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## Alternative oxidase (AOX) and phenolic metabolism in methyl jasmonate-treated hairy root cultures of *Daucus carota* L.

Debabrata Sircar<sup>a,\*</sup>, Hélia G. Cardoso<sup>a</sup>, Chiranjit Mukherjee<sup>b</sup>, Adinpunya Mitra<sup>b</sup>, Birgit Arnholdt-Schmitt<sup>a</sup>

<sup>a</sup> EU Marie Curie Chair, ICAAM, University of Évora, 7002-554 Évora, Portugal

<sup>b</sup> Agricultural and Food Engineering Department, Natural Product Biotechnology Group, Indian Institute of Technology Kharagpur, Kharagpur 721 302, India

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### ABSTRACT

Methyl-jasmonate (MJ)-treated hairy roots of *Daucus carota* L. were used to study the influence of alternative oxidase (AOX) in phenylpropanoid metabolism. Phenolic acid accumulation, as well as total flavonoids and lignin content of the MJ-treated hairy roots were decreased by treatment with salicylhydroxamic acid (SHAM), a known inhibitor of AOX. The inhibitory effect of SHAM was concentration dependent. Treatment with propyl gallate (PG), another inhibitor of AOX, also had a similar inhibitory effect on accumulation of phenolic acid, total flavonoids and lignin. The transcript levels of two *DcAOX* genes (*DcAOX2a* and *DcAOX1a*) were monitored at selected post-elicitation time points. A notable rise in the transcript levels of both *DcAOX* genes was observed preceding the MJ-induced enhanced accumulation of phenolics, flavonoids and lignin. An appreciable increase in phenylalanine ammonia-lyase (PAL) transcript level was also observed prior to enhanced phenolics accumulation. Both *DcAOX* genes showed differential transcript accumulation patterns after the onset of elicitation. The transcript levels of *DcAOX1a* and *DcAOX2a* attained peak at 6 hours post elicitation (hpe) and 12 hpe, respectively. An increase in the transcript levels of both *DcAOX* genes preceding the accumulation of phenylpropanoid-derivatives and lignin showed a positive correlation between AOX activity and phenylpropanoid biosynthesis. The results provide important new insight about the influence of AOX in phenylpropanoid biosynthesis.

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### Introduction

In plant mitochondria, the alternative respiratory pathway branches from the mitochondrial electron transport chain at the level of ubiquinone and bypasses electrons to an alternative oxidase (AOX) (Costa et al., 2010). AOX is a cyanide insensitive non-proton motive ubiquinol oxidase that directly transfers electrons from reduced ubiquinone to oxygen without generation of ATP (Albury et al., 2010; Carre et al., 2011). The energy passed to AOX is dissipated as heat (Gara et al., 2010), which helps in the reproductive process in certain plant species such as *Sauromatum guttatum* (Raskin et al., 1989). In addition to the general function of AOX in energy dissipation as heat, this enzyme is suggested to play a key role in stress adaptation (Arnholdt-Schmitt et al., 2006; Clifton et al., 2006). Involvement of AOX in regulating the oxygen

homeostasis within mitochondria is well documented, especially under stress conditions, when the production rate of reactive oxygen species (ROS) is increased by uncontrolled electron transfer to oxygen (Umbach et al., 2005; Blokhina and Fagerstedt, 2010). Additionally, in many plant species, a massive increase in AOX gene expression has been observed in response to various stress stimuli or stress inducing exogenous treatment (Lehmann et al., 2009; Vanlerberghe et al., 2009; Gara et al., 2010).

Several lines of evidence implicate a role of AOX in controlling cell growth, development and reprogramming (Clifton et al., 2005; Arnholdt-Schmitt et al., 2006). Treatment with salicylhydroxamic acid (SHAM), a known AOX inhibitor, can suppress various cell reprogramming processes, such as root initiation in *Olea europaea* (Macedo et al., 2009), and in *Helianthus tuberosus* (Hase, 1987) and somatic embryogenesis in *Daucus carota* (Frederico et al., 2009). It is presumed that AOX play a key role in regulating cell metabolism to changing external conditions (Arnholdt-Schmitt et al., 2006). However, the exact physiological and molecular details of such metabolic regulation remain largely unknown and represent an area of considerable research interest.

Phenolic acids, flavonoids and lignin play crucial roles in plant defense and stress tolerance (Dixon and Paiva, 1995). Phenolic acid derivatives can mimic the effects of cytokinins by regulating cell

Abbreviations: AOX, alternative oxidase; HPLC, high performance liquid chromatography; hpe, hours post elicitation; MJ, methyl-jasmonate; PAL, phenylalanine ammonia-lyase; PG, propyl gallate; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; SQ-RT-PCR, semi-quantitative reverse-transcription-PCR.

\* Corresponding author. Tel.: +351 926680372/+91 9332375828.

E-mail address: [dsircar.iitkgp@gmail.com](mailto:dsircar.iitkgp@gmail.com) (D. Sircar).

division and differentiation (Tamagnone et al., 1998). Biosynthesis of phenolic acids and lignin occurs through a series of reactions from intermediate metabolism of the shikimate pathway through the common phenylpropanoid pathway (Boudet et al., 1995). Phenylalanine ammonia-lyase (PAL) is the first key enzyme of the phenylpropanoid pathway, whose increase activity is known to be associated with enhanced phenylpropanoid biosynthesis (Olsen et al., 2008). A substantial allotment of carbon energy and reducing power is required for the operation of phenylpropanoid pathway (Booker and Miller, 1998). The potential of AOX to affect redox and energy metabolism may serve to balance energy change and constant growth under a variety of changing environments (Moore et al., 2002). Inhibitors of both the phenylpropanoid pathway and AOX are known to suppress cell division and differentiation processes (Cvikrová et al., 2003; Frederico et al., 2009; Palama et al., 2010), which suggests a positive association between AOX and phenylpropanoid biosynthesis.

In this study, we select elicited hairy roots of *D. carota* to gain new insight into the regulatory role of AOX in adaptive phenolic biosynthesis upon environmental changes. In addition to eliciting secondary metabolism in plants cultivated *in vitro* (Xiao et al., 2009; Sircar et al., 2011), methyl jasmonate (MJ) treatment also induces AOX gene expression in many plants (Fung et al., 2004, 2006). In *D. carota* hairy roots, phenylpropanoid metabolism was found to be increased with MJ treatment (Sircar et al., 2011). Further, the AOX gene family of *D. carota* is well explored (Costa et al., 2009; Cardoso et al., 2009, 2011), and the development-associated differential expression of both *DcAOX1a* and *DcAOX2a* genes has also been demonstrated (Campos et al., 2009). During the realization phase of carrot somatic embryogenesis, *DcAOX2a* was up-regulated (Frederico et al., 2009). *DcAOX2a* is polymorphic and is proposed as a candidate for functional marker identification linked to cell reprogramming under changing environmental conditions (Arnholdt-Schmitt et al., 2006; Cardoso et al., 2009). Elicitation and subsequent inhibition of a particular target enzyme may help to identify the regulatory role of the enzyme in the formation of target metabolites (Hartmann et al., 1988; Sircar and Mitra, 2009). In this paper, we report the role of AOX in the biosynthesis of phenylpropanoid-derivatives and lignin using MJ-treated hairy roots of *D. carota*. Two known inhibitors of AOX, SHAM and propyl gallate (PG) were fed in *D. carota* elicited hairy roots to find out any possible relationship between AOX activity and phenylpropanoid biosynthesis.

## Materials and methods

### Plant material

Hairy roots of *Daucus carota* were *in vitro* developed and maintained as described previously (Sircar and Mitra, 2008). Hairy root cultures growing in 100 mL Erlemyers provided with liquid 40 mL Gamborg's medium (Gamborg et al., 1968) were used for elicitation experiments and subsequent AOX inhibitor studies.

### Experimental design and data analyses

MJ, used as elicitor in this experiment, was diluted in ethanol prior to use. Hairy root cultures growing in the linear growth phase (15-day-old) were treated with MJ at 100  $\mu$ M, chosen as the most appropriate concentration for maximum phenolic accumulation (Sircar et al., 2011). Control roots were treated with equal concentrations of ethanol used to perform the MJ solution. Changes in phenolic acid content and total flavonoid content were monitored at defined post-elicitation time points: 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36 and 42 hours post elicitation (hpe). Lignin content was

monitored at 0, 6, 12, 18, 24, 30, 36, 42 and 48 hpe. Two to three biological repetitions were performed for each experiment

To study the involvement of AOX in phenylpropanoid metabolism, AOX inhibition studies were carried out using SHAM and PG, two well-known inhibitors of AOX. SHAM (Aldrich, St. Louis, MO, USA) stock solution was prepared in dimethyl sulfoxide (Fluka BioChemika, St. Louis, MO, USA) essentially as described by Frederico et al. (2009). From stock solution, different concentrations (0.1 mM, 0.2 mM, 0.4 mM, 0.5 mM and 0.8 mM) of SHAM were added to the liquid culture medium in conjugation with MJ (100  $\mu$ M). PG (Aldrich, St. Louis, MO, USA) stock solution was prepared in ethanol and used in two different concentrations (0.05 mM and 0.1 mM) together with MJ (100  $\mu$ M). Non-elicited control cultures were treated with equal volumes of solvents. Inhibitor-supplemented elicited hairy roots were harvested after 30 hpe and 36 hpe, respectively, and analyzed by high performance liquid chromatography (HPLC) for determination of soluble and wall-bound phenolic content, respectively. For analyses of flavonoids and lignin, inhibitor-treated hairy roots were harvested at 18 hpe and 42 hpe, respectively. Hairy roots treated with only MJ served as controls.

### Analyses of phenolics, flavonoids and lignin content

Soluble and cell wall bound phenolic compounds were extracted from elicited hairy roots as described by Sircar et al. (2007). Phenolic acid contents were analyzed by HPLC using a Waters Symmetry<sup>TM</sup> C<sub>18</sub> column, 75 mm  $\times$  4.6 mm  $\times$  3.5  $\mu$ m particle size (Waters, Milford, MA, USA). An isocratic linear solvent system of aqueous trifluoroacetic acid (68%) and methanol (32%) with a flow rate of 1 mL/min for 20 min was used to elute the phenolic compounds, as described previously (Sachan et al., 2004; Sircar et al., 2007). The extraction and analyses of total flavonoids followed the methods reported by Wang et al. (2008). Total flavonoid content was expressed as quercetin equivalent. Lignin content was estimated spectrophotometrically as thioglycolic acid (TGA) derivative. Lignin extraction and the estimation processes were similar to those described by Sircar and Mitra (2009).

### Assay of phenylalanine ammonia-lyase (PAL)

Cell-free extracts for the PAL assay were prepared exactly as described by Sircar and Mitra (2008). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (BSA) as a protein standard. PAL activity was assayed spectrophotometrically as described by Sung et al. (2005).

### Transcript quantification by semi-quantitative RT-PCR

Hairy root tissues at different post-elicited time points (0, 1, 2, 3, 6, 9, 12, 15, 18 and 24 hpe) were used for total RNA extraction by using the RNeasy<sup>®</sup> plant mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. To eliminate contaminating genomic DNA, DNase I (1 units/ $\mu$ L; Fermentas) treatment was performed as recommended by the manufacturer. Total RNA was eluted 4-times with the same 25–30  $\mu$ L of RNase-free water to enhance the yield. Total RNA content was measured using a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and integrity was analyzed by electrophoresis. DNase-treated RNA (1  $\mu$ g) was reverse transcribed with oligo (dT)18 primer using the RETROscript<sup>®</sup> kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Semi-quantitative RT-PCR (SQ-RT-PCR) was carried out to determine transcript abundance of *DcAOX2a*, *DcAOX1a* and *DcPAL* genes. RT-PCRs were normalized using the carrot actin gene (*DcActin*). SQ-RT-PCR was performed using Ready-To-Go RT-PCR beads (GE Healthcare, Little

**Table 1**

Primers used for SO-RT-PCR.

Gene	acc.	Forward primer (5'–3')	Reverse primer (5'–3')
<i>DcAOX2a</i>	EU286575	TGCTGCATCTGAGGTCTCTCC	GGAGCAGGAACATTTTCAATTG
<i>DcAOX1a</i>	EU286573	CTTCAACGCCTACTTCCTTG	ATCTCGCAATGTAGAGTCAGC
<i>DcPAL</i>	AB435640.1	GGGAGCCATTGTGAGGAGGTGA	ATCACCCAGTCGCTACTCGCC
<i>DcActin</i>	X17526	CACACGGTGCCAAATTTATGAA	GATCACGGCCAGCAAGGT

acc.: accession number in NCBI (National Center for Biotechnology Information).

Chalfont, England) with 2  $\mu$ L of cDNA and 0.2  $\mu$ M of each of primer pair specific for *DcAOX2a*, *DcAOX1a*, *DcPAL* and *DcActin* gene. The gene specific primers are indicated in Table 1. For all experiments, at least two biological and two technical repeats were performed. Technical repeats were performed using same thermocycler and electrophoresis apparatus. Specific primers generated PCR products of 306 bp (*DcAOX2a*), 196 bp (*DcAOX1a*), 186 bp (*DcPAL*) and 72 bp (*DcActin*), respectively.

For each of the three genes studies, the appropriate number of PCR cycles was optimized (by testing the different cycles between 20 and 38 cycles). For amplification of *DcAOX2a* and *DcAOX1a*, after initial denaturation at 94 °C for 5 min, 32 PCR amplification cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) were run and followed by a final extension for 10 min. A similar PCR condition with 30 amplification cycles was used for amplification of *DcPAL* and *DcActin* genes. SQ-RT-PCR products (10  $\mu$ L aliquots) were separated in 1.4% (w/v) agarose gel and subsequently analyzed after ethidium bromide staining (2 ng/mL) by a Gene Flash Bio Imaging system (Syngene, Cambridge, UK).

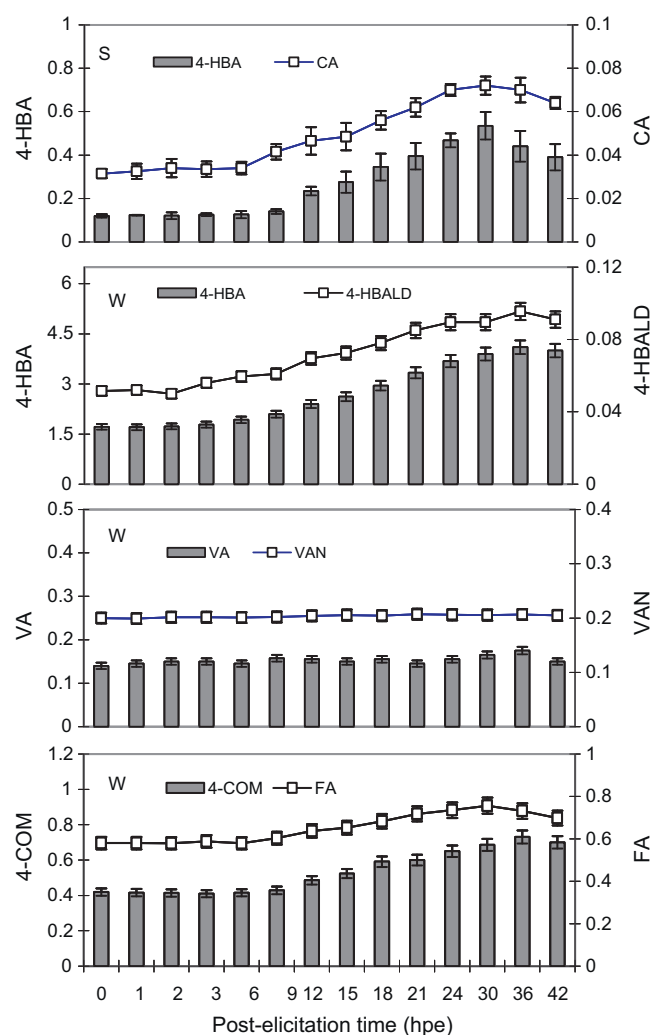
## Results

### Phenolic acid, lignin and flavonoid content in MJ elicited hairy roots

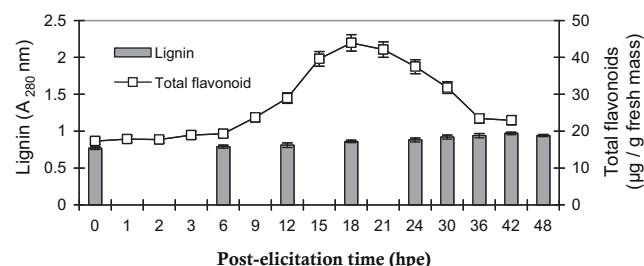
HPLC analyses showed that the predominant phenolics in the soluble fraction were 4-hydroxybenzoic acid followed by caffeic acid (chromatogram not shown). In the wall-bound fraction, the predominant phenolic acid was 4-hydroxybenzoic acid, followed by ferulic acid, 4-coumaric acid, vanillic acid, vanillin and 4-hydroxybenzaldehyde (chromatogram not shown). Time-course analyses revealed that the amounts of phenolics, both in the soluble and wall bound fraction, increased in response to MJ treatment. In the soluble fraction, maximum phenolic accumulation was detected 30 hpe. Maximum 4-hydroxybenzoic acid and caffeic acid accumulated in soluble fraction was 0.53 mg/g dry mass and 0.072 mg/g dry mass, respectively (Fig. 1), which were 4.4-fold and 2.2-fold higher than the control roots, respectively. In wall-bound fraction, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-coumaric acid and ferulic acid content (Fig. 1) significantly enhanced upon MJ treatment, whereas the content of vanillic acid and vanillin remained nearly unchanged (Fig. 1). Content of 4-hydroxybenzoic acid (4.1 mg/g dry mass), 4-hydroxybenzaldehyde (0.095 mg/g dry mass), 4-coumaric acid (0.73 mg/g dry mass) reached peaks at 36 hpe. Ferulic acid content (0.75 mg/g dry mass) attained a maximum at 30 hpe. Non-elicited hairy root lines did not show any appreciable change in phenolic acid content over the same time course studied.

Lignin (TGA-derivatives) accumulation was slightly enhanced by MJ treatment. Compared to non-elicited control roots, a maximum 1.25-fold increase in lignin content was observed after 42 hpe (Fig. 2).

The time course for total flavonoid accumulation in elicited hairy root cultures is presented in Fig. 2. Total flavonoid content was enhanced by MJ treatment. Up to 6 hpe, total flavonoid content remained nearly unchanged (with a basal value of 17  $\mu$ g/g

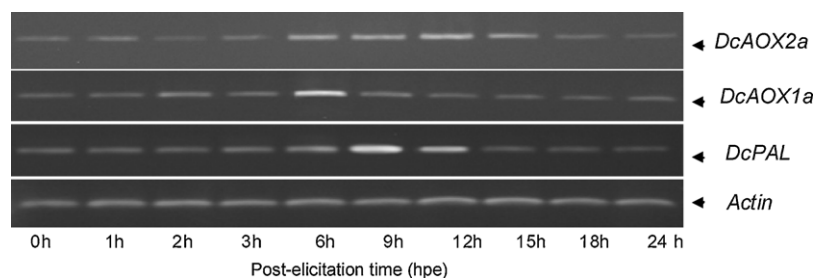


**Fig. 1.** MJ-induced changes in the phenolic accumulation in hairy roots of *D. carota*. Each value represents the mean of three independent determinations  $\pm$  SD. No significant changes in phenolic acid content were observed for non-elicited control roots during the time course studied. The term S and W denotes soluble and wall-bound phenolics, respectively. 4-HBA: 4-hydroxybenzoic acid; 4-HBALD: 4-hydroxybenzaldehyde; VA: vanillic acid; VAN: vanillin; 4-COM: 4-coumaric acid; FA: ferulic acid.



**Fig. 2.** Time-course changes in lignin (TGA-derivatives) and total flavonoid content in MJ-treated hairy roots of *D. carota*. Each value represents the mean of three independent determinations  $\pm$  SD.





**Fig. 3.** Differential transcript accumulation of *DcAOX2a*, *DcAOX1a* and *DcPAL* genes in MJ-treated hairy roots of *D. carota*. Transcript accumulation was analyzed by SQ-RT-PCR using *DcActin* gene for normalization.

fresh mass). Thereafter, total flavonoid content rapidly increased and reached a peak 18 hpe (44  $\mu$ g/g fresh mass). After 36 hpe, total flavonoid content had decreased nearly to the basal level.

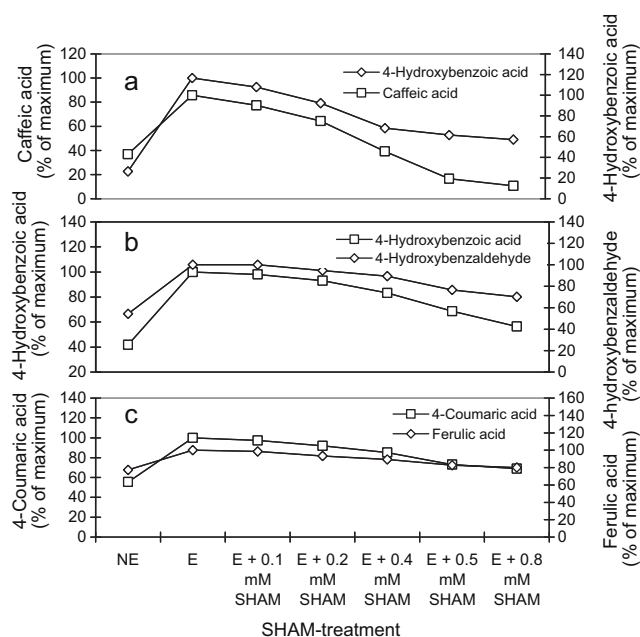
#### Changes in *DcAOX2a*, *DcAOX1a* and *DcPAL* transcript level and PAL activity after MJ-treatment

Upon MJ treatment, *DcAOX2a*, *DcAOX1a* and *DcPAL* genes displayed differential transcript accumulation. Both *DcAOX* genes showed similar transcript accumulation patterns. However, expression of *DcAOX2a* remained lower than *DcAOX1a*. A low basal transcript level was observed up to 3 hpe for both of the *DcAOX* genes. An increase in the transcript level of *DcAOX2a* was observed at 6 hpe, which remained high up to 12 hpe and then rapidly decreased to the basal level (Fig. 3). For *DcAOX1a*, the transcript level reached its peak at 6 hpe. Thereafter, the transcript level decreased to the basal level. *DcPAL* gene transcripts showed a basal level up to 6 hpe, reached a peak at 9 hpe, and after 12 hpe, again decreased to the basal level. Interestingly, the peak of *DcPAL* transcript levels was preceded by an increase in the transcript levels of both *DcAOX* genes.

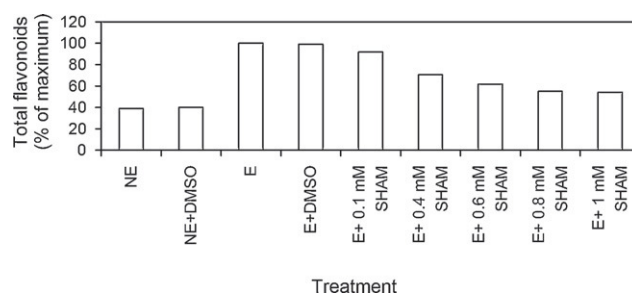
After MJ elicitation, a marked increase in PAL enzyme activity was observed, which attained a peak at 12 hpe (4.2 nkat/mg protein). At 24 hpe, PAL activity had decreased nearly to the basal level (Fig. S1, in supplementary data). Interestingly, an increase of phenolic acid, flavonoid and lignin accumulation was preceded by an increase in PAL activity. Non-elicited control roots did not show appreciable changes in PAL activity over the time course studied.

#### Effect of SHAM and PG treatment on phenolics, flavonoids and lignin accumulation

In an effort to investigate the potential role of AOX-mediated alternative respiration in phenylpropanoid biosynthesis, MJ-treated hairy roots were incubated in the presence of various concentrations of AOX inhibitors (SHAM and PG). SHAM treatment reduced the formation of phenolics (both in soluble and wall-bound fraction) in a concentration dependent manner, but a complete suppression was never observed, even at 1 mM SHAM concentration. Notably, SHAM concentrations above 1 mM had a detrimental effect on hairy root growth and viability. In the soluble fraction, 4-hydroxybenzoic acid and caffeic acid content decreased to 49% and 12.5%, respectively, when treated with 0.8 mM SHAM (Fig. 4a). Interestingly, caffeic acid content markedly decreased with higher SHAM concentration (0.8 mM). Phenolic acid content in MJ-elicited roots not submitted to SHAM treatment were considered as 100%. A similar decrease in soluble phenolics was also observed with PG treatment. 4-Hydroxybenzoic acid and caffeic acid content decreased to 56% and 18%, respectively, when treated with 0.1 mM PG. In the wall-bound fraction of SHAM treated roots, the content of 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-coumaric acid and ferulic acid decreased to maximum values of 56%, 70%, 69% and 80%, respectively, compared to elicited control roots (Fig. 4b and c). With the use of PG (0.1 mM), the corresponding decrease in the amount of wall-bound phenolics were 60%, 72%, 68% and 78%, respectively.



**Fig. 4.** Effect of SHAM on accumulation of phenolic acid in MJ-treated hairy roots of *D. carota*. Results are expressed in terms of percentage of maximum for making a simplified overview. Phenolic acid content of only elicited root was considered as 100%. The terms E and NE in the x-axis legend denote -with and -without elicitor, respectively. (a) Soluble phenolics, (b and c) wall-bound phenolics. Values are mean of three independent experiments.



**Fig. 5.** Inhibitory effect of SHAM on total flavonoid accumulation in MJ treated hairy roots of *D. carota*. Results expresses as percentage of maximum. Flavonoid content of only elicited root was considered as 100%. Values are mean of three independent experiments.

To examine whether total flavonoid accumulation required enhanced synthesis of early precursors in the glycolytic pathway primed by alternate oxidase, MJ-treated hairy roots were incubated in the presence of SHAM. SHAM treatment also reduced MJ-induced flavonoid accumulation in a concentration-dependent manner. However, a complete suppression was never observed, even with high concentrations of SHAM (1 mM). With 1 mM SHAM, a maximum 46% reduction in total flavonoid content was noted compared to elicited control roots (100%) (Fig. 5). A similar decrease in total flavonoid accumulation was also observed in PG-treated hairy roots. PG treatment in a final concentration of 0.1 mM resulted in a 54% reduction in total flavonoid content.

Lignin (TGA-derivative) accumulation was inhibited by SHAM treatment in a dose-dependent manner. Lignin content of only MJ-treated hairy root was considered as the maximum (100%) and other values were presented as a percentage of the maximum. A maximum 18% reduction in lignin content was observed when hairy roots were treated with 0.8 mM SHAM. With 0.1, 0.2, 0.4 and 0.5 mM SHAM, corresponding decrease in lignin contents were 4%, 9%, 11% and 16%, respectively. Treatment with PG (0.1 mM), another inhibitor of AOX also reduced the lignin content by 14%.

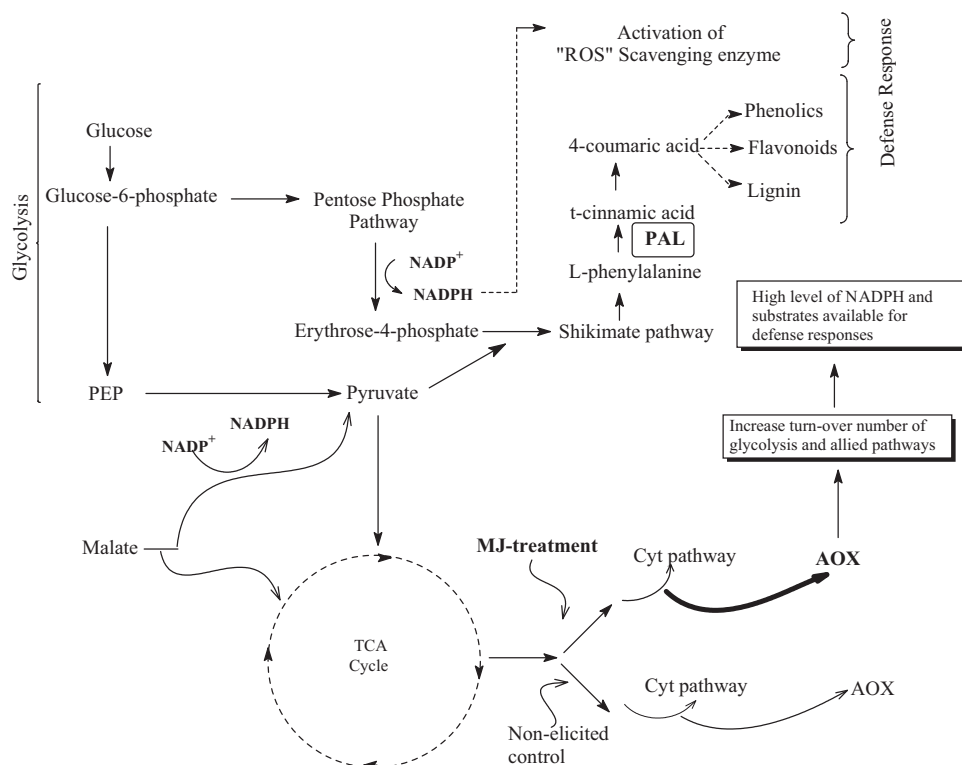
## Discussion

In this study, we report the role of AOX in phenylpropanoid biosynthesis. Two specific inhibitors of AOX (SHAM and PG) were used to inhibit AOX, and their effects on biosynthesis of phenylpropanoid derivatives were subsequently monitored. Furthermore, transcript levels of two *DcAOX* genes (*DcAOX2a* and *DcAOX1a*) were correlated with PAL activity and the subsequent formation of phenylpropanoid derivatives. Our results demonstrate that MJ treatment enhanced the transcript levels of *DcAOX2a* and *DcAOX1a*, which preceded the enhanced transcript level of *DcPAL*. This suggests a role of AOX in up-regulating PAL, the first dedicated enzyme in phenylpropanoid biosynthesis. An increase in the *DcPAL* transcript level was first followed by an increase in *DcPAL* activity and then the subsequent increase in phenolics, flavonoids and lignin content, which argues in support of possible involvement of the *DcAOX2a* and *DcAOX1a* genes in up-regulation of phenylpropanoid biosynthesis. Involvement of PAL in phenylpropanoid biosynthesis is well known (Cheng et al., 2001). MJ has widely been used in inducing phenolic metabolites (Xiao et al., 2009). Significant stimulation of general phenylpropanoid metabolism by MJ treatment has previously been reported in many plant systems (Gundlach et al., 1992; Pauwels et al., 2008). Recently, MJ treatment has been found to massively increase the accumulation of 4-hydroxybenzoic acid in hairy roots of *D. carota* (Sircar et al., 2011) and rosmarinic acid in hairy roots of *Salvia miltiorrhiza* (Xiao et al., 2009). MJ is also known to trigger up-regulation of monolignol biosynthesis, genes leading to the enhanced accumulation of monolignols (Pauwels et al., 2008). In addition to lignin, flavonoid biosynthesis is also known to be increased by MJ treatment (Rudell and Mattheis, 2008). This enhancement effect on flavonoid biosynthesis is mainly due to activation of certain transcription factors by MJ (Von Endt et al., 2002). Our experimental results showed that *DcAOX2a* and *DcAOX1a* displayed differential transcript accumulation after the onset of MJ treatment. Similar enhancement in AOX transcript accumulation upon MJ treatment was previously reported in *Cap-sicum annuum* (Fung et al., 2004) and in *Lycopersicon esculentum* (Fung et al., 2006). In the present study, the *DcAOX2a* and *DcAOX1a* genes were chosen for correlation studies with phenylpropanoid biosynthesis because of known involvement of these two AOX genes in the process of developmental and cell reprogramming (Campos et al., 2009; Frederico et al., 2009). Recently, Campos et al. (2009) showed developmental and tissue specific differential transcript

accumulation of *DcAOX2a* and *DcAOX1a* in *D. carota*. A parallel result was obtained in *Arabidopsis thaliana*, where *AtAOX2* was differentially expressed during developmental stages (Clifton et al., 2006). Marked changes in *DcAOX2a* transcript levels were observed during somatic embryogenesis in *D. carota* (Frederico et al., 2009).

The inhibitory effect of SHAM and PG on accumulation of phenolics, flavonoids and lignin, indicated the possible involvement of AOX in the biosynthesis of phenylpropanoid derivatives. The capacity of SHAM and PG as potential AOX inhibitors is well-documented (Nun et al., 2003; Macedo et al., 2009). After SHAM and PG treatment, caffeic acid content in the soluble fraction was dramatically decreased. In parallel, the levels of 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-coumaric acid and ferulic acid significantly decreased in the wall-bound fraction. This indicated that the whole phenylpropanoid pathway was down-regulated upon SHAM/PG treatment. Caffeic acid and 4-coumaric acid play central roles in the biosynthesis of lignin monomers (Dixon et al., 2001). Decreases in caffeic acid and 4-coumaric acid content probably influenced the decline in lignin content. Further, biosynthesis of flavonoids requires 4-coumaroyl-CoenzymeA (CoA ester of 4-coumaric acid) as a starter substrate. Thus, in our study, the lower level of 4-coumaric acid in SHAM or PG-treated hairy roots argues in support of decreased flavonoid content. It is worth noting that MJ treatment, on the one hand, enhanced the transcript level of both *DcAOX2a* and *DcAOX1a* genes together with enhanced accumulation of phenolics, flavonoids and lignin. On the other hand, use of SHAM in conjugation with MJ suppressed accumulation of phenolics, flavonoids and lignin, which suggests that enhanced AOX transcript accumulation corresponds with enhanced AOX activity. A parallel correlation between AOX transcript level and protein was previously reported in pathogen infected *A. thaliana* (Simons et al., 1999).

A positive association between AOX and biosynthesis of phenylpropanoid derivatives is further evident by the increase in the *DcPAL* transcript level and subsequent PAL activity following the enhanced *DcAOX* transcript level. A similar correlation between increased PAL activity and enhanced phenylpropanoid biosynthesis was reported previously in *A. thaliana* (Olsen et al., 2008). PAL is the first key enzyme in phenylpropanoid biosynthesis and known to be linked with enhanced phenylpropanoid biosynthesis (Gómez-Vásquez et al., 2004). It is likely that MJ treatment up-regulates various secondary metabolic pathways (Gundlach et al., 1992), which results in demand of high substrate requirements. Aromatic amino acid phenylalanine, the end product of the shikimic acid pathway, is the starter molecule for all phenolic metabolites, including flavonoids and lignin. Precursors of the shikimic acid pathway (phosphoenolpyruvate and erythrose-4-phosphate) arise from respiratory breakdown of glucose (Arcuri et al., 2004). It is possible that, to fulfill high substrate requirements, glycolysis and TCA-cycle turn-over number increases, which in turn saturates electron flow through the normal cytochrome pathway (Vanlerberghe and McIntosh, 1996; Vanlerberghe and Ordog, 2002). Saturation of the cytochrome pathway results in the production of more ROS (Maxwell et al., 1999). ROS generation may trigger the signal transduction pathway necessary for the activation of AOX (Amirsadeghi et al., 2006; Hanqing et al., 2010). AOX then directly accept electrons from reduced ubiquinone and prevent over-reduction of the cytochrome pathway, thus allowing glycolysis and TCA cycle to run in high turnover numbers (Fig. 6). The high turnover numbers of glycolysis and the TCA cycle not only provide precursors (phosphoenolpyruvate) of the shikimic acid pathway, but also rapid turnover of the pentose phosphate pathway, leading to the formation of erythrose-4-phosphate and NADPH. Erythrose-4-phosphate then enters the shikimic acid pathway for the production of aromatic amino acids, the building blocks of phenolics, flavonoids and lignin (Fig. 6). High NADPH



**Fig. 6.** Possible co-relation between enhance AOX activity and phenylpropanoid metabolism. MJ-treatment mimics pathogen attack and stimulates AOX genes. Enhanced AOX activity in turn increases the turn-over number of glycolysis, pentose phosphate pathway and shikimate pathway resulting into the higher production of phenolic acids, flavonoids and lignin.

content is used to activate the ROS scavenging enzyme system (Mittler, 2002). Moreover, the enhanced NADPH level is further utilized by phenolics, flavonoids and lignin biosynthetic pathways as reducing energy equivalent (Hanqing et al., 2010). In many plant species, enhanced accumulation of phenolic and lignin occurs upon pathogen attack, and plays an important role in plant defense (Bennett and Wallsgrove, 1994). It has been suggested that, in many cases, pathogen attack or abiotic stress have resulted in the accumulation of MJ in the infected cell (Xiao et al., 2009) which then triggers up-regulation of phenolic biosynthesis in a similar fashion as observed in the present study with exogenous application of MJ.

In conclusion, biosynthesis of phenylpropanoid-derivatives in MJ-treated carrot hairy root seems to be positively associated with AOX activity. This perception is based on two important observations: first, AOX inhibition by SHAM and PG reduced accumulation of phenolics as well as flavonoids and lignin. Second, transcript levels of *DcAOX2a* and *DcAOX1a* significantly increased preceding MJ-induced enhanced accumulation of phenolics, flavonoids and lignin. It is plausible that AOX plays a key role in adjusting cellular metabolism upon MJ treatment to cope with a changing cellular environment and as a consequence of metabolic re-adjustment AOX stimulates enhanced formation of phenylpropanoid derivatives. However, the exact mechanistic details underlying this metabolic adjustment process require further investigation and represent an area of future research interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.11.019.

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