carrot genotypes (Arnholdt-Schmitt 1999). The rapid observation of differences in *callus* growth behaviour between carrot genotypes makes primary cultures a promising system to test the functionality of polymorphisms in *AOX* gene sequences.

Overall, one can refer to the importance of the existence of genetic variability for a desired trait and for the related genes as prerequisites for FM development. Hence, the already identified polymorphisms in carrot *AOX* gene sequences (alleles, haplotypes) (Nogales et al. submitted; Cardoso et al. 2009; Cardoso et al. 2011), create an important basis for association studies between polymorphic DNA marker and a desired trait.

1.6. Research goals

This thesis aims to explore the *AOX* and *PTOX* as target genes for functional markers (FM) development for yield-determining growth performance in carrot. No analyses have been done so far to explore the role of PTOX in the regulation of cell division activity and relation to growth. Due to the similarity between both enzymes it is plausible that PTOX is also involved in some of the same functions of AOX, and therefore the gene arises as a potential source of functional markers.

To understand if and how these genes might associate to growth performance, the different *AOX* gene members and the single *PTOX* gene were isolated, and their expression patterns evaluated in diverse carrot plant systems. An *in-vitro* primary culture system with reproducible length in the lag phase during growth induction was selected to study basic principles of *AOX* and *PTOX* transcript changes during cell reprogramming and growth performance. *AOX* and *PTOX* transcript accumulation was analysed during early time points of *de novo* growth induction during the lag-phase and during exponential growth, in cultures subjected to different temperatures. The results obtained, with *AOX* differentially transcribed early after inoculation – thus early after an environmental change - led to new experiments in a first attempt to transpose insights from the PCS to plant level. For this a pot experiment was performed to study carrot *AOX* early response to chilling. Further, *AOX* gene expression and the role of different AOX gene family members were studied during tap root growth along plant development.

In order to study if PTOX is associated with yield-determining tap root growth performance, *PTOX* transcript accumulation was targeted in the central root meristem, using growth chamber pot experiments. To clarify if PTOX was associated with secondary growth and/or carotenoids accumulation, *PTOX* expression was evaluated in developing carrot tap

roots in an experiment that included cultivars with different carotenoids contents. To get insights of the involvement of *PTOX* on adaptive growth, the early effect of temperature decrease was evaluated, with *PTOX* transcript accumulation targeted in the root meristem.

1.7. Thesis format

This thesis is organised in chapters, each of them a stand-alone research work. To achieve the defined goals of the research line specific tasks were defined:

- Isolation and characterisation of *DcAOX1* gene (CHAPTER 3), *DcAOX2a* and *DcAOX2b* (CHAPTER 2) and *DcPTOX* (CHAPTER 5, cDNA level and CHAPTER 6, gDNA level).
- First expression studies of *DcAOX* genes during *de novo* growth induction in the primary culture system (CHAPTER 2).
- Expression of *DcAOX* genes and *DcPTOX* gene by higher time resolution during the early phases of *de novo* growth induction and exponential growth in the PCS (CHAPTER 3, *AOX* and CHAPTER 6, *PTOX*).
- Pot plant experiments to analyse early *DcAOX* response to chilling (CHAPTER 3).
- *DcAOX* transcript accumulation during carrot tap root growth (CHAPTER 4).
- *DcPTOX* transcript accumulation during carrot root secondary growth. In a first experiment the meristem was used for expression analysis (CHAPTER 6), and later *DcPTOX* was analysed during carrot storage root development involving cultivars with different carotenoids contents (CHAPTER 5).
- Pot plant experiment to analyse early *DcPTOX* response to mild cold stress (CHAPTER 6).
- Selection of suitable reference genes in different carrot experimental systems for accurate normalisation of data (CHAPTER 7).

Note: This doctoral thesis was designed to be based on a group of manuscripts that are published, were submitted or are in preparation, and give body to the research of the thesis. As all articles were written to stand alone, the reader may find repetition in some parts of the manuscripts, especially in the introduction and method sections, as well as the final discussion. This fact results from sharing samples, instruments and procedures amongst the series of articles. The results presented in CHAPTER 2 were achieved in frame of the Marie Curie Chair project ‘Stress adaptation in plants - a molecular approach of socio-economic interest´ and mark the beginning of the tasks developed in this PhD project.

1.8. References

Albury MS, Elliott C, Moore AL (2009) Towards a structural elucidation of the alternative oxidase in plants. Physiol Plant 137:316–327. doi: 10.1111/j.1399-3054.2009.01270.x

Aluru MR, Rodermel SR (2004) Control of chloroplast redox by the IMMUTANS terminal oxidase. In: Physiologia Plantarum. pp 4–11

Aluru MR, Yu F, Fu A, Rodermel S (2006) Arabidopsis variegation mutants: New insights into chloroplast biogenesis. In: Journal of Experimental Botany. pp 1871–1881

Amor Y, Chevion M, Levine A (2000) Anoxia pretreatment protects soybean cells against H_2O_2-induced cell death: Possible involvement of peroxidases and of alternative oxidase. FEBS Lett 477:175–180. doi: 10.1016/S0014-5793(00)01797-X

Andersen JR, Lübberstedt T (2003) Functional markers in plants. Trends Plant Sci. 8:554–560

Andersson ME, Nordlund P (1999) A revised model of the active site of alternative oxidase. FEBS Lett 449:17–22. doi: S0014-5793(99)00376-2

Archibald JM, Rogers MB, Toop M, et al (2003) Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigelowiella natans*. Proc Natl Acad Sci U S A 100:7678–7683. doi: 10.1073/pnas.1230951100

Arnholdt-Schmitt B (1993) Hormonsensitivitaet und genomische DNS - Variabilitaet in ihrem Bezug zur Ertragsbildung bei *Daucus carota* L. ssp. sativus : ein Beitrag zur Zellbiologie pflanzlichen Wachstums. Justus-Liebig-University Giessen, Germany

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 (2005) Functional markers and a “systemic strategy”: Convergency between plant breeding, plant nutrition and molecular biology. Plant Physiol Biochem 43:817–820. doi: 10.1016/j.plaphy.2005.08.011

Arnholdt-Schmitt B, Costa JH, de Melo DF (2006) AOX - a functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11:281–287. doi: 10.1016/j.tplants.2006.05.001

Arscott SA, Tanumihardjo SA (2010) Carrots of many colors provide basic nutrition and bioavailable phytochemicals acting as a functional food. Compr Rev Food Sci Food Saf 9:223–239. doi: 10.1111/j.1541-4337.2009.00103.x

Banga O (1963) Main types of the western carotene carrot and their origin. W. E. J. Tjeenk Willink, Netherlands

Baranski R, Maksylewicz-Kaul A, Nothnagel T, et al (2011) Genetic diversity of carrot (*Daucus carota* L.) cultivars revealed by analysis of SSR loci. Genet Resour Crop Evol 59:163–170. doi: 10.1007/s10722-011-9777-3

Barr J, White WS, Chen L, et al (2004) The GHOST terminal oxidase regulates developmental programming in tomato fruit. Plant, Cell Environ 27:840–852. doi: 10.1111/j.1365-3040.2004.01190.x

Bartoli CG, Gomez F, Gergoff G, et al (2005) Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions. J Exp Bot 56:1269–1276. doi: 10.1093/jxb/eri111

Belozerova NS, Baik a. S, Butsanets P A, et al (2014) Effect of salicylic acid on the alternative pathway of yellow lupine respiration. Russ J Plant Physiol 61:38–46. doi: 10.1134/S1021443714010026

Bennoun P (1982) Evidence for a respiratory chain in the chloroplast. Proc Natl Acad Sci USA 79:4352–4356. doi: 10.1073/pnas.79.14.4352

Berthold DA (1998) Isolation of mutants of the *Arabidopsis thaliana* alternative oxidase (ubiquinol:oxygen oxidoreductase) resistant to salicylhydroxamic acid. Biochim Biophys Acta - Bioenerg 1364:73–83. doi: 10.1016/S0005-2728(98)00015-2

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

Berthold DA, Stenmark P (2003) Membrane-bound diiron carboxylate proteins. Annu Rev Plant Biol 54:497–517. doi: DOI 10.1146/annurev.arplant.54.031902.134915

Boiteux LS, Hyman JR, Bach IC, et al (2004) Employment of flanking codominant STS markers to estimate allelic substitution effects of a nematode resistance locus in carrot. Euphytica 136:37–44. doi: 10.1023/B:EUPH.0000019508.78153.dd

Busch F, Hüner NPA, Ensminger I (2008) Increased air temperature during simulated autumn conditions impairs photosynthetic electron transport between photosystem II and photosystem I. Plant Physiol 147:402–414. doi: 10.1104/pp.108.117598

Campos MD, Cardoso HG, Linke B, et al (2009) Differential expression and co-regulation of carrot *AOX* genes (*Daucus carota*). Physiol Plant 137:578–591. doi: 10.1111/j.1399-3054.2009.01282.x

Cardoso H, Campos MD, Nothnagel T, Arnholdt-Schmitt B (2011) Polymorphisms in intron 1 of carrot *AOX2b* – a useful tool to develop a functional marker? Plant Genet Resour 9:177–180. doi: 10.1017/S1479262111000591

Cardoso HG, Arnholdt-Schmitt B (2013) Functional Marker Development Across Species in Selected Traits. In: T Lübberstedt T & RK Varshney (ed) Diagnostics in Plant Breeding. Springer Netherlands, pp 467–515

Cardoso HG, Campos MD, Costa AR, et al (2009) Carrot alternative oxidase gene *AOX2a* demonstrates allelic and genotypic polymorphisms in intron 3. Physiol Plant 137:592–608. doi: 10.1111/j.1399-3054.2009.01299.x

Cardoso HG, Nogales A, Frederico AM, et al (2015) Natural AOX gene diversity. In: Gupta KJ, Mur LAJ, Neelwarne B (eds) Alternative respiratory pathways in higher plants. John Wiley & Sons, Inc, Oxford, pp 241–254

Carol P, Kuntz M (2001) A plastid terminal oxidase comes to light: Implications for carotenoid biosynthesis and chlororespiration. Trends Plant Sci. 6:31–36.

Carol P, Stevenson D, Bisanz C, et al (1999) Mutations in the Arabidopsis gene IMMUTANS cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. Plant Cell 11:57–68. doi: 10.1105/tpc.11.1.57

Cavalcanti JHF, Oliveira GM, Saraiva KDDC, et al (2013) 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. J Plant Physiol 170:1609–1619. doi: 10.1016/j.jplph.2013.06.012

Chen CM, Ertl JR, Leisner SM, Chang CC (1985) Localization of cytokinin biosynthetic sites in pea plants and carrot roots. Plant Physiol 78:510–513. doi: 10.1104/pp.78.3.510

Clifton R, Lister R, Parker KL, et al (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. Plant Mol Biol 58:193–212. doi: 10.1007/s11103-005-5514-7

Clotault J, Geoffriau E, Lionneton E, et al (2010) Carotenoid biosynthesis genes provide evidence of geographical subdivision and extensive linkage disequilibrium in the carrot. Theor Appl Genet 121:659–672. doi: 10.1007/s00122-010-1338-1

Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? Plant Physiol 147:469–486. doi: 10.1104/pp.108.118117

Considine MJ, Holtzapffel RC, Day D a, et al (2002) Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol 129:949–953. doi: 10.1104/pp.004150

Constance L (1971) History of the classification of Umbelliferae (Apiaceae). Bot J Linn Soc 64:1–11.

Costa JH, Cardoso HG, Campos MD, et al (2009) *Daucus carota* L. - An old model for cell reprogramming gains new importance through a novel expansion pattern of alternative oxidase (AOX) genes. Plant Physiol Biochem 47:753–759. doi: 10.1016/j.plaphy.2009.03.011

Costa JH, Cardoso HG, Campos MD, et al (2014a) Corrigendum to "*Daucus carota* L. - An old model for cell reprogramming gains new importance through a novel expansion pattern of alternative oxidase (AOX) genes" [Plant Physiol. Biochem. 47 (2009) 753e759]. Plant Physiol Biochem 85:114.

Costa JH, McDonald AE, Arnholdt-Schmitt B, Fernandes de Melo D (2014b) A classification scheme for alternative oxidases reveals the taxonomic distribution and evolutionary history of the enzyme in angiosperms. Mitochondrion

Costa JH, Mota EF, Cambursano MV, et al (2010) Stress-induced co-expression of two alternative oxidase (*VuAox1 and 2b*) genes in Vigna unguiculata. J Plant Physiol 167:561–570. doi: 10.1016/j.jplph.2009.11.001

Cournac L, Redding K, Ravenel J, et al (2000) Electron flow between photosystem II and oxygen in chloroplasts of photosystem I-deficient algae is mediated by a quinol oxidase involved in chlororespiration. J Biol Chem 275:17256–17262. doi: 10.1074/jbc.M908732199

Esau K (1940) Developmental anatomy of the fleshy storage organ of *Daucus carota*. Hilgardia 13:175–209.

Fernandes Santos CA, Senalik D, Simon PW (2005) Path analysis suggests phytoene accumulation is the key step limiting the carotenoid pathway in white carrot roots. Genet Mol Biol 28:287–293. doi: 10.1590/S1415-47572005000200019

Fiorani F, Umbach AL, Siedow JN (2005) The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants. Plant Physiol 139:1795–1805. doi: 10.1104/pp.105.070789

Frederico AM, Campos MD, Cardoso HG, et al (2009) Alternative oxidase involvement in *Daucus carota* somatic embryogenesis. Physiol Plant 137:498–508. doi: 10.1111/j.1399-3054.2009.01278.x

Fu A, Liu H, Yu F, et al (2012) Alternative Oxidases (AOX1a and AOX2) can functionally substitute for plastid terminal oxidase in Arabidopsis Chloroplasts. Plant Cell 24:1579–1595.

Gray GR, Maxwell DP, Villarimo AR, McIntosh L (2004) Mitochondria/nuclear signaling of alternative oxidase gene expression occurs through distinct pathways involving organic acids and reactive oxygen species. Plant Cell Rep 23:497–503. doi: 10.1007/s00299-004-0848-1

Hedayati V, Mousavi A, Razavi K, et al (2015) Polymorphisms in the *AOX2* gene are associated with the rooting ability of olive cuttings. Plant Cell Rep 34:1151–1164. doi: 10.1007/s00299-015-1774-0

Ho LHM, Giraud E, Lister R, et al (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. doi: 10.1104/pp.106.091819

Hole CC, Dearman J (1991) Carbon economy of carrots during initiation of the storage root in cultivars contrasting in shoot:root ratio at maturity. Ann Bot 68:427–434.

Hole CC, Morris GEL, Cowper A. (1987a) Distribution of dry matter between shoot and storage root of field-grown carrots. III. Development of phloem and xylem parenchyma and cell numbers in the storage root. J Hortic Sci 62:351–358.

Hole CC, Morris GEL, Cowper A. (1987b) Distribution of dry matter between shoot and storage root of field-grown carrots. II. Relationship between initiation of leaves and storage roots in different cultivars. J Hortic Sci 62:343–349.

Hole CC, Morris GEL, Cowper AS (1987c) Distribution of dry matter between shoot and storage root of field-grown carrots. I. Onset of differences between cultivars. J Hort Sci 62:335–341.

Hole CC, Thomas TH, McKee JMT (1984) Sink development and dry matter distribution in storage root crops. Plant Growth Regul 2:347–358.

Holtzapffel RC, Castelli J, Finnegan PM, et al (2003) A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta - Bioenerg 1606:153–162. doi: 10.1016/S0005-2728(03)00112-9

Houille-Vernes L, Rappaport F, Wollman F-A, et al (2011) Plastid terminal oxidase 2 (PTOX2) is the major oxidase involved in chlororespiration in Chlamydomonas. Proc. Natl. Acad. Sci. 108:20820–20825.

Huang X, Von Rad U, Durner J (2002) Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in Arabidopsis suspension cells. Planta 215:914–923. doi: 10.1007/s00425-002-0828-z

Iorizzo M, Senalik DA, Grzebelus D, et al (2011) De novo assembly and characterization of the carrot transcriptome reveals novel genes, new markers, and genetic diversity. BMC Genomics 12:389.

Ito Y, Saisho D, Nakazono M, et al (1997) Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature. Gene 203:121–129. doi: 10.1016/S0378-1119(97)00502-7

Josse EM, Simkin AJ, Gaffé J, et al (2000) A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. Plant Physiol 123:1427–1436. doi: 10.1104/pp.123.4.1427

Just BJ, Santos CAF, Fonseca MEN, et al (2007) Carotenoid biosynthesis structural genes in carrot (*Daucus carota*): isolation, sequence-characterization, single nucleotide polymorphism (SNP) markers and genome mapping. Theor Appl Genet 114:693–704. doi: 10.1007/s00122-006-0469-x

Just BJ, Santos CAF, Yandell BS, Simon PW (2009) Major QTL for carrot color are positionally associated with carotenoid biosynthetic genes and interact epistatically in a domesticated ?? wild carrot cross. Theor Appl Genet 119:1155–1169. doi: 10.1007/s00122-009-1117-z

Kuntz M (2004) Plastid terminal oxidase and its biological significance. Planta 218:896–899. doi: 10.1007/s00425-004-1217-6

Laufer B. (1919) Sino-Iranica: Chinese contributions to the History of civilization in Ancient Iran with special reference to the History of cultivated plants and products. Field Museum of Natural History, Chicago, Chicago

Liao YWK, Shi K, Fu LJ, et al (2012) The reduction of reactive oxygen species formation by mitochondrial alternative respiration in tomato basal defense against TMV infection. Planta 235:225–238. doi: 10.1007/s00425-011-1483-z

Lübberstedt T, Varshney R. (2013) Diagnostics in Plant Breeding. Springer Netherlands, Dordrecht

Maass D, Arango J, Wüst F, et al (2009) Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. PLoS One. doi: 10.1371/journal.pone.0006373

Mackevic V. (1929) The carrot of Afghanistan. Bull Appl Bot Genet Plant Breed 20:517–562.

Martí MC, Florez-Sarasa I, Camejo D, et al (2011) Response of mitochondrial thioredoxin PsTrxo1, antioxidant enzymes, and respiration to salinity in pea (*Pisum sativum* L.) leaves. J Exp Bot 62:3863–3874. doi: 10.1093/jxb/err076

Matos AR, Mendes AT, Scotti-Campos P, Arrabaça JD (2009) Study of the effects of salicylic acid on soybean mitochondrial lipids and respiratory properties using the alternative oxidase as a stress-reporter protein. Physiol Plant 137:485–497. doi: 10.1111/j.1399-3054.2009.01250.x

McDonald AE, Ivanov AG, Bode R, et al (2011) Flexibility in photosynthetic electron transport: the physiological role of plastoquinol terminal oxidase (PTOX). Biochim. Biophys. Acta - Bioenerg. 1807:954–967.

McDonald AE, Vanlerberghe GC (2006) Origins, evolutionary history, and taxonomic distribution of alternative oxidase and plastoquinol terminal oxidase. Comp Biochem Physiol - Part D Genomics Proteomics 1:357–364. doi: 10.1016/j.cbd.2006.08.001

Meeuse BJD (1975) Thermogenic respiration in Aroids. 117–126.

Mercy L, Svensson JT, Lucic E, et al (2015) AOX gene diversity in arbuscular mycorrhizal fungi (AMF) products – a special challenge. In: Gupta KJ, Mur LAJ, Neelwarne B (eds) From AOX diversity to functional marker development. John Wiley & Sons, Inc, Oxford, pp 305–311

Mhadhbi H, Fotopoulos V, Mylona P V., et al (2013) Alternative oxidase 1 (*Aox1*) gene expression in roots of *Medicago truncatula* is a genotype-specific component of salt stress tolerance. J Plant Physiol 170:111–114. doi: 10.1016/j.jplph.2012.08.017

Moore AL, Shiba T, Young L, et al (2013) Unraveling the heater: new insights into the structure of the alternative oxidase. Annu Rev Plant Biol 64:637–63. doi: 10.1146/annurev-arplant-042811-105432

Mróz TL, Havey MJ, Bartoszewski G (2015) Cucumber possesses a single terminal alternative oxidase gene that is upregulated by cold stress and in the mosaic (MSC) mitochondrial mutants. Plant Mol Biol Report. doi: 10.1007/s11105-015-0883-9

Neale DB, Savolainen O (2004) Association genetics of complex traits in conifers. Trends Plant Sci 9:325–330. doi: 10.1016/j.tplants.2004.05.006

Niyogi KK (2000) Safety valves for photosynthesis. Curr Opin Plant Biol 3:455–460. doi: 10.1016/S1369-5266(00)00113-8

Okegawa Y, Kobayashi Y, Shikanai T (2010) Physiological links among alternative electron transport pathways that reduce and oxidize plastoquinone in Arabidopsis. Plant J 63:458–468. doi: 10.1111/j.1365-313X.2010.04252.x

Palussek K, Neumann K. (1982) Studies on the gibberellin and cytokinin in various stages of development of the carrot root. Z Pflanzenernähr Bodenkd 145:268–277.

Peltier G, Cournac L (2002) Chlororespiration. Annu Rev Plant Biol 53:523–550. doi: 10.1146/annurev.arplant.53.100301.135242

Peterson CE, Simon PW (1986) Carrot Breeding. In: Bassett MJ (ed) Breeding Vegetable Crops. AVI Publishing, Westport, CT.

Pimenov MG, Leonov MV (1993) The genera of the umbelliferae: a nomenclator. Royal Botanic Gardens, Kew, London, UK

Plaxton WC, Podestá FE (2006) The functional organization and control of plant respiration. CRC Crit Rev Plant Sci 25:159–198. doi: 10.1080/07352680600563876

Polidoros AN, Mylona P V., Arnholdt-Schmitt B (2009) *Aox* gene structure, transcript variation and expression in plants. Physiol Plant 137:342–353. doi: 10.1111/j.1399-3054.2009.01284.x

Renato M, Pateraki I, Boronat a., Azcon-Bieto J (2014) Tomato fruit chromoplasts behave as respiratory bioenergetic organelles during ripening. Plant Physiol 166:920–933. doi: 10.1104/pp.114.243931

Rhoads DM, Umbach AL, Sweet CR, et al (1998) Regulation of the cyanide-resistant alternative oxidase of plant mitochondria: Identification of the cysteine residue involved in α-keto acid stimulation and intersubunit disulfide bond formation. J Biol Chem 273:30750–30756. doi: 10.1074/jbc.273.46.30750

Rodermel S (2001) Pathways of plastid-to-nucleus signaling. Trends Plant Sci. 6:471–478.

Rosso D, Ivanov AG, Fu A, et al (2006) IMMUTANS does not act as a stress-induced safety valve in the protection of the photosynthetic apparatus of Arabidopsis during steady-state photosynthesis. Plant Physiol 142:574–585. doi: 10.1104/pp.106.085886

Rubatzky VE, Quiros CF, Simon PW (1999) Carrots and related vegetable Umbelliferae. CABI Publ., New York

Rumeau D, Peltier G, Cournac L (2007) Chlororespiration and cyclic electron flow around PSI during photosynthesis and plant stress response. Plant, Cell Environ. 30:1041–1051.

Saika H, Ohtsu K, Hamanaka S, et al (2002) *AOX1c*, a novel rice gene for alternative oxidase; comparison with rice *AOX1a* and *AOX1b*. Genes Genet Syst 77:31–38. doi: 10.1266/ggs.77.31

Saisho D, Nambara E, Naito S, et al (1997) Characterization of the gene family for alternative oxidase from *Arabidopsis thaliana*. Plant Mol Biol 35:585–596. doi: 10.1023/a:1005818507743

Santos C, Simon P (2002) QTL analyses reveal clustered loci for accumulation of major provitamin A carotenes and lycopene in carrot roots. Mol Genet Genomics 268:122–129. doi: 10.1007/s00438-002-0735-9

Santos Macedo E, Cardoso HG, Hernández A, et al (2009) Physiologic 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. doi: 10.1111/j.1399-3054.2009.01302.x

Santos Macedo E, Sircar D, Cardoso HG, et al (2012) Involvement of alternative oxidase (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism. Plant Cell Rep 31:1581–1590. doi: 10.1007/s00299-012-1272-6

Shahbazi M, Gilbert M, Labouré A-M, Kuntz M (2007) Dual role of the plastid terminal oxidase in tomato. Plant Physiol 145:691–702. doi: 10.1104/pp.107.106336

Shinohara S. (1984) Introduction and variety development in Japan. In: Vegetable seed production technology of Japan elucidated with respective variety development histories, particulars. Shinohara's Authorized Agricultural Consulting Engineer Office, pp 273–282

Siedow JN, Umbach AL (2000) The mitochondrial cyanide-resistant oxidase: structural conservation amid regulatory diversity. Biochim Biophys Acta - Bioenerg 1459:432–439. doi: 10.1016/S0005-2728(00)00181-X

Siedow JN, Umbach AL, Moore AL (1995) The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett 362:10–14. doi: 10.1016/0014-5793(95)00196-G

Simkin AJ, Moreau H, Kuntz M, et al (2008) An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*. J Plant Physiol 165:1087–1106. doi: 10.1016/j.jplph.2007.06.016

Simon PW, Goldman IL (2007) Carrot. In: Singh RJG (ed) Genetic Resources, Chromosome Engineering, and Crop Improvement Series, Volume 3. Boca Raton: CRC Press., pp 497–517

Sircar D, Cardoso HG, Mukherjee C, et al (2012) Alternative oxidase (AOX) and phenolic metabolism in methyl jasmonate-treated hairy root cultures of *Daucus carota* L. J Plant Physiol 169:657–663. doi: 10.1016/j.jplph.2011.11.019

Steward F, Caplin S MF (1952) Investigations on the growth and metabolism of plant cells. I. New techniques for the investigation on metabolism, nutrition and growth in undifferentiated cells. Ann Bot 16:57-77.

Streb P, Josse EM, Gallouët E, et al (2005) Evidence for alternative electron sinks to photosynthetic carbon assimilation in the high mountain plant species *Ranunculus glacialis*. Plant, Cell Environ 28:1123–1135. doi: 10.1111/j.1365-3040.2005.01350.x

Sun T, Simon PW, Tanumihardjo SA (2009) Antioxidant phytochemicals and antioxidant capacity of biofortified carrots (*Daucus carota* L.) of various colors. J Agric Food Chem 57:4142–4147. doi: 10.1021/jf9001044

Sun X, Wen T (2011) Physiological roles of plastid terminal oxidase in plant stress responses. J. Biosci. 36:951–956.

Surles RL, Weng N, Simon PW, Tanumihardjo SA (2004) Carotenoid profiles and consumer sensory evaluation of specialty carrots (*Daucus carota* L.) of various colors. J Agric Food Chem 52:3417–3421. doi: 10.1021/jf035472m

Tang H, Zhang DW, Yuan S, et al (2014) Plastid signals induce alternative oxidase expression to enhance the cold stress tolerance in Arabidopsis thaliana. Plant Growth Regul 275–283. doi: 10.1007/s10725-014-9918-8

Thirkettle-Watts D, McCabe TC, Clifton R, et al (2003) Analysis of the alternative oxidase promoters from soybean. Plant Physiol 133:1158–1169. doi: 10.1104/pp.103.028183

Umbach AL, Fiorani F, Siedow JN (2005) Characterization of transformed Arabidopsis with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. Plant Physiol 139:1806–1820. doi: 10.1104/pp.105.070763

Valadas V, Espada M, Nobre T, et al (2015) AOX in parasitic nematodes – a matter of lifestyle? In: Gupta KJ, Mur LAJ, Neelwarne B (eds) Alternative respiratory pathways in higher plants. John Wiley & Sons, Inc, Oxford, pp 315–319

Vanlerberghe GC (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J. Mol. Sci. 14:6805–6847.

Vanlerberghe GC, McIntosh L (1992) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. Plant Physiol 100:115–119. doi: 10.1104/pp.100.1.115

Vassileva V, Simova-Stoilova L, Demirevska K, Feller U (2009) Variety-specific response of wheat (*Triticum aestivum* L.) leaf mitochondria to drought stress. J Plant Res 122:445–454. doi: 10.1007/s10265-009-0225-9

Velada I, Ragonezi C, Arnholdt-Schmitt B, Cardoso H (2014) Reference genes selection and normalization of oxidative stress responsive genes upon different temperature stress conditions in *Hypericum perforatum* L. PLoS One 9:e115206. doi: 10.1371/journal.pone.0115206

Vijayraghavan V SK (2010) Effect of short- and long-term phosphate stress on the non-phosphorylating pathway of mitochondrial electron transport in *Arabidopsis thaliana*. Funct Plant Biol 37:455–466.

Vishwakarma A, Bashyam L, Senthilkumaran B, et al (2014) Physiological role of AOX1a in photosynthesis and maintenance of cellular redox homeostasis under high light in Arabidopsis thaliana. Plant Physiol Biochem 81:44–53. doi: 10.1016/j.plaphy.2014.01.019

Wang J, Sommerfeld M, Hu Q (2009) Occurrence and environmental stress responses of two plastid terminal oxidases in *Haematococcus pluvialis* (Chlorophyceae). Planta 230:191–203. doi: 10.1007/s00425-009-0932-4

Wang M, Goldman IL (1996) Resistance to root knot nematode (Meloidogyne hapla Chitwood) in carrot is controlled by two recessive genes. J Hered 87:119–123.

Watanabe CK, Hachiya T, Terashima I, Noguchi K (2008) The lack of alternative oxidase at low temperature leads to a disruption of the balance in carbon and nitrogen metabolism, and to an up-regulation of antioxidant defence systems in *Arabidopsis thaliana* leaves. Plant, Cell Environ 31:1190–1202. doi: 10.1111/j.1365-3040.2008.01834.x

Wu D, Wright DA, Wetzel C, et al (1999) The IMMUTANS variegation locus of Arabidopsis defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. Plant Cell 11:43–55. doi: 10.1105/tpc.11.1.43

Zidenga T, Leyva-Guerrero E, Moon H, et al (2012) Extending cassava root shelf life via reduction of reactive oxygen species production. Plant Physiol. 159:1396–1407.

CHAPTER 2 - DIFFERENTIAL EXPRESSION AND CO-REGULATION OF CARROT (*Daucus carota* L.) *AOX* GENES

This chapter is adapted from the manuscript:

Campos MD, Cardoso H, Linke B, Costa JH, Fernandes de Melo D, Justo L, Frederico AM, Arnholdt-Schmitt B (2009) Differential expression and co-regulation of carrot *AOX* genes (*Daucus carota*). Physiol Plant 137: 578–591

Abstract

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*. Here we report on the expression patterns of the carrot *AOX* genes *DcAOX1*, *DcAOX2a* and *DcAOX2b*. Our results demonstrate that all of the three 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. *DcAOX1* and *DcAOX*2a 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* showed 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 exon 1 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 subfamily evolution.

Keywords: *Daucus carota*; alternative oxidase; cell reprogramming; growth induction; *DcAOX2* gene characterisation

2.1. Introduction

Daucus carota L. was highlighted as an ideal model plant to progress alternative oxidase (AOX) stress research (Costa et al. 2009a). This was deduced from the easiness of cell reprogramming in this species upon stress, which led in 1958 to the first demonstration of totipotency in plants (Raghavan 2006). 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 signalling. 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).

The reason why these two gene subfamilies evolved and their functional importance across species are not understood. Recently, differences in conserved sequences between *AOX1* and *AOX2* genes were identified, covering also near-neighbour sequences of the CysI site (Costa et al. 2009a; Frederico et al. 2009). From *in silico* analysis it is known that considering neighbour 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, *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, it is important to study expression patterns and structural organization of the gene sequence. An *in-vitro* primary culture system was selected to study basic principles of *AOX* transcript changes during cell reprogramming and growth performance. The carrot primary culture system has been established originally by Steward et al. in 1952 to study mechanisms of growth. It was this group that also had succeeded (Steward et al. 1958), simultaneously with the group of Reinert (1958), to prove for the first time totipotency in plant cells in *D. carota* as an experimental confirmation of the ideas developed by Haberlandt (1902). The primary culture system has

been improved and maintained as an experimental system for studies on cell reprogramming in the group of Neumann (see review in Arnholdt-Schmitt 1993; 1999). In this system, after inoculation of the explants that consist on pieces of homogenous types of cells of quiescent, differentiated secondary root phloem tissue, tissue dedifferentiation can be induced and *callus* growth is initiated mainly through cell division growth (Arnholdt-Schmitt 1993). Due to a supplementation of cytokinin in the culture medium, no organogenesis is initiated during 28 days of the experiment. However, if depletion of cytokinin happens, at the prolonged stationary phase, rooting from the callogenic tissues can be observed. If cultured from the beginning without cytokinin, rooting starts around day 14 of culture (Neumann 1966; Arnholdt-Schmitt 1999). In cells of initial explants taken from secondary phloem of mature tap roots usually only carotene-containing chromoplasts appear to be present and neither chloroplasts nor other plastid structures were found; however, during the first 8 days in culture (lag-phase), restructuration of chromoplasts to chloroplasts is initiated via an intermediate state as amylo-chloroplasts (Kumar et al. 1983). Tissue dedifferentiation and induction to *callus* formation also take place during the lag-phase (Arnholdt-Schmitt 1999). After that, exponential *callus* growth starts and typically *callus* continues to proliferate during 28 days in culture.

Here, we report the differential expression of *DcAOX1*, *DcAOX2a* and *DcAOX2b* in various tissues or organs and during growth, and highlight within-gene differences between *DcAOX2* subfamily gene members. The results revealed expression of all three carrot *AOX* genes. Independent regulation of both genes in *DcAOX2* subfamily was accompanied by co-regulation of *DcAOX1* and *DcAOX2a*. The role of within-gene differences is discussed.

2.2. Materials and Methods

2.2.1. 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 16h photoperiod: 95-100µmol$m^{-2}s^{-1}$, 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. 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.

2.2.2. 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 2 individual plants (cv. Rotin) were cut and 5 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 $\mu molm^{-2}s^{-1}$, Philips) at 28 °C. During culture *callus* formation is induced. After a lag-phase of 6 to 8 days exponential *callus* growth starts mainly due to 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 (7 2h), 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. 36 h after inoculation marks the termination of a first cell cycle round, which is initiated synchronously in some cells of the explant. 6 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; Arnholdt-Schmitt 1999).

2.2.3. Reverse transcription semi-quantitative PCR (RT-sqPCR)

Primer design for RT-sqPCR expression analyses were based on the published carrot *AOX* gene sequences for *DcAOX1* (Acc. No EU286573), *DcAOX2a* (Acc. No EU286575) and *DcAOX2b* (Acc. No EU286576) (see Costa et al. 2009a).

Total RNA was 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, 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. RT-sqPCR 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 350bp: *DcAOX1F* and *DcAOX1R* for *DcAOX1*, *DcAOX2aF* and *DcAOX2aR* for *DcAOX2a*, *DAOX2b_133F and DAOX2b_402Rev* for *DcAOX2b* (Table 1*).* In these assays RT-sqPCR for each gene was normalized by *D. carota* L. *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 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.

Table 1. List of primers used in this study.

Primer Sequence	Application	Comments
DcA1F:5'-ATGTTGCTATCCAGGCTGTGC-3´	RT	Designed from *DcActin1*
DcA1R:5'-TCACGAACAATTTCCCGCTCG-3'	RT	Designed from *DcActin1*
DcAOX1F: 5'-GCAAGTCACTCAGGCGCTTTG-3'	RT	Designed from *DcAOX1*
DcAOX1R: 5'-CATGGTTTGACGAGGGATTT -3'	RT	Designed from *DcAox1*
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*

2.2.4. 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 by using GeneTools (Syngene, Cambridge, UK). The degenerated primer pair P1/P2

designed in exon 3 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.

2.2.5. 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).

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, 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).

In order to isolate the 5' end of both *DcAOX2* genes a cDNA library of *D. carota* L. cv. Marktgaertner M853 cloned into a Lambda gt22a phage vector (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 *P6* (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 2min (35 cycles).

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 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*) (Table1). PCRs were performed using 0.2 µM of each specific primers and the Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer 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.

2.2.6. 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, USA). Plasmid DNA was extracted from putative recombinant clones (Birnboim and Doly 1979) and analysed 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, USA). Sequence homology was explored in the NCBI (National Center for Biotechnology Information) data basis using BLAST algorithm (Karlin and Altschul 1993).

DNA and cDNA sequencing data were analysed with SeqMan from Lasergene 7 software (DNASTAR, Madison, WI), 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). 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.

2.3. Results

2.3.1. Expression of carrot *AOX* genes

Organ-specific expression

Fig. 1 shows expression patterns of *DcAOX1*, *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 *DcAOX1* 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.

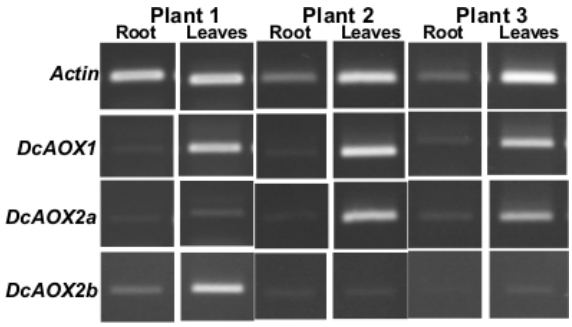


Fig. 1. Expression of *AOX* genes in carrot root and leaves. Transcript accumulation was analysed by RT-sqPCR using the *Actin1* gene for normalisation. Expression levels of *DcAOX1, DcAOX2a* and *DcAOX2b* in three different *in vitro* plants was evaluated. Differential expression amongst both genes and plants can be observed. The RT-sqPCR-products of root and leaves of all *AOX* genes were run together in the same electrophoresis.

De novo differentiation and tissue-specific expression

In order to focus on the dynamics of gene expression during growth, a primary culture experimental approach was chosen (Fig. 2A). *AOX* expression was studied after inoculation of differentiated secondary root phloem explants in a cytokinin-containing nutrient solution that induces tissue dedifferentiation 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). *DcAOX1* 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 levels of both *DcAOX1* and *DcAOX2a* were still higher than in the original, quiescent tissue. Expression of *DcAOX2b* remained overall low, and did not show specific patterns.

Due to the significance that *DcAOX1* and *DcAOX2a* demonstrated during growth induction, the expression of these genes was analysed *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. *DcAOX1* presented again the highest transcript accumulation (Fig. 2B).

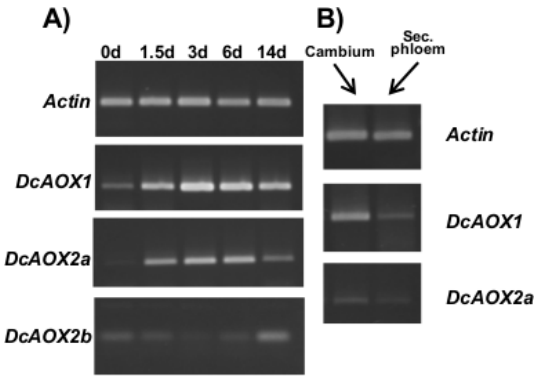


Fig. 2. Expression of *AOX* genes in carrot. Transcript accumulation was analysed by RT-sqPCR using the *Actin1* gene for normalisation. A) Primary cultures from the secondary phloem of carrot roots. The explants were collected at the inoculation moment in the culture medium (0) and 1.5, 3, 6 and 14 days after inoculation. Discrimination between *DcAOX1, DcAOX2a* and *DcAOX2b* gene expression was performed. B) Discrimination between *DcAOX1* and *DcAOX2a* expression in the meristematic tissues cambium and secondary phloem.

2.3.2. Characterisation of the full-length sequences of both *DcAOX2* genes

In search for differences between both extended genes of the *DcAOX2* subfamily, two full-length cDNA sequences were isolated from an individual plant of *D. carota* cv. Rotin. Fig. 3 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 Dc*AOX2* gene sequences show 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 due to exon 1 in the N-terminal region. Exon 1 has a size of 339 bp in *DcAOX2a* and of 282 bp in *DcAOX2b*.

Fig. 3. Nucleotide and deduced amino acid sequences of two cDNAs encodind *Daucus carota* alternative oxidase, *DcAOX2a* (NCBI accession number EU286575) and *DcAOX2b* (NCBI accession number EU286576). The sites of introns are indicated by filled triangles, and * indicates stop codons.

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 ESTs (expressed sequence tags) sequences (Fig. 4) the N-terminal regions (that comprises the exon 1) between the AOX2 sequences analysed 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. 4). 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. 4).

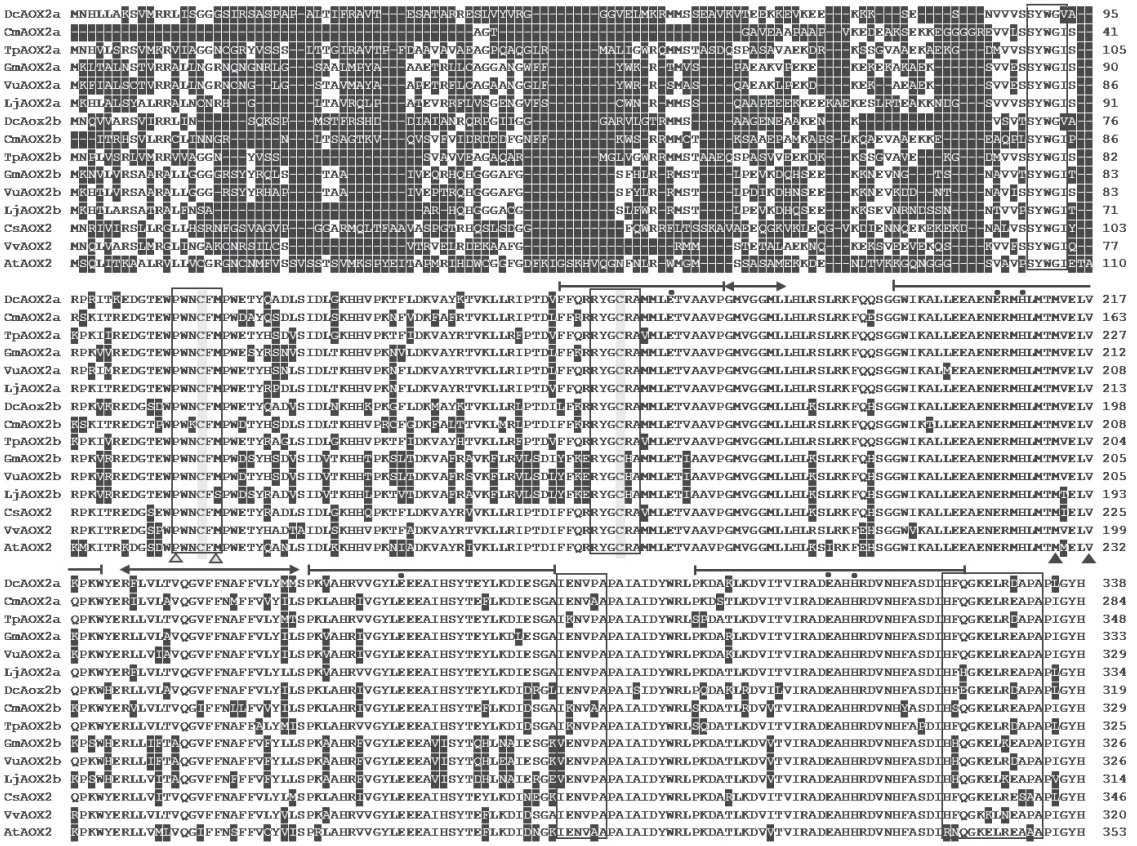


Fig. 4. Multiple alignment of the deduced amino acid complete sequences of *DcAOX2a* and *DcAOX2b* and 13 previously reported AOX2 proteins. The alignment was performed using the ClustalW method of Lasergene 7 software. The sites of two conserved cysteins (CysI and CysII) that are involved in dimerization of the AOX protein by S-S bond formation (Umbach and Siedow 1993) are indicated in grey boxes. Grey 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 (Anderson 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 (Anderson and Nordlund 1999 and 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).

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 neighbour-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. 5). 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 (see Fig. 4).

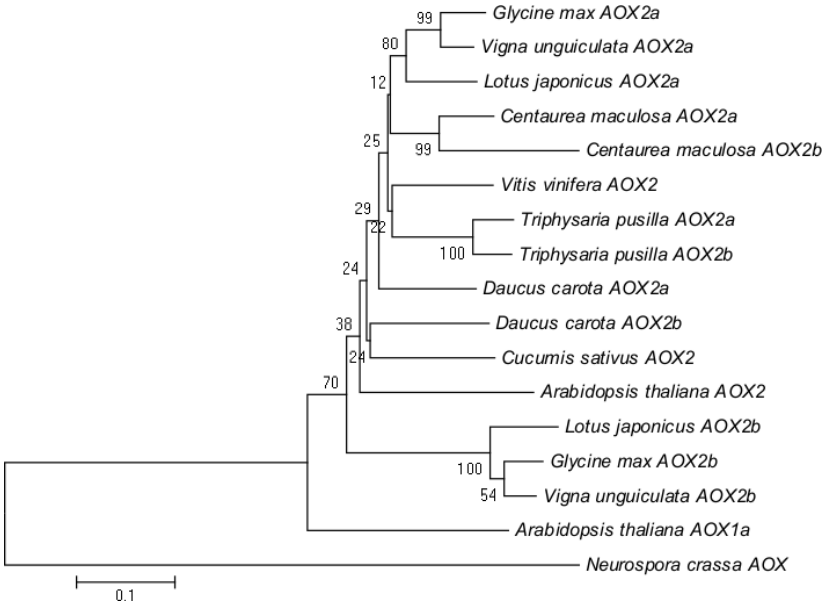


Fig. 5. Phylogenetic tree describing the relationship among AOX2 proteins from plants, including the two AOX2 sequences of *Daucus carota* L. The alignments were bootstrapped with 1000 replicates by the Neigbor-Joining method using the MEGA 3.1 software. The fungus *Neurospora crassa* and *Arabidopsis 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: *D. carota* AOX2a (EU286575), *D. 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).

In all three asterids two genes have been classified as belonging to subfamily AOX2. The AOX2a and AOX2b sequences exhibited higher similarity between each other in each of these plant species, as can be seen in Fig. 5. *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. 5). The plant species that show a single AOX2 sequence (*V. vinifera*, *Cucumis sativus* and *A. thaliana*) are included in a group together with AOX2a and AOX2b from *D. carota*, *C. maculosa* and *T. pussilla* (Fig. 5).

2.4. Discussion

Our results present evidence for the expression of all three carrot *AOX* genes. They were expressed in all studied organs (roots and leaves) and tissues (cambium and secondary phloem of the tap root), as well as in primary cultures induced to *callus* growth, and demonstrate differential expression in relation to organs, tissues and growth. *DcAOX1* played the most important role at transcript level in various organs or tissues and responses on changing environmental conditions that need acclimation. 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 of 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 (Holtzapfel et al. 2003; Cardoso et al. 2009; Costa et al. 2009b; Ferreira et al. 2009; Frederico et al. 2009; Polidoros et al. 2009; Santos 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 large number of studies with transgenic plants that the genomic background of plants can be expected to strongly interfere with gene expression patterns.

The expression data uncovers two typical patterns for carrot *AOX* genes. At first, a close link between *DcAOX1* and *DcAOX2a* expression was discovered. Both genes have been induced under the same conditions, although different transcript levels have been outlined. *DcAOX1* shows typically a higher transcript accumulation. Secondly, *DcAOX2a* and *DcAOX2b* are differentially expressed among each other and typically, *DcAOX2b* is less responsive.

AOX1 and A*OX2* 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. 4). 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 (see Fig. 4). 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 due to regulative elements outside gene sequences or due to 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 on cause-relationships for 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; Santos Macedo et al. 2009). A comparison of complete *DcAOX1* and *DcAOX2a* sequences can be expected in helping to reveal important within-gene motifs for co-regulation.

Contrarily, both *AOX2* subfamily member genes of *D. carota*, *DcAOX2a* and *DcAOX2b*, clearly indicated differential regulation patterns, despite the 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. 5), whereas AOX2a and AOX2b from *V. unguiculata, G. max* and *L. japonicus* were more similar to the according protein in the other species. 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 carrot *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* subfamily. The most pronounced difference was due to 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-termini (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* (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; Thirkette-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 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 (Santos 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 bp and 303 bp in the *AOX2a* and *AOX2b* of *G. m*ax, and 339 bp 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 bp, 489 bp 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. (Santos 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. 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 been observed also in *AOX* genes (Polidoros et al. 2005; Santos 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.

2.5. Conclusion

Results about transcript abundances in diverse carrot tissues, organs and a primary culture indicate a differential expression for all three identified *AOX* genes from *Daucus carota* L.. Similar expression profiles for *DcAOX1* 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 three *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.

It can be concluded that given the differential expression dynamics of carrot *AOX* genes, this species has a good potential as model to advance current insights in the functionality and evolutionary importance of both *AOX* subfamilies. A future transgenic

approach for functional genomics and association studies is expected to bring knowledge a significant step forward.

2.6. 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.

2.7. References

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

Arnholdt-Schmitt B (1993) Hormonsensitivität und genomische DNS - Variabilität in ihrem Bezug zur Ertragsbildung bei *Daucus carota* L. ssp. sativus. Ein Beitrag zur Zellbiologie pflanzlichen Wachstums. Justus-Liebig-University Giessen, Germany.

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 carrot – 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, et al (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, Lister R, Parker KL, et al (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, et 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, et al (2002) Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol 129: 949-953.

Costa JH, Cardoso HC, Campos MD, et al (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, et al. (2009b) The alternative oxidase family of *Vitis vinifera* reveals an attractive model for genomic design. Physiol Plant 137: 553-565.

Costa JH, Hasenfratz-Sauder MP, Pham-Thi AT, et al (2004) Identification in *Vigna unguiculata* (L.) Walp. of two cDNAs encoding mitochondrial alternative oxidase orthologous to soybean alternative oxidase genes *2a* and *2b*. Plant Sci 167: 233-239.

Ferreira A, Cardoso HG, Santos Macedo E, 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, et al (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. Plant Physiol 114: 455-466.

Frederico AM, Zavattieri MA, Campos MD, et al (2009) The gymnosperm *Pinus pinea* L. 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 and Organ Cult 22: 27-35.

Haberlandt G (1902) Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Math. Naturwiss. Kl. Kais. Akad. Wiss. Wien. 111: 69-92.

Hall, TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser41: 95-98.

Ho LHM, Giraud E, Lister R, Thirkettle-Watts D, et al (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: 1519-33.

Holtzapffel RC, Castelli J, Finnegan PM, et al (2003) A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta 1606: 153-162.

Ito Y, Saisho D, Nakazono M, et al (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: 5873-7.

Kumar A, Bender L, Pauler B, et al (1983) Ultrastructural and biochemical development of the photosynthetic apparatus during *callus* induction in carrot root explants. Plant Cell Tiss Org Cult 2:161-177.

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.* The 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.

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 bioassays 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. Les Phytohormones et L'Organogenèse 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, et al (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.

Reinert J (1958) Morphogenese und ihre kontrolle an gewebekulturen aus karotten. Naturwissenschaften 45: 34-345.

Saika H, Ohtsu K, Hamanaka S, et al (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, et al (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, et al (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.

Santos Macedo E, Hernández A, Cardoso HCG, et al (2009) Physiological responses and gene diversity indicate olive alternative oxidase (AOX) as a potential source for markers of efficient adventitious rooting induction. Physiol Plant 137: 532-552.

Schaffer S, Arnholdt-Schmitt B (2001) Characterization of genome variation in tissue cultures by RAPD fingerprinting – a methodological comment. Plant Biosystems 135: 115-120

Steward FC, Caplin SM, Millar FK (1952) Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. Ann. Bot 16: 57–77.

Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cell. II. Organization in culture grown freely suspended cells. Am J Bot 45: 705-708.

Thirkettle-Watts D, McCabe TC, Clifton R, Moore C, et al (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 diabets. Diabetes 54: 893-899.

Zottini M, Formentin E, Scattolin M, et al (2002) Nitric oxide affects plant mitochondrial functionality *in vivo*. FEBS Lett 515: 75–78.

CHAPTER 3 - STRESS-INDUCED ACCUMULATION OF *DcAOX1* AND *DcAOX2a* TRANSCRIPTS COINCIDES WITH CRITICAL TIME POINT FOR STRUCTURAL BIOMASS PREDICTION IN CARROT (*Daucus carota* L.) PRIMARY CULTURES

This chapter is adapted from the submitted manuscript:

Campos MD, Nogales A, Cardoso HG, Rajeev Kumar S, Nobre T, Sathishkumar R, Arnholdt-Schmitt B. Stress-induced accumulation of *DcAOX1* and *DcAOX2a* transcripts coincides with critical time point for structural biomass prediction in carrot primary cultures (*Daucus carota* L.). Submitted

Abstract

Stress-adaptive cell plasticity in target tissues and cells for plant biomass growth is important for yield stability. *In vitro* systems with reproducible cell plasticity can help to identify relevant metabolic and molecular events during early cell reprogramming. In carrot, regulation of the central root meristem is a critical target for yield-determining secondary growth. Calorespirometry, a tool previously identified as promising for predictive growth phenotyping has been applied to measure to measure the respiration rate in carrot meristem. In a carrot primary culture system (PCS), this tool allowed identifying an early peak related with structural biomass formation during lag phase of growth, around the 4th day of culture. In the present study, we report a dynamic and correlated expression of carrot alternative oxidase (*AOX*) genes (*DcAOX1* and *DcAOX2a*) during PCS lag phase and during exponential growth. Both genes showed an increase in transcript levels until 36 h after explant inoculation, and a subsequent down-regulation, before the initiation of exponential growth. In PCS growing at two different temperatures (21 °C and 28 °C), *DcAOX1* was also found to be more expressed in the highest temperature, and the transcript accumulation of both *AOX* genes did not unambiguously relate to growth performance. *DcAOX* genes' were further explored in pot plants in response to chilling, which confirmed the early *AOX* transcript increase prior to the induction of a specific anti-freezing gene (AFP). Our findings point to *DcAOX1* and *DcAOX2a* as being reasonable candidates for functional marker development related to early cell reprogramming. While the sequence of g*DcAOX2a* was previously described, we characterise the complete genomic sequence of *DcAOX1*.

Keywords: *Daucus carota*; alternative oxidase; cell reprogramming; growth induction; chilling; *DcAOX1* gene characterisation

3.1. Introduction

Plant breeding can use i*n vitro* systems not only for plant cloning and propagation, but also as a system to isolate scientific questions related to stress responsiveness for later up-scaling of the knowledge to plant level. Especially, early molecular plant responses during cell reprogramming upon abiotic stress can easily be targeted (Arnholdt-Schmitt 1993; Arnholdt-Schmitt et al. 1995 and references in: Arnholdt-Schmitt 2004; Arnholdt-Schmitt et al. 2006; Frederico et al. 2009; Zavattieri et al. 2010). In recent years, considerable progress has been made regarding the development and isolation of stress tolerant genotypes by using *in vitro* techniques (Pérez-Clemente and Gómez-Cadenas 2012). Phenotypic variability shown in *in vitro* culture systems is due to high genotype dependence, going from species level to the level of cultivar/variety and individual genotypes. It can vary between organs/tissues and developmental stages (Cardoso et al. 2010 and references therein). This variability in response, known as *in vitro* recalcitrance, could be described as varying capacity for plant cells to adapt to new environmental conditions, i.e. the capacity to develop and express new cell programs. This general capacity is important at plant level when environmental conditions are changing. For example, efficient transformation of trichoblasts (see Arnholdt-Schmitt 2004) to fine root hairs is important under changing phosphorus availability in the soil to guarantee access to the nutrient. Plant adaptive plasticity was recently proposed as a new trait in plant breeding (Nicotra et al. 2010; Cardoso and Arnholdt-Schmitt 2013), since it influences stability of plant biomass and yield production. Plant research for robust phenotypes that show stability in growth performance is crucial, but also the most critical and most expensive step in breeding. Efficient marker systems and reliable screening tools that can assist in identifying and selecting superior robust genotypes with differential adaptive plasticity are still important bottlenecks (Arnholdt-Schmitt et al. 2014; Arnholdt-Schmitt et al. 2015a).

In molecular plant breeding, candidate gene approaches for marker-assisted selection are considered a promising strategy (Collins et al. 2008). Good candidate genes for multi-stress tolerance and yield stability are genes involved in cell coordination and decision making in target cells. Alternative oxidase (AOX) is increasingly getting into the focus of research on stress acclimation and adaptation and seems to play a key role in regulating the process of cell reprogramming by ameliorating metabolic transitions related with the cellular redox state and flexible carbon balance (Arnholdt-Schmitt et al. 2006; Rasmusson et al. 2009; Arnholdt-Schmitt 2015). AOX is supposed to provide the respiratory system with built-in flexibility regarding the degree of coupling between carbon metabolism pathways, electron transport chain activity, and ATP turnover (Vanlerberghe 2013). For this reason, *AOX* genes were proposed and adopted as candidate genes for functional marker development related to multi-stress

tolerance and plant adaptive plasticity (Arnholdt-Schmitt et al. 2006; Polidoros et al. 2009; Cardoso and Arnholdt-Schmitt 2013). *AOX* genes were found to be differentially transcribed in various systems early during *in vitro* culture –induced morphogenic responses. This includes *de novo* growth from quiescent root phloem tissue (Campos et al. 2009) and somatic embryogenesis (Frederico et al. 2009a) in carrot and adventitious rooting in olive (Santos Macedo et al. 2009; Santos Macedo et al. 2012).

Nogales et al. (2013) developed calorespirometry as a new tool for breeding in a carrot *in vitro* primary culture system. This *in vitro* system, originated from quiescent secondary tap root phloem, was first established by Steward et al. (1952) and later proposed by Arnholdt-Schmitt (1999) as a mean for carrot yield prediction. Calorespirometry has shown useful to accurately monitor temperature dependent growth performance in terms of metabolic rates, respiratory rates, efficiency of biomass acquisition and growth rates over 21 days of *in vitro* cultures (Nogales et al. 2013). Those data showed a drastic increase in structural biomass formation until around the 4th day after inoculation during the lag phase of growth.

In the work here presented, we expanded the number of cultures tested by Nogales et al. (2013) and first demonstrate that the increase in structural biomass formation, showing an early peak during the lag phase of growth, is present in all the five tested cultures. We report that both carrot *AOX* genes, *DcAOX1* and *DcAOX2a*, previously demonstrated as the ones with major expression in the primary culture system (Campos et al. 2009), showed increased levels of transcripts until the 4th day of culture and subsequent down-regulation before exponential growth starts. As a first attempt to transpose these findings to plant level, we also show an early transcript accumulation for both *AOX* genes in a chilling pot plant experiment prior to the induction of a specific anti-freezing gene (*AFP*). This study identifies *DcAOX1* and *DcAOX2a* as being reasonable candidates for functional marker development on efficient cell reprogramming under changing environments in general. The isolation and characterisation of the complete genomic sequence of *DcAOX1* is further reported.

3.2. Materials and methods

3.2.1. Establishment of a primary culture system (PCS)

To study the dynamics of gene expression during growth, a primary culture approach was chosen. Ten weeks old plants of *D. carota* L. cv. Rotin grown in pots, containing commercial soil mixture maintained under greenhouse conditions were used (Campos et al. 2009). Five explants from the secondary tap root phloem of each plant were inoculated per Erlenmeyer containing 20 mL of NL liquid medium (Neumann 1966) supplemented with kinetin (1 mg·L^{-1}) and indoleacetic acid (2 mg·L^{-1}). Inoculation of explants from quiescent secondary

root phloem in a cytokinin-containing nutrient media induces cell program changes that imply the acquisition of a so-called 'undifferentiated' stage (*callus*) and subsequent cells multiplication (*callus* growth).

3.2.2. Calorespirometry measurements

In order to calculate specific growth rates (i.e. structural biomass formation rate, $R_{struct\_biomass}$) and efficiencies of biomass acquisition as described in Nogales et al. (2013), the respiratory metabolic heat rates and CO_2 emission rates were measured in PCS by calorespirometry, at different time points. To confirm reproducibility of the early peak for structural biomass formation reported by these authors, we expanded the existing data set and performed new measurements in three PCS growing at two different incubation temperatures (21 °C and 28 °C). A total of five PCS measurements are presented.

3.2.3. *DcAOX1* and *DcAOX2a* expression analysis

AOX response during tissue dedifferentiation and callus growth

We studied *DcAOX1* and *DcAOX2a* mRNA levels in an *in vitro* PCS by:

(i) Semi-quantitative PCR (RT-sqPCR) on both *AOX* genes, in order to shed light on transcript changes during the earliest events related to cell reprogramming and also in the later growth phase. Explants from 4 individual carrot plants (4 biological replicates) inoculated at 21 °C were collected at different time points: 0 h, 4 h, 8 h, 12 h, 36 h, 4 days, 8 days, 14 days, 21 days and 28 days post inoculation (hpi/dpi). From 0 hpi until 4 dpi, 30 explants were taken per time point. For the remaining time points, a maximum of 15 explants were taken. Samples were collected as bulked samples. Fresh weight (FW) of each *callus* was also determined at 0, 4, 8, 12, 14, 18, 21 and 28 dpi.

(ii) Quantitative real-time PCR (RT-qPCR), to compare the transcript changes of *AOX* on PCS under two incubation temperatures (21 °C and 28 °C, as in 2.2). Explants from five individual plants (five biological replicates) were collected at 0 and 14 dpi (T0 and T14). Samples consisted of bulked samples of about 50 explants. The five plants used on expression analysis (n = 5) resulted from a previous selection of 12 individual plants based on their *callus* growth behaviour under the two temperatures tested. Fresh weight of each *callus* was determined at respective time points in the 12 plants. Data were analysed by Student's *t* -test using the STATISTICA 8.0 statistical package (StatSoft Inc., Tulsa, USA).

AOX response to chilling exposure (CE) of carrot plants

Seeds of *D. carota* cv. Rotin were sowed in pots containing commercial soil mixture and maintained at controlled conditions for one month (23 °C, 70-75 % of air humidity and 16 h photoperiod with 200 ± 20 µmol m^{-2} s^{-1} light intensity).

Two CE experiments were conducted by:

(i) Semi-quantitative PCR (RT-sqPCR), to study the effect of chilling exposure on *AOX* expression of seedlings growing under controlled conditions (as described above), and exposed to 4 °C for five days. Samples were collected at different time points: 0, 1, 2, 3, 4 and 5 days post chilling exposure (dpce). Samples consisted of young leaves taken as bulked samples from three individual plants. Three bulked samples (biological replicates) were considered in a total of 54 plants.

(ii) Quantitative real-time PCR (RT-qPCR), to evaluate early *AOX* transcript levels on seedlings exposed to 4 °C for 24 h. Samples were collected at 0 h, 10 min, 45 min, 4 h, 6 h and 24 h post chilling exposure (minpce or hpce) and at 24 h and 48 h after transferring the plants back to the initial growth conditions, as described above (recovery period). Samples consisted of young leaves taken from single plants. Four plants (4 biological replicates) were considered per time point. Additionally, the expression of carrot antifreezing protein *(DcAFP)* was evaluated in this experiment at the referred time points.

Sample processing

Total RNA from all samples was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with on-column digestion of DNA using RNase-Free DNase Set (Qiagen, Hilden, Germany), according to manufacturer's instruction. DNase-treated total RNA (1 µg) were reverse transcribed with random decamer primer (PCS experiments) or the oligo d(T) primer (CE experiments), using the RETROscript® kit (Ambion, Austin, TX, USA) according to manufacturer's instruction. The maximum number of time points chosen to collect plant material for RNA extraction and for growth curve analysis was restricted by nature of root sizes.

Transcript analyses

(i) RT-sqPCR: all RT-sqPCR experiments were performed using Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England), 2 µL of cDNA (diluted 1:10) as template and 0.2 µM of each specific primer (Table 1). *Elongation factor 1 alpha* (*DcEF1α*) was previous selected (results not shown) as the reference gene for all RT-sqPCR experiments. PCR for *DcEF1α* and *DcAOX1* (for primers sequences see Table 1) was carried out for 32 cycles, each one consisting

of 30 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C. For *DcAOX2a* the PCR was of 35 cycles consisting in 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. An initial step at 94 °C for 5 min and a final extension at 72 °C for 5 min were performed in both cases. A previous experiment confirmed that disinfection did not have influence on *AOX* transcript levels in PCS (data not shown). RT-sqPCR products were analysed by electrophoresis in 2 % (w/v) agarose gel. For PCS, image analysis was carried out to normalise the expression level of *AOX* cDNA with the reference *DcEF1α* gene, by density band analyses using ImageJ 1.47v software (http://imagej.nih.gov/ij/) (Schneider et al. 2012). The results were expressed as mean ± standard error (SE) of four individual plants. Differences between time points were examined by one-way ANOVA using the STATISTICA 8.0 statistical package (StatSoft Inc.).

(ii) RT-qPCR: Transcript abundances of *DcAOX1, DcAOX2a* and *DcAFP* (Table 1) were determined by RT-qPCR on a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA) using Maxima SYBR Green q-PCR Master Mix (Fermentas, Ontario, Canada). Reaction (15 µL), consisted in 2 µL of first-strand cDNA (previously diluted 1:10) and 0.3 µM of each specific primer were used for the expression analysis*. DcEF1α* was selected for data normalisation based on previous experiments involving 12 candidate genes (Campos et al. 2015; data for PCS not shown). Amplicons of all genes were previously confirmed by direct sequencing. Standard curves of a 4-fold dilution series (1:1-1:125) (run in triplicate) of pooled cDNA from all samples were used for primer efficiency determination. Minus reverse transcriptase and no template controls were included to assess contaminations. RT-qPCR was performed for 40 cycles, each consisting of 15 s at 95 °C followed by 1 min at 60 °C. To analyse the dissociation curve profiles, an additional step at 95 °C during 15 s was added, followed by a constant increase of temperature between 60 and 95 °C. The $2^{-\Delta\Delta CT}$ methodology (Livak and Schmittgen 2001) was used to normalise expression data.

For PCS experiment (i) and CE experiment (ii), a One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to search for significant differences in gene expression between time points. Regarding the PCS experiment at different temperatures (ii), differences in transcript levels between temperatures at T14 were analysed by Student's *t* -test (n = 5). A Pearson's product–moment correlation (Zar 2010) was used to compare normalised expression data of *DcAOX1 versus DcAOX2a*. All statistical analyses were performed using the STATISTICA 8.0 statistical package (StatSoft Inc., Tulsa, USA). Significance levels were set at $P<0.05$.

Table 1. Primers used in RT-sqPCR and RT-qPCR.

Gene	[NCBI accession ID]	Primer sequence (5'→3')		AS (bp)	*E* (%) (r^2)	
					PCS	CE
DcEF1α	[GenBank:GQ380566]	Fw	TGGTGATGCTGGTTTCGTTAAG	75	97.0 (0.996)	97.7 (0.996)
		Rv	AGTGGAGGGTAGGACATGAAGGT			
DcAOX1	[GenBank:EU286573]	Fw	CTTCAACGCCTACTTCCTTG	196	99.2 (0.996)	87.7 (0.994)
		Rv	ATCTCGCAATGTAGAGTCAGC			
DcAOX2a	[GenBank:EU286575]	Fw	TCTTCAATGCTTTCTTTGTTCTT	200	92.9 (0.993)	87.7 (0.992)
		Rv	GACATCTTTTAGTTTGGCATCTTT			
DcAFP	[Genbank:AJ131340]	Fw	CGACAAGCAAGC TTTACT CCAA	80	-	94.1 (0.992)
		Rv	CGTCTGACACCCATGAGTCTGT			

Amplicons size. (AS); primers efficiency (*E*) and regression efficiency (r^2) for the primary culture experiment (PCS) and chilling experiments (CE).

3.2.4. *DcAOX1* gene isolation

Plant material

Seeds of *D. carota* L. cv. Rotin were germinated *in vitro* in MS basal media (Murashige and Skoog 1962) and maintained under controlled conditions (25 ± 1 °C, 16 h photoperiod with 34 $\mu molm^{-2}s^{-1}$ light intensity). Eight-week-old *in vitro* grown seedlings were used for genomic DNA (gDNA) and total RNA extraction. For gene identification, mixtures of several plants were used; for complete gene isolation at gDNA and cDNA level, single plants were taken.

Identification of DcAOX1

gDNA extraction was performed using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Degenerate primers (*P1/P2*, annealing at 60 °C for 2 min and extension at 72 °C for 2 min, Table 2), previously designed based on *A. thaliana* (Saisho et al. 1997) were used for *AOX* gene identification. PCR was performed with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) using 10 $ng \cdot \mu L^{-1}$ of gDNA as template and 0.2 µM of each primer.

Isolation of DcAOX1 complete sequence

To determine the 5' and 3' ends of the isolated *DcAOX1* fragment, 5' and 3' RACE-PCRs were performed. Total RNA was isolated using RNeasy Plant Mini Kit (20) (Qiagen, Hilden, Germany), with on-column digestion of DNA with the RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacture's protocol.

For 3'RACE-PCR, single cDNA strand was produced using the enzyme RevertAid^{Tm}HMinus M-MuLV Reverse (Fermentas, Ontario, Canada), with oligo (dT) primer *VIAL 8* (Roche, Mannheim, Germany) (Table 2), according to the manufacturer's instruction. For the synthesis of the second cDNA strand and subsequent 3' end amplification, the reverse

primer *VIAL 9* (Roche, Mannheim, Germany) (Table 2) was used in combination with gene-specific forward primer (*DcAOX1Fw*, annealing at 55 °C for 30 s, extension 72 °C for 60 s, see Table 2) designed based on previously isolated *AOX1 P1/P2* sequence. One µL of a 1:10 cDNA dilution of first strand PCR product was used as template for amplification.

To isolate the 5' end, a cDNA library of *D. carota* L. cv. Marktgaertner M853 (kindly provided by Dr. Bettina Linke, Humboldt University of Berlin, Germany) cloned into a Lambda gt22a phage vector (Invitrogen, Karlsruhe, Germany) was generated (Linke et al. 2003). 5' RACE-PCR was carried out using 1 µL of cloned library as template and vector specific forward primer *P6* (Table 2), combined with a gene-specific reverse primer designed based on the sequence previously isolated with *P1/P2 (DcAOX1R*, annealing at 55 °C for 30 s and extension at 72 °C for 2 min, see Table 2).

Table 2. Primers used for *DcAOX1* gene isolation.

Primer name	Sequence (5'→3')
P1	CTGTAGCAGCAGTVCCTGGVATGGT
P2	GGTTTACATCRCGRTGRTGWGCCTC
VIAL 8	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV
VIAL 9	GACCACGCGTATCGATGTCGAC
DcAOX1Fw	GCAAGTCACTCAGGCGCTTTG
P6	CGCGGAAGAAGGCACATGGCTGAATA
DcAOX1R	ATCTCGCAATGTAGAGTCAGCC
DcAOX1_25Fw	ATTTCTGGTACATTTTAGTTTTGA
DcAOX1_1304Rev	CATGGTTTGACGAGGGATTT
DcAOX1_24Fw	AAAATAACAATGATGATGACACG
DcAOX1_1032Rv	AACCAGAGATTCCTCCACTTCA

V = A, C or G; R= A or G; W=A or T

RACE-PCRs were performed using mix of 0.5 U of *Taq* DNA polymerase (Thermo Scientific, Wilmington, DE, USA) with 1X manufacturer supplied $(NH4)_2SO_4$ buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs (Fermentas, Ontario, Canada) and 0.2 µM of each primer.

For complete gene (cDNA) isolation, a gene-specific primer set (*DcAOX1_25Fw* and *DcAOX1_1304Rv*, annealing at 52 °C for 20 s and extension at 72 °C for 2 min, Table 2) was designed based on 5' and 3' UTR sequences previously isolated with RACE-PCRs. For gDNA complete gene isolation, another gene-specific primer set (*DcAOX1_24Fw* and *DcAOX1_1032 Rv*, annealing at 64 °C for 30 s and extension at 72 °C for 2 min, Table 2) was designed. Ten $ng \cdot \mu L^{-1}$ of gDNA and a 1:10 cDNA dilution from a single plant were used as templates. PCRs were performed using Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland)

according to the manufacturer's instruction, using 0.2 µM of each specific primer. All PCR products were analysed in 1.4 % (w/v) agarose gel.

Cloning and sequence analysis

All PCR fragments were purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, England) according to the manufacture's protocol, and cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA). Reaction products were transformed to *E. coli* JM109 (Promega Madison, WI, USA) competent cells. Plasmid DNA was extracted from putative recombinant clones (Bimboim and Doly 1979) and confirmed by using *Eco*RI restriction enzyme (Fermentas, Ontario, Canada). Selected recombinant clones were sequenced (Macrogen, Seoul, Korea: www.macrogen.com) using *T7* and *SP6* primers (Promega, Madison, WI, USA). Sequence homology was confirmed in NCBI GenBank database (National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov/) using BLASTn and BLASTp algorithm (Karlin and Altschul 1993).

SeqMan and EditSeq softwares (LASERGENE 7, GATC Biotech, Konstanz) were used to edit the obtained *AOX* sequence. Phylogenetic studies included data retrieved from public web-based databases, freely available (NCBI: http://www.ncbi.nlm.nih.gov/), Plaza (http://bioinformatics.psb.ugent.be/plaza/); e!EnsemblPlants (http://plants.ensembl.org /Multi/Search/New?db=core) and IPK Barley Blast Server: (http://webblast.ipk-gatersleben.de/barley/). Non-annotated *AOX* sequences used for phylogenetic studies were previously identified (Cardoso et al. 2015).

Sequences were aligned using MAFFT software (online version: http://mafft.cbrc.jp/alignment/server/) under the model G-INS-1 (Slow; progressive method with an accurate guide tree), all other variables left as default. The best-fit model of amino acid replacement was selected by Akaike Information Criterion (AIC) according to the software ProtTest (Darriba et al. 2011). The selected model of protein evolution (probability of change from a given amino acid to another over a period of time, given some rate of change) was JTT+I+G [the JTT empirical model (Jones et al. 1992), considering an invariable fraction of amino acids (+I) and assigning each site a probability to belong to a different category of rate change (Darriba et al. 2011)]. Phylogenetic reconstruction was done through maximum likelihood as implemented in the software MEGA v.6, under the above referred model, and bootstrapped with 1000 replicates. The phylogenetic tree was rooted with *Chlamydomonas reinhardtii* alternative oxidase (*AOX1*) sequence.

MITOPROT software (Claros and Vincens 1996) was used to predict mitochondrial targeting sequence and cleavage site. Gene draw was performed in FancyGene 1.4 (Rambaldi

and Ciccarelli 2009). For intron identification, the Spidey software (http://www.ncbi.nlm.nih.gov/spidey/) was used.

3.3. Results

3.3.1. Calorespirometry in primary cultures

Fig. 1 shows the results for $R_{struct\_biomass}$ calculated from calorimetrically measured R_q and R_{CO2}, from day 0 to day 21 after inoculation. An increase on $R_{struct\_biomass}$ could already be observed at day 2 in all PCS, and in most cases reached a maximum at day 4. In PCS1 and PCS4 grown at 21 °C the peak on $R_{struct\_biomass}$ is reached at day 7, since the speed (slope when a linear regression is fitted between two data points) of increase is slower.

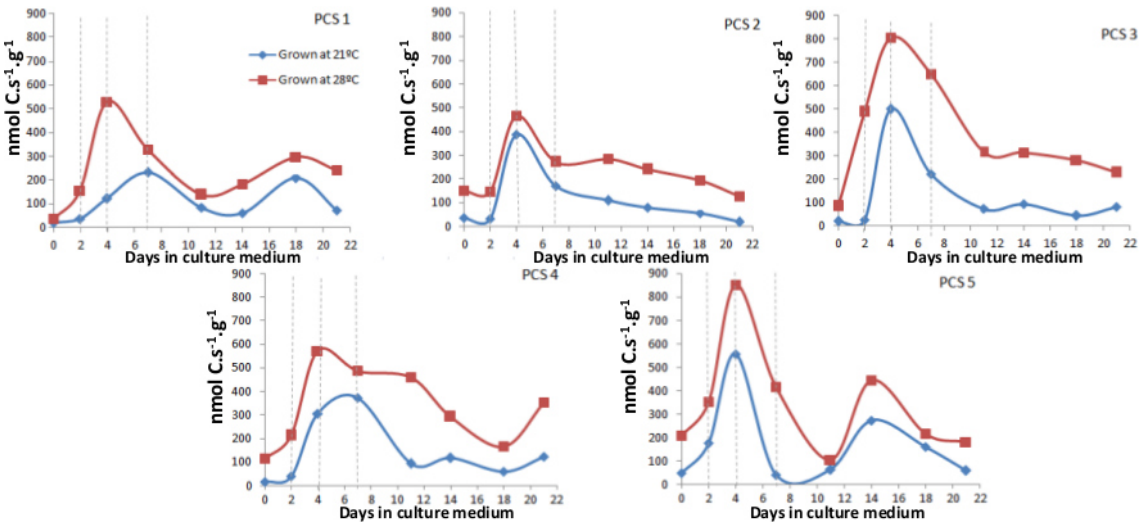


Fig. 1. $R_{structural\ biomass}$ in *callus* grown at 21 °C and 28 °C in primary cultures during 21 days. Each data point represents the average of 3 measurements performed using 200-300 mg of *callus.*

3.3.2. Expression of carrot *AOX* genes

PCS de novo differentiation

The growth curve and aspect of *callus* along the 28 days of *in vitro* culture can be observed in Suppl. Fig. 1. During the first 8 days in culture (lag-phase), growth of the explants was not visible. Then, exponential cell division started and *callus* proliferate until 28 days. During growth, *callus* lost the original orange colour of the explants and progressively acquired a green colour (Suppl. Fig. 1).

Transcript levels for both *AOX* genes were found to slightly increase from the early beginning (4 hpi) of the lag-phase until 36 hpi (Fig. 2). The time points showing highest level of transcripts (P>0.05) were 4 hpi until 4 dpi for *DcAOX1*, and 8 hpi until 36 hpi for *DcAOX2a*.

However, it was also observed that the timings of higher or lower expression were somewhat unphased between individual explants (not shown), thus reducing the possibility of observing significant differences between time points. At 4 dpi (lag phase), while the level of expression was still high for *DcAOX1*, *DcAOX2a* was already down regulated to values near the ones measured at 0 hpi. At the end of the lag phase and at initiation of exponential growth (8 dpi), expression of both genes achieved the lowest levels and remained relatively stable until 28 dpi, with values similar to the original, quiescent tissue (0 h). Expression patterns of *DcAOX1* and *DcAOX2a* significantly correlated (*P*=0.01).

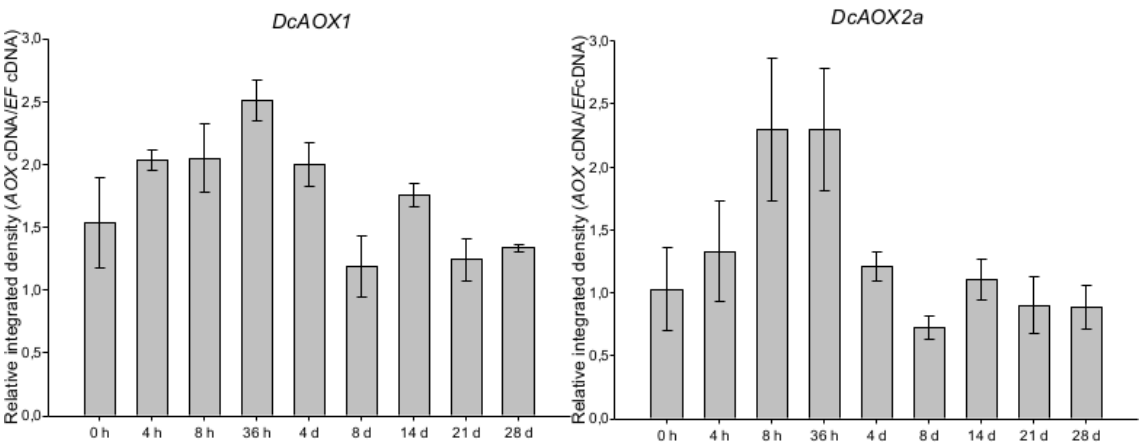


Fig. 2. Transcript levels of *DcAOX1* and *DcAOX2a* in primary cultures from secondary phloem of carrot roots. Samples were collected at 0 h, 4 h, 8 h, 36 h, 4 days, 8 days 14 days, 21 days and 28 days post *in vitro* inoculation. Cultures were maintained at 21 °C. Transcript levels were analysed by RT-sqPCR using *Elongation factor 1 alpha (DcEF1α*) as reference gene. Normalisation of the quantity of *DcAOX* transcripts was performed through the ratio of integrated densities *DcAOX* cDNA and *DcEF1α* cDNA bands. Data are the mean values ± SE of four individual plants.

PCS response to temperature

Fig. 3 shows expression levels for both *DcAOX* genes during exponential growth, at 14 dpi (T14), at two different growing temperatures (21 °C and 28 °C), in 5 explants with independent origins. *DcAOX1* was significantly higher expressed (5-fold) at 28 °C than at 21 °C (*P*<0.05). No significant differences were observed for *DcAOX2a* between temperatures, and this gene showed low expression levels at both temperatures (Fig. 3). These five plants had been selected for these studies on *AOX* gene expression variation from a larger group of 12 plants, because they showed variation in growth (Table 3). *Callus* FWs of explants were taken per single plants, grown at 21 °C and 28 °C (Table 3). As expected, temperature influenced *callus* FWs. At 28 °C, the mean value for FWs exceeded significantly the achieved at 21 °C (Table 3). A clear genotype effect was visible, allowing thus identification of temperature-

dependent growth variants. From 12 plants used, 8 showed significantly higher production of *callus* biomass at 28 °C, 3 did not show significant differences between both temperatures and one even had a significantly higher *callus* FW at 21 °C.

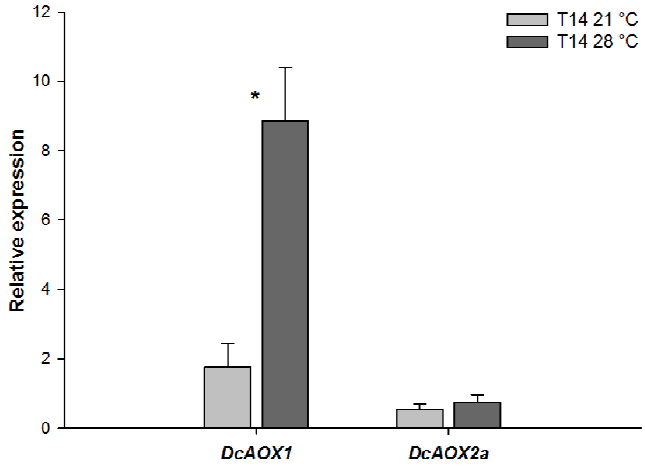


Fig. 3. Transcript levels of *DcAOX1* and *DcAOX2a* in primary cultures derived from secondary phloem of carrot roots grown at different temperatures. The expression was normalised with *Elongation factor 1 alpha* (*DcEF1α*). T14-21 °C: explants after 14 days in culture, growing at 21 °C; T14-28 °C: explants after 14 days in culture, growing at 28 °C. T0 (explants before inoculation) was used as calibrator. Data are the mean values ± SE of five individual plants. Student's *t* test was applied to test differences between temperatures for each gene. Significant differences are marked with *.

The five selected plants revealed the following variation in growth (Table 3): two showed significantly higher *callus* biomass at 28 °C (R2 and R5), two had no significant differences between both temperatures (R1 and R3) and one showed a significantly higher *callus* FW at 21 °C (R4). R3 was also characterised by low growth at both temperatures. However, concerning *AOX* transcript accumulation, at T14 during the exponential growth phase no direct link was detected between *callus* FW and individual *DcAOX1* or *DcAOX2a* transcript accumulation.

Table 3. *Callus* fresh weight (FM) from a primary culture system of *Daucus carota* L. cv. Rotin at exponential growth phase, grown at 21 °C or 28 °C.

Plant number	*Callus* fresh weight (mg)				
	n	28 °C	n	21 °C	
1	53	42.4	50	39.9	**R1**
2	47	64.1***	55	24.8	
3	76	62.2***	62	26.3	
4	35	28.7	38	25.5	
5	64	55.0***	77	21.4	**R2**
6	34	28.5***	36	9.0	
7	65	25.7	61	23.6	**R3**
8	62	28.0	65	32.8*	**R4**
9	60	21.8***	60	14.6	
10	57	59.0***	55	33.5	**R5**
11	40	51.5***	45	27.0	
12	38	82.3***	49	32.3	
mean plant/temperature		45.5***		26.0	

Due to their different growth behaviour, R1, R2, R3, R4 and R5 were the plants selected for the RT-qPCR expression analysis.
n: number of obtained *callus* from each plant. Differences between *callus* FW in the two temperatures were analysed by Student's *t* test, and separatly for each root. Significant differences between temperatures are indicated by *($p < 0.05$) or *** ($p < 0.001$).

Plant response to chilling

One month old carrot seedlings exposed to 4 °C for five days showed a similar induction pattern between both *DcAOX1* and *DcAOX2a* (Fig. 4). The level of transcripts detected in *DcAOX1* was clearly higher than that of *DcAOX2a*. Expression levels of *DcAOX1* were high from day 1 to day 3 and decreased from day 4 to 5.

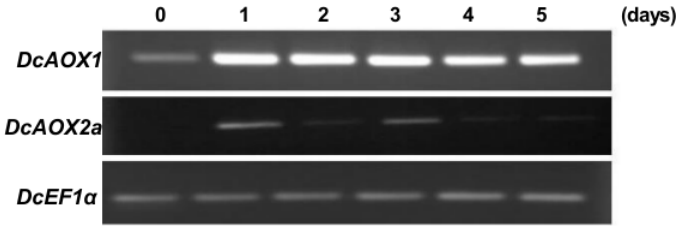


Fig. 4. Transcript levels of *DcAOX1* and *DcAOX2a* genes in leaves of *D. carota* cv Rotin seedlings exposed to chilling (4 °C) for five days. *Elongation factor 1 alpha* (*EF1α*) was used as reference gene and the gel profiles are representative of three independent RT reactions from 3 biological replicates.

When RT-qPCR analysis was performed with a higher time resolution until 24 hpce, both *AOX* genes showed very early responses to chilling exposure, since significantly higher mRNA levels were found at 45 min comparing to 0 hpce ($P < 0.05$) (Fig. 5). In case of *DcAOX1*, an increase was observed immediately after 10 min of exposure with 1.2-fold difference in RE, followed by a 1.5-fold increment after 45 min. A slight transcript level reduction was observed, followed by a significant increase until 24 hpce ($P < 0.05$) (Fig. 5). *DcAOX2a* increased 2.4-fold at 45 min of cold exposure relatively to 0 hpce ($P < 0.05$) (Fig. 5). Transcript levels of *DcAOX2a* then decreased, showing constant levels until the 24 h of the recovery phase. By 48 h of recovery, a further reduction in mRNA levels was observed (Fig. 5). Expression patterns of *DcAOX1* and *DcAOX2a* significantly correlated ($P < 0.001$).

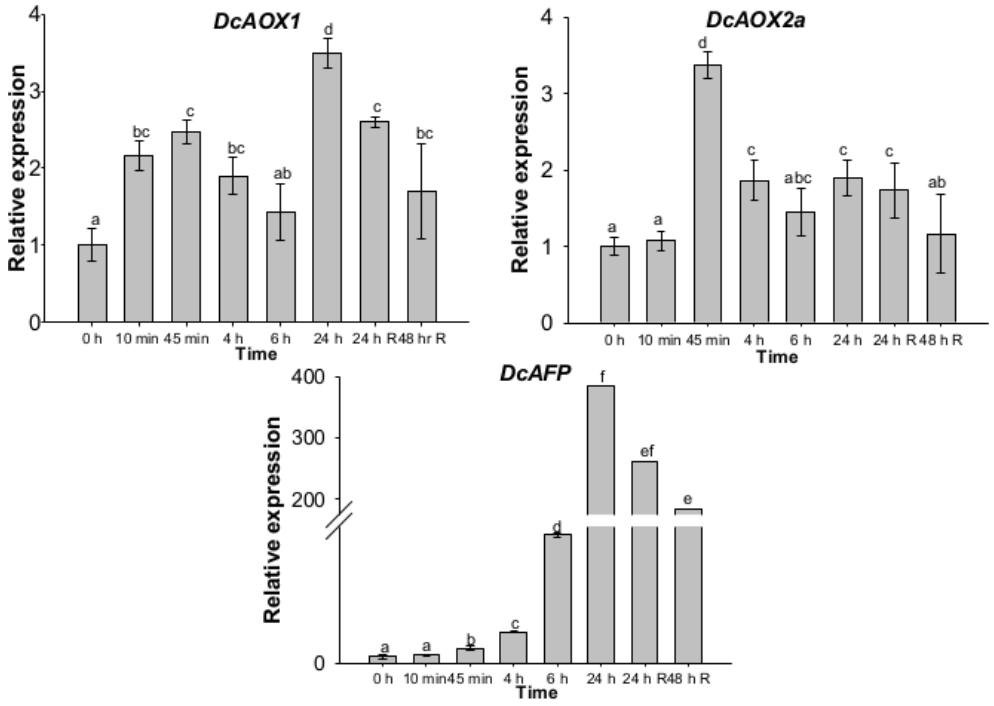


Fig. 5. Transcript levels of *DcAOX1*, *DcAOX2a* and *DcAFP* during chilling exposure in one-month-old carrot (*Daucus carota* L. cv. Rotin) seedlings. Samples were harvested at 0 hpce (immediately before chilling exposure) and after 10 min, 45 min, 4 h, 6 h, and 24 h of exposure to at 4 °C and after 24 h and 48 h after transferring plants back to the initial growth conditions (recovery period). Expression data was analysed by RT-qPCR using *Elongation factor 1 alpha* (*DcEF1α*) as reference gene. Data are the mean values ± SE of four plants considered per time point. Statistical analysis (one-way ANOVA with Tukey's post hoc test) was applied to each gene separately. Different superscript letters indicate significant differences between sampling points.

Compared to both *AOX* genes, cold-responsive gene *AFP* in carrot showed a later but much higher level of transcripts in plants subjected to chilling stress. After 10 min and 40 min

of cold stress, the increase was only of 0.15 and 1.17-fold difference in RE from the 0 hpce respectively (Fig. 5). However, *DcAFP* expression then highly increased, particularly at 24 hpce, showing an almost 400-fold difference comparing to 0 hpce (Fig. 5).

3.3.3. Analysis of complete *DcAOX1* sequence

A single band of expected size (ca. 450 bp) was obtained with primer combination P1/P2 and identified as *D. carota AOX1* based on high similarity with *AOX* gene sequences from other plant species available at NCBI database. Sequenced clones obtained were of 444 bp, and showed similarity between 75 % and 99 % with *AOX* from different plant species. For 5' end isolation, reverse specific primer was used in combination with vector specific primer, which led to the amplification of a fragment near 1000 bp. For 3' end isolation, the use of a forward specific primer in combination with the oligo d(T) primer, led to the amplification of fragments between 670-827 bp (described below). Based on match of 5' and 3' UTR sequences with initial partial exon 3 sequence, *in silico* full-length cDNA of *D. carota AOX1* (*DcAOX1*) was predicted.

At genomic level, *DcAOX1* of *D. carota* L. cv. Rotin has 1812 bp, consisting of three exons (exon 1: 432 bp, exon 2: 489 bp and exon 3: 57 bp) interrupted by two introns (intron 1: 630 bp and intron 2: 173 bp). Gene structure of *DcAOX1* and structure of previously identified *AOX1* genes in several other plant species are shown in Suppl. Table 1. At transcript level, it has 1366 bp in length (Fig. 6) with a continuous open reading frame (ORF) of 981 bp, which encodes a putative polypeptide of 326 amino acid residues. The homologous identity score performed in NCBI with deduced amino acid residues showed that *DcAOX1* shares a high degree of similarity with AOX1 proteins from other plant species such as *Nicotiana benthamiana* (78 %), *Brassica juncea* (73 %), *Gossypium hirsutum* (72 %) and *Arabidopsis lyrata* (70 %). Different lengths of 3' UTR were identified on *DcAOX1* cDNA sequences (3' end isolation), varying between 185 to 344 bp (data not shown). The *in silico* sequence presented in Fig.6 was the longest reconstructed sequence.

DcAOX1 revealed structural features usually found in AOX1 sub-family members, with highly variable N-terminal region. DcAOX1 also showed two conserved cysteines (CystI and CystII) and di-iron-binding sites (Fig. 7). DcAOX1 protein was predicted to be localized in mitochondria (mTP score of 0.868). The predicted length of the cleavage site of the mitochondrial targeting sequence (from the beginning of the protein) is 14 amino acids. Prediction of mitochondrial transit peptide for sequences used in the alignment of Fig. 7 shows no conservation across protein sequences and species, with DcAOX1 presenting the smallest predicted length. AOX1 sequences from *O. sativa* showed a predicted length of the

mitochondrial targeting peptide of 67 (BGIOSGA008063), 58 (BGIOSGA005788), 54 (BGIOSGA014422) or 51 (BGIOSGA014421) amino acids. *A. thaliana* AOX1 predicted length of mitochondrial targeting peptide displayed 52 (AT3G22360), 63 (AT3G22370), 53 (AT3G27620) or 50 (AT1G32350) amino acids.

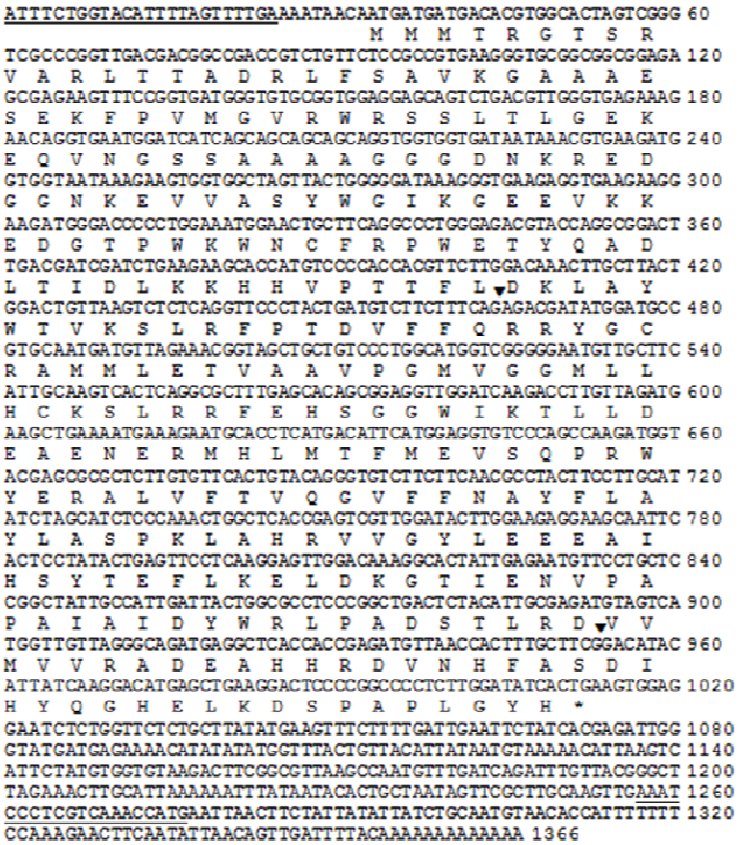


Fig. 6. Nucleotide and deduced amino acid sequences of cDNA encoding *Daucus carota* L. cv. Rotin *AOX1* (*DcAOX1)* (NCBI accession number EU286573.2). Underlines indicate location of primers used for amplification of complete gene sequence. The sites of introns are indicated by filled triangles, and * indicates stop codon.

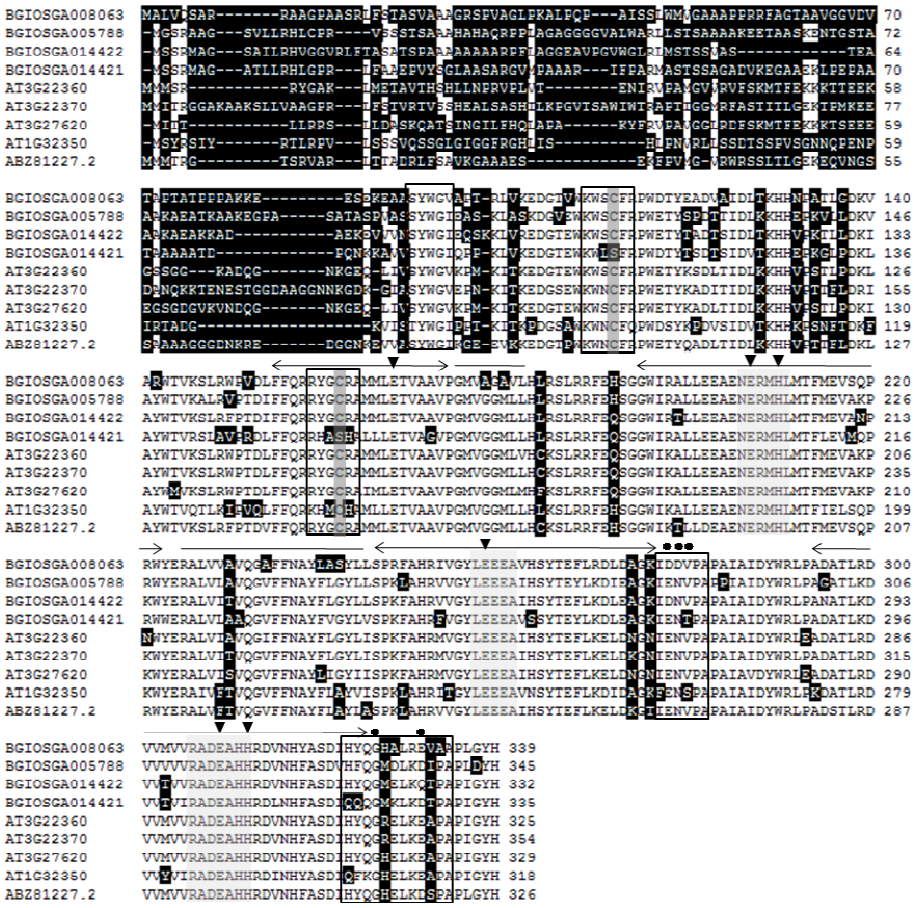


Fig. 7. Multiple alignment of previously reported AOX1 proteins from *Oryza sativa* (BGIOSGA008063, BGIOSGA005788, BGIOSGA14422, BGIOSGA14421), *Arabidopsis thaliana* (AT3G22360, AT3G22370, AT3G27620, AT1G32350) (Cardoso et al. 2015) and DcAOX1 (ABZ81227.2). Data retrieved from public web-based databases, freely available (Plaza: http://bioinformatics.psb.ugent.be/plaza/; e!EnsemblPlants: http://plants.ensembl.org/Multi/Search /New?db=core; and NCBI: http://www.ncbi.nlm.nih.gov/). Amino acids residues differing are shown on a black background, deletions are shown by minus signs. The sites of two conserved cysteins (CysI and CysII) involved in dimerization of the AOX protein by S–S bond formation (Umbach and Siedow 1993) are indicated in dark grey boxes. Amino acid in light grey boxes are three regions defined by Berthold et al. (2000) as highly conserved in AOX. E (glutamate) and H (histidine) amino acids residues involved in iron-binding are indicated by filled triangles. Black boxes indicate the structural elements proposed to influence AOX regulatory behaviour (Crichton et al., 2005), residues potentially involved in regulation of AOX activity are indicated by filled circles. Helical regions assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund 1999; Berthold et al. 2000) are shown by two-headed arrows above the amino acid sequences. Possible membrane-binding domains center (Andersson and Nordlund 1999; Berthold et al. 2000) are shown with a line above amino acid sequences. The peptide sequences presented in this figure refer to the ORF translation of the sequences given in Fig. 6.

The identified *D. carota AOX1* sequence clearly nests within the AOX1 clade, and within the eudicots (Fig. 8). Indeed, AOX1 sequences could be separated into two different groups: one including all eudicots sequences and other with the monocots (Fig. 8A). Both within eudicots and monocots, the AOX1d clade was identified although not in an ancestral position (see Costa et al. 2014 for details). Within the eudicots, the Solanaceae, the Brassicaceae and the Fabaceae formed distinct monophyletic groups (Fig. 8B).

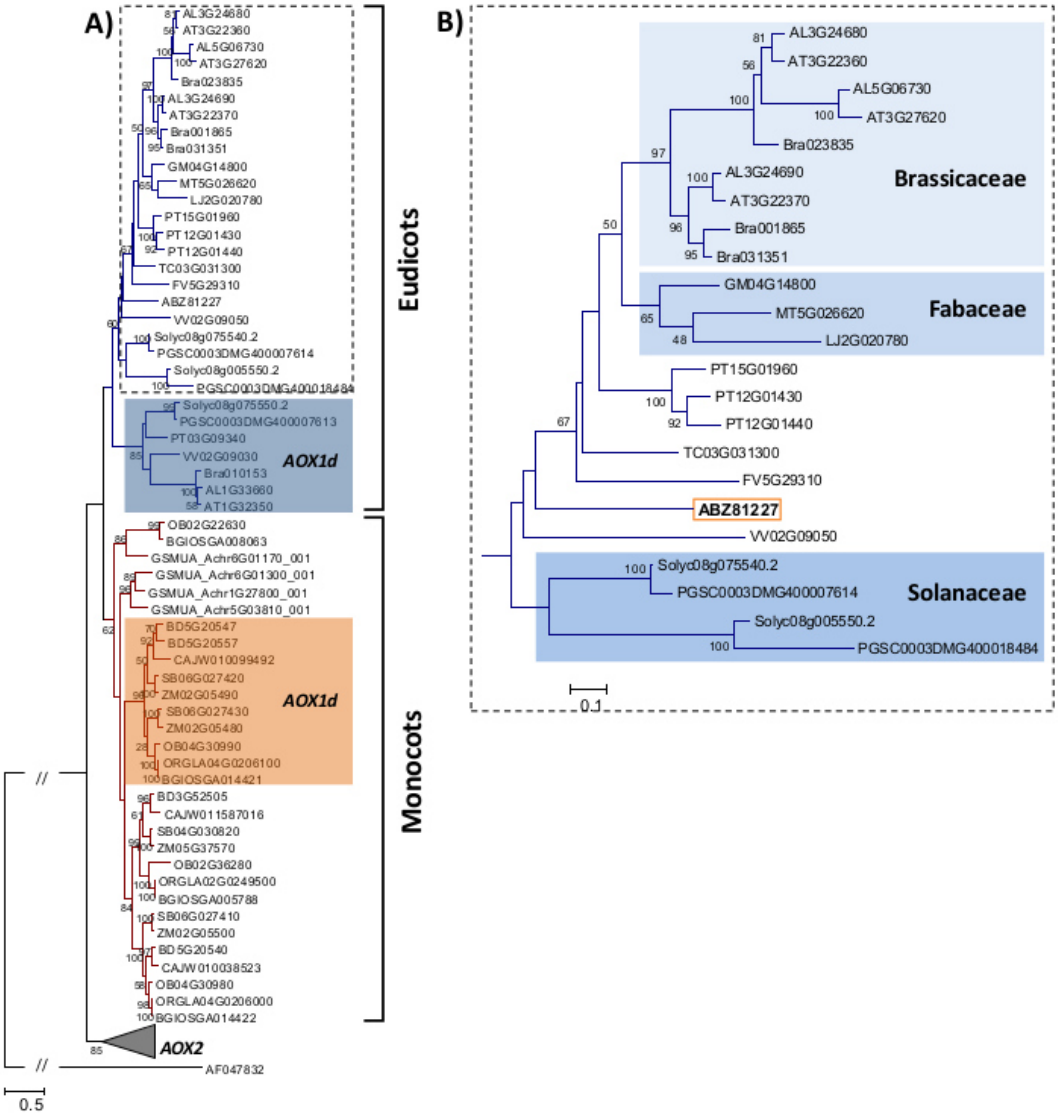


Fig. 8. A) Maximum likelihood (ML) tree showing the relationships among translated amino acid sequences of previously reported AOX1 proteins from plants, including the DcAOX1 sequence (ABZ81227.2) of *D. carota* L. cv. Rotin. Phylogeny reconstruction was done following the parameters described in the Material and methods section. Data retrieved from public web-based databases, freely available (Plaza: http://bioinformatics.psb.ugent.be/plaza/; e!EnsemblPlants: http://plants.ensembl.org /Multi/Search/New?db=core; and NCBI: http://www.ncbi.nlm.nih.gov/). *AOX* sequences were annotated by Cardoso et al. (2015) and *AOX1* sequences are identified in Suppl. Table 1. B) AOX eudicot clade, without AOX1d representatives, showing the position of DcAOX1 sequence (ABZ81227.2).

3.4. Discussion

3.4.1. *AOX*, cell reprogramming and temperature-dependent growth

Cell reprogramming upon external stress initiates cascades of events including dedifferentiation and *de novo* differentiation (see Nagl 1987; 1989; 1992; Arnholdt-Schmitt 2004; Fehér 2015 and references there in; Grafi and Barak 2015). Dedifferentiation can be rapidly induced as shown by severe stress through protoplastation (within 24 hours) (Fehér 2015 and references therein). Since its beginning, plant tissue culture has substantially contributed to the current understanding of inducibility of differentiation events and the role of associated stress (e.g.Bassi 1990; Arnholdt-Schmitt 2001; Zavattieri et al. 2010; Grafi et al. 2011). The carrot PCS used in our experiments was established originally by Steward et al. (1952) to study mechanisms of growth, and was later improved and maintained as an experimental system for studies on cell reprogramming (see review in Arnholdt-Schmitt 1993b; 1999). In PCS, tissue dedifferentiation is induced in cells from quiescent secondary phloem followed by *callus* growth initiation, mainly due to cell divisions (Arnholdt-Schmitt 1993b). Cells from secondary phloem are quiescent adult cells that recently had developed by switching fate from meristem to phloem cells. The cambial root cells are considered target cells for both yield formation and environmental responses (Arnholdt-Schmitt 1999). New meristem in the *callus* are unregularly distributed across the explant, beneath the periphery. Such cell fate switching can happen via stress-induced endogenous hormone regulation directly in perivascular stem cells, or indirectly via dedifferentiation in differentiated, competent cells, as it was shown for the well-studied process of somatic embryogenesis (e.g. Grieb et al. 1997).

The efficiency by which cell reprogramming can occur is of special interest, as this process is important for applied systems such as breeding or commercial propagation. In a given system, efficiency might be limited during phase of induction or during initiation, or both. Fehér (2015) pointed that genetic differences for efficiency are more likely to be found during initiation. The usefulness of calorespirometry to study morphogenic responses (i.e. cell reprogramming) in *in vitro* cultures was first demonstrated by Kim et al. (2006) and later, this system was also used by Nogales et al. (2013) in *D. carota* cv. Rotin PCS to study temperature dependent growth performance at 21 and 28°C. In their study, an early peak around day 4 for $R_{struct\_biomass}$ was observed, which was coincident with the cell reprogramming process that happens in this system. In the present work, using this same system, we studied potential genotype differences on the early increase of $R_{struct\_biomass}$ associated with cell reprogramming process, and we found that the peak appeared homogeneously in all five tested PCS. However,

the different slopes found in the curve of $R_{struct\_biomass}$ from day 0 to day 4 were indeed plant dependent.

AOX genes seem to have a role during earliest events of cell reprogramming upon environmental changes (Arnholdt-Schmitt et al. 2006). For somatic embryogenesis, Frederico et al. (2009a) showed an early expression of *AOX* genes during initiation of the embryonic development ('realisation phase' after depletion of auxin from the medium), being *DcAOX1* gene the one responding at higher level than *DcAOX2a*. In olive microshoots that were induced to rooting, *callus* growth that originated from cells close to the xylem and preceded rooting could not be inhibited by salicylhydroxamic acid (SHAM), a known inhibitor of AOX, while in the same system at the identical plants and morphologic region root induction was suppressed by SHAM (Santos Macedo et al. 2012). This observation suggests that AOX is not required for *callus* growth *per se*. However, the induction of root organogenesis seemed to be linked to AOX activity. Therefore, a role for AOX may be seen in the early control of signaling and metabolic homeostasis for carbon and energy metabolism as a prerequisite for later balancing growth and development according to the available environmental conditions. This view confirms the proposal of Vanlerberghe (2013) and seem also to fit to the observations of Petrussa et al. (2009), who pointed the role of AOX as an anti-apoptotic factor in neighbour cells that have critical role for the reprogramming to somatic embryogenesis in *Abies alba* (see also Smertenko and Bozhkov 2014; Arnholdt-Schmitt et al. 2015b). In carrot PCS, our results suggest a role of *DcAOX1* and *DcAOX2a* genes during the first hours of induced *de novo* differentiation of secondary phloem explants. These genes showed a modest though consistent increase in transcript levels until 36 h after inoculation and subsequent down-regulation before the beginning of the exponential growth. Due to the *a priori* unknown high intrinsic variability of the explants, future experiments must however include a higher number of samples.

AOX has been shown to be especially active in meristematic tissues (Hilal et al. 1997) and several studies have indicated links between AOX activity and plant growth (Arnholdt-Schmitt et al. 2006; Vanlerberghe 2013 and references therein). Strong support for this view was found by experiments performed under various nutrient conditions in transgenic tobacco cells with silenced *AOX1a*. Sieger et al. (2005) demonstrated that *AOX1a* knockdown led to the incapacity of the cells for down-regulating growth under P- and N-deficiency, and concluded that AOX activity provides a mechanism for adjusting growth and counteracting nutrient imbalance. When maintaining the knockdown of *AOX1a*, tobacco cell growth was connected to more stable carbon use efficiency. Arnholdt-Schmitt et al. (2006) also hypothesized on the importance of considering down-regulation of *AOX* as a potential tool for molecular breeding

on higher nutrient efficiency. This led us to explore the hypothesis that differential *AOX* gene regulation relates to growth rates, with expected higher levels of *AOX* connected to suppressed growth or to lower growth rates. This seems to fit to our observation in PCS: during the lag phase of growth, particularly until 8 days, where we observed a dynamic behaviour of both *AOX* genes, but no increase in FW (although substantial amounts of nutrients were provided) were found. During lag phase, cells are thought to be prepared for the new fate and in PCS, first cells were reported to enter into the S-phase of cell cycling from 12 hpi to 24 hpi (Gartenbach-Scharrer et al. 1990). According to its known effect on cell homeostasis (Vanlerberghe et al. 2009; Vanlerberghe 2013), AOX could have contributed to suppressing growth during lag phase.

In the present work it was also investigated the effect of temperature on *AOX* expression during PCS exponential growth phase. Temperature is a major environmental constraint and can influence the molecular mechanisms controlling growth. Despite the generally higher growth in the PCS from 28 °C comparing to 21 °C, in the individual plants (Table 3), and being *DcAOX1* responsive to the highest growing temperature (Fig. 3), the expression of both *AOX* genes in each individual plant was not unambiguously related to *callus* FW (not shown).

In a first attempt to transpose these findings to plant level, we investigated both *AOX* genes in a chilling pot plant experiment, and compared it with the expression of the gene encoding the anti-freezing protein (*AFP*). Interestingly, the two *AOX* genes were co-regulated in both PCS and pot experimental systems, which is in agreement with previous findings (Campos et al. 2009; Van Aken et al. 2009; Vanlerberghe 2013). Clifton et al. (2005) analysed the response of plant cells from *A. thaliana* at 3 h and up to 24 h post exposure upon various treatments designed to induce abiotic stress, and identified alternative respiration pathway components as the most important ones for early responses. The components of normal respiration were shown to be more stable during early times of acclimation without pronounced variations in transcript abundance. Low temperature stress - either chilling or freezing - is one of the most important abiotic stresses, with plants showing reduced yield (Beck et al. 2004). Our results indicated *DcAOX1* as the highest/rapidly responsive *AOX* gene during cold stress in carrot (Fig. 4 and Fig. 5). Nevertheless, the patterns of transcript abundance over time course also revealed a further prevalent response of *DcAOX2a*, which was basal in control conditions (Fig. 4). In general, *AOX1* is reported as a stress-responsive gene, whereas *AOX2* sub-family members were considered during long time as housekeeping genes or related to developmental events (Considine et al. 2002). Later on, *AOX2* members were found also to be involved in plastid-dependent signalling (Clifton et al. 2005) and in

response upon several stress factors, including cold stress (Costa et al. 2010). *AOX2* stress response during chilling was also seen in the present study. In *A. thaliana*, Fiorani et al. (2005) reported a significantly lower leaf area in an *AOX1a* anti-sense line growing upon low temperature when comparing with the wild type. This phenotype was associated with reduced amount of *AOX* transcripts (almost entirely suppressed) and consequently low level of AOX protein. The authors suggested that the lower level of AOX1a protein could point to a reduced ability of the plant to cope with low temperature throughout the whole vegetative growth period. Taken these results together, it can be concluded that differential expression and co-regulation of diverse *AOX* family member genes might contribute to fine-tuning the metabolic-physiological cell responses upon stress towards deciding for growth and/development.

3.4.2. *DcAOX1* sequence and phylogenetic analyses

Plant plasticity allows coping with stressful environmental conditions. Rapid acclimation and adaptation are desired plant characteristics, and target traits for which we aim to develop functional markers. It was thus our interest to look for genetic variability in a target gene, which could be linked with the desired trait. In this frame, the existence of polymorphisms in *AOX* gene sequences (alleles, haplotypes) is an essential basis for association studies to find links to achieve breeding goals (Arnholdt-Schmitt 2015). Complete information about gene sequences is essential, since it is known that important/relevant differences between genotypes often occur not only in the coding region, but also in introns or UTRs. *DcAOX2a* gene isolation and variability in intronic regions among genotypes was already described (Campos et al. 2009; Cardoso et al. 2009). *DcAOX1* gene sequence and structural organization were still unknown and are here reported for the first time.

At transcript level, *DcAOX1* is 1366 bp in length, encoding a putative polypeptide of 326 amino acid residues. Variability on the *DcAOX1* 3' end was observed, ranging from 185 bp to 344 bp, with 294 bp as the average size. The involvement of alternative polyadenylation (production of mature transcripts with 3' ends of variable length) in oxidative stress response in plants has been pointed out (Xing and Li 2011). *AOX* genes members present variability at the 3' end, with a length ranging between 111-313 bp in maize (Polidoros et al. 2005) and between 76-301 bp in olive (Santos Macedo et al. 2009). In *AOX1* genes, previous transcript analysis revealed also the existence of length variation between *Arabidopsis* and *O. sativa* (Loke et al. 2005; Shen et al. 2008).

Analysis of the deduced amino acid sequence revealed structural features usually found in most of the higher plant AOXs (Fig. 6). The role of some of these residues in AOX activity have been previously studied using site-directed mutagenesis in plants and other

organisms such as protists, which revealed that many residues are critical for activity (Ajayi et al. 2002; Albury et al. 2002; Berthold et al. 2002; Crichton et al. 2005; Nakamura et al. 2005; Crichton et al. 2010; Moore et al. 2013). Multiple sequence alignment showed a highly variable N-terminus of AOX1 sub-family as a result of low similarity amongst exon 1 sequences. Lack of homology in mitochondrial targeting signals is common and typical for proteins which require N-terminal signals for mitochondrial import (Finnegan et al. 1997). Despite the variability in length of the transit peptide, its presence is vital for targeting the peptide to mitochondria.

The conserved cysteine residues assumed to be involved in post-translational regulation of AOX activity (Umbach and Siedow 1993; Rhoads et al. 1998; Grant et al. 2009) were also identified in DcAOX1. In some plant species, the conserved CysI in the N-terminal region of protein is replaced by SerI (Umbach and Siedow 1993; Costa et al. 2009). This leads to regulation by succinate instead of pyruvate (Holtzapffel et al. 2003; Grant et al. 2009). Substitution of CysII by SerII can be observed in the alignment presented in the present work (Fig. 6). However, physiological consequences of such changes are still unknown. Within AOX1 family monocots can be separated from eudicots and within the last ones, some groups form separated clades highlighting their common evolutionary history. Differential regulation on *AOX* gene subfamilies described by several authors may come from different positions in the plant genomes related to chromosomal territories (Arnholdt-Schmitt 2004; Costa et al. 2009). The common distribution of *AOX* members is on at least two different chromosomes, at one gene per chromosome, occurring either in sense or antisense orientation (Cardoso et al. 2015). Furthermore, the presence of iron-binding motifs within four helical regions suggests that AOX might be a member of di-iron carboxylate protein family (Berthold et al. 2002; Berthold and Stenmark 2003; Moore et al. 2008). Four conserved α-helical regions rich in histidine and glutamate were identified in *DcAOX1,* involved in the formation of hydroxyl bridged binuclear iron center (Siedow et al. 1995).

The predominant structure of genomic *AOX* sequences, including also both carrot *DcAOX2* genes (*DcAOX2a* and *DcAOX2b*), consists of four exons interrupted by three introns at well-conserved positions (Campos et al. 2009; Cardoso et al. 2015). Genes sharing this structure usually show exon size conservation for last three exons (Campos et al. 2009). Although not conserved, exon 1 presents a size around 300 bp (Campos et al. 2009; Cardoso et al. 2015). This feature is responsible for the formation of similar AOX proteins across different plant species. However, in *DcAOX1* loss of intron 1 was identified (Suppl. Table 1). Hence, a fusion of exon 1 and 2 could have consequently resulted in increase in exon size (432 bp) as compared to the common size of around 300 bp for exon 1 in genes showing the 4 exon structure. Nevertheless, it was observed that the last two exons showed a conserved size of

489 bp and 57 bp respectively. Evolutionary intron loss and gain have resulted in the variation of intron numbers in some *AOX* members, with alterations in exons size (Considine et al. 2002; Polidoros et al. 2009; Cardoso et al. 2015) Suppl. Table 1. For instance, Cardoso et al. (2015) reported the existence of an *AOX* gene of *Oryza brachyantha* with six exons and five introns and showed the existence of a *Hordeum vulgare AOX* gene completely devoid of introns. Recent findings also showed the absence of introns in an *AOX* gene member of *Triticum urartu* (EnsemblPlants acc. nº TRIUR3_12374) (data not shown).

3.5. Conclusions

With this study, calorespirometry arises as a suitable technology for the identification of cell reprogramming events related to metabolic and respiratory activity in carrot and shows a great potential to be used for phenotyping yield stability in *in vitro* systems. Our results are comparable with previous results showing an early peak in structural biomass formation during the lag phase of growth in the PCS, and show that *DcAOX1* and *DcAOX2a* were co-expressed in the earliest events in cell reprogramming upon environmental changes (inoculation or chilling). *DcAOX1* responded also to a higher growing-temperature in the exponential phase of the PCS. For a better understanding of these results, the complete gene sequence of *DcAOX1* and its structural organisation were also analysed. High throughput genotype screening on complete *DcAOX1* and *DcAOX2a* genes could help on future identification of important within-gene motifs for co-regulation and differential transcript accumulation in view of novel resources for functional marker identification.

3.6. Acknowledgements

This work was funded by FEDER Funds through the Operational Program for Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the Strategic Project PEst-C/AGR/UI0115/2011 and PEst-OE/AGR/UI0115/2014. The authors are thankful to the Portuguese FCT - Foundation for Science and Technology for the support provided to MDC (SFRH/BD/65354/2009) and given under the program POPH – Programa Operacional Potencial Humano to BAS and HC (Ciência 2007 and Ciência 2008: C2008-UE/ICAM/06). TN was supported by a Marie Curie fellowship (FP7-PEOPLE-2012-CIG Project Reference 321725) and by the Portuguese Foundation for Science and Technology –FCT- (SFRH/BCC/52187/2013). We further acknowledge fellowships provided to SRK within the project PTDC/EBB-BIO/099268/2008 and to AN within the project PTDC/AGR-GPL/111196/2009. The authors want to thank Bettina Linke from Humboldt University, Berlin, Germany for providing the cDNA library used for gene isolation, and

appreciate language corrections and important comments by Catarina Campos (Universidade de Évora, Portugal).

3.7. References

Ajayi WU, Chaudhuri M, Hill GC (2002) Site-directed mutagenesis reveals the essentiality of the conserved residues in the putative diiron active site of the trypanosome alternative oxidase. J Biol Chem 277:8187–8193. doi: 10.1074/jbc.M111477200

Albury MS, Affourtit C, Crichton PG, Moore AL (2002) Structure of the plant alternative oxidase. Site-directed mutagenesis provides new information on the active site and membrane topology. J Biol Chem 277:1190–1194. doi: 10.1074/jbc.M109853200

Arnholdt-Schmitt B (1993a) Rapid changes in amplification and methylation pattern of genomic DNA in cultured carrot root explants (Daucus carota L.). Theor Appl Genet 85:793–800. doi: 10.1007/BF00225021

Arnholdt-Schmitt B (2015) From AOX diversity to functional marker development. In: Gupta KJ, Mur LAJ, Neelwarne B (eds) Alternative respiratory pathways in higher plants. John Wiley & Sons, Inc, Oxford,

Arnholdt-Schmitt B (1993b) Hormonsensitivitaet und genomische DNS - Variabilitaet in ihrem Bezug zur Ertragsbildung bei *Daucus carota* L. ssp. sativus : ein Beitrag zur Zellbiologie pflanzlichen Wachstums. Justus-Liebig-University Giessen, Germany

Arnholdt-Schmitt B (2004) Stress-induced cell reprogramming. A role for global genome regulation? Plant Physiol 136:2579–2586. doi: 10.1104/pp.104.042531

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, Costa JH, de Melo DF (2006) AOX - a functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11:281–287. doi: 10.1016/j.tplants.2006.05.001

Arnholdt-Schmitt B, Hansen LD, Nogales A (2015a) Calorespirometry, oxygen isotope analysis and functional-marker-assisted selection (’CalOxy-FMAS') for genotype screening: A novel concept and tool kit for predicting stable plant growth performance and functional marker identification. Brief Funct Genomics 1–6. doi: 10.1093/bfgp/elv008

Arnholdt-Schmitt B, Herterich S, Neumann KH (1995) Physiological aspects of genome variability in tissue culture. I. Growth phase-dependent differential DNA methylation of the carrot genome (*Daucus carota* L.) during primary culture. Theor Appl Genet 91:809–815. doi: 10.1007/BF00220964

Arnholdt-Schmitt B, Ragonezi C, Cardoso HG (2015b) Do mitochondria play a central role in stress-induced somatic embryogenesis? I*n vitro* embryogenesis in higher plants. Springer-Humana Press.

Arnholdt-Schmitt B, Valadas V, Doring M (2014) Functional marker development is challenged by the ubiquity of endophytes--a practical perspective. Brief Funct Genomics 1–6. doi: 10.1093/bfgp/elu049

Bassi P (1990) Quantitative variations of nuclear DNA during plant development: a critical analysis. Biol Rev 65:185-225

Beck EH, Heim R, Hansen J (2004) Plant resistance to cold stress: mechanisms and environmental signals triggering frost hardening and dehardening. J Biosci 29:449–459. doi: 10.1007/BF02712118

Berthold DA, Stenmark P (2003) Membrane-bound diiron carboxylate proteins. Annu Rev Plant Biol 54:497–517. doi: DOI 10.1146/annurev.arplant.54.031902.134915

Berthold DA, Voevodskaya N, Stenmark P, et al (2002) EPR studies of the mitochondrial alternative oxidase: Evidence for a diiron carboxylate center. J Biol Chem 277:43608–43614. doi: 10.1074/jbc.M206724200

Bimboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523. doi: 10.1093/nar/7.6.1513

Campos MD, Cardoso HG, Linke B, et al (2009) Differential expression and co-regulation of carrot AOX genes (*Daucus carota*). Physiol Plant 137:578–591. doi: 10.1111/j.1399-3054.2009.01282.x

Campos MD, Frederico AM, Nothnagel T, et al (2015) Selection of suitable reference genes for reverse transcription quantitative real-time PCR studies on different experimental systems from carrot *(Daucus carota* L.). Sci Hortic (Amsterdam) 186:115–123. doi: 10.1016/j.scienta.2014.12.038

Cardoso HG, Arnholdt-Schmitt B (2013) Functional Marker Development Across Species in Selected Traits. In: T Lübberstedt T & RK Varshney (ed) Diagnostics in Plant Breeding. Springer Netherlands, pp 467–515

Cardoso HG, Campos MC, Pais MS, Peixe A (2010) Use of morphometric parameters for tracking ovule and microspore evolution in grapevine (*Vitis vinifera* L., cv. “Aragonez”) and evaluation of their potential to improve in vitro somatic embryogenesis efficiency from gametophyte tissues. Vitr Cell Dev Biol - Plant 46:499–508. doi: 10.1007/s11627-010-9295-6

Cardoso HG, Campos MD, Costa AR, et al (2009) Carrot alternative oxidase gene *AOX2a* demonstrates allelic and genotypic polymorphisms in intron 3. Physiol Plant 137:592–608. doi: 10.1111/j.1399-3054.2009.01299.x

Cardoso HG, Nogales A, Frederico AM, et al (2015) Natural *AOX* gene diversity. In: Gupta KJ, M.L. NB (ed) Alternative respiratory pathways in higher plants., John Wiley. Oxford, pp 241–254

Claros MG, Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241:779–786. doi: 10.1111/j.1432-1033.1996.00779.x

Clifton R, Lister R, Parker KL, et al (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. Plant Mol Biol 58:193–212. doi: 10.1007/s11103-005-5514-7

Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? Plant Physiol 147:469–486. doi: 10.1104/pp.108.118117

Considine MJ, Holtzapffel RC, Day D a, et al (2002) Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol 129:949–953. doi: 10.1104/pp.004150

Costa JH, Cardoso HG, Campos MD, et al (2009) *Daucus carota* L. - An old model for cell reprogramming gains new importance through a novel expansion pattern of alternative oxidase (*AOX*) genes. Plant Physiol Biochem 47:753–759. doi: 10.1016/j.plaphy.2009.03.011

Costa JH, McDonald AE, Arnholdt-Schmitt B, Fernandes de Melo D (2014) A classification scheme for alternative oxidases reveals the taxonomic distribution and evolutionary history of the enzyme in angiosperms. Mitochondrion 19:172-183. doi: 10.1016/j.mito.2014.04.007

Costa JH, Mota EF, Cambursano MV, et al (2010) Stress-induced co-expression of two alternative oxidase (*VuAox1 and 2b*) genes in *Vigna unguiculata*. J Plant Physiol 167:561–570. doi: 10.1016/j.jplph.2009.11.001

Crichton PG, Affourtit C, Albury MS, et al (2005) Constitutive activity of *Sauromatum guttatum* alternative oxidase in Schizosaccharomyces pombe implicates residues in addition to conserved cysteines in α-keto acid activation. FEBS Lett 579:331–336. doi: 10.1016/j.febslet.2004.10.107

Crichton PG, Albury MS, Affourtit C, Moore AL (2010) Mutagenesis of the *Sauromatum guttatum* alternative oxidase reveals features important for oxygen binding and catalysis. Biochim Biophys Acta - Bioenerg 1797:732–737. doi: 10.1016/j.bbabio.2009.12.010

Darriba D, Taboada GL, Doallo R, Posada D (2011) ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27:1164-1165.

Fehér A (2015) Somatic embryogenesis - Stress-induced remodeling of plant cell fate. Biochim Biophys Acta 1849:385-402

Finnegan PM, Whelan J, Millar AH, et al (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. Plant Physiol 114:455–466. doi: 10.1104/pp.114.2.455

Fiorani F, Umbach AL, Siedow JN (2005) The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis *AOX1a* transgenic plants. Plant Physiol 139:1795–1805. doi: 10.1104/pp.105.070789

Frederico AM, Campos MD, Cardoso HG, et al (2009a) Alternative oxidase involvement in *Daucus carota* somatic embryogenesis. Physiol Plant 137:498–508. doi: 10.1111/j.1399-3054.2009.01278.x

Frederico AM, Zavattieri MA, Campos MD, et al (2009b) The gymnosperm *Pinus pinea* contains both AOX gene subfamilies, *AOX1* and *AOX2*. Physiol Plant 137:566–577. doi: 10.1111/j.1399-3054.2009.01279.x

Gartenbach-Scharrer U, Habib S, Neumann K-H (1990) Sequential synthesis of some proteins in cultured carrot explant (*Daucus carota*) cells during *callus* induction. Plant Cell Tissue Organ Cult 22:27–35.

Grafi G, Florentin A, Ransbotyn V, Morgenstern Y (2011) The stem cell state in plant development and in response to stress. Front Plant Sci 2:53.

Grant N, Onda Y, Kakizaki Y, et al (2009) Two cys or not two cys? That is the question; alternative oxidase in the thermogenic plant sacred Lotus. Plant Physiol 150:987–995. doi: 10.1104/pp.109.139394

Grieb B, Schafer F, Imani J, et al (1997) Changes in soluble proteins and phytohormone concentrations of cultured carrot petiole explants during induction of somatic embryogenesis (*Daucus carota* L.). J Appl Bot Bot 71:94–103.

Hilal M, Castagnaro A, Moreno H, Massa EM (1997) Specific localization of the respiratory alternative oxidase in meristematic and xylematic tissues from developing soybean roots and hypocotyls. Plant Physiol 115:1499–1503. doi: 10.1104/pp.115.4.1499

Holtzapffel RC, Castelli J, Finnegan PM, et al (2003) A tomato alternative oxidase protein with altered regulatory properties. Biochim Biophys Acta - Bioenerg 1606:153–162. doi: 10.1016/S0005-2728(03)00112-9

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282. doi: 10.1093/bioinformatics/8.3.275

Karlin S, Altschul SF (1993) Applications and statistics for multiple high-scoring segments in molecular sequences. Proc Natl Acad Sci USA 90:5873–5877. doi: 10.1073/pnas.90.12.5873

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. doi: 10.1006/meth.2001.1262

Loke JC, Stahlberg EA, Strenski DG, Haas BJ, Wood PC, Li QQ (2005) Compilation of mRNA polyadenylation signals in *Arabidopsis* revealed a new signal element and potential secondary structures. Plant Physiol 138: 1457-1468

Moore AL, Carré JE, Affourtit C, et al (2008) Compelling EPR evidence that the alternative oxidase is a diiron carboxylate protein. Biochim Biophys Acta - Bioenerg 1777:327–330. doi: 10.1016/j.bbabio.2008.01.004

Moore AL, Shiba T, Young L, et al (2013) Unraveling the heater: new insights into the structure of the alternative oxidase. Annu Rev Plant Biol 64:637–63. doi: 10.1146/annurev-arplant-042811-105432

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. doi: DOI: 10.1111/j.1399-3054.1962.tb08052.x

Nagl W (1987) Replication. Progr Bot 49:181-191

Nagl W (1989) Replication. Progr Bot 51:173-180

Nagl W (1992) Replication. Progr Bot 53:166-180

Nakamura K, Sakamoto K, Kido Y, et al (2005) Mutational analysis of the *Trypanosoma vivax* alternative oxidase: The E(X)6Y motif is conserved in both mitochondrial alternative oxidase and plastid terminal oxidase and is indispensable for enzyme activity. Biochem Biophys Res Commun 334:593–600. doi: 10.1016/j.bbrc.2005.06.131

Neumann K (1966) Wurzelbildung und nukleinsäuregehalt bei phloem-gewebekulturen der karottenwurzel auf synthetischem nährmedium. Congr Coll Univ Liege 38:96–102.

Nicotra AB, Atkin OK, Bonser SP, et al (2010) Plant phenotypic plasticity in a changing climate. Trends Plant Sci. 15:684–692.

Nogales A, Muñoz-Sanhueza L, Hansen LD, Arnholdt-Schmitt B (2013) Calorespirometry as a tool for studying temperature response in carrot (*Daucus carota* L.). Eng Life Sci 13:541–548. doi: 10.1002/elsc.201200197

Pérez-Clemente RM, Gómez-Cadenas A (2012) *In vitro* tissue culture , a tool for the study and breeding of plants subjected to abiotic stress conditions. Recent Adv Plant Vitr Cult 91–108. doi: 10.5772/52760

Petrussa E, Bertolini A, Casolo V, et al (2009) Mitochondrial bioenergetics linked to the manifestation of programmed cell death during somatic embryogenesis of *Abies alba.* Planta 231:93–107. doi: 10.1007/s00425-009-1028-x

Polidoros AN, Mylona P V, Pasentsis K, et al (2005) The maize alternative oxidase 1a (*Aox1a*) gene is regulated by signals related to oxidative stress. Redox Rep 10:71–78. doi: 10.1179/135100005X21688

Polidoros AN, Mylona P V., Arnholdt-Schmitt B (2009) *Aox* gene structure, transcript variation and expression in plants. Physiol Plant 137:342–353. doi: 10.1111/j.1399-3054.2009.01284.x

Rambaldi D, Ciccarelli FD (2009) FancyGene: Dynamic visualization of gene structures and protein domain architectures on genomic loci. Bioinformatics 25:2281–2282. doi: 10.1093/bioinformatics/btp381

Rasmusson AG, Fernie AR, Van Dongen JT (2009) Alternative oxidase: a defence against metabolic fluctuations? Physiol. Plant. 137:371–382.

Rhoads DM, Umbach AL, Sweet CR, et al (1998) Regulation of the cyanide-resistant alternative oxidase of plant mitochondria: Identification of the cysteine residue involved in α-keto acid stimulation and intersubunit disulfide bond formation. J Biol Chem 273:30750–30756. doi: 10.1074/jbc.273.46.30750

Saisho D, Nambara E, Naito S, et al (1997) Characterization of the gene family for alternative oxidase from *Arabidopsis thaliana*. Plant Mol Biol 35:585–596. doi: 10.1023/a:1005818507743

Santos Macedo E, Cardoso HG, Hernández A, et al (2009) Physiologic 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. doi: 10.1111/j.1399-3054.2009.01302.x

Santos Macedo E, Sircar D, Cardoso HG, et al (2012) Involvement of alternative oxidase (*AOX*) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism. Plant Cell Rep 31:1581–1590. doi: 10.1007/s00299-012-1272-6

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675.

Shen Y, Ji G, Haas BJ, Wu X, Zheng J, Reese GJ, Li QQ (2008) Genome level analysis of rice mRNA 3'-end processing signals and alternative polyadenylation. Nucleic Acids Res 36: 3150-3161

Siedow JN, Umbach AL, Moore AL (1995) The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett 362:10–14. doi: 10.1016/0014-5793(95)00196-G

Sieger SM, Kristensen BK, Robson CA, et al (2005) The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. J Exp Bot 56:1499–1515. doi: 10.1093/jxb/eri146

Smertenko A, Bozhkov P V. (2014) Somatic embryogenesis: Life and death processes during apical-basal patterning. J. Exp. Bot. 65:1343–1360.

Steward F, Caplin S MF (1952) Investigations on the growth and metabolism of plant cells. I. New techniques for the investigation on metabolism, nutrition and growth in undifferentiated cells.

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.

Van Aken O, Giraud E, Clifton R, Whelan J (2009) Alternative oxidase: a target and regulator of stress responses. Physiol. Plant. 137:354–361.

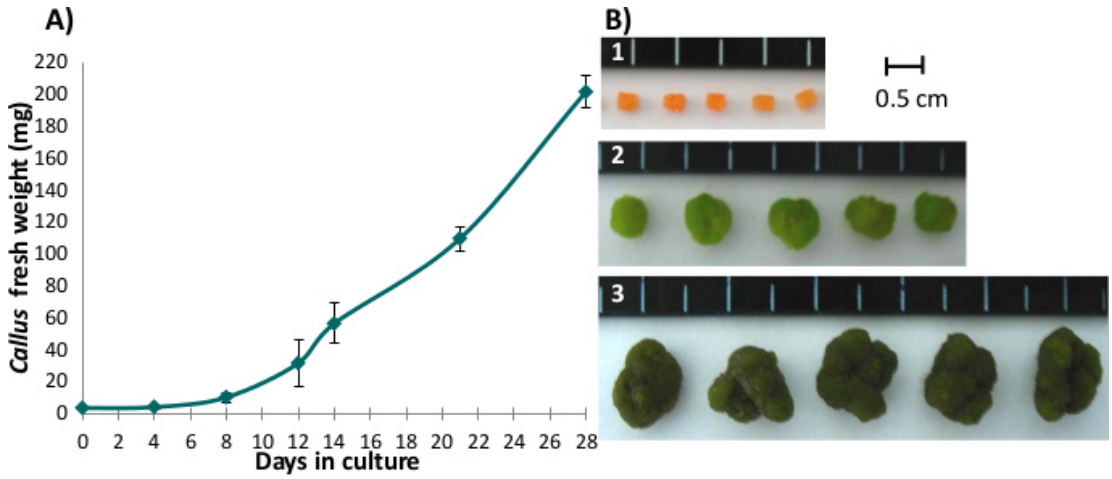
Vanlerberghe GC (2013) Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J. Mol. Sci. 14:6805–6847.

Vanlerberghe GC, Cvetkovska M, Wang J (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? Physiol. Plant. 137:392–406.

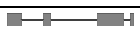
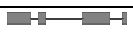
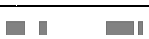
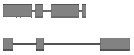
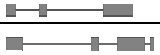
Xing D, Li QQ (2011) Alternative polyadenylation and gene expression regulation in plants. Wiley Interdiscip. Rev. RNA 2:445–458.

Zar JH (2010) Biostatistical Analysis, Fifth edition, Prentice-Hall, UpperSaddleRiver, NJ, pp. 226-244.

Zavattieri MA, Frederico AM, Lima M, et al (2010) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron. J. Biotechnol. 13.



Supplemental Fig. 1. A) Growth curve of primary cultures system of *Daucus carota* L. cv. Rotin during 28 days in culture at 21 °C in four individual plants. Data are shown as *callus* fresh weight values and represented as mean±S.D. B) Explants from the secondary phloem of carrot tap roots (primary culture system). Aspect of the explants (1) before (T0), (2) 14 days and (3) 28 days after *in vitro* inoculation.

	Species	Gene_id	Gene size	Protein lenght	Exon-intron gene structure
Eudicots	*Arabidopsis lyrata*	AL1G33660	1319	315	
		AL3G24680	1237	324	
		AL3G24690	1518	354	
		AL5G06730	1290	330	
	Arabidopsis thaliana	AT1G32350	1333	318	
		AT3G22360	1229	325	
		AT3G22370	1527	354	
		AT3G27620	1307	329	
	Brassica rapa	Bra010153	3045	319	
		Bra001865	2110	346	
		Bra031351	2078	360	
		Bra023835	1804	324	
	Daucus carota	KJ669723	1366	326	
	Fragaria vesca	FV5G29310	1323	361	
	Glycine max	GM04G14800	3196	321	
	Lotus japonicus	LJ2G020780	2471	314	
	Medicago truncatula	MT5G026620	2287	330	
	Populus trichocarpa	PT03G09340	1310	329	
		PT12G01430	2149	352	
		PT12G01440	2091	350	
		PT15G01960	2140	351	
	Solanum lycopersicum	Solyc08g005550.2	2198	366	
		Solyc08g075540.2	1402	358	
		Solyc08g075550.2	2711	318	
	Solanum tuberosum	PGSC0003DMG400007613	2518	321	
		PGSC0003DMG400007614	1524	356	
		PGSC0003DMG400018484	2333	279	
	Theobroma cacao	TC03G031300	2729	326	
	Vitis vinifera	VV02G09030	1252	322	
		VV02G09050	1245	320	
Monocots	*Brachypodium distachyon*	BD3G52505	1308	343	
		BD5G20540	1907	333	
		BD5G20547	1165	324	
		BD5G20557	1186	330	
	Hordeum vulgare	CAJW010038523	1815	281	
		CAJW011587016	1163	270	
		CAJW010099492	975	324	
	Musa acuminata	GSMUA_Achr5G03810_001	1277	324	
		GSMUA_Achr6G01170_001	1225	328	
		GSMUA_Achr6G01300_001	1397	317	
		GSMUA_Achr1G27800_001	1458	327	
	Oryza brachyantha	OB02G22630	2617	316	
		OB02G36280	4950	806	
		OB04G30980	2009	331	
		OB04G30990	1219	331	
	Oryza glaberrima	ORGLA02G0249500	1344	345	
		ORGLA04G0206000	2216	331	
		ORGLA04G0206100	1220	335	
	Oryza sativa	BGIOSGA008063	2061	339	
		BGIOSGA005788	1344	345	
		BGIOSGA014421	1227	335	
		BGIOSGA014422	2191	332	
	Sorghum bicolor	SB04G030820	1398	346	
		SB06G027410	1779	331	
		SB06G027420	1179	314	
		SB06G027430	1210	332	
	Zea mays	ZM02G05480	1215	332	
		ZM02G05490	1178	329	
		ZM02G05500	2287	329	
		ZM05G37570	2136	347	

Supplemental Table 1. Diversity of *AOX1* in exon-intron pattern across higher plants. Data retrieved from public web-based databases, freely available (Plaza: http://bioinformatics.psb.ugent.be/plaza/; e!EnsemblPlants: http://plants.ensembl.org/Multi/Search/New?db=core; IPK Barley Blast Server: http://webblast.ipk-gatersleben.de/barley/; NCBI: http://www.ncbi.nlm.nih.gov/). Gene draw was performed in FancyGene 1.4 (Rambaldi and Ciccarelli 2009). (Adapted from Cardoso et al. 2014).

❖ loss of intron 1, **Δ** loss of intron 3, ● loss of intron 2, ▲ loss of all introns, ♦ gain of intron in exon 1

CHAPTER 4 - DYNAMICS OF CARROT ALTERNATIVE OXIDASE EXPRESSION IN DEVELOPING STORAGE ROOTS

Abstract

Plant alternative oxidase (AOX) is a nuclear encoded mitochondria-targeted inner membrane enzyme involved in alternative respiration. The expression of *AOX* genes can be tissue–specific and/or developmentally regulated, and affected by several stress factors. For a better understanding of the putative role of AOX in carrot tap root secondary growth, the expression patterns of carrot *AOX* gene family (*DcAOX1, DcAOX2a* and *DcAOX2b)* were analysed in five carrot cultivars, starting at the beginning of the secondary growth (5 weeks post sowing) until 13 weeks post sowing. Root fresh weight and root length were measured. While the levels of *DcAOX1* transcripts were generally low and *DcAOX2b* transcripts were not detected, *DcAOX2a* mRNA levels changed during the experiment, with the highest values detected at the initial time points. That period was characterised by a strong increase in root length and to the very beginning of the secondary growth. At the end of the experiment, when *DcAOX2a* expression was lowest and tap root secondary growth (determined by root fresh weight) was highest, no increase in root length was observed. Such changes in *DcAOX2a* transcript levels during carrot tap root development are discussed.

Keywords: *Daucus carota*; alternative oxidase; development; growth

4.1. Introduction

The alternative oxidase (AOX) is a cyanide-resistant terminal oxidase that participates in the electron transport chain, found in the inner mitochondrial membrane of plants and several other lineages (Siedow et al. 1995). AOX accepts electrons directly from ubiquinol, diverting electrons from two of the three proton translocation sites (Complex III and Complex IV). Hence, electrons flowing through the AOX pathway contribute less to the generation of ATP than those flowing through the cytochrome oxidase pathway and are not subjected to control by cellular adenylate energy status (Siedow et al. 1995; Moore et al. 2013). The free energy that is released by electron flow through AOX is released as heat, a phenomenon which, together with the very large amounts of AOX and consequent rapid rate of uncoupled respiration, underlies the prominent role of AOX (Finnegan et al. 2004; Watling et al. 2006; Grant et al. 2008; Wagner et al. 2008). In higher plants, AOX is nuclear encoded by a small multigene family, comprising one to six gene members (Cardoso et al. 2015).

The presence of AOX provides the respiratory system with built-in flexibility regarding the degree of coupling between carbon metabolism pathways, electron transport chain activity, and ATP turnover (Vanlerberghe 2013). AOX can play a role in response to stress and on the maintenance of cellular and mitochondrial homeostasis (reviewed in Finnegan et al. 2004) and numerous studies have focused on the role of AOX under stressful growth conditions (Thirkettle-Watts et al. 2003; Clifton et al. 2005; Umbach et al. 2005; Costa et al. 2010; Cavalcanti et al. 2013; Belozerova et al. 2014; Tang et al. 2014; Vishwakarma et al. 2014). Interestingly, *AOX* genes have also showed differential expression in plant organs and tissues (Macherel et al. 2007; Campos et al. 2009; Cavalcanti et al. 2013), as well during post-germination development (McCabe et al. 1998; Saisho et al. 2001). Nevertheless, understanding the role of AOX during normal plant growth and development has been a relatively slow process. The use of transgenic plant approaches have contributed however to unravel the involvement of AOX in several biological processes, including physiological and morphologic changes (Fiorani et al. 2005; Chai et al. 2012; Cvetkovska et al. 2014; Ivanova et al. 2014), and on AOX implication in plant vegetative growth and reproductive performance (Lennon et al. 1995; McCabe et al. 1998; Fiorani et al. 2005; Murakami and Toriyama 2008; Chai et al. 2012). *AOX* genes have been also associated with fruit ripening, especially in climacteric fruits such as tomatoes and mangoes (see in Kumar et al. 1990; Considine et al. 2001; Xu et al. 2012).

The present work aimed to explore the role of carrot (*Daucus carota* L.) *AOX* gene family (namely *DcAOX1*, *DcAOX2a*, *DcAOX2b*) during storage roots development, in five carrot cultivars, growing under typical greenhouse growth conditions.

4.2. Materials and methods

4.2.1. Plant material

Five different cultivars of *Daucus carota* L. were used in the experiment: white (711-1), yellow (207-1), red (203-1), and purple (purple phloem with yellow xylem) (699-1) (cultivated carrot breeding stocks developed by the USDA carrot breeding program), and the orange coloured cv. Rotin. Seeds of each cultivar were sown in pots containing commercial substrate and maintained under greenhouse conditions. Three pots with a total of 10 plants per pot were considered per cultivar. Harvest was performed arbitrarily (from 4 to 6 plants, biological replicates) at different time points: 5, 7, 9 and 13 weeks post sowing (wps). Fresh weight (g) and root length (cm) of each tap root were annotated and samples were snap frozen in liquid nitrogen and stored at -80 °C for further expression analyses. Samples consisted of complete

roots (for samples collected at 5 and 7 wps) or pieces from the upper third-part of the root (for samples collected at 9 and 13 wps).

4.2.2. RNA extraction and cDNA synthesis

Total RNA was isolated 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 protocol. The quantification of RNA and evaluation of its quality were determined in a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity was evaluated by denaturing gel electrophoresis and visualised using a Gene Flash Bio Imaging system (Syngene, Cambridge, UK) after staining in an EtBr solution (2 $ng \cdot mL^{-1}$). DNase-treated total RNA (1 µg) was reverse transcribed with random decamer primers, using the RETROscript® kit (Ambion, Austin, TX, USA) according to manufacturer's instruction.

4.2.3. Reverse transcription quantitative real-time PCR (RT-qPCR)

Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, USA). Target transcripts were *DcAOX1, DcAOX2a* and *DcAOX2b.* Carrot *EF-1A, GAPDH*, and the Ribosomal 5.8S (*5.8S rRNA*) genes were used for selection of the most appropriate reference genes. Primer sequences and amplicon sizes are shown in Table 1.

Quantification of gene expression was performed by RT-qPCR with SYBR Green q-PCR Master Mix (Fermentas, Ontario, Canada) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). 18 µl reaction volume containing 5 µL of first-strand cDNA (previously diluted 1:10) and 560 nM of each specific primer was used for expression analysis. The identity of each amplicon was confirmed by Sanger sequencing and specificity of RT-qPCR reactions was evaluated by melting curve analysis. Efficiencies were calculated using a 4-point standard curves from a 4-fold dilution series (1:1-1:125) (run in triplicate) of pooled cDNA. RT-qPCR was conducted for 40 cycles, each consisting in 15 s at 95 °C followed by 1 min at 60 °C. To analyse the dissociation curve profiles, an additional step at 95 °C during 15 s was added, followed by a constant increase of temperature between 60 °C and 95 °C. All samples were run in duplicate. Minus reverse transcriptase and no template controls were included for all genes. Cq values were acquired for each sample with the Applied Biosystems 7500 software (Applied Biosystems, Foster City, CA, USA) with a fluorescence threshold arbitrarily set at 1.

Evaluation of expression stability of reference genes and selection of the most appropriate combination of genes to be used as reference was done using the statistical application *geNorm* (Vandesompele et al. 2002).

Table 1. Primers used in RT-qPCR. AS corresponds to amplicon size and E corresponds to primer efficiency.

[NCBI accession ID]	Gene	Primer sequence (5'→3')	AS (bp)	E (%)
[GenBank:D12709]	*EF-1A*	Fw: TGGTGATGCTGGTTTCGTTAAG	75	99
		Rv: AGTGGAGGGTAGGACATGAAGGT		
[GenBank:AY491512]	*GAPDH*	Fw: GGGAGGTGCAAAGAAAGTTATCA	79	96
		Rv: TTCCTTTTCATTGACACCAACAA		
[GenBank:X17534]	*5.8S*	Fw: AATGACTCTCGGCAACGGATAT	73	102
		Rv: TCACACCAAGTATCGCATTTCG		
[GenBank: EU286573]	*DcAOX1*	Fw: CTTCAACGCCTACTTCCTTG	196	97
		Rv: ATCTCGCAATGTAGAGTCAGC		
[GenBank: EU286575]	*DcAOX2a*	Fw: TCTTCAATGCTTTCTTTGTTCTT	200	93
		Rv: GACATCTTTTAGTTTGGCATCTTT		
[GenBank: EU286576]	*DcAOX2b*	Fw: CAAGAGAGAAAATGAATCAAGTGGTAG	92	110
		Rv: ATGACTCCGAAATGTACTCATAGGTG		

4.2.4. Statistics

A One-way analysis of variance (ANOVA) and the Tukey's post hoc test was used to test for significant differences in gene expression, fresh weight and length between time points. Statistical analyses were performed using the STATISTICA 8.0 statistical package (StatSoft Inc., Tulsa, USA). Significance levels were set at $P<0.05$.

4.3. Results

Carrot tap root fresh weight and length measurements were taken from developing roots at 5, 7, 9 and 13 wps. The general aspect of tap roots at the defined time points is shown in Fig. 1. The part of the tap root used in the length determination is also indicated (Fig. 1).

Carrot tap root fresh weight increased from week 5 to week 13, when it reached a maximum. A similar behaviour was detected in all five cultivars, as can be seen in Table 2. Within each cultivar, fresh weight slightly increased from 5 to 7 wps, without significant differences detected ($P>0.05$), and was followed by a marked increase until 13 wps, being significantly higher than at 5 wps ($P<0.05$). Fresh weight mean value vary according to the cultivar, with the white and yellow cultivars reaching the highest and the red the lowest value at 13 wps (Table 2).

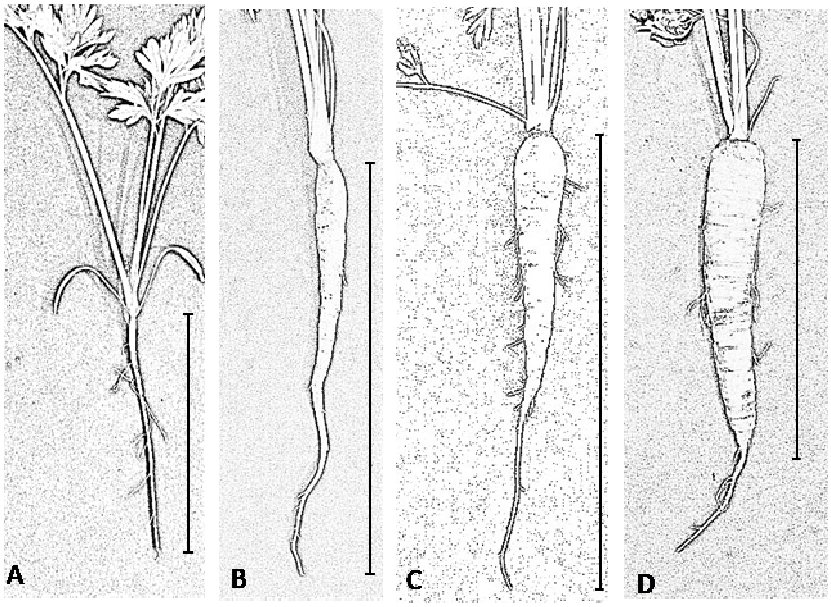


Fig. 1. Scheme representing the general aspect of carrot tap roots at (A) 5, (B) 7, (C) 9 and (D) 13 weeks post sowing. The vertical bar indicates the part of the tap root used for length measurement.

Contrarily to the fresh weight, the root length sharply increased at the beginning of the experiment (Table 2). Between 5 wps and 7 wps a great increase occurred in all studied cultivars, with significant differences of at least $P < 0.05$ (Table 2). At the end of the experiment (between 9 and 13 wps) a decrease on root length mean values was observed in almost all cultivars (exception on yellow), and significantly different in the red cultivar (Table 2). This resulted from that in the last time point only the clearly distinguishable storage tap root was considered to take the length value. The final part of carrot tap root was not taken in account, since in some roots it was noticed that this part was broken during harvest (see Fig. 1D).

Table 2. Fresh weight (g) and root length (cm) measurements of carrot tap roots at 5, 7, 9 and 13 weeks post sowing (wps).

Cultivar	Time point (wps)	Root fresh weight (g)	Root length (cm)
White	5	0.2 ± 0.06[a]	7.9 ± 0.6[a]
	7	4.2 ± 0.68[a,b]	21.0 ± 1.2[b]
	9	32.5 ± 10.25[b]	21.0 ± 1.9[b]
	13	51.0 ± 9.15[b]	19.3 ± 2.3[b]
Yellow	5	0.2 ± 0.02[a]	5.6 ± 1.2[a]
	7	5.7 ± 1.46[a,c]	18.7 ± 1.1[b]
	9	23.3 ± 3.00[b,c]	20.0 ± 0.6[b]
	13	49.2 ± 5.36[b]	20.5 ± 0.8[b]
Orange	5	0.1 ± 0.01[a]	6.0 ± 0.3[a]
	7	4.6 ± 0.95[a,c]	15.9 ± 1.4[b]
	9	13.0 ± 1.76[b,c]	16.5 ±1.0[b]
	13	32.5 ± 5.47[b]	11.8 ± 1.0[a,b]
Red	5	0.1 ± 0.03[a]	8.3 ± 0.9[a]
	7	3.4 ± 0.62[a,c]	14.6 ± 0.8[b,c]
	9	15.8 ± 2.63[b,c]	17.3 ± 0.4[b]
	13	27.1 ± 4.13[b]	13.6 ± 0.8[c]
Purple	5	0.2 ± 0.05[a]	6.9 ± 0.9[a]
	7	4.1 ± 1.29[a,b]	16.0 ± 2.7[b]
	9	20.3 ± 2.10[b]	21.1 ± 0.4[b]
	13	40.0 ± 11.16[b]	16.1 ± 1.2[b]

White (711-1), yellow (207-1), orange (Rotin), red (203-1), and purple (purple phloem with yellow xylem) (699-1), correspond to the different carrot cultivars used in the experiment. Data is presented as the mean ± SE of four to six tap roots independent measurements. Different superscript letters in the same column indicate significant differences of at least $P<0.05$ between time points, within each cultivar.

Expression analyses of carrot *AOX* genes revealed that both *DcAOX1* and *DcAOX2a* genes were expressed throughout the investigated period, in the five different cultivars (Fig. 2). In contrast, no transcripts of *DcAOX2b* were detected at any time point and cultivar.

DcAOX1 expression remained generally low, with few significant differences amongst time points observed in the white, yellow, orange and purple cultivars (Fig. 2A-E). On the contrary, the red cultivar presented a higher expression at the beginning, with significant changes during the course of the experiment ($P < 0.05$). However, it is worth to notice a high variability in *DcAOX1* expression among individual plants, particularly in the last time point (5 biological replicates were considered), which is reflected by a high standard error (Fig. 2D). Overall, no specific trend was detected in *DcAOX1* transcript levels during carrot root development. Different results were observed regarding the highly expressed *DcAOX2a* (Fig. 2A-E). A clear expression pattern, in all five cultivars, was observed during the time course of

the experiment. *DcAOX2a* presented a marked decrease on transcript levels, particularly between either the first or second time points (5 and 7 wps) and the two final time points (9 and 13 wps), with significant differences (P<0.05) in all the studied cultivars (Fig. 2). Those differences reached a maximum of approximately 5-fold for purple and red, 3-fold for white and 2-fold for yellow and orange cultivars (Fig. 2).

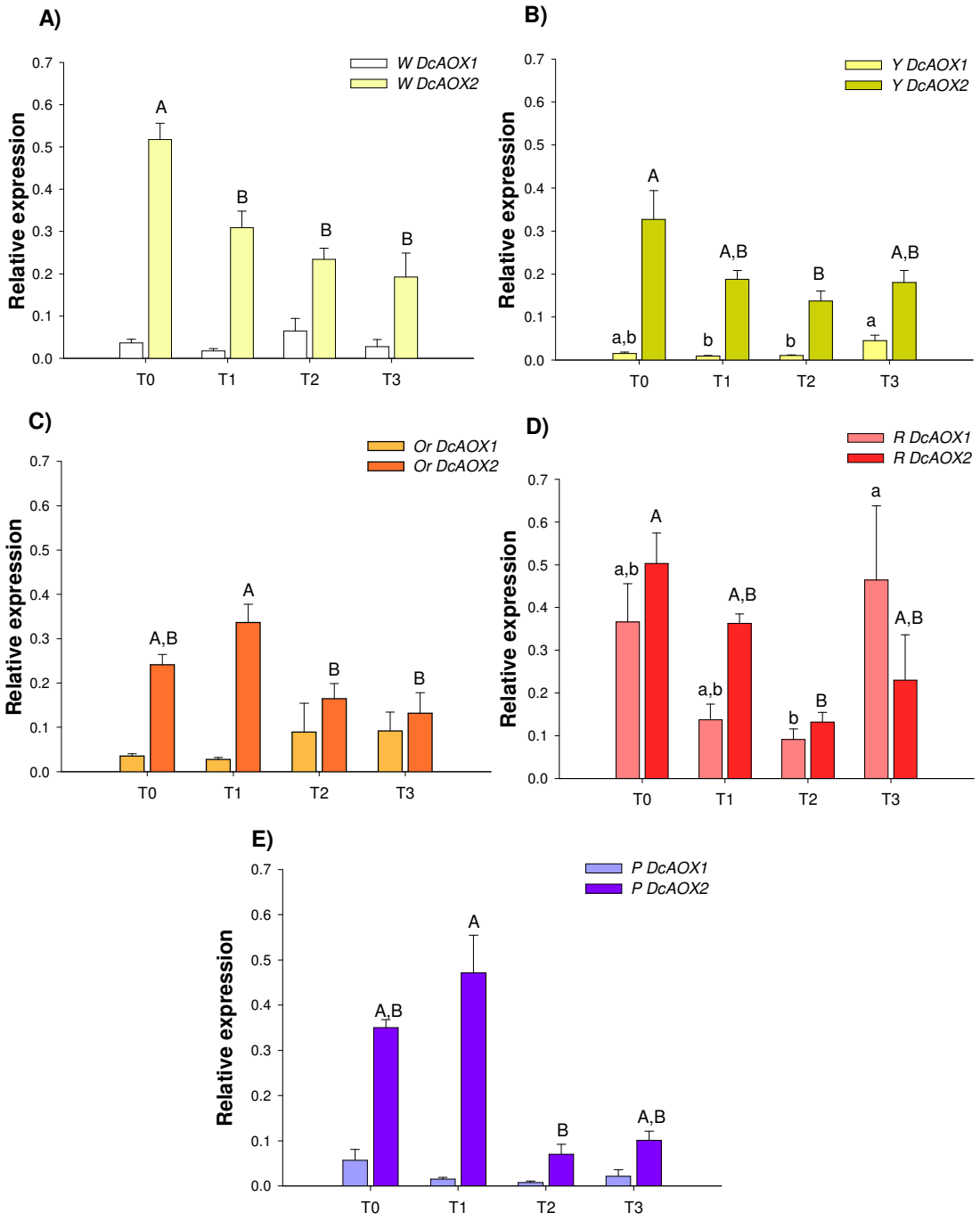


Fig. 2. Expression patterns of *DcAOX1 and DcAOX2a* during carrot root secondary growth in the cultivars white (711-1) (A), yellow (207-1) (B), orange (Rotin) (C), red (203-1) (D), and purple (purple phloem with yellow xylem) (699-1) (E). Transcript levels were determined by RT-qPCR. In each harvest time point, 4-6 biological replicates were considered per cultivar. Error bars indicate the standard error of the mean. Different superscript letters indicate significant differences between the stages of development for *DcAOX1* (small letters), or for *DcAOX2a* (capital letters). W: white; Y: yellow; Or: orange; R: red; P: purple.

4.4. Discussion

Root meristems located in the cambium ring are the main tissue responsible for secondary growth in carrot tap roots (Nogales et al. 2014) and, it is known that, independently of the cultivar, the cambium ring starts its development around 4-7 weeks after sowing (Hole et al. 1987). Located between the primary xylem and the phloem, the cambium ring produces phloem tissue on the outside and xylem tissue on the inside (Hole et al. 1987). Therefore, it is very likely that at the initial time point presented in this work (5 wps), the secondary growth of carrot tap roots was at the very beginning, since a pronounced increase in root biomass was only observed after 7 wps. On the contrary, during initial stages (between 5 and 7 wps) the root length sharply increased. Our observation is in accordance with Palussek and Neumann (1982), which referred that root length is determined previously to the root secondary growth. Regarding the expression data, *DcAOX2a* presented the highest values between 5 and 7 wps, just before the initiation of the secondary growth. During that period no significant increase of fresh weight was detected, while root length greatly increased in all the studied cultivars. At a later stage, when *DcAOX2a* expression was reduced (at 9 and 13 wps), and when higher cell division rates are likely to take place in the meristem (reflected as secondary growth), the increment on root length stopped. Therefore, and differently to *DcAOX1*, *DcAOX2a* followed a concrete trend during carrot storage root growth.

AOX has been shown to be especially active in meristematic tissues (Hilal et al. 1997) and several studies have indicated links between AOX activity and plant growth (Arnholdt-Schmitt et al. 2006; Vanlerberghe 2013 and references therein). Strong support for this view was found on experiments performed under various nutrient conditions with transgenic tobacco cells with silenced *AOX1a*. Sieger et al. (2005) demonstrated that *AOX1a* knockdown led to the incapacity of cells for down-regulating growth under P- and N-deficiency, and concluded that AOX activity provides a mechanism for adjusting growth and counteracting nutrient imbalance. When maintaining the knockdown of *AOX1a*, tobacco cell growth has been connected to more stable carbon use efficiency. Another single cell system in which the relation between AOX and growth has been critically examined is in the green alga

Chlamydomonas reinhardtii (Mathy et al. 2010). Similar to the case with tobacco suspension cells (although not requiring nutrient-limiting growth conditions), transgenic *Chlamydomonas* lacking AOX displayed a large increase in biomass accumulation comparing to wild type cultures. In general, while the single cell systems referred above show that removing AOX had a positive impact on growth, the relation between AOX and growth will undoubtedly be much more complex in whole plants. Several examples illustrate the AOX effect on plant vegetative growth and reproductive performance. It can be pointed out the work of Fiorani and co-workers (2005), who observed a reduced leaf area and rosette size through the antisense suppression of *AtAOX1a* in *Arabidopsis* plants grown for 21 days at 12 °C. Such differences diminished as the plants approached flowering, suggesting that *AOX* played a role in the acclimation of shoot growth to low temperature during early vegetative development. Focusing on the *AOX2* subfamily, several reports point to its involvement on development and growth processes. Chai et al. (2012) reported the impact of altered expression of an *AOX2* gene on growth of soybean. In this case, an antisense knockdown of *Aox2b* was shown to compromise both vegetative growth and seed yield under typical greenhouse growth conditions. Other study showed that during seedling development, the relative abundance of *AOX2* in soybean transcripts decreased, whereas the transcript abundance of other *AOX* genes increased (McCabe et al. 1998). Saisho et al. (2001) observed that *AOX2* expression in *Arabidopsis* was high in dry seeds and subsequently decreased during early germination, whereas *AOX1a* was less abundant at the beginning of the process and only increased in a later stage.

It seems therefore that the involvement of *AOX* genes on growth and development is highly species-specific, and it is not only related with the gene sub-family (*AOX1* or *AOX2*) but also with the specific function of each gene inside the sub-family, which can differ amongst plant species.

4.5. References

Arnholdt-Schmitt B, Costa JH, de Melo DF (2006) AOX - a functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11:281–287. doi: 10.1016/j.tplants.2006.05.001

Belozerova NS, Baik AS, Butsanets PA, et al (2014) Effect of salicylic acid on the alternative pathway of yellow lupine respiration. Russ J Plant Physiol 61:38–46. doi: 10.1134/S1021443714010026

Campos MD, Cardoso HG, Linke B, et al (2009) Differential expression and co-regulation of carrot AOX genes (*Daucus carota*). Physiol Plant 137:578–591. doi: 10.1111/j.1399-3054.2009.01282.x

Cardoso HG, Nogales A, Frederico AM, et al (2015) Natural AOX gene diversity. In: Gupta KJ, M.L. NB (ed) Alternative respiratory pathways in higher plants., John Wiley. Oxford, pp 241–254.

Cavalcanti JHF, Oliveira GM, Saraiva KDDC, et al (2013) 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. J Plant Physiol 170:1609–1619. doi: 10.1016/j.jplph.2013.06.012

Chai TT, Simmonds D, Day DA, et al (2012) A *GmAOX2b* antisense gene compromises vegetative growth and seed production in soybean. Planta 236:199–207. doi: 10.1007/s00425-012-1601-6

Clifton R, Lister R, Parker KL, et al (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. Plant Mol Biol 58:193–212. doi: 10.1007/s11103-005-5514-7

Considine MJ, Daley DO, Whelan J (2001) The expression of alternative oxidase and uncoupling protein during fruit ripening in mango. Plant Physiol 126:1619–1629. doi: 10.1104/pp.126.4.1619

Costa JH, Mota EF, Cambursano MV, et al (2010) Stress-induced co-expression of two alternative oxidase (*VuAox1* and *2b*) genes in *Vigna unguiculata*. J Plant Physiol 167:561–570. doi: 10.1016/j.jplph.2009.11.001

Cvetkovska M, Dahal K, Alber NA, et al (2014) Knockdown of mitochondrial alternative oxidase induces the “stress state” of signaling molecule pools in *Nicotiana tabacum*, with implications for stomatal function. New Phytol 203:449–461. doi: 10.1111/nph.12773

Finnegan P, Soole KL, Umbach AL (2004) Alternative mitochondrial electron transport proteins in higher plants. In: Day, DA, Millar, AH, Whelan J (ed) Advances in Photosynthesis and Respiration. Plant Mitochondria: From Genome to Function., Springer. Dordrecht, pp 163–230

Fiorani F, Umbach AL, Siedow JN (2005) The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants. Plant Physiol 139:1795–1805. doi: 10.1104/pp.105.070789

Grant NM, Miller RE, Watling JR, Robinson SA (2008) Synchronicity of thermogenic activity, alternative pathway respiratory flux, AOX protein content, and carbohydrates in receptacle tissues of sacred lotus during floral development. J Exp Bot 59:705–714. doi: 10.1093/jxb/erm333

Hilal M, Castagnaro A, Moreno H, Massa EM (1997) Specific localization of the respiratory alternative oxidase in meristematic and xylematic tissues from developing soybean roots and hypocotyls. Plant Physiol 115:1499–1503. doi: 10.1104/pp.115.4.1499

Hole CC, Morris GEL, Cowper AS (1987) Distribution of dry matter between shoot and storage root of field-grown carrots. I. Onset of differences between cultivars. J Hort Sci 62:335–341.

Ivanova A, Law SR, Narsai R, et al (2014) A functional antagonistic relationship between auxin and mitochondrial retrograde signaling regulates alternative oxidase 1a Expression in Arabidopsis. Plant Physiol 165:1233–1254. doi: 10.1104/pp.114.237495

Kumar S, Patil BC, Sinha SK (1990) Cyanide resistant respiration is involved in temperature rise in ripening mangoes. Biochem Biophys Res Commun 168:818–822. doi: 10.1016/0006-291X(90)92394-F

Lennon AM, Pratt J, Leach G, Moore AL (1995) Developmental regulation of respiratory activity in pea leaves. Plant Physiol 107:925–932.

Macherel D, Benamar A, Avelange-Macherel MH, Tolleter D (2007) Function and stress tolerance of seed mitochondria. In: Physiologia Plantarum. pp 233–241

Mathy G, Cardol P, Dinant M, et al (2010) Proteomic and functional characterization of a chlamydomonas reinhardtii mutant lacking the mitochondrial alternative oxidase. J Proteome Res 9:2825–2838. doi: 10.1021/pr900866e

McCabe TC, Finnegan PM, Harvey Millar A, et al (1998) Differential expression of alternative oxidase genes in soybean cotyledons during postgerminative development. Plant Physiol 118:675–682. doi: 10.1104/pp.118.2.675

Moore AL, Shiba T, Young L, et al (2013) Unraveling the heater: new insights into the structure of the alternative oxidase. Annu Rev Plant Biol 64:637–63. doi: 10.1146/annurev-arplant-042811-105432

Murakami Y, Toriyama K (2008) Enhanced high temperature tolerance in transgenic rice seedlings with elevated levels of alternative oxidase, OsAOX1a. Plant Biotechnol 25:361–364.

Nogales A, Muñoz-Sanhueza L, Hansen LD, Arnholdt-Schmitt B (2014) Phenotyping carrot (*Daucus carota* L.) for yield-determining temperature response by calorespirometry. Planta 241:525–538. doi: 10.1007/s00425-014-2195-y

Palussek K, Neumann K. (1982) Studies on the gibberellin and cytokinin in various stages of development of the carrot root. Z Pflanzenernähr Bodenkd 145:268–277.

Saisho D, Nakazono M, Lee KH, et al (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. doi: 10.1266/ggs.76.89

Siedow JN, Umbach AL, Moore AL (1995) The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett 362:10–14. doi: 10.1016/0014-5793(95)00196-G

Sieger SM, Kristensen BK, Robson CA, et al (2005) The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. J Exp Bot 56:1499–1515. doi: 10.1093/jxb/eri146

Tang H, Zhang DW, Yuan S, et al (2014) Plastid signals induce alternative oxidase expression to enhance the cold stress tolerance in *Arabidopsis thaliana*. Plant Growth Regul 275–283. doi: 10.1007/s10725-014-9918-8

Thirkettle-Watts D, McCabe TC, Clifton R, et al (2003) Analysis of the alternative oxidase promoters from soybean. Plant Physiol 133:1158–1169. doi: 10.1104/pp.103.028183

Umbach AL, Fiorani F, Siedow JN (2005) Characterization of transformed Arabidopsis with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. Plant Physiol 139:1806–1820. doi: 10.1104/pp.105.070763

Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034

Vanlerberghe GC (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J. Mol. Sci. 14:6805–6847.

Vishwakarma A, Bashyam L, Senthilkumaran B, et al (2014) Physiological role of AOX1a in photosynthesis and maintenance of cellular redox homeostasis under high light in *Arabidopsis thaliana*. Plant Physiol Biochem 81:44–53. doi: 10.1016/j.plaphy.2014.01.019

Wagner AM, Krab K, Wagner MJ, Moore AL (2008) Regulation of thermogenesis in flowering Araceae: The role of the alternative oxidase. Biochim Biophys Acta - Bioenerg 1777:993–1000. doi: 10.1016/j.bbabio.2008.04.001

Watling JR, Robinson S a, Seymour RS (2006) Contribution of the alternative pathway to respiration during thermogenesis in flowers of the sacred lotus. Plant Physiol 140:1367–1373. doi: 10.1104/pp.105.075523

Xu F, Yuan S, Zhang DW, et al (2012) The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. J Exp Bot 63:5705–5716. doi: 10.1093/jxb/ers226

CHAPTER 5 - ISOLATION AND CHARACTERIZATION OF PLASTID TERMINAL OXIDASE GENE FROM CARROT AND ITS RELATION TO CAROTENOID ACCUMULATION

This chapter is adapted from the submitted manuscript:

Campos MD, Campos C, Cardoso HG, Simon PW, Oliveira M, Nogales A, Arnholdt-Schmitt B. Isolation and characterization of plastid terminal oxidase gene from carrot and its relation to carotenoid accumulation. Submitted

Abstract

Carrot (*Daucus carota* L.) is a biennial plant that accumulates considerable amounts of carotenoid pigments in the storage root. To better understand the molecular mechanism for carotenoid accumulation in developing storage roots, plastid terminal oxidase (*PTOX*) cDNA was isolated and selected for reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Present in photosynthetic species, PTOX is a plastid-located, nucleus encoded plastoquinone (PQ)-O2 oxidoreductase (plastioquinol oxidase). The enzyme is known to play a role as a cofactor for phytoene desaturase, and consequently plays a key in the carotenoid biosynthesis pathway. In carrot a single *PTOX* gene copy was identified (*DcPTOX*). *DcPTOX* encodes a putative protein with 366 amino acids that contains the typical structural features of PTOXs from higher plants. The expression of *DcPTOX* was analysed during the development of white, yellow, orange, red, and purple carrot roots, along with five genes known to be involved in the carotenoid biosynthesis pathway, *PSY2*, *PDS*, *ZDS1*, *LCYB1*, and *LCYE.* Expression analysis revealed the presence of *DcPTOX* transcripts in all cultivars, and an increase of transcripts during the time course of the experiment, with differential expression among cultivars in early stages of root growth. Our results demonstrated that *DcPTOX* showed a similar profile to that of other carotenoid biosynthetic genes with high correlation to all of them. The preponderant role of *PSY* in the biosynthesis of carotenoid pigments was also confirmed.

Keywords: *Daucus carota*; *DcPTOX*; gene isolation; expression analysis; carotenoid biosynthesis; development

5.1. Introduction

The plastid terminal oxidase (PTOX) is a nucleus encoded plastid-located plastoquinone (PQ)-O_2 oxidoreductase (plastoquinol oxidase) that occurs widely in photosynthetic species,

including algae and higher plants (Cournac et al. 2000; Carol and Kuntz 2001; Archibald et al. 2003; Kuntz 2004). *PTOX* is present in some eukaryotic algae as a small multigene family, composed by two members (*PTOX1* and *PTOX2*). In higher plants *PTOX* appears as a single gene (Wang et al. 2009; Houille-Vernes et al. 2011), which is involved in chlororespiration, chromorespiration and carotenoid biosynthesis (Josse et al. 2000; Carol and Kuntz 2001; Joet et al. 2002; Aluru and Rodermel 2004; Kuntz 2004; Shahbazi et al. 2007). PTOX is the terminal oxidase of chlororespiration, regulating the redox state of the PQ pool (Peltier and Cournac 2002; Aluru and Rodermel 2004). It transfers the excess of electrons to O_2, in order to maintain the relative redox balance in the photosynthetic electron transport chain (ETC), and reduces by this means the possibility for oxidative damage (McDonald et al. 2011). PTOX is considered to play a role in minimizing the generation of reactive oxygen species (ROS) when induced under environmental stresses (McDonald et al. 2011). Sun and Wen (2011) suggested a protective function with stress-induced inhibition of photosynthetic ETC.

Carotenoid pigments are important compounds in human health because they serve as both vitamin A precursors as well as having antioxidant properties. Carrot (*Daucus carota* L.) is a biennial plant that in its storage root provides an important source of carotenoids in the human diets. Carotenoids play essential biological roles in plants and the genes coding for enzymes in the carotenoid pathway have already been subject of intensive studies in many species. However, the molecular regulation of carotenoid accumulation in the storage root of carrot has not been extensively explored.

Recent reports point to PTOX as a key enzyme in the carotenoid biosynthesis pathway. Using a transgenic approach, Carol and Kuntz (2001) showed that the lack of PTOX blocks carotenoid synthesis. PTOX absence gives rise to the *immutans* phenotype in *Arabidopsis thaliana* and to the *ghost* phenotype in *Solanum lycopersicum* (also known as *Lycopersicon esculetum*) (Carol et al. 1999; Wu et al. 1999; Josse et al. 2000; Carol and Kuntz 2001; Rodermel 2001; Aluru et al. 2006). These phenotypes are characterized by variegated leaves with green and bleached sectors and additionally -in *S. lycopersicum*- by a yellow-orange ripe fruit. In *immutans*, the variegated phenotype might thus be due to a block in the desaturation of phytoene in the carotenoid biosynthetic pathway, as a result of insufficient oxidized PQ, which is needed as an electron acceptor for this reaction (Wu et al. 1999; Carol and Kuntz 2001), leading to photobleaching of green tissues. PTOX has also a preponderant role in carotenoid biosynthesis in fruit chromoplasts (Josse et al. 2000), as observed in the yellow-orange *S. lycopersicum* fruit, which is characterized by reduced carotenoid content (Barr et al. 2004). In *S. lycopersicum*, a dual role for PTOX in efficient carotenoid desaturation as well as in chlororespiration in green tissues is refereed by Shahbazi et al. (2007). However, *PTOX*

transcript levels and carotenoid accumulation are not correlated in all tissues and organs (Aluru et al. 2001).

Protein sequence analysis shows that PTOX shares sequence similarity with the stress-inducible mitochondrial alternative oxidase (AOX) in a number of plant species (Berthold and Stenmark 2003; Carol et al. 1999; Wu et al. 1999). As with AOX proteins, PTOX sequence analysis reveals the existence of several conserved domains, such as iron-binding residues (McDonald et al. 2011). In both enzymes the sequences exhibit at their C-terminus the iron-binding motifs typical of Type II di-iron carboxylate proteins (Carol and Kuntz 2001).

In this work the *PTOX* gene was isolated from *D. carota* (*DcPTOX*) and its expression was investigated in relation to carotenoid content in the developing storage root of white, yellow, orange, red, and purple cultivars. These results were compared with the expression of five genes encoding carotenoid biosynthesis enzymes. To our knowledge this is the first report about the isolation of *PTOX* in *D. carota* and the analysis of its expression.

5.2. Material and Methods

5.2.1. Plant materials

For *DcPTOX* gene isolation seeds of *D. carota* L. cv. Rotin were *in vitro* germinated in pots containing MS solid medium (Murashige and Skoog 1962) maintained under controlled conditions (25±1 °C at 16 h photoperiod: 34 $\mu molm^{-2}s^{-1}$ light intensity, provided by day light Philips lamps). cDNA from a pool of eight week-old *in vitro* grown seedlings was used for gene identification, while cDNA from a single plant was used for complete gene isolation.

To study the involvement of *DcPTOX* in carotenoid accumulation, an experiment with five cultivars representing a wide range of pigmented carrot material was performed under greenhouse conditions. White (711-1), yellow (207-1), red (203-1), and purple (purple phloem with yellow xylem) (699-1) (cultivated carrot breeding stocks developed by the USDA carrot breeding program), and the orange colored cv. Rotin. Seeds of each cultivar were sown in three pots with a total of 10 plants per pot. Samples of each cultivar were collected arbitrarily from 4 to 6 plants (biological replicates) at different time points: 5, 7, 9 and 13 weeks post sowing (wps). Samples consisted of complete roots (for samples collected at 5 and 7 wps) or pieces from the upper third root part (for samples collected at 9 and 13 wps). The appearance of the roots during the time course of the experiment can be observed on Suppl. Fig. 1.

All collected samples were ground to a fine powder using liquid nitrogen and stored at -80 °C until further analysis.

5.2.2. Total RNA isolation

Total RNA was 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 protocol. The quantification of RNA and the evaluation of its quality were determined in a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity was evaluated by denaturing gel electrophoresis and visualized using a Gene Flash Bio Imaging system (Syngene, Cambridge, UK) after staining in an EtBr solution (2 $ng \cdot mL^{-1}$).

5.2.3. Identification of *DcPTOX* and Rapid Amplification of the cDNA Ends (RACE)

Single strand cDNA was produced with RevertAid^{Tm}HMinus M-MuLV *Reverse* enzyme (Fermentas, Ontario, Canada) using the oligo (dT) primer *VIAL 8* (Roche, Mannheim, Germany) (Table 1), according to the manufacturer's instruction. The degenerate primer pair (*ptox_613fw* and *ptox_1023rv*, see sequence in Table 1) was designed by choosing the two most conserved regions on an alignment performed with plant *PTOX* gene sequences available at NCBI data bases (National Center for Biotechnology Information, Bethesda, USA) (not shown) and was used for *D. carota PTOX* (*DcPTOX*) gene identification. PCR was performed with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) using 1 µL of cDNA as template and 0.2 µM of each primer. Based on the *DcPTOX* cDNA partial sequence, new primer pairs were designed to conduct Rapid Amplification of the cDNA Ends (RACE) to isolate the 5' and 3' end of the gene. To determine the 5' end of *DcPTOX* gene a cDNA library of *D. carota* cv. Marktgaertner M853 (kindly provided by Dr. Bettina Linke, Humboldt University of Berlin, Germany) cloned into a Lambda gt22a phage vector (Invitrogen, Karlsruhe, Germany) was generated (Linke et al. 2003). 5' RACE-PCR was carried out using 1 µL of cloned library as template and the vector specific forward primer *P6* (Table 1) combined with a gene-specific reverse primer (*DcPTOX_24Rv,* annealing at 58 °C for 30 s and extension at 72 °C for 60 s, see Table 1). For *DcPTOX* 3' end isolation, 3'RACE-PCR was conducted using the reverse primer *VIAL 9* (Roche, Mannheim, Germany) in combination with a gene-specific forward primer (*DcPTOX_364Fw*, annealing at 58 °C for 30 s and extension at 72 °C for 60 s, see Table 1). One µl of a 1:10 cDNA dilution of the first strand PCR product was used as template for amplification. RACE-PCRs were performed with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) and 0.2 µM of each primer. For complete gene isolation one gene-specific primer set (*DcPTOX_13Fw* and *DcPTOX_1183Rv*, annealing at 55 °C for 15 s and extension at 72 °C for 60 s, Table1) was designed based on the 5' and 3' UTR sequences previously isolated with RACE-PCRs. One µl of a 1:10 cDNA dilution from a single plant were used as templates.

All PCRs were performed in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). PCRs products were separated in 1.4 % agarose gel and subsequently analysed, after EtBr staining (2 ng·mL^{-1}) on a Gene Flash Bio Imaging system (Syngene, Cambridge, UK). Fragments showing the expected size were purified from agarose gel using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, England) according to the manufacturer's protocol. For cloning, PCR fragments were inserted into a pGem®-T Easy vector (Promega, Madison, WI,USA) and used to transform *E. coli* JM109 (Promega Madison, WI, USA) competent cells. Plasmid DNA was extracted from putative recombinant clones (Birnboim and Doly, 1979) and confirmed by restriction enzyme analysis using *EcoR*I (Fermentas, Ontario, Canada). Sense and antisense strands were sequenced (Macrogen company: www.macrogen.com) in selected recombinant clones using *T7* and *SP6* primers (Promega, Madison, WI, USA).

Table 1. Primers used for cDNA *DcPTOX* gene isolation.

Primer name	Sequence (5'→3')
ptox_613fw	GYTTTGGYTGGTGGAGAMGRG
ptox_1023rv	CTCKGCTTCRTCRTCTCTRATG
VIAL 8	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV
DcPTOX_364Fw	ACACACGAAGACCAGTGATAG
VIAL 9	GACCACGCGTATCGATGTCGAC
P6	CGCGGAAGAAGGCACATGGCTGAATA
DcPTOX_24Rv	CAAAATGGACTTTCAGATAGTC
DcPTOX_13Fw	GTCCGTCATTATTCAAACTTCAA
DcPTOX_1183Rv	ATCATCCTACTTGCCTAATATC

(V = A, C or G)

5.2.4. Bioinformatic analysis of the full-length *DcPTOX* cDNA and putative protein sequence

Sequence homology was explored at the NCBI database using the BLAST algorithm (Karlin and Altschul 1993) (http://www.ncbi.nlm.nih.gov/) (BLASTn). To edit *DcPTOX* sequence data, SeqMan and EditSeq softwares (LASERGENE 7, GATC Biotech, Konstanz) were used. Phylogenetic studies included the PTOX proteins from a group of 21 eudicot and 5 monocots plant species retrieved from the genomic database freely available Plaza (Plaza 3.0: http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_monocots/ and http:// bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/), and were based on a ClustalW Multiple alignment made in BioEdit software (Hall 1999); the alignment was bootstrapped with 1000 replicates by the Neighbor-Joining (NJ) method using the MEGA 4 software. For protein sequence comparison, a ClustalW Multiple alignment was performed using the CLC Main

Workbench 6.7.1 software (CLC bio). TargetP 1.1 software (Emanuelsson et al. 2000) (http://www.cbs.dtu.dk/services/TargetP/) was used to predict the chloroplast targeting sequence cleavage site.

5.2.5. Reverse transcription quantitative real-time PCR (RT-qPCR)

DNase-treated total RNA (1 µg) was reverse transcribed with the random decamer primers provided by the RETROscript® kit (Ambion, Austin, TX, USA) according to manufacturer's instruction. RT-qPCR was used to investigate the involvement of *DcPTOX* with carotenoid accumulation in five cultivars of carrot with different root colors. Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, USA). The genes considered for normalisation were: elongation factor-1alpha (*EF-1A*), glyceraldeyde 3-phosphate dehydrogenase (*GAPDH*), and the ribosomal RNA 5.8S (*5.8S rRNA*) (previous selected by Campos et al. 2015). The target genes selected for RT-qPCR were *DcPTOX*, and the carotenoid biosynthetic genes: phytoene synthase 2 (*PSY2*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), lycopene β-cyclase 1*(LCYB1*), lycopene ε-cyclase (*LCYE*). Primer sequences and amplicon sizes are shown in Table 2.

Quantification of gene expression was performed by RT-qPCR with SYBR Green q-PCR Master Mix (Fermentas, Ontario, Canada) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). 15 µl reaction volume containing 5 µL of first-strand cDNA (previously diluted 1:10) and 560 nM of each specific primer was used for expression analysis. The identity of each amplicon was confirmed by Sanger sequencing and specificity of qPCR reactions was evaluated by melting curve analysis. Efficiencies were calculated using a 4-point standard curves from a 4-fold dilution series (1:1-1:125) (run in triplicate) of pooled cDNA. RT-qPCR was conducted for 40 cycles, each consisting of 15 s at 95 °C followed by 1 min at 60 °C. To analyse dissociation curve profiles, an additional step at 95 °C during 15 s was added, followed by a constant increase of temperature between 60 and 95 °C. All samples were run in duplicate. Minus reverse transcriptase and no template controls were included to assess contaminations. Cq values were acquired for each sample with the Applied Biosystems 7500 software (Applied Biosystems, Foster City, CA, USA) with a fluorescence threshold arbitrarily set at 1.

Table 2. Primers used in RT-qPCR.

[NCBI accession ID]	Gene	Primer sequence (5'→3')		AS (bp)	E (%)
[GenBank:D12709]	*EF-1A*	Fw	TGGTGATGCTGGTTTCGTTAAG	75	99
		Rv	AGTGGAGGGTAGGACATGAAGGT		
[GenBank:AY491512]	*GAPDH*	Fw	GGGAGGTGCAAAGAAAGTTATCA	79	96
		Rv	TTCCTTTTCATTGACACCAACAA		
[GenBank:X17534]	*5.8S*	Fw	AATGACTCTCGGCAACGGATAT	73	102
		Rv	TCACACCAAGTATCGCATTTCG		
[GenBank:DQ192187]	*PSY2*	Fw	GGTTGGGTTCCCGGGATA	67	81
		Rv	TCCGCAGCTTACCCTTCTCA		
[GenBank:DQ222429]	*PDS*	Fw	TAACATGGCCTGAGAAAGTCAAGT	71	105
		Rv	CACGTAGGCTTGTCCACCAA		
[GenBank:DQ222430]	*ZDS1*	Fw	CCGAAGCTAAAAGTGGCTATTATAGG	77	93
		Rv	TGGCCCTGATCTAGAAGCTCAA		
[GenBank:DQ192190]	*LCYB1*	Fw	CCAGTTGTTGCCAATGCAAT	63	84
		Rv	GTTTCCCAAAGCGCCTTTC		
[GenBank:DQ192192]	*LCYE*	Fw	CATTCCATGCAGGCTTGCTA	70	94
		Rv	CCCAACCTCATACTGCAAAAGTT		
[GenBank: EU331420]	*DcPTOX*	Fw	CGTTTTCGTGTAATTCGTTGAGAT	142	93
		Rv	TCCACAACTACCTTCTCCTCATTCT		

AS corresponds to amplicon size; E corresponds to primer efficiency. Primers sequences of *PDS*, *ZDS1* and *LCYE* were previously published in Clotault et al. (2008).

Evaluation of expression stability of reference genes and selection of the most appropriate combination of genes to be used for data normalisation was done using the statistical application *geNorm* (Vandesompele et al. 2002). Error bars shown in RT-qPCR data represent the standard error of the means of 4 to 6 biological replicates per time point.

5.2.6. Statistics

The gene expression values were transformed by using a $\log_e$ transformation to ensure a Normal distribution and homogeneity of variance (tested by Shapiro–Wilk and the Levene's tests for normality and homocedasticity respectively). A two-way analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) for unequal N (Spjotvoll and Stoline 1973) were used to test for significant differences in gene expression between time points and/or cultivars. Statistical analyses were performed by using software SAS commercial software (Statistical Analysis System) v.9.2. In all the analyses we considered significant a *P-value* < 0.05.

The correlation between transcript levels of *PTOX* and the other studied genes in all cultivars and time points was evaluated by a parametric correlation test using SPSS v. 16.0.

5.3. Results

5.3.1. Characterisation of *DcPTOX* cDNA

Specific primers allowed the isolation of the complete *PTOX* gene of *D. carota* L. cv. Rotin (*DcPTOX*). *DcPTOX* full-length cDNA sequence of 1338 bp (Fig. 1) includes a continuous open reading frame (ORF) with 1098 bp, which encodes a putative polypeptide consisting of 366 amino acid residues, with a predicted molecular weight (mol wt.) of 41.73 kDa and a hypothetical isoelectric point (pI) of 5.57. The homologous identity score performed in NCBI with the deduced amino acid residues sequence shows that DcPTOX shares high degree of similarity with PTOX from other plant species such as C*apsicum annuum* (72 % identity, 93 % coverage), *S. lycopersicum* (71 % identity, 98 % coverage) and *Coffea canephora* (70 % identity, 94 % coverage). In most of the analysed sequences, *PTOX* appears as a single gene. Nevertheless, in five species (*Cucumis melo*, *Eucalyptus grandis*, *Glycine max*, *Populus trichocarpa* and *Zea mays*) two *PTOX* genes were identified (see CHAPTER 6).

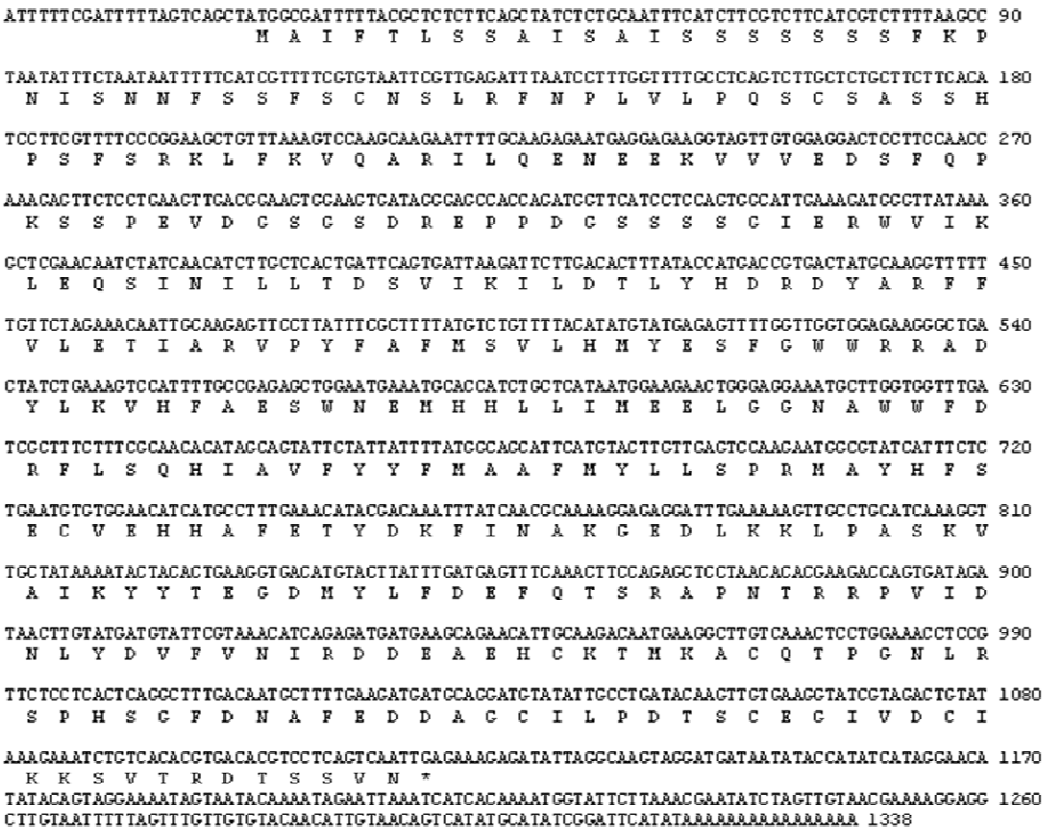


Fig. 1. Nucleotide and deduced amino acid sequences of cDNA encoding *Daucus carota* L. cv. Rotin plastid terminal oxidase, *DcPTOX*. * indicates stop codon. (GenBank: EU331420)

A multiple sequence alignment of 10 complete protein-coding regions of several PTOX plant sequences, including DcPTOX was used to highlight similarities and differences in the

protein sequences (Fig. 2): DcPTOX revealed structural features usually found in most of the higher plants' PTOX. These perfectly conserved residues are four glutamates and two histidines, also conserved in DcPTOX at the positions: E151, E190, H193, E242, E312, H315 (position at the alignment sequence, see in Figure 2). The conservation of 6 cysteines at the C-terminal region was also noted, as in other plant species (C240, C316, C322, C346, C354, C360 in Fig. 2). A conserved 16-amino acid residues insertion, identified by Fu et al. (2005), which corresponds to exon 8 of the higher plant genomic *PTOX* gene sequence was located near de C-terminus (Fig. 2).

PTOXs from the eudicot group can be clearly distinguished from the monocots by specific residues at the positions E86, M204 and W218 (Fig. 2). PTOX sequences from monocots (Fig. 2) can be also distinguished from the remaining plants by specific residues, F191, R213, F214, F217, A225, R243, L255, A269, N276 and E345. These results were confirmed in an alignment with a larger group of sequences (results not shown). PTOX encoded peptides from *G. max* (GM09G01130 and GM15G11950) and *Z. mays* (ZM02G01080 and ZM02G01090) were included in the alignment. Few differences can be detected between both *G. max* PTOX sequences, only differing in 5 amino acids (Fig. 2). *Z. mays* presented many amino acids differences between the two PTOX sequences (Fig. 2).

DcPTOX protein was predicted to be located in the chloroplast, with a predicted length of the chloroplast transit peptide (cTP) of 49 amino acids residues (cTP score of 0.988). Prediction of cTP for the sequences used in the alignment of Fig. 2 shows no conservation across species. PTOX sequences from *G. max* (GM09G01130 and GM15G11950) showed a conserved predicted length of the cTP of 36 amino acid residues. *A. thaliana* (AT4G22260) cTP displayed 56 amino acids. The members of Solanaceae *S. lycopersicum* (SL11G011990) and *Solanum tuberosum* (ST11G007260) showed a cTP of 65 amino acids. PTOX sequences from *Z. mays* (ZM02G01080 and ZM02G01090) present a cTP of 94 and 46 respectively.

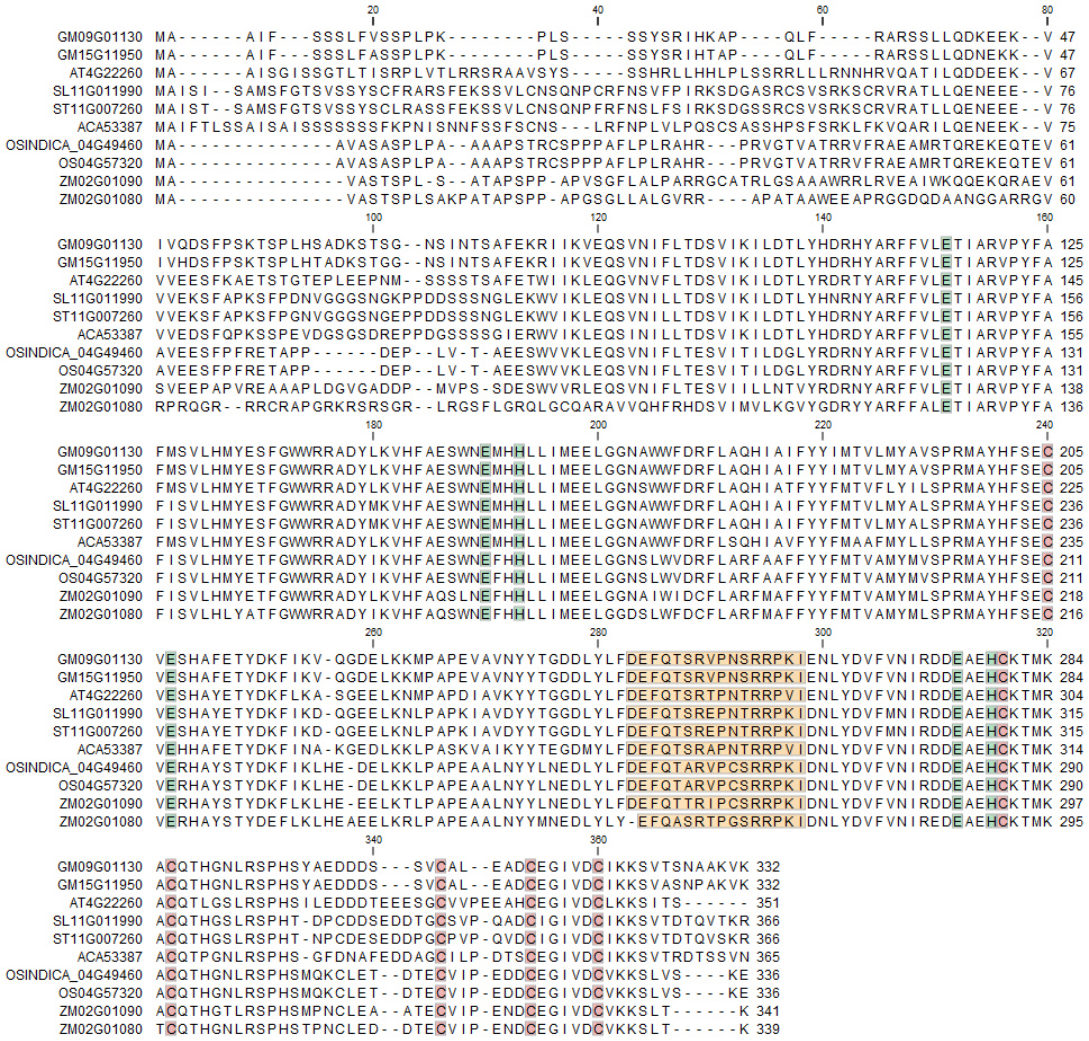


Fig. 2. Multiple alignment of the deduced amino acid sequence from *Daucus carota* L. cv. Rotin PTOX (DcPTOX) and 9 other plant PTOX sequences. The alignment was performed using CLC Main Workbench 6.7.1 software. Six conserved cysteines identified by Josse et al. (2000) are indicated in red. The amino acids required for iron binding are indicated in green (Berthold et al. 2000). The location of the exon 8 identified by Fu et al. (2005) is boxed in yellow. The predicted amino acid sequences used for comparison were DcPTOX (GeneBank: ACA53387) and other PTOX sequences from Plaza databases as follows: GM09G01130 and GM15G11950, *Glycine max*; AT4G22260, *Arabidopsis thaliana*; SL11G011990, *Solanum lycopersicum*; ST11G007260, *S. tuberosum*; OSINDICA_04G49460*, Oryza satica indica;* OS04G57320*, O. sativa japonica;* ZM02G01090 and ZM02G01080*, Zea mays.*

To determine the relationship between DcPTOX and PTOX from other plant species a NJ tree was constructed (Fig. 3), using the identified PTOX sequences. In plants, the PTOX can be separated into two different groups: one group includes all eudicots sequences and other the monocots (Fig. 3). A clade that comprises members of Brassicaceae (AL7G19990, AT4G22260, BR01G12490, CRU_007G18140, TP7G20380) can also be clearly identified within the eudicots.

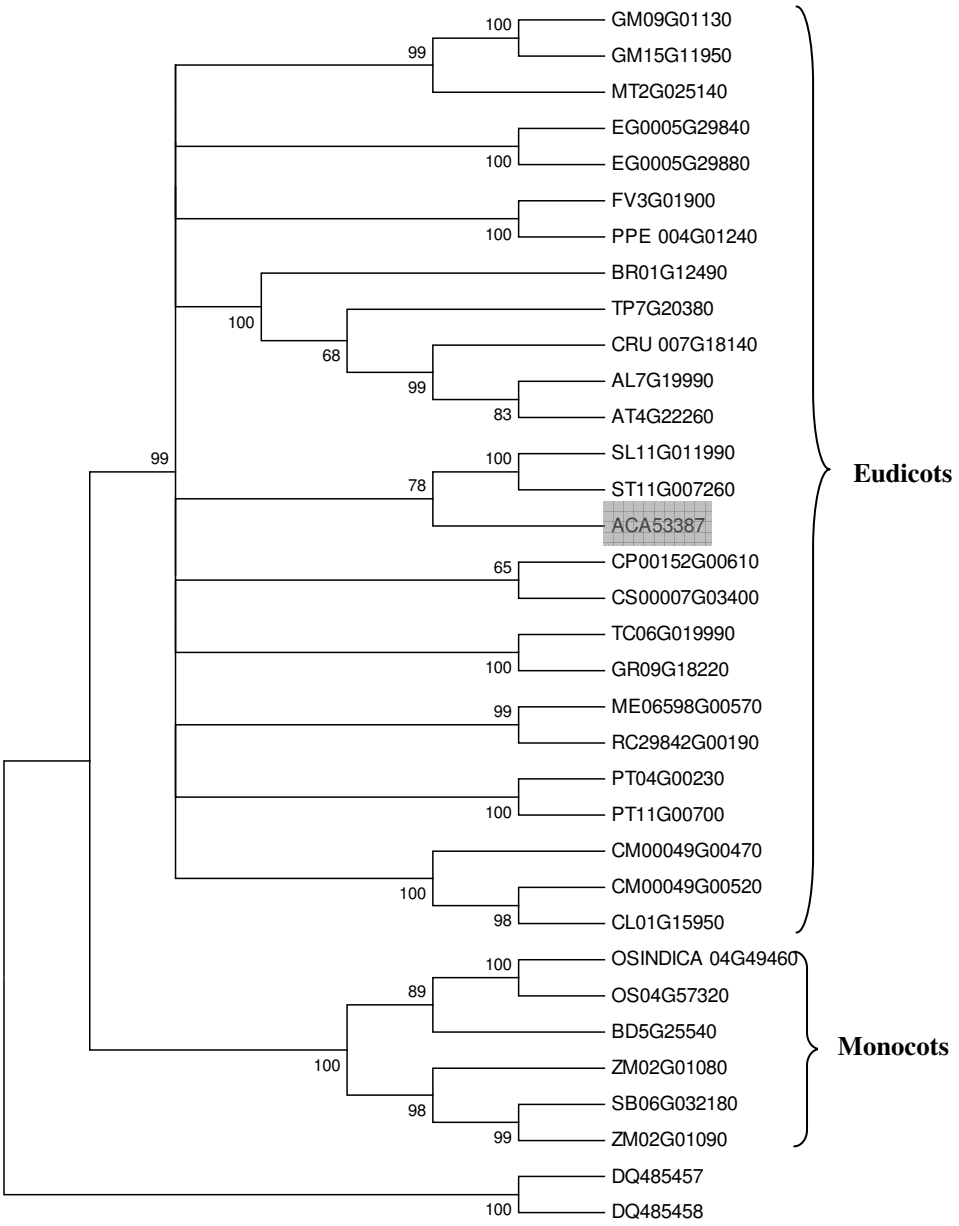


Fig. 3. Neighbor-Joining (NJ) tree showing the relationships among 32 deduced PTOX sequences from plants. The shaded area indicates DcPTOX. The NJ tree was obtained using the complete PTOX peptide sequences. The alignments were bootstrapped with 1000 replicates by the NJ method using the MEGA 4 software. PTOX sequences from the green algae *Haematococcus pluvialis* (GenBank: DQ485457 and DQ485458), were used as outgroups. The scale bar indicates the relative amount of change along branches. The predicted amino acid sequences used for comparison were *Daucus carota* PTOX (GeneBank: ACA53387) and other PTOX sequences from Plaza databases as follows: GM09G01130 and GM15G11950, *Glycine max*; MT2G025140, *Medicago truncatula*; EG0005G29840 and EG0005G29880, *Eucalyptus grandis*; FV3G01900, *Fragaria vesca*; PPE_004G01240, *Prunus persica*; BR01G12490, *Brassica rapa*; TP7G20380, *Thellunginela parvula*; CRU_007G18140, *Capsella rubella*; AL7G19990, *Arabidopsis lyrata*; AT4G22260, *A. thaliana*; SL11G011990, *Solanum lycopersicum*; ST11G007260, *S. tuberosum;*

CP00152G00610, Carica papaya; CS00007G03400, *Citrus sinensis*; TC0006G22260, *Theobroma cacao*; GR09G18220, *Gossypium raimondii*; ME06598G00570, *Manihot esculenta*; RC29842G00190, *Ricinus communis*; PT04G00260 and PT11G02180, *Populus trichocarpa*; CM00049G00470 and CM00049G00520, *Cucumis melo*; CL01G15950*, Citrullus lanatus;* OSINDICA_04G49460*, Oryza sativa indica;* OS04G57320*, O. sativa japonica;* BD5G25540, *Brachypodium distachyon;* ZM02G01090 and ZM02G01080*, Zea mays;* SB06G032180, *Sorghum bicolor.*

5.3.2. *DcPTOX* gene expression and its relation to carotenoid biosynthesis

To examine whether carotenoid accumulation during carrot root development and differences in carotenoid composition between carrot cultivars could be related to the expression of *PTOX*, transcript levels of *DcPTOX* and five carotenoid biosynthetic genes were analysed. *EF-1A* and *GAPDH* were selected to normalize target gene expression. Carotenoids analysis confirmed that the dominant carotenoids that accumulate in the analysed carrot roots vary according to root color and increased during carrot developmental age (results not shown).

RT-qPCR analysis performed in carrot tap roots during secondary root growth showed that all the analysed genes were expressed between the 5th and 13th week of plant growth in the five cultivars, including the white carrot, where very little or no carotenoid pigments were detected (Fig. 4).

Target gene transcript accumulation in the different cultivars throughout the investigated period can be observed in Fig. 4 (for differences during development for each genotype see uppercase letters). As for other carotenoid biosynthetic genes (Fig. 4 B, C, D) *DcPTOX* presented an increased transcript level during the time course of the experiment, with significant (P<0.05) differences during early root development (5-9 wps) (approximately 3-fold for white, yellow and purple; 2-fold for red and 4-fold for orange, Fig. 4 A). This is consistent with the accumulation of total carotenoids in colored cultivars during root development. Carotenoid accumulation and levels of most transcripts seemed to be less correlated during the late rapid carotenoid accumulation stage (9-13 wps). For most genes studied in all cultivars transcript levels were stable over this period, with *LCYB1* being the only exception. Although the white cultivar did not contain carotenoids (see below), transcript levels of *DcPTOX*, *PDS*, *ZDS1* and *LCYB1* increased globally throughout the investigation period. *PSY2* remained constantly low in this cultivar (Fig. 4) with a slight increase in the period between 5-7 wps.

Differences in transcript levels among cultivars detected per time point can also be seen in Figure 4 (see lowercase letters). At 7, 9 and 13 wps *PSY2* presented differential expression between the white and the remaining cultivars with a maximum of 11.6-fold

difference from the orange cultivar, achieved 9 wps. *LCYE* was low in the white cultivar in all studied time points, and significantly different from all cultivars at 13 wps. *PDS*, *PTOX* and *ZDS* presented a similar profile, with some differences between different root colors only detected at 7 wps.

Transcript accumulation of *DcPTOX* was correlated with the transcript accumulation of *PSY2* (Pearson Coefficient: 0,747, $P< 0.001$), *PDS* (Pearson Coefficient: 0,810, $P< 0.001$), *ZDS* (Pearson Coefficient: 0,721, $P< 0.001$), *LCYB1* (Pearson Coefficient: 0,791, $P< 0.001$) and *LCYE* (Pearson Coefficient: 0,631, $P< 0.001$).

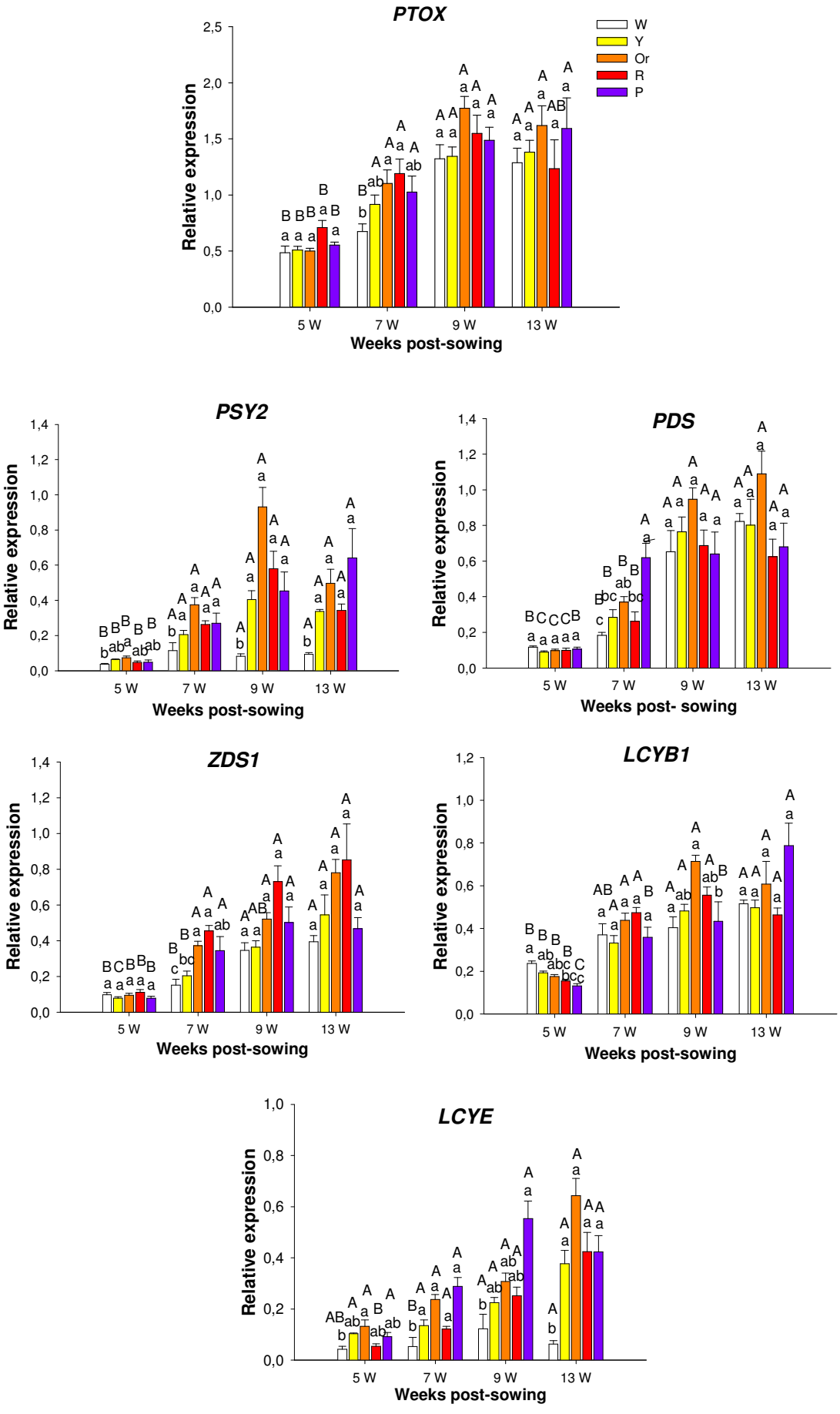

PTOX
PSY2
PDS
ZDS1
LCYB1
LCYE
W
Y
Or
R
P
Relative expression
Weeks post-sowing
Weeks post- sowing
5 W
7 W
9 W
13 W

Fig. 4. Transcript accumulation of *DcPTOX* and five carotenoid biosynthetic genes during carrot root development in five different carrot cultivars, growing under greenhouse conditions. Transcript levels were determined by RT-qPCR. In each harvest time point, 4-6 biological replicates were considered per cultivars. Error bars indicate the standard error of the mean. Different superscript letters indicate significant differences between the stages of development for each cultivars (capital letters), or between cultivars within each time point (small letters). W: white; Y: yellow; Or: orange; R: red; P: purple cultivars.

5.4. Discussion

5.4.1. *DcPTOX* sequence analysis

A single gene encoding the PTOX was identified on *D. carota* L. cv. Rotin (*DcPTOX*), as reported in higher plants such as *S. lycopersicum*, *C. annuum* and *Arabidopsis* (Scolnik et al. 1987; Carol et al. 1999; Wu et al. 1999; Josse et al. 2000), as well as in several plant species phylogenetic distant included in this study like *Brachypodium distachyon* and *Sorghum bicolor.* Nevertheless, it was possible to identify, by analysis of sequences available from projects of all genome sequencing, the existence of two *PTOX* genes in some plant species (including mono and dicot plant species) (Fig. 2, detailed information in CHAPTER 6) e.g. in *C. melo*, *E. grandis*, *G. max*, *P. trichocarpa* and *Z. mays*. The deduced amino acid sequence of *DcPTOX* shared high sequence similarity with PTOXs from other higher plants, including the conserved iron binding residues described by several authors (Josse et al. 2000; Finnegan et al. 2003; Fu et al. 2009; McDonald et al. 2011). PTOX is a member of the non-heme diiron carboxylate (DOX) protein family. The DOX domain is composed of a four-helix bundle that provides six ligands for binding the diiron center (Andersson and Nordlund 1999; Berthold et al. 2000). Fu et al. (2005) demonstrated by using *in vitro* and *in planta* site-directed mutagenesis, that the iron ligands of PTOX are essential for activity and that they do not tolerate changes. These mutagenesis experiments also showed a highly conserved 16 amino acid domain located near the C-terminus, encoded by exon 8, required for PTOX activity and stability. That domain, characteristic of PTOX (McDonald et al. 2003; Fu et al. 2005; Fu et al. 2009; McDonald et al. 2011), could exceptionally be absent. That is the case of *A. thaliana*, in which an allele of *IM* gene (*PTOX* gene for *A. thaliana)* was characterized by the lack of exon 8 (Aluru et al. 2006). That mutation did not affect gene regulation at the transcriptional level but had a strong role on post-transcriptional gene regulation; normal levels of mRNA were produced, but no IM protein was accumulated. Six previously reported conserved cysteines on the C-terminal region (Josse et al. 2000) were also identified in DcPTOX.

PTOX chloroplast localisation was confirmed by *in organelle* import assays in *A. thaliana* (Wu et al. 1999; Carol et al. 1999). PTOX has a N-terminal cTP, like most of the precursor proteins targeted to chloroplasts, and its length influences the efficiency of translocation (Bionda et al. 2010). In PTOX of higher plants, no conservation across species was identified when a prediction of cTP was done using bioinformatics tools. The biological reason for those differences remains unknown. Nevertheless, there are other examples of no conservation of TP across plant species. It is the case of the mitochondrial alternative oxidase (AOX) where the predicted length of the mitochondrial targeting sequence cleavage site is highly variable, not only across plant species but also within the same plant species across proteins encoded by different members of the *AOX* gene family (Campos et al. 2009).

5.4.2. *DcPTOX*, carrot development and carotenoid accumulation

Carotenoids are common pigments and their compositions and contents are important indexes to evaluate the nutritional and commercial values. For a better understanding of the involvement of *PTOX* on the carrot carotenoid biosynthesis pathway, an experiment involving five cultivars with variable root colors and different developmental stages was performed. *DcPTOX* transcripts were ubiquitously detected between the 5^{th} and 13^{th} week of plant growth for all cultivars. Beside *DcPTOX*, five other genes directly involved in carotenoid biosysthesis pathway, with their transcripts detected in carrot roots were selected (Just et al. 2007; Clotault et al. 2008; Fuentes et al. 2012). *DcPTOX* presented a similar expression profile to these genes, and highly correlated with all, especially with *PDS*. PTOX is known to play a role as a cofactor for PDS and is consequently a key enzyme in carotenoid biosynthesis. In the carotenoid biosynthetic pathway, the colored carotenoids are synthesized within plastids from phytoene, a non-colored precursor that results from two geranylgeranyl diphosphate (GGPP) molecules, catalyzed by PSY. Desaturation of phytoene by the sequential activity of the enzymes PDS and ZDS (Carol and Kuntz 2001; Simkin et al. 2008) results in the production of lycopene, a substrate for the formation of both α- and β-carotene. Catalytic activity of PDS and ZDS has been shown to require several redox components including PQ and O_2. As a PQ/O_2 oxidoreductase, plant PTOX has been regarded as an important co-factor of carotenoid biosynthesis by transferring the electrons derived from PDS and ZDS steps to O_2 via the PQ pool (Carol and Kuntz 2001; Aluru and Rodermel 2004). PTOX is responsible for oxidizing PQH_2 and making PQ available for reduction allowing the process to continue (Josse et al. 2000; McDonald et al. 2011). Lack of PTOX will block carotenoid synthesis at the PDS step due to over reduction of the PQ pool (Carol and Kuntz 2001). Due to its position in metabolism, PDS activity could serve to regulate both the carotenoid and chlorophyll biosynthetic pathways

(McDonald et al. 2011). In tomato, the loss of PTOX function in the *ghost* mutant leads to carotenoid accumulation defects in both leaves (chloroplasts) and fruit (chromoplasts), indicating that PTOX is a major cofactor involved in phytoene desaturation in both photosynthetic and non-photosynthetic tissues (Josse et al. 2000).

Carrot root colour is a result of various pigments that serve as intermediate products in the carotenoid pathway. Different root colors are the result of differences in carotenoid composition. The orange color is due to α- and β-carotene, whereas the red color is due mostly to an accumulation of lycopene. The purple carrots contain anthocyanins, and the yellow color in carrot roots is due to xanthophylls, downstream of β-carotene. White-colored roots are low in total carotenoids. However a poor link was detected in our experiment among carotenoid accumulation and the relative transcript abundance of *DcPTOX* as well as other studied carotenoid biosynthetic genes. In fact, only in the early stages of root growth differences on *DcPTOX* transcript accumulation were detected among cultivars. However, differential expression of *PSY2* was detected when comparing the carrot storage root tissue of the white and the other cultivars. Our results are in agreement with Maass et al. (2009), where they suggest that *PSY* is the major driver for accumulation of carotenoids, and with Santos et al. (2005) that refer an important role of *PSY* in regulating carotenoid accumulation in a carrot population segregating for white, yellow and orange root color. From the analysed carotenoid biosynthetic genes in different carrot storage root colors (*PTOX* not included), also Bowman et al. (2014) observed differential expression only on *PSY* genes, suggesting these authors its key role in the biosynthesis of carotenoid pigments. These authors hypothesized that differential expression of one or more genes in that pathway may account for, or at least contribute to, the large differential accumulation of carotenoids observed by comparing white and orange carrots. Evidence of carotenoid gene expression in non-pigmented carrot root (without carotenoid accumulation), with a similar profile of several carotenoid genes of colored roots (including *DcPTOX*) was observed in our experiments, as well as by several other authors (Bowman et al. 2014; Clotault et al. 2008). Several mechanisms were already suggested to explain this apparent paradox such as the existence of non-functional alleles, tissue-specific isoforms, impaired enzyme activity, or increased carotenoid degradation in white carrots, but no direct experimental evidence supporting any of them was presented (Rodriguez-Conception and Stange 2013; Clotault et al. 2008). Recently, the identification of post-transcriptional mechanisms with influence on carotenoid accumulation can also explain this poor correlation (Ruiz-Sola and Rodríguez-Concepción 2012). As an example of this post-transcriptional mechanisms we can refer to the orange curds of the cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* variety, in which chromoplast-like plastids with inclusions of membranous

compartments develop due to a mutation in a gene that results in the accumulation of much higher carotenoid (β-carotene) levels compared to uncolored varieties without changes in the expression of carotenoid biosynthetic genes (Li et al. 2001). Although the biological role of this mutation is still unclear, these studies illustrate how carotenoid accumulation can be boosted by triggering the synthesis of a plastid deposition sink to store carotenoids (Giuliano and Diretto 2007; Li and Van Eck 2007). From the analysis of our results we can suggest an additional explanation for the increase of *PTOX* transcripts in the carrot white cultivar along the experiment, which is based on the association of *DcPTOX* not only with carotenoid biosynthesis, but also with development and growth.

As a complement to gene expression, *DcPTOX* genomic sequence analysis with the identification of single nucleotide polymorphisms (SNPs) and InDels (insertion and deletions) in all parts of the gene (exons, introns, promoter regions and UTRs) will be of further interest to determine if these features are associated with storage root pigmentation. DNA sequence analysis in carotenoid biosynthesis genes has already been associated with accumulation of various carotenoid pigments. In maize, the natural variation of DNA sequence of the carotenoid gene *LCYE* has been found to control the flux of carotenoid metabolism towards either α-carotene or β-carotene accumulation, and can be used as a source of genetic variation for maize biofortification (Harjes et al. 2008). Sequence variability in *DcPTOX* could also contribute to the diversity in carotenoid accumulation in *D. carota*. Association of these polymorphisms will help bridge the gap between genomics and phenomics.

5.5. Conclusions

Our results showed that *DcPTOX* expression has a similar profile to that of other carotenoid biosynthetic genes, with a high correlation to all, suggesting an involvement of *DcPTOX* in carotenoid biosynthesis pathway during carrot storage root development. Our results also reaffirm previous experiments regarding the preponderant role of *PSY* in the biosynthesis of carotenoid pigments. We also propose that *DcPTOX* might be associated not only with carotenoid biosynthesis, but also with development and growth.

5.6. Acknowledgements

The authors are thankful to Bettina Linke from Humboldt University, Berlin, Germany for providing the cDNA library used for gene isolation. We are also thankful to the Portuguese FCT - Foundation for Science and Technology for the support given under the program POPH – Programa Operacional Potencial Humano to B.AS. and H.G.C. (Ciência 2007 and Ciência 2008: C2008-UE/ICAM/06), and for the support given to M.D.C. (SFRH/BD/65354/2009). This work is

funded by FEDER Funds through the Operational Program for Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the Strategic Project PEst-C/AGR/UI0115/2011 and PEst-OE/AGR/UI0115/2014.

5.7. References

Aluru MR, Bae H, Wu D, Rodermel SR (2001) The Arabidopsis immutans mutation affects plastid differentiation and the morphogenesis of white and green sectors in variegated plants. Plant Physiol 127 (1):67-77.

Aluru MR, Rodermel SR (2004) Control of chloroplast redox by the IMMUTANS terminal oxidase. Physiol Plant 120 (1):4-11.

Aluru MR, Yu F, Fu A, Rodermel S (2006) Arabidopsis variegation mutants: new insights into chloroplast biogenesis. J Exp Bot 57 (9):1871-1881.

Andersson ME, Nordlund P (1999) A revised model of the active site of alternative oxidase. Febs Letters 449 (1):17-22.

Archibald JM, Rogers MB, Toop M,et al (2003) Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigelowiella natans*. Proc Natl Acad Sci USA 100 (13):7678-7683.

Atteia A, van Lis R, van Hellemond JJ, et al (2004) Identification of prokaryotic homologues indicates an endosymbiotic origin for the alternative oxidases of mitochondria (AOX) and chloroplasts (PTOX). Gene 330:143-148.

Barr J, White WS, Chen L, et al (2004) The GHOST terminal oxidase regulates developmental programming in tomato fruit. Plant Cell and Environment 27 (7):840-852.

Berthold DA, Andersson ME, Nordlund P (2000) New insight into the structure and function of the alternative oxidase. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1460 (2-3):241-254.

Berthold DA, Stenmark P (2003) Membrane-bound diiron carboxylate proteins. Annu Rev Plant Biol 54:497-517.

Bionda T, Tillmann B, Simm S, et al (2010) Chloroplast import signals: the length requirement for translocation in vitro and in vivo. J Mol Biol 402 (3):510-523.

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

Bowman MJ, Willis DK, Simon PW (2014) Transcript abundance of phytoene synthase 1 and phytoene synthase 2 is associated with natural variation of storage root carotenoid pigmentation in carrot. J Amer Soc Hort Sci 139:63-68.

Campos MD, Cardoso HG, Linke B, et al (2009) Differential expression and co-regulation of carrot AOX genes (*Daucus carota*). Physiol Plant 137 (4):578-591.

Campos MD, Frederico AM, Nothnagel T, et al (2015) Selection of suitable reference genes for reverse transcription quantitative real-time PCR studies on different experimental systems from carrot (*Daucus carota* L.). Sci Hort 186:115-123 DOI:10.1016/j.scienta.2014.12.038.

Carol P, Kuntz M (2001) A plastid terminal oxidase comes to light: implications for carotenoid biosynthesis and chlororespiration. Trends in Plant Science 6 (1):31-36.

Carol P, Stevenson D, Bisanz C, et al (1999) Mutations in the Arabidopsis gene IMMUTANS cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. Plant Cell 11 (1):57-68.

Clotault J, Peltier D, Berruyer R, et al (2008) Expression of carotenoid biosynthesis genes during carrot root development. J Exp Bot 59 (13):3563-3573.

Cournac L, Redding K, Ravenel J, et al (2000) Electron flow between photosystem II and oxygen in chloroplasts of photosystem I-deficient algae is mediated by a quinol oxidase involved in chlororespiration. J Biol Chem 275 (23):17256-17262.

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300 (4):1005-1016.

Finnegan PM, Umbach AL, Wilce JA (2003) Prokaryotic origins for the mitochondrial alternative oxidase and plastid terminal oxidase nuclear genes. FEBS Letters 555 (3):425-430.

Fu A, Aluru M, Rodermel SR (2009) Conserved active site sequences in Arabidopsis plastid terminal oxidase (PTOX): in vitro and in planta mutagenesis studies. J Biol Chem 284 (34):22625-22632.

Fu A, Liu H, Yu F, et al (2012) Alternative oxidases (AOX1a and AOX2) can functionally substitute for plastid terminal oxidase in Arabidopsis chloroplasts. Plant Cell 24 (4):1579-1595.

Fuentes P, Pizarro L, Moreno JC, et al (2012) Light-dependent changes in plastid differentiation influence carotenoid gene expression and accumulation in carrot roots. Plant Mol Biol 79 (1-2):47-59.

Giuliano G, Diretto G (2007) Of chromoplasts and chaperones.Trends Plant Sci 12: 529-531.

Houille-Vernes L, Rappaport F, Wollman FA, et al (2011) Plastid terminal oxidase 2 (PTOX2) is the major oxidase involved in chlororespiration in Chlamydomonas. Proc Natl Acad Sci U S A 108 (51):20820-20825.

Joet T, Genty B, Josse EM, et al (2002) Involvement of a plastid terminal oxidase in plastoquinone oxidation as evidenced by expression of the *Arabidopsis thaliana* enzyme in tobacco. J Biol Chem 277 (35):31623-31630.

Josse EM, Simkin AJ, Gaffe J, et al (2000) A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. Plant Physiol 123 (4):1427-1436.

Just BJ, Santos CA, Fonseca ME, et al (2007) Carotenoid biosynthesis structural genes in carrot (*Daucus carota*): isolation, sequence-characterization, single nucleotide polymorphism (SNP) markers and genome mapping. Theor Appl Genet 114 (4):693-704.

Khachik F, Beecher GR, Goli MB, Lusby W (1992) Separation and quantification of carotenoids in foods. Methods Enzymol 213:347-359.

Kong J, Gong JM, Zhang ZG, et al (2003) A new AOX homologous gene OsIM1 from rice (*Oryza sativa* L.) with an alternative splicing mechanism under salt stress. Theor Appl Genet 107 (2):326-331.

Kuntz M (2004) Plastid terminal oxidase and its biological significance. Planta 218 (6):896-899.

Lennon AM, Prommeenate P, Nixon PJ (2003) Location, expression and orientation of the putative chlororespiratory enzymes, Ndh and IMMUTANS, in higher-plant plastids. Planta 218 (2):254-260.

Li L, Paolillo DJ, Parthasarathy MV, et al (2001) A novel gene mutation that confers abnormal patterns of beta-carotene accumulation in cauliflower (*Brassica oleracea var. botrytis*). Plant J 26:59-67.

Li L, Van Eck J (2007) Metabolic engineering of carotenoid accumulation by creating a metabolic sink. Transgenic Res 16:581-585.

Linke B, Nothnagel T, Borner T (2003) Flower development in carrot CMS plants: mitochondria affect the expression of MADS box genes homologous to GLOBOSA and DEFICIENS. Plant J 34 (1):27-37.

Maass D, Arangi J, Wüst F, et al (2009) Carotenoid crystal formation in arabidopsis and carrot roots caused by increased phytoene synthase protein levels. PLoS one 4 (7): e6373.

McDonald AE, Ivanov AG, Bode R, et al (2011) Flexibility in photosynthetic electron transport: the physiological role of plastoquinol terminal oxidase (PTOX). Biochim Biophys Acta 1807 (8):954-967.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-97.

Peltier G, Cournac L (2002) Chlororespiration. Annu Rev Plant Biol 53:523-550.

Rodermel S (2001) Pathways of plastid-to-nucleus signaling. Trends Plant Sci 6 (10):471-478.

Rodrígues-Conceptión M, Stange C (2013) Biosynthesis of carotenoids in carrot: An underground story comes to light. Arch Biochem Biophys 539:110-116.

Ruiz-Sola MÁ, Rodríguez-Concepción M (2012) Carotenoid biosynthesis in Arabidopsis: a colorful pathway. Arabidopsis Book 10, 158-185.

Santos CAF, Senalik DA, Simon PW (2005). Path analysis suggests phytoene accumulation is the key step limiting the carotenoid pathway in white carrot roots. Genet Molec Biol 28: 287-293

Scolnik PA, Hinton P, Greenblatt IM, et al (1987) Somatic instability of carotenoid biosynthesis in the tomato ghost mutant and its effect on plastid development. Planta 171:11–18.

Shahbazi M, Gilbert M, Laboure AM, Kuntz M (2007) Dual role of the plastid terminal oxidase in tomato. Plant Physiol 145 (3):691-702.

Simkin AJ, Moreau H, Kuntz M, et al (2008) An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*. J Plant Physiol 165 (10):1087-1106.

Simon PW, Wolff XY (1987) Carotenes in typical and dark orange carrots. J. Agr. Food Chem. 35:1017–1022.

Spjotvoll E, Stoline MR (1973) An extension of the T-method of multiple comparison to include the cases with unequal sample sizes. J. Am. Stat. Assoc. 68:976–978.

Sun X, Wen T (2011) Physiological roles of plastid terminal oxidase in plant stress responses. J Biosci 36 (5):951-956.

Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3 (7):RESEARCH0034.

Wang J, Sommerfeld M, Hu Q (2009) Occurrence and environmental stress responses of two plastid terminal oxidases in *Haematococcus pluvialis* (Chlorophyceae). Planta 230 (1):191-203.

Wu D, Wright DA, Wetzel C, et al (1999) The IMMUTANS variegation locus of Arabidopsis defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. Plant Cell 11 (1):43-55.

Supplemental Fig. 1. Aspect of the roots during the time course of the experiment (5, 7, 9 and 13 weeks post-sowing). Cultivars: white (711-1), yellow (207-1), Rotin, red (203-1) and purple (699-1) (from left to right).

CHAPTER 6 - DIFFERENTIAL EXPRESSION OF CARROT PLASTID TERMINAL OXIDASE (*DcPTOX*) DURING STORAGE ROOT GROWTH, AND IN EARLY RESPONSE TO GROWTH INDUCTION

Abstract

The nuclear plastid terminal oxidase gene (*PTOX*) is present in photosynthetic species and functions in the oxidation of the plastoquinone pool. Expression analysis on diverse carrot experimental systems showed a dynamic expression of carrot *PTOX* (*DcPTOX)*. First, an increase on transcript accumulation during carrot tap root secondary growth in a pot plant growth chamber (GC) experiment was detected, which suggests the involvement of *DcPTOX* in tap root secondary growth and/or in the carotenoid biosynthesis pathway. A short-term early response in *DcPTOX* transcript accumulation upon temperature decrease was found, probably associated with adaptive growth. After the beginning of the mild-cold treatment the storage root growth (measured by root biomass, length and thickness) was suppressed. However, the mild-cold stress had a positive effect on the final storage root growth. Next, the expression of *DcPTOX* was studied by high time resolution in a primary culture system (PCS) during early phases of *de novo* growth induction and exponential growth at 21 °C/28 °C. The PCS results showed that *DcPTOX* gene was differentially transcribed during earliest events in cell reprogramming and exponential growth. Differential *DcPTOX* expression was also observed between genotypes and dependent of temperature. Furthermore, the thorough analysis of the complete *DcPTOX* gene revealed it as a single gene, with an exceptionally large genomic sequence (9422 bp) in comparison to other species, comprising nine exons interrupted by eight introns. *In silico* analysis based on data from whole genome sequencing projects revealed that some plant species present two *PTOX* genes. Prediction of pre-miRNA sequences in intronic regions of *DcPTOX* showed two putative locations coding for precursors of miRNAs, one located at intron 2 and other located at intron 6. Regarding future development of functional markers, the search for sequence variability at genomic level was performed. *DcPTOX* revealed intron length polymorphisms (ILPs) in intron 2, due to the occurrence of two insertion/deletions (InDels) events.

Keywords: *Daucus carota*; plastid terminal oxidase; cell reprogramming; growth; *DcPTOX* gene characterisation

6.1. Introduction

The nuclear plastid terminal oxidase gene, initially identified through transposon tagging (Carol et al. 1999) codes for a plastid quinol:oxygen oxidoreductase termed plastid terminal oxidase (PTOX). PTOX is the terminal oxidase of the chlororespiration, and regulates the redox state of the plastoquinone (PQ) pool (Peltier and Cournac 2002; Aluru and Rodermel 2004) by transferring excess electrons to O_2, in order to maintain the relative redox balance in the photosynthetic electron transport chain (ETC), and reducing by this means the possibility for oxidative damage (McDonald et al. 2011). This catalytic function was deduced from its protein sequence similarity to mitochondrial alternative oxidase (AOX). Several conserved domains such as iron-binding residues that are essential for activity have been identified on its amino acid sequence (McDonald et al. 2011; Fu et al. 2012). Such as AOX,PTOX is a member of the non-heme diiron carboxylate (DOX) protein family (Carol et al. 1999; Wu et al. 1999; Berthold and Stenmark 2003) and both enzymes have been modeled as interfacial membrane proteins with an active site (DOX) domain (McDonald et al. 2011). PTOX in chloroplasts has been suggested to be functionally analogous to AOX in mitochondria (Aluru and Rodermel 2004; Kuntz 2004) and for instance a role in minimizing the generation of reactive oxygen species (ROS) when induced under environmental stresses has been attributed to PTOX (McDonald et al. 2011). The “safety valve” function of PTOX, which is a protective function against over-reduced states under stress conditions, has frequently been put forward for e.g. in the alpine plant *Ranunculus glacialis* under light stress at increasing altitudes (Streb et al. 2005), in the halophyte *Thellungiella halophila* under salt stress (Stepien and Johnson 2009) and in *Brassica fruticulosa* under temperature and light stress (Díaz et al. 2007). All these studies seem to indicate a metabolic role for plant PTOX upon a diversity of environmental stresses. However, other authors argue that PTOX does not act as a “safety valve” for photosynthesis during stressful conditions but more likely plays important roles during plant development (Rosso et al. 2006; Shahbazi et al. 2007; Busch et al. 2008; Okegawa et al. 2010). In fact, it is likely that PTOX is involved on stress response of certain plants, but does not act as a universal or essential safety valve in the whole plant kingdom. An important function on carotenoids biosynthesis pathway is also attributed to PTOX (Carol et al. 1999; Wu et al. 1999; Josse et al. 2000; Carol and Kuntz 2001; Rodermel 2001; Aluru et al. 2006; see CHAPTER 5)

Interestingly, recent findings on the interaction between AOX and PTOX were referred in *Arabidopsis thaliana* by Fu et al. (2012). According to these authors, AtAOX2 was imported into chloroplasts using its own transpeptide and so, it was proposed that AtAOX2 is able to function in chloroplasts to supplement PTOX activity during early events of chloroplast biogenesis. Similar results were obtained when AtAOX1a was reengineered to target the

plastid. The ability of AtAOX1a and AtAOX2 to substitute PTOX in the correct physiological and developmental contexts is a striking example of the capacity of a mitochondrial protein to replace the function of a chloroplast protein and illustrates the plasticity of the photosynthetic apparatus (Fu et al. 2012).

The involvement of *AOX* genes in plant stress responses towards phenotype plasticity and the relation of AOX activity to growth have put forward the idea of its use as a source for functional marker (FM) candidates in molecular plant breeding on adaptive stress behaviour (Arnholdt-Schmitt et al. 2006; Arnholdt-Schmitt 2009; Polidoros et al. 2009; Cardoso and Arnholdt-Schmitt 2013; CHAPTER 3). Due to the similarity between both enzymes it is plausible that PTOX is also involved in at least some of those functions, and therefore the gene arises as a potential source of functional markers.

In the present work it is described the dynamics of *DcPTOX* transcript accumulation in two experimental systems: (a) during carrot tap root secondary root growth, in a growth chamber experiment (GC) and (b), during growth in an *in vitro* primary culture system (PCS). The effect of temperature was evaluated in both systems. Considering the possible involvement of *DcPTOX* on important agronomical traits in carrot (yield or pigmentation of storage roots), the idea of future development of functional markers could be of high interest to assist carrot plant breeding. In this context the isolation and characterization of the *DcPTOX* complete genomic sequence is presented, as well the evaluation of polymorphisms at intronic level.

6.2. Material and methods

6.2.1. *DcPTOX* expression analysis

Growth chamber experiment (GC)

In order to study the involvement of *DcPTOX* in plant growth and plant response upon mild-cold stress, growth chamber experiments were performed. Seeds of *D. carota* cv. Rotin were sown in pots in order to have 10 plants *per* pot, and three pots *per* time point. Pots were distributed in two growth chambers maintained at 25 °C day and 20 °C night, under 16 h photoperiod with 202.5 µmol m^{-2} s^{-1} (15000 lux) of light intensity. These experiments run at the German 'Federal Centre for Breeding Research on Cultivated Plants (BAZ)' in Quedlinburg within the frame of a bilateral cooperation. The plant material was harvested, properly stored and sent to PhD candidate for the expression analyses.

To study the involvement of *DcPTOX* on plant growth, samples were collected from tap roots growing under 25/20 °C (day/night) at 28, 42, 70 and 98 days post-sowing.

To study the involvement of *DcPTOX* upon cold stress, the temperature conditions of one chamber changed at 84 days after sowing from 25/20 °C (day /night) to 15/10 °C (day/night), and samples were collected from tap roots at 84 days after sowing (before exposure to cold stress), and at 3 h, 8 h, 24 h and 4 days after exposure to cold stress.

Samples consisted of small pieces of the cambium tissue plus adjacent secondary phloem cells, taken from 18 plants as a bulked sample *per* time point.

The parameters root biomass (root fresh weight), root length and root thickness were evaluated in 6 individual storage roots from both chambers at the described temperature conditions at the time points: 28, 42, 56, 70, 84, 85, 86, 88, 90, 92, 98 and 125 days after sowing.

Establishment of a primary culture system (PCS)

To study the dynamics of *DcPTOX* gene expression during growth, a primary culture system approach was chosen. For complete and detailed description of the procedure see Campos et al. (submitted, see in CHAPTER 3. Explants from secondary tap root phloem from 10 weeks old plants of *D. carota* L. cv. Rotin were inoculated in NL liquid medium (Neumann 1966) supplemented with kinetin (1 $mg \cdot L^{-1}$) and indole acetic acid (2 $mg \cdot L^{-1}$). Inoculation of explants from quiescent secondary root phloem in a cytokinin-containing nutrient media induces cell program changes that imply the acquisition of a so-called 'undifferentiated' stage (*callus*) and subsequent growth. To get novel insights about role of *PTOX* on tissue dedifferentiation and *callus* growth, explants from 4 individual carrot plants (4 biological replicates) were inoculated at 21 °C and were collected at different time points: 0 h, 8 h, 12 h, 36 h, 4 days, 8 days, 14 days, 21 days and 28 days post inoculation (hpi/dpi). Samples consisted in a bulked of 30 explants taken at time points from 0 hpi until 4 dpi, and about 15 explants in the remaining time points. The maximum number of time points chosen to collect plant material for RNA extraction and for growth curve analysis was restricted by root sizes (for growth curve analysis results and the aspect of the *callus* during growth see CHAPTER 3).

To compare the transcript changes of *DcPTOX* on PCS under two different incubation temperatures (21 °C and 28 °C), explants from five individual plants (five biological replicates previously selected from 12 individual plants based on *callus* growth behaviour, under the two temperatures tested, see details in CHAPTER 3) were collected before inoculation (T0), and from *callus* from exponentially growing primary cultures at 14 dpi. Samples consisted of bulked samples of about 50 explants.

Sample processing

All samples were immediately grounded to fine powder using liquid nitrogen and stored at -80 °C until further analysis. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with on-column digestion of DNA using RNase-Free DNase Set (Qiagen, Hilden, Germany), according to manufacturer's instruction. The integrity of RNA samples were analysed in denaturing gel after stained with EtBr solution (2 $ng \cdot mL^{-1}$) and visualized in a Gene Flash Bio Imaging system (Syngene, Cambridge, UK). RNA concentration was determined in a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNase-treated total RNA (1 µg) were reverse transcribed with random decamer primer using the RETROscript® kit (Ambion, Austin, TX, USA) according to manufacturer's instruction.
In GC experiment two RTs were performed per time point. In the PCS bulked samples from individual plants were taken and a single RT was performed per time point.

Transcript analysis

The availability of complete *DcPTOX* gene sequences at the cDNA level in *Daucus carota* (see in CHAPTER 5) allowed the design of specific primers to study transcript accumulation in both experimental systems.

Transcript analyses of *DcPTOX* were performed by semi-quantitative PCR (RT-sqPCR) and by quantitative PCR (RT-qPCR). Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, USA) (Table 1). For RT-sqPCR experiments the gene considered for normalisation was elongation factor-1alpha (*EF-1A*) (selection based on previous experiments, see technical details on CHAPTER 7). For RT-qPCR experiments the reference genes selection was previously made, and vary according to the experimental system: *EF-1A* and *alpha-tubulin* (*TBA*) for GC (to evaluate plant growth, see CHAPTER 7), and *EF-1A* and *Actin* for GC (to evaluate the effect of cold treatment, previously selected from a set of 4 tested reference genes, results not shown) and for PCS (see CHAPTER 7).

RT-sqPCR experiment was performed only in PCS using Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England), 2 µL of cDNA (diluted 1:10) as template and 0.2 µM of each specific primer (Table 2). PCR for *EF-1A* and *DcPTOX* was carried out in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA) for 35 cycles, each one consisting of 30 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C. RT-sqPCR products were analysed by electrophoresis in 2 % (w/v) agarose gel. Image analysis was carried out to normalize the expression level of *PTOX* cDNA with the reference *DcEF1α* gene. The densities of the bands were analysed with using ImageJ 1.47v (http://imagej.nih.gov/ij) (Schneider et al. 2012) . The results were expressed as mean ± standard error (SE) of four individual plants. Differences

between time points were examined by one-way ANOVA using the STATISTICA 8.0 statistical package (StatSoft Inc.).

RT-qPCR gene expression was performed with SYBR Green q-PCR Master Mix (Fermentas, Ontario, Canada) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). Fifteen µl reaction volume containing 5 µL of first-strand cDNA (previously diluted 1:10) and specific primers adjusted to the adequate concentration (see primer details on Table 1) were used. The identity of each amplicon was confirmed by Sanger sequencing and specificity of qPCR reactions was evaluated by melting curve analysis. Efficiencies were calculated using a 4-point standard curves from a 4-fold dilution series (1:1-1:125) (run in triplicate) of pooled cDNA. RT-qPCR was conducted for 40 cycles, each consisting in 15 s at 95 °C followed by 1 min at 60 °C. To analyse the dissociation curve profiles, an additional step at 95 °C during 15 s was added, followed by a constant increase of temperature between 60 and 95 °C. All samples were run in duplicate. Minus reverse transcriptase and no template controls were included to assess contaminations. Cq values were acquired for each sample with a fluorescence threshold arbitrarily set at 1. Evaluation of expression stability of reference genes and selection of the most appropriate combination of genes to be used as reference was done using the statistical application *geNorm* (Vandesompele et al. 2002). The results were expressed as mean ± standard error (SE), and examined by One-Way ANOVA or by Student's *t* tests, using the STATISTICA 8.0 statistical package (StatSoft Inc., Tulsa, USA).

Table 1. Primers used in sq-qPCR and RT-qPCR.

[NCBI accession ID]	Gene	Primer sequence (5'→3')	AS (bp)	[µM]
[GenBank:X17525]	*ACT*	Fw: CACACGGTGCCAATTTATGAA Rv: GATCACGGCCAGCAAGGT	73	300
[GenBank:D12709]	*EF-1A*	Fw: TGGTGATGCTGGTTTCGTTAAG Rv: AGTGGAGGGTAGGACATGAAGGT	75	900
[GenBank:D12709]	*TBA*	Fw: TCTGGTGCCATACCCAAGGA Rv: ATAGGCCTTCTCAGCGGAGAT	73	500
[GenBank: EU331420]	*DcPTOX*	Fw: GGAAATCGGCCACATTAAAATTA Rv: CCCAACATTCCCCACTTGTC	142	500

6.2.2. *DcPTOX* genomic sequence isolation and sequence variability

Plant material

For genomic *DcPTOX* sequence isolation, 8 weeks old *in vitro* growing seedlings of *D. carota* L. cv. Rotin were used (for detailed information related with *in vitro* establishment see CHAPTER 2). A complete single plant was considered for genomic DNA (gDNA) extraction and gene sequence isolation.

For intron length polymorphism studies (ILP), there were considered 10 weeks old plants of *D. carota* cv. Rotin growing under greenhouse conditions. Leaf samples were taken from individual plants and used for gDNA extraction.

For the identification of polymorphisms that could be associated with carotenoids' content, 5 cultivars representing a wide range of pigmented carrot material (3 plants/cultivar, 15 plants in total) were used: white (711-1), yellow (207-1), red (203-1), and purple (purple phloem with yellow xylem) (699-1) (cultivated carrot breeding stocks developed by the USDA carrot breeding program), and the orange coloured cv. Rotin. Samples were taken from leaves of 13 weeks old plants growing under greenhouse conditions and were further used for gDNA extraction.

gDNA extraction

All samples were grounded to fine powder using liquid nitrogen and stored at -80 °C until further analysis. gDNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. gDNA integrity analysis was performed by electrophoresis in 1 % (w/v) agarose gel and visualised using GeneTools (Syngene, Cambridge, UK) after staining with EtBr solution (2 $ngmL^{-1}$). gDNA concentration was determined in a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Isolation of genomic sequence

For complete gene isolation several gene-specific primers sets were designed (Table 2) based on *DcPTOX* cDNA sequence (GenBank acc nº EU331420.2), and on contigs and scaffolds from the draft assembly of inbred line 493 of the carrot genome sequencing program (provided by Dr Philipp Simon, University of Wisconsin-Madison, USA). Considering this previous information, primers covering seven partially overlapping genomic regions were obtained. PCRs were performed using 0.2 µM of each specific primer and the Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) following manufacturer's instruction. Ten ng of gDNA from a single plant were used as template. PCRs were performed in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). PCR products were separated in 1.4 % (w/v) agarose gel, stained in EtBr (2 $ng \cdot mL^{-1}$) and subsequently visualised on a Gene Flash Bio Imaging system (Syngene, Cambridge, UK). Fragments showing the expected size were purified from the agarose gel using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, England) according to the manufacturer's protocol. For cloning, PCR fragments were separately inserted into a pGem®-T Easy vector (Promega, Madison, WI, USA). Reaction

product was used to transform *E. coli* JM109 (Promega Madison, WI, USA) competent cells. Plasmid DNA was extracted from putative recombinant clones following an alkaline lysis protocol (Bimboim and Doly 1979) and the insert was confirmed by restriction using *EcoR*I (Fermentas, Ontario, Canada). Selected recombinant clones were sequenced (Macrogen company: www.macrogen.com) in the directions of sense and antisense strands using *T7* and *SP6* primers.

Table 2. Sequence of primers used for *DcPTOX* amplification.

Amplified fragment	Sequence (5'→3')	Primer TM (°C)	Amplicon size (bp)	Cloned/direct sequenced
① Exon 1-Exon 4	170F: CGTTTTCGTGTAATTCGTTGAGAT 24R: CAAAATGGACTTTCAGATAGTC	58 60	3059	Cloned
② Exon 4-Exon 5	int4F1: GAAAGTCCATTTTGCCGAGA int4R: CAAACCACCAAGCATTTCCT	60 60	926	Cloned
③ Exon 5-Exon 9	int6F1: AGGAAATGCTTGGTGGTTTG int6R1: AACGGAGGTTTCCAGGAGTT	60 60	5522	direct sequenced
④ Intron 6-begin	int6bF: TCATTAGAGTTGCAAGGTTCCA int6bR: AGGATTTGGCAGCAAACATGT	58 59	1341	Cloned
⑤ Intron 6-middle	int6mF: TTGAGTGCATCCCTTAGCCA int6mR: TTGGTTGCACTGCTTTCGTT	59 59	927	Cloned
⑥ Intron 6-end	int6eF: GGGGCTCGGTAAAATATGCT Int6eR: CCGAGTTCCTGTCCCTCAAT	58 59	1781	Cloned
⑦ Exon 8 -3'UTR	364F: ACACACGAAGACCAGTGATAG 1301R: AGCCTCCTTTTCGTTACA	62 45	581	cloned

Sequence analysis

Sequence homology was explored at the NCBI database using the BLAST algorithm (Karlin and Altschul 1993) (http://www.ncbi.nlm.nih.gov/) (BLASTn). To edit the *DcPTOX* sequence data, SeqMan and EditSeq softwares (LASERGENE 7, GATC Biotech, Konstanz) were used.

PTOX genes from a group of 21 eudicot and 5 monocots plant species were retrieved from the genomic database freely available (Plaza 3.0: http://bioinformatics .psb.ugent.be/plaza/versions/plaza_v3_monocots/ and http://bioinformatics.psb.ugent.be /plaza/versions/plaza_v3_dicots/). Sequences were checked at the NCBI database using the BLAST algorithm (Karlin and Altschul 1993) (http://www.ncbi.nlm.nih.gov/) (BLASTn) and exon/intron borders annotations were corrected when needed. Gene draw was performed using FancyGene 1.4 (Rambaldi and Ciccarelli 2009).

In silico identification of regulatory elements located at the DcPTOX intronic regions

For identification of putative miRNA precursors in *DcPTOX* introns, the publicly available software miR-*abela* (http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi) was

used. For validation of potential pre-miRNAs, the software MiPred was applied (http://www.bioinf.seu.edu.cn/miRNA/) (Jiang et al. 2007). Prediction of the secondary structure of pre-miRNA was run on the web-based software Mfold, (available at http://mfold.rit.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker 2003). The criteria applied for screening the candidates of potential pre-miRNA have been previously described (Xie et al. 2007). To screen potential candidates for miRNAs, the validated pre-miRNAs were run with the software miRBase (http://microrna.sanger.ac.uk/sequences/search.shtml). BLASTx from NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find the potential target genes (Mathews et al. 1999; Zuker 2003).

Studies of DcPTOX sequence variability

To study the sequence variability of *DcPTOX* within a cultivar, two regions of the gene were selected: intron 2 and intron 6. To easily check for the occurrence of polymorphisms associated with insertion/deletion (InDels) events, the Exon Priming Intron Crossing-PCR (EPIC-PCR) approach was followed. Specific primers were designed in the exon boundaries of the intron 2 (Fw: 5'-ACCAGATGGTTCATCCTCCA-3', Rv: 5'-TGCATAGTCACGGTCATGGT-3'; annealing at 60 °C for 20 s and extension at 72 °C for 2 min) and intron 6 (Fw: 5'-TGGAACATCATGCCTTTGAA-3', Rv: 5'-AACGGAGGTTTCCAGGAGTT-3'; annealing at 60 °C for 20 s and extension at 72 °C for 2 min). PCRs were performed with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) according to the manufacturer's instruction.

To check for the occurrence of specific polymorphisms associated with the carotenoids content across 5 different cultivars representing a wide range of pigmented carrot material, the analysis of *DcPTOX* sequence variability was focused only on intron 2 (same primers as above). PCRs were performed using Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instruction.

PCRs were performed in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA) using 10 ng of gDNA as template and 0.2 µM of each primer. Ten µl of each PCR product were analysed by 2 % (w/v) agarose gel electrophoresis. Gel staining and visualisation were performed as described above. Amplicon identity was confirmed by cloning and Sanger sequencing following the above described procedure.

6.3. Results

6.3.1. Expression analysis on *DcPTOX*

In vivo carrot GC experimental system

RT-qPCR analysis performed on carrot tap root cambium during secondary root growth allowed detecting *PTOX* mRNA at all time points.

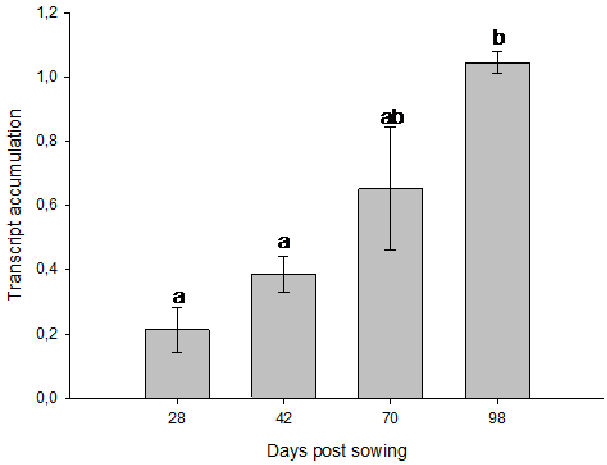


Fig. 1. Expression of *DcPTOX* measured in samples taken from the cambium tissue plus adjacent secondary phloem cells of carrot (*Daucus carota* L. cv. Rotin) plants growing at 25 °C day/20 °C night under 16 h photoperiod. Transcript levels were determined by RT-qPCR. In each harvest time point, samples were collected as a bulked sample of 30 plants, and two RTs were performed. Error bars indicate the standard error of the mean. Different superscript letters indicate significant differences between days post sowing.

The expression analysis was performed during storage root growth at 28, 42, 70 and 98 days post sowing (Fig. 1). The profile of *DcPTOX* through carrot development showed a marked increase (Fig. 1). Expression levels at 98 days post sowing were significantly higher (P<0.05) than at 28 days (almost 6-fold) and at 42 days post sowing (almost 3-fold). This result suggests *DcPTOX* involvement in carrot tap root growth (aspect of carrots during development and the heterogeneity observed between roots can be seen on Suppl. Fig. 1). Forty two days after sowing, the tap roots can be easily identified, and the orange-pale colour becomes visible. At the end of the experiment roots presented the size and colour of the commercialized carrot tap roots.

In order to evaluate *DcPTOX* gene expression involvement in storage root responses upon cold stress, at 84 days post sowing the temperature conditions were changed in one growth chamber from 25 °C/20 °C (day/night) to 15 °C/10 °C (day/night).

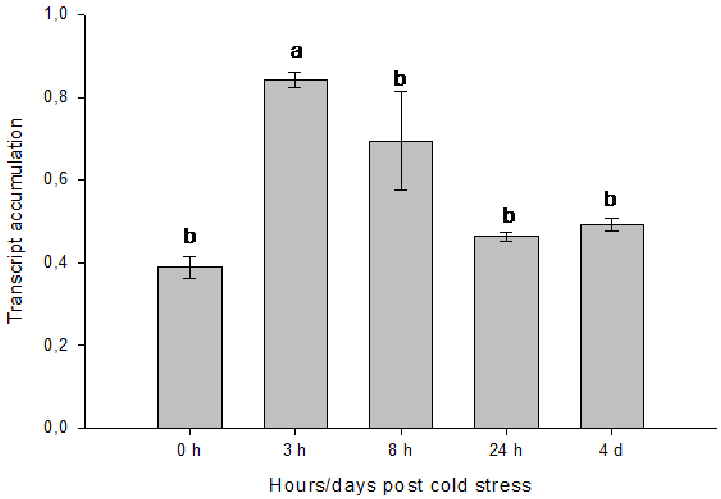


Fig. 2. Transcript accumulation of *DcPTOX* measured in the cambium tissue plus adjacent secondary phloem cells, on carrot (*Daucus carota* L. cv. Rotin) plants upon cold stress. Until 84 days post sowing the growth chamber was at 25 °C day/20 °C night; after that time the temperature decreased to 15 °C day/10 °C night, until the end of the experiment. In each harvest time point, samples were collected as a bulked sample of 18 plants. Two RTs were performed. Error bars indicate the standard error of the mean. Significant differences between the initial time point (0h) and the remaining sampling points are indicated by different superscripts.

A short-term early response in *DcPTOX* transcript accumulation could be observed 3 h after cold stress (Fig. 2). Eight hours after cold exposure, the transcript levels decreased and at 24 h the levels were similar to the ones observed at 0 h, maintaining that level until the end of the experiment. The peak of transcript accumulation was observed at 3 h post cold stress with significant differences ($P < 0.05$) between 0 h and 3 h, between 3 h and 24 h and between 3 h and 4 days after cold stress.

The parameters root biomass, root length and root thickness were also measured during the 125 days of the experiment (Fig. 3). While in chamber 1 plants were maintained at 25 °C/20 °C (day /night) during all experiment (control treatment), in chamber 2 the temperature conditions changed at 84 days after sowing (marked with a vertical arrow in Fig. 3) from 25 °C/20 °C (day /night) to 15 °C/10 °C (day/night). After the beginning of the cold treatment the storage root growth was suppressed, with the biomass, length and thickness values presenting significant lower results than the control, at 88, 92 and 88 days respectively. However, at the end of the experiment, the mild cold stress treatment had a positive impact in all measured parameters (Fig. 3).

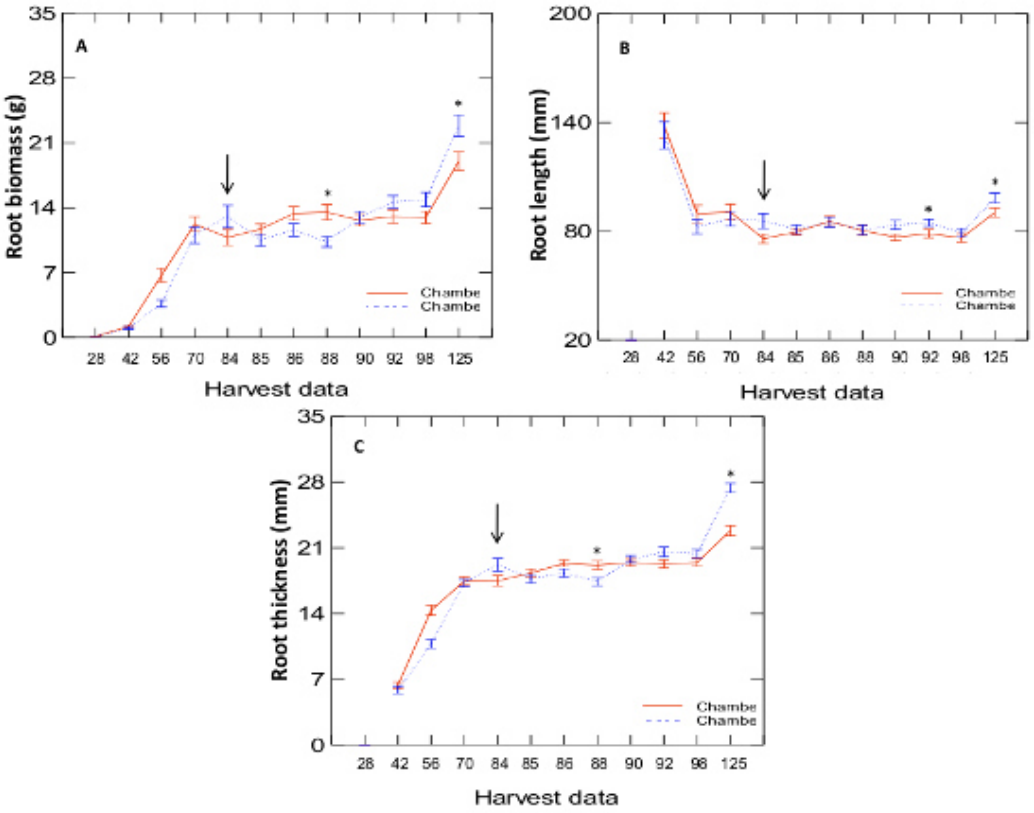


Fig. 3. Measurements of root biomass (A), root length (B) and root thickness (C) of carrot plants (*Daucus carota* cv Rotin) during secondary growth under controlled temperature conditions. Chamber 1: plants maintained at 25/20 °C (day /night) during time course of the experiment; Chamber 2: the temperature conditions changed at 84 days after sowing (marked with a vertical arrow) from 25/20 °C (day /night) to 15/10 °C (day/night). Harvest date refers to the number of days post sowing. Data is presented as the mean ± SE of six tap roots independent measurements. * indicate significant differences ($P < 0.05$) between treatments.

De novo differentiation

To study the potential role of *PTOX* in early cell reprogramming and induction of growth, primary cultures of four carrot roots (*D. carota* cv Rotin) were established at 21 °C. The inoculation of differentiated secondary root phloem explants in a cytokinin-containing nutrient media lead to a cell program change related with the acquisition of an undifferentiated stage (*callus*) and subsequent growth. To shed light on involvement of *DcPTOX* in the earliest events related to cell reprogramming and in a later growth phase, a high temporal resolution in the initial phase and a long observation time was chosen (Fig. 4).

Increased transcript accumulation for *DcPTOX* gene was observed at the early beginning (8 hpi) of the lag-phase during growth induction (Fig. 4). The a priori unknown high variability of individual response of each single plant (4 biological replicates were considered)

led to the absence of statistical significance for differences between time points. Nevertheless, it was possible to distinguish a pattern of expression. At the end of the lag phase and at initiation of exponential growth (8 dpi) transcript accumulation was down regulated to values near the ones measured at 0 hpi followed by an increase at 14 dpi (exponential growth phases) and slight decrease at 21 dpi which was maintained at 28 dpi to values measured for the original, quiescent tissue (Fig. 4).

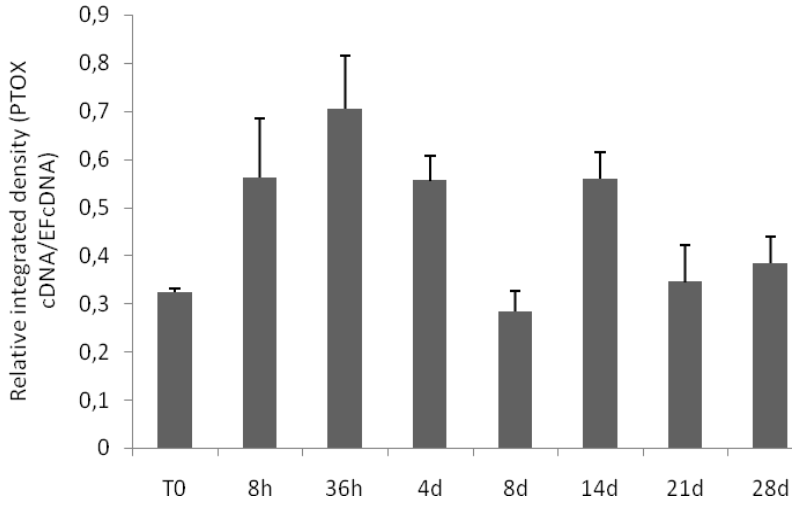


Fig. 4. Transcript levels of *DcPTOX* in primary cultures from secondary phloem of carrot roots. Samples were collected at 0 h, 8 h, 36 h, 4 days, 8 days, 14 days, 21 days and 28 days post *in vitro* inoculation. Cultures were maintained at 21 °C. Transcript accumulation was analysed by RT-sqPCR using *EF-1A* as reference gene. Normalisation of the quantity of *DcPTOX* transcripts was performed through the ratio of integrated densities of *DcPTOX* cDNA and *EF1-1A* cDNA bands. Data are the average values ± SE of four individual plants.

To study transcript accumulation changes of *DcPTOX* on growth acclimation upon temperature, a second PCS experiment was performed considering two growth temperatures (21 °C and 28 °C). The five plants selected for this study presented different temperature-dependent *callus* growth performance (for *callus* growth information see Table 3 in CHAPTER 3): two showing a significantly higher production of *callus* biomass at 28 °C (R2 and R5), two without significant differences between both temperatures (R1 and R4) and only one showing a significantly higher *callus* fresh weight at 21 °C. *DcPTOX* is expressed in the initial explants before *in vitro* inoculation (T0), as well as after 14 days of cell division growth (Fig. 5), initiating a trend for differential expression. Differences for differential gene transcript accumulation between individual plants can be detected, however without a clear tendency between them. Interestingly in R3, the inferior *callus* fresh weight at 28 °C compared with 21 °C (see Table 3 in CHAPTER 3), is coincident with a lower *DcPTOX* transcript accumulation at 28 °C.

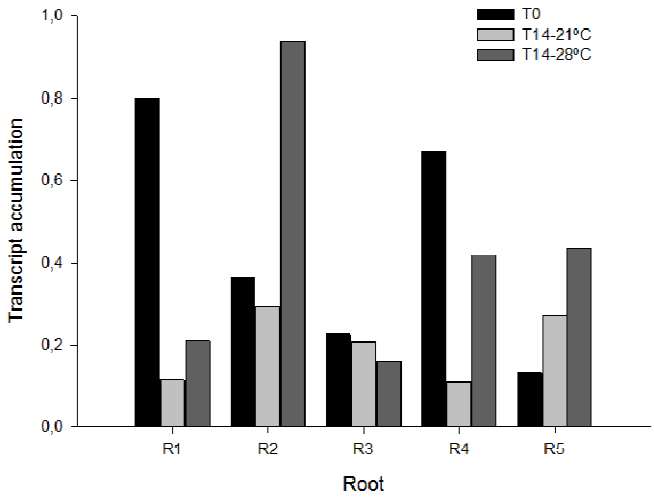


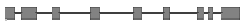
Fig. 5. Transcript accumulation of *DcPTOX* in a primary culture system of *Daucus carota* L. cv. Rotin established under different temperatures. Five biological replicates (single plants) were considered (R1 to R5). T0: explants before inoculation; T14-21 °C: explants after 14 days in culture, growing at 21 °C; T14-28 °C: explants after 14 days in culture, growing at 28 °C

6.3.2. *DcPTOX* sequence analysis and *PTOX* gene diversity across higher plant species

Specific primers allowed the isolation of the complete *PTOX* gene of *D. carota* L. cv. Rotin (*DcPTOX*) at gDNA level. *DcPTOX* has 9422 bp, consisting of nine exons (exon 1: 173 bp; exon 2: 196 bp; exon 3: 94 bp; exon 4: 116 bp; exon 5: 109 bp; exon 6: 69 bp; exon 7: 72 bp; exon 8: 48 bp; exon 9: 221 bp) interrupted by eight introns (intron 1: 1931 bp; intron 2: 474 bp; intron 3: 207 bp; intron 4: 843 bp; intron 5: 120 b p; intron 6: 4474 bp; intron 7: 78 bp; intron 8: 197 bp).

The diversity in terms of orientation, size and exon-intron pattern across 27 plant species is presented on Table 3. The general structure of plant *PTOX* is of nine exons interrupted by eight introns. A single exception was detected in *Medicago truncatula* , with a total of 8 exons and 7 introns due to intron 1 loss and consequent fusion of exon 1 and exon 2. A single gene was identified in most of the plant species, showing a sense or antisense orientation. Nevertheless, five species presented two *PTOX* genes (*Cucumis melo*, *Eucalyptus grandis*, *Glycine max*, *Populus trichocarpa* and *Zea mays*), in three of them appearing as tandem duplications (*C. melo*, *E. grandis*, *Z. mays*). Exon size conservation across plant species was verified at the level of exons 3, 4, 5, 6, 7 and 8 (results not shown). That conservation is responsible by the low variability of the ORF size and consequently of the encoded peptide. Exons 1, 2 and 9 are the regions responsible by the variability identified across plant species (exon 1 range from 101 to 177 bp; exon 2 from 172 to 220 bp; exon 9 from 200 to 233 bp) (Table 3).

Table 3. Diversity of *PTOX* in terms of gene location, orientation, size and exon-intron pattern across 27 higher plant species. Data was retrieved from public web-based databases, freely available (Plaza 3.0: http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_monocots/ and http://bioinformatics.psb. ugent.be/plaza/versions/plaza_v3_dicots/). *Daucus carota PTOX* at GenBank: KM514737 (NCBI: http://www.ncbi.nlm.nih.gov/). Gene draw was performed in FancyGene 1.4 (Rambaldi and Ciccarelli 2009).

Species	Gene_id	Chromosome location	Gene orientation	Gene size	Protein lenght	Exon-intron (box-line) gene structure
Arabidopsis lyrata	AL7G19990	Scaffold_7	+	2292	352	
Arabidopsis thaliana	AT4G22260	4	-	2384	352	
Brassica rapa	BR01G12490	A01	-	1834	355	
Capsella rubella	CRU_007G18140	Scaffold_7	+	2338	354	
Carica papaia	CP00152G00610	Supercontig_152	-	7586	357	
Citrullus lanatus	CL01G15950	1	-	3594	355	
Citrus sinensis	CS00007G03400	Scaffold00007	-	3722	350	
Cucumis melo	CM00049G00470	CM3.5_scaffold00049	+	6058	360	
	CM00049G00520	CM3.5_scaffold00049	+	3112	356	
Daucus carota	KM514737	2	nd	9422	366	
Eucalyptus grandis	EG0005G29840	Scaffold_5	+	4359	352	
	EG0005G29880	Scaffold_5	+	4344	362	
Fragaria vesca	FV3G01900	LG3	-	2908	366	
Glycine max	GM09G01130	9	-	4215	333	
	GM15G11950	15	-	6953	333	
Gossypium raimondii	GR09G18220	9	+	2746	361	
Manihot esculenta	ME06598G00570	Scaffold06598	+	5485	360	
Medicago truncatula	MT2G025140	2	-	3946	343	
Populus trichocarpa	PT04G00260	4	+	3616	363	
	PT11G02180	11	-	3437	359	
Prunus persica	PPE_004G01240	Scaffold_4	+	3112	355	
Ricinus communis	RC29842G00190	29842	+	3229	357	
Solanum lycopersicum	SL11G011990	11	-	4686	367	
Solanum tuberosum	ST11G007260	11	+	4402	367	
Thellungiella parvula	TP7G20380	7-4	-	2387	346	
Theobroma cacao	TC0006G22260	Scaffold_6	-	3060	359	
Brachypodium distachyon	BD5G25540	5	-	2906	369	
Oryza sativa indica	OSINDICA_04G49460	4	-	2440	337	
Oryza sativa japonica	OS04G57320	4	-	2440	337	
Sorghum bicolor	SB06G032180	6	-	2516	352	
Zea mays	ZM02G01080	2	+	3530	340	
	ZM02G01090	2	+	3106	342	

Prediction of pre-miRNA sequences in intronic regions of *DcPTOX* revealed two putative locations coding for precursors of miRNAs, one located at intron 2 and other located at intron 6. In both cases a high homology with previously described miRNAs was found (see Table 4). Searches for sequence homologies regarding putative miRNAs target genes identified, for both introns, disease resistance genes (in intron 2 a 100 % homology with *Citrus sinensis* GenBank acc. XM_006495173.1 and in intron 6 a 100 % homology with *Phaseolus vulgaris* GenBank acc. HQ632856.1).

Table 4. Computational prediction of intronic miRNA precursors in *PTOX* of *Daucus carota* cv. Rotin.

Intron	Putative pre-miRNA sequence	bp	MFE	Prob (%)
1	UAUAUAACAAUUACCCGGACUGGGUUUAUAAUCUUAUGAGA AAUUGGGAUGGCAAACUAGUUUACGGCGUCAAGAAAUGUAU ACAUCUAUGUGUCUUGUAUAUGUG	106	-19.72	No
2	UUCCUACUCAAGUUUCUGUGUGUAGGAUGUCAGCAUUCUGA UUAAUGUUGGUAUUGUAUGAUAGUAAGGAA	71	-15.90	72.8
3	No pre-miRNAs were identified	-	-	-
4	No pre-miRNAs were identified	-	-	-
5	GACGUAUUUCUUUGAUUAUAAAUUAUCUUCUUGGGUUCUAC GACUUAUAUAGAUAUGUAUAUAACAAGAUCUCAUGUU	78	-13.10	No
6	GUCUAUACCUCCUAGAUAUCAUCUAUCUUCUAGUUGCUGGCC CAUGAGCGUAUGAGUCAUUCAAAUUAGUCAGUUAAGUACGU ACUAGGUUCUCUCGGUGUAGAU	105	-25	69.2
7	GAAGAACAAUUGGGUUUGAAAGUAAACUUACAUUUUUACUU CCAUCGCUUC	51	-8.9	No
8	No pre-miRNAs were identified	-	-	-

Pre-miRNAs were determined with miR-abela software (http://www.mirz.unibas.ch/cgi/pred miRNA genes.cgi.). bp: length of the pre-miRNA sequence in bp, MFE: minimal free energy in kcal/mol, Prob: probability to be a real pre-miRNA sequence calculates at MiPred software (http://www.bioinf.seu.edu.cn/miRNA/) (Jiang et al. 2008).

The identification of regulatory elements at intron 2 and 6 led us to investigate the occurrence of gene variability on those regions by following the EPIC-PCR approach. The existence of ILP in *D. carota* cv. Rotin was detected only in intron 2, both at genotype level (heterozygosis) and across genotypes. In the agarose gel two fragments were visualised: a short (*S*) fragment with 0.6 Kb and a large (*L*) fragment with 1.3 Kb (Fig. 6a). The *S* fragment is common to all genotypes tested. The *L* fragment appeared with a significant lower frequency: from the 22 individual cv Rotin plants analysed, it appeared only in 3 heterozygous plants. The *L* fragment was never found alone. Sequence analysis revealed that the ILP identified by electrophoresis in agarose gel is due to the existence of two InDel events, one of 118 bp and

another with 560 bp (Fig. 6b). However, any of the polymorphisms identified at sequence level were located at both predicted pre-miRNA encoding sites.

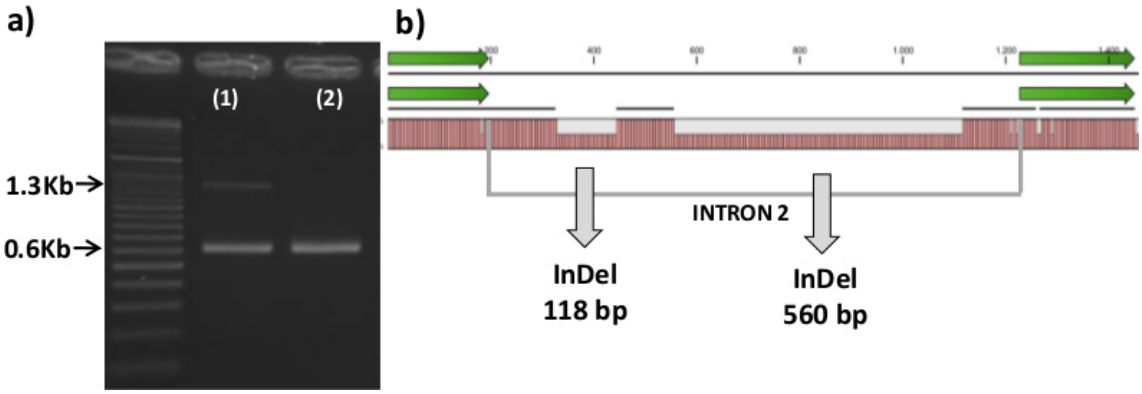


Fig. 6. Results of the analysis of intron 2 of *DcPTOX* in individual plants of *D. carota* cv. Rotin. a) Variants identified by electrophoresis in agarose gel (1) heterozygous genotype with allele *S* and *L*, (2) homozygous genotype showing only allele *S* ; b) Scheme representing the location of InDels on the intron 2 which give rise to two different sizes of intron 2 (two variants).

When intron 2 was analysed in plants belonging to a range of pigmented carrot material (3 plants/cultivar, an exception to the Red with a single genotype analysed), only the *S* fragment was identified. Five recombinant clones were sequenced from each plant, and in most genotypes, a single intron 2 sequence (variant) was identified. The exceptions were W-1, W-3, Y-2 and P-2 that presented two different variants (Table 5). The variability identified among all those sequences was restricted to three SNPs (single nucleotide polymorphisms) and those were also not located at any of the pre-miRNAs encoding sites (Table 5).

Table 5. Polymorphic nucleotide positions in intron 2 of *DcPTOX.*

Genotype	Nucleotide position		
	50	127	426
W-1-var 1	G	T	T
W-1-var 2	T	T	C
W-2	G	T	T
W-3-var 1	T	C	T
W-3-var 2	T	T	T
Y-1	G	T	T
Y-2-var 1	G	T	T
Y-2-var 2	T	T	C
Y-3	G	T	T
Or-1	T	C	T
Or-2	T	C	T
Or-3	T	T	C
R-1	T	T	T
P-1	T	T	C
P-2-var1	G	T	T
P-2-var2	T	T	C
P-3	T	T	T

The nucleotide position is reoffered according the beginning of the intron 2 short sequence. The genotypes correspond to White (W), Yellow (Y), Orange (Or), Red (R) and Purple (P) carrot cultivars. The genotypes W-1, W-3, Y-2 and P-2 presented two different variants (var1 and var2).

6.4. Discussion

6.4.1. *DcPTOX* expression analysis

DcPTOX transcripts were ubiquitously detected in the systems studied. The carrot growth chamber experimental system allowed the study of the involvement of *PTOX* gene on carrot root secondary growth (Fig. 1). The tissue collected for transcript analysis was the cambium ring, where the root meristems are located and therefore is the main responsible tissue for secondary growth in carrot tap roots. The meristematic tissue is the metabolically most active tissue in the tap root and is responsible for the yield production (Hole et al. 1984; Arnholdt-Schmitt 1999). However, due to the small cell layers of this tissue (approximately 10 cell thickness) the immediately next secondary phloem that is originated directly from the meristem was picked together with the cambium ring. An increase of *DcPTOX* transcripts was detected during tap root secondary growth, with the highest levels observed 14 weeks (98 days) after sowing, which relates with the roles in development already attributed to PTOX (Shahbazi et al. 2007; Busch et al. 2008; Okegawa et al. 2010). Nevertheless, this increase is coincident with the accumulation of carotenoids in the root, which relates to the appearance

of the yellow colour visible 28 days after sowing. It is known that young carrot roots are pale and start to accumulate carotenoids after the first month of growth (Phan and Hsu 1973; Clotault et al. 2008; Fuentes et al. 2012). Under good cultivation conditions, the total carotenoid content in carrot roots sharply increases at the 14th week after germination and shortly before secondary growth is completed (Nicolle et al. 2004; Surles et al. 2004; Clotault et al. 2008). A model integrating *PTOX* as a component of an electron transport chain associated to carotenoid desaturation, as well as to respiratory activity within plastids, was proposed in *A. thaliana* (Carol and Kuntz 2001). The biosynthesis of carotenoid pigments has been extensively reviewed (Bartley and Scolnik 1995; Cunningham and Gantt 1998; Carol and Kuntz 2001; Hirschberg 2001; Just et al. 2007), and recent reports point to the involvement of PTOX on that biosynthetic pathway (Simkin et al. 2008; Simkin et al. 2010; Sun and Wen 2011) (see CHAPTER 5). Several genes coding for carotenoid biosynthesis enzymes have been identified and their transcripts were detected in carrot roots (Just et al. 2007; Clotault et al. 2008; Fuentes et al. 2012; CHAPTER 5). Transcript levels of several of those genes globally increased during carrot root development, in parallel with carotenoid accumulation rate (Clotault et al. 2008). Therefore, it is proposed that the increase of *DcPTOX* transcript accumulation is due to both carrot root secondary growth and carotenoid accumulation.

When the analysis of the effect of mild-cold stress on *DcPTOX* gene expression in the carrot tap root cambium during secondary growth was performed, an immediate increase in transcript accumulation was observed, suggesting an involvement of *DcPTOX* in adaptive growth (Fig. 2). In fact, after few days from the beginning of the mild-cold treatment, the storage root growth (measured by root biomass, length and thickness) was suppressed. However, the mild-cold stress had a positive effect on the final storage root growth. Although without enough quality and reliability to be here presented, the same results as *DcPTOX* were observed with the homologous *DcAOX* genes in this same experiment (not shown). When the effect of cold treatments was evaluated on *PTOX* transcript accumulation in carrot plants from two inbreed lines, with the high time resolution of 6 h, 9 h, 24 h and 48 h after cold stress, *DcPTOX* gene was found to be early responsive to chilling exposure (results not shown, publication in preparation). In this experiment and for both inbreed lines, *DcPTOX* presented a higher mean mRNA level in cold stress than in the respective controls in all time points, being some of them statistically significant. The expression of *DcPTOX* in the control treatment remained constantly low throughout the investigation period.

In the PCS experiments the explants were taken directly from the secondary phloem tissue, immediately next to the cambium ring. This culture system was established by Steward et al. (1952) to study cell reprogramming and growth and has previously been proposed as a

test system for the genetic potential of yield production in carrots (Arnholdt-Schmitt 1999). The results here presented point to a role of *DcPTOX* during earliest events of cell reprogramming demonstrated during the first hours of induced *de novo* differentiation of secondary phloem explants from carrot tap roots in a medium containing auxin and cytokinin, with the same tendency observed with the homologous *AOX* (CHAPTER 3). Our results are also in accordance with previous findings achieved with the functionally analogous AOX (Van Aken et al. 2009; Vanlerberghe 2013). Cell reprogramming upon external stresses initiate a cascade of events including dedifferentiation and *de novo* differentiation. A discussion on these processes is also referred in CHAPTER 3. Thus, the results here presented showed that *DcPTOX* is differentially transcribed during growth, but at individual plant level no direct link can be suggested between *callus* fresh weight and *DcPTOX* transcript accumulation (data on *callus* fresh weight were included on CHAPTER 3). Differential transcript accumulation induced by primary culture between genotypes and temperatures was also detected, being the differences between the *callus* growing at 21 °C and at 28 °C mainly related with differences in biomass due to cell division growth (Arnholdt-Schmitt 1999).

Other processes can also be pointed to explain *DcPTOX* expression dynamics in PCS. In this system a reversible differentiation of the chromoplasts into chloroplasts takes place, and the chlorophyll content of explants increases continuously during the culture period (Kumar et al. 1983). *Callus* lose the orange colour of the original explants and acquire progressively a green colour. In cells of freshly cut explants from the secondary phloem of mature tap roots of *D. carota*, only chromoplasts containing carotene usually occur and neither chloroplasts nor other plastid structures can be found (Kumar et al. 1983). The cells along the cambium ring are the youngest (Baranska et al. 2006), and the highest levels of carotene are accumulated in the cells of the secondary phloem of carrot roots (Koch and Goldman 2005; Baranska et al. 2006; Kim et al. 2010). This observation agrees with the results achieved on *DcPTOX* transcript accumulation at secondary phloem collected before inoculation (T0), (Fig. 5). Also in *Capsicum annuum* and *Solanum lycopersicum* fruits it was shown an involvement of PTOX in carotenoid desaturation during chromoplasts differentiation, when carotenoid production is enhanced (Josse et al. 2000). However during the PCS two different processes overlap and can explain *DcPTOX* accumulation: i) the presence of chromoplasts in the initial explants, which accumulate carotenoids and ii), the differentiation of chloroplast during the exponential phase of growth, and its consequent implication in the chlororespiration of the *callus* green tissues.

6.4.2. *DcPTOX* sequence analysis

A single gene encoding the PTOX was already identified on *D. carota* L. cv. Rotin (*DcPTOX*) (CHAPTER 5). A blast search on the whole genome reference using the complete or fragments of the isolated *DcPTOX* gene sequence confirmed that *PTOX* is clearly a single copy gene in carrot, located at chromosome 2 (Dr Philipp Simon, Wisconsin University, USA, personal communication). A single gene encoding PTOX was also recognized in the tomato mutant *ghost* (Scolnik et al. 1987; Josse et al. 2000) and *Arabidopsis* mutant *immutans* (Carol et al. 1999; Wu et al. 1999), as well as in most of the plant species included in the *PTOX* gene diversity study here presented (Table 3). Nevertheless, several plant species (*C. melo*, *E. grandis*, *G. max*, *P. trichocarpa* and *Z. mays*) with two *PTOX* genes, located in the same or in different chromosomes, were here reported for the first time. Two *PTOX* genes were also detected in eukaryotic algae (at least in the genera *Chlamydomonas*, *Haematococcus*, *Ostreococcus* and *Cyanidioschyzon*) genomes (Wang et al. 2009). Although present in cyanobacterial strains, the marine cyanobacterium *Acaryochloris marina* is unique in that it possesses two *PTOX* genes (McDonald et al. 2011).

A typical 9 exons/8 introns structure was found in *PTOXs* from higher plant species, with *DcPTOX* presenting the largest size (see Table 3). In the same way as its homologue *AOX* (CHAPTER 2), *PTOX* from higher plant presents conservation in the size of exons: in this case in 6 of the 9 exons. Events of intron loss and gain are responsible by the variability in gene structure and consequently in exons size. The *PTOX* gene identified in *Medicago truncatula* presents the largest intron 1 size, which resulted from intron 1 loss and consequent fusion of exon 1 and 2.

The involvement of introns in the regulation of gene expression is getting high interest in diverse research fields (Rose 2008; Goebels et al. 2013; Heyn et al. 2015). The encoding of important regulatory elements in introns makes them of great interest for the identification of polymorphisms that could be responsible for phenotypical differences, precisely due to differences on gene regulation induced by such elements. miRNAs are an example of regulatory elements that can be encoded at intronic regions, playing its control by negatively regulating gene expression at post-transcription level. In plants, miRNAs play a critical role in almost all biological and metabolic processes, including plant development - and there is evidence for the regulation of root growth (Wang et al. 2005), leaf development (Mallory et al. 2004), flower development and fertility (Achard et al. 2004) – and stress response playing vital roles in plant resistance to abiotic as well as biotic factors (see reviews of Khraiwesh et al. 2012; Kumar 2014). Many miRNA families are evolutionarily conserved across all major lineages of plants, including bryophytes, lycopods, ferns and monocots and dicots (Axtell and

Bartel 2005). This conservation makes it possible to identify homologous miRNAs in other species (Axtell and Bartel 2005; Zhang et al. 2006). Considering this knowledge, the *in silico* identification of two pre-miRNAs which possess a high similarity with miRNAs already identified in other plant species (*Glycine max*_ gma-miR1520p and *Oryza sativa*_ osa-miR5494), lead us to the hypothesis that *PTOX* gene could be involved in the regulation of genes related with plant response upon biotic stresses.

The location of polymorphic sites on regions encoding for pre-miRNAs could be related with phenotypical differences due to differences on the mechanism of regulation of genes directly linked with a specific phenotype. The identification of an InDel event on a region encoding for a pre-miRNA was previously reported in a gene belonging to the *AOX* gene family (Cardoso et al. 2009), on that case might be related with a gene involved in the somatic embryogenesis. The search for polymorphic sites at the intron 2 and intron 6 allowed to the identification of two InDels located at intron 2 and few SNPs. Nevertheless, none of those polymorphic sites were located at the pre-miRNA encoding regions, with no expected differential gene regulation.

6.5. Conclusion

Besides the already known involvement of PTOX on chlororespiration, it is suggested that this enzyme also participates in both carrot storage root growth and carotenoid accumulation, as well on adaptive growth. The results here presented at transcriptomic level showed that *DcPTOX* is differentially transcribed during the likely earliest events of cell reprogramming. Differential *DcPTOX* transcript accumulation in the PCS between individual plants and between temperatures was also found. Therefore, the results achieved are encouraging to strengthen future efforts on the identification of polymorphic motifs in *DcPTOX* for the development of functional markers related to agronomic traits of interest such as yield or pigmentation of storage roots.

6.6. References

Achard P, Herr A, Baulcombe DC, Harberd NP (2004) Modulation of floral development by a gibberellin-regulated microRNA. Development 131:3357–3365. doi: 10.1242/dev.01206

Aluru MR, Rodermel SR (2004) Control of chloroplast redox by the IMMUTANS terminal oxidase. Physiol Plant 120:4–11

Aluru MR, Yu F, Fu A, Rodermel S (2006) Arabidopsis variegation mutants: New insights into chloroplast biogenesis. J Exp Bot 57:1871–1881

Arnholdt-Schmitt B (2009) Alternative oxidase (AOX) and stress tolerance-approaching a scientific hypothesis. Physiol Plant 137:314–315. doi: 10.1111/j.1399-3054.2009.01311.x

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, Costa JH, de Melo DF (2006) AOX - a functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11:281–287. doi: 10.1016/j.tplants.2006.05.001

Axtell MJ, Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. Plant Cell 17:1658–1673. doi: 10.1105/tpc.105.032185

Baranska M, Baranski R, Schulz H, Nothnagel T (2006) Tissue-specific accumulation of carotenoids in carrot roots. Planta 224:1028–1037. doi: 10.1007/s00425-006-0289-x

Bartley GE, Scolnik PA (1995) Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell 7:1027–1038. doi: 10.1105/tpc.7.7.1027

Berthold DA, Stenmark P (2003) Membrane-bound diiron carboxylate proteins. Annu Rev Plant Biol 54:497–517. doi: DOI 10.1146/annurev.arplant.54.031902.134915

Bimboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523. doi: 10.1093/nar/7.6.1513

Busch F, Hüner NPA, Ensminger I (2008) Increased air temperature during simulated autumn conditions impairs photosynthetic electron transport between photosystem II and photosystem I. Plant Physiol 147:402–414. doi: 10.1104/pp.108.117598

Cardoso HG, Arnholdt-Schmitt B (2013) Functional marker development across species in selected traits. In: Diagnostics in Plant Breeding. pp 467–515

Cardoso HG, Campos MD, Costa AR, et al (2009) Carrot alternative oxidase gene *AOX2a* demonstrates allelic and genotypic polymorphisms in intron 3. Physiol Plant 137:592–608. doi: 10.1111/j.1399-3054.2009.01299.x

Carol P, Kuntz M (2001) A plastid terminal oxidase comes to light: Implications for carotenoid biosynthesis and chlororespiration. Trends Plant Sci. 6:31–36.

Carol P, Stevenson D, Bisanz C, et al (1999) Mutations in the Arabidopsis gene IMMUTANS cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. Plant Cell 11:57–68. doi: 10.1105/tpc.11.1.57

Clotault J, Peltier D, Berruyer R, et al (2008) Expression of carotenoid biosynthesis genes during carrot root development. J Exp Bot 59:3563–3573. doi: 10.1093/jxb/ern210

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:557–583.

Díaz M, De Haro V, Muñoz R, Quiles MJ (2007) Chlororespiration is involved in the adaptation of Brassica plants to heat and high light intensity. Plant, Cell Environ 30:1578–1585. doi: 10.1111/j.1365-3040.2007.01735.x

Fu A, Liu H, Yu F, et al (2012) Alternative Oxidases (AOX1a and AOX2) can functionally substitute for plastid terminal oxidase in arabidopsis chloroplasts. Plant Cell 24:1579–1595.

Fuentes P, Pizarro L, Moreno JC, et al (2012) Light-dependent changes in plastid differentiation influence carotenoid gene expression and accumulation in carrot roots. Plant Mol Biol 79:47–59. doi: 10.1007/s11103-012-9893-2

Goebels C, Thonn A, Gonzalez-Hilarion S, et al (2013) Introns regulate gene expression in *Cryptococcus neoformans* in a Pab2p dependent pathway. PLoS Genet. doi: 10.1371/journal.pgen.1003686

Heyn P, Kalinka AT, Tomancak P, Neugebauer KM (2015) Introns and gene expression: Cellular constraints, transcriptional regulation, and evolutionary consequences. BioEssays 37:148–154. doi: 10.1002/bies.201400138

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol 4:210–218. doi: 10.1016/S1369-5266(00)00163-1

Hole CC, Thomas TH, McKee JMT (1984) Sink development and dry matter distribution in storage root crops. Plant Growth Regul 2:347–358.

Jiang P, Wu H, Wang W, et al (2007) MiPred : classification of real and pseudo microRNA precursors using random forest prediction model with combined features. 35:339–344. doi: 10.1093/nar/gkm368

Josse EM, Simkin AJ, Gaffé J, et al (2000) A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. Plant Physiol 123:1427–1436. doi: 10.1104/pp.123.4.1427

Just BJ, Santos CAF, Fonseca MEN, et al (2007) Carotenoid biosynthesis structural genes in carrot (*Daucus carota*): Isolation, sequence-characterization, single nucleotide polymorphism (SNP) markers and genome mapping. Theor Appl Genet 114:693–704. doi: 10.1007/s00122-006-0469-x

Karlin S, Altschul SF (1993) Applications and statistics for multiple high-scoring segments in molecular sequences. Proc Natl Acad Sci USA 90:5873–5877. doi: 10.1073/pnas.90.12.5873

Khraiwesh B, Zhu J-K, Zhu J (2012) Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochim. Biophys. Acta - Gene Regul. Mech. 1819:137–148.

Kim JE, Rensing KH, Douglas CJ, Cheng KM (2010) Chromoplasts ultrastructure and estimated carotene content in root secondary phloem of different carrot varieties. Planta 231:549–558. doi: 10.1007/s00425-009-1071-7

Koch TC, Goldman IL (2005) Relationship of carotenoids and tocopherols in a sample of carrot root-color accessions and carrot germplasm carrying Rp and rp alleles. J Agric Food Chem 53:325–331. doi: 10.1021/jf048272z

Kumar A, Bender L, Pauler B, et al (1983) Ultrastructural and biochemical development of the photosynthetic apparatus during callus induction in carrot root explants. Plant Cell Tissue Organ Cult 2:161–177. doi: 10.1007/BF00043361

Kumar R (2014) Role of MicroRNAs in biotic and abiotic stress responses in crop plants. Appl. Biochem. Biotechnol. 174:93-115.

Kuntz M (2004) Plastid terminal oxidase and its biological significance. Planta 218:896–899. doi: 10.1007/s00425-004-1217-6

Mallory AC, Reinhart BJ, Jones-Rhoades MW, et al (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. EMBO J 23:3356–3364. doi: 10.1038/sj.emboj.7600340

Mathews DH, Sabina J, Zuker M, Turner DH (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol 288:911-940.

McDonald AE, Ivanov AG, Bode R, et al (2011) Flexibility in photosynthetic electron transport: The physiological role of plastoquinol terminal oxidase (PTOX). Biochim. Biophys. Acta - Bioenerg. 1807:954–967.

Neumann K (1966) Wurzelbildung und nukleinsäuregehalt bei phloem-gewebekulturen der karottenwurzel auf synthetischem nährmedium. Congr Coll Univ Liege 38:96–102.

Nicolle C, Simon G, Rock E, et al (2004) Genetic Variability In fl uences Carotenoid, Vitamin, Phenolic, and Mineral Content in White, Yellow, Purple, Orange, and Dark-orange Carrot Cultivars. J Am Soc Hortic Sci 129:523–529.

Okegawa Y, Kobayashi Y, Shikanai T (2010) Physiological links among alternative electron transport pathways that reduce and oxidize plastoquinone in Arabidopsis. Plant J 63:458–468. doi: 10.1111/j.1365-313X.2010.04252.x

Peltier G, Cournac L (2002) Chlororespiration. Annu Rev Plant Biol 53:523–550. doi: 10.1146/annurev.arplant.53.100301.135242

Phan CT, Hsu H (1973) Physical and chemical changes occurring in carrot root during growth. Can J Plant Sci 53:629-634.

Polidoros AN, Mylona P V., Arnholdt-Schmitt B (2009) Aox gene structure, transcript variation and expression in plants. Physiol Plant 137:342–353. doi: 10.1111/j.1399-3054.2009.01284.x

Rambaldi D, Ciccarelli FD (2009) FancyGene: Dynamic visualization of gene structures and protein domain architectures on genomic loci. Bioinformatics 25:2281–2282. doi: 10.1093/bioinformatics/btp381

Rodermel S (2001) Pathways of plastid-to-nucleus signaling. Trends Plant Sci. 6:471–478.

Rose AB (2008) Intron-mediated regulation of gene expression. Curr. Top. Microbiol. Immunol. 326:277–290.

Rosso D, Ivanov AG, Fu A, et al (2006) IMMUTANS does not act as a stress-induced safety valve in the protection of the photosynthetic apparatus of Arabidopsis during steady-state photosynthesis. Plant Physiol 142:574–585. doi: 10.1104/pp.106.085886

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675.

Scolnik PA, Greenblatt IM, Giuliano G, et al (1987) Planta and its effect on plastid development. Planta 171:11–18.

Shahbazi M, Gilbert M, Labouré A-M, Kuntz M (2007) Dual role of the plastid terminal oxidase in tomato. Plant Physiol 145:691–702. doi: 10.1104/pp.107.106336

Simkin AJ, Kuntz M, Moreau H, McCarthy J (2010) Carotenoid profiling and the expression of carotenoid biosynthetic genes in developing coffee grain. Plant Physiol Biochem 48:434–442. doi: 10.1016/j.plaphy.2010.02.007

Simkin AJ, Moreau H, Kuntz M, et al (2008) An investigation of carotenoid biosynthesis in Coffea canephora and *Coffea arabica*. J Plant Physiol 165:1087–1106. doi: 10.1016/j.jplph.2007.06.016

Stepien P, Johnson GN (2009) Contrasting responses of photosynthesis to salt stress in the glycophyte Arabidopsis and the halophyte thellungiella: role of the plastid terminal oxidase as an alternative electron sink. Plant Physiol 149:1154–1165. doi: 10.1104/pp.108.132407

Steward F, Caplin S MF (1952) Investigations on the growth and metabolism of plant cells. I. New techniques for the investigation on metabolism, nutrition and growth in undifferentiated cells. Ann Bot 16:57-77.

Streb P, Josse EM, Gallouët E, et al (2005) Evidence for alternative electron sinks to photosynthetic carbon assimilation in the high mountain plant species *Ranunculus glacialis*. Plant, Cell Environ 28:1123–1135. doi: 10.1111/j.1365-3040.2005.01350.x

Sun X, Wen T (2011) Physiological roles of plastid terminal oxidase in plant stress responses. J. Biosci. 36:951–956.

Surles RL, Weng N, Simon PW, Tanumihardjo SA (2004) Carotenoid profiles and consumer sensory evaluation of specialty carrots (*Daucus carota* L.) of various colors. J Agric Food Chem 52:3417–3421. doi: 10.1021/jf035472m

Van Aken O, Giraud E, Clifton R, Whelan J (2009) Alternative oxidase: A target and regulator of stress responses. Physiol Plant 137:354–361

Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034

Vanlerberghe GC (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J. Mol. Sci. 14:6805–6847.

Wang J, Sommerfeld M, Hu Q (2009) Occurrence and environmental stress responses of two plastid terminal oxidases in *Haematococcus pluvialis* (Chlorophyceae). Planta 230:191–203. doi: 10.1007/s00425-009-0932-4

Wang J-W, Wang L-J, Mao Y-B, et al (2005) Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. Plant Cell 17:2204–2216. doi: 10.1105/tpc.105.033076

Wu D, Wright DA, Wetzel C, et al (1999) The IMMUTANS variegation locus of Arabidopsis defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. Plant Cell 11:43–55. doi: 10.1105/tpc.11.1.43

Xie FL, Huang SQ, Guo K, et al (2007) Computational identification of novel microRNAs and targets in *Brassica napus*. FEBS Lett 581:1464–1474. doi: 10.1016/j.febslet.2007.02.074

Zhang B, Pan X, Cannon CH, et al (2006) Conservation and divergence of plant microRNA genes. Plant J 46:243–259. doi: 10.1111/j.1365-313X.2006.02697.x

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. 31:3406–3415. doi: 10.1093/nar/gkg595

Supplemental Fig. 1. Aspect of the roots (*Daucus carota* L. cv. Rotin) 28 days after sowing (A), 42 days after sowing (B), 68 days after sowing (C) and 95 days after sowing (D). The heterogenity between roots is evident (D). Bar 5 cm.

CHAPTER 7 SELECTION OF SUITABLE REFERENCE GENES FOR REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR STUDIES ON DIFFERENT EXPERIMENTAL SYSTEMS FROM CARROT (*Daucus carota* L.)

7.1. Selection of suitable reference genes for RT-qPCR studies during carrot tap root secondary growth and on a somatic embryogenesis system

This sub-chapter refers to the manuscript:

Campos MD, Frederico AM, Nothnagel T, Arnholdt-Schmitt B, Cardoso H (2015) Selection of suitable reference genes for reverse transcription quantitative real-time pcr studies on different experimental systems from carrot (*Daucus carota* L.). Sci Hortic 186: 115-123

Abstract

Reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) is a preferred method for rapid and accurate quantification of gene expression studies. Appropriate application of RT-qPCR requires accurate normalisation through the use of suitable reference genes. This study aimed selecting robust and reliable reference genes which are constitutively and equally expressed for accurate RT-qPCR normalisation analysis in two different experimental systems with carrot. A systematic comparison of 12 selected candidate genes for carrot is presented. These included seven genes commonly used as reference gene: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), *18S* ribosomal RNA *(18S rRNA),* ubiquitin (*UBQ*), alpha-tubulin (*TUA*)*,* beta-tubulin (*β-TUB)* and elongation factor-1alpha (*EF-1A*). Additionally, other five genes were here presented as potential candidates: other ribosomal RNA (*25S* and *5.8S rRNA)* and ribosomal protein L2 encoding gene (*rpL2)*, the transcriptional initiation factor (*TIF1*), and the heat shock protein 70 (*hsp70*). Expression stability was evaluated in: i) *in vivo* growth experiment based on carrot tap root secondary growth carried out in growth chambers and ii) realization phase of somatic embryogenesis. During carrot tap root secondary growth, two reference genes *GAPDH* and *5.8S rRNA,* were stably expressed. In the somatic embryogenesis realization experiment, two ribosomal RNAs were selected as reference genes, the *5.8S* and the *25S rRNA*. Additionally, the expression profile of the mitochondrial alternative oxidase gene *DcAOX1* was conducted in the *in vivo* growth experiment, to show the impact of reference genes selection. Taken together, our results provide a systematic analysis for the selection of superior reference genes for accurate transcript normalisation in carrot, under different experimental conditions. We reinforce the idea that the validation of reference genes for the conditions under study is essential, as

emphasized by the expression analysis of *DcAOX1* as target gene. These results show that a previous selection of reference genes for each experimental system is crucial to achieve accurate and reliable RT-qPCR gene expression data, avoiding low precision or misleading results.

Keywords: carrot; reverse transcription quantitative real-time PCR; reference gene; normalisation

---

7.1.1. Introduction

Reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) is a preferred method for rapid and accurate quantification of gene expression in various biological systems (Bustin et al. 2005). The accuracy of the results depends strongly on several variables, including the RNA integrity, cDNA synthesis efficiency, enzyme and primer performance, reference genes used and method chosen for data analysis (Bustin 2002; Bustin and Nolan 2004; Pfaffl 2001; Skern et al. 2005). All these variables are crucial for relative quantification of gene expression by RT-qPCR. However, because quantification is based on the expression ratio of a target gene versus a reference gene (Pfaffl and Hageleit 2001), the choice of reference gene(s) has a direct and strong impact, given all other variables optimal and constant. A vast number of reference genes has been proposed for gene expression analysis (Gu et al. 2014; Jin et al. 2013; Kumar et al. 2011; Warrington et al. 2000). Traditional reference genes, used to examine gene expression in plants, usually code for proteins or ribosomal RNAs (rRNA) that function in basic cellular processes such as cell structure maintenance or primary metabolism. Some of the best known and most frequently used reference transcripts for RT-qPCR in plants include those of *18S rRNA*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor-1alpha (*EF-1A*), ubiquitin (*UBQ*), actin (*ACT*), alpha-tubulin and beta-tubulin (*TUA* and *β-TUB*, respectively) genes (Dheda et al. 2004; Goidin et al. 2001; Kim et al. 2003; Kumar et al. 2011; Radonic et al. 2004). On *in vitro* somatic embryogenesis studies in *Dimocarpus longan*, *Pinus pinaster* and *Picea abies*, *TUA, EF-1A*, *Histone H3* and *UBQ* are referred as suitable reference genes (de Vega-Bartol et al. 2013; Lin and Lai 2010). Any substitution of traditional references will only be available after screening and identification of novel reference genes. The availability of plant genome and transcriptome sequences in an increasing number of plant species are source of miscellaneous genes for several experimental conditions. These have the potential to be used in RT-qPCR and aid in fixing the difficulties of gene expression variability (Kumar et al. 2011; Czechowski et al. 2005; Lee et al. 2010; Manoli et al. 2012). It is highly

unlikely that an ideal universal reference gene exists (Bustin et al. 2005; Gutierrez et al. 2008; Vandesompele et al. 2002); many studies have used reference genes without proper validation of their presumed stability of expression, even though their transcript levels can vary considerably (Die et al. 2010; Radonic et al. 2004). Reference genes should be transcribed at a relatively constant level across various conditions -such as developmental stage or tissue type- and their expression is assumed to be unaffected by experimental parameters.

Different statistical procedures or software packages have been reported to assess the expression stability of candidate reference genes, such as geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004) and 'Stabiliy index' (Brunner et al. 2004), with the ranking of candidate reference genes depending upon the selected software (Liu et al. 2012; Qi et al. 2010; Rivera-Vega et al. 2012). These algorithms have simplified the researchers' choice of appropriate reference genes and the optimal number of candidate genes required for normalisation. Methods and tools that integrate the rankings provided by different software have been developed (Goulao et al. 2012; Xie et al. 2012) and provide relevant insights.

In the present study we were interested in selecting robust and reliable reference genes which are constitutively and equally expressed for accurate RT-qPCR normalisation analysis in two different experimental systems with carrot: i) an *in vivo* growth experiment based on carrot tap root secondary growth and ii) the realization phase of somatic embryogenesis. The studied genes included commonly used reference genes and other potential gene candidates. The expression of 12 selected candidate genes were carefully evaluated, specifically for each of the above referred experimental systems, aiming to identify the most suitable and stable reference genes for data normalisation. Furthermore, in order to illustrate the impact of the reference genes choice on the biological interpretations, expression analyses of a mitochondrial alternative oxidase gene (*DcAOX1*) in an *in vivo* growth experiment is discussed using several combinations of candidate reference genes. Alternative oxidase (*AOX*) was selected as target gene since it belongs to a gene family related with growth (see Arnholdt-Schmitt et al. 2006; Campos et al. 2009), with the prevention oxidative stress under various forms of abiotic or biotic stress (Umbach et al. 2005) and, in specific cases, it is involved in morphogenic responses (see review in Cardoso and Arnholdt-Schmitt 2013), being suggested to play an important role in efficient cell reprogramming under stress (Arnholdt-Schmitt et al. 2006; Clifton et al. 2006). To the authors' knowledge, this work provides the first systematic analysis for the selection of superior reference genes for accurate transcript normalisation in carrot.

7.1.2. Material and methods

Plant material and experimental conditions

Two different experimental systems were carried out: (i) based on plant growth in carrot plants growing *in vivo* in growth chambers; (ii) focused on the realization phase of somatic embryogenesis system, which is related with the differentiation of somatic embryos from auxin-induced embryogenic *callus* material. This last system is related to morphogenic responses as a consequence of cell reprogramming upon stress.

In the growth chamber experiment (i), seeds of carrot (*D. carota* cv. Rotin) were sown in pots, with 10 plants/pot, and incubated in a growth chamber under 16 h photoperiod with 202.5 $\mu molm^{-2}s^{-1}$ (15000 lux) of light intensity and 25 °C day and 20 °C night temperature. Samples were collected from 30 plants as a bulked sample (3 pots, 10 plants/pot) at eight time-points: 28, 56, 84, 85, 88, 92, 98, and 125 days post sowing (one bulked sample/time point). Each sample consisted of small pieces of the cambium tissue plus adjacent secondary phloem cells.

In the assay on early realization phase of somatic embryogenesis (ii), the embryogenic cell line L5.S.R of *D carota* L. cv. Rotin was inoculated in 20 mL of B_5^- medium without 2,4-D (see protocol details on Frederico et al. 2009). Cells and/or embryos were collected through sieving with 54 µm mesh pore polyester precision woven screens (Sefar Petex, Thal, Switzerland) during a 10 days' time course experiment with a total of 12 time points (see the description of the time points on Suppl. Table 1).

The information about all the experimental conditions mentioned above is summarized in Table 1.

Sample processing

After sampling, plant material was immediately frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with on-column digestion of DNA applying the RNase-Free DNase Set (Qiagen, Hilden, Germany), according to manufacturer's protocol. RNA integrity was verified by electrophoresis in 0.1 % DEPC (diethyl pyrocarbonate) (Sigma-Aldrich, St. Louis, MO, USA). RNA concentration was determined with the NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNase-treated total RNA (1 µg) was reverse transcribed with RETROscript® kit (Ambion, Austin, TX, USA) using random decamer primers, in a 20 µl reaction volume according to manufacturer's instruction. In both experiments two RTs were performed per time point (Table 1).

Table 1. Summary of the experimental systems and samples used in present study.

Experimental system	Tissue type	No treatments	Biological replicates	Sampling dates	RTs per time point	Total of cDNA samples (treatmentsxreplicatesxdatesxRTs)
Growth chamber	pieces of cambium tissue plus adjacent secondary phloem	1	1 bulked sample	8	2	16
Somatic embryogenesis	cells and/or embryos	1	1 bulked sample	12	2	24
Total						40

Candidate reference gene selection and primer design

A set of 12 candidate genes were selected, comprising several conventional and/or most commonly used reference genes in plants, and others based on previous reports: *GAPDH*,*TBA*, *β-TUB*, *ACT*, *UBQ*, *EF-1A*, heat shock protein 70 (*hsp70*), ribosomal protein L2 (*rpL2*), transcriptional initiation factor (*TIF1*) and the ribosomal *18S*, *25S* and *5.8S* RNAs (Table 2).

Table 2. List and description of the selected candidate reference genes.

Gene name	Gene symbol	Biological process
Actin	*ACT*	Cytoskeletal structural protein.
Elongation factor 1-alpha	*EF-1A*	Translational elongation, from the formation of the first peptide bond to the formation of the last one. Also binds/disunites microtubules.
Heat shock protein 70	*hsp70*	Protection of cells from thermal or oxidative stress.
Translational initiation factor 1	*TIF1*	Seems to be required for maximal rate of protein biosynthesis.
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Important glycolytic pathway enzyme.
Alpha-tubulin and Beta-tubulin	*TBA and β-TUB*	Structural constituent of cytoskeleton.
Ubiquitin	*UBQ*	Postranslational attachment of ubiquitin monomers: proteasomal degradation, control of stability, function, and intracellular localization of proteins.
18S, 25S, 5.8S Ribosomal subunits	*18S, 25S, 5.8S rRNAs*	Component of small ribosomal subunits.
Ribosomal protein L2	*rpL2*	Association of the ribosomal subunits and tRNA binding.

Primers were designed on the basis of known sequences and sequence stretches from NCBI GenBank sequence data for carrot (Table 3). All primer pairs for RT-qPCR amplification were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA) with default parameters. Amplicon length ranges were set to be between 62 and 97 bp. All primer

sets were checked for amplification specificity and annealing temperature by endpoint PCR using synthesized cDNA.

Table 3. Primer sequences and amplicon characteristics for each of the 12 candidate reference genes under evaluation.

[NCBI accession ID]	Gene	Primer sequence (5'→3')		AS (bp)	*E* (%) (r^2)	
					Growth chamber	Somatic embryogenesis
[GenBank:X17525]	*ACT*	Fw	CACACGGTGCCAATTTATGAA	73	105.7 (0.997)	84.7 (0.972)
		Rv	GATCACGGCCAGCAAGGT			
[GenBank:D12709]	*EF-1A*	Fw	TGGTGATGCTGGTTTCGTTAAG	75	103.2 (0.998)	81.2 (0.978)
		Rv	AGTGGAGGGTAGGACATGAAGGT			
[GenBank:X60088]	*hsp70*	Fw	GAAGTTTGAGCTCACGGGAATT	77	93.2 (0.998)	85.4 (0.987)
		Rv	TCGCATCAATGTCGAACACA			
[GenBank:ABI32458]	*TIF1*	Fw	GGTATGTTCCGTATTCGCTTAG	97	89.1 (0.999)	82.4 (0.987)
		Rv	TATCGCCTGGTAGTATCC			
[GenBank:AY491512]	*GAPDH*	Fw	GGGAGGTGCAAAGAAAGTTATCA	79	93.4 (0.999)	89.0 (0.997)
		Rv	TTCCTTTTCATTGACACCAACAA			
[GenBank:AY007250]	*TBA*	Fw	TCTGGTGCCATACCCAAGGA	73	85.3 (0.993)	91.5 (0.993)
		Rv	ATAGGCCTTCTCAGCGGAGAT			
[GenBank:AAB64308]	*β-TUB*	Fw	GGGCTCTCTATGTCTTCCACATTC	78	102.9 (0.988)	85.0 (0.981)
		Rv	AAACTGTTCACTAACTCGTCGAAACA			
[GenBank:U68751]	*UBQ*	Fw	TCTTCGCCGGCAAGCA	67	82.5 (0.997)	61.6 (0.996)
		Rv	GTGGACTCCTTCTGGATGTTGTAGT			
[GenBank:X17534]	*5.8S*	Fw	AATGACTCTCGGCAACGGATAT	73	94.5 (0.998)	83.1 (0.990)
		Rv	TCACACCAAGTATCGCATTTCG			
[GenBank:X17534]	*18S*	Fw	GACTACGTCCCTGCCCTTTG	62	96.1 (0.998)	86.0 (0.995)
		Rv	TCACCGGACCATTCAATCG			
[GenBank:X17534]	*25S*	Fw	AGTCGGGTTGTTTGGGAATG	67	94.5 (0.996)	91.3 (0.991)
		Rv	TCGCCTGTATTTAGCCTTGGA			
[GenBank:ABI32465]	*rpl2*	Fw	GGAAATCGGCCACATTAAAATTA	92	98.0 (0.993)	91.2 (0.994)
		Rv	CCCAACATTCCCCACTTGTC			

AS corresponds to the amplicons size; Primers efficiency (*E*) and regression coefficient (r^2) for the growth chamber and somatic embryogenesis experimental systems.

Reverse transcription quantitative real time PCR

RT-qPCR experiments were performed on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using Maxima SYBR Green q-PCR Master Mix (Fermentas, Ontario, Canada). 15 µl reaction volume containing 4 µl of previously diluted cDNA (1:10) and specific primers previously adjusted to the concentration, was applied in 96-well plates (Thermo Scientific) and sealed with adhesives (Applied Biosystems, Foster City, CA, USA). All samples were run in duplicate and no-template controls were included in all plates. RT-qPCR was conducted for 40 cycles, each consisting in 15 s at 95 °C followed by 1 min at 60 °C. To analyse dissociation curve profiles, an additional step at 95 °C during 15 s was added, followed by a constant increase of the temperature between 60 and 95 °C. Single peaks were obtained for every primer pair and one single band on agarose gels indicated that one single PCR product was amplified with each primers pair.

Standard curves of a 4-fold dilution series (1:1 to 1:125) (run in triplicate) from pooled cDNA (mix of cDNA from all samples in each experiment) were generated for each primer pair.

The PCR efficiency (*E*) is given by the equation: $E(\%)=(10^{-(1/slope)}-1)x100$ (Radonic et al. 2004), with the slope of linear regression model fitted over log-transformed data of the input cDNA concentrations versus cycle threshold (Ct) values.

Data analyses

Global variability of gene expression for each gene was analysed using the standard statistical parameters provided in the *SigmaStat* statistical package (Systat software, London, UK). Three publically available software tools were used to rank expression stability of reference genes in all experimental sets: geNorm v3.5 (medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004). The geNorm algorithm assumes that expression variation of the ratio of two ideal reference genes is identical in all samples. Based on pairwise variation of candidate reference genes, the stability measure (M value), which reflects instability in expression levels of one gene, is calculated. M values lower than 0.5 are typically observed for stably expression genes in relatively homogeneous sample panels, however, M values of 1 are acceptable for heterogeneous sample panels (Hellemans et al. 2007). NormFinder is based on a variance estimation approach, which calculates an expression stability value (SV) for each gene analysed. It enables estimation of the overall variation of the reference genes, taking into account intra and intergroup variations of the sample set. BestKeeper is able to compare expression levels of up to 10 reference genes. As a set of 12 candidate genes were selected in our experiments, the two worst ranked by geNorm and NormFinder in each experimental system were removed from BestKeeper analysis. According to this algorithm, genes with lowest SV will be top ranked. BestKeeper tool uses the Ct values of all candidate reference genes to calculate their standard deviation (SD). Genes with a SD value of less than 1 are generally considered stable. A pairwise correlation coefficient of variation between each gene and the BestKeeper index (geometric mean between Ct values of stable genes) was calculated in BestKeeper.

The three methods described above are implemented in RefFinder (Xie et al. 2012; http://www.leonxie.com/referencegene.php), a web-based comprehensive tool developed for evaluating and screening the stability of reference genes. It integrates the currently available major computational programs: geNorm (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the comparative delta-Ct method (Silver et al. 2006). Mean Ct values were entered to the RefFinder web page. Based on the rankings provided by each of the software products, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking

(RefFinder recommended comprehensive ranking). The three experimental systems were analysed individually as subsets, and also together as a single set.

In addition to the ranking of a set of reference genes and the expression stability (M) for each gene, geNorm calculates the pairwise variation (V) to determine the optimal number of reference genes needed for building an accurate normalisation factor (NF). Pairwise variations of $NF_n$and NF_{n+1} were calculated, reflecting the effect of including additional (n+1) gene. A V value with a cut-off of 0.15 as threshold was used to select the optimal number of reference genes (Vandesompele et al. 2002).

Normalisation of DcAOX1

DcAOX1 encoding a carrot alternative oxidase was obtained from NCBI [GenBank: EU286573.2] and used as a target gene to demonstrate the usefulness of the validated candidate reference gene in RT-qPCR. Gene expression levels of *DcAOX1* were quantified in samples from the growth chamber experiment (described above) at five time-points: 56, 85, 92, 98, and 125 days post sowing (one bulked sample/time point, two RTs). RT-qPCR reaction and cycling conditions were as above. Primer pair (Fw: 5'-CTTCAACGCCTACTTCCTTG-3', Rv: 5'-ATCTCGCAATGTAGAGTCAGC-3') of *DcAOX1* were also verified by melting curve analysis and sequencing as described for reference genes.

Three normalisation strategies were followed to determine the expression of *DcAOX1*: (a) using the 2 top reference genes given by RefFinder recommended comprehensive ranking; (b) using the optimal number of reference genes based on geNorm pairwise variation; and finally (c) *DcAOX1* was normalized with the worst candidate gene ranked in all programs.

Calculations associated with the delta-Ct method incorporating the amplification efficiency (*E*) for each primer pair (Pfaffl 2001) for RT-qPCR, were applied using the qCalculator version 1.0 (available at: http://www.genequantification.de/qCalculator.zip). The time-point 56 days was selected as calibrator. Results were given as mean of duplicated Ct values from each RT.

7.1.3. Results

Amplification specificity and efficiency

To examine the expression stability of the potential reference genes selected, transcript levels of the 12 candidate reference genes (Table 2) were measured by RT-qPCR using gene-specific primer pairs (Table 3). Gene-specific amplification of each of the 12 genes was confirmed by the presence of a single peak in the dissociation curve (Suppl Fig. 1). Amplification efficiencies of every candidate reference gene were calculated individually for

each experimental system, to check for variation in primer efficiency. All calibration curves exhibited linear relationships (regression coefficient -r^2- varying from 0.97 to 0.99; with only two values below 0.98) between the fractional cycle number and the log of the initial copy number (Table 3).The amplification efficiency (*E*) of the reactions ranged from 61.6 % for U*BQ* on somatic embryogenesis to 105.7 % for *ACT* on growth chamber experiment (Table 3).

Expression profiling of candidate reference genes

RT-qPCR was designed for transcript profiling of the 12 candidate reference genes in two experimental systems in a total of 40 diverse samples. For an overview of the relative abundance of candidate reference genes, the calculated cycle threshold (Ct) values were determined for each gene across both experimental systems. Analysis of the raw expression levels across samples identified some variation amongst candidate reference genes (Table 4). The *18S*, *25S* and *5.8S rRNAs* were the candidate genes with the highest expression, with *β-TUB* being the one with the lowest expression values. All the other candidate reference genes were expressed at moderate levels with mean Ct values from 17.89 to 26.93. *rpL2* showed the narrowest Ct ranges among all samples, whereas *UBQ* was the most variable one. None of the tested reference genes exhibited a constant expression level across experimental systems.

Table 4. Expression levels of 12 candidate reference genes across all samples (overall) and for each experimental system.

Genes	Overall	Growth chamber	Somatic embryogenesis
ACT	22.79 ± 1.39	23.94 ± 1.56	22.45± 0.25
EF-1A	21.43 ± 2.03	23.18 ± 1.36	20.20 ± 0.41
hsp70	25.04 ± 2.25	24.71 ± 3.08	25.31 ± 1.04
TIF1	18.57 ± 1.32	17.19 ± 1.59	18.71 ± 0.27
GAPDH	21.19 ± 1.41	22.10 ± 1.50	20.79 ± 0.41
TBA	24.42 ± 1.92	26.16 ± 1.46	23.35 ± 0.48
β-TUB	32.42 ± 1.77	31.57 ± 2.28	32.78 ± 0.68
UBQ	26.93 ± 2.90	29.77 ± 1.55	24.84 ± 0.57
5.8S	8.63 ± 1.54	9.85 ± 1.58	8.19 ± 0.19
18S	12.77 ± 1.81	11.10 ± 2.51	13.01 ± 0.24
25S	9.28± 2.23	11.48 ± 1.75	8.18 ± 0.13
rpL2	17.89 ± 1.28	18.91 ± 1.62	17.77 ± 0.24

The mean raw cycle threshold (Ct) RT-qPCR data of each reference gene and the standard deviation (SD) are presented. Values are mean ± SD.

A more detailed analysis of individual gene expression levels in each experimental system revealed that candidate reference genes were expressed in the two experimental systems albeit with different expression patterns (Table 4). The standard deviation of Ct values

can be used as a surrogate for the expression stability of each candidate reference gene. In this way, the somatic embryogenesis system shows the highest expression stability for all the candidate genes. On the growth chamber system, due to dramatic changes on transcriptional activity, it is particularly evident that a straightforward statistical analysis of raw Ct values is not suitable to select the best reference genes for normalisation of these RT-qPCR data.

Gene expression stability analysis

The expression stability was determined by geNorm, NormFinder and BestKeeper packages. The choice of the best genes from the 12 candidate reference genes, separately by experimental systems was not totally consistent between softwares and showed a high variability of the expression stability depending on the experimental conditions (Table 5).

In the growth chamber experiment, (i) geNorm ranked *EF-1A* and *TBA* as the most stable genes, which together with *GAPDH* had the highest expression stability values (the lowest M value, <0.5, Table 5). As geNorm, Bestkeeper revealed the same three most stable genes in the growth chamber experiment, even though there was some difference in their rank (Table 5). NormFinder however, selected *5.8S* as the most stable gene in this system followed by *GAPDH* and *25S rRNA*. Considering BestKeeper analysis, only the most stable gene (*EF-1A*) presented a SD<1. The least stable genes in this system were *18S* rRNA, *β-TUB* and *hsp70*.

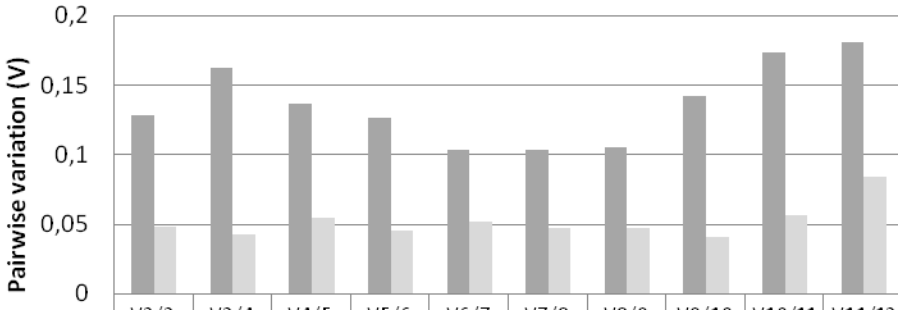
In the somatic embryogenesis experimental system (ii) all the 12 genes were highly stable, with geNorm M values ranging from 0.13 to 0.53. Bestkeeper returned SD values lower than 1.0 in all candidate genes. *25S* and *5.8S rRNAs* were in the top three most stable genes considering all programs. *UBQ*, *β-TUB* and *hsp70* were ranked as the least stable genes in this system.

RefFinder comprehensive ranking considered *GAPDH*, *5.8SrRNA*, *EF-1A* and *TBA* as the first four recommended reference genes for the growth chamber experiment, and *5.8S rRNA*, *25S rRNA*, *ACT* and *18S rRNA* for the somatic embryogenesis system (Suppl. Fig. 2 a, b). If no discrimination is done between the experimental system, RefFinder returns the genes *5.8S rRNA*, *GAPDH*, *ACT* and *rpL2* as the most stable four candidates, while *hsp70*, *β-TUB* and *UBQ* were in the last positions of the rank (Suppl. Fig. 2 c).

geNorm package was also applied on the determination of the optimal number of reference genes for normalisation. Figure 1 shows the pairwise variation analysis, which suggests that normalisation requires the use of only two reference genes for both experimental systems, since $V_{2/3}$ values were under the cut-off level (0.05 in somatic embryogenesis and 0.13 in growth experiments).

Table 5. Ranking of the 12 candidate reference genes in two different experimental systems (growth chamber and somatic embryogenesis) according to their expression stability values as given by geNorm, NormFinder, and BestKeeper.

	Growth chamber						Somatic embryogenesis					
Rank	**geNorm**	**M**	**NormFinder**	**SV**	**BestKeeper**	**std dev [+/-CP]**	**geNorm**	**M**	**NormFinder**	**SV**	**BestKeeper**	**std dev [+/-CP]**
1	*EF-1A|TBA*	0.430	*5.8S*	0.094	*EF-1A*	0.88	*5.8S|18S*	0.128	*5.8S*	0.020	*25S*	0.10
2			*GAPDH*	0.315	*GAPDH*	1.00			*25S*	0.053	*58S*	0.16
3	*GAPDH*	0.436	*25S*	0.340	*TBA*	1.02	*25S*	0.148	*ACT*	0.104	*rpl2*	0.19
4	*ACT*	0.562	*TIF1*	0.391	*TIF1*	1.09	*ACT*	0.166	*18S*	0.114	*18S*	0.19
5	*5.8S*	0.641	*TBA*	0.416	*5.8S*	1.10	*TIF1*	0.214	*rpl2*	0.162	*TIF1*	0.21
6	*25S*	0.709	*ACT*	0.513	*ACT*	1.15	*rpl2*	0.242	*TIF1*	0.164	*ACT*	0.21
7	*UBQ*	0.752	*UBQ*	0.527	*rpl2*	1.19	*GAPDH*	0.286	*GAPDH*	0.256	*EF-1A*	0.32
8	*TIF1*	0.803	*EF-1A*	0.591	*UBQ*	1.21	*TBA*	0.318	*EF-1A*	0.276	*GAPDH*	0.33
9	*rpl2*	0.859	*rpl2*	0.598	*25S*	1.28	*EF-1A*	0.353	*TBA*	0.278	*TBA*	0.40
10	*18S*	0.992	*18S*	0.963	*18S*	1.79	*UBQ*	0.377	*UBQ*	0.297	*UBQ*	0.47
11	*ß-TUB*	1.176	*ß-TUB*	1.372	*ß-TUB*	-	*ß-TUB*	0.431	*ß-TUB*	0.424	*ß-TUB*	-
12	*hsp70*	1.360	*hsp70*	1.453	*hsp70*	-	*hsp70*	0.534	*hsp70*	0.698	*hsp70*	-



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10	V10/11	V11/12
Growth chamber	0,128	0,162	0,136	0,126	0,103	0,103	0,105	0,142	0,173	0,181
Somatic embryogenesis	0,048	0,042	0,054	0,045	0,052	0,047	0,047	0,041	0,056	0,084

Fig. 1. Optimal number of reference genes required for effective normalisation. The pairwise variation (*Vn/Vn+1*) between the normalisation factors NFn and NFn+1 was analysed by geNorm program to determine the minimum number of reference genes required for RT-qPCR data normalisation in two experimental systems: growth chamber experiment; somatic embryogenesis.

Evaluation of selected reference genes

For the growth chamber system, *DcAOX1* transcript accumulation was evaluated at 56 days post sowing, corresponding to the initial explants (used as calibrator), and at 85, 92, 98 and 125 days post sowing. Three normalisation strategies were followed to determine the expression of *DcAOX1*: (a) using the 2 top reference genes given by RefFinder recommended comprehensive ranking - *GAPDH* and *5.8S rRNA*; (b) using the 2 best reference genes selected by geNorm that correspond to the V value $V_{2/3}$=0.13 (below the 0.15 cut-off value) - *EF-1A* and *TBA*; and finally (c) using the worst gene ranked in all programs -*hsp70.*

The analyses revealed that expression of *DcAOX1* increased from the 56 days post sowing until the end of the experiment. Similar results were obtained between combinations (a) and (b) (Fig. 2). However, when *hsp70* was used as reference gene (the worst ranked gene), a substantial higher increase on *DcAOX1* transcript accumulation took place (Fig. 2).

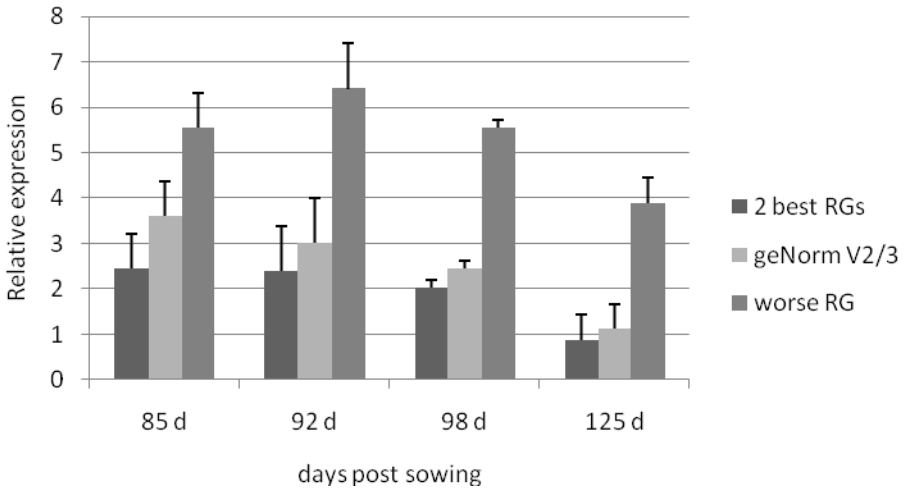


Fig. 2. Relative quantification of *DcAOX1* expression after 56, 85, 92, 98, and 125 days post sowing in a growth chamber experiment. Initial *DcAOX1a* expression (56 days post-sowing) from each plant was selected as calibrator. Three normalisation strategies are presented: the two best RGs from the RefFinder comprehensive ranking; GeNorm V value from pairwise variation analysis and the worst RG from the comprehensive ranking (*hsp70)*. RG: reference gene. Mean and SD deviation values of two RTs are presented.

7.1.4. Discussion

Several genes including *GAPDH*, *ACT*, *18S rRNA*, *UBQ*, *TBA*, *β-TUB* and *EF* have already been used as reference genes for expression studies in many plant species (Goulao et al. 2012; Kumar et al. 2011; Meng et al. 2013; Monteiro et al. 2013; Yi et al. 2012). Besides those, we proposed *25S*, *5.8S* rRNA, *TIF1*, *hsp70* and the *rpL2* as candidates due to their specific roles in several cellular processes. Furthermore, the availability of the carrot sequence transcriptome (Iorizzo et al. 2011) can allow the identification of additional candidate genes, which are potentially steadily expressed and involved in diverse molecular functions, biological processes or forming part of cellular components.

Recent studies suggest that some of the most traditionally used reference genes display unacceptable high expression variability, limiting their use as internal controls (Exposito-Rodriguez et al. 2008; Jain et al. 2006; Zhu et al. 2012). Validating a set of candidate reference genes for specific experimental conditions should be a main concern, as the expression level of those genes cannot be assumed to remain constant under all possible conditions (Hruz et al. 2011; Hu et al. 2009). It is thus currently accepted that the stability of potential reference genes must be systematically determined prior to their use (Guenin et al. 2009; Gutierrez et al. 2008). Failure to do so has been considered the most common error in RT-qPCR experiments, which, together with normalisation based on a single reference gene,

lead to erroneous expression differences (Vandesompele et al. 2002) and thus faults on the biological understanding of the mechanisms under study. The results of the present study further support this statement, as we clearly have shown that each experimental system requires a specific set of reference genes. A good example is the *GAPDH* gene, ranked in the first positions in the growth chamber experimental system (i) which clearly contrasts with its lower stability in the somatic embryogenesis experimental system (ii). In coffee (Barsalobres-Cavallari et al. 2009) or field mustard (Qi et al. 2010), *GAPDH* was also reported as a stable reference gene. However the expression stability of *GAPDH* was found to be unsuitable for normalisation in some experiments with soybean, tomato and petunia (Exposito-Rodriguez et al. 2008; Jian et al. 2008; Mallona et al. 2010). Ribosomal RNAs have been suggested to be good reference genes as they are expressed in all cell types to direct biogenesis of ribosomes; a large number of *rRNAs* have been validated as suitable reference genes in plants (Barsalobres-Cavallari et al. 2009; Luo et al. 2014; Zhang and Hu 2007). In the two experimental systems here tested, the highly expressed ribosomal *5.8S, 25S* and *18S* rRNA genes appear as well ranked genes. Also in other plant species and experimental conditions, ribosomal genes are expressed at very high levels (Barsalobres-Cavallari et al. 2009; Jain et al. 2006; Saha and Vandemark 2012). Furthermore, the commonly used reference gene *β-TUB* was repeatedly ranked in the last positions, regardless the experimental system analysed. On somatic embryogenesis, *hsp70* unsuitability as reference gene can be explained by the involvement of this gene in the somatic embryogenesis process (Gyorgyey et al. 1991). In fact *hsp70*, normalized by the stable *5.8S* rRNA, presents a progressive increase during somatic embryogenesis realization phase (results not shown). Previous gene expression studies on carrot used *EF-1A* or *UBQ* for normalisation of carotenoids biosynthesis genes (Clotault et al. 2008; Fuentes et al. 2012) or *GAPDH* on carrot allergens experiments (Zagon et al. 2010). On carrot embryogenic cell lines, the reference genes *TUB* or *UBQ* (Frederico et al. 2009; Milioni et al. 2001) have also been used. However, under the conditions here assayed, the ribosomal genes were the best ranked in the somatic embryogenesis system. Our results emphasize the importance of reference genes validation for each experimental condition, especially when samples belong to very different sets.

Differences in the stability ranking between the different computational programs (geNorm, NormFinder, BestKeeper) were also observed, as they make use of different approaches and algorithms. It has been argued that co-regulation of genes may compromise geNorm approach as the software tends to select genes with a similar expression profile (Andersen et al. 2004; Exposito-Rodriguez et al. 2008; Paolacci et al. 2009). It is worth to notice that the main difference observed between approaches was on the rank of the most stable

genes, being all programs in agreement on the least stable genes (Table 5). Due to the variability among softwares, RefFinder comprehensive ranking can represent a strategy on the selection of candidate genes. It was previously used to rank candidate reference genes in several other experimental systems in different plant species (Castro-Quezada et al. 2013; Figueiredo et al. 2013; Ovesna et al. 2012; Yeap et al. 2014; Zhu et al. 2012).

Based on geNorm data, two reference genes were recommended for somatic embryogenesis and growth chamber experimental systems. Even though geNorm recommended the use of *EF-1A* and *TBA* on growth chamber experiment (i) and of *ACT* and *5.8S* rRNA on somatic embryogenesis (ii), we would like to propose the ones recommended by RefFinder comprehensive ranking: *GAPDH* and *5.8S rRNA* for (i) and *5.8S* and *25S* rRNAs for (ii), due to the similarity between the M values of these genes (see Table 5). In fact, when the suitability of the reference genes identified on the growth chamber experiment was verified on a member of the alternative oxidase gene family, *DcAOX1,* similar results were found when data were normalized using the best ranked candidate genes selected by RefFinder comprehensive ranking or selected by geNorm pairwise variation. We have clearly shown that wrong data interpretation can result from the use of a wrong reference gene - normalisation was obscured when the least stable reference gene (*hsp70)* was chosen.

Although no single reference gene had an optimal performance across all the experimental systems, *5.8S rRNA* and *GAPDH* can be proposed as a good starting point for gene expression studies on carrot, whereas *hsp70*, *β-TUB* and *UBQ* should not be used as reference genes.

7.1.5. Conclusions

In this study we evaluated 12 genes for potential use as reference genes for RT-qPCR analysis on gene expression in carrot, in two different experimental systems. To the best of our knowledge, this is the first report on the selection of appropriate reference genes in carrot. The magnitude of samples and experimental conditions contribute for a more accurate use of RT-qPCR in the analysis of gene expression in this species. We provide a clear indication of reference genes to be used on carrot expression analysis, but we also reinforce the idea that the validation of reference genes for the experimental conditions under study is essential. This need was emphasized by the presented expression analysis of *DcAOX1* as target gene, where the adverse effect of using unsuitable reference genes for normalisation were unarguably shown. Our results demonstrate that a previous selection of reference genes for a specific experimental setup is crucial to achieve accurate and reliable RT-qPCR gene expression data, avoiding low precision or misleading results.

7.1.6. Acknowledgements

The authors are thankful to Tânia Nobre, Isabel Velada and Catarina Campos (ICAAM, University of Évora, Portugal) for reading the manuscript and for giving suggestion to improve it, and to Carlos Noceda (CIBE-ESPOL, Guayaquil, Ecuador) for important advices on software analysis. We are also thankful to the Portuguese Foundation for Science and Technology for providing the fellowships to MD Campos (SFRH/BD/65354/2009) and to AM Frederico (SFRH/BD/64987/2009) and for the support given under the program POPH – Programa Operacional Potencial Humano (Ciência 2007 and Ciência 2008: C2008-UE/ICAM/06). Collaboration with Germany was supported by a bilateral cooperation contract GRICES/FCT and DAAD. This work is funded by FEDER Funds through the Operational Program for Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the Strategic Project PEst-C/AGR/UI0115/2011.

7.1.7. References

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64, 5245-5250.

Arnholdt-Schmitt B, Costa JH, de Melo DF (2006) AOX - a functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11, 281-287.

Barsalobres-Cavallari CF, Severino FE, Maluf MP, Maia IG (2009) Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions. BMC Mol Biol 10, 1.

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 4, 14.

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29.

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR - a perspective. J Mol Endocrinol 34, 597-601.

Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 15, 155-166.

Cardoso HG, Arnholdt-Schmitt B (2013) Functional marker development across species in selected traits, Diagnostics in Plant Breeding, Lübberstedt T & Varshney RK ed. Springer Netherlands, pp. 467-515. doi: 10.1007/978-94-007-5687-8_21

Campos MD, Cardoso HG, Linke B, Costa JH, de Melo DF, Justo, L., Frederico AM, Arnholdt-Schmitt B (2009) Differential expression and co-regulation of carrot AOX genes (*Daucus carota*). Physiol Plant 137, 578-591.

Castro-Quezada P, Aarrouf J, Claverie M, Favery B, Mugniery D, Lefebvre V, Caromel B (2013) Identification of reference genes for normalizing RNA expression in potato roots infected with *Cyst* nematodes. Plant Mol Biol Rep 31, 936-945.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome wide identification and testing of superior reference genes for transcript in Arabidopsis. Plant Physiol 139, 5–17.

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. Bba-Bioenergetics 1757, 730-741.

Clotault J, Peltier D, Berruyer R, Thomas M, Briard M, Geoffriau E (2008) Expression of carotenoid biosynthesis genes during carrot root development. J Exp Bot 59, 3563-3573.

de Vega-Bartol JJ, Santos RR, Simoes M, Miguel CM (2013) Normalizing gene expression by quantitative PCR during somatic embryogenesis in two representative conifer species: *Pinus pinaster* and *Picea abies*. Plant Cell Rep 32, 715-729.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 37, 112-114, 116, 118-119.

Die JV, Roman B, Nadal S, Gonzalez-Verdejo CI (2010) Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. Planta 232, 145-153.

Exposito-Rodriguez M, Borges AA, Borges-Perez A, Perez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol 8, 131.

Figueiredo A, Loureiro A, Batista D, Monteiro F, Varzea V, Pais MS, Gichuru EK, Silva MC (2013) Validation of reference genes for normalization of qPCR gene expression data from *Coffea* spp. hypocotyls inoculated with *Colletotrichum kahawae*. BMC Res Notes 6, 388.

Frederico AM, Campos MD, Cardoso HG, Imani J, Arnholdt-Schmit B (2009) Alternative oxidase involvement in *Daucus carota* somatic embryogenesis. Physiol Plantarum 137, 498-508.

Fuentes P, Pizarro L, Moreno JC, Handford M, Rodriguez-Concepcion M, Stange C (2012) Light-dependent changes in plastid differentiation influence carotenoid gene expression and accumulation in carrot roots. Plant Mol Biol 79, 47-59.

Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 295, 17-21.

Goulao LF, Fortunato AS, Ramalho JC (2012) Selection of reference genes for normalizing quantitative Real-Time PCR gene expression data with multiple variables in *Coffea* spp. Plant Mol Biol Rep 30, 741-759.

Gu C-S, Liu L-Q, Deng Y-M, Zhu X-D, Lu X-Q, Huang S-Z (2014) Validation of reference genes for RT-qPCR normalization in *Iris. lactea* var. *chinensis* leaves under differente experimental conditions. Sci Hort 175, 144-149.

Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L (2009) Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 60, 487-493.

Gutierrez L, Mauria M, Guenin S, Pelloux J, Lefebvre JF, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, Van Wuytswinkel O (2008) The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J 6, 609-618.

Gyorgyey J, Gartner A, Nemeth K, Magyar Z, Hirt H, Heberle-Bors E, Dudits D (1991) Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol 16, 999-1007.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8, R19.

Hruz T, Wyss M, Docquier M, Pfaffl MW, Masanetz S, Borghi L, Verbrugghe P, Kalaydjieva L, Bleuler S, Laule O, Descombes P, Gruissem W, Zimmermann P (2011) RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genomics 12.

Hu R, Fan C, Li H, Zhang Q, Fu YF (2009) Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 10, 93.

Iorizzo M, Senalik DA, Grzebelus D, Bowman M, Cavagnaro PF, Matvienko M, Ashrafi H, Van Deynze A, Simon PW (2011) *De novo* assembly and characterization of the carrot transcriptome reveals novel genes, new markers, and genetic diversity. BMC Genomics 12, 389.

Jain M, Nijhawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 345, 646-651.

Jian B, Liu B, Bi YR, Hou WS, Wu CX, Han TF(2008) Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Mol Biol 9.

Jin XH, Fu JX, Dai SL, Sun Y, Hong Y (2013) Reference gene selection for qPCR analysis in cineraria developing flowers. Sci Hort 153, 64-70.

Kim BR, Nam HY, Kim SU, Kim SI, Chang YJ (2003) Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnol Lett 25, 1869-1872.

Kumar V, Sharma R, Trivedi PC. Vyas GK. Khandelwal V (2011) Traditional and novel references towards systematic normalization of qRT-PCR data in plants. Aust J Crop Sci 5, 1455-1468

Lee JM, Roche JR, Donaghy DJ, Thrush A, Sathish P (2010) Validation of reference genes for quantitative RT-PCR studies of gene expression in perennial ryegrass (*Lolium perenne* L.). BMC Mol Biol 11, 8–21.

Lin YL, Lai ZX (2010) Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree. Plant Sci 178, 359-365.

Liu CL, Wu GT, Huang XH, Liu SH, Cong BL (2012) Validation of housekeeping genes for gene expression studies in an ice alga *Chlamydomonas* during freezing acclimation. Extremophiles 16, 419-425.

Luo H, Luo K, Luo L, Li E, Guan B, Xiong D, Sun B, Peng K, Yang B (2014) Evaluation of candidate reference genes for gene expression studies in *Cymbidium kanran*. Sci Hort 167, 43-48.

Mallona I, Lischewski S, Weiss J, Hause B, Egea-Cortines M (2010) Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*. BMC Plant Biol 10.

Manoli A, Sturaro A, Trevisan S, Quaggiotti S, Nonis A (2012) Evaluation of candidate reference genes for qPCR in maize. J Plant Physiol 169, 807–815.

Meng Y, Li N, Tian J, Ghao J, Zhang C (2013) Identification and validation of reference genes for gene expression studies in postharvest rose flower (*Rosa hybrida*). Sci Hort 158, 16-21.

Milioni D, Franz G, Sung R, Hatzopoulos P (2001) Gene expression during heat-shock in embryogenic carrot cell lines. Plant Cell Tis Org Cult 65, 221-228.

Monteiro F, Sebastiana M, Pais MS, Figueiredo A (2013) Reference gene selection and validation for the early responses to downy mildew infection in susceptible and resistant *Vitis vinifera* cultivars. PLoS One 8, e72998.

Ovesna J, Kucera L, Vaculova K, Strymplova K, Svobodova I, Milella L (2012) Validation of the beta-amy1 transcription pProfiling assay and selection of reference genes suited for a RT-qPCR assay in developing barley caryopsis. Plos One 7, e41886.

Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10, 11.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Pfaffl MW, Hageleit M (2001) Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett 23, 275-282.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 26, 509-515.

Qi JN, Yu SC, Zhang FL, Shen XQ, Zhao XY, Yu YJ, Zhang DS (2010) Reference gene selection for real-time quantitative polymerase chain reaction of mRNA transcript levels in Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*). Plant Mol Biol Rep 28, 597-604.

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313, 856-862.

Rivera-Vega L, Mamidala P, Koch JL, Mason ME, Mittapalli O (2012) Evaluation of reference genes for expression studies in Ash (*Fraxinus* spp.). Plant Mol Biol Rep 30, 242-245.

Saha GC, Vandemark GJ (2012) Evaluation of expression stability of candidate references genes among green and yellow pea cultivars (*Pisum sativum* L.) subjected to abiotic and biotic stress. Am J Plant Sci 3, 235-243.

Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7, 33.

Skern R, Frost P, Nilsen F (2005) Relative transcript quantification by quantitative PCR: roughly right or precisely wrong? BMC Mol Biol 6.

Umbach AL, Fiorani F, Siedow JN (2005) Characterization of transformed *Arabidopsis* with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. Plant Physiol 139, 1806-1820.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3, research0034.0031-0034.0011.

Warrington JA, Nair A, Mahadevappa M, Tsyganskaya M (2000) Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiol Genomics 2, 143-147.

Xie FL, Xiao P, Chen DL, Xu L, Zhang BH (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80, 75-84.

Yeap W-C, Loo J, Wong Y, Kulaveerasingam H (2014) Evaluation of suitable reference genes for qRT-PCR gene expression normalization in reproductive, vegetative tissues and during fruit development in oil palm. Plant Cell Tiss Org Cult 116, 55-66.

Yi S, Qian Y, Han L, Sun H, Fan C, Liu J, Ju G (2012) Selection of reliable reference genes for gene expression studies in *Rhododendron micranthum* Turcz. Sci Hort 138, 128-133.

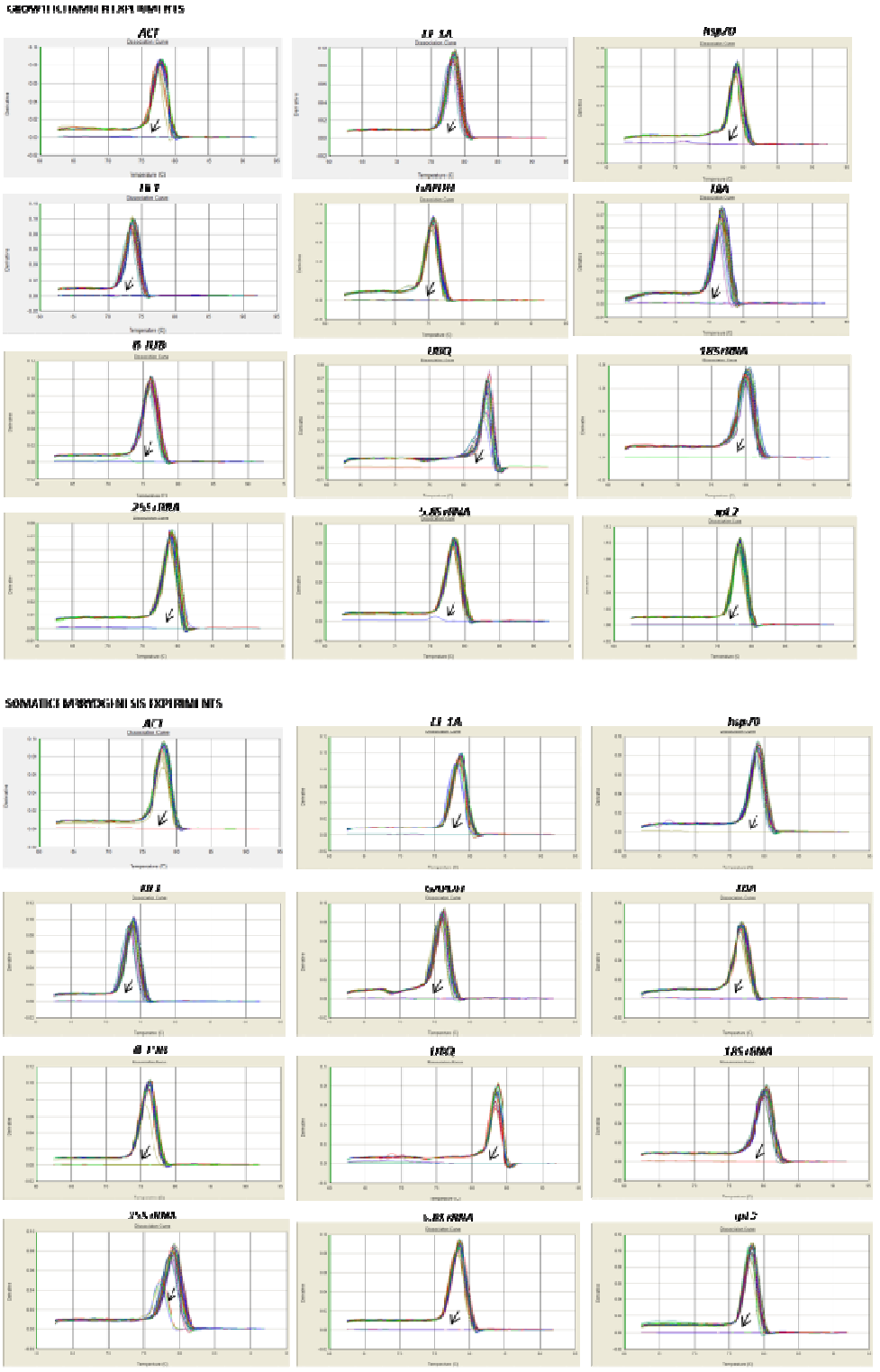
Zagon J, Jansen B, Knoppik M, Ehlers A, Kroh LW, Holzhauser T, Vieths S, Broll H (2010) Gene transcription analysis of carrot allergens by relative quantification with single and duplex reverse transcription real-time PCR. Anal Bioanal Chem 396, 483-493.

Zhang Z, Hu J (2007) Development and validation of endogenous reference genes for expression profiling of medaka (*Oryzias latipes*) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. Toxicol Sci 95, 356-368.

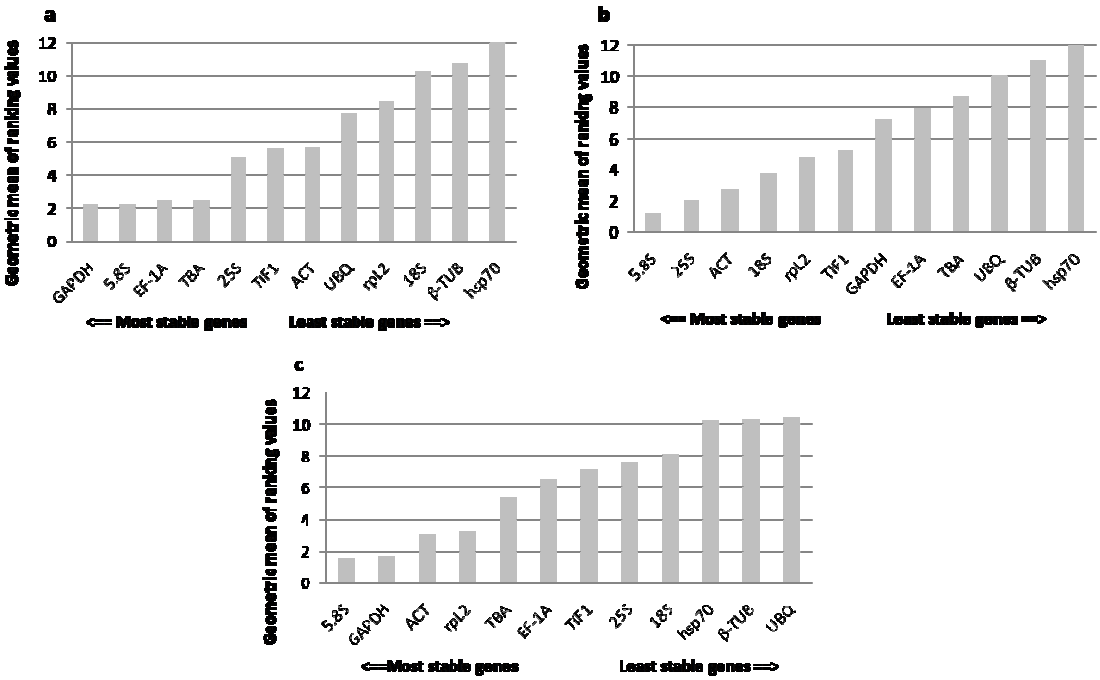
Zhu XY, Li XP, Chen WX, Chen JY, Lu WJ, Chen L, Fu DW (2012) Evaluation of new reference genes in papaya for accurate transcript normalization under different experimental conditions. Plos One 7, e44405.

Supplemental Table 1. Sampling times during somatic embryogenesis realization phase (*Daucus carota* L. cv. Rotin, cell line L5.S.R) (adapted from Frederico et al. 2009).

Sample name	Description
T-2	Flow-through cells sieved with a 200 µm mesh pore screen in B_5^+
T-1	Flow-through cells sieved with a 95 µm mesh pore screen in B_5^+
0 h	Washed cells with B_5^- by centrifugation (Somatic embryogenesis start)
1 h	1 hour after inoculation in B_5^-
4 h	4 hours after inoculation in B_5^-
12h	12 hours after inoculation in B_5^-
24 h	24 hours after inoculation in B_5^-
2 days	2 days after inoculation in B_5^-
3 days	3 days after inoculation in B_5^-
5 days	5 days after inoculation in B_5^-
7 days	7 days after inoculation in B_5^-
10 days	10 days after inoculation in B_5^-



Supplemental Fig. 1. Primer specificity test through dissociation curve analysis collected from 7500 System SDS software ver. 1.3.1 (Applied Biosystems). *ACT*, *EF-1A*, *hsp70*, *TIF1*, *GAPDH*, *TBA*, *β-TUB*, *UBQ*, *18S rRNA, 25S rRNA*, *5.8 S rRNA* and *rpL2*, in the two tested experimental systems. Non-template control is indicated by a black arrow.



Supplemental Fig. 2. Ranking of expression stability values in the two analysed experimental systems (12 candidate reference genes). The ranking was calculated with RefFinder software. **a** recommended comprehensive ranking on the growth chamber experiment, **b** recommended comprehensive ranking on somatic embryogenesis system, **c** recommended comprehensive ranking on the overall experiments.

7.2. Selection of suitable reference genes for RT-qPCR studies on a carrot primary culture system

Abstract

This study aimed selecting robust and reliable reference genes which are constitutively and equally expressed for accurate normalisation of RT-qPCR data from a carrot primary culture system. A systematic comparison of 12 selected candidate genes is presented. These include seven commonly used (*GAPDH*, *ACT*, *18S rRNA*, *UBQ*, *TBA*, *β-TUB, EF*) and other five potential candidates (*25S* and *5.8S rRNA*, *TIF1*, *hsp70*, *rpL2*). Additionally, the expression profile of the mitochondrial alternative oxidase gene *DcAOX1* was conducted in the primary culture system, to demonstrate the importance of selection of reference genes. Although all candidate reference genes did not reach the minimum values to be selected as valid for data normalisation, *EF-1A* and *rpL2* were the most stable genes. The results obtained on *DcAOX1* expression using those two genes as reference genes, in comparison with the genes identified as the less stable, reinforce the idea that the validation of reference genes for the conditions under study is essential. These results demonstrate that a previous selection of reference genes is crucial to achieve accurate and reliable RT-qPCR gene expression data, avoiding low precision or misleading results.

Keywords: carrot; reverse transcription quantitative real-time PCR; reference gene; normalisation

7.2.1. Aim of the study

The aim of this study was the selection of robust and reliable reference genes which are constitutively and equally expressed for accurate RT-qPCR normalisation analysis in the carrot primary culture *in vitro* system (PCS). This system is related to morphogenic responses as a consequence of cell reprogramming upon stress. The expression stabilities of twelve selected candidate genes were carefully evaluated, aiming to identify the most suitable and stable reference genes for data normalisation. These genes included commonly used reference genes and other potential candidates. Furthermore, in order to illustrate the usefulness of the reference genes, expression analysis of a mitochondrial alternative oxidase gene (*DcAOX1*) in the PCS is presented using several combinations of reference genes.

7.2.2. Material and methods

Plant material and experimental conditions

Tap roots of 10 weeks old carrot plants (*Daucus carota* cv. Rotin) growing under greenhouse conditions were used. Five explants, which consisted in small pieces of secondary phloem, taken from three individual plants (biological replicates), were inoculated per Erlenmeyer containing 20 mL of NL liquid medium (Neumann 1966) supplemented with kinetin (1 mgL^{-1}) and indoleacetic acid (2 mgL^{-1}) (for protocol details see Campos et al. 2009). Samples were collected per plant as bulked sample of about 50 explants (10 Erlenmeyers) before (T0) and after 14 days in culture (T14), growing at 21 °C and 28 °C.

Sample processing

After sampling, plant material was immediately frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with on-column digestion of DNA applying the RNase-Free DNase Set (Qiagen, Hilden, Germany), according to manufacturer's protocol. RNA integrity was verified by electrophoresis on a 1 % agarose gel using DEPC (diethyl pyrocarbonate) (Sigma-Aldrich®, St. Louis, MO, USA) treated water, stained with ethidium bromide (2 $ng \cdot mL^{-1}$) and visualized in a Gene Flash Bio Imaging system (Syngene, Cambridge, UK). RNA concentration was determined with the NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNase-treated total RNA (1 µg) was reverse transcribed with RETROscript® kit (Ambion, Austin, TX, USA) using random decamer primers, in a 20 µl reaction volume according to manufacturer's instruction. Nine cDNA samples were synthesized. From each biological replicate the time points considered were the referred above: T0, T14 at 21°C, and T14 at 28 °C.

Candidate reference gene selection and primer design

See detailed description in 7.1. The complete list of the selected candidate reference genes, and respective primer sequences tested in the PCS are included in Table 1.

Quantitative Real Time PCR

See detailed description in 7.1.

Data analysis

See detailed description in 7.1.

Table 1. List and description of the selected candidate reference genes under evaluation in the primary culture system.

Gene name	Gene symbol	NCBI accession ID	Primer sequence (5'→3')	AS (bp)
Actin	*ACT*	[GenBank:X17525]	Fw: CACACGGTGCCAATTTATGAA Rv: GATCACGGCCAGCAAGGT	73
Elongation factor 1-alpha	*EF-1A*	[GenBank:D12709]	Fw: TGGTGATGCTGGTTTCGTTAAG Rv: AGTGGAGGGTAGGACATGAAGGT	75
Heat shock protein 70	*hsp70*	[GenBank:X60088]	Fw: GAAGTTTGAGCTCACGGGAATT Rv: TCGCATCAATGTCGAACACA	77
Translational initiation factor 1	*TIF1*	[GenBank:ABI32458]	Fw: GGTATGTTCCGTATTCGCTTAG Rv: TATCGCCTGGTAGTATCC	97
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	[GenBank:AY491512]	Fw: GGGAGGTGCAAAGAAAGTTATCA Rv: TTCCTTTTCATTGACACCAACAA	79
Alpha-tubulin	*TBA*	[GenBank:AY007250]	Fw: TCTGGTGCCATACCCAAGGA Rv: ATAGGCCTTCTCAGCGGAGAT	73
Beta-tubulin	*β-TUB*	[GenBank:AAB64308]	Fw: GGGCTCTCTATGTCTTCCACATTC Rv: AAACTGTTCACTAACTCGTCGAAACA	78
Ubiquitin	*UBQ*	[GenBank:U68751]	Fw: TCTTCGCCGGCAAGCA Rv: GTGGACTCCTTCTGGATGTTGTAGT	67
18S Ribosomal subunits	*18S rRNAs*	[GenBank:X17534]	Fw: GACTACGTCCCTGCCCTTTG Rv: TCACCGGACCATTCAATCG	62
25S Ribosomal subunits	*25S rRNAs*	[GenBank:X17534]	Fw: TCACCGGACCATTCAATCG Rv: TCGCCTGTATTTAGCCTTGGA	67
5.8S Ribosomal subunits	*5.8S rRNAs*	[GenBank:X17534]	Fw: AATGACTCTCGGCAACGGATAT Rv: TCACACCAAGTATCGCATTTCG	73
Ribosomal protein L2	*rpL2*	[GenBank:ABI32465]	Fw: GGAAATCGGCCACATTAAAATTA Rv: CCCAACATTCCCCACTTGTC	92

Primer sequence sets and amplicon characteristics for each of the twelve candidate reference genes are also presented. AS corresponds to the amplicons size.

Normalisation of DcAOX1

DcAOX1 encoding a carrot alternative oxidase was obtained from NCBI [GenBank:EU286573.2] and used as a target gene to demonstrate the usefulness of the validated candidate reference gene in RT-qPCR. RT-qPCR reaction and cycling conditions were in point 2.1. Primer pair (Fw: 5'-CTTCAACGCCTACTTCCTTG-3', Rv: 5'-ATCTCGCAATGTAGAGTCAGC-3') of *DcAOX1* were also verified by melting curve analysis and sequencing as described for reference genes.

Four normalisation strategies were followed to determine the expression of *DcAOX1*: (i) using the 2 top reference genes given by RefFinder recommended comprehensive ranking; (ii) using the 3 top reference genes given by RefFinder recommended comprehensive ranking; (iii) using the optimal number of reference genes based on geNorm pairwise variation; and finally (iv) *DcAOX1* was normalized with the worst candidate gene ranked in all programs.

Calculations associated with the delta-Ct method incorporating the amplification efficiency (*E*) for each primer pair (Pfaffl, 2001) for RT-qPCR, were applied using the freeware qCalculator version 1.0 (freely available at: www.genequantification.de/qCalculator.zip). The T0 sample was selected as the calibrator for each tap root. Results were given as the mean of duplicated Ct values from each sample.

7.2.3. Results

Amplification specificity and efficiency

To examine the expression stability of the potential reference genes selected, transcript levels of the twelve candidate genes were measured by RT-qPCR using gene-specific primer pairs (Table 1). Gene-specific amplification of each of the twelve candidates was confirmed by the presence of a single peak in the dissociation curve analysis (Fig. 1). Amplification efficiencies of every candidate gene were calculated individually (Table 2). All calibration curves exhibited linear relationships (regression coefficient r^2 varying from 0.971 to 0.999) between the fractional cycle number and the log of the initial copy number (Table 2). The amplification efficiency (*E*) of the reactions ranged from 85.7 % for U*BQ* to 106.1 % for *GAPDH* (Table 2).

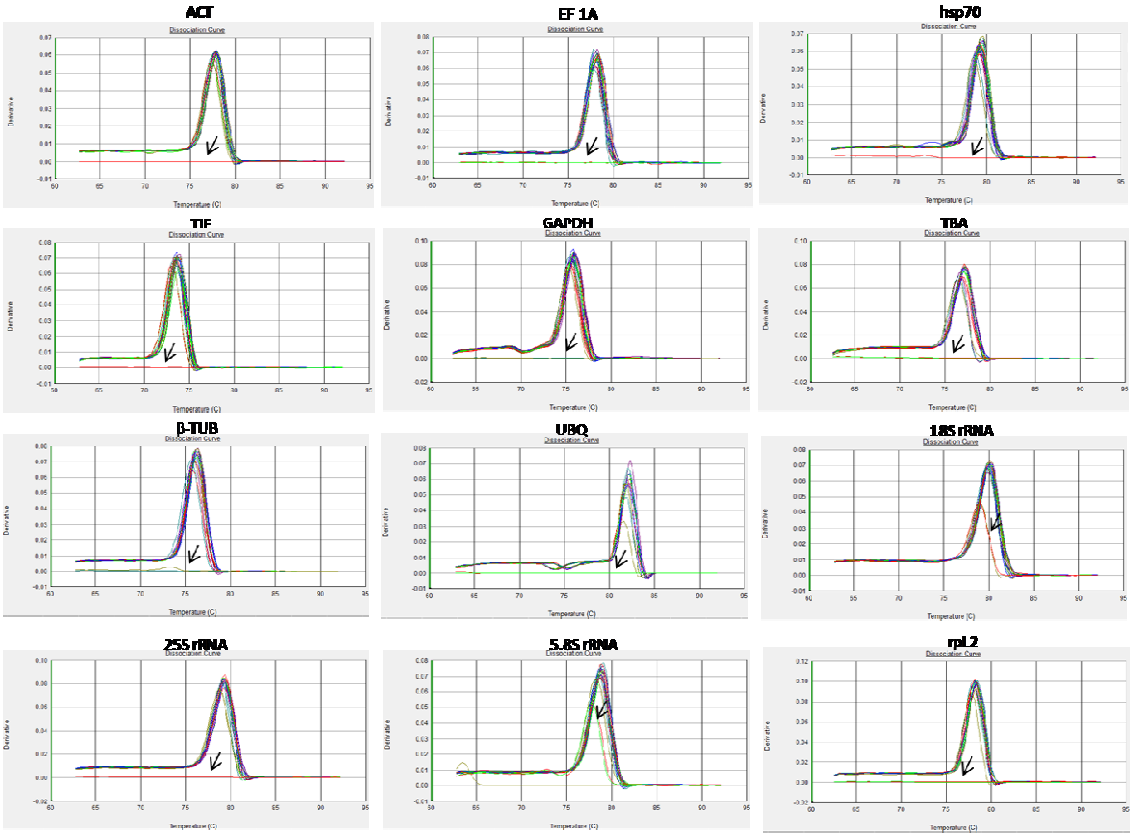


Fig. 1. Primer specificity test through dissociation curve analysis collected from 7500 System SDS software ver. 1.3.1 (Applied Biosystems). *ACT*, *EF-1A*, *hsp70*, *TIF1*, *GAPDH*, *TBA*, *β-TUB*, *UBQ*, *18S rRNA*,

25S rRNA, *5.8 S rRNA* and *rpL2*, in the primary culture system. Non-template control is indicated by a black arrow.

Expression profiling of candidate reference genes

The standard deviation of Ct values indicates the expression stability of each candidate reference gene (Table 2). In the PCS, several candidate genes displayed a high variability, being evident that a straight forward statistical analysis of raw Ct values is not suitable to select the best reference genes for normalisation of these RT-qPCR data.

Table 2. Expression levels of 12 candidate reference genes for the primary culture system.

Genes	*E* (%) (r^2)	Mean Ct ± SD
ACT	98.2 (0.999)	22.18 ± 1.61
EF-1A	102.0 (0.999)	19.26 ± 1.43
hsp70	102.2 (0.994)	27.28 ± 1.81
TIF1	102.3 (0.992)	20.97 ± 1.08
GAPDH	106.1 (0.997)	21.22 ± 1.99
TBA	100.7 (0.992)	21.86 ± 1.60
β-TUB	85.7 (0.971)	29.95 ± 3.41
UBQ	104.9 (0.989)	26.15 ± 2.32
5.8S	100.8 (0.992)	9.50 ± 3.27
18S	100.5 (0.980)	13.55 ± 3.33
25S	99.9 (0.998)	9.20 ± 3.28
rpL2	90.1 (0.987)	20.96 ± 0.89

Primers efficiency (E) and regression coefficient (r^2) are indicated. The mean raw cycle threshold (Ct) RT-qPCR data of each reference gene and the standard deviation (SD) are presented.

Gene expression stability analysis

The expression stability of the 12 candidate reference genes was determined by geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004) packages. The results for best reference genes were not totally consistent between softwares, as it can be seen in Table 3.

The geNorm software revealed M values ranging from 0.854 to 2.488, and BestKeeper displayed SD values lower than 1.0 only in the two more stable genes (*rpl2* and *TIF1*). The RefFinder software (Xie et al. 2012), that integrates the computational programs geNorm, Normfinder, BestKeeper and the comparative delta-Ct method, and which calculates the geometric mean of their weights for the overall final ranking (RefFinder recommended

comprehensive ranking), was also used to evaluate the expression stability of candidate reference genes. Accordingly, RefFinder comprehensive ranking considered *EF-1A*, *rpL2*, *TIF1* and *ACT* (Fig. 2).

Table 3. Ranking of the 12 candidate reference genes in the primary culture system according to their expression stability values as given by geNorm, NormFinder, and BestKeeper.

Rank	geNorm	M	NormFinder	SV	BestKeeper	std dev [+/-CP]
1	*EF-1A*|*TIF1*	0.854	*rpl2*	0.566	*rpl2*	0.65
2			*TBA*	0.642	*TIF1*	0.90
3	*ACT*	0.887	*ACT*	0.724	*EF-1A*	1.03
4	*GAPDH*	0.998	*EF-1A*	0.744	*TBA*	1.17
5	*TBA*	1.118	*TIF1*	0.954	*ACT*	1.26
6	*rpl2*	1.243	*GAPDH*	1.156	*hsp70*	1.55
7	*UBQ*	1.450	*UBQ*	1.320	*GAPDH*	1.68
8	*hsp70*	1.711	*hsp70*	1.399	*UBQ*	1.75
9	*5.8S*	2.035	*5.8S*	1.662	*5.8S*	2.54
10	*25S*	2.198	*25S*	1.683	*25S*	2.55
11	*18S*	2.318	*18S*	1.869	*18S*	-
12	*β-TUB*	2.488	*β-TUB*	2.052	*β-TUB*	-

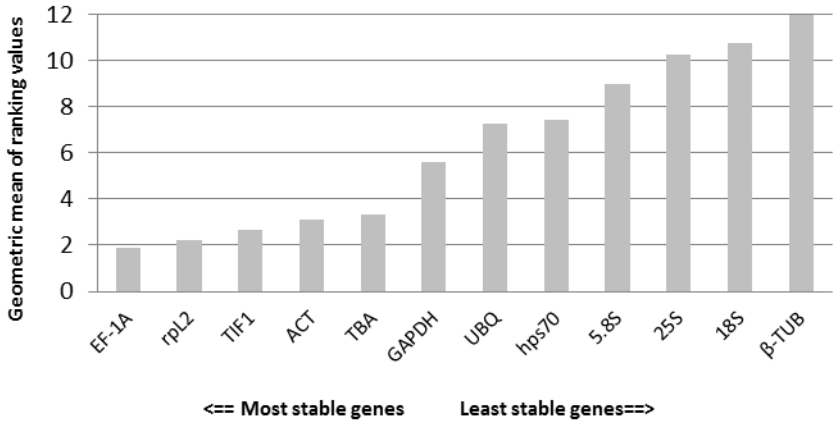


Fig. 2. Ranking of expression stability values in the primary culture system (12 candidate reference genes). The ranking was calculated with RefFinder software.

Determination of the optimal number of reference genes for normalisation by GeNorm

The geNorm v3.5 software was applied to calculate the pairwise variations $V_{n/n+1}$ in order to determine the optimal number of reference genes required for building an accurate NF in the different experimental systems. A V value below 0.15 was suggested as the limit

under which it was unnecessary to add an additional gene for normalisation (Vandesompele et al. 2002). As shown in figure 3, all the V values exceeded the cut-off value, being five genes ($V_{5/6}$=0.221) the best combination for an accurate normalisation.

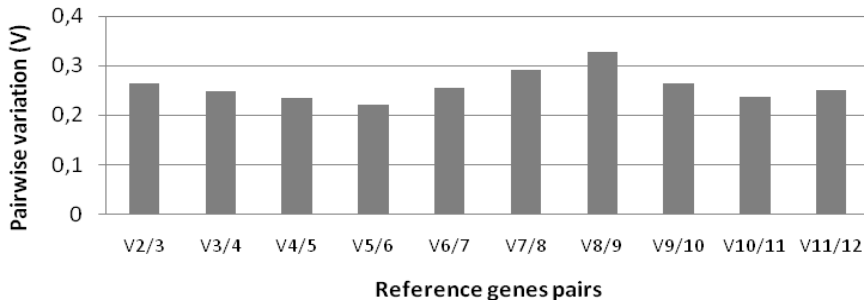


Fig.3. Optimal number of reference genes required for effective normalisation in the primary culture system. The pairwise variation (*Vn/Vn+1*) between the normalisation factors NFn and NFn+1 was analysed by geNorm program to determine the minimum number of reference genes required for RT-qPCR data normalisation.

Evaluation of selected reference genes

Four normalisation strategies were followed to determine the expression level of *DcAOX1* in a PCS. In this system the pairwise variation threshold value was not achieved, and the effect of selecting different combination of reference genes for *DcAOX1* normalisation was evaluated.

DcAOX1 transcript accumulation was evaluated at T0, corresponding to the initial explants (used as calibrator), and in 14 days *callus*, under two tested temperatures (Fig. 4). Four normalisation strategies were followed to determine the expression of *DcAOX1*: (i) using the 2 top reference genes given by RefFinder recommended comprehensive ranking; (ii) using the 3 top reference genes given by RefFinder recommended comprehensive ranking; (iii) using the 5 best reference genes selected by geNorm that correspond to the lowest V value (V5/6 =0.221) (the V did not reach the 0.15 cut-off value on PCS); and finally (iv) using the worst ranked in all programs on PCS. Therefore, *DcAOX1* was normalised based on the expression of (i*) EF-1A* and *rpL2*; (ii) *EF-1A*, *rpL2* and *TIF1*; (iii) *EF-1A*, *TIF1*, *ACT*, *GAPDH* and *TBA* and (iv) *β-TUB.*

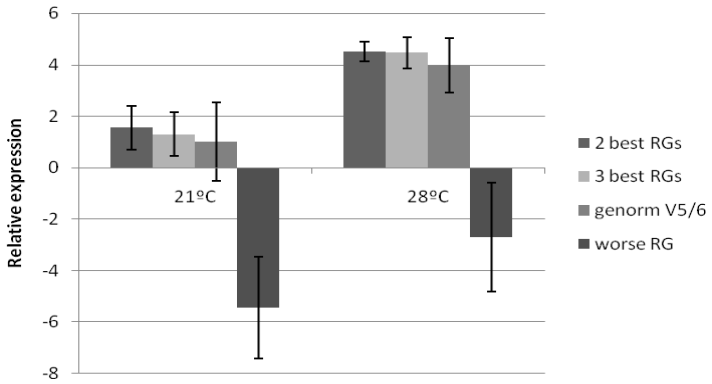


Fig. 4. Relative quantification of *DcAOX1* expression after 14 days (T14) of *in vitro* culture from carrot primary culture system. *Callus* grew at 21 °C and 28 °C. Four normalisation strategies are presented: the two and the three best reference genes from the RefFinder comprehensive ranking; GeNorm V value from pairwise variation analysis and the worst reference gene from the comprehensive ranking (*β-TUB)*. Initial *DcAOX1* expression (T0) from each plant was selected as calibrator. Mean and SD deviation values of two biological replicates are presented.

The analysis using the three first normalisation strategies revealed that expression levels of *DcAOX1* increased from T0 to T14 under both temperatures tested (Fig. 4), and the highest value was observed in tissue growing at 28 ^{o}C. However, when *β-TUB* was used as reference gene, the worst ranked gene in all programs, a significant decrease on *DcAOX1* transcript accumulation between T0 and T14 took place (Fig. 4).

7.2.4. Discussion

General discussion is included in 7.1. Here the focus in on the PCS.

With geNorm M threshold values ranging from 0.842 to 2.533, none of the studied genes could be considered as valid reference gene in the PCS. It seems therefore evident that PCS samples have an intrinsic high variability, which might be explained by its proliferative nature. In this system, the inoculation of differentiated secondary root phloem explants in a cytokinin-containing nutrient media leads to a cell program change related with the acquisition of an undifferentiated stage (*callus*) and subsequently growth. Moreover, the samples selected for this study presented different *callus* growth behaviour for the two different temperatures tested (for growth results see CHAPTER 3). Therefore, this was a very heterogeneous sample panel. Nevertheless, it is very likely that additional reference genes, which have not been used so far, will improve the normalisation outcome. For instance, the availability of the carrot sequence transcriptome (Iorizzo et al. 2011) can allow the identification of additional

candidate genes, which are potentially steadily expressed and involved in diverse molecular functions, biological processes or forming part of cellular components.

When the suitability of the reference genes identified on the PCS was verified on a member of the alternative oxidase gene family, *DcAOX1,* similar results were found when data were normalized using the best ranked candidate genes selected by RefFinder comprehensive ranking or selected by geNorm pairwise variation. However, the results showed that normalisation was obscured when the least stable reference gene (*β-TUB)* was chosen. In this sense, here it is proposed the top two *EF-1A* and *rpl2* reference genes selected by the RefFinder comprehensive ranking for normalisation of PCS transcript data. The *β-TUB* unsuitability as reference gene in the PCS can be explained by the involvement of tubulin genes in processes related with cell division activity (Stotz and Long, 1999; Dumontet et al., 1996; Setter and Flannigan, 2001). In fact, *β-TUB* normalized by *EF-1A*, presents an increase during the PCS between the initial explants (T0) and the growing *callus* after 14 days in culture (results not shown).

In conclusion, our analysis on a PCS, although presenting limitations, illustrates the adverse effect of using unsuitable reference genes for normalisation. These results are a very important starting point for further primary culture transcript analysis. Our results demonstrate that a previous selection of reference genes for a specific experimental setup is crucial to achieve accurate and reliable RT-qPCR gene expression data, avoiding low precision or misleading results.

7.2.5. References

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64, 5245-5250.

Campos MD, Cardoso HG, Linke B, et al (2009). Differential expression and co-regulation of carrot *AOX* genes (*Daucus carota*). Physiol Plant 137, 578-591.

Dumontet C, Durán GE, Steger KA, et al (1996) Differential expression of tubulin isotypes during the cell cycle. Cell Motil Cytoskeleton 35, 49-58.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 26, 509-515.

Setter TL, Flannigan BA (2001) Water deficit inhibits cell division and expression of transcripts involved in cell proliferation and endoreduplication in maize endosperm. J Exp Bot 52, 1401-1408.

Stotz HU, Long SR (1999) Expression of the pea (*Pisum sativum* L.) alpha-tubulin gene TubA1 is correlated with cell division activity. Plant Mol Biol 41, 601-14.

Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3, research0034.0031-0034.0011.

Xie FL, Xiao P, Chen DL,et al (2012). miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80, 75-84.

CHAPTER 8 - GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

8.1. General discussion

In the past two decades routine protocols were developed to genetically identify and characterise the loci contributing to a quantitative trait, referred to as quantitative trait loci (QTL). In contrast to the feasibility of QTL mapping studies, zooming into natural segregating loci (quantitative or qualitative effect) to find their molecular bases was until now not straightforward. Recently, the availability of high-throughput sequencing technologies with reduced prices allowed accelerating the analysis of natural sequence variation, at different levels, going from single gene level or even exome analysis, to whole genome sequencing. However, with the huge amount of data that those technologies provide, the bottleneck is represented nowadays by the ability to genetically dissect complex traits and identify the genes underlying them. One example of a quantitative trait is the yield, which strongly depends on the capacity of plants to efficiently adapt their growth to varying conditions, recently defined as a new trait - plant plasticity (see Cardoso and Arnholdt-Schmitt 2013). The ability for growth adaptation to new environmental conditions depends on the existence of phenotypic variation inside a species, cultivar or variety. This variation, known as natural variation is defined as phenotypic variation caused by spontaneously arisen genetic polymorphisms that are maintained either by natural or human selection (Alonso-Blanco et al. 2005). The identification of genes and polymorphisms within these genes that affect protein function and consequently natural variation assumed high importance in plant breeding due to the development of functional markers (FM). These markers within the target gene provide an absolute differentiation between phenotypes (Andersen and Lübberstedt 2003). Genes of interest for FM development can be identified by high-throughput differential gene analyses or in hypothesis-driven research approaches (Arnholdt-Schmitt 2005). Candidate gene approaches for marker-assisted selection are actually rated as the most promising strategies in molecular plant breeding (Collins et al. 2008). *AOX* was previously proposed in a hypothesis-driven approach as target to develop FMs for efficient cell reprogramming, to assist breeding for robust plants with individual or multi-stress tolerance linked to traits such as yield stability (Arnholdt-Schmitt et al. 2006; Arnholdt-Schmitt 2009; Polidoros et al. 2009; Arnholdt-Schmitt 2015).

Different studies have been demonstrating the involvement of *AOX* genes in plant response upon a diversity of biotic and abiotic stresses (see reviews of Plaxton and Podestá 2006; Vanlerberghe 2013), including morphogenic responses (Fiorani et al. 2005; Ho et al. 2007; Campos et al. 2009; Frederico et al. 2009; Santos Macedo et al. 2009; Santos Macedo et

al. 2012). Several authors also report the key role of AOX in regulating the process of cell-reprogramming by ameliorating metabolic transitions related with the cellular redox state and the flexible carbon balance (Arnholdt-Schmitt et al. 2006; Rasmusson et al. 2009). Clifton et al. (2005; 2006) pointed to the importance of this pathway as an early-sensing system for cell programming.

Contrarily to *AOX*, in which there are a vast number of reports showing the involvement of the different genes in diverse physiological and morphological processes in plants (stress response, development and cell-reprogramming) and the existence of sequence variation across genotypes (Cardoso et al. 2009; Ferreira et al. 2009; Santos Macedo et al. 2009), PTOX has very limited literature describing its role, and no studies have be done so far on the existence of sequence variability. Nevertheless, the similarities at protein sequence level that PTOX has with AOX (Atteia et al. 2004) led to the possibility that the enzyme encoded by *PTOX* could also be involved in some of the same functions that *AOX*. This assumption prompted us to select this latter gene as candidate for further FM development related with yield stability. The work presented in this thesis aimed therefore to explore the potential role of both *AOX* and *PTOX* as target genes for FM development for yield-determining growth performance in carrot. This research is embedded in a broader Competence Focus on 'AOX Research and Functional Marker Development' across diverse organisms and applied systems, and has been also linked to several industrial pilot projects on carrot breeding. Thus, the results of the thesis contribute to the overall knowledge at that focus and provide the basis for advancing in the state-of-the art for ongoing and future projects.

Focusing on the defined research line fully explained in CHAPTER 1, several were the advances attained with the present work. For transcript analysis the first step was the isolation of the different genes. Through CHAPTER 2, CHAPTER 3, CHAPTER 5 and CHAPTER 6 a detailed characterisation of *AOX* and *PTOX* genes' is made. The expression of *AOX* and *PTOX* was then evaluated in different experimental system and conditions, with the main results here discussed.

8.1.1. *AOX* and *PTOX* on cell reprogramming and growth performance

Daucus carota L. was the first organism where cell totipotency was proved (Steward et al. 1958), and up-to-now it is the most used species to study cell programming (Harada et al. 1990; Imani et al. 2001; Kikuchi et al. 2006), due to the easiness to reprogram cells. The *in vitro* primary culture system (PCS) of secondary phloem explants of carrot tap roots was established to study the processes of cell reprogramming and growth performance. This *in vitro* system

had a preponderant role on the experiments presented in this thesis, and it was used to study changes on the expression of *AOX* genes and of *PTOX* gene on cell dedifferentiation process and growth adaptation to different temperatures.

The first results obtained with this system (CHAPTER 2), showed a dynamic expression of *AOX* genes during *de novo* induction of secondary root phloem explants, with a similar pattern observed between *DcAOX1* and *DcAOX2a*. It was found an increase in transcript levels as early as 36 h, followed by a reduction at exponential growth phase (after 14 days in culture). To shed light on even earlier events related to cell reprogramming, the expression of both *DcAOX1* and *DcAOX2a* genes was then determined in a higher temporary resolution in the initial phase, with an earlier start, and a longer observation time (CHAPTER 3). These results revealed that *DcAOX1* and *DcAOX2a* transcript levels increased until 36 h after explant inoculation, with a subsequent down-regulation, before the initiation of exponential growth. At the end of the lag phase and at initiation of exponential growth (8 dpi), expression of both genes reached the lowest levels and remained relatively stable until 28 dpi, with values similar to the original, quiescent tissue. Although not directly in the frame of this thesis, the calorespirometry measurements presented in CHAPTER 3 arise as a crucial complement for the interpretation of the expression results. This tool previously identified as promising for predictive growth phenotyping (Nogales et al. 2014; Arnholdt-Schmitt et al. 2015), was developed by Nogales et al. (2013) for breeding in carrot *in vitro* primary culture systems. Calorespirometry has shown useful to accurately monitor temperature dependent growth performance in terms of metabolic rates, respiratory rates, efficiency of biomass acquisition and growth rates over 21 days of *in vitro* cultures (Nogales et al. 2013). The structural biomass formation showed a drastic increase until around the 4th day after inoculation (CHAPTER 3), during the lag phase of growth (without any visible growth detected), in all five PCS tested. This peak was associated with the cell reprogramming process occurring in this system. Additionally, the callus FW from 12 individual PCS allowed the identification of different growth behaviour in PCS at two different temperatures (21 °C and 28 °C), during the exponential growth phase. However, regarding *AOX* expression, no direct link was detected between callus FW and individual *DcAOX1* or *DcAOX2a* transcript levels during the exponential growth phase. Nonetheless, those results allowed identifying *DcAOX1* as the gene responsive to a higher growing temperature in the PCS, during the exponential growth phase.

Following the same trend as *DcAOX* genes, a rather similar role for *DcPTOX* during the earliest events of cell reprogramming was suggested (CHAPTER 6). However other processes to explain *DcPTOX* expression dynamics during PCS cannot be discarded. In the PCS a reversible differentiation of the chromoplasts into chloroplasts takes place, and the chlorophyll content

of explants increases continuously during the culture period (Kumar et al. 1983). Therefore, it was proposed in CHAPTER 6 that during the PCS two different processes overlap and can also explain *DcPTOX* accumulation pattern: i) the presence of chromoplasts in the initial explants, which accumulate carotenoids (PTOX involvement on carotenoids biosynthesis described below), and ii) the differentiation of chloroplast during the exponential phase of growth, and consequent implication of PTOX in chlororespiration of the *callus* green tissues. In the same way to *DcAOX*, no direct link was detected between *callus* FW at exponential growth phase and *DcPTOX* expression.

8.1.2. *AOX* early response to chilling

As an attempt to transpose the *in vitro* PCS results to plant level, a pot plant experiment was performed to analyse how early *DcAOX1* and *DcAOX2a* would respond to chilling (CHAPTER 3). Low temperature stress is one of the most important abiotic stresses in plants, with the alternative respiration pathway components referred as the most important ones for early responses to abiotic stresses (Clifton et al. 2005). The presented results revealed that *DcAOX1* and *DcAOX2a* are both early transcribed upon chilling stress, with differences detected from the control as soon as after 45 min of exposure. *DcAOX* genes' response was prior to the induction of the cold responsive *AFP* gene, although the latter presented a substantially higher mRNA level.

8.1.3. *AOX* and storage root growth during plant development

In order to get insights on the role of the different *DcAOX* genes in tap root growth during plant development, gene expression studies were performed (CHAPTER 4). In this experiment, performed using different carrot cultivars, the expression dynamics of all *DcAOX* gene family members (*DcAOX1*, *DcAOX2a* and *DcAOX2b*) were assessed. Growth was evaluated by measurements of root length and root secondary growth (indicated by fresh weight) in the developing storage roots (from the 5th to the 13th wps). A substantial increase on tap root length was detected at the beginning of the experiment, right after the formation of the cambium ring (Hole et al. 1987), when secondary growth (evaluated by fresh weight) was almost inexistent. In later stages of development there was a halt on root length increase concomitantly to an increment on root secondary growth. Expression analysis revealed that during storage root development, the only *AOX* gene that followed a concrete trend was *DcAOX2a*, with the highest levels of transcripts detected at the initial time points, followed by a pronounced decreased. Combining the growth information with the expression analysis it is clear that *DcAOX2a* is somehow associated with carrot tap root development: a high

expression was observed during early stages, coincident with a high root length increase, and a decrease in transcript levels was observed concomitantly to the increment of secondary growth.

A positive impact on growth related with the lack of AOX was already emphasised on studies using single cell systems (Sieger et al. 2005; Mathy et al. 2010). Sieger et al. (2005) demonstrated that *AOX1a* knockdown led to the incapacity of cells for down-regulating growth under P- and N-deficiency, and concluded that AOX activity provides a mechanism for adjusting growth and counteracting nutrient imbalance. These results let Arnholdt-Schmitt (2006) hypothesize on the importance of considering down-regulation of *AOX* as a potential tool for molecular breeding on higher nutrient efficiency.

However the relation between AOX and growth is undoubtedly much more complex in whole plants systems. In this sense it cannot be totally discarded the positive relationship observed in the first time points of the experiment, between *DcAOX2a* transcripts and tap root primary growth (evaluated by root length). Nevertheless, AOX was shown to be especially active in meristematic tissues (Hilal et al. 1997; see also CHAPTER 2), and expression studies made directly on carrot root meristem will for sure allow a complete clarification on the role of AOX on root secondary growth.

8.1.4. *PTOX* association with secondary growth and/or carotenoids accumulation

In order to study if PTOX is associated with yield-determining tap root growth performance, *PTOX* expression was investigated in central root meristem (cambium ring), using pot plant experiments (CHAPTER 6). An increase on *DcPTOX* transcripts was detected during storage root development. The meristematic tissue is the metabolically most active tissue in the tap root and is responsible for secondary growth, and consequently on yield production (Hole et al. 1984; Arnholdt-Schmitt 1999). However, due to the small cell layers of the cambium ring (approximately 10 cell thickness), the immediately next secondary phloem that is originated directly from the meristem was picked together. Although in a first approach the results point to a relation between *PTOX* and root secondary growth, it is necessary to consider that cells from secondary phloem contain carotenoids, and previous studies already indicated the involvement of PTOX on the metabolic pathway of carotenoids biosynthesis (Simkin et al. 2008; Simkin et al. 2010; Sun and Wen 2011).

In this sense, and to understand whether PTOX is associated with secondary growth and/or carotenoids accumulation, *DcPTOX* expression was evaluated in developing carrot tap roots (in complete roots or in pieces from the upper part), in an experiment that included cultivars with different carotenoids contents (CHAPTER 5). It was found an increase of

transcripts during the time course of the experiment, in all cultivars, including the white one where very little or no carotenoid pigments are detected. *DcPTOX* showed a similar transcript profile to other carotenoid biosynthetic genes and highly correlated to all of them.

Taken together, our data indicate an association of *DcPTOX* with both carotenoid biosynthesis and storage root secondary growth mechanisms. A first analysis on *DcPTOX* sequence variability was also performed, with encouraging results to strengthen future efforts on the identification of polymorphic motifs in *DcPTOX* for the development of FM related to agronomic traits of interest such as yield or pigmentation of storage roots.

8.1.5. Early *DcPTOX* response to mild cold stress

To get insights on the involvement of *PTOX* on adaptive growth, the early effect of temperature decrease was further evaluated in tap root meristems (CHAPTER 6). In this experiment, a short-term early response was detected in *DcPTOX* expression levels upon temperature decrease, most probably associated with adaptive growth. After the beginning of the mild-cold treatment, the storage root growth (measured by root biomass, length and thickness) was suppressed. However, the mild-cold stress had a positive effect on the final storage root growth.

8.1.6. Selection of suitable reference genes in different carrot experimental systems for accurate data normalisation

Clearly, throughout the research work leading to the present thesis, transcript analysis was the main approach. The importance of the selection of suitable reference genes in different carrot experimental systems was highlighted and demonstrated. The somatic embryogenesis system results, although not in the frame of this PhD project, were included as an additional experimental system for analysis. An indication of reference genes to be used on those experimental systems of carrot was provided, and the idea that the validation of reference genes for each experimental condition is essential for accurate and reliable RT-qPCR gene expression measurements was reinforced.

8.2. Conclusions

The studies performed in this thesis represent an important contribution towards the exploitation of *AOX* and *PTOX* as target genes for FM development for yield-determining growth performance in carrot. The following main conclusions can be highlighted from the results presented:

- *DcAOX1* and *DcAOX2a* can be considered reasonable candidates for FM development on efficient cell reprogramming under changing environments in general (*in vitro* inoculation and chilling). Additionally, calorespirometry measurements allowed assessing the efficiency by which cell reprogramming occurs. *DcPTOX* follows the same trend as *DcAOX* genes during earliest events of cell reprogramming. However, the involvement of *DcPTOX* in processes such as carotenoids biosynthesis and chlororespiration in green tissues must be further considered. No direct link was detected between *callus* fresh weight at exponential growth phase and *DcAOX* and *DcPTOX* transcript accumulation.
- *DcAOX2a* was associated with tap root growth during carrot development, following a concrete trend.
- *DcPTOX* was associated with both carotenoid biosynthesis and secondary growth during storage root development.
- A short-term early response was detected in *DcPTOX* transcript accumulation in tap root meristem upon temperature decrease. However, the mild cold stress treatment had a positive effect on the final root biomass, length and thickness.
- The selection of suitable reference genes for accurate RT-qPCR analysis for each carrot experimental system was performed and its importance highlighted.

8.3. Future perspectives and work in progress

In view to continue the work here described, several tasks are planned or already in course. The existence of sequence variability within a gene linked to a desired trait and consequently related with differences at phenotypic level (the basis of association studies) is a fundamental requisite for FM development. The identification of polymorphisms in *AOX* and *PTOX* sequences considering the different regions of the gene (promoter, UTRs, exons and introns) are of higher interest to continue FM research. Studies on *DcAOX* gene variability and on the identification of regulatory elements were the focus of several research papers (Nogales et al. submitted; Cardoso et al. 2009; Cardoso et al. 2011), with further analysis still in course. First results on *DcPTOX* sequence variability were performed and included in CHAPTER 6, but additional *in silico DcPTOX* sequence analysis to search for sequences coding for regulatory elements or involved in regulation of gene expression will be essential. Also, it is necessary to ascertain whether the presented results on *AOX* and *PTOX* gene expression are reflected at protein level and if polymorphic sites are related with protein functionality. The association of selected polymorphisms with the trait of interest is as well a point of high interest for carrot FM development.

As a first attempt to elucidate the functionality of each carrot *AOX* gene, a work related with AOX-silencing and overexpression following a transgene approach is in course. This work is being developed in the frame of a bilateral cooperation between Portugal (University of Évora) and India (University of Coimbatore), under the project 'Study of *DcAOX* genes functionality associated with cell reprogramming under abiotic stresses'. A similar procedure related with gene silencing and overexpression would be important on PTOX research.

Additionally, a methodology to test the functionality of a selected polymorphism, this meaning to understand if a specific polymorphic site has consequences on protein functionality, has been recently established in the frame of a FCT project (EXCL/BEX-GMG/0038/2012) under the consultancy of Dr. Anthony Moore group (University of Sussex/UK), and it is possible to investigate all polymorphisms located at the ORF. This methodology is based on gene expression in a yeast test system and allows the investigation of the effect of each single polymorphism in protein functionality by measuring AOX capacity/activity.

As a final step in FM development and previous to field trials for its validation, appropriate screening tools to identify the final trait need to be used. Several advanced tools are being developed for this (reviewed by Furbank and Tester 2011), including calorespirometry (Nogales et al. 2013) which was used in CHAPTER 3. This technology has recently been presented as a novel tool for efficient phenotype screening related to temperature responses and related to growth potentials (Nogales et al. 2013). Preliminary results point to its great potential on detecting functional *AOX* gene polymorphisms for molecular breeding in carrot (Arnholdt-Schmitt et al. 2015). Besides its application in plants, this methodology is under establishment for yeast. This will allow to focus in a specific polymorphism, independently of other polymorphisms that can occur simultaneously, and prevent the interference of the different gene family members.

8.4. References

Alonso-Blanco C, Mendez-Vigo B, Koornneef M (2005) From phenotypic to molecular polymorphisms involved in naturally occurring variation of plant development. Int. J. Dev. Biol. 49:717–732.

Andersen JR, Lübberstedt T (2003) Functional markers in plants. Trends Plant Sci. 8:554–560.

Arnholdt-Schmitt B (2015) From AOX diversity to functional marker development. In: Gupta KJ, Mur LAJ, Neelwarne B (eds) Alternative respiratory pathways in higher plants. John Wiley & Sons, Inc, Oxford.

Arnholdt-Schmitt B (2005) Functional markers and a “systemic strategy”: Convergency between plant breeding, plant nutrition and molecular biology. Plant Physiol Biochem 43:817–820. doi: 10.1016/j.plaphy.2005.08.011

Arnholdt-Schmitt B (2009) Alternative oxidase (AOX) and stress tolerance-approaching a scientific hypothesis. Physiol Plant 137:314–315. doi: 10.1111/j.1399-3054.2009.01311.x

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, Costa JH, de Melo DF (2006) AOX - a functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11:281–287. doi: 10.1016/j.tplants.2006.05.001

Arnholdt-Schmitt B, Hansen LD, Nogales A (2015) Calorespirometry, oxygen isotope analysis and functional-marker-assisted selection (’CalOxy-FMAS') for genotype screening: A novel concept and tool kit for predicting stable plant growth performance and functional marker identification. Brief Funct Genomics 1–6. doi: 10.1093/bfgp/elv008

Atteia A, Van Lis R, Van Hellemond JJ, et al (2004) Identification of prokaryotic homologues indicates an endosymbiotic origin for the alternative oxidases of mitochondria (AOX) and chloroplasts (PTOX). Gene 330:143–148. doi: 10.1016/j.gene.2004.01.015

Campos MD, Cardoso HG, Linke B, et al (2009) Differential expression and co-regulation of carrot *AOX* genes (*Daucus carota*). Physiol Plant 137:578–591. doi: 10.1111/j.1399-3054.2009.01282.x

Cardoso H, Doroteia Campos M, Nothnagel T, Arnholdt-Schmitt B (2011) Polymorphisms in intron 1 of carrot *AOX2b* – a useful tool to develop a functional marker? Plant Genet Resour 9:177–180. doi: 10.1017/S1479262111000591

Cardoso HG, Arnholdt-Schmitt B (2013) Functional marker development across species in selected traits. In: Diagnostics in Plant Breeding. pp 467–515.

Cardoso HG, Campos MD, Costa AR, et al (2009) Carrot alternative oxidase gene AOX2a demonstrates allelic and genotypic polymorphisms in intron 3. Physiol Plant 137:592–608. doi: 10.1111/j.1399-3054.2009.01299.x

Clifton R, Lister R, Parker KL, et al (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. Plant Mol Biol 58:193–212. doi: 10.1007/s11103-005-5514-7

Clifton R, Millar A, 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 1757:730–41. doi: 10.1016/j.bbabio.2006.03.009

Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? Plant Physiol 147:469–486. doi: 10.1104/pp.108.118117

Ferreira AO, Cardoso HG, MacEdo ES, et al (2009) Intron polymorphism pattern in *AOX1b* of wild St John's wort (*Hypericum perforatum*) allows discrimination between individual plants. Physiol Plant 137:520–531. doi: 10.1111/j.1399-3054.2009.01291.x

Fiorani F, Umbach AL, Siedow JN (2005) The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants. Plant Physiol 139:1795–1805. doi: 10.1104/pp.105.070789

Frederico AM, Campos MD, Cardoso HG, et al (2009) Alternative oxidase involvement in *Daucus carota* somatic embryogenesis. Physiol Plant 137:498–508. doi: 10.1111/j.1399-3054.2009.01278.x

Furbank RT, Tester M (2011) Phenomics : technologies to relieve the phenotyping bottleneck. Trends Plant Sci 16:635–644. doi: 10.1016/j.tplants.2011.09.005

Harada H, Kiyosue T, Kamada H, Kobayashi T (1990) Stress-induced carrot somatic embryogenesis and its application to synthetic seeds. In: Sangwan RS, Sangwan-Norreel R. (eds) The impact of Biotechnology in Agriculture. Kluwer Academic Publishers, The Neetherlands, pp 129–157.

Hilal M, Castagnaro A, Moreno H, Massa EM (1997) Specific localization of the respiratory alternative oxidase in meristematic and xylematic tissues from developing soybean roots and hypocotyls. Plant Physiol 115:1499–1503. doi: 10.1104/pp.115.4.1499

Ho LHM, Giraud E, Lister R, et al (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. doi: 10.1104/pp.106.091819

Hole CC, Morris GEL, Cowper A. (1987) Distribution of dry matter between shoot and storage root of field-grown carrots. III. Development of phloem and xylem parenchyma and cell numbers in the storage root. J Hortic Sci 62:351–358.

Hole CC, Thomas TH, McKee JMT (1984) Sink development and dry matter distribution in storage root crops. Plant Growth Regul 2:347–358.

Imani J, Tran Thi L, Langen G, et al (2001) Somatic embryogenesis and DNA organization of genomes from selected *Daucus* species. Plant Cell Rep 20:537–541. doi: 10.1007/s002990100363

Kikuchi A, Sanuki N, Higashi K, et al (2006) Abscisic acid and stress treatment are essential for the acquisition of embryogenic competence by carrot somatic cells. Planta 223:637–645. doi: 10.1007/s00425-005-0114-y

Kumar A, Bender L, Pauler B, et al (1983) Ultrastructural and biochemical development of the photosynthetic apparatus during callus induction in carrot root explants. Plant Cell Tissue Organ Cult 2:161–177. doi: 10.1007/BF00043361

Mathy G, Cardol P, Dinant M, et al (2010) Proteomic and functional characterization of a chlamydomonas reinhardtii mutant lacking the mitochondrial alternative oxidase. J Proteome Res 9:2825–2838. doi: 10.1021/pr900866e

Nogales A, Muñoz-Sanhueza L, Hansen LD, Arnholdt-Schmitt B (2014) Phenotyping carrot (Daucus carota L.) for yield-determining temperature response by calorespirometry. Planta 241:525–538. doi: 10.1007/s00425-014-2195-y

Nogales A, Muñoz-Sanhueza L, Hansen LD, Arnholdt-Schmitt B (2013) Calorespirometry as a tool for studying temperature response in carrot (*Daucus carota* L.). Eng Life Sci 13:541–548. doi: 10.1002/elsc.201200197

Plaxton WC, Podestá FE (2006) The functional organization and control of plant respiration. CRC Crit Rev Plant Sci 25:159–198. doi: 10.1080/07352680600563876

Polidoros AN, Mylona P V., Arnholdt-Schmitt B (2009) *Aox* gene structure, transcript variation and expression in plants. Physiol Plant 137:342–353. doi: 10.1111/j.1399-3054.2009.01284.x

Rasmusson AG, Fernie AR, Van Dongen JT (2009) Alternative oxidase: a defence against metabolic fluctuations? Physiol. Plant. 137:371–382.

Santos Macedo E, Cardoso HG, Hernández A, et al (2009) Physiologic 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. doi: 10.1111/j.1399-3054.2009.01302.x

Santos Macedo E, Sircar D, Cardoso HG, et al (2012) Involvement of alternative oxidase (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism. Plant Cell Rep 31:1581–1590. doi: 10.1007/s00299-012-1272-6

Sieger SM, Kristensen BK, Robson CA, et al (2005) The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. J Exp Bot 56:1499–1515. doi: 10.1093/jxb/eri146

Simkin AJ, Kuntz M, Moreau H, McCarthy J (2010) Carotenoid profiling and the expression of carotenoid biosynthetic genes in developing coffee grain. Plant Physiol Biochem 48:434–442. doi: 10.1016/j.plaphy.2010.02.007

Simkin AJ, Moreau H, Kuntz M, et al (2008) An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*. J Plant Physiol 165:1087–1106. doi: 10.1016/j.jplph.2007.06.016

Steward FC, Mapes MO, Smith J (1958) Growth and organized development of cultured cells. I. growth and division of freely suspended cells. Am. J. Bot. 45:693.

Sun X, Wen T (2011) Physiological roles of plastid terminal oxidase in plant stress responses. J. Biosci. 36:951–956.

Vanlerberghe GC (2013) Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J. Mol. Sci. 14:6805–6847.