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RESEARCH PAPER

Expression of carotenoid biosynthesis genes during carrot root development

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Abstract

Carotenogenesis has been extensively studied in fruits and flower petals. Transcriptional regulation is thought to be the major factor in carotenoid accumulation in these organs. However, little is known about regulation in root organs. The root carotenoid content of carrot germplasm varies widely. The present study was conducted to investigate transcriptional regulation of carotenoid biosynthesis genes in relation to carotenoid accumulation during early carrot root development and up to 3 months after sowing. HPLC carotenoid content analysis and quantitative RT-PCR were compared to quantify the expression of eight genes encoding carotenoid biosynthesis enzymes during the development of white, yellow, orange, and red carrot roots. The genes chosen encode phytoene synthase (*PSY1* and *PSY2*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS1* and *ZDS2*), lycopene ε-cyclase (*LCYE*), lycopene β-cyclase (*LCYB1*), and zeaxanthin epoxidase (*ZEP*). All eight genes were expressed in the white cultivar even though it did not contain carotenoids. By contrast with fruit maturation, the expression of carotenogenic genes began during the early stages of development and then progressively increased for most of these genes during root development as the total carotenoid level increased in coloured carrots. The high expression of genes encoding LCYE and ZDS noted in yellow and red cultivars, respectively, might be consistent with the accumulation of lutein and lycopene, respectively. The results showed that the accumulation of total carotenoids during development and the accumulation of major carotenoids in the red and yellow cultivars might partially be explained by the transcriptional level of genes directing the carotenoid biosynthesis pathway.

Key words: Carotenoid accumulation, *Daucus carota*, gene expression, root development.

Introduction

The regulation of carotenoid biosynthesis has been intensively investigated in crops. Many studies have dealt with carotenogenesis during development and/or amongst cultivars showing different organ colours. Such investigations have been conducted in fruits like tomato (Giuliano *et al*., 1993; Pecker *et al*., 1996; Ronen *et al*., 1999), pepper (Hugueney *et al*., 1996; Ha *et al*., 2007), *Citrus* (Kato *et al*., 2004), apricot (Marty *et al*., 2005), Japanese apricot (Kita *et al*., 2007), and in petals of flowers like *Gentiana lutea* (Zhu *et al*., 2002), marigold (Moehs *et al*., 2001), chrysanthemum (Kishimoto and Ohmiya, 2006), and orchid species (Hieber *et al*., 2006). The only study on underground organs was focused on potato tubers (Morris *et al*., 2004). Very little is therefore known about the control of carotenogenesis in roots. Carrot (*Daucus carota* ssp. *sativus*) is one of the rare species that accumulates large amounts of carotenoids in roots. Differences in carotenoid composition result in white, yellow, orange, and red root colours (Surles *et al*., 2004). Carrot is therefore a good model system to study the regulatory mechanisms underlying carotenoid biosynthesis in root organs.

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Abbreviations: BDV, cv. Blanche demi-longue des Vosges; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; BLR, cv. Bolero; CHXB, β-ring hydroxylase; CHXE, ε-ring hydroxylase; DXP, 1-deoxy-D-xylulose-5-phosphate; EF1α, elongation factor 1 α; FW, fresh weight; GGPP, geranylgeranyl diphosphate; HPLC, high performance liquid chromatography; LCYB, lycopene β-cyclase; NTR, cv. Nutrired; PDS, phytoene desaturase; PSY, phytoene synthase; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction; YLS, cv. Yellowstone; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase.

Carotenoids play a major role in plants. In green tissues, carotenoids located in chloroplasts are involved as accessory pigments in light-harvesting antennae (Lee and Thornber, 1995) and as photo-oxidation protectants (Ma *et al*., 2003). In flowers and fruits, carotenoid accumulation in chromoplasts gives rise to colours that attract animals to enhance pollen and seed dispersal (Howitt and Pogson, 2006). Carotenoids are also apocarotenoid precursors which play a role as hormones (e.g. abscisic acid) and are involved in flavours, aromas, defence compounds, and plant development (Giuliano *et al*., 2003).

Carotenoids are synthesized by all photosynthetic organisms (plants, algae, and cyanobacteria) and also by some non-photosynthetic bacteria and fungi (Britton *et al*., 2004). Vertebrates cannot synthesize carotenoids—they have to acquire them from dietary sources for health purposes. Carotenoids are retinal precursors (vitamin A). Deficiency in retinal affects many children throughout the world (xerophthalmia, blindness, and premature death) (Fraser and Bramley, 2004). By their antioxidant properties, intake of carotenoid-rich food can prevent some cancers, UV-induced skin damage, coronary heart disease, cataracts, and macular degeneration (Botella-Pavia and Rodriguez-Concepcion, 2006). Carotenoids are also used as a food supplement for aquaculture-grown salmon, eggs, and poultry production. Carotenoids are utilized as natural pigments for industrial food preparation, cosmetics, and the pharmaceutical industry. In horticultural crops (flowers, fruits, and vegetables), product colour represents an important quality trait, thus enhancing product diversity and market segmentation opportunities.

Carotenoid biosynthesis is now well characterized (Hirschberg, 2001; Fraser and Bramley, 2004; Taylor and Ramsay, 2005; Römer and Fraser, 2005; DellaPenna and Pogson, 2006; Howitt and Pogson, 2006) (Fig. 1). In plants, carotenoids are produced in plastids by nuclear-encoded enzymes (Cunningham and Gantt, 1998). The carotenoid precursor C20-geranylgeranyl diphosphate (GGPP) is synthesized via the plastidial 1-deoxy-D-xylulose-5-phosphate (DXP) pathway (Eisenreich *et al*., 2001). GGPP is also the precursor of gibberellins, plastoquinones, terpenes, chlorophylls, tocopherols, and phylloquinones (Bouvier *et al*., 2005). Colourless phytoene results from the condensation of two GGPP molecules by a phytoene synthase (PSY). Desaturation of phytoene into lycopene via ζ-carotene, the first yellow carotenoid, is catalysed by both phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). Carotenoid isomerase (CRTISO) is involved in the conversion of poly-*cis* lycopene to *trans*-lycopene (Isaacson *et al*., 2002; Park *et al*., 2002). Subsequently, the ends of the linear carotenoid lycopene can be cyclized. The co-ordinated action of lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE) allows synthesis of α-carotene from lycopene. The single action of LCYB leads to β-carotene.

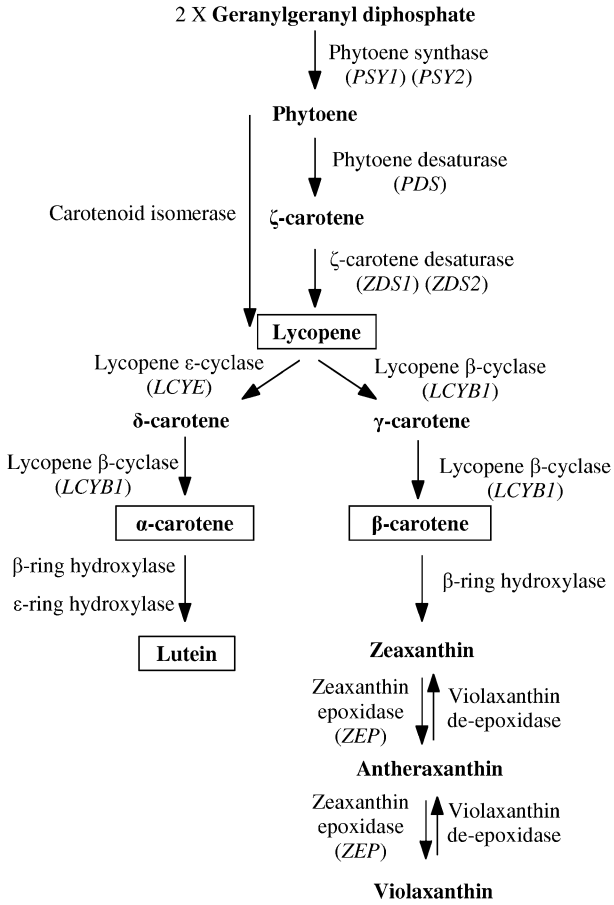

Fig. 1. Carotenoid biosynthetic pathway in higher plants. Boxes indicate main carotenoids found in carrot.

α- and β-carotene can then be hydroxylated into lutein by both β-ring hydroxylase (CHXB) and α-ring hydroxylase (CHXE), and into zeaxanthin by CHXB. Zeaxanthin is then transformed into violaxanthin via antheraxanthin by zeaxanthin epoxidase (ZEP). The final product of the pathway is abscisic acid. Along the pathway, carotenoids could be cleaved by carotenoid cleavage dioxygenases into apocarotenoids (Auldridge *et al*., 2006).

During plant development, two main mechanisms have been described to regulate carotenoid biosynthesis and accumulation in chromoplasts: (i) variation in the abundance of carotenogenic gene transcripts, and (ii) the presence of structures sequestering carotenoids in plastids (Howitt and Pogson, 2006). In tomato, which represents the main model of carotenogenesis in fruits, the huge accumulation of pigments (especially lycopene) during ripening was found to be correlated with a 10–20-fold increase in *PSY1* and *PDS* transcripts (Giuliano *et al*., 1993) and a decrease in *LCYE* and *LCYB* transcripts (Pecker *et al*., 1996; Ronen *et al*., 1999). In mandarin and orange (Kato *et al*., 2004), the switch from β-ε-carotenoids (α-carotene and lutein) to β-β-carotenoids (β-carotene, β-cryptoxanthin, zeaxanthin, violaxanthin) was related to decreased *LCYE*

expression and increased *PSY*, *PDS*, *ZDS*, *LCYB*, *CHXB*, and *ZEP* expression. Precursors from a carotenoid pathway were mobilized from one branch into another. In pepper fruits (Hugueney *et al*., 1996), Japanese apricot fruits (Kita *et al*., 2007), and yellow chrysanthemum flowers (Kishimoto and Ohmiya, 2006), transcriptional regulation of the carotenoid biosynthesis pathway explains the pattern of accumulation of carotenoids during development. Depending on the chromoplast type (Vishnevetsky *et al*., 1999), carotenoids can also be stored in sequestering structures. In *Capsicum*, esterification of xanthophylls was found to be increased during ripening and was thought to be induced during xanthophyll accumulation (Hornero-Mendez and Minguez-Mosquera, 2000).

Mechanisms underlying differences in carotenoid content patterns in different cultivars have also been investigated. Some mutations were shown to directly affect the amino acid sequence of a carotenogenic enzyme. As an example, watermelon yellow- and red-fleshed cultivars exhibited *LCYB* allelic sequence variations (Bang *et al*., 2007). Carotenoid patterns in orange fruits of tomato mutants *Delta* (Ronen *et al*., 1999) and *Beta* (Ronen *et al*., 2000) were shown to result from an alteration in carotenoid biosynthesis gene expression. The 100-fold differential accumulation of total carotenoids in marigold germplasm was related to expression differences in many carotenoid pathway genes (Moehs *et al*., 2001). On the other hand, transcriptional regulation of the carotenoid biosynthesis pathway did not explain differences in colouration between orange and white apricot fruits (*Prunus armeniaca*) (Marty *et al*., 2005). The *Or* gene causing β-carotene accumulation in curds of a cauliflower mutant was shown to act in plastids differentiation (Lu *et al*., 2006).

In carrot root, the colour trait mainly depends on the carotenoid content variability. If carotenoid composition has been investigated in mature roots of various coloured cultivars (Nicolle *et al*., 2004; Surles *et al*., 2004), variations of individual carotenoid content during root development of coloured carrots have not yet been studied. Genetic mechanisms underlying the colour trait have been widely studied. Seven genes involved in colour determination were found (Peterson and Simon, 1986). *Y* and *Y2* genes underlying the orange intensity of xylem/phloem were mapped (Bradeen and Simon, 1998). None of these genes have so far been shown to correspond to a specific carotenoid biosynthesis gene. Phytoene synthesis was shown to be the limiting step of carotenoid accumulation in white wild carrot (Santos *et al*., 2005). Carotenoid content mapping resulted in 20 quantitative trait loci (QTL) (Santos and Simon, 2002) and was completed by minimum gene number estimation (Santos and Simon, 2006). However, the molecular basis of carotenoid accumulation is still unknown in carrot. Twenty-four genes involved in the carotenoid biosynthesis pathway were recently cloned and mapped in carrot and represent good candidates (Just *et al*., 2007).

The goal of the present study was to investigate the role of transcriptional regulation of carotenoid biosynthesis pathway genes in carotenoid accumulation in carrot roots. The transcript levels of eight genes, i.e. *PSY1*, *PSY2*, *PDS*, *ZDS1*, *ZDS2*, *LCYE*, *LCYB1*, and *ZEP*, were analysed during the development of white, yellow, orange, and red carrot roots. In addition, carotenoid root content was assessed. The global kinetics of transcript accumulation and total carotenoid accumulation, and the correlations between transcript levels and the accumulation of specific carotenoids were studied.

Materials and methods

Plant materials

Four cultivars of carrot were selected on the basis of their different root colour: Blanche demi-longue des Vosges (white), Yellowstone (yellow), Bolero (orange), and Nutrired (red). Plants were grown in greenhouses at the Institut National d'Horticulture (Angers, France). Seeds were sown in March in a mix of sand and peat. For each cultivar, eight plants were harvested weekly at 10.00 h. between the 4th and the 9th week after sowing. These early stages were particularly targeted in order to detect the onset of carotenogenesis. Two extra samplings were then added at the 11th and 14th weeks after sowing (i.e. 3 months of development). The sampled roots are depicted in Fig. 2. Root pieces from the eight plants were pooled, frozen in liquid nitrogen, and powdered. Both carotenoids and RNA were extracted from the same root samples.

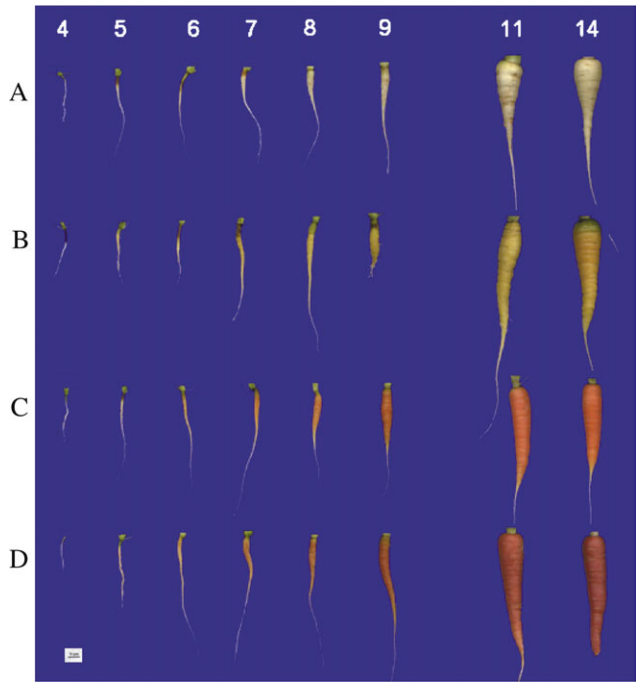

Fig. 2. Photographs of carrot root development stages. Values indicate the number of weeks after sowing. (A) Blanche demi-longue des Vosges (white), (B) Yellowstone (yellow), (C) Bolero (orange), and (D) Nutrired (red).

Carotenoid extraction, quantification, and HPLC analysis

Extraction of carotenoids was carried out in duplicate. The following protocol was adapted from Nicolle *et al*. (2004). Approximately 500 mg of powdered frozen material were mixed with 7 ml $MgCO_3$ 0.57%, 3,5-di-*tert*-butyl-4-hydroxytoluene BHT 0.1% in methanol. After vortexing, 7 ml of 0.1% BHT-containing chloroform was added and samples rested in darkness for 15 min. Seven millilitres of ultrapure water was added followed by vortex mixing and then centrifugation at 236 *g* for 10 min at 8 °C). The lower layer was concentrated under vacuum evaporation and the dry extract was dissolved in 300 µl of acetonitrile/dichloromethane (50:50, v/v) containing 0.1% BHT. Ten µl were injected onto an HPLC column (C18 interchim Silice Optisphere 120A 5 µM ODB). The mobile phase was composed of acetonitrile:dichloromethane:methanol (70:20:10, by vol.) containing 0.1% BHT and 0.1% triethylamine. The flow rate was 1.8 ml min^{-1}. Elution was monitored with a Shimadzu diode-array detector. Compounds were detected at 450 nm (lutein, lycopene, α-carotene, β-carotene) or 400 nm (ζ-carotene) and quantified according to their respective standard curves, except for α-carotene (β-carotene standard curve).

Total RNA isolation

Root and leaf tissues were ground in liquid nitrogen. Total RNA was extracted from frozen powder using a hot-phenol extraction procedure and selective precipitation with 4 M LiCl to remove DNA (Verwoerd *et al*., 1989).

Reverse transcription

Genomic DNA traces were first eliminated by a 30 min RNase-free DNase I treatment at 37 °C followed by enzyme inactivation at 80 °C for 5 min. The reaction mixture contained 2 µg of total RNA, 1 U DNase I, 1.7 mM $MgCl_2$ (Promega), and 1× RT Promega buffer and was adjusted to 15 µl with diethyl pyrocarbonate (DEPC)-treated water. The reverse transcription reaction was then performed by adding 1 mM of each dNTP, 1.3 µM $Oligo(dT)_{15}$ (Promega), 1 U reverse-transcriptase MMLV (Promega), and 1× RT Promega buffer and the final volume was adjusted to 30 µl with DEPC-treated water. Reverse transcription was carried out at 37 °C for 1 h and 80 °C for 10 min. Specific primers of the gene encoding elongation factor 1α (*EF1*α) were designed on both sides of the *EF1*α intron in order to discriminate the size of amplicons generated from cDNA or gDNA templates. The absence of amplification from gDNA in cDNA samples was tested by comparison of PCR products obtained from them and from genomic DNA templates with *EF1*α primers. These PCR tests confirmed the absence of gDNA in all cDNA samples.

Quantitative PCR

For the eight target carotenogenic genes and the *EF1*α housekeeping gene, primers were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA). Specific primers targeting carotenoid biosynthesis genes (*PSY1*, *PSY2*, *PDS*, *ZDS1*, *ZDS2*, *LCYE*, *LCYB1*, and *ZEP)* (Table 1) were designed on the basis of published cDNA sequences (Just *et al*., 2007). Orthologue-specific primers were used to discriminate between the two genes *PSY1* and *PSY2*. A consensus primer pair was used to monitor the expression of both *ZDS1* and *ZDS2* genes.

Real-time PCR reactions were performed in triplicate using about 5 µl of RT product, 0.3 µM of each primer, and 1× SYBR green PCR master mix (Eurogentec) in a 25 µl volume. The PCR mixtures were preheated at 50 °C for 2 min and then at 95 °C for 10 min to activate the HotGoldStar DNA polymerase, followed by 40 amplification cycles (95 °C for 15 s; 60 °C for 1 min). Amplification specificity was verified by a final dissociation of PCR products. The levels of PCR products were monitored with an ABI PRISM 7000 sequence detection system and analysed with ABI PRISM 7000 SDS software (Applied Biosystems). Relative expression levels of the target genes were calculated using the mathematical method of Pfaffl (2001). This method normalizes the Ct data obtained for a target gene with Ct values for a housekeeping gene in order to take into account experimental sample differences (quantity of reverse transcripted mRNA, reverse transcription efficiency, pipetting errors). The *EF1*α housekeeping gene was used as the reference gene after checking the homogeneity of Ct variations with a second housekeeping gene, *AC1*. Standard error was calculated using the three repetitions for each sample.

Results

Analysis of carotenoid levels during root development

Several studies have reported carotenoid levels in mature roots from a range of carrot germplasm (Nicolle *et al*., 2004; Surles *et al*., 2004). To our knowledge, no surveys have been published on carotenoid biosynthesis patterns during carrot root development.

Carotenoid content was analysed by HPLC between the 4th and 14th weeks of development in four cultivars. Carotenoid levels remained under the detection threshold throughout the experiment in cv. Blanche demi-longue des Vosges roots (Fig. 3A).

At the 4th week, all cultivars exhibited small uncoloured roots (Fig. 2). HPLC measurements at this stage showed very low total carotenoid levels (Fig. 3A): 0.9 µg g^{-1} fresh weight (FW) in cv. Yellowstone, 0.6 µg g^{-1} in Bolero, and 3.6 µg g^{-1} in Nutrired. The three coloured cultivars showed carotenoid accumulation during root

Table 1. *Primer sequences used for qRT-PCR*

Locus	Forward primer	Reverse primer	Accession number
*EF1*α	5′-GGAACCTCTCAGGCTGATTGTG-3′	5′-TTGGAGATACCAGCTTCAAAACC-3′	D12709
AC1	5′-TGCC CCCAGTAGCATGAAG-3′	5′-CCAATCCAGAGATCGCTGTACTT-3′	X17526
PSY1	5′-TTGGGCAATCTATGTGTGGTGTAG-3′	5′-GCCTTGGGCGTGATATGG-3′	DQ192186
PSY2	5′-TCAGTTGCTATGTCCTGGATTGTT-3′	5′-CCTCTCGGGCAGTCTCCAA-3′	DQ192187
PDS	5′-TAACATGGCCTGAGAAAGTCAAGT-3′	5′-CACGTAGGCTTGTCCACCAA-3′	DQ222429
ZDS1, *ZDS2*	5′-CCGAAGCTAAAAGTGGCTATTATAGG-3′	5′-TGGCCCTGATCTAGAAGCTCAA-3′	DQ222430 & DQ192189
LCYB1	5′-AGTGGGAATTGGCGGTACAG-3′	5′-GCAGCTAGAGTTCTTGCTACCATATATC-3′	DQ192190
LCYE	5′-CATTCCATGCAGGCTTGCTA-3′	5′-CCCAACCTCATACTGCAAAAGTT-3′	DQ192192
ZEP	5′-TGGCTGCTTTGGAAGCTATTG-3′	5′-TCACCAGTAATACAACCAGCTTTCA-3′	DQ192197

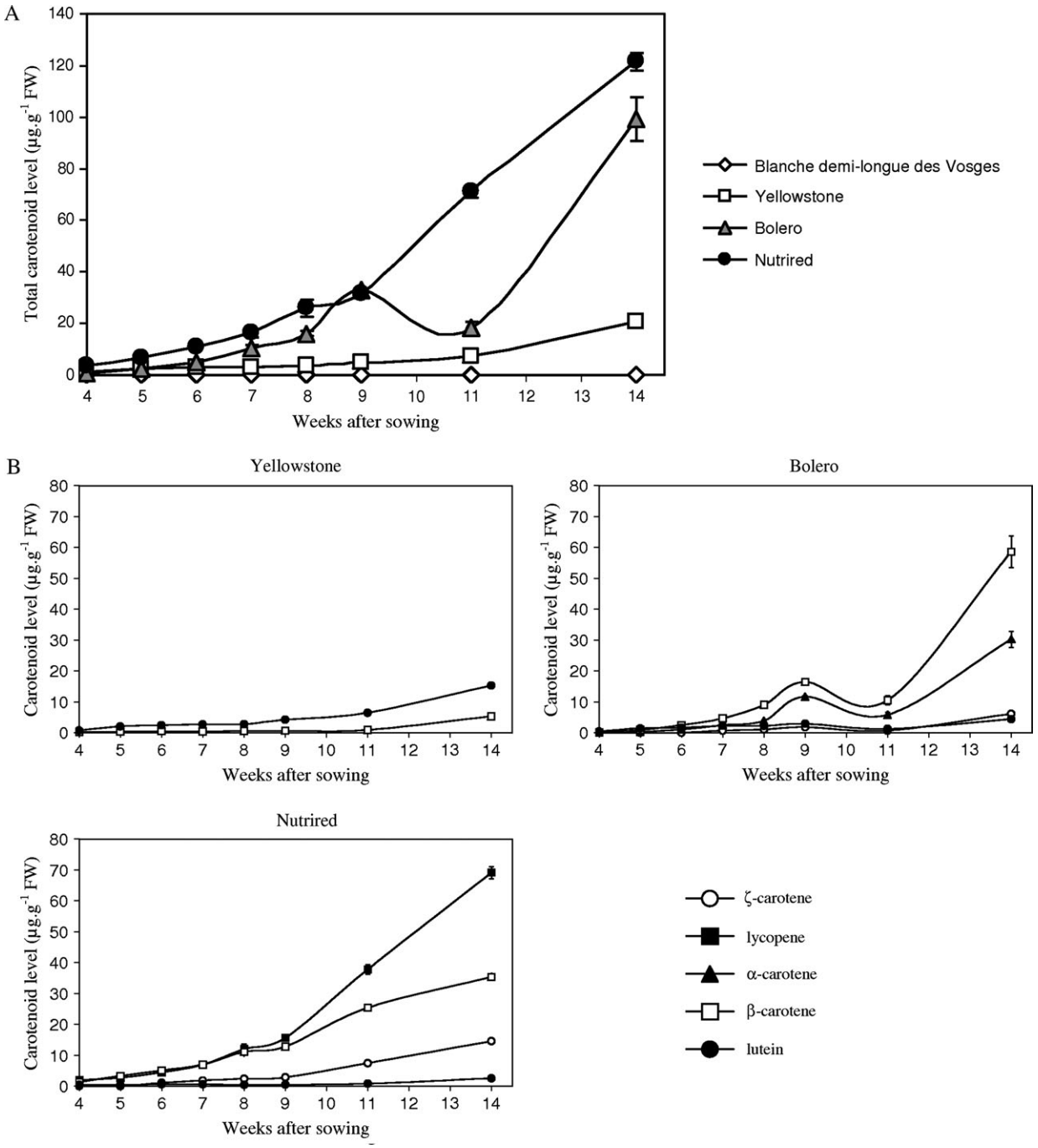

Fig. 3. Carotenoid content patterns during carrot root development in four cultivars. (A) Carotenoid levels during root development were summed to compare total carotenoid levels between the four cultivars. The carotenoid content was under the detection threshold in the white cv. Blanche demi-longue des Vosges. (B) Carotenoid content during root development is shown for yellow Yellowstone, orange Bolero, and red Nutrired. Values are means ±SE (n=2).

development which could be separated into two stages. From the 4th to the 8th week of development, total carotenoid accumulation was slow and regular (Fig. 3A). Carotenoid accumulation became faster after the 8th week of development. An unexplained decrease around the 11th week after sowing was observed in cv. Bolero. At the 14th week, the total carotenoid content in roots rose to 21 µg g^{-1} FW in cv. Yellowstone roots, 99 µg g^{-1} FW in Bolero, and 121 µg g^{-1} FW in Nutrired. These values are consistent with the results of previous studies in mature roots (Surles *et al*., 2004; Nicolle *et al*., 2004).

At the 14th week after sowing, the three coloured cultivars exhibited different carotenoid content patterns (Fig. 3B). Yellow roots of cv. Yellowstone predominantly

contained lutein (74% of total carotenoids on average) along with lower amounts of β-carotene (26%). In cv. Bolero orange roots, the major carotenoid was β-carotene (59%). This cultivar was the only one to contain α-carotene (30%). Small amounts of ζ-carotene (6%) and lutein (4%) were also identified in cv. Bolero. Red roots in cv. Nutrired were the only ones containing lycopene (57%), followed by β-carotene (29%), ζ-carotene (12%), and lutein (2%). For these three cultivars, accumulation was co-ordinated between specific carotenoids even though the accumulation rates differed.

Analysis of transcript abundance

To examine whether carotenoid accumulation during carrot development and differences in carotenoid composition between carrot cultivars could be related to the expression of carotenoid biosynthetic genes, transcript levels of eight carotenogenic genes were analysed by qRT-PCR (Fig. 4) in the same samples as used in the HPLC analysis.

Transcripts were detected in all 224 measurements, showing that all the investigated genes were expressed between the 4th and 14th weeks of plant growth for the four cultivars, including the white cv. Blanche demi-longue des Vosges, even though no carotenoid was detected in the roots of this cultivar.

Most of the genes showed an increasing transcript level during early root development, which is consistent with the accumulation of total carotenoids in coloured cultivars during root development. The transcript level of most carotenogenic genes increased during the early low carotenoid accumulation stage in coloured cultivars (4–8/9 weeks after sowing), especially *PSY1*, *LCYE*, and *LCYB1* in the three coloured cultivars and *ZDS* genes in Nutrired.

Carotenoid accumulation and levels of most transcripts seemed to be less correlated during the late fast total carotenoid accumulation stage (8–14 weeks after sowing). For some genes, transcript levels were stable over this period (*PSY1* in cv. Yellowstone and Bolero; *PDS* in the four cultivars; *ZDS* genes and *LCYE* in Nutrired). In other cases, transcript levels even decreased despite the acceleration of carotenoid accumulation (*PSY1* and *LCYB1* in cv. Nutrired; *LCYB1* in Bolero). Transcript levels, nevertheless, increased during this period for *PSY2*, and *ZDS* genes, *LCYE* and *ZEP* in cv. Yellowstone and Bolero. The carotenoid-poor Yellowstone cultivar showed a 20-fold increase in *LCYE* transcript level whereas the lutein level increased by 3.6-fold. Although white cv. Blanche demi-longue des Vosges did not contain carotenoids, transcript levels of all genes, except *PSY2,* increased globally throughout the investigation period.

Carotenogenic transcript levels between cultivars and their specific carotenoid patterns are summarized in Fig. 5. The two most relevant differences in transcript levels were consistent with the extent of accumulation of the major carotenoid in given cultivars. More *LCYE* transcripts were detected in cv. Yellowstone, on average, during the experiment as compared to Nutrired, Blanche demi-longue des Vosges, and Bolero, i.e. 8.6-, 7.9-, and 4.8-fold more, respectively. This gene is involved in lycopene channelling into the pathway branch ending at lutein, which is the major carotenoid in Yellowstone. Similarly, cv. Nutrired showed more *ZDS* gene transcripts, on average, during the experiment than Bolero, Blanche demi-longue des Vosges, and Yellowstone, i.e. 4.6-, 3.1-, and 3.0-fold more, respectively. ZDS is the enzyme located directly upstream from lycopene, which was specifically accumulated in cv. Nutrired. *LCYB1* transcript levels were about 1.7–2.7-fold higher in Nutrired than in the other cultivars between 4–14 weeks after sowing, which was in line with the high β-carotene content in Nutrired. The absence of α-carotene and the low levels of lutein in cv. Nutrired were consistent with the low levels of *LCYE* transcripts in this cultivar. Comparatively, cv. Bolero did not exhibit any particular transcript level pattern for the eight investigated genes, notably for *LCYB1* and *LCYE*. This result was consistent with the accumulation, in cv. Bolero, of carotenoids from the two branches downstream from lycopene, i.e. β-carotene and α-carotene.

Less than 2-fold average *PSY1* and *PSY2* transcript level differences were noted between cultivars. The uncoloured Blanche demi-longue des Vosges cultivar had the lowest transcript levels for these two genes in most of the measurements. Nevertheless, it did not show a dramatic difference with respect to the coloured cultivars, which could have explained the absence of carotenoids.

Discussion

In this study, HPLC and qRT-PCR were used in parallel to characterize carotenogenesis during carrot root development and amongst cultivars exhibiting different colours. Quantitative RT-PCR was used on account of the high sensitivity of this method to detect transcripts of genes with low expression (Gachon *et al*., 2004), such as some carotenogenic genes in other models (Cunningham and Gantt, 1998). *PSY1* and *PSY2* encode the carotenogenic key enzyme phytoene synthase (Hirschberg, 2001). *PDS*, *ZDS1*, *ZDS2*, *LCYE*, and *LCYB1* are coding for enzymes located downstream and/or upstream from carotenoids accumulated in carrot (Nicolle *et al*., 2004; Surles *et al*., 2004).

Among coloured cultivars, root colours were explained by qualitative and quantitative differences in carotenoid accumulation, linked with differential carotenogenesis gene expression levels. Meanwhile, no carotenoids were detected in the roots of the white Blanche demi-longue des Vosges cultivar. Nevertheless, transcripts were

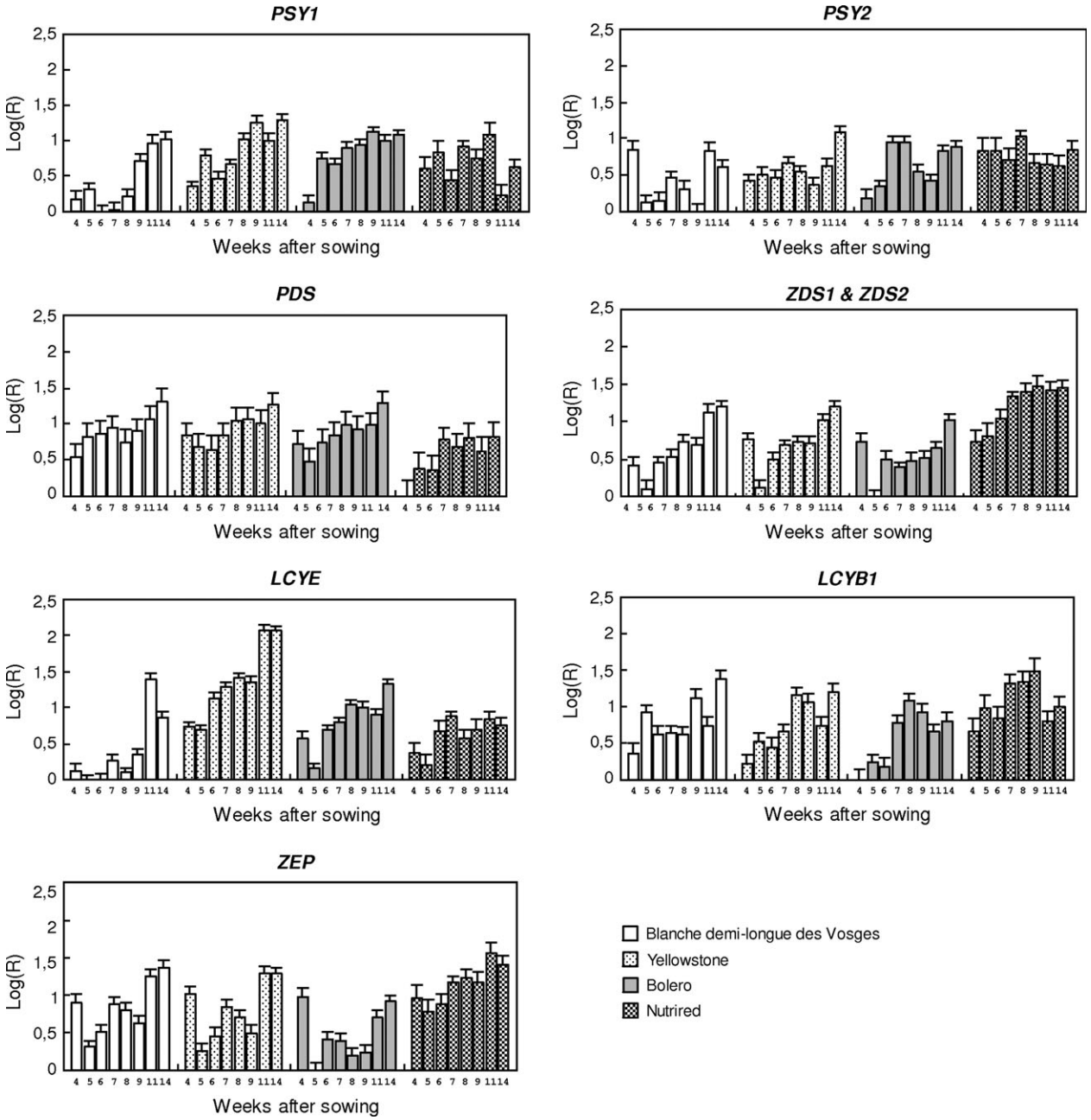

Fig. 4. Relative transcript accumulation of eight carotenoid biosynthesis genes during carrot root development. Transcript levels were evaluated by qRT-PCR. Numbers below the *x*-axis are weeks after sowing. Data are log of ratio (log(*R*)) of Pfaffl (2001) ±SE (n=3). For each gene, transcript levels are expressed relative to the sample in which the lowest transcript levels were measured (base value=0). Log(*R*) for different genes are unrelated.

detected for all the genes investigated and their level was not dramatically altered when compared with coloured cultivars. Carotenogenic transcript levels were even increased for most genes during plant development. This indicates that the differential transcriptional regulation of these genes was not the cause of the absence of pigmentation of this white cultivar. However, carotenogenic gene expression does not always lead to a functional carotenogenic pathway. Deleterious mutation could produce unfunctional alleles. Nevertheless, the crucial photosynthetic protection function of carotenoids imposes the expression of at least one functional allele in white carrots leaves. Then the existence of several tissue-specific genes could explain the presence of one functional enzyme in leaves and one non-functional enzyme in roots leading to the absence of carotenoid biosynthesis. Supporting this hypothesis two *PSY* genes exist in carrot. Nevertheless, transcripts of these two genes were recorded in the white

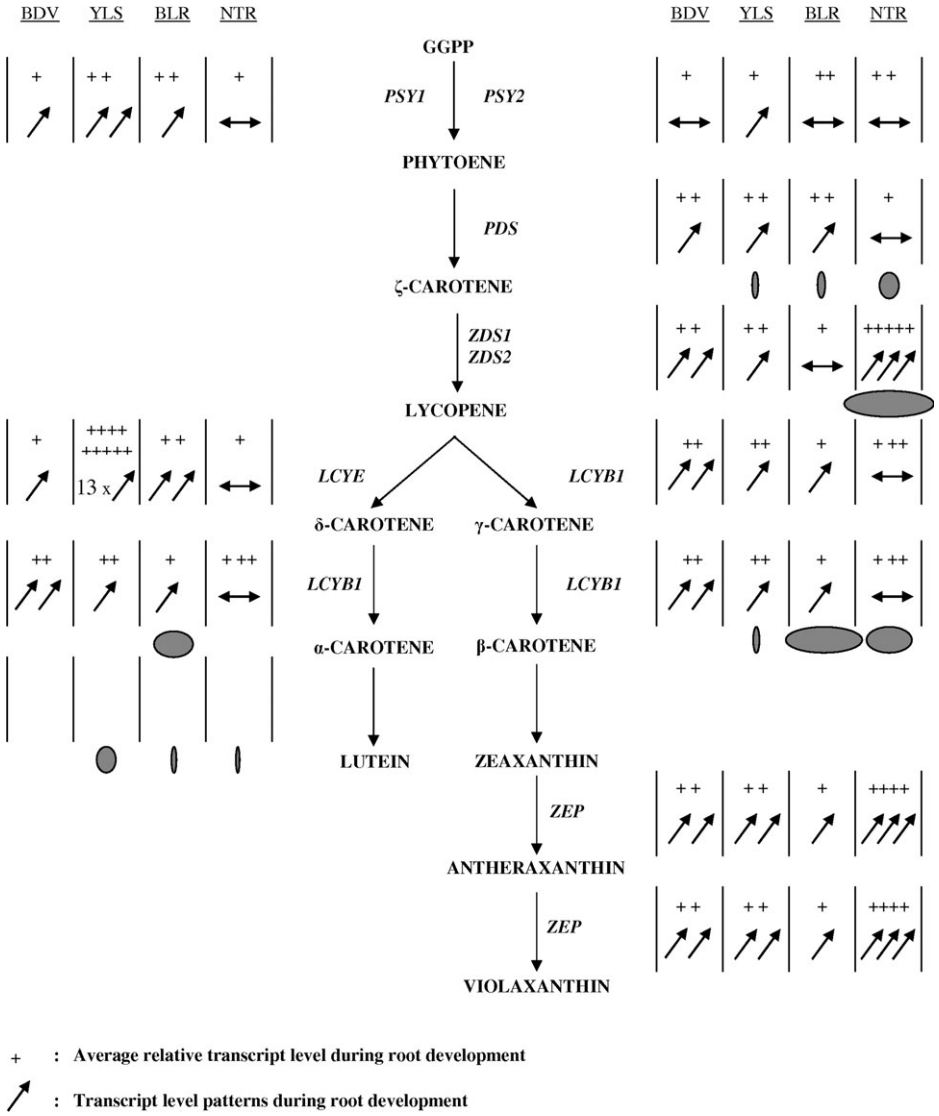

Fig. 5. Schematic view of the accumulation of carotenoids and transcripts during root development in four cultivars. Carotenoid content is symbolized by an ellipse. The width of the ellipse is proportional to the quantity of the compound at the 14th week after sowing. The average relative transcript level between the 4th and 14th weeks of development is represented by '+' symbols. For each gene, a single '+' symbol was attributed to the cultivar with the lowest average. For the three other cultivars, the number of '+' symbols is proportional to the average relative transcript level for this gene. Variations in transcript levels throughout the investigation period are indicated by arrows. From relative transcript levels, linear regression slopes were calculated and are represented by arrows. The '↔' symbol means a null value; the number of arrows is proportional to the slope. BDV: Blanche demi-longue des Vosges. YLS: Yellowstone. BLR: Bolero. NTR: Nutrired.

cultivar. Finally post-transcriptional regulation could lead to the absence of PSY enzymes in roots.

Apart from the transcript regulation level of the carotenoid biosynthesis pathway, the absence of precursors could explain the absence of carotenoids in white cultivars: Santos *et al*. (2005) have suggested that the lack of phytoene synthesis is the step which limits the carotenoid pathway in white carrot roots. Regulation of genes upstream from phytoene, particularly genes involved in the DXP pathway, could be the focus of further studies to explain the absence of carotenoids in white carrots.

The disappearance of synthesized carotenoids could be another hypothesis. If phytoene is synthesized and carotenoids subsequently produced in the white cultivar, the action of carotenoid cleavage enzymes could explain the absence of carotenoid. In chrysanthemum petals, a carotenoid cleavage dioxygenase (*CmCCD4a*) was found to be more expressed in white cultivars than in yellow cultivars (Ohmiya *et al*., 2006). In white petals, carotenoids were thought to be produced but subsequently degraded into colourless compounds. The regulation of DXP pathway genes and carotenoid cleavage genes should be tested in white carrots in

order to gain further insight into the absence of carotenoids.

Through a carotenogenesis investigation in coloured carrots, the present study generated information on carotenogenesis regulation in a root organ. During the development of coloured carrot roots, apart from a single measurement in Bolero, total carotenoid levels increased, with two stages differing slightly with respect to the carotenoid accumulation rate. In parallel, transcript levels of the investigated carotenogenic genes globally increased during carrot root development. Carotenoid accumulation in coloured carrots was therefore concomitant with the increase in transcripts levels of carotenogenic genes, thus suggesting that global carotenoid accumulation during root development may be regulated by carotenogenic gene expression.

This relatively linear phenomenon differs from the contrasting stages in ripening fruit models. In juvenile fruits, carotenoids that accumulate before the breaker stage (mainly lutein, violaxanthin, and β-carotene) are involved in photosynthesis. After the breaker stage, specific carotenoids are accumulated in maturing fruits as attractants to promote seed dissemination. Carotenoid patterns are thus closely related to the ripening stage. Simultaneously, in tomato (Bramley, 2002) and *Citrus* (Kato *et al*., 2004) fruits, expression was found to be increased for some carotenogenic genes acting upstream from accumulated carotenoids and decreased for others acting downstream from accumulated carotenoids during ripening. These modifications were explained by a shift from a chloroplastic-type of carotenogenesis into a chromoplastic-type of carotenogenesis. The contrast of regulation of gene expression in fruits with the linear increase of carotenoid gene expression in carrot roots could be related to the difference of development of these organs. In carrot roots, early development involves differentiation of proplastids, with a low carotenoid level, into chromoplasts (Ben-Shaul and Klein, 1965). Contrasting with fruit maturation, this absence of chloroplast/chromoplast shift could explain the absence of gene repression during carrot root development.

This study also aimed to assess the extent to which transcriptional regulation could be involved in the marked variations in root carotenoid content in carrot germplasm. The high *LCYE* transcript levels might be consistent with the accumulation of lutein in yellow cv. Yellowstone. The high *ZDS1* and/or *ZDS2* transcript levels might be consistent with the specific accumulation of lycopene in red cv. Nutrired. The high expression of specific genes in cultivars could account for differences in accumulated carotenoid levels in carrot roots. This would be in accordance with other models in which colouration differences are often related to differential carotenogenic gene expression.

Nevertheless the very high carotenoid accumulation noted in the late stages of development was less correlated with the carotenogenic transcript level than with the accumulation observed in the early stages. It is also hard to interpret the high carotenoid content of cv. Bolero through its transcript level pattern. Other mechanisms would need to be assessed to explain the differential accumulation of carotenoids in carrot roots. The expression of a second gene encoding LCYB (*LCYB2*), as well as genes encoding two hydroxylases acting downstream from β- and α-carotene (*CHXB1*, *CHXB2*, *CHXB3*, and *CHXE*) and genes involved in carotenoid cleavage (Just *et al*., 2007), should now be studied to gain further insight into this phenomenon. Cloning of genes known to control carotenoid accumulation in carrot, like *Y*, Y_1, and Y_2 (differential distribution of α- and β-carotene in phloem and xylem), *A* (α-carotene accumulation) or L_1 and L_2 (lycopene accumulation), could generate new candidates to enhance the overall understanding of differential carotenoid accumulation in carrot.

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