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Alternative oxidase involvement in *Daucus carota* somatic embryogenesis

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Plant alternative oxidase (AOX) is a mitochondrial inner membrane enzyme involved in alternative respiration. The critical importance of the enzyme during acclimation upon stress of plant cells is not fully understood and is still an issue of intensive research and discussion. Recently, a role of AOX was suggested for the ability of plant cells to change easily its fate upon stress. In order to get new insights about AOX involvement in cell reprogramming, quantitative real-time polymerase chain reaction (PCR) and inhibitor studies were performed during cell redifferentiation and developmental stages of *Daucus carota* L. somatic embryogenesis. Transcript level analysis shows that *D. carota AOX* genes (*DcAOX1a* and *DcAOX2a*) are differentially expressed during somatic embryogenesis. *DcAOX1a* shows lower expression levels, being mainly down-regulated, whereas *DcAOX2a* presented a large up-regulation during initiation of the realization phase of somatic embryogenesis. However, when globular embryos start to develop, both genes are down-regulated, being this state transient for *DcAOX2a*. In addition, parallel studies were performed using salicylhydroxamic acid (SHAM) in order to inhibit AOX activity during the realization phase of somatic embryogenesis. Embryogenic cells growing in the presence of the inhibitor were unable to develop embryogenic structures and its growth rate was diminished. This effect was reversible and concentration dependent. The results obtained contribute to the hypothesis that AOX activity supports metabolic reorganization as an essential part of cell reprogramming and, thus, enables restructuring and de novo cell differentiation.

Introduction

Somatic embryogenesis (SE) is the most prominent example of cell reprogramming. *Daucus carota* L. has been the first species where totipotency through SE had been proven (see discussion in Costa et al. 2009, Raghavan 2006, Steward et al. 1958). In *D. carota*, SE is auxin induced and the realization of the induced program depends on the depletion of auxin in the medium (Grieb et al. 1997). Energetic or metabolic restrictions for the realization process can be expected to be as important as the induction itself for successful embryo formation. However, genotypic differences exist even in *D. carota* as a very 'easy to induce' plant

Abbreviations – 2,4-D, 2,4-dichlorophenoxyacetic acid; AOX, alternative oxidase; *AtAOX, Arabidopsis thaliana alternative oxidase; DcAOX, Daucus carota alternative oxidase*; DEPC, diethyl pyrocarbonate; DMSO, dimethyl sulfoxide; IBA, indole-3-butyric acid; MMLV-RT, moloney murine leukemia virus-reverse transcriptase; QTL, quantitative trait locus; qRT-PCR, quantitative real-time polymerase chain reaction; SE, somatic embryogenesis; SHAM, salicylhydroxamic acid; SNP, single nucleotide polymorphism.

species. Thus, a strong genetic control for the capacity of cells to form somatic embryos is given. All *D. carota* genotypes seem to be inducible, but differ in the timing of embryo formation (Arnholdt-Schmitt B, personal communication). Identification of markers for easy-embryo formation from *D. carota* is expected to help developing functional markers for efficient biotechnological propagation of recalcitrant genotypes.

Plants as sessile organisms can respond with high flexibility to environmental constraints and opportunities. This plasticity includes adaptive biochemical, physiological and morphological reactions and is related to high flexibility in metabolism (Foyer and Noctor 2009, McDonald and Vanlerberghe 2006, Plaxton and Podestá 2006) and adaptive reprogramming of gene activities (e.g. Clifton et al. 2006, Scheible et al. 2004). SE can be interpreted as a stress-induced morphogenic response (Fehér 2005, Kikuchi et al. 2006, Pasternak et al. 2002, Potters et al. 2007). Such reactions depend on biochemical sensors and networks for the integration of bioenergetics, signal transduction cascades and metabolism (Sweetlove et al. 2007, Zeng et al. 2007). Mitochondria are increasingly accepted to play a central role as a physical platform for networks, signal perception and signal canalization (Amirsadeghi et al. 2007, Fernie et al. 2004, Kohler and Villar 2008, Noctor et al. 2007, Raghavendra and Padmasree 2003, Rhoads and Subbaiah 2007, Sweetlove et al. 2007). The significance of mitochondria for cell fate decisions through dedifferentiation and de novo differentiation is recognized (Amirsadeghi et al. 2007, Sheahan et al. 2005). The alternative respiration pathway is localized in mitochondria and is increasingly getting into the focus of research on stress acclimation and adaptation. Clifton et al. (2005, 2006) pointed to the importance of this pathway as an early sensoring system for cell programming. AOX is the critical component in the alternative pathway that transfers electrons from reduced ubiquinone directly to oxygen. The enzyme is related to all types of abiotic and biotic stress and known to be involved in growth responses and development (Giraud et al. 2008, Ho et al. 2007, Sieger et al. 2005, Sugie et al. 2007, Umbach et al. 2005). *AOX* is proposed as a functional marker candidate for efficient cell reprogramming upon stress (Arnholdt-Schmitt et al. 2006, see also: www.aox2008.uevora.pt). Induced single nucleotide polymorphism (SNP) was shown to affect a quantitative trait locus (QTL) for thermo tolerance in *Oryza sativa* L. (Abe et al. 2002). *AOX* genes demonstrate a rich natural source for polymorphic within-gene sequences in various species that might be explored in the future for functional marker candidates (Cardoso et al. 2009, Ferreira et al. 2009, Holtzapffel et al. 2003, Macedo et al. 2009).

Recently, *AOX* genes have been isolated from *D. carota* (Campos et al. 2009, Cardoso et al. 2009, Costa et al. 2009). The *D. carota AOX* (*DcAOX*) multigene family consists of two genes from the subfamily *AOX1* and two genes from the subfamily *AOX2*. All four genes were expressed in various tissues and during development and growth. However, both genes of each subfamily demonstrated differential activities among each other. Typically, higher transcript accumulation and responsiveness were found for the two genes *DcAOX1a* and *DcAOX2a* and co-regulation of both was demonstrated during de novo growth induction (Campos et al. 2009).

The paper presents indications for the importance of AOX activity in SE and the specific involvement of *DcAOX* genes during realization. *DcAOX2a* is outlined as a promising gene candidate for functional marker development related to the easy-embryo formation of *D. carota*.

Material and methods

Plant material

Embryogenic cell line selection

Seeds from *D. carota* cv. Rotin were surface desinfected for 5 min with ethanol at 75% (v/v), followed by an immersion in a solution at 20% (v/v) commercial blench for 20 min and subsequently washed twice with sterilized bidistilled water. Seeds were placed in Petri dishes ($Ø = 9$ cm) onto solidified B_5^+ culture medium (modified from Gamborg et al. 1968, see in Grieb et al. 1997) supplemented with 2.3 µ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) in order to induce embryogenic calli development (induction phase).

Selected embryogenic calli were isolated from the original seed material and propagated individually as cell lines. These stock cell lines were maintained in the dedifferentiated state in the same B_5^+ medium as it was used for embryogenic calli induction and subcultured every 2 weeks. The cultures were maintained at 25°C and under a 16 h photoperiod (approximately 95–100 µmol m^{-2} s^{-1} of light intensity provided by fluorescent lamps; Philips, Amsterdam, The Netherlands). To select the cell line with the highest efficiency for the development of somatic embryos, the obtained lines were subcultured on solid B_5^- medium under the same conditions (realization phase). B_5^- contains the same basal components as B_5^+, but lacks 2,4-D. Selection parameters were: required time to develop somatic embryos and somatic embryos quality (normal and abnormal development). The cell line L5.S.R. showed the highest rank related to rapid induction and high

degrees of normal development and was selected to study AOX activities involvement in *D. carota* SE during the realization phase in cell suspension cultures.

L5.S.R. suspension culture and realization experiments

The cell line L5.S.R. was used to initiate cell suspension cultures in Erlenmeyer flasks containing 33% (v/v) B_5^+ liquid culture medium supplemented with 0.9 µ*M* 2,4-D. Subcultures were made every 10 days. Cell diameter homogeneity was maintained through periodic sieving at subculture, using 95 µm mesh pore polyester precision woven screens (Sefar Petex, Thal, Switzerland). The embryogenic competence was evaluated periodically as described above. The cultures were kept at 28°C, continuous light (approximately 95–100 µmol m^{-2} s^{-1} provided by fluorescent lamps; Philips, Amsterdam, The Netherlands) and 90–95 rpm on an orbital shaker (SM-30, Edmund Buhler, Hechingen, Germany).

For realization experiments, the L5.S.R. cell line was taken during the exponential growth phase, between 4 and 6 days after subculture and sieved to achieve homogeneity (see Fig. 1). Flow-through cells were washed twice by centrifugation at 300 G with fresh B_5^- medium. Experiments were performed by using equal packed cell volumes of 0.5% (v/v) in Erlenmeyer flasks filled with 33% (v/v) B_5^- culture medium at the physical conditions referred above.

To investigate the influence of the AOX inhibitor salicylhydroxamic acid (SHAM) on cell growth rates and the ability of embryogenic cells to develop embryogenic structures, a realization experiment was performed during 12 days. Seven repetitions were performed: three were used to evaluate the development of embryogenic structures (4th, 8th and 12th day after inoculation) and four were taken to determine dry weights at the 12th day after inoculation.

To quantify *AOX* transcript levels during the realization process, 12 samples were collected during a 10 days' time course experiment (see Fig. 1). Cells and/or embryos were collected through sieving with 54 µm mesh pore polyester precision woven screens (Sefar Petex, Thal, Switzerland), immediately disrupted in liquid nitrogen and stored at −80°C until RNA extraction.

The use of SHAM in the AOX inhibition studies

A 500 m*M* SHAM (Aldrich, St. Louis, MO, USA) stock solution was prepared freshly. Dimethyl sulfoxide (DMSO) (Fluka BioChemika, St. Louis, MO, USA) (50% v/v) was the primary solvent and bidistilled water was added (50% v/v). The pH of the SHAM stock solution was adjusted to culture medium pH (5.72) and sterilized by filtration using a 0.22 µm mesh pore filter (Sarstedt, Nümbrecht, Germany).

SHAM concentrations were optimized by considering its effect on cell growth and embryo development. The final SHAM concentrations tested were: 0.2 m*M*, 0.4 m*M*, 0.6 m*M*, 0.8 m*M*, 1 m*M* and 1.5 m*M*. The control treatment was performed without SHAM supplementation.

To exclude any negative effect of the solvent DMSO as it was observed in other cell culture systems (Randhawa 2008, Rodríguez-Burford et al. 2003, Wang et al. 2002), parallel realization experiments were performed by supplementing suspension cultures only with DMSO.

Hydroxamic acids such as SHAM are able to chelate ferric iron (Rich et al. 1978) and browning was observed in the *D. carota* suspension cultures after SHAM application. Therefore, control experiments without iron in the medium were conducted to exclude the possibility of negative effects by iron depletion during the realization experiments.

AOX qRT-PCR studies

RNA extraction and cDNA synthesis

Total RNA was extracted by using the RNeasy® plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. After DNase I (Fermentas, Ontario, Canada) treatment, RNA integrity was analyzed on 1.4% DEPC (diethyl pyrocarbonate) (Sigma, St. Louis, MO, USA) agarose gel and total RNA was quantified in a spectrophotometer (DU 530, Beckman Coulter Inc., Fullerton, CA, USA) using a 50 µl microcell. For first-strand cDNA synthesis, 2 µg of DNA-free RNA was used with the moloney murine leukemia virus-reverse transcriptase (MMLV-RT) and oligo $(dT)_{18}$ primer according the RETROscript® kit (Ambion, Austin, TX, USA) manufacturer's instruction.

qRT-PCR primers design

For *AOX* transcript quantification by qRT-PCR, specific primers were designed. The forward (5′-CTTCAACGCCTACTTCCTTG) and the reverse primer (5′-ATCTCGCAATGTAGAGTCAGC) were designed for the amplification of a 196 bp size fragment of *DcAOX1a* (EU286573). For the amplification of the *DcAOX2a* (EU286575) 200 bp size fragment, the forward (5′-TCTTCAATGCTTTCTTTGTTCTT) and the reverse primer (5′-GACATCTTTTAGTTTGGCATCTTT) were applied. As housekeeping, the *D. carota ubiquitin-carboxyl extension* gene (U68751) was used. The specific primers to amplify a 152 bp size fragment for this gene were:

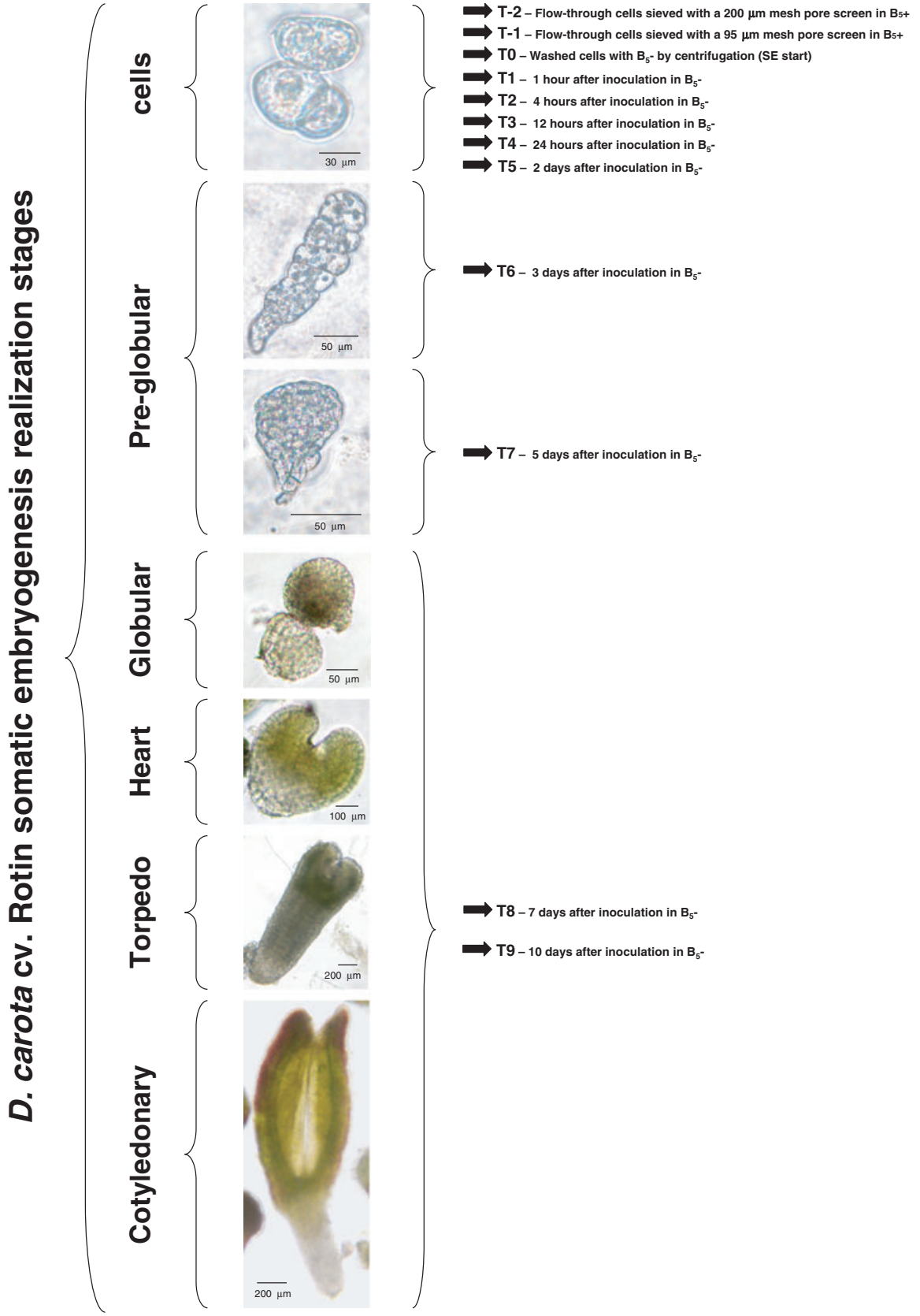

Fig. 1. Legend on next page

forward (5′-AAGCCCAAGAAGATCAAGCA) and reverse (5′-TCAAAATGATTGGCCATGAA).

qRT-PCR conditions

qRT-PCR was performed in a Mx3000P® qPCR system (Stratagene, La Jolla, CA, USA). About 15 µl PCR was applied using 10 ng of cDNA and SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, St. Louis, MO, USA) in 96-well plates. Specific primers were adjusted to the final concentration of 166 n*M*. An initial step at 95°C during 7 min for initial template denaturation was run followed by 40 cycles consisting each one in 95°C during 30 s for denaturation; 60, 52 or 51°C during 30 s for primers annealing (*DcAOX1a*, *DcAOX2a* and *ubiquitin*, respectively); and finally the extension step at 72°C during 30 s. To analyze dissociation curve profiles, an additional step at 95°C during 1 min was added, followed by a constant increase of the temperature between 60 and 95°C. For each sample, PCRs were ran in triplicate.

Data analyses

The statistical analyses and graphics applied to calculate the effect of SHAM on growth rates were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Growth of cells and embryo structures was measured as dry weights. Dry weights are presented in a boxplot analysis from four repetitions of each applied treatment including the control. Maximum and minimum dry weights, the median and the 50% quartile were the statistical parameters used to illustrate the results.

Somatic embryo stages were named in accordance with Yeung (1995). Images representing the cells and embryo developmental stages (see Fig. 1 and 3) were taken using a digital camera (PowerShot A630, Canon Inc., Tokyo, Japan). The images representing cells or embryos were selected as representing the most common developmental stage observed in three samples collected from different suspension cultures of each treatment or experiment using an inverted microscope (CK40, Olympus, Tokyo, Japan). The effect of SHAM on embryo structure development was evaluated by the presence or absence of embryo structures.

Statistical calculations associated with the $2^{-\Delta\Delta C_T}$ methodology (Livak and Schmittgen 2001) for qRT-PCR were applied for the triplicated C_T values obtained for each sample using the freeware qCalculator version 1.0 (freely available at: www.gene-quantification.de/qCalculator.zip). The sample collected at T-2 was selected as the calibrator. The results are presented as the average of the triplicated C_T values from each sample. Standard deviation was residual among the sample triplicates.

Results

The effect of SHAM on the realization of SE

The effect of AOX pathway inhibition on SE was studied by applying SHAM permanently to an embryogenic cell line during the realization phase of the embryogenic programme induced by auxin. Fig. 2 demonstrates the dry weights of the embryogenic cells and structures achieved after 12 days with and without SHAM treatments. Under the control treatment, the dry weights were in average 9.6 times higher (approximately 106 mg) than for the initially inoculated cells (approximately 11 mg) (data not shown). When the culture medium was supplemented with SHAM, the highest value of the dry weight at the 12th day was always lower than in the control treatment. At the three lowest SHAM concentrations tested (0.2 m*M*, 0.4 m*M* and 0.6 m*M*), the variation in dry weight between the four repetitions was higher in comparison with the three upper concentrations (0.8 m*M*, 1 m*M* and 1.5 m*M*). From the six SHAM concentrations tested, the highest dry weight variation was found at 0.6 m*M*, ranging from 32 to 86 mg dry weight (Fig. 2). However, the lowest dry weight variation was detected at 1.5 m*M* SHAM, ranging from 16 mg dry weight to 23 mg.

In Fig. 3, the development of embryo structures at the 4th, 8th and 12th day of the experiment is shown at different concentrations of SHAM. When SHAM was supplied at the lower concentrations of 0.2 and 0.4 m*M*, a small delay in the appearance of embryogenic structures (globular and heart shaped embryos) was observed at the fourth and eighth day in relation to the control treatment. However, the ability to develop embryogenic structures was not suppressed. At the 12th day, it was not possible to detect by the applied methodology any difference among these two SHAM-treated suspension cultures and the control in relation to the development of embryogenic structures. At concentrations higher than 0.6 m*M*, the ability of the

Fig. 1. Experimental design to study *AOX* transcript accumulation during SE realization (*Daucus carota* L. cv. Rotin, cell line L5.S.R.). SE developmental stages are schematically presented in relation to the sampling times for qRT-PCR analyses. The images are showing the most advanced stages of development in the suspension culture at the time of sampling. Samples T-2 and T-1 were harvested from B_5^+ medium. All other samples were taken from B_5^- medium. T0 represents the beginning of the SE realization phase.

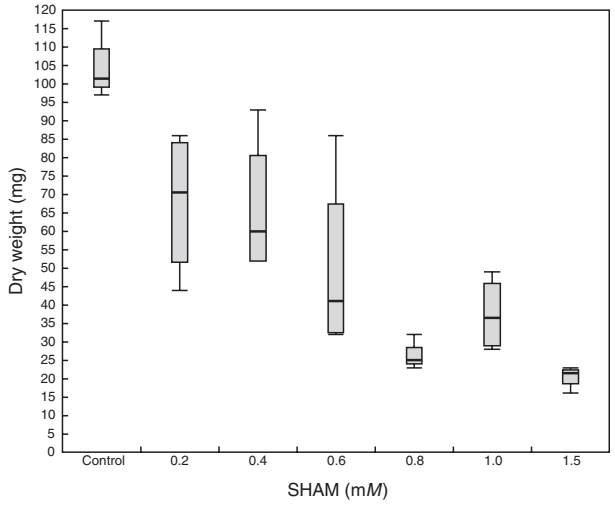

Fig. 2. The effect of SHAM on dry weights of cells and embryogenic structures during SE realization *(Daucus carota* L. cv. Rotin, cell line L5.S.R.). Cells were inoculated in liquid B_5^- medium supplemented with different SHAM concentrations ranging from 0.2 to 1.5 m*M*. The dry weight of cells and embryos were measured after 12 days in the SE realization medium. A boxplot analysis was performed for four repetitions.

embryogenic cells to form embryogenic structures was completely suppressed. No globular-shaped structures were visible in all three repetitions of all treatments.

SHAM-treated cells that are suppressed in their development to form embryogenic structures at higher concentrations do not lose permanently this ability. If subcultured in B_5^- medium without SHAM they can recover and develop embryogenic structures (data not shown).

When applied alone DMSO, at the applied volumes, did not have any measurable or visible effect on cell growth rates or the ability to develop embryogenic structures. Also, the lack of medium iron did not affect normal suspension culture growth or embryo structure formation during the realization phase (data not shown). Globular, heart and torpedo-shaped embryos developed as in untreated iron-supplemented suspension cultures (data not shown).

Transcription of DcAOX1a and DcAOX2a during the realization phase

Figure 4 shows the result of qRT-PCR analysis of *DcAOX1a* and *DcAOX2a* expression during the realization phase of SE. The accumulation of transcripts is given in reference to the initial level for each gene (see T-2 in Fig. 1). The two genes were differentially expressed. The level of transcript accumulation of *DcAOX1a* was lower during the realization of the embryogenic program than at the beginning of the experiment (Fig. 4A), whereas *DcAOX2a* was up-regulated and remained high during the experiment in comparison with the initial level at the transfer to B_5^- medium (Fig. 4B).

Sieving had no direct effect on *DcAOX1a* transcript levels. However, a one-fold increase was observed when 2,4-D was removed from the culture medium and the cells were centrifuged (T0, Fig. 4A). This minimal change remained visible also 1 h later (T1, Fig. 4A). *DcAOX2a* transcripts accumulated the same amount of transcripts as *DcAOX1a* already at sieving (T-1, one-fold change, Fig. 4) and remained at almost the same level during centrifugation (T0, 1.5-fold, Fig. 4B).

Four hours after inoculation in B_5^- medium, *DcAOX1a* transcript accumulation level decreased largely (> 2.5-fold) (T2, Fig. 4A). *DcAOX2a* decreased also, but only slightly and earlier at 1 h after inoculation (T1, Fig. 4B). The clear difference in regulation between the two genes starts to become obvious 4 h after inoculation (T2, Fig. 4A, B). *DcAOX2a* showed a 2.5-fold up-regulation in comparison with the experimental start. Contrarily, *DcAOX1a* is clearly down-regulated (approximately 1.5-fold) at that stage. Both genes show a slightly increased transcript accumulation between 24 and 48 h and a down-regulation at 72 h (T6, Fig. 4A and B) after inoculation, which is especially pronounced for *DcAOX2a* (two-fold). This stage is characterized by the appearance of globular structures (see T6, Fig. 1). Down-regulation of *DcAOX1a* resulted in a level of transcripts that remained more or less stable during the following periods until the end of the experiment. The large decrease in transcripts at T6 observed for *DcAOX2a* was transient with a subsequent up-regulation during the following 5–10 days (up to 2.5-fold at day 7).

Discussion

Campos et al. (2009) reported that both *DcAOX1a* and *DcAOX2a* showed the highest level of transcript accumulation in tissues and organs and had been co-regulated during the first days of de novo growth induction of secondary phloem tissues from *D. carota* tap roots. However, co-regulation seems to be system- and development dependent. Here, we present the expression of these two genes in another biological system during the realization of SE from auxin-induced embryogenic cells. The initiation of somatic embryo formation from auxin-induced embryogenic cells is accompanied by important differential *AOX* transcription of both genes. However, the two genes revealed also phases of co-regulation. Similar regulation was observed at critical points for transitions such as directly when auxin is removed from the medium as well as when globular

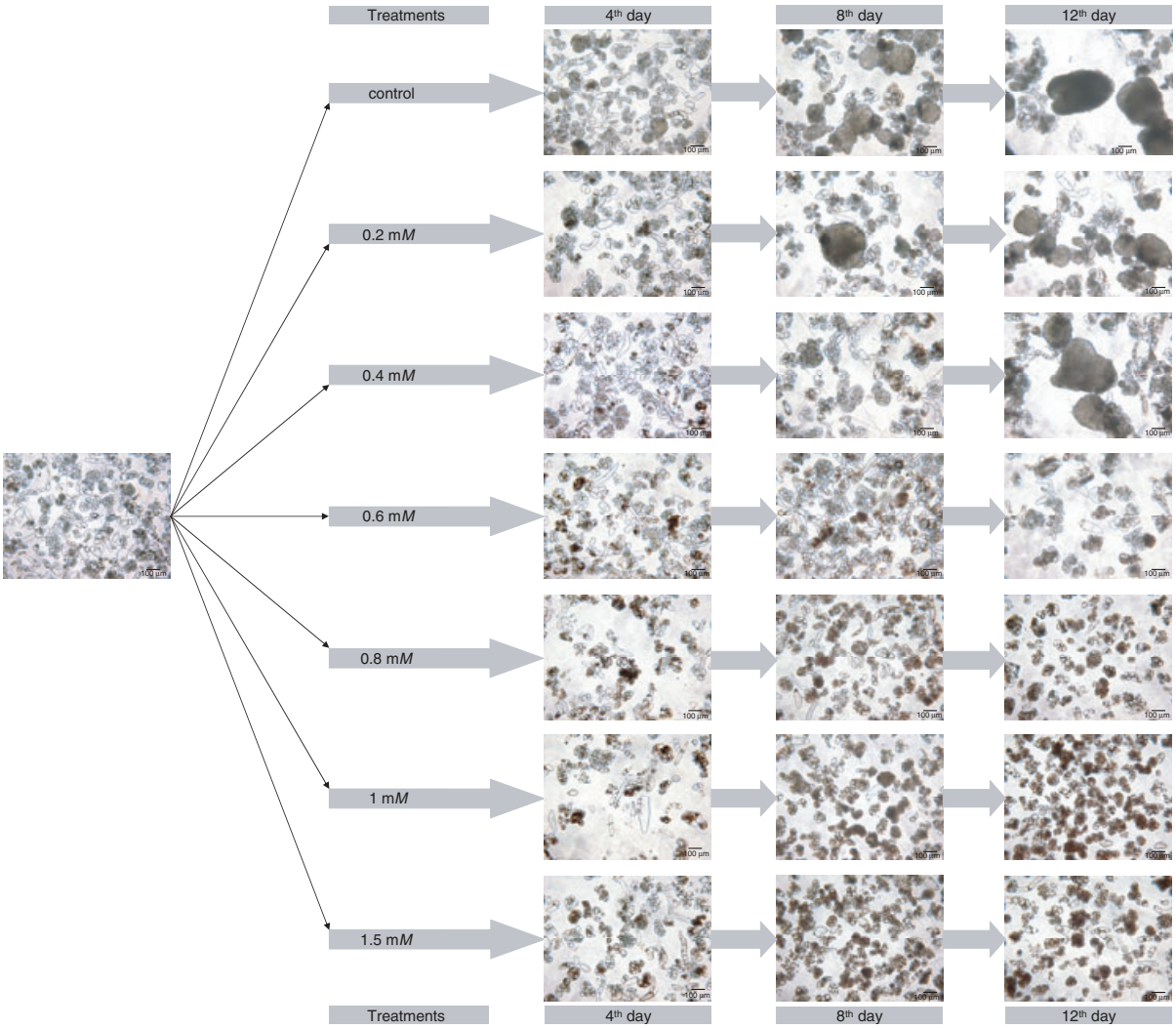

Fig. 3. The effect of SHAM on embryo structure formation during SE realization *(Daucus carota* L. cv. Rotin, cell line L5.S.R.). The figure shows representative photos taken at various times during the SE realization phase of cell line L5.S.R. in B_5^- medium: initial cell suspension before inoculation into B_5^- medium (left side); cells and embryogenic structures at the 4th, 8th and 12th day, during SE realization in the control treatment without SHAM and at increasing supplementations of SHAM from 0.2 to 1.5 m*M*.

structures start to be formed. Differential regulation was especially obvious during the first hours of acclimation to the auxin-depleted medium. The transcript level of *DcAOX2a* was 2.5-fold up-regulated and *DcAOX1a* was around 1.5-fold down-regulated in comparison with the initial transcript levels of each gene. Interestingly, the down-regulation of *DcAOX1a* and the up-regulation of *DcAOX2a* between the 5th and the 10th days of embryo formation are ranging both around a two-fold change in transcript accumulation, but in the opposite direction.

Up-regulation of both genes upon sieving and the depletion of auxin can be interpreted as a general stress response. It shows the immediate reaction upon changes of the environmental conditions. However, acclimation to auxin-depletion during the very first hours seems to be related to a more specific response of both *AOX* genes. Campos et al. (2009) studied *AOX* transcript levels starting from 1.5 days after de novo growth induction and found co-regulation of both genes. However, the main point where differential expression between both genes seems to play a role during embryo formation is already earlier during the first 12 h. During that time, the 2.5-fold up-regulated and 1.5-fold down-regulated transcript levels from both genes together count in the balance for an overall up-regulation of *AOX* transcripts (one-fold). This observation can be important in view of the current discussion about the effect of transcript abundance variability as a response upon environmental

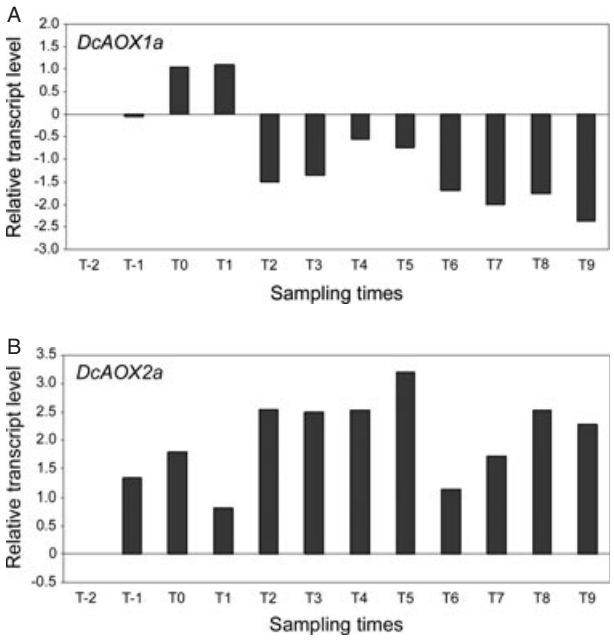

Fig. 4. Relative transcript levels of *DcAOX1a* and *DcAOX2a* during SE realization *(Daucus carota* L. cv. Rotin, cell line L5.S.R.). Relative transcript levels were measured during the first 10 days in B_5^- medium. *AOX* transcript levels were normalized by the endogenous control (*ubiquitin*) and T-2 was selected as calibrator. (A) Relative transcript levels of *DcAOX1a*. (B) Relative transcript levels of *DcAOX2a*.

changes. To understand the significance of AOX, it can be critical to consider the expression of all *AOX* genes at the same time. In a primary culture system of secondary phloem explants of *D. carota* tap roots, *DcAOX1a* and *DcAOX2a* were both responsive and co-regulated, whereas *DcAOX1b* and *DcAOX2b* did not respond during de novo growth induction (Campos et al. 2009). In this context, the effect of down- and up-regulation of the two genes *DcAOX1a* and *DcAOX2a* at the same time and to the same degree during the final phases of embryo formation may indicate that the overall AOX activity was unchanged in relation to the initial activities. This interpretation suggests that AOX activities can be substituted by other *AOX* genes. At this final culture stages, embryo structure development was already initiated and development took place. The observation is of interest as a basis for further studies in this system to understand the relationship between *AOX* gene expression or transcript abundance and AOX activity. Oxygen isotope fractionation is currently the only available method to study in vivo electron partitioning in plant respiration and the method would need to be adapted to the system (Ribas-Carbo et al. 2005). However, SHAM application was highly effective to suppress cell growth and embryo formation. This should already indicate a role for AOX activity for the developmental process. However, this does not mean that other genes and especially those related to respiration cannot also be involved. The inhibiting effect of SHAM was concentration dependent and inhibition could be reverted by a subculture to SHAM-free medium. The cells did not loose their ability to form embryos. The importance of a critical early peak of AOX activity for the realization of a new program gets confirmation from experiments with *Olea europaea* L. root initiation (Macedo et al. 2009). *O. europaea* root development can be induced by indole-3-butyric acid (IBA). However, the degree of rooting could be reduced to the control level with almost no rooting when SHAM was applied only for 20 s during the auxin treatment.

Clifton et al. (2005) analyzed the stress response of plant cells from *Arabidopsis thaliana* L. after 3 h and up to 24 h and identified alternative respiration pathway components as most important 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. The authors have interpreted their data from qRT-PCR as large effects, when the transcript levels varied from the untreated levels by two-fold and as dramatic changes, when it was around five-fold. Obviously, the somatic embryo formation is related to large, but not dramatic effects of *AOX* transcript level changes. Differential expression between *A. thaliana AOX* (*AtAOX*) members was highlighted. However, *AtAOX1a* and *AtAOX2* exhibited both dramatic increases after stress treatments (Clifton et al. 2005). *AtAOX1a* peaked earlier than *AtAOX2* after 3 h and either decreased or remained at a level of transcript abundance. *AtAOX1a* and *AtAOX1d* are among the most responsive genes in *A. thaliana* upon stress. *AtAOX1a* and *AtAOX1c* transcripts were the predominant transcripts in cell and callus cultures (Clifton et al. 2006). *AtAOX2* was reported to peak around 12 h on some stress treatments, but responded typically by a decrease in transcript accumulation upon the applied stress-inducers. Overall, a comparison of the results confirms the current view that *AOX* gene expression pattern need to be studied at species and also at plant systems level.

Conclusion

This study was conducted to reveal whether AOX can be involved in the realization of programs for embryo formation in *D. carota*. The observed results are sufficient to confirm the involvement. In view of functional marker development for defined stress behavior they are encouraging to study in future experiments whether

within-gene polymorphisms can be related to changes in differential gene regulation and expression patterns that can influence the efficiency of the realization of embryo formation. *DcAOX2a* can be highlighted as a promising gene marker candidate. The genomic sequence of *DcAOX2a* is already known (Campos et al. 2009, Cardoso et al. 2009) and important polymorphisms in intron 3 have been indicated. Interestingly, Cardoso et al. (2009) predicted a pre-miRNA site in the polymorphic sequence in intron 3 that seems to be related to embryogenesis. Studies are in progress to validate this site as a functional marker candidate in the presented system. In addition, *DcAOX2a* was mapped to linkage group 7 of the segregating population DM19 and will be explored for a correlation to other markers present in the same linkage group (Cardoso et al. 2009).

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