Plant Science 291 (2020) 110327

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Differential role of the two ζ-carotene desaturase paralogs in carrot (*Daucus carota*): *ZDS1* is a functional gene essential for plant development and carotenoid synthesis

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ARTICLE INFO

Keywords:
Z-carotene desaturase
Carrot
Plant development
Carotenoid regulation
Storage root
Post transcriptional gene silencing

ABSTRACT

Daucus carota is a biennale crop that develops an edible storage root. Orange carrots, the most consumed cultivar worldwide, accumulate high levels of β-carotene and α-carotene in the storage root during secondary growth. Genes involved in β-carotene synthesis have been identified in carrots and unlike most species, *D. carota* has two ζ-carotene desaturase genes, named *ZDS1* and *ZDS2*, that share 91.3 % identity in their coding regions. *ZDS1* expression falls during leaf, but not root development, while *ZDS2* is induced in leaves and storage roots of a mature plant. In this work, by means of post-transcriptional gene silencing, we determined that *ZDS1* is essential for initial carrot development. The suppression of the expression of this gene by RNAi triggered a reduction in the transcript levels of *ZDS2* and *PSY2* genes, with a concomitant decrease in the carotenoid content in both, leaves and storage roots. On the contrary, transgenic lines with reduced *ZDS2* transcript abundance maintain the same levels of expression of endogenous *ZDS1* and *PSY*2 and carotenoid profile as wild-type plants. The simultaneous silencing of *ZDS1* and *ZDS2* resulted in lines with a negligible leaf and root development, as well as significantly lower endogenous *PSY*2 expression. Further functional analyses, such as a plastidial subcellular localization of ZDS1:GFP and the increment in carotenoid content in transgenic tobacco plants overexpressing the carrot *ZDS1*, confirmed that *ZDS1* codifies for a functional enzyme. Overall, these results lead us to propose that the main ζ-carotene desaturase activity in carrot is encoded by the *ZDS1* gene and *ZDS2* gene has a complementary and non essential role.

1. Introduction

Carotenoids are a group of isoprenoid molecules composed of 40 carbons that are synthesized by all photosynthetic organisms including plants and algae, and some non-photosynthetic organisms such as fungi and bacteria [1]. The conjugated double bonds that are present in each molecule give them chromophore properties and color within the yellow and red range. In plants, carotenoids are synthesized in plastids through the non-mevalonate pathway (MEP) [2] by nuclear-encoded enzymes and has been described elsewhere [3]. The first committed step in carotenoid biosynthesis is the production of the colorless carotenoid 15-*cis*-phytoene catalyzed by phytoene synthase (PSY) (Fig. 1). Then, 15-*cis* phytoene is desaturated by phytoene desaturase (PDS) to produce 9,15,9′-*tri*-*cis*-ζ-carotene, which is then isomerized by the ζ-carotene isomerase (Z-ISO) and/or light to originate 9,9′-*di*-*cis*-ζ-carotene [4]. Fig. 1 also shows a representation of the two sequential desaturations mediated by ζ-carotene desaturase (ZDS) to synthesized 7,9,7′,9′ *tetra*-*cis*-lycopene (prolycopene) *via* 7,9,9′-*tri*-*cis*-neurosporene [5]. Next, lycopene β-cyclase (LCYB) produces β-carotene, by the generation of β-rings at both ends of the lycopene molecule, whereas LCYB together with lycopene ε-cyclase (LCYE) are required to form α-carotene. Hydroxylation of β-carotene by carotenoid β-hydroxylase (CHYB), leads to the production of zeaxanthin, whereas hydroxylation of α-carotene carried out by β- and ε-hydroxylase (CHYB and CHYE), results in the production of lutein. Several xanthophyll molecules are produced by zeaxanthin epoxidase (ZEP) and neoxanthin synthase (NSY). At last, xanthophylls are cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes to produce abscisic acid (ABA) [6].

Carotenoids are located in the photosynthetic membranes in the form of chlorophyll–carotenoid–protein complexes and some carotenogenic enzymes have been described to be membrane-associated [7,8]. In photosynthetic tissue, carotenoids accumulate in chloroplasts

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https://doi.org/10.1016/j.plantsci.2019.110327
Received 14 June 2019; Received in revised form 16 September 2019; Accepted 21 October 2019
Available online 26 November 2019
0168-9452/

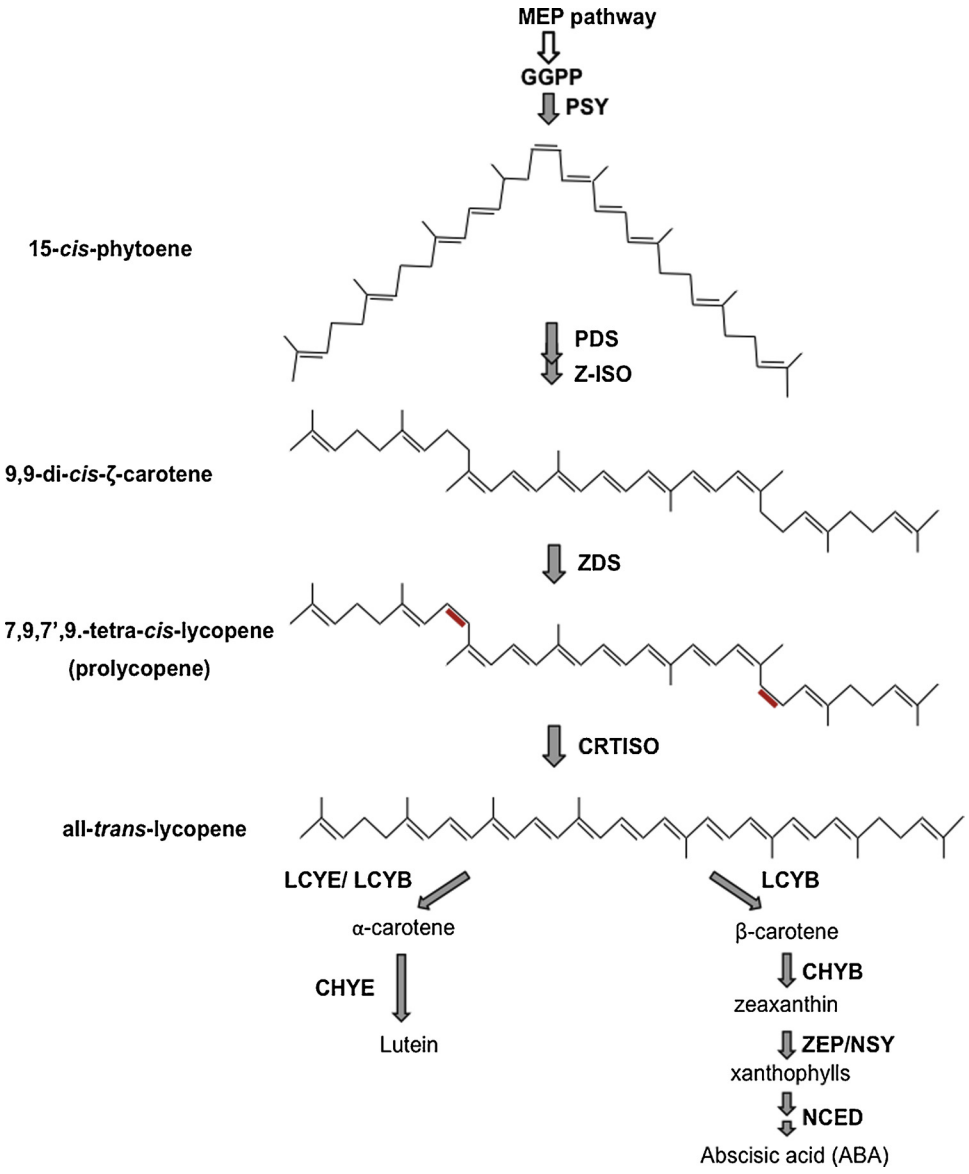

Fig. 1. The Carotenoid Biosynthetic Pathway in Plants. The route gives rise to several isoprenoid molecules starting from the non-mevalonate pathway (MEP). Abbreviations: geranylgeranyl pyrophosphate (GGPP), phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS), carotenoid isomerase (CRTISO), lycopene β-cyclase (LCYB), lycopene ε-cyclase (LCYE), β-carotene hydroxylase (CHYB), ε-carotene hydroxylase (CHYE), zeaxanthin epoxidase (ZEP), violaxanthin deepoxidase (VDE), neoxanthin synthase (NSY), 9-cis-epoxycarotenoid dioxygenase (NCED). White arrows indicate multiple enzymatic reactions in the MEP pathway for generating GGPP. Light gray arrows represent different steps of the carotenogenic pathway. Red bar highlight specific desaturations carried out by ZDS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and participate in the absorption of light during photosynthesis. Moreover, they protect plants from photooxidative damage [9,10] through the xanthophyll cycle [11,12]. Consequently, changes in the carotenogenic pathway, often lead to an irregular plastid development, which is associated with an abnormal or lethal phonotype [13,14].

High levels of carotenoids are also found in chromoplasts, the main plastids specialized in the synthesis and accumulation of carotenoids providing color to flowers, fruits, seeds or storage roots [15–17]. Animals, are unable to synthesize carotenoids and have to include them in their diet because carotenoids are precursors of vitamin A [18–20], and contribute to prevent macular degeneration, some types of cancer, cardiovascular disease, and aging [21,22].

Daucus carota subsp. *sativus*, carrot (2n = 18) belongs to the botanical group Umbelliferae (or Apiaceae). Currently, a wide range of carrot colors and cultivars exist, but the orange carrot is the most popular and is consumed worldwide. Orange cultivars accumulate mainly β-carotene and α-carotene in the storage root [23–27]. Storage root development takes around three months [28,29] and is accompanied by a dramatic increase in size and carotenoid levels [30]. The complete cDNA sequences of putative carotenoid biosynthetic genes linked to QTLs for carotenoid accumulation in *D. carota* subsp. *sativus* have been mapped and cloned [31,32] which has been complemented with the assembled carrot genome [33]. During carrot storage root development, the expression of most carotenogenic genes is upregulated in correlation with the increase in carotenoid levels [28,30]. The paralog genes for *PSY*, *ZDS* and *LCYB* present differential expression patterns in leaves and roots during development, which may be associated with their role in carrot carotenoid synthesis [31,34,35]. Functional characterization of *LCYB1* concluded that this gene participates in carotenoid and chlorophyll synthesis in carrot leaves and storage roots [36] and its heterologous expression in tobacco induces an increment in gibberellins and plant fitness [37]. However, the functionality of the other paralogous genes remains to be established.

In general plant species present only one ZDS enzyme (ZDS) normally associated with a single corresponding *ZDS* gene. Interestingly, in a few species more than one *ZDS* gene has been described, potentially codifying for more than one ZDS protein. In carrot and apple (*Malus domestica*), two *ZDS* have been reported, whereas in sweet potato (*Citrus sinensis)* 11 putative ZDS unigenes were identified although transcriptional and functional characterization remains to be determined (Chen et al. 2010). [31,32,38]. The increment in conjugated double bonds changes the spectrum of absorption of the molecule, resulting in the synthesis of the reddish lycopene and yellow-orange neurosporene from the pale-yellow ζ-carotene [7]. Mutants in the unique *ZDS* gene have

been reported in sunflower [39], *Arabidopsis thaliana* [40,41], orange [42] and maize [43,44]. Most of these mutants are not viable in homozygosis, displaying carotenoid deficiency and giving rise to colorless leaves, abnormal development of chloroplasts, high production of superoxide, reduced activity of photosystem II and lower synthesis of ABA [39,40,42,43,45]. The Arabidopsis allelic *ZDS* mutant variant, *spc1-1* has a weak phenotype presenting pigment defective embryos, leaf bleaching, an increment in reactive oxygen species (ROS) and mosaic cell death [40]. Moreover, *A. thaliana zds/zlb5* mutant, exhibited a disrupted ZDS activity, which has been associated with alterations in leaf and chloroplast development [41]. In maize, the vp-wl2 viviparous mutant caused by the insertion of Mu9 in the first intron of the zeta-carotene desaturase (*ZDS*) gene, produces white or pale-yellow kernels and a dramatic reduction in carotenoid and ABA content (Chen et al., 2017).

In carrot, the expression level of *ZDS1* is high in leaves at the initial stages of development and does not change during root development, while *ZDS2* is induced in leaves and roots at the mature stage [30], showing a difference in plastidial and developmental response of the two paralog *ZDS* genes in carrot leaves and roots. In the current work we determined that although *ZDS2* has a higher transcript abundance in carrot leaves and storage roots in the mature stage, transgenic carrot plants that present 98 % reduced transcript levels of *ZDS2* develop normally and accumulate similar carotenoid levels in comparison to wild-type plants. On the other hand, the suppression of the expression of *ZDS1* by RNAi affects initial plant development and triggers a significant reduction in the expression of other carotenogenic genes such as *ZDS2* and *PSY2* with a concomitant reduction in carotenoid levels of 50%–60%. In addition, during the induction of simultaneous silencing of *ZDS1* and *ZDS2* aberrant phenotype in seedling development is observed in direct correlation with reduced *ZDS1* expression, similar than in ZDS1 RNAi. Indeed, the plastidial localization of ZDS1:GFP and the increment in carotenoid content in ZDS1 transgenic *Nicotiana tabacum* (tobacco) plants confirmed that this gene codifies for a functional enzyme. Overall, these results, lead us to propose that *ZDS1* has a more predominant role in carotenoid synthesis and in early seedling development in carrot.

2. Materials and methods

2.1. Phylogenetic analysis

Protein motif analysis was performed with InterProScan [46]. The motifs in ZDS sequences were evaluated in PFAM, TIGRFAMs, Gene3D, PRINTS and PANTHER databases. The phylogenetic analysis was carried out using MEGA5.2.2 (www.megasoftware.net) [47]. The alignment was generated with MUSCLE [48] and the phylogenetic tree by means of the neighbor-joining method with a bootstrap of 500. The outgroup consists of a ZDS from the algae *Auxenochlorella protothecoides* (Accession Nº GU269622.1) [49]. The prediction of the signal peptide to chloroplast was performed using the ChloroP.1.1 Server [50].

2.2. Plant material

Carrot (*Daucus carota* L.) cultivar Nantes and *N. tabacum* cultivar *Xanthi NN* seeds were sown *in vitro* in solid MS (4.4 g/L MS salts, 20 g/L sucrose and 0.7 % agar, pH 5.8) [51]. For *Agrobacterium tumefaciens* mediated transformation, carrot and *N. tabacum* plants were cultivated for three and eight weeks, respectively. *In vitro* regenerated carrot and tobacco transformants were transferred to a mix of soil and vermiculite (2:1) in a greenhouse when they reached 5 cm. All transgenic plants were cultivated in a 16 h day photoperiod illuminated with cool-white fluorescent light (115 μmol m^{-2} s^{-1}) at 20–23 °C until molecular and functional analysis.

2.3. Plasmid constructs

Primers within the 3′UTR were designed for the specific post-transcriptional gene silencing (PTGS) of ZDS1 (Accession Nº DQ222430, primers FZDS1 and RZDS1) and ZDS2 (Accession Nº DQ192189, primers FZDS2 and RZDS2). Total RNA (RNA-Solv, Omega Bio-tek, USA) was extracted from leaves of 12-week-old plants. Fragments of the 3′UTR of ZDS1 (295 bp) and of ZDS2 (271 bp) were amplified from carrot cDNA obtained by using Improm II Reverse Transcriptase (Promega). Cloning was carried out according to the Gateway® (Invitrogen) standard procedure. The ZDS1 and ZDS2 fragments were cloned into pCR®8/GW/TOPO®. Positive ZDS1 clones were evaluated for antisense orientation of the fragment and then recombined into the binary vector pMDC32 [52]. The resulting pASZ1 construction for ZDS1 was obtained for the expression of the fragment in antisense orientation with respect to the double 35S CaMV promoter. ZDS2 was recombined into the binary vector pHellsgate12 [53], obtaining the genetic construct pASZ2, where the fragment is cloned in tandem in a sense and antisense orientation with respect to the 35S CaMV promoter for expressing the double strand hairpin to trigger ZDS2 silencing. For ZDS1 expression in tobacco, the complete ZDS1 cDNA sequence including the 3′UTR region (1933 bp) was amplified using F*ZDS*1atg and R*ZDS*1 primers, cloned into pCR®8/GW/TOPO®and recombined into the binary vector pGWB2, generating the construct termed pGZ1 in which ZDS1 expression is directed by the 35S CaMV promoter. For subcellular localization, the complete ZDS1 cDNA sequence (1719 bp) was amplified without the stop codon using F*ZDS*1atg and RZDS1ST primers and recombined into the binary vector pGWB5 [54] to obtain the construct termed pGZ1GFP, which produces the chimeric protein ZDS1:GFP directed by the 35S CaMV promoter. For the generation of the polyclonal ZDS1 antibody, ZDS1 cDNA was cloned into pMAL-c2E (New England Biolabs) producing the construct pMAL-ZDS1. This construct generates a fusion protein with ZDS1 upstream of the Maltose-binding Protein (MBP). All constructs were sequenced (Macrogen Corp. USA), and the primers mentioned above, and all other primers used during this work are detailed in Supplemental Table S1.

2.4. Carrot and tobacco transformation

Binary constructs pASZ1, pASZ2, pGZ1 and pGZ1GFP were transformed into *A. tumefaciens* (strain GV3101). *D. carota* transformation with pASZ1 and pASZ2, and the regeneration procedure, were carried out following the standard protocol [36,55]. Briefly, wild-type hypocotyls of two-week old seedlings growing *in vitro* were co-cultivated with *A. tumefaciens* carrying the construct of interest and then placed on solid MS media in darkness for two days. Subsequently, the explants were transferred to solid MS medium supplemented with 1 mg L^{-1} 2,4D, and maintained in darkness for five weeks for somatic embryogenesis induction in the presence of antibiotics to eliminate *A. tumefaciens* (300 mg L^{-1} cefotaxime) and to select transformed explants (25 mg L^{-1} kanamycin). Hypocotyls transformed with pASZ1 were cultivated in kanamycin free media under dim light (1 μmol m^{-2} s^{-1} at 20–23 °C [39]) as the only way to obtain seedlings. Antibiotic-resistant embryos transformed with pASZ2 and thus obtained with pASZ1were transferred to solid MS (solid MS supplemented with 0.5 mg L^{-1} 2,4D) in a 16 h light photoperiod (115 μmol m^{-2} s^{-1} at 20–23 °C) for four weeks. Explants were transferred to MS media, without hormones, to induce both shoot and root development. Six months later, the transformed plantlets were transferred to soil under controlled greenhouse conditions. Transgenic lines were selected by PCR amplification of the antibiotic resistant gene.

For tobacco transformation, leaf explants were co-cultivated with *Agrobacterium* carrying pGZ1 and placed on solidified MS media supplemented with 1 mg L^{-1} BAP, 0.5 mg L^{-1} IBA, 25 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin [37,56]. After four weeks, the explants were placed on solidified MS medium without hormones containing

50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin. When rooted seedlings reached 5 cm in height, they were transferred to plastic pots (20 × 10 cm) containing a mix of soil and vermiculite (2:1). Seven transgenic Z1OE T0 lines were analyzed at the molecular level, and two of them were used for western blotting and carotenoid quantification.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was obtained from 200 mg of leaves or storage roots of three-month old transgenic and of 4-, 8- or 12-week old wild-type *D. carota*. RNA extractions were performed using the RNA-Solv protocol (RNA-Solv, Omega Bio-tek, USA). For cDNA synthesis, 1 nM oligo-dT primer was mixed with 2 µg total DNA-free RNA and treated with Improm II reverse transcriptase. Relative quantification of transcript levels (qRT-PCR) was carried out as described in Fuentes et al., 2012 [30] using a Stratagene Mx3000 P thermocycler and SYBR Green double strand DNA binding dye. For carrot PTGS analysis, *ZDS1* primers F5N.Z1q and R5N.z1q (0.95 efficiency) that amplify a 191 bp fragment from the 5′UTR region, *ZDS2* primers F5N.Z2q and R5N.Z2q (1.07 efficiency) that amplify a 122 bp fragment from the 5′UTR region and *PSY*2 primers Fpsy2q and Rpsy2q (0.95 efficiency) that amplify a 112 bp fragment from the coding sequence were used. Untransformed wild-type carrot, plants were selected as a calibrator. Raw fluorescence values were processed using the equation described by Pfaffl, 2001 including previously-determined primer efficiency values. Each qRT-PCR reaction was performed with three biological replicates and each sample was analyzed in duplicate. The reaction specificities were evaluated by the melting gradient dissociation curves. Significant differences between the means were assessed based on a 1-way ANOVA and a Tukey post-test using the statistical software "R" [57]. Differences were considered significant at $P < 0.05$.

2.6. Carotenoid quantification

Pigments from carrot leaves and roots, and tobacco leaves were extracted from 150 mg of tissue. The tissue was frozen in liquid N_2, ground and homogenized with 1 ml hexane/acetone/ethanol (2:1:1 v/v) as described in Fuentes et al., (2012) [30]. The extraction procedure was repeated at least twice until the tissue was blanched. Finally, carotenoids were dried using N_2 gas and stored at −80 °C. All the procedures were carried out in cold and dark conditions to avoid photodegradation, isomerization or structural changes of carotenoids. Total pigment concentration was determined by adding 1 ml acetone to the dried pigments and measuring the absorbance at 474 nm in a spectrophotometer (Shimadzu). Specific carotenoid composition was determined by means of HPLC (Shimadzu, LC 20AT) with a diode array detector (SPD-M20A). A LiChroCART® C-18 reverse phase column (Merckmillipore®) and an acetonitrile: methanol: isopropanol (85:10:5 v/v) solution as mobile phase were used. The flow rate was 1.5 ml min^{-1} at room temperature in isocratic conditions. Carotenoids were determined comparing absorption spectra and retention times with specific pigment standards (Carotenoids Handbook, Britton 2008). Data analysis was carried out using LCsolutions® software.

2.7. Subcellular localization

pGZ1GFP, and the control constructs, 35SCaMV:GFP and 35SCaMV:recA-YFP [58] were transiently expressed in leaves of two-month old *Nicotiana tabacum* plants by agroinfiltration according to [36]. Lower epidermal peels of transformed leaves were visualized after four days in an inverted epifluorescence microscope (IX-70, Olympus America Inc., Melville, NY) and images processed with LSM5 Image Browser and Adobe Photoshop software [59].

2.8. Plant protein extraction and immunoblot analysis

Total protein extracts were obtained from leaves and roots of 4-, 8- and 12-week old wild-type carrots, and from two-months old tobacco (wild-type and Z1OE transgenic lines) by homogenizing 0.5 g tissue in extraction buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride and 10 % glycerol). After centrifugation at 22,000 *g* for 30 min, supernatants were transferred to clean tubes and stored at −80 °C. Protein concentration was determined by the Bradford method, using the BioRad Protein Assay [60]. For immunoblot analysis, 40 µg protein extract per line was resolved by 12 % SDS–PAGE and transferred to nitrocellulose membrane by semi-dry electro blotting (Transblot SD semi-dry transfer cell, BioRad). The polyclonal anti-ZDS antiserum was raised in mice against recombinant ZDS1-MBP purified from *Escherichia coli* strain BL21 (DE3) transformed with pMAL-ZDS1. The over-expression of ZDS1-MBP was induced by the addition of 0.5 mM IPTG. For the purification of the recombinant protein, the cells were sonicated, centrifuged and the cytosolic phase was purified by amylose affinity chromatography in a PD10 column (BioRad). ZDS antibody specificity was evaluated by western blot with ZDS-His (cloned previously in pET28a and used as a control) and *E. coli* protein enriched fractions containing ZDS-MBP.

For western blotting, the membrane was incubated for 16 h in TBS-T with 5 % non-fat dried milk at 4 °C and then blocked for 1 h at 22 °C in TBS-T and 3 % BSA, followed by incubation in anti-ZDS antiserum (1:500 dilution) overnight at 4 °C in TBS-T/1 % BSA. Goat anti-mouse IgG alkaline phosphatase-conjugate solution was used as secondary antibody (1:10,000 dilution in TBS-T/3 % BSA). The immunocomplexes were visualized using an alkaline solution (100 mM Tris − HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) containing 0.33 mg mL^{-1} nitroblue tetrazolium and 0.17 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate.

2.9. Detection of reactive oxygen species

Detection of H_2O_2 was carried out incubating leaves with 3,3′-diaminobenzidine (DAB) adapting the method described previously [61]. Briefly, leaves were submerged in a 1 mg mL^{-1} DAB solution during 5 h with gently agitation. Then, chlorophyll was removed with a solution of ethanol, acetic acid and glycerol (3:1:1) at 78C. An image of the leaves was digitalized and pseudo-color scale was modified using imageJ [62] software.

3. Results

3.1. Phylogenetic analysis of ZDS genes

In carrot, two ζ-carotene desaturase genes (*ZDS1* and *ZDS2*) have been identified [31,32] which share 91.3 % nucleotide identity in the coding region. In the 5′UTR and the 3′UTR, they possess 24 % and 53 % nucleotide identity, respectively. The predicted proteins encoded by *ZDS1* and *ZDS2* are 573 and 575 amino acids in length, respectively, with predicted molecular masses of 63.2 kDa. In order to analyze the amino acid sequence with a functionally-characterized ZDS [40,63], a protein sequence alignment with *A. thaliana* ZDS was performed *in silico*. The analysis showed a high percentage of identity between all three sequences; 81.9 % and 81.7 % between *A. thaliana* ZDS and *D. carota* ZDS1 and ZDS2 respectively (Fig. 2a). The percentage of similarity is 98 % when comparing DcZDS1 and DcZDS2. In addition, protein signature analysis revealed that *A. thaliana* and *D. carota* share many of the same motifs, including the amino oxidase (PF01593, amino acids 66–530 in the *A. thaliana* sequence), ζ-carotene desaturase (TIGR2732, amino acids 58–531) and FAD/NAD(P)-binding domain (SSF51905, amino acids 36–536). When comparing the sequences, there are nine amino acids shared between ZDS of *A. thaliana* and ZDS1 of *D. carota,* but which are different in ZDS2 (Fig. 2a, positions

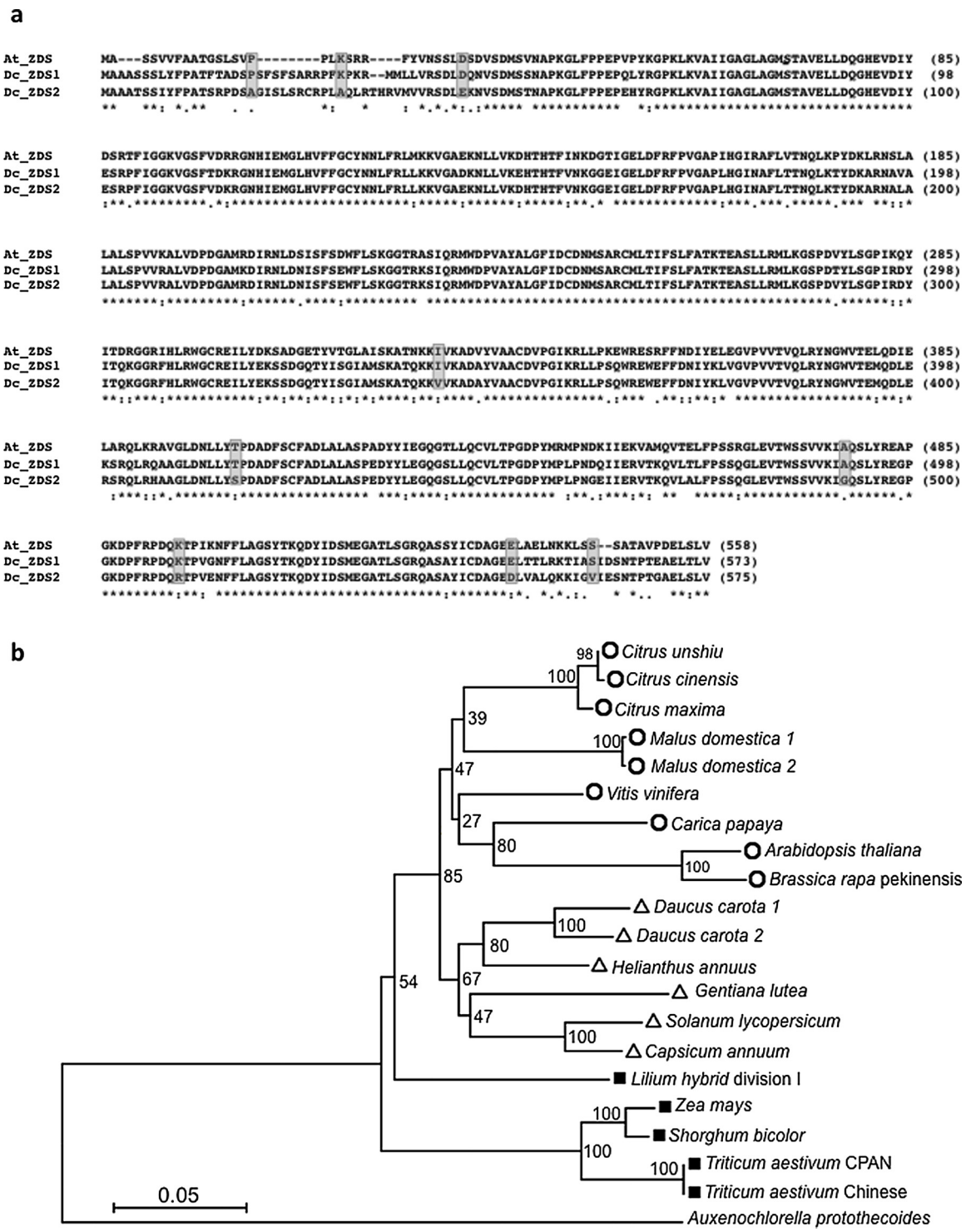

Fig. 2. Phylogenetic analysis based on the amino acid sequences of ZDS. (a) Sequence alignment of ζ-carotene desaturases from *A. thaliana* (AtZDS) and *D. carota* (DcZDSs). Identical amino acids in all sequences are marked with a star, conserved and semi-conserved substitutions are marked with two dots and a single dot, respectively. Conserved motifs are indicated above the sequences, Amino Oxidase (+), zeta-carotene desaturase (×), and FAD/NAD(P) binding (^). Identical amino acids between ZDS of *A. thaliana* and ZDS1 of *D. carota* but different in ZDS2 of *D. carota* are highlighted in gray. **(b)** Phylogenetic tree of ZDS proteins from different organisms. The analysis was carried out using MEGA 5.2.2. The sequence alignment was generated using MUSCLE and the phylogenetic tree was created by means of the neighbor-joining method with bootstrap values of 500 replicates to assess the robustness of the tree. The algae *Auxenochlorella protothecoides* was used as an outgroup. Eudicotyledonous Asterids are marked with a triangle (Δ), Eudicotyledonous Rosids with a circle (○) and Monocotyledonous Commelinids with a square (□). The accession numbers of the sequences included in the phylogenetic tree are the following: *Citrus unshiu* (ABC33728), *Citrus sinensis* (NP_001275793.1), *Citrus maxima* (ACE79169.1), *Malus domestica* ZDS1 (Q5W5X7), *Malus domestica* ZDS2 (AAQ04225), *Vitis vinifera* (AFP28797), *Carica papaya* (ACO40527), *Arabidopsis thaliana* (OAP05892), *Brassica rapa* pekinensis (ACM68701), *Daucus carota* ZDS1 (ABB52083), *Daucus carota* ZDS2 (ABB52070), *Helianthus annuus* (AHA36972), *Gentiana lutea* (ACF21785), *Solanum lycopersicum* (ABD67160), *Capsicum annuum* (NP_001311497), *Lilium hybrid* division I (BAH10588), *Zea mays* (ALF62627), *Sorghum bicolor* (AAX56323), *Triticum aestivum* CPAN (CAX36915), *Triticum aestivum* Chinese (ACI04664), *Auxenochlorella protothecoides* (ADR82202). The numbers above each node represent the support of the node as a result of a bootstrapping of 500 replicates. The 0.05 scale bar represents the number of substitutions per site over time.

highlighted in gray boxes). Three of them are in the putative plastidial signal peptide and importantly, four of these amino acids (Ile340, Thr415, Ala490 and Lys508) are located within the conserved ζ-carotene desaturase motif (which comprises from amino acid 57 until amino acid 530), which is a putative essential motif for the ζ-carotene desaturase activity (predicted by MEGA Software).

To assess the degree of conservation of ZDS protein in carrot in comparison with the ZDS protein in other species, a boot-strapped neighbor-joining phylogenetic tree was generated using 21 predicted or characterized ZDS sequences (Fig. 2b). As expected, the tree grouped monocots and eudicots as two main clades. Within the eudicots clade, Asterid and Rosid groups share a more recent common ancestor. *D. carota* ZDS1 and ZDS2 group together in a monophyletic clade with ZDS of *Helianthus annuus* (sunflower) that diverged recently from ancestral ZDS enzymes [39]. Interestingly, two out of the four potentially essential amino acids mentioned above, Thr415 and Lys508 are conserved in all the 20 analyzed sequences, and only different in ZDS2 from *D. carota* (Supplemental Fig. 1). Although, the first amino acid change cannot be ruled out, this analysis suggests that these two amino acids could be essential and therefore relevant candidates for further analysis.

3.2. Transcript and protein expression analysis of ZDS1 and ZDS2 in carrot leaves and roots

An increment in transcript levels of carotenogenic genes has been reported during de-etiolation, fruit ripening and flower development, correlating with an increase in carotenoid levels [64–69]. In carrot, the expression pattern of most carotenogenic genes correlates with a rise in carotenoid content during storage root development [28–30,36]. In order to make direct comparison between the expression of *ZDS1* and *ZDS2* in carrot roots and leaves, we re-analyzed the transcriptional data reported in Fuentes et al. [30]. The expression profiles of *ZDS1* and *ZDS2* in both, leaves and storage roots during carrot development were dissimilar. Specifically, in leaves, the transcript levels of *ZDS1* and *ZDS2* showed a significant reduction from four- to eight-week old plants. Afterwards, *ZDS2* levels increased more than seven times in 12-week old plants, whilst *ZDS1* levels remained constant. In roots, *ZDS1* and *ZDS2* levels were similar and did not exhibit alterations between weeks 4 and 8. Interestingly, only *ZDS2* exhibited a significant 5-fold increase in 12-week old plants (Fig. 3a). Thus, the differences in the expression profile between leaves and roots suggested a differential regulation for each gene during carrot development. In terms of proteins, by means of a polyclonal antiserum raised against carrot ZDS1-MBP, we observed the presence of ZDS protein(s) in leaves and roots, especially in the later stages of carrot development (Fig. 3b). An expected single 63 kDa band for ZDS protein(s) was obtained in leaves. Surprisingly, multiple bands ranging from 55 kDa to 63 kDa were observed exclusively in storage roots (Fig. 3b). This could be due to a degradation resulting in a shorter, and probably nonfunctional, version of ZDS. However, considering that the multiple bands were present exclusively in roots at 8- and 12-weeks old, it is feasible to ascribe this to an organ-specific post-translational modification associated with root development.

3.3. Post-transcriptional gene silencing of ZDS genes in Daucus carota

In order to evaluate the functionality of *ZDS* genes in carrot, we aimed to trigger post-transcriptional gene silencing (PTGS) as this approach has proved successful in demonstrating the molecular functionality of *LCYB1* in this plant [36].

Epicotyls transformed with pASZ1 did not induce the generation of embryos when using antibiotic to select transformed seedlings. In order to increase the chances of obtaining transformants, the explants were exposed to dim light conditions and the antibiotic was removed during the first eight weeks, when the pre-embryonic tissue is generated. This approach generated both transgenic but also non-transgenic seedlings, which were discriminated by means of PCR analyses. Transgenic seedlings exhibited a dwarf phenotype and a delayed development in comparison with wild-type plants. Nonetheless, once they reached maturity, they developed a normal but paler-orange storage root (Fig. 4a). Transcript level assessment determined by qRT-PCR revealed that all three transgenic lines had remarkably lower *ZDS1* levels in comparison with wild-type plants, reaching silencing levels of 90 % or above (Fig. 4b). Interestingly, transcript levels of *ZDS2* and *PSY2* genes were also reduced to a similar extent reaching also about 90 % of silencing. *PSY2* was selected among the other key carotenogenic genes, as its transcript abundance was affected after PTGS of *LCYB1* in carrot [36]. Consistently with the reduction in the transcript levels, carotenoid content exhibited a reduction in all three transgenic lines in both leaves and roots (Fig. 4c). Analysis in leaves revealed that all transgenic lines

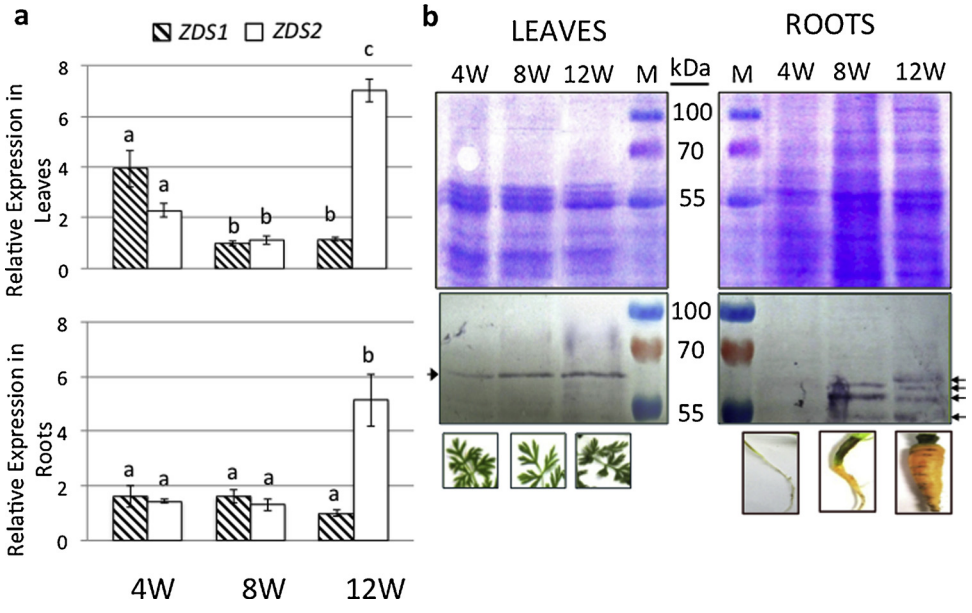

Fig. 3. Transcript and protein expression of *ZDS1* and *ZDS2* in leaves and roots during *D. carota* development. (a) Transcript levels of *ZDS1* and *ZDS2* in leaves and storage roots of 4-, 8- and 12-week-old plants were determined by using gene-specific primers to amplify fragments of the 5′UTR region of each gene. Different letters indicate significant differences between developmental stages ($P \leq 0.05$). *Ubiquitin* was used as normalizer. The gene in the developmental stage with the lowest expression value was used as calibrator; *ZDS1* at 8 weeks and *ZDS1* at 12 weeks in leaves and roots, respectively. **(b)** The detection of ZDS protein(s) was carried out by using a polyclonal anti-carrot ZDS antisera and 40 µg of total protein extracted from leaves and roots of 4-, 8- and 12-week-old plants. Upper panels show Coomassie-stained gels, middle panels western blots and lower panels show representative pictures of each organ corresponding to the weeks of development. The arrow in leaves indicates the expected size of ZDS proteins (63 kDa). In roots, different isoforms ranging from 55 kDa to 63 kDa were detected (arrows). M: Pre-stained molecular weight protein marker.

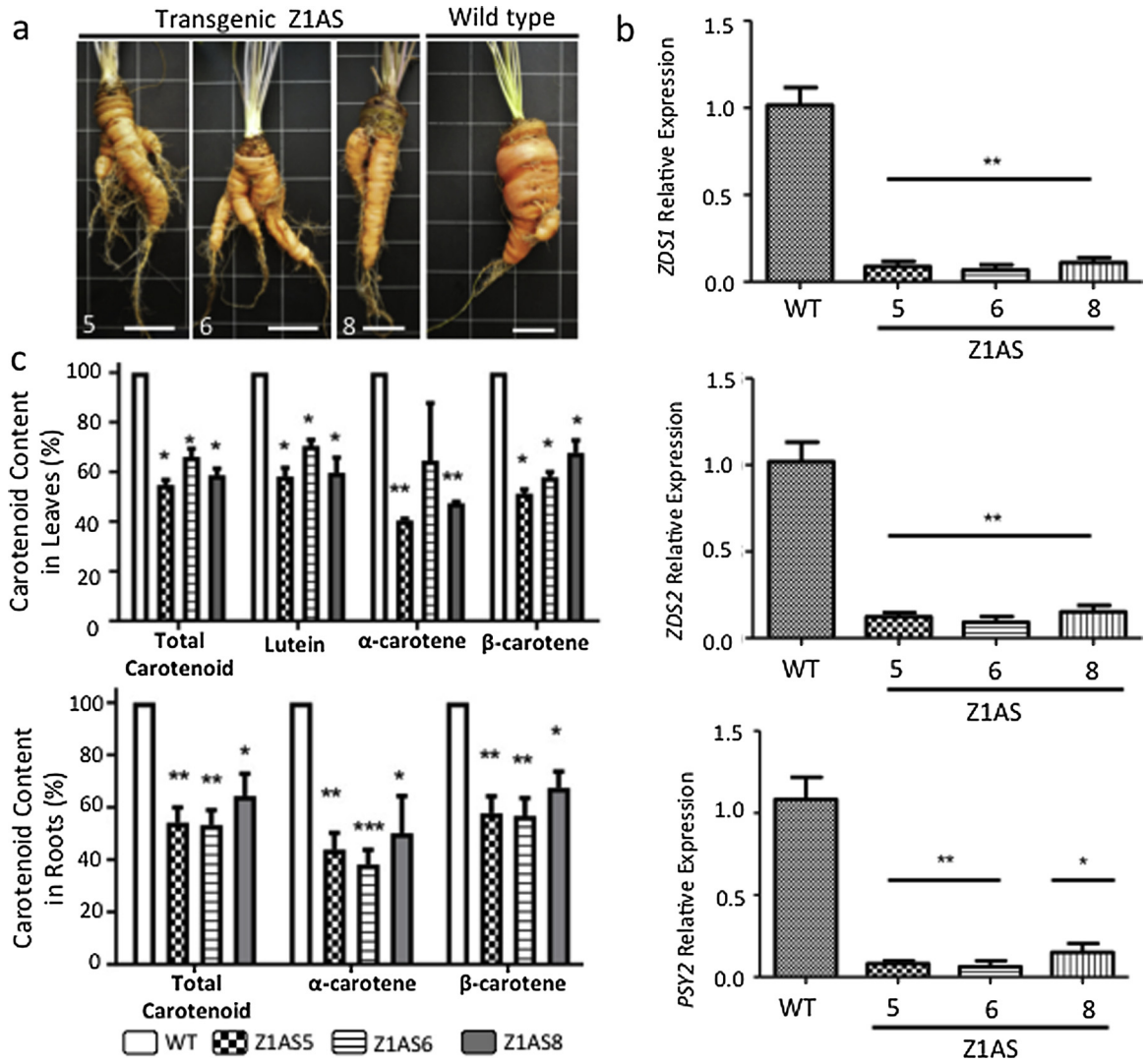

Fig. 4. Post transcriptional gene silencing of *ZDS1* in carrot. (a) Phenotype of 12 weeks-old fully-developed storage roots of transgenic lines Z1AS5, Z1AS6, Z1AS8 and wild-type plants. Scale bar = 3 cm. **(b)** qRT-PCR analysis of *ZDS1*, *ZDS2*, and *PSY2* in wild-type (WT) and transgenic lines (Z1AS5, Z1AS6 y Z1AS8) using actin as normalizer. WT condition was used as calibrator. Significant differences are indicated with stars (* P < 005; ** P < 001). **(c)** Carotenoid composition in leaves and roots of 12 weeks-old WT and transgenic lines with reduced levels of *ZDS1* expression.

reached 60 % of total carotenoid content in comparison with wild-type plants. Further analysis of the carotenoid composition showed that lutein reaches also 60 % in all transgenic lines, while α-carotene and β-carotene ranged from 40 %–65 %. Line Z1AS5 exhibited the stronger decrease reaching 40 % and 50 % of α-carotene and β-carotene respectively. Line Z1AS6 had about 60 % of β-carotene in comparison with wild-type plants and although the reduction in α-carotene was not significant, it showed a consistent trend with lower levels of this pigment. Z1ASS8 line had about 50 % of α-carotene content and a reduction of 30 % in β-carotene levels respect to wild-type plants (Fig. 4c). Carotenoid content in roots showed a similar reduction pattern in all transgenic lines. Total carotenoid levels and β-carotene reached on average 60 % in comparison with wild-type plant whereas the decrease was slightly higher in α- carotene reaching 40 %–50 % on average between the transgenic lines (Fig. 4c). Considering that carotenoids also act as photoprotectors, and carotenoid-deficient mutants present high photooxidative stress [70], we assess the oxidative (H_2O_2) status of the silenced *ZDS1* carrots plants, through a physiological analysis using DAB staining. These experiments showed that leaves of Z1AS transgenic plants had higher levels of H_2O_2, suggesting that transgenic plants were stressed (Supplemental Figure S2). This is consistent with the lower carotenoid content of the *ZDS1* RNAi transgenic plants.

Then, we carried out PTGS to assess the *in vivo* role of ZDS2 in carrot. Epicotyls transformed with pASZ2 developed normal embryos and carrot plants that were obtained under antibiotic selection (Fig. 5a). By means of qRT-PCR it was determined that all four independent lines (Z2AS1, Z2AS2, Z2AS3 and Z2AS6) had a specific and significant reduction in *ZDS2* transcript levels (by between 70 % and 98 %) whereas the expression of *ZDS1* and *PSY*2 remained constant and similar to WT plants (Fig. 5b). Carotenoid quantification showed that there were no significant differences in the content of total carotenoids, lutein, α-carotene or β-carotene in leaves between the silenced lines and the wild-type plants (Fig. 5c). In roots, only the α-carotene level in Z2AS2 showed a significant increase (Fig. 5c). Considering that a decrease in the transcript levels of *ZDS2* does not affect the normal development and carotenoid accumulation in carrot, it suggests that *ZDS2* is not an essential gene and it may not be involved in the carotenogenic pathway or *ZDS1* may be capable of compensating the deficiency in *ZDS2*.

For a further understanding of the role of ZDSs in carrot, the simultaneous PTGS of *ZDS1* and *ZDS2* was performed. Stems from Z2AS1 transgenic carrot plants silenced for *ZDS2* were transformed with pASZ1 in order to force the decrease in *ZDS1* expression in transgenic

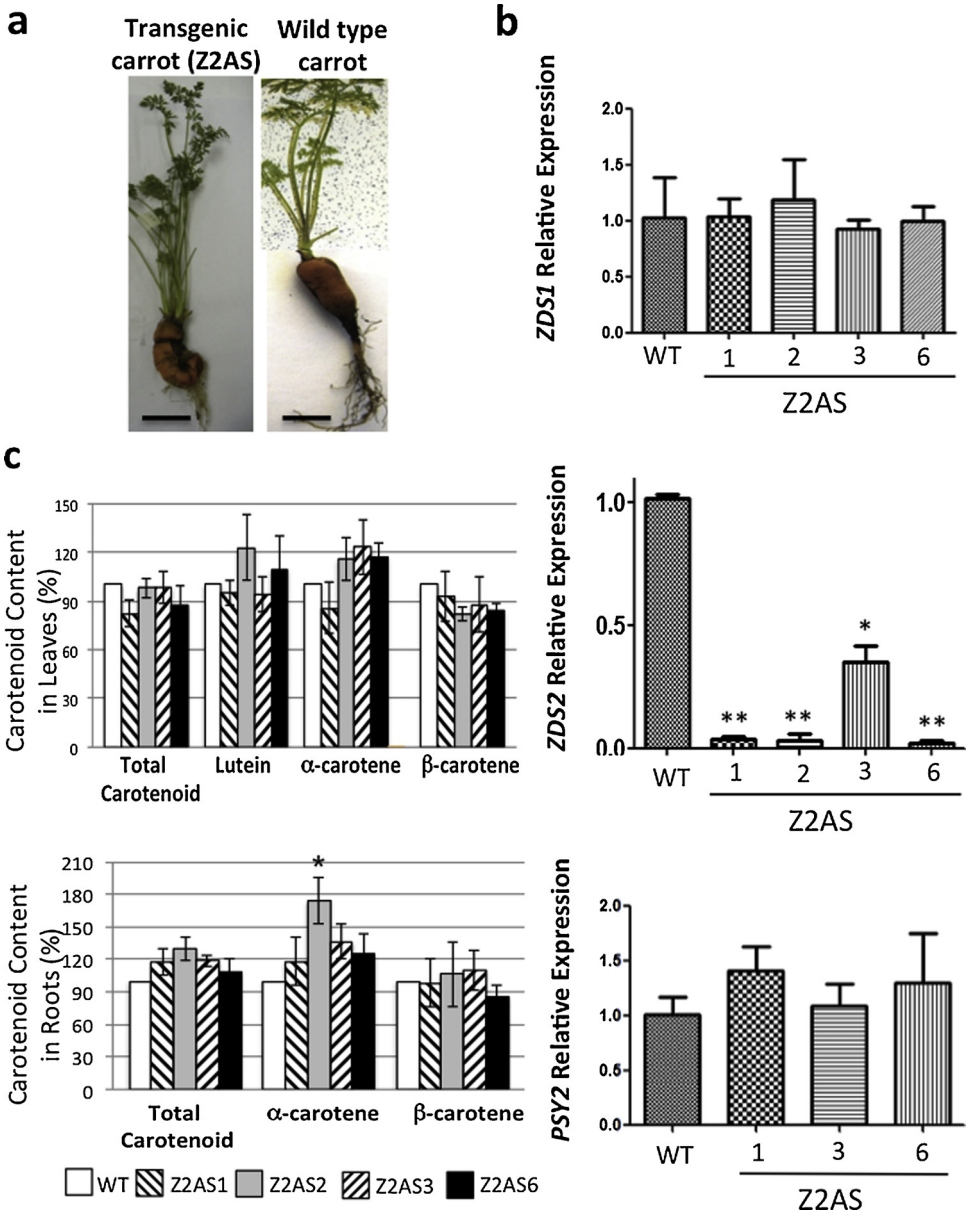

Fig. 5. Post transcriptional gene silencing of *ZDS2* in carrot. (a) Phenotype of representative 12 weeks-old transgenic lines transformed with pASZ2 (left panel) and wild-type plants (right panel). Scale bar = 2 cm. **(b)** qRT-PCR of *ZDS1*, *ZDS2* and *PSY2* in wild-type (WT) and transgenic lines (Z2AS1, Z2AS2, Z2AS3 and Z2AS6), using *18S* as normalizer. WT condition was used as calibrator. Significant differences are indicated with stars (* P < 005; ** P < 001). **(c)** Carotenoid composition in leaves and roots of 12 weeks-old WT and transgenic lines with reduced levels of *ZDS2* expression.

lines. Similar than during PTGS of *ZDS1* alone, embryos were viable only when somatic embryogenesis was carried out in dim light and crucially in the absence of antibiotic. Under these conditions, four Z2Z1-RNAi plantlets (Z2Z1AS1, Z2Z1AS2, Z2Z1ASS3 and Z2Z1AS6) were obtained after longer time period in culture than WT (Fig. 6a). Moreover the phenotype of these plantlets was similar to the phenotype exhibited by the Z1AS lines, at the same developmental stage; including a negligible development of leaves, an incipient and weak root, and a length no longer than 3 cm, which is considerably shorter in comparison with a wild-type plant of a similar age grown in comparable conditions, which reach 12 − 14 cm (Fig. 6a).

At this stage of development, and due to limited amount of tissue, molecular analyses were only possible for *ZDS1*, *ZDS2* and *PSY2* genes in Z2Z1AS1 and Z2Z1AS4 lines. *PSY2* gene was selected considering that previous studies in carrots, overexpressing *LCYB1*, revealed a tighter correlation, especially in carrot root, between its transcript abundance and the total carotenoid content in comparison with PSY1 [36]. Moreover, PSY has been thoroughly reported as a key regulatory gene in the carotenogenic pathway [14,43,65,68,71,75,76]. Thus, in order to have a consistent analysis, *PSY2* was also selected as a representative/marker gene for the Z1AS and Z2AS transgenic lines as well.

In these lines, the level of silencing of *ZDS2* remained at 98 % and *ZDS1* levels were reduced by 59 % and 89 % in Z2Z1AS1 and Z2Z1AS4 lines, respectively, showing a robust correlation between the phenotype and the transcript level of *ZDS1* (Fig. 6a and b). Interestingly and similar to the Z1AS plants, Z1Z2AS lines also showed a decrease of about 90 % in the transcript level of *PSY*2 in comparison to wild-type plants. Therefore, a similar phenotype and expression level of *ZDS1*, *ZDS2* and *PSY2* was obtained for Z1AS and Z1Z2AS. This result strongly suggests that *ZDS1* is required and essential for the initial steps of carrot development, and a reduction in its expression levels affects the expression of the key carotenogenic gene, *PSY*2 together with a reduction in the carotenoid level.

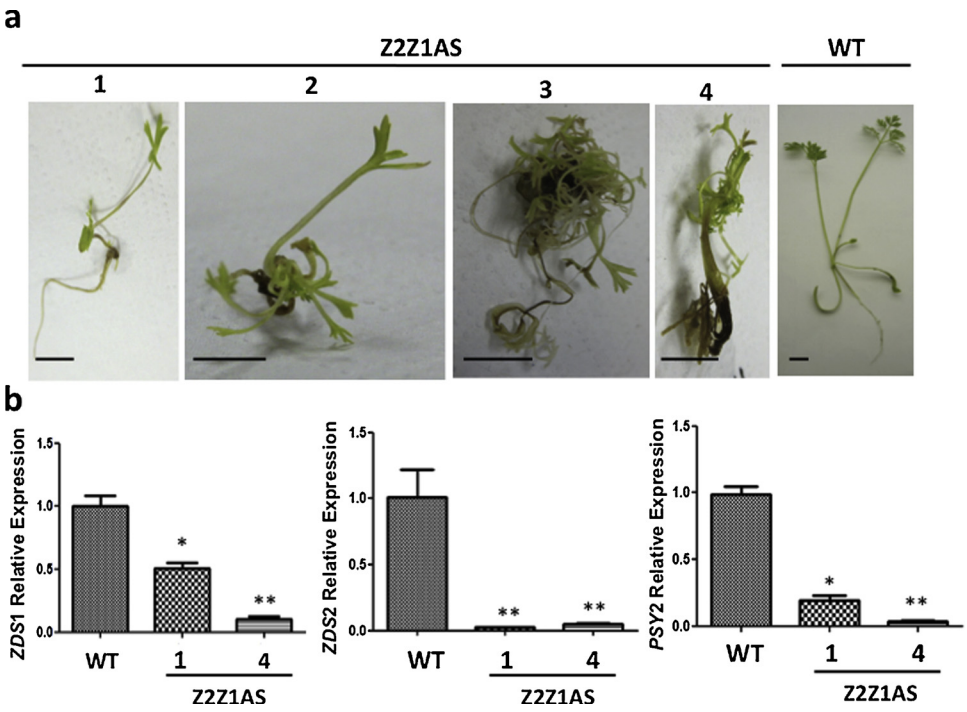

Fig. 6. Simultaneous post transcriptional gene silencing of *ZDS1* and *ZDS2* in carrot. **(a)** Phenotype of 4 weeks-old double transgenic lines (Z2Z1AS1, Z2Z1AS2, Z2Z1AS3, Z2Z1AS4) and WT carrots obtained through somatic embryogenesis. Scale bar = 1 cm. **(b)** qRT-PCR of *ZDS1*, *ZDS2* and *PSY2* in WT and transgenic lines (Z2Z1AS1 and Z2Z1AS4) of carrot, using *18S* as normalizer. WT condition was used as calibrator. Significant differences are indicated with stars (* P < 005; ** P < 001).

3.4. *ZDS1* subcellular localization and ectopic expression in N. tabacum

Taken into account that *ZDS1* has an essential role in carrot, we evaluate the subcellular localization of ZDS1 and its functionality by overexpressing in a heterologous plant. As shown in Fig. 7a, the fluorescence from the ZDS1:GFP fusion protein exhibited a distribution pattern with defines speckles grouped in discrete regions within the cells, which suggests ZDS1 is a plastid-localized protein. On the contrary, the control of 35S:GFP showed a continuous fluorescence distribution along the entire cell, which agrees with the soluble cytosolic localization described for GFP (Fig. 7b). A discrete fluorescence pattern was also observed in the positive control (35S:recA:YFP, Fig. 7c), which has plastidial localization.

Additionally, two transgenic tobacco lines that express *ZDS1* were generated. RT-PCR analyses showed that the transgene was expressed in Z1OEL1 and Z1OEL11 but not in the wild-type plants (Supplemental Figure S3a). The polyclonal ZDS antibody recognized faintly the endogenous ZDS in tobacco (Supplemental Figure S3b), which is expected as these two proteins share 82 % amino acid identity. However, the signal obtained for both transgenic plants was much stronger, confirming that these tobacco lines had higher levels of carrot ZDS. Ultimately, by means of HPLC it was determined that the transgenic

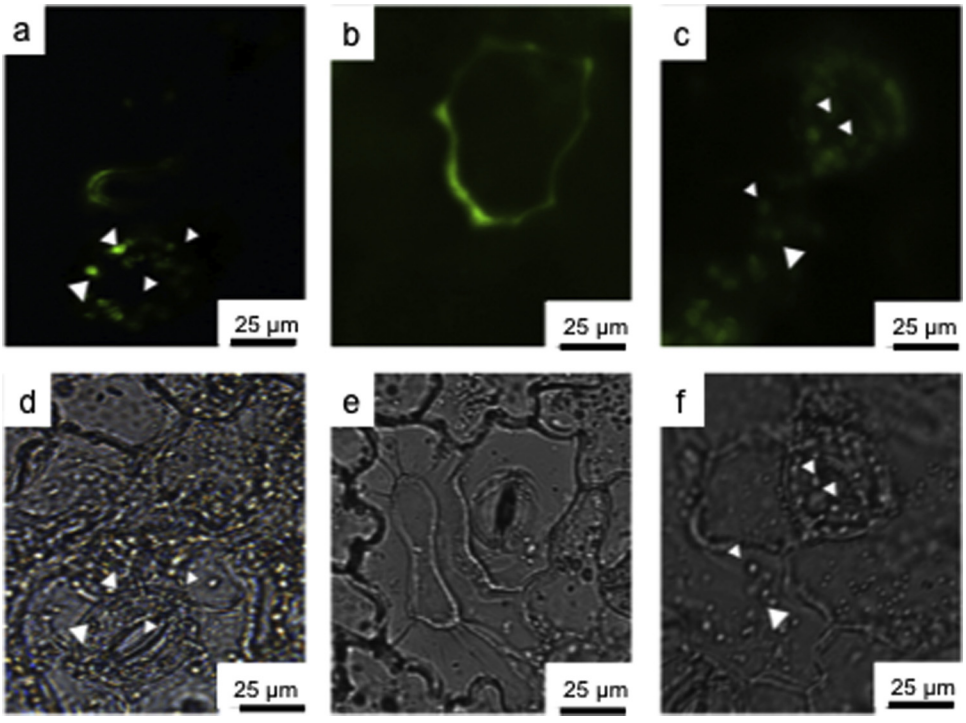

Fig. 7. Subcellular localization of ZDS1:GFP. Transient transformation with *A. tumefaciens* of two-month epidermal tobacco leaves with fluorescent-tagged proteins. Agroinfiltration with **(a)** 35SCaMV:ZDS1:GFP exhibited a punctuate fluorescence indicative of a plastidial localization of ZDS1:GFP **(b)** Control assay with 35SCaMV:GFP reveled the expected cytosolic localization of soluble GFP. **(c)** Transformation with 35SCaMV:recA-YFP (positive control), which has been characterized as a protein with plastidial localization. **(d, e, f)**. Bright field images of a, b, c, respectively. All images were taken with 40x and fluorescence was observed after excitation at 489 nm.

lines presented a significant increment in total carotenoids and chlorophyll contents (Supplemental Figure S3c). All together, these results support the findings that *ZDS1* codifies for a functional plastid-targeted enzyme that promotes carotenoid synthesis in heterologous systems.

4. Discussion

4.1. Sequence and expression analysis of carrot ZDS1 and ZDS2

In carrot, two ZDS have been identified, *ZDS1* (DQ222430) and *ZDS2* (DQ192189) which map to different linkage groups; *ZDS1* maps to carrot linkage group 4 (chromosome number 2), while *ZDS2* maps to group 5 (chromosome number 7), together with *ZEP* (chromosome number 7) [31,33]. As expected, due to the high identity of the amino acid sequences between ZDS1 and ZDS2 (91 %), both grouped together in a phylogenetic tree that includes ζ-carotene desaturases from other plant species. This suggests that one of the genes may have originated from the other in a recent duplication event. Similar evidence supporting the duplication hypothesis as a relevant mechanism in carrot evolution includes examples of other duplicated genes such as phytoene synthase (*PSY1*, *PSY2*), lycopene β-cyclase (*LCYB1*, *LCYB2*) [36] and β-ring carotene hydroxylase (*CHXB1*, *CHXB2*) [31]. Additionally, the first genome assembly of the orange carrot has shown that several gene families have expanded as a consequence of gene duplication [33].

Sequence analysis led us to identify that there are four conserved amino acids in the ζ-carotene desaturase motif between ZDS1 and the functional AtZDS [40,63], but different from the predicted ZDS2 sequence. Thus, a different functionality between the carrot paralog enzymes ZDS1 and ZDS2 may be ascribed to these differences in the amino acids. The expression profile of both genes in leaves and roots during carrot development is different but not directly associated with ZDS protein abundance (Fig. 3) and carotenoid accumulation [30]. Indeed, as expected, ZDSs protein abundance in leaves and roots agrees with carotenoid level, especially in the root at 8 and 12 weeks when the storage root begins to swell and to accumulate α-carotene and β-carotene [30]. However, both genes are expressed in leaves and storage roots, in contrast to ZDS paralogs in *Malus domestica*, another of the few plants that presents two ZDS paralogs. In apple, *MdZDS1* is expressed in flowers, buds, senescing leaves and fruitlet whilst *MdZDS2* expression is higher in fruits and buds [38]. Moreover, *MdZDS1* (AF429983) expression is higher in the carotenoid enriched variety 'Aotea', whilst *MdZDS2* (GO546818) expression is higher in the M9 rootstock and 'Royal Gala', varieties with low levels of carotenoids [38].

Regarding ZDS protein accumulation, the several bands exhibited exclusively in storage roots at 8- and 12-weeks old could be due to ZDS isoforms with a differential degree of processing related with the subcellular dynamics of carotenogenic protein. The multiple bands were obtained accordingly with carotenoid accumulation, and not in leaves of carrot or in transgenic tobaccos overexpressing *ZDS1*. Multiple bands were also reported in *A. thaliana* overexpressing PSY [71]. Therefore, it is feasible to propose that this phenomenon is an organ-specific post-translational modification associated with chromoplast differentiation and/or carotenoid accumulation in the dark-grown root during storage root development.

4.2. Transgenic carrot plants with reduced expression level of ZDS2 present a normal phenotype

We have previously established that either silencing or over expressing a carotenogenic gene in carrot has an impact on several parameters such as the carotenoid composition and gene expression [36]. Therefore, PTGS strategy was selected to generate *ZDS1* and *ZDS2* knock-down mutants instead of knock-out mutants, which might be lethal, as was determined in other plant models [39,43]. Since carotenoids in plants have a fundamental role chloroplasts as structural components of the photosynthetic apparatus but also as photosynthetic and photoprotective pigments [8,72,73], it is expected that mutants exhibited several issues if not lethal in homozygosis [40]. Sunflower and maize ZDS mutants, named *non dormant-1* (*nd-1*) and *viviparous9* (*vp9)* respectively, are only viable under weak light, exhibit an accumulation of ζ-carotene and also a viviparous phenotype [39,43]. The *Oryza sativa* mutant phs2 accumulates ξ-carotene in light-grown seedlings [74], as do maize ZDS vp9 mutants [43,75]. The *A. thaliana spc1* mutant presents a spontaneous cell death phenotype. The stronger allelic variant, *spc1-2* presents a complete arrest of development after germination leading to a lethal phenotype [40].

Lines with 98 % of PTGS for *ZDS2* exhibited normal development from somatic embryogenesis until adult plants and did not reveal any abnormal phenotype in comparison to wild-type carrots (Fig. 4a). Moreover, the carotenoid composition and the expression of endogenous *ZDS1* and *PSY2* in these transgenic plants did not change with respect to wild-type plants, suggesting that *ZDS1* may compensate the deficiency of *ZDS2*, thus denoting a less prominent role of *ZDS2* in embryogenesis, carrot development and carotenoid synthesis. However, considering that the transcript abundance of DcZDS2, increases at 12-weeks, it is feasible to speculate that DcZDS2 could have a role in a fully developed carrot, perhaps not directly related with the carotenoid biosynthesis, but associated with a broader physiological process involving carotenoid such as the stress response [76].

Considering that ζ-carotene desaturase activity has been confirmed for *A. thaliana* ZDS [63], the four putative crucial amino acids within the ζ-carotene desaturase conserved motif between *A. thaliana* and *D. carota* ZDS1 but different from *D. carota* ZDS2, may interfere with the ZDS2 activity and could be evaluated by site directed mutagenesis. Such analysis has been performed for phytoene synthase in tomato [77], and a similar approach in carrot would elucidate whether functional differences between ZDS1 and ZDS2 are a consequence of these amino acid substitutions.

4.3. ZDS1 codifies for a functional enzyme required for plant development and carotenoid synthesis in D. carota

The plant desaturases, PDS and ZDS are membrane-associated enzymes that are part of a multienzymatic complex [7]. In most plant species, ZDS is encoded by a single gene [40] and the proteins are localized in both the envelope membrane and stroma of chloroplasts [2,78,79]. In this work, we demonstrated the plastidial localization of ZDS1 and its *in vivo* functionality by the expression of *ZDS1* in tobacco, which confirms that *ZDS1* from *D. carota* is functionally active and plays a role in the biosynthesis of carotenoids *in planta* (Fig. 7 and Supplemental Figure S3).This result was expected, as a putative N-terminal signal peptide for targeting to chloroplasts was predicted for carrot ZDS1 using ChloroP/TargetP, with a potential cleavage site between amino acids 20 and 21. The location and length of the signal peptide of carrot ZDS is consistent with that of other plant ZDSs (normally between the first 30–60 aa) such as sunflower [39] and *Arabidopsis* [80] among others.

In our initials attempt to silence *ZDS1* in carrot by means of somatic embryogenesis, embryos were arrested at the globular stage, but any of them originated plants. This result is consistent with the strong *spc1-2* allelic variant obtained in *A. thaliana*, in which the loss of function of the *ZDS* encoded by ZDS/CLB5/SPC1/PDE181 is lethal [40] and exhibits dramatic alterations in leaf morphology and chloroplast biogenesis at a very early stage of development [41]. More recently, a viviparous mutant in maize was identified as a result of a mutation in the *ZDS* gene [44]. Considering that carotenoids are precursors for ABA, an essential hormone for both carrot somatic embryo development and dormancy, which is significantly accumulated during embryo maturation process [81,82], it was reasonable to expect that exogenous application of this hormone would restore the normal development of the antisense *ZDS1* transformed embryos. However, even after treatments with different concentrations of ABA (1–500 μM) [83,84], the embryo

remained arrested at the initial globular stage (data not shown), suggesting that instead of ABA, an upstream molecule could be involved in blocking the somatic embryogenesis at an early stage. According to this proposal, Avendano-Vazquez et al. [41] present biochemical and genetic evidence that an apocarotenoid generated by carotenoid cleavage dioxygenases (CCD4) from phytofluene or ξ-carotene is responsible for altering early leaf development.

Considering these initial results, we assess several alternatives in order to overcome the blockage during the somatic embryogenesis. Thus, we obtained transgenic lines with reduced levels of *ZDS1* exclusively when the somatic embryogenesis process was carried out in favorable conditions for the plant cell development, including media without antibiotic and low light conditions, which suggest that ZDS1 is required for the initial steps of seedling development, similarly that was proposed by Avendaño et al. 2014 [41].

Pigments analysis of Z1AS transgenic plants showed a trend between the decrease in the carotenoid content in leaves and storage roots and the reduction in the transcription levels of *ZDS1*, *ZDS2* and *PSY2*. These results indicate that the carotenogenic pathway was severely affected by the PTGS of *ZDS1* with a concomitant decrease in the transcript levels of the paralog gene and *PSY2*, a key and highly regulated carotenogenic gene [2,3]. This represent robust evidence supporting that *ZDS1* gene has a predominant role in the carotenogenic pathway. Although, the decrease in the carotenoid content of about 50 % was not as dramatic as expected considering PTGS in transgenic Z1AS lines reached about 90 %. Similar result was reported in transgenic carrot plants with reduced levels of *LCYB1* gene [36]. Data showing that the expression of *ZDS2* was also reduced in *ZDS1* RNAi lines, led us to consider that another enzyme is carrying out the desaturation of ζ-carotene and therefore compensating the deficit in either ZDS1 and/or ZDS2. *In vitro* analysis showed that ZDS is capable of carry out the desaturation normally associated with PDS [43]. Hence, it is feasible to consider that another desaturase could carry out the ζ-carotene desaturation. Blast analysis, using the available genomic sequence of *D. carota* in the Phytozome Plant Comparative Genomics portal (https://phytozome.jgi.doe.gov/pz/portal.html) allows us to rule out the possibility of a putative third ZDS enzyme.

In terms of stress response, carotenoids also act as photoprotectors, and carotenoid-deficient mutants present high photooxidative stress [70]. The allelic variant *spc1-1* has a weaker phenotype and possesses a pigment defective embryo, leaf bleaching, an increment in ROS and mosaic cell death [40]. Moreover, a direct link between *ZDS* and ABA was observed in *Ipomea batatas* L, sweetpotatoe [85]. Authors established that *IbZDS* overexpression led to a significant up-regulation of key carotenogenic genes and increment in ABA content which correlated with an enhance tolerance to salt [85]. Here we show that the oxidative status of the silenced *ZDS1* lines is higher than wild type plants, which is consistent with the lower carotenoid content (Supplemental Figure S2).

To give further insight on the pivotal role of ZDS1 in carrot, a previously-silenced ZDS2 adult line was transformed with *ZDS1*-RNAi, and similar to the Z1AS, embryos had to be grown under low light intensities to promote the regeneration of silenced seedlings probably due to the lower photo-oxidative damage produced in dim light conditions. Transgenic plantlets presented aberrant phenotypes that correlated positively with the degree of *ZDS1* silencing and begin with embryo arrest and extend to leaf and plant development, similar with the initial stage of Z1AS. Arabidopsis *ZDS* mutants are also affected in leaf anatomy and chloroplast biogenesis [40] and supports the presence of one or more plastid-derived signals that regulate nuclear genes involved in embryo and leaf development. Therefore, considering that Z1AS as well as Z1Z2AS presented a similar reduction in *ZDS1*, *ZDS2* and *PSY2* expression as well as abnormalities at the initial stages of plant development, let us to propose that *ZDS1* has a major role in the synthesis of carotenoids and in carrot embryo development.

5. Conclusions

Our results indicate that *ZDS1* is required for carrot development and plays an essential role in the carotenoid biosynthetic pathway. Moreover, *ZDS2* appears to have a non-essential or complementary role in carrot physiology.

Funding source

This work was financed by the International Foundation for Science (IFS C4784-1) and the National Fund for Scientific and Technological Development, Chile (Fondecyt 1130245 and 1180747- C.S.).

Authors contribution statement

CS and CF conceived and designed the experiments. CF performed molecular and biochemical analyses of Z2AS, Z1Z2AS and Z1OE lines. LMA carried out the subcellular localization of ZDS1. FD and DA generated Z1AS lines and AU performed molecular and biochemical analyses of Z1AS. GW obtained ZDS antibody. CF and CS wrote the manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of commercial or financial relationships that could be construed as potential conflict of interest.

Acknowledgements

We would like to thank Dr María Rosa Bono and Dr Leonardo Vargas (Department of Biology, Faculty of Science, University of Chile) for the production of the anti-ZDS antisera.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2019.110327.

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