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ORIGINAL ARTICLE

# A de novo transcriptome analysis revealed that photomorphogenic genes are required for carotenoid synthesis in the dark-grown carrot taproot

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**Abstract**
Carotenoids are terpenoid pigments synthesized by all photosynthetic and some non-photosynthetic organisms. In plants, these lipophilic compounds are involved in photosynthesis, photoprotection, and phytohormone synthesis. In plants, carotenoid biosynthesis is induced by several environmental factors such as light including photoreceptors, such as phytochromes (PHYs) and negatively regulated by phytochrome interacting factors (PIFs). *Daucus carota* (carrot) is one of the few plant species that synthesize and accumulate carotenoids in the storage root that grows in darkness. Contrary to other plants, light inhibits secondary root growth and carotenoid accumulation suggesting the existence of new mechanisms repressed by light that regulate both processes. To identify genes induced by dark and repressed by light that regulate carotenoid synthesis and carrot root development, in this work an RNA-Seq analysis was performed from dark- and light-grown carrot roots. Using this high-throughput sequencing methodology, a de novo transcriptome model with 63,164 contigs was obtained, from which 18,488 were differentially expressed (DEG) between the two experimental conditions. Interestingly, light-regulated genes are preferably expressed in dark-grown roots. Enrichment analysis of GO terms with DEGs genes, validation of the transcriptome model and DEG analysis through qPCR allow us to hypothesize that genes involved in photomorphogenesis and light perception such as *PHYA, PHYB, PIF3, PAR1, CRY2, FYH3, FAR1* and *COP1* participate in the synthesis of carotenoids and carrot storage root development.

**Keywords** Carrot · De novo transcriptome · Carotenoid biosynthesis · Photomorphogenic genes · Carrot storage root

## Introduction

Carotenoids are the second most abundant natural pigments worldwide with more than 750 structurally different compounds (Nisar et al. 2015). In photosynthetic tissues, carotenoids accumulate in chloroplasts and participate in photosynthesis, light harvesting and photoprotection (Grotewold 2006; DellaPenna and Pogson 2006; Johnson et al. 2007; Rosas and Stange 2016) and are the essential precursors of the phytohormones abscisic acid (ABA, Walter and Strack 2011) and strigolactons (SL, Alder et al. 2012; Beltran and Stange 2016). In addition, they possess antioxidant properties acting as scavengers of reactive oxygen species. Carotenoids are also found at very high levels in chromoplasts-enriched organs providing yellow, orange and red color to flowers, fruits and seeds (Hirschberg 2001; Fraser and Bramley 2004; Tanaka and Ohmiya 2008; Walter and Strack 2011). Daily intake is the mode for animals to obtain the necessary carotenoids for vitamin A synthesis, which plays essential roles in preventing age-related macular degeneration among other functional roles (Abdel-Aal et al. 2013). For humans, carotenoids are also essential as antioxidants for preventing aging and several diseases such as cancer (Zu et al. 2014; Delcourt et al. 2006).

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Carotenoid biosynthesis has been extensively investigated for several years in plant models (Ruiz-Sola and Rodriguez-Concepción 2012) and some economically relevant crops have been improved in carotenoid content using metabolic engineering (Lado et al. 2016). In plant models such as *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco) and *Solanum lycopersicum* (tomato), carotenoid synthesis has been described to be regulated by internal factors such as developmental cues, epigenetic, circadian clock and by external factors, i.e., light (Ruiz-Sola and Rodriguez-Concepción 2012; Sun et al. 2018). Light and development are important cues that regulate the expression of carotenogenic genes during photomorphogenesis and fruit ripening (Rodriguez-Concepción and Stange 2013; Llorente et al. 2017; Sun et al. 2018). During photomorphogenesis and de-etiolation, different light qualities are sensed by phytochromes (PHYs), cryptochromes (CRY) and phototropins inducing their translocation to the nucleus (Stange and Flores 2012). Through the direct binding of PHYs to phytochrome interacting factors (PIFs), PIFs are subjected to degradation, leading to the expression of isoprenoid and carotenogenic genes, and increasing the increase in carotenoids and chlorophyll levels (Stange and Flores 2012; Woitsch and Römer 2003; Ronen et al. 1999, 2000; Giuliano et al. 1993; Pecker et al. 1996; Toledo-Ortiz et al. 2010; von Lintig et al. 1997). In addition, the Arabidopsis gene *AtPAR1* (Phytochrome-rapidly regulated 1), which participates in the shade avoidance syndrome (SAS) and photomorphogenesis (Bou-Torrent et al. 2008), also recruit PIFs in shade that permits the expression of *AtPSY* gene for carotenoid synthesis (Bou-Torrent et al., 2015). PIFs that are expressed in the dark and shade bind to light-responsive elements (LREs) located in promoters of carotenogenic and photomorphogenic genes impairing the expression of genes, such as *PSY* in the dark and shade (Toledo-Ortiz et al. 2010; Bou-Torrent et al. 2015).

Carrot (*Daucus carota*), which belongs to the Apiaceae family, accumulates many nutrients, such as carotenoids, vitamins, anthocyanins, fiber, and minerals, in the carrot fresh taproot (Simpson et al. 2016a; Que et al. 2019). At present, carrot is one of the ten most widely cultivated root vegetables in the world. This crop was originally cultivated in Afghanistan and Turkey around the ninth century (Mackevic 1929; Simpson et al. 2016a) and the domestication and selective breeding of yellow carrots led to the appearance of the orange carrot in the seventeenth century in Netherlands. Analysis of individual carotenoids and carotenogenic gene expression in six different carrot cultivars revealed that *CHXB2, CHXE* y *CYP97a3* are most expressed in the yellow cultivar, in correlation with the high lutein level (Ma et al. 2017). On the contrary, the high α- and β- carotene levels could be the result of the low expression of these genes in the orange varieties (Ma et al. 2017). The orange varieties that are the most worldwide consumed are enriched in mostly α- and β- carotene (Fraser and Bramley 2004; Baranska et al. 2006). Contrary to other plants, carrots accumulate high levels of carotenoids in the storage root grown in dark (underground). Young roots are pale and thin and start accumulating carotenoids at 8 weeks of culture, reaching the biggest size and the highest level of carotenoids after 3 months which correlates with carotenoid gene expression in the orange carrot (Clotault et al. 2008; Fuentes et al. 2012), but not in other varieties (Clotault et al. 2008). But, in contrast to other plants, when the root is grown in light (long day photoperiod), the root develops into a thin and greenish root where proplastids differentiate into chloroplasts instead of chromoplasts, which is also accompanied by a reduction in carotenoids and the expression of carotenogenic genes involved in α and β-carotene synthesis (Fuentes et al. 2012) (Fig. 1). Therefore, light is an environmental factor that negatively regulates secondary root growth and the synthesis and/or accumulation of carotenoids in the carrot root, modulating in some way the expression of carotenogenic genes such as *DcPSY1* and *DcPSY2* (Fuentes et al. 2012), suggesting the existence of new mechanisms repressed by light that regulate both processes. To identify genes induced by dark and repressed by light that regulate carotenoid synthesis and carrot root development, we performed a transcriptome assembly and RNA-Seq analysis considering that the development of next-generation sequencing strategies allowed to obtain several de novo carrot transcriptomes (Iorizzo et al. 2011; Rong et al. 2014; Xu et al. 2014a, b; Chen et al. 2015; Huang et al. 2015; Tian et al. 2015; Wang et al. 2015a, b) and subsequently the high-quality genome sequencing of

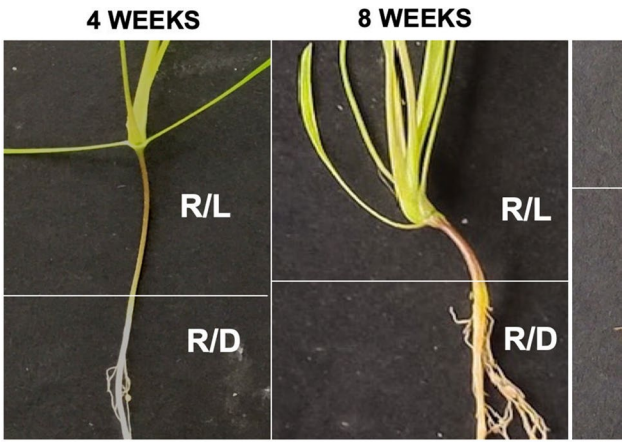


**Fig. 1** Phenotype of carrot roots grown in the presence of light (R/L) and in darkness (R/D) at 4, 8 and 12 weeks. In the dark, thickening and accumulation of carotenoids in the root were promoted, which gave the characteristic orange color to this plant organ. However, the root that grows in light (R/L) remains thin, and the accumulation of carotenoids is negatively affected. Chlorophyll and chloroplasts accumulate in R/L, which causes the greenish phenotype

*Daucus carota* ssp. sativus L. (Iorizzo et al. 2016) and new transcriptome analysis (Ma et al. 2018).

Using this high-throughput sequencing methodology in 8 weeks old dark (R/D) and light-grown carrot roots (R/L), we obtained a de novo transcriptome model with 63,164 contigs, from which 18,488 genes are differentially expressed (DEG) between the two experimental conditions. Among genes regulated by light/dark, surprisingly we found that light-regulated genes such as *PHYA, PHYB, PIF3, PAR1, CRY2, FYH3, Far1* and *COP1* are preferably expressed in dark-grown roots. We also presented enrichment analysis of GO terms with DEGs genes and the validation of the transcriptome model and DEG analysis through qRT-PCR. Contrary to conventional plant models, our results permit proposing that genes involved in light perception mechanism participate in the synthesis of carotenoids and carrot root development in darkness.

## Materials and methods

### Plant material and RNA isolation

Carrot roots were cultivated 8 weeks in dark (R/D) and in light (R/L) based on Fuentes et al. (2012). Briefly, seeds of commercially acquired carrot (*Daucus carota* L.) cultivar Nantaise were sown in a mix of soil and vermiculite (2:1). Plants were cultivated in a growth chamber with a 16 h long day photoperiod illuminated with cool-white fluorescent light (115 µmol $m^{-2}$ $s^{-1}$) at 20-23 °C. Carrots were grown with the down section of the root in darkness (R/D) and the upper section in the presence of light (R/L, long day photoperiod, Fig. 1). Roots were harvested at 8 weeks when carotenoid synthesis and secondary root growth begin (Fuentes et al. 2012). Therefore, each section was taken from a pool of plants subjected to both, light and dark conditions for RNA extraction. Total RNA was extracted from the harvested R/D and R/L roots using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. Three independent RNAs replicates were treated with DNasa I (Invitrogen™) to remove DNA trace from the samples according to the manufacter's instructions. Then, ribosomal RNA was depleted using RiboMinus™ Plant kit for RNA-Seq (Invitrogen™) according to the manufacturer's instructions. The quality and quantity of extracted RNA were determined by electrophoresis gel in denaturant conditions and using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was stored at − 80 °C until further use.

### Carrot root transcriptome sequencing and assembly

RNA was sequenced by OMIC Solution (Santiago, Chile) using the Ion Torrent PGM technology (chip P1.1.17) with IonXpress RNA kit (Thermo Fisher Scientific) after library profile analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Data were processed as described in Vizoso et al. (2009). A quality analysis of the raw sequences was performed with CLC Genomics Workbench version 7.0.3. (CLC Bio) and FastQC version 0.10.1 software packages. Then, adapter sequences were removed, and a de novo transcriptome assembly was carried out following CLC Genomics Workbench default parameters including a quimera analysis.

### Gene functional annotation and classification

Gene functional annotation and Gene Ontology classification was done using the Blast2GO platform version 5.2.5 (Götz et al. 2008). The BlastX results obtained against the Viridiplantae subset of the NR database and Interproscan results obtained against all available databases were combined and merged to perform a classification with a bigger GO coverage. Only those blast hits whose e-value were greater than $10^{-6}$ and whose score was greater than 150 bits were considered for gene functional annotation.

### Gene Ontology enrichment analysis

Functional enrichment analysis was performed using Blast2GO software version 5.2.5 (Götz et al. 2008) with the Fisher's exact test and a FDR cutoff of 0.05.

### Differential gene expression analysis

A differential expression analysis was performed between R/D and R/L conditions using the de novo assembled contigs as a reference transcriptome. Each read was mapped against the reference sequences using the RNA-Seq tool of CLC Genome Workbench software version 7.0.3 with the following parameters: mismatch cost = 2; insertion cost = 3; deletion cost = 3; minimum fraction length = 0.6; minimum similarity fraction = 0.9, and maximum number of hits per read = 10. Gene expressions were based on reads per kilobase per million mapped read (RPKM) values and were analyzed with the Gaussian-based *T* test ('t Hoen et al. 2008) with an FDR correction of *p* values. An FDR of less than 0.05 (95% confidence) and a selection of genes with up to twofold more expression in R/D or R/L were considered to determine significant differences in gene expression.

### qRT-PCR and gene expression analysis

For RNA-Seq validation and differential gene expression analysis, RNA was obtained from the roots of a new group of 8 weeks old seedlings grown in dark (R/O) or in light (R/L). Total RNA was extracted from a frozen powder of

100 mg of *Daucus carota* roots using Trizol® Reagent (Invitrogen). For cDNA synthesis, 7 µg of total RNA sample was treated with DNase I and then incubated with 1 mM of Oligo-AP primer (5′-CGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) and Improm II reverse transcriptase (Promega®) according to the manufacturer's recommendations. Quantitative RT-PCR (qRT-PCR) experiments were performed in a Stratagene Mx3000P thermocycler and Brilliant II SYBR® Green QPCR Master Mix binding dye (Agilent Technologies) as described previously (Fuentes et al. 2012). cDNA was eventually diluted to use a total of 10 ng for each qRT-PCR reaction. Different primers were designed to amplify a specific fragment of 20 differentially expressed contigs that presented at least eight to ten reads (Table S1). The coding sequences of the ubiquitin and myosin contigs were selected as the normalizers (Table S1). qRT-PCR data were processed using the equation described by PfaffI (2001). Each qRT-PCR reaction was performed with three biological replicates and each sample was analyzed in duplicate (technical replicate). In all cases, the reaction specificities were tested with melting gradient dissociation curves and electrophoresis gels. To test for significant differences in gene expression, the results were analyzed using the General Linear Models option in the Statistical Software Package GraphPad Prism. The one and two-tailed Student's *t* test ($p < 0.05$, confidence interval 95%) was used.

## Results

### De novo carrot transcriptome assembly, functional annotation and gene count

Carrot roots were cultivated 8 weeks in dark (R/O) or in light (R/L) considering that at this time the storage root in darkness begins to swell and to accumulate carotenoids (Clotault et al. 2008; Stange et al. 2008; Fuentes et al. 2012) and the R/L presented a thin and green phenotype with reduced carotenoids (Fig. 1, Fuentes et al. 2012). As these phenotypes are also accompanied by a differential expression of carotenogenic genes, we selected this stage for RNA-Seq for identifying non-carotenogenic genes that are repressed by light and induced by dark that could participate in storage root development and carotenoid synthesis. The RNA-Seq analysis was done before the carrot genome was published (Iorizzo et al. 2016), and therefore in a first stage, our transcriptome was assembled de novo. As seen in Table 1, the mapped reads in R/D and R/L were between 8,093,029 and 9,926,046 for R/D and 8,908,618 and 14,137,605 for R/L with a mapped rate between 87 and 92% for R/D and 96% and 98% for R/L. When the mapping of reads was assessed against predicted genes from the published carrot genome (Iorizzo et al. 2016), between 1,173,177 and 1,726,834

**Table 1** Mapping results against de novo transcriptome, *Daucus carota* genome-predicted genes and whole genome

| Sample | Total bases (Gb) | Read count # | Average length (bp) | Inputs reads # | Average length (bp) | De novo transcriptome ($n = 64{,}129$) | | Genome-predicted genes ($n = 32{,}118$) | | Whole genome | | Genome-predicted ribosomal genes ($n = 340$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mapped reads # | Rate (%) | Mapped reads # | Rate (%) | Mapped reads # | Rate (%) | Mapped reads # | Rate (%) |
| R/D.r1 | 1.16 | 10,145,629 | 114 | 9,114,327 | 120 | 8,093,029 | 89 | 1,429,453 | 16 | 8,474,739 | 93 | 36,495 | 0.40 |
| R/D.r2 | 1.26 | 11,350,416 | 111 | 10,138,489 | 116 | 8,807,547 | 87 | 1,726,834 | 17 | 9,338,143 | 92 | 44,936 | 0.44 |
| R/D.r3 | 1.73 | 11,459,435 | 151 | 10,778,329 | 156 | 9,926,046 | 92 | 1,173,177 | 11 | 9,911,566 | 92 | 32,032 | 0.30 |
| R/L.r1 | 1.73 | 12,922,474 | 134 | 12,391,130 | 135 | 11,944,455 | 96 | 403,498 | 3 | 11,503,443 | 93 | 2,058 | 0.02 |
| R/L.r2 | 2.17 | 14,804,376 | 147 | 14,408,909 | 148 | 14,137,605 | 98 | 286,458 | 2 | 13,818,118 | 96 | 917 | 0.01 |
| R/L.r3 | 1.32 | 9,556,534 | 138 | 9,202,640 | 139 | 8,908,618 | 97 | 382,070 | 4 | 8,631,962 | 94 | 1,387 | 0.02 |

Non-specific match handling was set to "random mapping"

mapped reads were obtained for R/D and 286,458 and 403,498 mapped reads for R/L, which represents a mapped rate of 11–17% for R/D and 2–3% for R/L (Table 1). Nevertheless, when reads mapping was assesed against the whole carrot genome sequences, between 8,474,738 and 9,911,566 mapped reads were obtained for R/D and between 8,631,962 and 13,818,118 were obtained for R/L, which represents a mapped rate of 92–93% for R/D and 93–96% for R/L (Table 1). Interestingly, a high number of reads mapped outside the gene-predicted regions (intergenic regions) that, when revised, contains sequences attributable to new coding regions or extension to existing structures. As genome-predicted genes do not represent our transcriptomic data, we decide to continue with our de novo transcriptome as the reference. Reads were assembled into contigs using the CLC Genomics Workbench 7.0.3. software which leads to obtain a de novo transcriptome of 63,164 contigs with an average length of 416 bp (Tables 2, S9). 43,297 contigs (67%) were associated with an annotation assignment by BlastX against the Viridiplantae subset of the NR database. Of them, 32,934 contigs could be associated with a Gene Ontology functional category using Blast2GO (Table S2). Finally, the number of reads in R/D and R/L through read mapping was estimated for each contig (Table 1). Considering at least a twofold change and a maximum FDR of 0.05, we obtained that 15,427 contigs were over-represented in R/D (and downregulated in R/L) (Table S3), while 3,022 contigs were over-represented in R/L (and downregulated in R/D) (Table S4). As R/D is the normal (control condition), both experimental conditions are opposite and interdependent, meaning that the contigs overexpressed in R/D are down-expressed in R/L and vice versa.

## Gene expression of selected genes validate the de novo transcriptome

To correlate the RNA-Seq results and the in vivo expression, a group of 20 contigs differentially expressed between R/D and R/L were selected for qRT-PCR expression analysis. Among the sequences most expressed in R/D, we selected some that code for genes that participate in the synthesis of carotenoids such as *DXS1*, *LCYB2*, *PDS* and *ZDS* (Table 3). We also selected *PSY2* and *CRTISO* which showed higher expression level in R/L conditions (Table 3). These results are similar to those already reported previously in 8 weeks old seedlings cultivated in R/L and R/D (Fuentes et al. 2012; Rodriguez-Concepción and Stange 2013). As shown in Fig. 2, the relative expression level of these genes correlated with the RNA-Seq results, with the exception of *CRTISO* that showed a higher expression level in R/D than in R/L (Fig. 2). Other contig sequences were also tested for transcriptome assembly validation such as some involved in light-mediated signaling (*PHYA, PHYB, PIF4, PAR1, FAR1,* and *COP1*), genes involved in chloroplasts and chromoplasts development (*DAG* and *OR*), the abiotic stress-inducible transcription factors ALFINs (*ALF1* and *ALF5*), myosin 1 (*MIO* 1) and sequences randomly chosen that participate in different biological processes (*ARF6, ARC6* and *PAR1p*) (Fig. 2, Table 3). The result shows a correlation between the relative expression levels of the selected genes and the RNA-Seq prediction (Fig. 2, Table 3). *MIO1* does not present variations in its relative expression levels nor by RNA-Seq, which makes it an excellent candidate as a housekeeping gene in the evaluated conditions. This result as a whole shows an 85% correlation between the predictions made by RNA-Seq and the qRT-PCR analysis, which validate the de novo transcriptome assembly.

**Table 2** Summary of de novo RNA assembly data obtained for *Daucus carota*

| | Count | Average length (bp) | Total bases (bp) |
|---|---|---|---|
| Reads | 88,772,699 | 133.1 | 11,812,971,831 |
| Matched | 80,856,892 | 134.7 | 10,888,925,029 |
| Not matched | 7,915,807 | 116.7 | 924,046,802 |
| Raw Contigs | 64,129 | 412.0 | 26,431,310 |
| Contigs[a] | 63,164 | 415.7 | 26,256,541 |

[a]Quimera checked

## Differential expression analysis

Considering that the carrot is an unusual plant model and that the information regarding how the carotenoid biosynthesis is regulated in its root in the absence of light is limited, certain parameters were established that allowed us to obtain as much information as possible. First, a false discovery rate (FDR) less than 0.05 was established. Then, for differentially expressed genes (DEGs), all those sequences with a fold change equal or greater than twice between R/D and R/L were considered. Under these criteria, we found that 15,427 genes were more expressed (denoted over-represented) in R/D (Table S3), whereas only 3022 genes presented a lower expression in this condition (denoted over-represented in R/L, Table S4). Considering that these two experimental conditions (R/D vs. R/L) are contrasting and opposite, all upregulated genes in the control condition, R/D, are downregulated in R/L and vice versa.

To have a global view respect to DEGs and the biological processes most represented in them, a GO enrichment analysis was performed in the upregulated sequences in R/D and R/L (Fig. 3). In R/D all the GO terms of "Biological Processes" were enriched (Fig. 3a), due exclusively to that R/D is the normal growth condition of the plant. Thus, all the metabolic or physiological machinery is under the

**Table 3** List of genes used for in vivo validation of the transcriptome de novo assembly and for the expression levels predicted by RNA-Seq

| Upregulated genes in carrot root grown in dark (R/D) | | |
|---|---|---|
| Gene | Contig ID | Fold change (R/D with respect to R/L) |
| *PA1p* | Contig 10351 | 2863 |
| *PHYA* | Contig 7257 | 1135 |
| *ALF1* (ALFIN 1) | Contig 1983 | 1022 |
| *ARF6* | Contig 3577 | 465 |
| *ALF5* (ALFIN 5) | Contig 22603 | 447 |
| *PDS* | Contig 6437 | 328 |
| *ZDS* | Contig 11017 | 208 |
| *PIF4* | Contig 3423 | 103 |
| *PAR1* | Contig 42760 | 100 |
| *ARC6* | Contig 17614 | 78 |
| *LCYB* | Contig 29892 | 76 |
| *FAR1-5* | Contig 28971 | 67 |
| *COP1* | Contig 1906 | 51 |
| *DXS 1* | Contig 457 | 34 |
| *PHYB* | Contig 1318 | 22 |
| *OR* | CONTIG 15664 | 9 |
| *DAG* | Contig 12990 | 6 |
| Upregulated genes in carrot root grown in light (R/L) | | |
| Gene | Contig ID | Fold change (R/L with respect to R/D) |
| *CRTISO* | Contig 33224 | 11 |
| *PSY2* | Contig 3840 | 3 |
| Genes with no change in its expression between R/D and R/L | | |
| Gene | Contig ID | Fold change |
| *MIO1* (MIOSIN 1) | Contig 5184 | 1 |

It shows the name of the gene, its identification (contig ID) and expression fold change

usual development. On the contrary, in the R/L there are enriched terms associated with photosynthetic processes as 'photosynthesis and light reaction', which refer to light-mediated reactions that take place in photosystems II and I during photosynthesis (Fig. 3b). This result is reasonable considering that this segment is exposed to light and presents a green coloration due to the enrichment by chloroplasts instead of chromoplasts and carotenoids (Fig. 1, Fuentes et al. 2012).

In addition, we observed other highly enriched categories that are related to the production of energy from metabolites and metabolic process named as 'generation of precursor metabolites and energy' and 'multi-organism metabolic process', respectively. These categories involve the processes that make up the central metabolism, processes that are carried out in all the structures of a plant and that may be enriched due to the new photosynthetic activity in the R/L (Fig. 3b).

To further investigate and search for genes that are normally expressed in R/D but affected by light (R/L downregulated, Table S4), a manual analysis of the 15,427 upregulated sequences in R/D was performed. As expected, we found several carotenogenic genes, such as those that were selected for RNA-seq validation (Fig. 2, Tables 3 and S5) in accordance with the results reported in Fuentes et al. (2012). In addition, many sequences related to hormone signaling and some that respond to phytohormones were more expressed in R/D and repressed in R/L (Table S6), according to the fact that at 8 weeks the carrot root begins to thicken. For secondary root development, phytohormones such as auxins, gibberellins, among others are required; therefore, it was projected that different genes regulated by hormones were actively expressed during the development of carrot root in darkness. Some of them correspond to sequences involved in auxin response such as several *ARFs* (Auxin Response Factor), AUX1 (Auxin resistant 1), auxin-responsive *GH3*

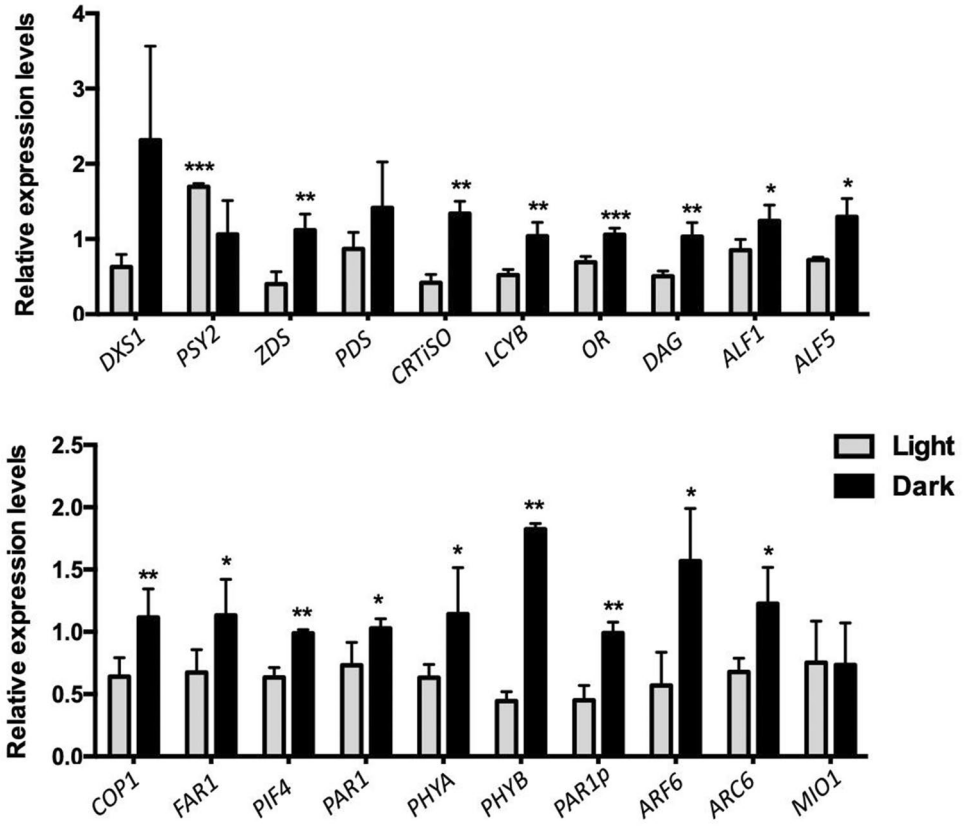


**Fig. 2** Relative expression levels of genes chosen for the de novo transcriptome assembly validation and the in vivo relative expression levels predicted by RNA-Seq analysis. The qRT-PCR was normalized with respect to the levels of ubiquitin gene expression. Comparisons of relative expression levels for each gene were made between R/L and R/D. The significant differences indicated with asterisks were calculated by *T* test, with $p < 0.05$ and Welch's correction

family protein and *SAUR-like* (Small Auxin Up RNAs-like) (Table S6). All these sequences participate in some way in the signaling activated by auxin, a hormone that regulates a wide range of processes in the plant, such as the development of the root and its architecture (Sozzani and Iyer-Pascuzzi 2014; Takatsuka and Umeda 2014; Fukaki et al. 2007; van Gelderen et al. 2018). The ARFs are transcriptional factors that modulate the early auxin response gene expression such as *AUX/IAAs* and *SAURs* (Guilfoyle and Hagen 2007; Fukaki et al. 2007; Ren and Gray 2015; Li et al. 2016), and the Arabidopsis *AUX1* and *IAA8* genes are involved in the formation of lateral roots (Marchant et al. 2002; Arase et al. 2012; Fukaki et al. 2007).

Sequences involved in different plant biological processes, denoted as "other biological processes", are more expressed in R/D (Table S7). In this group, we found genes that codify factors involved in chloroplast development and chromoplast differentiation such as '*plastid developmental protein*' (designated here as *DAG*), which are composed of a family of genes involved in chloroplast RNA editing. The *ARC6* (Accumulation and Replication of Chloroplasts 6) gene that codifies for a component of the plastid division machinery (Glynn et al. 2008) and the *OR* (Orange) gene were also upregulated in R/D (Table S7). The OR protein participates in the differentiation of chromoplasts and is the major post-translational regulator of PSY that through direct binding stabilizes the enzyme-promoting carotenoid production (Zhou et al. 2015; Park et al. 2016; Chayut et al. 2017).

Unexpectedly, we found 63 contigs belonging to genes involved in light perception, which are normally not induced in dark-grown roots in other species (Toledo-Ortiz et al. 2010) (Table S8). Our results showed the over-representation in R/D of genes involved in photomorphogenesis such as *PAR1, PIF4, FHY3, COP1, PHYA, PHYB*, and *CRY1* (Table 3). PAR1 (PHYTOCHROME-RAPIDLY REGULATED 1) is a transcriptional co-factor participating in the shade-avoidance syndrome (SAS), a light-responsive response in which PHYs are involved (Bou-Torrent et al. 2008). Both PHYA (PHYTOCHROME A), PHYB (PHYTOCHROME B) and CRY (CRYPTOCHROME) are photoreceptors that participate and fulfill essential roles in plant photomorphogenesis (Wu 2014; Gommers and Monte 2018). PHYA is activated and stabilized by far-red light (FR), CRY by blue light and PHYB by red light (R), and then translocated to the nucleus (Flores and Stange 2012; Oka et al. 2012). Likewise, PIFs are transcription factors that negatively regulate photomorphogenesis in darkness and in SAS, promoting hypocotyl elongation (Toledo-Ortiz et al. 2010). COP1 (Constitutive Photomorphogenic 1) as a ubiquitin E3 ligase sequesters transcription factors that positively regulate photomorphogenesis when plants are in the dark, thus inhibiting photomorphogenesis in SAS and the dark condition

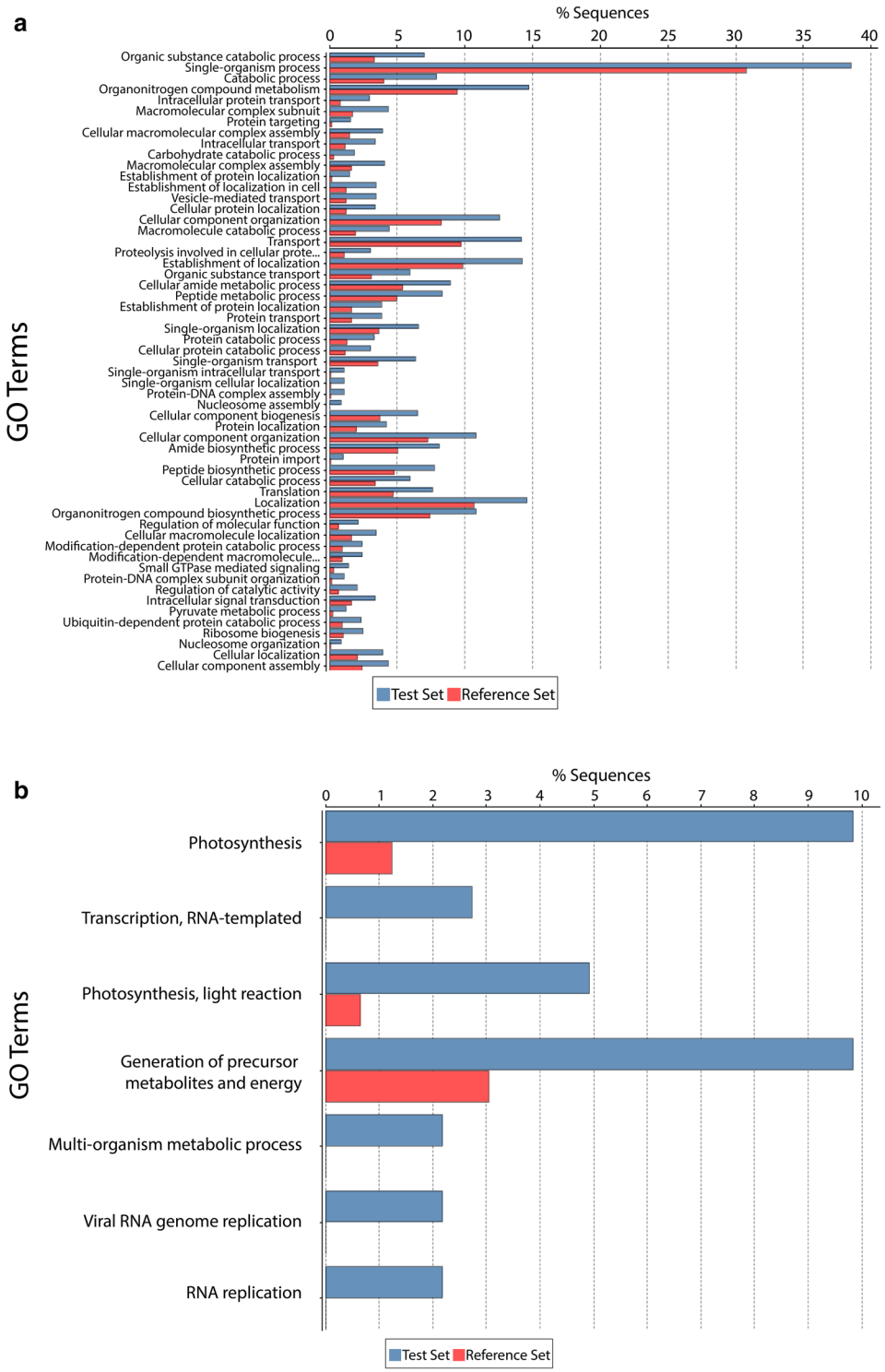


**Fig. 3** Gene Ontology (GO) enrichment analysis, category "Biological processes" of differentially expressed genes (DEGs) between R/D and R/L. **a** Enrichment of R/D DEGs with respect to all genes expressed in R/D. **b** Enrichment of R/L DEGs with respect to all the genes expressed in R/L. The red bars correspond to the percentage of sequences that were used as a reference to perform the enrichment analysis (all genes expressed in R/D or in R/L). The blue bars correspond to the percentage of sequences that were enriched for a given sub-category (GO-Terms) of the main category (Biological Processes). The sub-categories (Go-Terms) correspond to those indicated in the vertical part of the graph

(Wu 2014; Gommers and Monte 2018). FHY3 (FAR-RED ELONGATED HYPOCOTYL 3) is a transcription factor that induces the expression of *FHY1*, a gene that codifies a chaperone that translocates the active form of PHYA to the nucleus (Siddiqui et al. 2016), thus participating in FR-PHYA-mediated signaling during photomorphogenesis. All these light-regulated factors participate in the repression or induction of photomorphogenesis, contributing to the regulation of carotenoid and chlorophyll synthesis in the aerial part of plants and in general they do not have a carotenoid synthesis-related role in roots. Contrary to that expected, their expression was lower in R/L than in R/D and therefore

the functional role of these genes in carotenoid synthesis in the dark-grown carrot storage root remains to be established.

## Discussion

Carrot synthesizes and accumulates large amounts of carotenoids in its root that grows in the absence of light (approximately, 1000 ng/gDW) (Clotault et al. 2008; Rodriguez-Concepción and Stange 2013; Simpson et al. 2016a). Given this, carrot has been used as a plant model to understand how these pigments are synthesized and accumulated, as well as how this process is regulated (Rodriguez-Concepción and Stang, 2013; Simpson et al. 2016b). In this context, the complete cDNA sequences of most carotenogenic genes of carrot have been deposited in the NCBI database (Just et al. 2007, 2009). This has allowed the characterization of the potential key genes controlling the carotenogenic pathway in carrot (Moreno et al. 2013, 2016; Simpson et al. 2016b; Wang et al. 2014; Flores-Ortiz et al. 2020). However, these studies do not fully explain the mechanisms that control the synthesis or accumulation of carotenoids in carrot roots. Using two mapping populations with contrasting carotenoid accumulation, Iorizzo et al. (2016) reported that *Y loci* regulates the high carotenoid accumulation in yellow as well as in dark orange roots. Within *Y*, a mutated version of DCAR_032551, was the only one that segregates with high carotenoid pigmentation. Arabidopsis mutants of the DCAR_032551 homolog, named PSEUDO-ETIOLATION IN LIGHT (PEL), have an etiolated phenotype (Iorizzo et al., 2016). In our de novo transcriptome, the DCAR_032551 presents a high identity with contig 9951 which has an annotation as "Protein kinase superfamily protein" that shows 99.77% of identity to a serine/threonine-protein kinase SAPK1-like (LOC108224679) of *Daucus carota* subsp. sativus (access XM_017399365.1) when compared to Viridiplantae in a Blastn. Although contig 9951 presents more reads in R/L than in R/D, it does not pass the FDR filter < 0.05. However, given the results reported by Iorizzo et al. (2016), it would be interesting to study its role in the synthesis and/or accumulation of carotenoids in the carrot root.

With the advent of massive sequencing technologies, the transcriptomic research emerged as an approach for the study of gene expression and search for genetic markers or new genes that explain carrot domestication (Iorizzo et al. 2011; Rong et al. 2014; Que et al. 2019). On the other hand, researchers developed a transcriptomic and genomic database of carrot named CarrotDB (http://apiaceae.njau.edu.cn/carrotdb/) (Xu et al. 2014a), as well as the published de novo high-quality genome assembly of carrot (Iorizzo et al. 2016), which facilitates functional studies in carrot. Using the information provided by Iorizzo et al. (2016) and CarrotDB platform (Xu et al. 2014b), Ma et al. (2018) presents another transcriptomic analysis comparing DEGs between leaves and roots of the orange carrot variety Kuroda. Similar to that in Fuentes et al. (2012), they found that several carotenogenic genes and those related to photosynthesis are upregulated in leaves with respect to roots. These results show again that the carotenogenic genes level expression in carrots is regulated by light and that it is highly probable that the carotenoid synthesis regulation is different depending on the organ of the plant. Therefore, there is currently a good amount of genomic information that can be used to continue investigating the mechanisms that regulate and/or command the carotenoid biosynthesis in carrot roots.

Given the tremendous effect that produces light in carrot root development, carotenoid synthesis and carotenogenic gene expression (Stange et al. 2008; Fuentes et al. 2012), we carried out a sequencing of mRNAs of carrot roots developed in the dark (R/D) and in the presence of light (R/L) of the orange Nantaise cultivar with a subsequent RNA-Seq analysis to identify gene candidates inhibited by light, encoding molecular factors that could regulate chromoplast differentiation and carotenoid accumulation in dark-grown roots.

Due to the fact that no reference genome was available during the beginning of this work, we chose a de novo transcriptome assembly, an approach that had already been used successfully in carrot (Iorizzo et al. 2011; Rong et al. 2014). After the publication of the carrot genome (Iorizzo et al. 2016), we made a gene structure comparison of de novo assembled contigs and carrot genome through a BLASTN homology search. This analysis allowed us to confirm that 55,935 contigs (87% of total contigs) were present on the carrot genome with an identity over 95%. Nevertheless, only 14,528 contigs (22% of total contigs) had genome-predicted CDSs with a percent identity over 95% and a query coverage over 90%. This result correlates with the read mapping rate differences against the whole genome, but not against the genome-predicted CDSs. Similar results were obtained by Machaj et al. (2018), with a high percent of RNA reads mapping outside the gene-predicted regions. This suggests the need of an improvement on gene predictions over carrot genome using new expression data as released by us.

Regarding carotenogenic genes, the expression predicted by RNA-seq is not only in agreement with the qRT-PCR of *DXS1, DXR, PDS, ZDS2* and *LCYB2* genes in R/D and R/L, but also with previously published results (Fuentes et al. 2012) and consistent with carotenoid accumulation in orange varieties (Clotault et al. 2008; Stange et al. 2008; Fuentes et al. 2012). A recent work (Wang et al. 2020) concluded that light also may be an important cue for modulating gene expression in carrot leaves and roots in the orange, red and purple varieties, giving rise to a different pigment accumulation. Complementing our results, by means of an RNA-seq, *DcPSY1*, *DcZ-ISO*,

*DcLCYB*, and *DcLCYE* were upregulated in leaves with respect to roots (Ma et al. 2018). This information, together with the results of this work, strongly suggests that the regulation mechanisms of carotenoid biosynthesis or accumulation in *Daucus carota* are different in leaves and root, but both of them depend on the absence or presence of light.

As carotenoid synthesis in carrot roots occurs in chromoplast, Fuentes et al. (2012), was fortunate to find out genes involved in plastid differentiation in R/D (chromoplast enriched organ) more represented than in R/L (chloroplast enriched segment). The *OR* gene isolated from an orange cauliflower mutant encodes a DnaJ cysteine-rich zinc finger protein and is the only one known today that participates in triggering chromoplast biogenesis in plants (Li et al. 2001, 2012; Lu et al. 2006; Ellison et al. 2018). OR interacts and stabilizes PSY in Arabidopsis and sweet potato (Li et al. 2001; Lu et al. 2006; Zhou et al. 2015; Park et al. 2016). It is possible that the OR protein stabilizes or regulates carrot PSY, besides promoting the formation of chromoplast in the root grown in darkness. According to our results, light negatively regulates the *OR* gene expression causing a decrease of its transcripts in R/L, which would explain the lower abundance of chromoplasts in this root segment and the decrease in carotenoid levels (Fuentes et al. 2012). A GWAS analysis showed that the *OR* gene was associated with carotenoid synthesis in the orange carrot root (Ellison et al. 2018). Domesticated colored carrots with high carotenoid level present a T/T (LEU/LEU) genotype; cultivars with less carotenoid content present, in a heterozygote condition, a non-synonymous mutation caused by the change from serine to leucine C/T (SER/LEU); and the white cultivar has the homozygote C/C (SER/SER) genotype (Ellison et al. 2018). In our de novo transcriptome, we found the *OR* gene with a C mutation, suggesting that our variety is heterozygote for this mutation. The next step is to determine how light regulates *OR* (either at the transcriptional or post-transcriptional level) and its functional role in carotenoid synthesis in the dark-grown carrot root.

For secondary root development, hormones such as auxins and gibberellins are required and therefore genes such as *ARF6* (contig 3577) involved in auxin signaling (Li et al. 2016) may play a functional role. Auxin participates in root development, promotion of cell differentiation and elongation, and in the induction of lateral roots (van Gelderen et al. 2018), and therefore genes such as *ARF6*, *AUX*, *IAA* and *SAUR-like* were found mostly expressed in R/D in correlation with normal root development. On the other hand, light negatively affects the development of carrot root. The R/L presents alterations in its development, is thinner and accumulates chlorophyll and chloroplasts, probably due, among other facts, to the decrease in the expression level of phytohormones regulated genes.

Arabidopsis roots grown in darkness presents a longer main root and higher number of lateral roots than roots grown in light. In addition, light affects auxin-mediated signaling in roots that grew in light (Silva-Navas et al. 2015). Although light is an essential resource for plants, its direct detection by roots causes stress that impairs normal root development. In Arabidopsis roots, light causes oxidative burst, showing a fast accumulation of ROS (Yokawa et al. 2011). However, photosynthetic related-genes are most expressed in R/L than in R/D, which suggests that light promotes the activation of the photosynthetic metabolism than the photomorphogenic development of plants, and can be the reason for chloroplast instead of chromoplast establishment (Fuentes et al. 2012). Similar results were obtained in the orange carrot Kuroda, in which genes involved in photosynthesis are upregulated in leaves with respect to roots (Ma et al. 2018). These results suggest that light activates the photosynthetic machinery in a tissue that normally does not perform photosynthesis, at the expense of the processes that regulate the synthesis and accumulation of carotenoids and secondary root development.

According to this proposal, in R/D there is no induction of photomorphogenesis mediated by light; on the contrary, there is another mechanism induced by the dark condition that activates the synthesis of carotenoids in the carrot root. However, in R/D, surprisingly genes involved in photomorphogenesis were most expressed. This result agrees with a transcriptomic analysis done between high and low carotenoid accumulated carrots, which showed higher expression of light-regulated genes in the first one (Iorizzo et al. 2016). PHYA, PHYB, and CRY are photoreceptors that play fundamental roles in chlorophyll and carotenoid synthesis during photomorphogenesis (Wu 2014; Gommers and Monte 2018) and carotenoid synthesis in tomato ripening (Bianchetti et al. 2017). But there are also evidences about the expression of photoreceptors and their activity in Arabidopsis roots (Salisbury et al. 2007; Lee et al. 2016). Phytochromes participate in the control of phototropism and gravitropism in the roots as well as in root hair development, primary root elongation and lateral root formation (Reed et al. 1993; Ruppel et al. 2001; Correl et al. 2003; Correl and Kiss 2005; Salisbury et al. 2007; Lee et al. 2016), but a direct functional role in the synthesis of carotenoids in roots has not been reported yet.

A group of bHLH-transcription factors called PIFs repress photomorphogenesis in darkness and in simulated shade (Wu 2014; Gommers and Monte 2018; Bou-Torrent et al. 2015). PIFs bind to *PSY* promoter preventing its expression, thus reducing the synthesis of carotenoids when Arabidopsis is grown in darkness (Toledo-Ortiz et al. 2010) or simulated shade (Bou-Torrent et al. 2015). However, in Arabidopsis roots, PIFs do not contribute significantly to the regulation of *PSY* expression and carotenoid synthesis

(Ruiz-Sola et al. 2014). On the contrary, PIFs are potent regulators of cell expansion promoting hypocotyl elongation in etiolation, shade or the night/day rhythm conferred by the circadian clock regulation (Nozue et al. 2007; Niwa et al. 2009). Thus, the role of PIFs in the carrot root could be related to the development and secondary growth (Quail 2002; Gyula et al. 2003; Xu et al. 2015), which would also involve the action of phytohormones such as auxin, whose transport from the stem to the root is stimulated by the activation of the phytochromes in the shoot apex (Salisbury et al. 2007).

*PAR1*, which was also more expressed in R/D, codes for a transcriptional co-factor involved in SAS and photomorphogenesis (Roig-Villanova et al. 2006, 2007; Bou-Torrent et al. 2008; Hao et al. 2012; Zhou et al. 2014; Roig-Villanova and Martínez-García 2016). SAS refers to all development changes in plants when they are in shade (greater proportion of FR with respect to R light) (Roig-Villanova and Martínez-García, 2016). For example, when the R:FR ratio decreases, shade-intolerant plants such as Arabidopsis elongate their hypocotyl and decrease their photosynthetic metabolism (including the synthesis of photosynthetic pigments), among other changes (Roig-Villanova et al. 2006, 2007; Roig-Villanova and Martínez-García 2016). The hypocotyl elongation is promoted by PIFs, so these transcription factors are considered positive regulators of SAS (Roig-Villanova and Martínez-García 2016). On the contrary, AtPAR1 is a negative regulator of SAS and positively modulates the de-etiolation and photomorphogenesis, stimulating consequently the activation of the photosynthetic machinery after its transcription is induced, and preventing the excessive elongation of the hypocotyl promoted by PIFs (Roig-Villanova et al. 2007; Hao et al. 2012; Zhou et al. 2014). In this scenario, PAR1 binds PIF1, allowing the photomorphogenic positive transcription factors, such as HY5, to bind to *AtPSY* promoter inducing its transcription in shaded conditions (W + FR) and stimulating carotenoid biosynthesis in the photosynthetic tissue thereof (Toledo-Ortiz et al. 2014; Bou-Torrent et al. 2015). Then, *PAR1* appears as a good candidate that may regulate carotenoid biosynthesis in the carrot taproot that grows in darkness.

Interestingly, light that is sensed in Arabidopsis leaves is transported as photons through the stem, allowing to reach to the roots a reduced amount of R/FR which is able to activate PHYB in the root (Lee et al. 2016). Although it remains to be determined in carrot root, it is probable that the concept of "darkness", referring to the growth of carrot root underground, may correspond to a reduced level of R/FR and supports the finding of *PAR1, PHYA, PHYB* and *PIF*s genes.

Hence, it is highly probable that, as occurs in photosynthetic tissue during photomorphogenesis, the synthesis and accumulation of carotenoids in the dark-grown carrot root are regulated by the activation of genes involved in light signaling such as *PHYA, PHYB, PIFs* and *PAR1*. Therefore, these results together with those in which light-regulated genes were also mostly expressed in colored carrot roots than in white ones (Iorizzo et al. 2016) suggest that photomorphogenic genes also participate in the regulation of carotenoid biosynthesis in carrot root.

All this information led us to propose that carrot roots accumulate high levels of carotenoids together with chromoplast differentiation, because they are exposed to low R/FR in which several photomorphogenic-inducible factors such as PHYA, PHYB, CRY, COP1, FHY3 and PAR1 are highly expressed. But, why should it happen in carrot taproot and not in other roots? Future work will focus on the functional evaluation of these candidate genes to give rise to novel evidence toward clarifying whether and how light-induced genes are responsible for carotenoid biosynthesis and chromoplast differentiation in dark-grown carrot storage roots.

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**Availability of data** The nucleotide sequences of raw reads from this study were submitted to NCBI's Sequence Read Archive through the BioProject ID: PRJNA626692. Contigs of the de novo transcriptome assembly are available in table S9.

## Compliance with ethical standards

**Conflict of interest** Dr. Daniela Arias, Dr. Jonathan Maldonado, Dr. Herman Silva and Dr. Claudia Stange declare that they do not have conflict of interest.

**Ethical approval** This article does not contain any studies with human participants performed by any of the authors.

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