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Carrot plastid terminal oxidase gene (*DcPTOX*) responds early to chilling and harbors intronic pre-miRNAs related to plant disease defense

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ABSTRACT

The nuclear-encoded plastid terminal oxidase gene (*PTOX*) is present in photosynthetic species and functions in the oxidation of the plastoquinone pool. It plays an important role on chlororespiration, chromorespiration and carotenoid biosynthesis. Here, we show short-term early response of carrot *PTOX* (*DcPTOX*) in leaves of two carrot inbred lines upon chilling. Analysis of the complete gene confirmed *DcPTOX* as a single gene and revealed 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 discovered that some plant species present two *PTOX* genes. A search for sequence variability at genomic level was performed in the heterogeneous, ancient carrot cultivar 'Rotin'. *DcPTOX* revealed intron length polymorphisms (ILPs) in intron 2, due to the occurrence of two insertion/deletions (InDels) events. Prediction of pre-miRNA sequences in intronic regions of *DcPTOX* showed two putative locations coding for putative miRNAs, one located at intron 2 and other located at intron 6. Both putative miRNAs revealed high homology with miRNAs described in *Glycine* max (gma-miR1520p) and *Oryza sativa* (osa-miR5494), respectively. Plant disease resistance genes were identified as miRNAs target genes.

The nuclear-encoded plastid terminal oxidase gene (*PTOX*) was initially identified through transposon tagging (Carol et al., 1999). It codes for the plastid quinol:oxygen oxidoreductase active in chlororespiration that regulates the redox state of the plastoquinone (PQ) pool by transferring excess electrons to O_2 (Aluru and Rodermel, 2004). Thus, the relative redox balance in the photosynthetic electron transport chain (ETC) is maintained and the possibility for oxidative damage can be reduced (McDonald et al., 2011). This catalytic function was deduced from its protein sequence similarity to mitochondrial alternative oxidase (AOX) (McDonald et al., 2011; Fu et al., 2012). Such as AOX, PTOX is a member of the non-heme diiron carboxylate (DOX) protein family (Berthold and Stenmark, 2003). Both enzymes have been modeled as interfacial membrane proteins with an active site (DOX) domain exposed to the matrix (case of AOX) or stroma (case of PTOX) (McDonald et al., 2011). For this reason, PTOX in chloroplasts has been suggested to be functionally analogous to AOX in mitochondria (Aluru and Rodermel, 2004). 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 high light and other stress conditions, has frequently been put forward (Streb et al., 2005; Stepien and Johnson, 2009; Díaz et al., 2007). Other studies argue that PTOX does not act as a safety valve for photosynthesis during stressful conditions but more likely plays important roles in plant development (Okegawa et al., 2010). In fact, it is likely that PTOX is involved in stress response of certain plants, but does not act as a universal or essential safety valve in the whole plant kingdom. Several other reports point to PTOX as a key enzyme in the carotenoid biosynthesis pathway (Aluru et al., 2006; Campos et al., 2016a).

In the present work we describe the dynamics of carrot *PTOX* gene (*DcPTOX*) transcript accumulation during *Daucus carota* L. plants exposure to chilling. For this analysis leaves of two carrot inbred lines (B0493 and B5280), with ten weeks old and growing under controlled temperature conditions (25 °C day/15 °C night) were used (see Table S1). Half of the plants were exposed to cold treatment (5 °C) during 48 h, while the other half was kept at the same temperature conditions as before. Samples were taken at 6 h (T6), 9 h (T9), 24 h (T24) and 48 h (T48) post chilling exposure. *DcPTOX* gene was found to be

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very early responsive to chilling exposure in the inbred line B5280 (at T6) while B0493 presented a slightly later increase on transcript accumulation (at T24) (Fig. 1). For both inbred lines, higher mean levels of transcripts were observed in all time points during chilling exposure comparing to the respective controls. Statistically significant differences were observed at T24 for B0493 and at T6 and T9 for B5280 ($P < 0.05$) (Fig. 1). An increase of relative expression was shown 6 h post chilling in both inbred lines, which increased at T9 by approximately 2-fold and 3-fold difference, when compared with control plants, for B0493 and B5280, respectively. The highest peak was achieved at T24 for B0493 and T9 for B5280 (Fig. 1). Transcript accumulation of *DcPTOX* then slightly decreased but mean values remained always higher than for control plants. Besides the involvement of PTOX on chlororespiration and carotenoids biosynthesis (*e.g.* Aluru and Rodermel, 2004), the results suggest a role for *DcPTOX* on the response of carrot plants to low temperatures.

Photosynthesis in chloroplasts involves a vectorial electron transfer from water in the lumen to $NADP^+$ in the stroma, which is achieved by means of redox carriers (Paredes and Quiles, 2015). Besides major photosynthetic complexes of oxygenic photosynthesis, there is a chlororespiratory pathway (Bennoun, 1982), 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 PTOX. PTOX as the terminal oxidase of chlororespiration, regulates the redox state of the PQ pool (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). Plants grown in moderate light under non-stress conditions have low PTOX concentrations (about 1 PTOX protein per 100 PSII; Lennon et al., 2003). Low temperature stress - either chilling or freezing - is a critical abiotic stress, leading to reduced crop yield (Beck et al., 2004). The safety valve function of PTOX, which is a protective function against over-reduced states under stress conditions, was already put forward, *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), in *Brassica fruticulosa* under temperature and light stress (Díaz et al., 2007), in *Avena sativa* under high temperature, high light and drought (Quiles, 2006), and in *Arabidopsis thaliana* under low temperatures and high light stress (Ivanov et al., 2012). All those reports show an increasing of PTOX protein levels indicating a metabolic role of PTOX upon a diversity of environmental stresses. As referred above, PTOX in chloroplasts has been suggested to be functionally analogous to the stress-induced mitochondrial alternative oxidase (AOX) (Aluru and Rodermel, 2004). *AOX* genes and encoded proteins are known to be responsive to several stress conditions (see review in Vanlerberghe, 2013; Campos et al., 2016b). Several studies developed in many species show a sharp increase in *AOX* transcript and/or protein after exposure or during growth at low temperature (Vanlerberghe, 2013; Velada et al., 2014). Alternative oxidase expression under cold stress and plant tissue respiration has also been the focus of several studies. Enhancement of endogenous *AOX1a* expression *via* low temperature stress was verified in wild type and in transgenic plants (overexpressing *Waox1a*) from *A. thaliana*, together with an increase of alternative respiratory capacity (Sugie et al., 2006). Also, cold-grown plants from *Vigna radiata* that up-regulated the level of AOX protein, maintained greater electron partitioning to AOX when measured after a decrease in temperature, supporting a role for the alternative pathway in response to low temperatures (Gonzàlez-Meler et al., 1999). Nevertheless, this response was not observed in *Glycine max* cotyledons, in which high levels of alternative pathway activity and unchanged amount of AOX were seen at both high and low temperature (Gonzàlez-Meler et al., 1999). In carrot leaves, an early *AOX* transcript increase upon chilling, prior to the induction of a specific anti-freezing gene coding for antifreezing protein (*AFP*), was detected. The expression of *AFP* showed a later but much higher level of transcripts in carrot plants subjected to chilling stress than *AOX* genes (Campos et al., 2016b). Our results indicate that *DcPTOX* is also a highly/rapidly responsive gene during cold stress in carrot leaves. Interestingly, the interaction between AOX and PTOX were reported in *A. 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

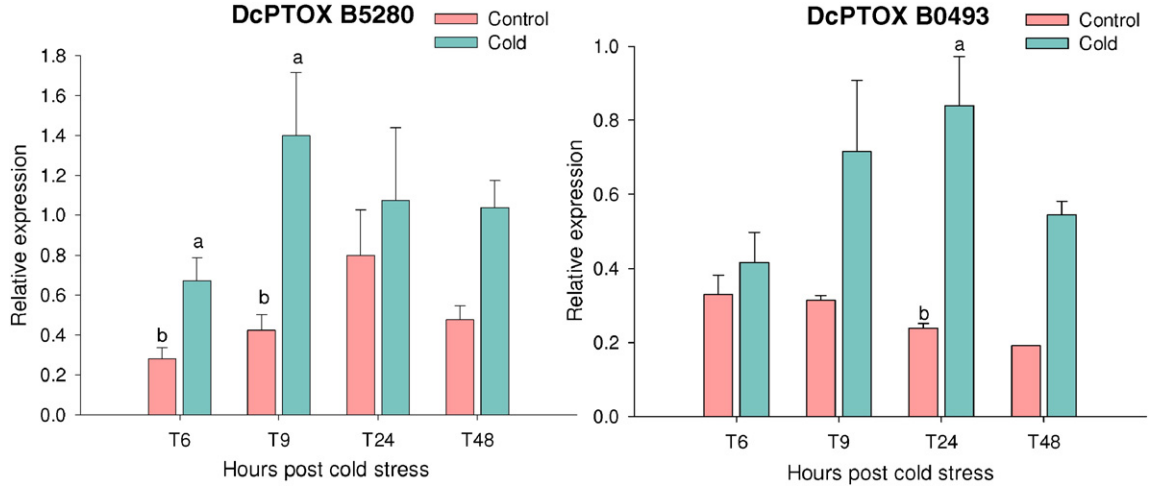

Fig. 1. Transcript levels of *DcPTOX* during chilling exposure in leaves of 10 weeks-old carrot inbred lines B5280 and B0493. Samples were harvested 6 h (T6), 9 h (T9), 24 h (T24) and 48 h (T48) post chilling exposure at 5 °C. For each time point control samples were taken. Transcript accumulation was analysed by RT-qPCR using elongation factor-1alpha (GenBank: D12709) and glyceraldehyde 3-phosphate dehydrogenase (GenBank: AY491512) for normalization (selected by Campos et al., 2015). Data are the average values ±SE of three plants considered per time point. Student's *t*-test was applied to test differences between control and cold treated samples in each time point. Different superscript letters indicate significant differences between sampling points. Plants were maintained under controlled conditions (25/15 °C day/night, 70–75% of air humidity, with 16 h photoperiod with 200 ± 20 μmol m^{-2} s^{-1} light intensity) for 10 weeks after sowing until the beginning of the experiment. Half of the plants were maintained under same conditions (control), and the other half was exposed to cold treatment (5 °C) during 48 h. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNase-treated total RNA (1 μg) was reverse transcribed with random decamer primers using the RETROscript® kit (Ambion, Austin, TX, USA). Gene-specific primers were design using Primer Express Software (Applied Biosystems, Foster City, USA) (Table S1). 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). Evaluation of expression stability of genes to be used as reference was done using the statistical application *geNorm* (Vandesompele et al., 2002).