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(54) Title: ARTIFICIAL CHROMOPLAST BIOGENESIS

(57) Abstract: In the present invention, we show for the first time that the supply of phytoene to feed the carotenoid pathway functions as a primary determinant of chromoplast biogenesis in green tissues. We provide evidence that exceeding a threshold level of phytoene, the first committed intermediate of the carotenoid pathway, is sufficient to induce chloroplast-to- chromoplast differentiation in leaves. The present invention thus reveals a novel aspect of carotenoid biology and additionally presents a powerful tool for biotechnological applications of the modulation of plastid identity, that could be used in the development of new biofortified crops, food or as a source of extracts for a variety of industries.

Artificial chromoplast biogenesis

FIELD OF THE INVENTION

The present invention generally relates to the field of plant molecular biology and synthetic biology and concerns both a method for the induction of artificial chromoplasts from chloroplasts cells, and products derived therefrom.

BACKGROUND OF THE INVENTION

Plastids comprise a group of morphologically and functionally diverse plant organelles capable of differentiating from one plastid type to another in response to developmental and environmental stimuli (Jarvis and Lopez-Juez 2013, Liebers, Grubler et al. 2017). Such plastidial conversions are essential to sustain many fundamental biological processes and largely contribute to cell specialization in the different plant tissues (Kleffmann, von Zychlinski et al. 2007, Barsan, Sanchez-Bel et al. 2010, Barsan, Zouine et al. 2012, Liebers, Grubler et al. 2017). Among the different plastid types, chromoplasts are plastids of great importance in nature and agriculture because of their capacity to accumulate high levels of carotenoids, plant pigments of isoprenoid nature that impart color in the yellow to red range (Rodriguez-Concepcion et al. 2018). Besides health-promoting carotenoids such as beta-carotene (pro-vitamin A), chromoplasts accumulate other isoprenoids of nutritional interest such as tocopherols (vitamin E). Both carotenoids and tocopherols are essential nutrients that animals canno t synthesize and therefore must be taken in the diet (Fraser and Bramley 2004, Brehelin, Kessler et al. 2007, Giuliano 2017, Gramegna, Rosado et al. 2018, Rodriguez-Concepcion, Avalos et al. 2018). They are also added-value compounds widely used in several industries, including cosmetic, food, and animal feed industries (Ogbonna 2009, Rodriguez-Concepcion, Avalos et al. 2018). Therefore the ability to promote the differentiation of chromoplasts from chloroplasts (in chlorophyll containing photosynthetically active cells) has several potential biotechnological applications including but not restricted to food, feed, agriculture, cosmetic or nutraceutical, either alone or in combination with other inventions.

Chromoplasts can differentiate from preexisting plastids such as proplastids (i.e., undifferentiated plastids) and amyloplasts (i.e., starch-storing plastids), but most often they develop from chloroplasts (i.e., photosynthetic, chlorophyll containing plastids). Transformation of chloroplasts into chromoplasts occurs during the development of many flower petals and fruits but only a few plant species differentiate true chromoplasts in chlorophyll containing leaves. The yellow to red colors that some leaves acquire as they senesce (e.g. in the autumn or when they are exposed to continuous darkness) are due to carotenoids becoming visible when the chlorophylls degrade. This senescence process, however, does not involve the

transformation of chloroplasts into chromoplasts but into a completely different type of plastids named gerontoplasts (Koiwa, Ikeda et al. 1986, Hormaetxe, Hernandez et al. 2004, Egea, Barsan et al. 2010, Jarvis and Lopez-Juez 2013, Liebers, Grubler et al. 2017).

The most prominent changes during chloroplast to chromoplast differentiation are the remodeling of the internal plastid structures, together with a concurrent loss of photosynthetic competence and over-accumulation of carotenoid pigments (Egea, Barsan et al. 2010, Jarvis and Lopez-Juez 2013, Liebers, Grubler et al. 2017, Llorente, Martinez-Garcia et al. 2017, Gramegna, Rosado et al. 2018). The remodeling of the internal plastid structures generates increased metabolic sink capacity for carotenoid biosynthesis and storage, and it is, therefore, a critical developmental mechanism regulating carotenoid production in plants (Yuan, Zhang et al. 2015, Llorente, Martinez-Garcia et al. 2017). Due to its unique capacity to accumulate high amounts of carotenoids and other dietary nutrients such as tocopherols, the control of chromoplast differentiation has been proposed as a very promising strategy for improving the nutritional and health benefits of crops, and for other biotechnological endeavors (Yuan, Owsiany et al. 2015, Yuan, Zhang et al. 2015, Giuliano 2017, Llorente, Martinez-Garcia et al. 2017, Wurtzel 2019). However, the experimental manipulation of chromoplast differentiation for biotechnological applications has been hampered by our limited understanding of the mechanisms regulating this process. The only regulator of chromoplast development identified to date is ORANGE (OR), a chaperone found to localize in both plastids and nuclei. The specific molecular mechanism by which ORANGE triggers chromoplast differentiation remains unclear, as it independently promotes carotenoid biosynthesis, stability, and storage. As described below, the phytoene inductive threshold mechanism for artificial biogenesis was found to be independent of ORANGE, that is, does not require ORANGE dependent functions.

Here, we show for the first time that the supply of a sufficiently large amount of phytoene into chlorophyll containing plastids (chloroplasts) functions as a primary determinant of chromoplast artificial or synthetic biogenesis in green tissues (i.e., leaves or any other chloropyll containing, photosynthetic organs, tissues or cells). We provide evidence that **exceeding a threshold level** of phytoene, the first committed intermediate of the carotenoid pathway, is sufficient to induce chloroplast-to-chromoplast differentiation in green tissues. The present invention thus reveals a novel aspect of carotenoid biology and additionally presents a powerful tool for biotechnological applications of the artificial or synthetic differentiation of plastids into chromoplasts that could be used in new biofortified food and feed crops or nutraceutical and pharmaceutical compositions.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Chromoplast-like plastids develop from chloroplasts in leaves producing crtB. A, B, C, E, TEM images of representative plastids from the indicated species and treatments.

Bars, 1 µm. D, Immonoblot analysis of plastidial proteins.

Figure 2. Plant-produced crtB stays in the cytosol but can also enter chloroplats. Subcellular localization of the indicated constructs in *N. benthamiana* leaf cells. Bars, 20 µm.

Figure 3. Bacterial crtB needs to enter the chloroplast to induce chromoplast differentiation. A, Phenotypes of N. benthamiana leaf sections expressing the indicated constructs. B, Photosynthetic pigment levels in the sections shown in B. C, Photosynthetic parameters of the sections shown in B.

Figure 4. Chromoplast differentiation in *N. benthamiana* leaves ressembles that in tomato fruit. Heatmap represents of GO term enrichment values (Z-scores) in calculated from log2FC values in *N. benthamiana* leaves (crtB vs. GFP, NbB), tomato fruit (light ripe vs. mature green, SLO, and red ripe vs. mature green, SlR) and Arabidopsis leaves (30D senescent vs. 16D controls, AtS).

Figure 5. Chromoplast differentiation induces glycolytic and oxidative energy metabolism. Heatmap represents statistically significant fold-change values of metabolite levels in *N. benthamiana* leaves (crtB vs. GFP). Inset represents respiration rates.

Figure 6. Phytoene levels need to reach an activation threshold to induce chloroplast-to-chromoplast differentiation. A, Phytoene and downstream carotenoid contents in *N. benthamiana* leaves infiltrated with serial dilutions of *A. tumefaciens* cultures to express (p)crtB at different levels. B, Phytoene and downstream carotenoid contents at different time points after agroinfiltration of *N. benthamiana* leaves with constructs to express (p)crtB. In all cases, values correspond to the mean and SD values.

Figure 7. Time-course of chloroplast-to-chromoplast differentiation. A, Effective quantum yield in leaf sections at different time points after agroinfiltration with the indicated constructs. Representative images are shown. B, Non-photochemical quenching in leaf sections at different time points after agroinfiltration with (p)crtB. C, D1 protein contents at different time points after agroinfiltration with the indicated constructs. Values correspond to the mean and SD values.

Figure 8. Carotenoid overaccumulation is necessary but not sufficient to transform leaf chloroplasts into chromoplasts. Effective quantum yield in leaf sections at different time points after agroinfiltration with the indicated constructs. Samples treated with norflurazon (NF) are boxed in white. At 96 hpi, carotenoid contents were quantified. Values correspond to the mean and SD values.

Figure 9. Comparison of crtB-triggered phenotypes in different plants. A, MGDG levels in *N. tabacum*. B, Photosynthetic pigment levels in *N. tabacum* and *N. benthamiana*. C, *SAG12* gene expression in *N. tabacum*. D, Representative images of crtB-infected leaves from an Arabidopsis wild-type Columbia (WT Col) plant and an OR-defective double *ator ator-like* mutant.

Figure 10. Plastid-targeted GFP does not affect chloroplast development. *N. benthamiana* leaves were agroinfiltrated with constructs to express a plastid-targeted version of GFP (P-GFP) or the untargeted GFP protein (upper left picture). At 96 hpi, agroinfiltrated tissue was examined under a confocal laser scanning microscope to confirm the localization of P-GFP in chloroplasts and GFP in the cytosol (lower left pictures). Effective quantum yield of PSII was identical in both samples (plot in the right).

Figure 11. Chromoplast differentiation in *N. benthamiana* leaves does not require changes in the expression of carotenoid-related genes. Heatmap represents log2FC values in *N. benthamiana* leaves (crtB vs. GFP, NbB), tomato fruit (light ripe vs. mature green, SLO, and red ripe vs. mature green, SlR) and Arabidopsis leaves (30D senescent vs. 16D controls, AtS).

Figure 12. Plant PSY enzymes do not induce chromoplast differentiation in leaves. A, Phenotypes of leaf tissue expressing AtPSY, SlPSY1, or crtB(p). B, Phytoene content in the leaf sections shown in A.

Figure 13. crtB-mediated chromoplast differentiation impairs the xanthophyll cycle. DES, de-epoxidation state calculated as (Zx+0.5×Ax)/(Zx+Ax+Vx), where Zx, Ax and Vx are the concentrations of zeaxanthin, antheraxanthin and violaxanthin, respectively.

DESCRIPTION OF THE INVENTION

Definitions

A "recombinant nucleic acid" designates a nucleic acid which has been engineered and is not found as such in wild type organisms. In some particular embodiments, this term may refer to a gene operably linked to a promoter that is different from its naturally occurring promoter.

The term "gene" designates any nucleic acid encoding a protein. The term gene encompasses DNA, such as cDNA or gDNA, as well as RNA. The gene may be first prepared by e.g., recombinant, enzymatic and/or chemical techniques, and subsequently replicated in a host cell

or an in vitro system. The gene typically comprises an open reading frame encoding a desired protein. The gene may contain additional sequences such as a transcription terminator or a signal peptide.

The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to a coding sequence, in such a way that the control sequence directs expression of the coding sequence.

The term "control sequences" means nucleic acid sequences necessary for expression of a gene. Control sequences may be native or heterologous. Well-known control sequences and currently used by the person skilled in the art will be preferred. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. Preferably, the control sequences include a promoter and a transcription terminator.

The term "expression cassette" denotes a nucleic acid construct comprising a coding region, i.e. a gene, and a regulatory region, i.e. comprising one or more control sequences, operably linked. Preferably, the control sequences are suitable for plant host cells.

As used herein, the term “signal peptide”, may refer to sequences that when in translated into functional protein enhance the traffic of said protein into specific sub-cellular compartments, in the specific case of the present invention, into plastids, and more specifically into chloroplasts. Many signal peptides have been identified to date (from Rubisco i.e., Ribulose-1,5-bisphosphate carboxylase/oxygenase EC 4.1.1.39 , or in US20020178467A1, or in https://doi.org/10.1016/j.tplants.2013.04.003).

As used herein, the term "expression vector" means a DNA or RNA molecule that comprises an expression cassette. Preferably, the expression vector is a linear or circular double stranded DNA molecule.

As used herein, the term "native" or "endogenous", with respect to a specific organism (e.g. bacterium or plant), refers to a genetic element or a protein naturally present in said organism. The term "heterologous", with respect to a specific organism, refers to a genetic element or a protein that is not naturally present in said organism.

As used herein, the term "sequence identity" or "identity" refers to the number (%) of matches (identical amino acid residues) in positions from an alignment of two polypeptide sequences. The sequence identity is determined by comparing the sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. In particular, sequence identity may be determined using any of a number of mathematical global or local alignment algorithms,

depending on the length of the two sequences. Sequences of similar lengths are preferably aligned using a global alignment algorithms (e.g. Needleman and Wunsch algorithm; Needleman and Wunsch, 1970) which aligns the sequences optimally over the entire length, while sequences of substantially different lengths are preferably aligned using a local alignment algorithm (e.g. Smith and Waterman algorithm (Smith and Waterman, 1981) or Altschul algorithm (Altschul et al., 1997; Altschul et al., 2005)). Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software available on internet web sites such as http://blast.ncbi.nlm.nih.gov/ or http://www.ebi.ac.uk/Tools/emboss/). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, % amino acid sequence identity values refers to values generated using the pair wise sequence alignment program EMBOSS Needle that creates an optimal global alignment of two sequences using the Needleman-Wunsch algorithm, wherein all search parameters are set to default values, i.e. Scoring matrix = BLOSUM62, Gap open = 10, Gap extend = 0.5, End gap penalty = false, End gap open = 10 and End gap extend = 0.5

As used in this specification, the term "about" refers to a range of values ± 10% of the specified value. For example, "about 20" includes ± 10 % of 20, or from 18 to 22. Preferably, the term "about" refers to a range of values ± 5 % of the specified value.

As used herein, the term "CrtB" or "phytoene synthase" refers to a phytoene synthase enzyme (EC 2.5.1.32) encoded by a *crtB* gene which catalyzes the condensation of two molecules of geranylgeranyl diphosphate (GGPP) to give phytoene. According to the organism, the nomenclature of the above identified enzyme and encoding gene may vary. However, for the sake of clarity, in the present specification, these terms are used independently from the origin of the enzymes or genes. Preferably, the term “CrtB” may refer to any of the sequences identified in table 1 below:

Table 1. 250 crtb sequences

Entry	Entry name	Gene names	Organism	Organism ID	Protein names	Length
P21683	**CRTB_PANAN**	**crtB**	**Pantoea ananas (Erwinia uredovora)**	**553**	**15-cis-phytoene synthase**	**309**
D4GFK9	D4GFK9_PANAM	**crtB** PANA_4162	Pantoea ananatis (strain LMG 20103)	706191	CrtB	309
A0A2P5IKE2	A0A2P5IKE2_9GAMM	C3408_19145	Pantoea sp. PSNIH6	2080494	Phytoene synthase	309

D5KXJ0	CRTB_ENTAG	**crtB**	Enterobacter agglomerans (Erwinia herbicola) (Pantoea agglomerans)	549	**15-cis-phytoene synthase**	295
A0A2P5GS98	A0A2P5GS98_9ENTR	CHU32_07085	Superficieibacter electus	2022662	Phytoene synthase	310
A0A0U5L0T8	A0A0U5L0T8_9GAMM	**crtB** EM595_0217	Erwinia gerundensis	1619313	Phytoene synthase CrtB	308
A0A0J8VQ29	A0A0J8VQ29_9ENTR	ACH50_10685	Franconibacter pulveris	435910	Phytoene synthase	308
A0A2N5EQI9	A0A2N5EQI9_9GAMM	CYR34_06205	Yersiniaceae bacterium 2016Iso3	2060066	Phytoene synthase	309
A0A2P8VGN3	A0A2P8VGN3_9ENTR	C7G83_16955	Siccibacter turicensis	357233	Phytoene synthase	308
A0A3R8ZPG5	A0A3R8ZPG5_9GAMM	EGK14_15735	Erwinia sp. 198	2022746	Phytoene/squalene synthase family p...	318
P22872	CRTB_ESCVU	**crtB**	Escherichia vulneris (Pseudescherichia vulneris)	566	**Phytoene synthase**	309
V5CM68	V5CM68_ENTCL	EDP2_323	Enterobacter cloacae S611	1399146	Squalene/phytoene synthase family p...	310
A0A2S9IBS8	A0A2S9IBS8_9GAMM	CQW29_12335	Pantoea coffeiphila	1465635	Phytoene synthase	309
A0A1X1D9H8	A0A1X1D9H8_9GAMM	HA48_10250	Pantoea wallisii	1076551	Phytoene synthase	309
A0A1S2DFG8	A0A1S2DFG8_9GAMM	BC443_00345	Salinicola sp. MIT1003	1882734	Phytoene synthase	308
A0A1W6B2R9	A0A1W6B2R9_9GAMM	B1H58_04565	Pantoea alhagi	1891675	Phytoene synthase	316
A0A090VNH4	A0A090VNH4_ESCVU	**crtB**EV102420_02_02370	Pseudescherichia vulneris NBRC 102420	1115515	Phytoene/squalene synthase	309
U3TN93	U3TN93_9ENTR	E05_04190	Plautia stali symbiont	891974	Phytoene synthase	309
A0A2I0FS50	A0A2I0FS50_9GAMM	CIG19_17290	Enterobacterales bacterium CwR94	2025587	Phytoene synthase	316
A0A0Q5HG39	A0A0Q5HG39_9BURK	ASF77_14330	Massilia sp. Leaf139	1736272	Phytoene synthase	326
A0A0J8YHL0	A0A0J8YHL0_9GAMM	AI29_03700	bacteria symbiont BFo2 of Frankliniella occidentalis	1628856	Phytoene synthase	308
A0A0L6VXG6	A0A0L6VXG6_9BURK	AB595_23635	Massilia sp. WF1	1406431	Phytoene synthase	327

A0A285N5W6	A0A285N5W6_9PSED	SAMN05660463_00761	Pseudomonas sp. URIL14HWK12:I9	1261633	Phytoene synthase	313
A0A1H8PBA5	A0A1H8PBA5_9BURK	SAMN05428959_107148	Duganella sp. CF517	1881038	Phytoene synthase	323
H5V4V1	H5V4V1_ATLHE	**crtB**EH105704_11_00060	Atlantibacter hermannii NBRC 105704	1115512	Phytoene synthase	308
K9DBF5	K9DBF5_9BURK	HMPREF9710_03393	Massilia timonae CCUG 45783	883126	Uncharacterized protein	331
A0A1E7WJF5	A0A1E7WJF5_9BURK	**crtB_3** DUPY_29190	Duganella phyllosphaerae	762836	All-trans-phytoene synthase/15-cis-...	331
A0A1H2PNS6	A0A1H2PNS6_9BURK	SAMN05216551_104353	Burkholderia sp. JS23	1770053	Phytoene synthase	339
A0A1S9AKN2	A0A1S9AKN2_9BURK	B0920_12090	Massilia sp. KIM	1955422	Phytoene synthase	323
A0A2R4CH02	A0A2R4CH02_9BURK	C9I28_27250	Massilia armeniaca	2072590	Phytoene synthase	323
A0A086WFW7	A0A086WFW7_9BURK	JN27_02885	Massilia sp. BSC265	1549812	Phytoene synthase	324
Q7MZE9	Q7MZE9_PHOLL	**crtB** plu4343	Photorhabdus luminescens subsp. laumondii (strain DSM 15139 / CIP 105565 / TT01)	243265	Phytoene synthase	308
A0A098T037	A0A098T037_9PSED	LT42_06025	Pseudomonas lutea	243924	Phytoene synthase	329
F6AD53	F6AD53_PSEF1	Psefu_2397	Pseudomonas fulva (strain 12-X)	743720	Phytoene synthase	332
A0A098U8I8	A0A098U8I8_9BURK	IA69_16815	Massilia sp. JS1662	1519190	Phytoene synthase	315
A0A0X8BW05	A0A0X8BW05_9PSED	APT63_09595	Pseudomonas monteilii	76759	Phytoene synthase	330
A0A1I7IY64	A0A1I7IY64_9GAMM	SAMN04487955_108147	Halomonas korlensis	463301	Phytoene synthase	311
A0A1H1Z0E0	A0A1H1Z0E0_9PSED	SAMN05216421_3335	Pseudomonas xinjiangensis	487184	Phytoene synthase	307
A0A290XFG2	A0A290XFG2_9GAMM	CNR27_10975	Luteimonas sp. 100111	2006110	Phytoene synthase	311
A0A1I2YQ18	A0A1I2YQ18_9GAMM	SAMN04487959_10239	Halomonas xianhensis	442341	Phytoene synthase	312
N2JAK4	N2JAK4_9PSED	HMPREF1487_04794	Pseudomonas sp. HPB0071	1203578	Uncharacterized protein	308
A0A1H2ZR06	A0A1H2ZR06_9PSED	SAMN05216287_2410	Pseudomonas kuykendallii	1007099	Phytoene synthase	308

A0A383RUF0	A0A383RUF0_9PSED	**crtB** CCOS865_02936	Pseudomonas reidholzensis	1785162	All-trans-phytoene synthase/15-cis-...	360
A0A0U4WG37	A0A0U4WG37_9PSED	APT59_08110	Pseudomonas oryzihabitans	47885	Phytoene synthase	311
A4VR94	A4VR94_PSEU5	PST_3872	Pseudomonas stutzeri (strain A1501)	379731	Phytoene synthase	308
A0A0C4WLC4	A0A0C4WLC4_9GAMM	**crtB** Achr_15270	Azotobacter chroococcum NCIMB 8003	1328314	Phytoene synthase crtB	321
A0A078LWK5	A0A078LWK5_9PSED	BN1079_02002	Pseudomonas saudiphocaensis	1499686	Phytoene synthase	308
A0A1T4L878	A0A1T4L878_9RHIZ	SAMN05428963_101108	Consotaella salsifontis	1365950	Phytoene synthase	320
A0A3L7AE21	A0A3L7AE21_9RHIZ	D9R14_11840	Xanthobacter tagetidis	60216	Phytoene/squalene synthase family p...	311
F5XXP8	F5XXP8_RAMTT	Rta_07700	Ramlibacter tataouinensis (strain ATCC BAA-407 / DSM 14655 / LMG 21543 / TTB310)	365046	Candidate phytoene synthase	310
A0A0Q6CNX1	A0A0Q6CNX1_9RHIZ	ASG43_04300	Aureimonas sp. Leaf454	1736381	Phytoene synthase	321
A0A0Q5LK63	A0A0Q5LK63_9BURK	ASF94_07960	Acidovorax sp. Leaf160	1736280	Phytoene synthase	315
A0A0Q6EHK4	A0A0Q6EHK4_9RHIZ	ASG54_15465	Aureimonas sp. Leaf460	1736384	Phytoene synthase	317
A0A3A9JHA4	A0A3A9JHA4_9PROT	D6Z83_17175	Roseomonas sp. Z24	2382222	Phytoene/squalene synthase family p...	308
Q93CI6	Q93CI6_XANP2	**crtB** Xaut_3578	Xanthobacter autotrophicus (strain ATCC BAA-1158 / Py2)	78245	Phytoene synthase	311
A0A0Q4G9T3	A0A0Q4G9T3_9BURK	ASE76_09585	Xylophilus sp. Leaf220	1735686	Phytoene synthase	317
A0A0Q5GZX1	A0A0Q5GZX1_9RHIZ	ASF65_09280	Aureimonas sp. Leaf324	1736336	Phytoene synthase	308
A0A0Q6F2D1	A0A0Q6F2D1_9RHIZ	ASG48_00690	Aurantimonas sp. Leaf443	1736378	Phytoene synthase	314
X5MES2	X5MES2_9RHIZ	BN1012_Phect1195	Candidatus Phaeomarinobacter ectocarpi	1458461	Phytoene synthase	317
A0A2D2AWL2	A0A2D2AWL2_9CAUL	CSW64_08135	Caulobacter mirabilis	69666	Phytoene synthase	311

Q1YGX7	Q1YGX7_AURMS	SI859A1_00462	Aurantimonas manganoxydans (strain ATCC BAA-1229 / DSM 21871 / SI85-9A1)	287752	Phytoene synthase	329
A0A371X1S1	A0A371X1S1_9RHIZ	DYI37_14280	Fulvimarina sp. 85	2293836	Phytoene/squalene synthase family p...	344
Q0G4C2	Q0G4C2_9RHIZ	FP2506_14039	Fulvimarina pelagi HTCC2506	314231	Phytoene synthase	352
A0A1K2HUI7	A0A1K2HUI7_9RHIZ	SAMN02983003_0371	Devosia enhydra	665118	Phytoene synthase	314
A0A1H0DQ75	A0A1H0DQ75_9RHIZ	SAMN05192530_101901	Aureimonas jatrophae	1166073	Phytoene synthase	314
A0A0Q5V890	A0A0Q5V890_9CAUL	ASG17_11235	Brevundimonas sp. Leaf363	1736353	Phytoene synthase	310
A0A0F5L7I4	A0A0F5L7I4_9RHIZ	VW35_11040	Devosia soli	361041	Phytoene synthase	307
A0A1Y6ES84	A0A1Y6ES84_9RHIZ	SAMN06295905_1144	Devosia lucknowensis	1096929	Phytoene synthase	307
A4Z1U2	A4Z1U2_BRASO	**crtB** BRADO6506	Bradyrhizobium sp. (strain ORS 278)	114615	Phytoene synthase	335
A0A175RT03	A0A175RT03_9RHIZ	NS365_09075	Aureimonas ureilytica	401562	Phytoene synthase	320
A0A1X0T982	A0A1X0T982_9RHIZ	ATO4_01980	Aurantimonas sp. 22II-16-19i	1317114	Phytoene synthase	341
A0A433XF97	A0A433XF97_9RHIZ	EMQ25_06370	Arsenicitalea aurantiaca	1783274	Phytoene/squalene synthase family p...	310
A0A346YPK6	A0A346YPK6_9RHIZ	CYG48_12265	Neorhizobium sp. SOG26	2060726	Phytoene synthase	310
A0A2P1NMV8	A0A2P1NMV8_9BURK	C7H73_12405	Melaminivora sp. SC2-7	2116657	Phytoene synthase	322
A0A239L348	A0A239L348_9BURK	SAMN06265795_11924	Noviherbaspirillum humi	1688639	Phytoene synthase	308
D9QK03	D9QK03_BRESC	Bresu_2278	Brevundimonas subvibrioides (strain ATCC 15264 / DSM 4735 / LMG 14903 / NBRC 16000 / CB 81) (Caulobacter subvibrioides)	633149	Phytoene synthase	313
A0A2R7L7D6	A0A2R7L7D6_9CAUL	DBR10_15595	Caulobacter sp. HMWF025	2056860	Phytoene synthase	309
A0A397Q5C8	A0A397Q5C8_9RHIZ	BXY53_1361	Dichotomicrobium thermohalophilum	933063	Phytoene synthase	322

A0A0V2F4S2	A0A0V2F4S2_CAUVI	AS593_07995	Caulobacter vibrioides (Caulobacter crescentus)	155892	Phytoene synthase	318
A0A1W2EFJ9	A0A1W2EFJ9_9RHIZ	SAMN06297251_1251	Fulvimarina manganoxydans	937218	Phytoene synthase	344
A0A2G8T1X4	A0A2G8T1X4_9BURK	CR103_09820	Massilia psychrophila	1603353	Phytoene synthase	317
A0A1G9EQX8	A0A1G9EQX8_9RHOB	SAMN04487971_10396	Paracoccus chinensis	525640	Phytoene synthase	303
A0A418ZVE0	A0A418ZVE0_9RHOB	D3P06_11200	Paracoccus aestuarii	453842	Phytoene/squalene synthase family p...	303
B0T3F4	B0T3F4_CAUSK	Caul_1711	Caulobacter sp. (strain K31)	366602	Squalene/phytoene synthase	309
A0A2S7K4N9	A0A2S7K4N9_9PROT	CW354_11695	Marinicaulis flavus	2058213	Phytoene synthase	325
A0A0D6T9X0	A0A0D6T9X0_9RHOB	SY26_13460	Paracoccus sp. 228	1192054	Uncharacterized protein	304
A0A1I7NV16	A0A1I7NV16_9RHIZ	SAMN05216456_3433	Devosia crocina	429728	Phytoene synthase	307
A0A0Q5PF57	A0A0Q5PF57_9SPHN	ASG07_08310	Sphingomonas sp. Leaf343	1736345	Phytoene synthase	315
P54975	CRTB_PARSN	**crtB**	Paracoccus sp. (strain N81106 / MBIC 01143) (Agrobacterium aurantiacum)	81397	**15-cis-phytoene synthase**	304
A0A1G5HZE2	A0A1G5HZE2_9RHOB	SAMN05660710_02407	Paracoccus tibetensis	336292	Phytoene synthase	303
A0A1I4BAN5	A0A1I4BAN5_9RHIZ	SAMN05444581_11411	Methylocapsa palsarum	1612308	Phytoene synthase	324
A0A0W1QPN2	A0A0W1QPN2_9SPHN	ATB93_04250	Sphingomonas sp. WG	1592629	Phytoene synthase	307
A0A0Q7SEW8	A0A0Q7SEW8_9CAUL	ASD25_22640	Brevundimonas sp. Root1423	1736462	Phytoene synthase	314
E3I5T7	E3I5T7_RHOVT	Rvan_0150	Rhodomicrobium vannielii (strain ATCC 17100 / ATH 3.1.1 / DSM 162 / LMG 4299)	648757	Phytoene synthase	324
A0A3S4CM38	A0A3S4CM38_9RHOB	**crtB** PARHAE_03900	Paracoccus sp. M1-83	2491866	15-cis-phytoene synthase	303
A0A1S4ENJ6	A0A1S4ENJ6_DIACI	**LOC103519589**	Diaphorina citri (Asian citrus psyllid)	121845	uncharacterized protein LOC10351958...	272
A0A0N0K3C6	A0A0N0K3C6_9PROT	IP88_03220	alpha proteobacterium AAP81b	1523432	Uncharacterized protein	310

A0A257EPT4	A0A257EPT4_9PROT	CFE37_03910	Alphaproteobacteria bacterium PA4	2015572	Phytoene synthase	310
A0A1E3LV90	A0A1E3LV90_9SPHN	BFL28_16930	Sphingomonas turrisvirgatae	1888892	Phytoene synthase	312
A0A1G5UKG4	A0A1G5UKG4_9SPHN	SAMN03159340_03048	Sphingomonas sp. NFR15	1566282	Phytoene synthase	314
A0A0Q4KET8	A0A0Q4KET8_9SPHN	ASE86_01900	Sphingomonas sp. Leaf33	1736215	Phytoene synthase	315
A0A238WF06	A0A238WF06_9RHOB	SAMN06265378_104140	Paracoccus sediminis	1214787	Phytoene synthase	305
A0A099F0H6	A0A099F0H6_9RHOB	IC63_12700	Paracoccus sphaerophysae	690417	Uncharacterized protein	302
B8EQ05	B8EQ05_METSB	Msil_2069	Methylocella silvestris (strain DSM 15510 / CIP 108128 / LMG 27833 / NCIMB 13906 / BL2)	395965	Squalene/phytoene synthase	355
A0A2A2SC16	A0A2A2SC16_9SPHN	CKY28_12235	Sphingomonas sp. 1PNM-20	1141887	Phytoene synthase	315
A0A2A3N073	A0A2A3N073_9SPHN	SxD43FB_02845	Sphingobium sp. D43FB	2017595	Phytoene synthase	318
A0A426RUJ8	A0A426RUJ8_9SPHN	D7D48_07035	Sphingorhabdus wooponensis	940136	Phytoene/squalene synthase family p...	307
T0GT04	T0GT04_9SPHN	L288_15200	Sphingobium quisquiliarum P25	1329909	Phytoene synthase	321
A0A1Y1SSK0	A0A1Y1SSK0_9RHOB	ATO13_10161	Stappia sp. 22II-S9-Z10	1317123	Squalene/phytoene synthase	309
A0A0Q4LMR4	A0A0Q4LMR4_9SPHN	ASE97_14625	Sphingomonas sp. Leaf42	1736219	Phytoene synthase	308
A0A243PM03	A0A243PM03_9SPHN	CA262_16950	Sphingobium sp. GW456-12-10-14-TSB1	1987165	Phytoene synthase	318
A0A126R3S0	A0A126R3S0_9SPHN	K663_01410	Sphingobium sp. MI1205	407020	Phytoene synthase	319
A0A430DUK7	A0A430DUK7_9SPHN	CA235_02705	Sphingomonas sp. ABOLF	1985879	Phytoene/squalene synthase family p...	317
A0A1L3J911	A0A1L3J911_9SPHN	LPB140_00865	Sphingopyxis sp. LPB0140	1913578	Phytoene synthase	313
A0A495RY84	A0A495RY84_SPHMI	DFR51_1399	Sphingosinicella microcystinivorans	335406	Phytoene synthase	320
A0A0P6ZPY7	A0A0P6ZPY7_9SPHN	AAJ72_13300	Citromicrobium sp. RCC1885	1647104	Phytoene synthase	336
A0A0J7Y0Y8	A0A0J7Y0Y8_9SPHN	V473_02115	Sphingobium czechense LL01	1420583	Phytoene synthase	316
A0A2R7JVN6	A0A2R7JVN6_9SPHN	DBR17_03295	Sphingomonas sp. HMWF008	2056845	Phytoene synthase	319

A0A494VVZ2	A0A494VVZ2_9SPHN	SAMIE_1000640	Sphingobium amiense	135719	Phytoene/squalene synthase family p...	321
A0A430BH61	A0A430BH61_9SPHN	BRX43_10450	Sphingomonas sp. S-NIH.Pt15_0812	1920129	Phytoene/squalene synthase family p...	321
A0A167JN79	A0A167JN79_9SPHN	A3736_03600	Erythrobacter sp. HI0063	1822240	Phytoene synthase	340
A0A1V0GYN7	A0A1V0GYN7_9RHOB	A6J80_22060	Paracoccus yeei	147645	Phytoene/squalene synthase family p...	308
E0THV7	E0THV7_PARBH	PB2503_11014	Parvularcula bermudensis (strain ATCC BAA-594 / HTCC2503 / KCTC 12087)	314260	Phytoene synthase	321
A0A418PZT2	A0A418PZT2_9SPHN	D3M59_07935	Sphingomonas sp. DAC4	2315689	Phytoene/squalene synthase family p...	335
A0A097EIE4	A0A097EIE4_9SPHN	MC45_14215	Sphingomonas taxi	1549858	Phytoene synthase	316
M2U509	M2U509_9SPHN	C725_1676	Pacificimonas flava	1234595	Phytoene synthase	340
D4YXE1	D4YXE1_SPHJU	**crtB** SJA_C1-01890	Sphingobium japonicum (strain DSM 16413 / CCM 7287 / MTCC 6362 / UT26 / NBRC 101211 / UT26S)	452662	Phytoene/squalene synthetase	317
A0A1Y2QRI3	A0A1Y2QRI3_9SPHN	CAP39_08625	Sphingomonas sp. IBVSS1	1985171	Uncharacterized protein	322
A0A345WN99	A0A345WN99_9SPHN	DM480_06150	Sphingomonas sp. FARSPH	2219696	Phytoene/squalene synthase family p...	316
A0A1A7QJ13	A0A1A7QJ13_9SPHN	A9995_08555	Erythrobacter sp. QSSC1-22B	1860125	Phytoene synthase	340
W1S3H7	W1S3H7_9SPHN	C100_11835	Sphingobium sp. C100	1207055	Phytoene synthase	317
A0A2N0I4B4	A0A2N0I4B4_9SPHN	B0I00_1213	Novosphingobium kunmingense	1211806	Phytoene synthase	307
A0A371REQ8	A0A371REQ8_9PROT	DX908_00745	Parvularcula sp. SM1705	2292771	Phytoene/squalene synthase family p...	326
A0A196M954	A0A196M954_9SPHN	A7Q26_08955	Sphingobium sp. TCM1	453246	Phytoene synthase	316
A0A147IY03	A0A147IY03_9SPHN	NS355_03645	Sphingomonas yabuuchiae	172044	Phytoene synthase	316
F6EUT5	F6EUT5_SPHCR	Sphch_1941	Sphingobium chlorophenolicum L-1	690566	Phytoene synthase	317
A0A371BF47	A0A371BF47_9SPHN	DXH95_01930	Sphingorhabdus sp. GY_G	2292257	Phytoene/squalene synthase family p...	308

A0A175Y1F2	A0A175Y1F2_9SPHN	AVM11_08125	Sphingomonas melonis TY	621456	Uncharacterized protein	314
A0A285QG69	A0A285QG69_9SPHN	SAMN06297144_1101	Sphingomonas guangdongensis	1141890	Phytoene synthase	316
A0A0Q4FTS3	A0A0Q4FTS3_9SPHN	ASE75_09645	Sphingomonas sp. Leaf17	1735683	Phytoene synthase	320
A0A165QP88	A0A165QP88_9SPHN	A3711_04890	Erythrobacter sp. HI00D59	1822215	Phytoene synthase	340
Q1NEY9	Q1NEY9_SPHSS	SKA58_15537	Sphingomonas sp. (strain SKA58)	314266	Squalene/phytoene synthase	318
A0A2K1EBQ1	A0A2K1EBQ1_9SPHN	BA950_01295	Erythrobacter sp. SAORIC-644	1869314	Phytoene synthase	340
A0A3S0RHF3	A0A3S0RHF3_9SPHN	EJC47_05525	Sphingomonas sp. TF3	2495580	Phytoene/squalene synthase family p...	315
A0A2A4I6W5	A0A2A4I6W5_9SPHN	COA07_12690	Sphingomonas adhaesiva	28212	Phytoene/squalene synthase family p...	303
A0A0P6WGV5	A0A0P6WGV5_9SPHN	SZ64_10920	Erythrobacter sp. SG61-1L	1603897	Phytoene synthase	317
A0A173KVT4	A0A173KVT4_9SPHN	EP837_01648	Sphingobium sp. EP60837	1855519	15-cis-phytoene synthase	319
A0A1Y6F456	A0A1Y6F456_9SPHN	SAMN06297468_1916	Altererythrobacter xiamenensis	1316679	Phytoene synthase	326
A0A1L6J797	A0A1L6J797_9SPHN	BDW16_2415, BRX40_04525, DAH55_03615, DAH56_00070	Sphingomonas koreensis	93064	Phytoene synthase	313
A0A1H9NI87	A0A1H9NI87_9SPHN	SAMN05518866_109103	Sphingobium sp. YR768	1884365	Phytoene synthase	316
A0A1H7RTY5	A0A1H7RTY5_9SPHN	SAMN05216382_2350	Sphingomonas sp. JS21-1	1855283	Phytoene synthase	317
G2IMR1	G2IMR1_SPHSK	**crtB** SLG_31750	Sphingobium sp. (strain NBRC 103272 / SYK-6)	627192	Phytoene/squalene synthase	321
A0A1H8HLP5	A0A1H8HLP5_9SPHN	SAMN05192583_3080	Sphingomonas sp. S6-262	1166340	Phytoene synthase	317
A0A345YCW4	A0A345YCW4_9SPHN	DVR09_04930	Erythrobacter sp. YH-07	2182384	Phytoene/squalene synthase family p...	340
A0A220W370	A0A220W370_9SPHN	**crtB**SPHFLASMR4Y_00463	Sphingorhabdus flavimaris	266812	All-trans-phytoene synthase/15-cis-...	311
A0A0N1LIX0	A0A0N1LIX0_9SPHN	IP83_16905	Novosphingobium sp. AAP93	1523427	Phytoene synthase	321
A0A0Q4GF22	A0A0Q4GF22_9SPHN	ASE72_07730	Sphingomonas sp. Leaf20	1735685	Phytoene synthase	316

A0A369VYY5	A0A369VYY5_9SPHN	DVW87_14475	Sphingomonas sp. WZY 27	2283317	Phytoene/squalene synthase family p...	334
A0A0N0K090	A0A0N0K090_9SPHN	IP68_11255	Blastomonas sp. AAP25	1523416	Phytoene synthase	293
J8SJA3	J8SJA3_9SPHN	LH128_11738	Sphingomonas sp. LH128	473781	Phytoene synthase	323
A0A2A4B9S7	A0A2A4B9S7_9SPHN	COC42_09205	Sphingomonas spermidinifaciens	1141889	Phytoene synthase	311
A0A1I5TJR0	A0A1I5TJR0_9SPHN	SAMN04488241_10891	Sphingomonas rubra	634430	Phytoene synthase	315
A0A1N6EQ33	A0A1N6EQ33_9SPHN	SAMN02745824_2034	Sphingorhabdus marina DSM 22363	1123272	Phytoene synthase	309
A0A3D9FH47	A0A3D9FH47_9SPHN	DFR46_2012	Parasphingopyxis lamellibrachiae	680125	Phytoene synthase	300
A0A2P7QGQ2	A0A2P7QGQ2_9SPHN	C7I55_23335	Sphingosinicella sp. GL-C-18	2116704	Phytoene synthase	309
F6IJD0	F6IJD0_9SPHN	PP1Y_AT8258	Novosphingobium sp. PP1Y	702113	Phytoene synthase	317
G6E9P6	G6E9P6_9SPHN	NSU_1067	Novosphingobium pentaromativorans US6-1	1088721	Phytoene synthase	317
A0A3R9WTJ5	A0A3R9WTJ5_9SPHN	HMF7854_11995	Sphingomonas sp. HMF7854	2315330	Phytoene/squalene synthase family p...	331
A0A0T0PYJ1	A0A0T0PYJ1_9SPHN	ASG29_01100	Sphingomonas sp. Leaf412	1736370	Phytoene synthase	311
A0A0Q8XE94	A0A0Q8XE94_9SPHN	ASE13_12025	Sphingomonas sp. Root241	1736501	Phytoene synthase	318
A0A420WJI4	A0A420WJI4_9RHOB	DES40_0400	Litorimonas taeanensis	568099	Phytoene synthase	307
A0A074MHB2	A0A074MHB2_ERYLO	EH31_05910	Erythrobacter longus	1044	Phytoene synthase	315
A0A0T2Q5L4	A0A0T2Q5L4_9SPHN	ASD76_17695	Altererythrobacter sp. Root672	1736584	Phytoene synthase	360
A0A0C5LBH7	A0A0C5LBH7_9SPHN	TS85_20435	Sphingomonas hengshuiensis	1609977	Phytoene synthase	311
W0A662	W0A662_9SPHN	NX02_00980	Sphingomonas sanxanigenens DSM 19645 = NX02	1123269	Uncharacterized protein	312
A0A147EF90	A0A147EF90_9SPHN	NS277_10930	Novosphingobium barchaimii	1420591	Phytoene synthase	323
A0A0M4M609	A0A0M4M609_9SPHN	AMC99_00377	Altererythrobacter epoxidivorans	361183	Phytoene synthase	365
A0A1W7MCK9	A0A1W7MCK9_9SPHN	NMD1_03712	Novosphingobium sp. MD-1	1630648	Phytoene synthase	334

A0A418NGM2	A0A418NGM2_9SPHN	D2V04_13375	Altererythrobacter sp. Ery1	2306995	Phytoene/squalene synthase family p...	348
A0A1B6ZB79	A0A1B6ZB79_9SPHN	A8B75_00795	Sphingomonadales bacterium EhC05	1849171	Phytoene synthase	313
A0A411ZGL9	A0A411ZGL9_9SPHN	BPTFM16_02528	Altererythrobacter sp. BPTF-M16	2169535	15-cis-phytoene synthase	333
Q2N8C0	Q2N8C0_ERYLH	ELI_09895	Erythrobacter litoralis (strain HTCC2594)	314225	Phytoene synthase	361
A0A437NDA7	A0A437NDA7_9SPHN	EOE18_02140	Novosphingobium sp. FSY-9	1908524	Phytoene/squalene synthase family p...	314
A0A1Y2QN53	A0A1Y2QN53_9SPHN	CAP40_02935	Sphingomonas sp. IBVSS2	1985172	Phytoene synthase	314
A0A074MDM0	A0A074MDM0_9SPHN	EH32_02875	Erythrobacter litoralis	39960	Phytoene synthase	322
A0A419MYR2	A0A419MYR2_9SPHN	D5I55_07375	Sphingomonadaceae bacterium KCTC 52944	2339262	Phytoene/squalene synthase family p...	294
A0A0Q5R0A9	A0A0Q5R0A9_9SPHN	ASG11_01840	Sphingomonas sp. Leaf357	1736350	Phytoene synthase	317
A0A109LQH6	A0A109LQH6_9SPHN	AUC45_12300	Erythrobacter sp. YT30	1735012	Phytoene synthase	322
A0A0Q6KJ92	A0A0Q6KJ92_9SPHN	ASG67_10245	Sphingomonas sp. Leaf339	1736343	Phytoene synthase	321
A0A0H0XRZ9	A0A0H0XRZ9_9SPHN	AAV99_13175	Erythrobacter marinus	874156	Phytoene synthase	353
A0A1H7AUW7	A0A1H7AUW7_9SPHN	SAMN05428950_102427	Sphingomonas sp. OV641	1881068	Phytoene synthase	315
A0A3N2QFK9	A0A3N2QFK9_9SPHN	EB810_12790	Altererythrobacter sp. FM1	2484537	Phytoene/squalene synthase family p...	350
N1MKG3	N1MKG3_9SPHN	EBBID32_17900	Sphingobium japonicum BiD32	1301087	Phytoene synthase	290
A0A239BTL3	A0A239BTL3_9SPHN	SAMN06295912_101379	Sphingomonas laterariae	861865	Phytoene synthase	309
A0A328ACM6	A0A328ACM6_9CAUL	DJ018_09820	Phenylobacterium deserti	1914756	Phytoene/squalene synthase family p...	311
I9C345	I9C345_9SPHN	WSK_3197	Novosphingobium sp. Rr 2-17	555793	Phytoene synthase	321
A0A2L0ACJ1	A0A2L0ACJ1_9SPHN	C1T17_16820	Sphingobium sp. SCG-1	2072936	Phytoene synthase	310
A0A1X9YG98	A0A1X9YG98_9SPHN	KC8_11320	Sphingomonas sp. KC8	1030157	Uncharacterized protein	316
A0A1X7G805	A0A1X7G805_9SPHN	SAMN06295910_1317	Sphingomonas indica	941907	Phytoene synthase	310

A0A0Q4C1H0	A0A0Q4C1H0_9SPHN	ASE49_14715	Novosphingobium sp. Leaf2	1735670	Phytoene synthase	333
A0A0F5PC85	A0A0F5PC85_9SPHN	WP12_10495	Sphingomonas sp. SRS2	133190	Phytoene synthase	309
A0A437LYG0	A0A437LYG0_9SPHN	EOD43_19555	Sphingomonas sp. CCP-7	1979270	Phytoene/squalene synthase family p...	311
A0A2K2FV15	A0A2K2FV15_9SPHN	A8V01_08485	Novosphingobium guangzhouense	1850347	Phytoene synthase	317
A0A2Z5Z1W0	A0A2Z5Z1W0_9SPHN	AEB_P3493	Altererythrobacter sp. B11	2060312	Phytoene synthase	353
A0A1I6LKX9	A0A1I6LKX9_9SPHN	SAMN05192580_2879	Sphingomonas jatrophae	1166337	Phytoene synthase	317
U2YNY3	U2YNY3_9SPHN	**crtB** NT2_10_00500	Novosphingobium tardaugens NBRC 16725	1219035	Phytoene/squalene synthase	327
F1ZA52	F1ZA52_9SPHN	Y88_0597	Novosphingobium nitrogenifigens DSM 19370	983920	Farnesyl-diphosphate farnesyltransf.. .	314
A0A2A8I0D5	A0A2A8I0D5_9SPHN	B2G71_02150	Novosphingobium sp. PC22D	1962403	Phytoene synthase	321
A0A0M6YJL0	A0A0M6YJL0_9RHOB	**crtM_2**JDO7802_01471	Jannaschia donghaensis	420998	Dehydrosqualene synthase	313
A0A0Q4J1X3	A0A0Q4J1X3_9SPHN	ASE85_05005	Sphingobium sp. Leaf26	1735693	Phytoene synthase	289
A0A0Q4L9F4	A0A0Q4L9F4_9SPHN	ASF00_03950	Sphingomonas sp. Leaf34	1736216	Phytoene synthase	315
T0J6D2	T0J6D2_9SPHN	L284_00540	Novosphingobium lindaniclasticum LE124	1096930	Phytoene synthase	340
A0A0N0KE23	A0A0N0KE23_9SPHN	IP81_12215	Novosphingobium sp. AAP83	1523425	Phytoene synthase	326
A0A1B2AGK0	A0A1B2AGK0_9SPHN	**crtB** A6F68_02787	Altererythrobacter dongtanensis	692370	All-trans-phytoene synthase/15-cis-...	358
A0A1V2EVJ7	A0A1V2EVJ7_9SPHN	**crtB** SPHI_13020	Sphingomonas jeddahensis	1915074	All-trans-phytoene synthase/15-cis-...	309
A0A3A1PIF8	A0A3A1PIF8_9SPHN	D2V17_00730	Erythrobacter xanthus	1784712	Phytoene/squalene synthase family p...	348
Q2G7B9	Q2G7B9_NOVAD	Saro_1814	Novosphingobium aromaticivorans (strain ATCC 700278 / DSM 12444 / CIP 105152 / NBRC 16084 / F199)	279238	Farnesyl-diphosphate farnesyltransf.. .	323

A0A1I2SC57	A0A1I2SC57_9SPHN	SAMN05518801_1326	Novosphingobium sp. CF614	1884364	Phytoene synthase	335
A0A0B9AG51	A0A0B9AG51_9SPHN	NJ75_00990	Novosphingobium subterraneum	48936	Farnesyl-diphosphate farnesyltransf..	321
A0A0F7KXU4	A0A0F7KXU4_9SPHN	**crtB** WYH_03017	Altererythrobacter atlanticus	1267766	All-trans-phytoene synthase/15-cis-...	353
A0A437JCA0	A0A437JCA0_9SPHN	ENE74_02460	Sphingobium sp. TLA-22	2008318	Phytoene/squalene synthase family p...	321
A0A192D489	A0A192D489_9SPHN	A9D12_07620	Porphyrobacter neustonensis	1112	Phytoene synthase	321
A0A418WQ24	A0A418WQ24_9SPHN	D3876_03070	Sphingomonas sp. K2R01-6	2320861	Phytoene/squalene synthase family p...	321
A0A0N0M211	A0A0N0M211_9SPHN	ADT71_10055	Novosphingobium sp. ST904	1684385	Phytoene synthase	337
A3WEG9	A3WEG9_9SPHN	NAP1_10293	Erythrobacter sp. NAP1	237727	Phytoene synthase	336
A0A420EC17	A0A420EC17_9SPHN	D6851_15445	Altererythrobacter sp. HN-Y73	2320269	Phytoene/squalene synthase family p...	337
A0A1C7D6Z5	A0A1C7D6Z5_9SPHN	**crtB** A6F65_00929	Altererythrobacter namhicola	645517	All-trans-phytoene synthase/15-cis-...	360
A0A494TBB3	A0A494TBB3_9SPHN	D3Y57_11030	Sphingomonas sp. YZ-8	2319844	Phytoene/squalene synthase family p...	310
A0A0G9MNI0	A0A0G9MNI0_9SPHN	AAW01_10680	Erythrobacter gangjinensis	502682	Phytoene synthase	340
A0A396RSP8	A0A396RSP8_9SPHN	D1610_00335	Sphingomonas sp. ZDH117	2305907	Phytoene/squalene synthase family p...	287
A0A369Q8B6	A0A369Q8B6_9SPHN	HME9302_01927	Altererythrobacter sp. HME9302	2161824	15-cis-phytoene synthase	326
A0A1Z1FC14	A0A1Z1FC14_9SPHN	A9D14_09345	Croceicoccus marinus	450378	Phytoene synthase	334
A0A0G9MVX5	A0A0G9MVX5_9SPHN	AAW00_11290	Erythrobacter luteus	1581420	Phytoene synthase	340
J3A5E2	J3A5E2_9SPHN	PMI02_03066	Novosphingobium sp. AP12	1144305	Phytoene/squalene synthetase	324
A0A0B1ZL61	A0A0B1ZL61_9SPHN	LK12_19160	Novosphingobium malaysiense	1348853	Phytoene synthase	332
A0A0S9CFM5	A0A0S9CFM5_9SPHN	ASE95_06645	Sphingomonas sp. Leaf231	1736301	Phytoene synthase	311
A0A3N5CYQ0	A0A3N5CYQ0_9SPHN	EG799_11545	Erythrobacter sp. HN-E23	2488860	Phytoene/squalene synthase family p...	349
A0A0G3X6L4	A0A0G3X6L4_9SPHN	AM2010_155	Altererythrobacter marensis	543877	Phytoene synthase	363

A0A0G3XHM3	A0A0G3XHM3_9SPHN	AB433_09240	Croceicoccus naphthovorans	1348774	Phytoene synthase	328
A0A0S6WVG2	A0A0S6WVG2_9SPHN	MBENS4_2381	Novosphingobium sp. MBES04	1206458	Phytoene synthase	336
A0A419RX85	A0A419RX85_9SPHN	D6201_11720	Erythrobacter aquimixticola	1958945	Phytoene/squalene synthase family p...	340
A0A2T5G385	A0A2T5G385_9SPHN	CLG96_04775	Sphingomonas oleivorans	1735121	Phytoene synthase	291
T0J841	T0J841_9SPHN	M529_06435	Sphingobium ummariense RL-3	1346791	Uncharacterized protein	261
A0A2V5BIA2	A0A2V5BIA2_9SPHN	C7493_114111	Novosphingobium sp. GV010	2135477	Phytoene synthase	328
A0A2Z5E8D2	A0A2Z5E8D2_9SPHN	TQ38_003970	Novosphingobium sp. P6W	1609758	Phytoene/squalene synthase family p...	335
A0A124JYQ4	A0A124JYQ4_9SPHN	AQZ50_09295	Novosphingobium sp. Fuku2-ISO-50	1739114	Phytoene synthase	304
A0A1W1Y718	A0A1W1Y718_9SPHN	SAMN06272759_101461	Novosphingobium sp. B1	1938756	Phytoene synthase	327
A0A437H299	A0A437H299_9SPHN	EKN06_00185	Croceicoccus sp. GM-16	2217664	Phytoene/squalene synthase family p...	329
A0A418NS70	A0A418NS70_9SPHN	D2V07_10530	Erythrobacter sp. V18	2307003	Phytoene/squalene synthase family p...	345
A0A102DFS7	A0A102DFS7_9SPHN	AQZ49_01020	Novosphingobium sp. FSW06-99	1739113	Phytoene synthase	317

Preferably, the present invention refers to the following CrtB sequences corresponding to the phytoene synthase of *Pantoea ananatis* (acessions AXU24940; AHG94989; WP_013027995; WP_110015463; WP_105080745; WP_042676315; WP_105077524; WP_041931046 or WP_028722638), or to the phytoene synthase of *Pantoea stewarti* (AAN85600), or to the phytoene synthase of *Pantoea conspicua* (WP_094119067), or to the phytoene synthase of *Pantoea agglomerans* (AF289043), or to the phytoene synthase of *Pantoea eucalypti* (WP_140915947), or to the phytoene synthase of *Pantoea vagans* (WP_135910885), or to the phytoene synthase of *Pantoea deleyi* (WP_140916915) or more generally to the phytoene synthase of *Pantoea sp.* defined through the GenBank accession HAB23577.

As used herein, the term "phytoene" refers to any phytoene isomer, and preferably to 15-cis phytoene.

The references herein are included here in its full extent.

Description

The regulation of plastids is a core process in plants that remains poorly defined. In this invention, we have shown that the supply of phytoene is a primary determinant of chloroplast-to-chromoplast differentiation. Based on our results, we propose that a metabolic threshold switch mechanism promotes the loss of chloroplast identity when phytoene is supplied in excess. This result also allows explaining why previous attempts using plant PSY enzymes (Maass, Arango et al. 2009, Yuan, Owsiany et al. 2015), which presumably did not reach this activation threshold, have not resulted in chromoplast differentiation in leaves. Our results are consistent with the existence of a two-step process responsible for the transformation of leaf chloroplasts into chromoplasts. First, inducing the production of a sufficiently large amount of phytoene elicits the initial changes, and secondly, increased accumulation of carotenoids due to sustained phytoene availability but also to phytoene-triggered changes in chloroplast membranes completes the differentiation of chloroplasts into chromoplasts. While our conclusions are based on an artificial system (i.e. the expression of a bacterial gene in chloroplast containing cells), the similarity of transcriptomic profiles between this process and fruit ripening strongly points to a general mechanism for chloroplasts to become chromoplasts without the need of a developmental signal. We propose that an initial boost of phytoene production might be necessary to break chloroplast identity in primary photosynthetic tissues such as leaves. However, it might not be required in organs or/and stages in which chloroplast identity is weak (e.g. when photosynthesis is not a functional requirement as in green fruits) or non-existent (e.g. in dark-grown calli, tubers, or roots). Consistent with this conclusion, constitutive overexpression of tomato or Arabidopsis genes encoding PSY enzymes resulted in chromoplast-like structures arising in mature green fruit of tomato (Fraser et al. 2007) and non-photosynthetic tissues of Arabidopsis (Maass et al. 2009) but had no effect in the leaves of these transgenic tomato and Arabidopsis plants (Fraser et al. 2007; Maass et al. 2009). A lower competency of leaf chloroplasts to develop into chromoplasts would also explain why upregulation of ORANGE (OR) triggers carotenoid overaccumulation and chromoplast differentiation in tomato fruit, potato tubers, cauliflower curds, or Arabidopsis calli, but does not work in the leaves of any of these plants (Li et al. 2001; Lopez et al. 2008; Yuan et al. 2015; Yazdani et al. 2019).

Phytoene-induced loss of chloroplast identity might be caused by direct effects of this uncolored carotenoid on the photosynthetic pigment–protein complexes responsible for harvesting sunlight and transferring excitation energy to the photosystems (Domonkos et al. 2013; Liguori et al. 2017). Another possibility is an indirect effect derived from retrograde signals generated when phytoene levels override the threshold. These unknown signals would trigger changes in nuclear gene expression presumably causing the remodeling or pre-conditioning of chloroplast membrane structures in a first phase of the chromoplast differentiation process.

In a second phase, which likely overlaps with the first one, conversion of phytoene into downstream carotenoids in pre-conditioned chloroplasts is required to eventually generate a chromoplast. Without pre-conditioning, carotenoids levels can increase but chloroplasts stay photosynthetically active and chromoplasts do not differentiate, (Roig-Villanova et al. 2007; Bou-Torrent et al. 2015). Moreover, without carotenoids downstream of phytoene, pre-conditioned chloroplasts remain unchanged and chromoplasts do not differentiate. It is likely that carotenoid accumulation occurs concomitantly with the remodeling of the internal plastid structures and that both factors synergistically activate each other. The new structures created following the disassembly of the photosynthetic grana and thylakoids likely contribute to reach high carotenoid levels by accommodating increasing amounts of carotenoids and by preventing their degradation (Sadali et al. 2019; Jarvis and Lopez-Juez 2013; Sun et al. 2018; Egea et al. 2010). They might additionally enhance carotenoid production by stimulating the activity of endogenous carotenoid biosynthetic enzymes (including PSY), many of which are membrane-associated (Ruiz-Sola and Rodriguez-Concepcion 2012).

The structural changes associated with the crtB-mediated chloroplast to chromoplast transformation involve reorganization of the plastidial proteome but also require global reprogramming of nuclear gene expression and primary metabolism. We also show that shut down of photosynthesis relies on enhanced respiration for an enhanced supply of energy and carbon precursors to synthesize carotenoids. It is likely that implementing these changes relies on retrograde signals produced by transforming plastids. Carotenoids can function as precursors for the biosynthesis of signaling molecules that regulate many developmental processes in plants, including plastid development (Avendano-Vazquez et al. 2014; Van Norman et al. 2014; Hou et al. 2016; Wang et al. 2019). The observation that Arabidopsis mutants defective in carotenoid cleavage dioxygenase activity in the cytosol and the plastids are not affected in the crtB-dependent leaf chromoplast differentiation process suggests that signals independent of these enzymes or of carotenoids are responsible for eliciting the changes in nuclear gene expression and cell metabolism supporting chromoplast biogenesis.

Other studies have shown that increasing the supply of phytoene and/or promoting the metabolic activity of the carotenoid pathway can induce the differentiation of leucoplasts (i.e., plastids devoid of pigments) to chromoplasts in non-colored tissues such as calli, tubers, and roots (Lopez, Van Eck et al. 2008, Maass, Arango et al. 2009, Bai, Rivera et al. 2014, Yuan, Owsiany et al. 2015, Schaub, Rodriguez-Franco et al. 2018). We discovered that producing high enough phytoene levels is a necessary first step towards transforming leaf chloroplasts into carotenoid-overaccumulating chromoplasts.

Recent work also showed that transplastomic plants with a synthetic metabolic pathway for producing the algae carotenoid astaxanthin differentiated leaf plastids that lost most of their

endogenous carotenoids and chlorophylls and hence their chloroplast identity (Lu, Stegemann et al. 2017). No information was reported on the levels of phytoene in these lines. However, it is possible that the substitution of endogenous carotenoids by asthaxanthin might interfere with the activity of photosynthetic pigment–protein complexes similarly to phytoene overaccumulation. Chromoplast biogenesis has long been thought to be differently regulated by tissue-specific mechanisms subjected to a tight developmental control (Egea, Barsan et al. 2010). However, these emerging descriptions, together with results reported here, clearly point toward a common regulatory mechanism linking the activity of the carotenoid pathway (i.e. metabolic rather than developmental cues) to the regulation of chromoplast biogenesis.

In summary, we reveal a tool that, for the first time, allows the differentiation of chromoplasts from leaf chloroplasts. This very simple tool that we describe here to induce chromoplast biogenesis on demand can be applied to improve the nutritional quality of crops or other biotechnological applications. For example, our system could be used to increase carotenoid and tocopherol contents in leaf vegetables and/or forage crops for animal feed and/or cell cultures by placing crtB expression under the control of a specific agrochemical such as Routine (Okada, Nemoto et al. 2017), or other induction systems known in the prior-art AlcR/AlcA (ethanol inducible); GR fusions, GVG, and pOp/LhGR (dexamethasone inducible); XVE/OlexA (beta-estradiol inducible); or heat shock induction (Borghi, 2010). These and other agrochemicals or methods could be applied before harvest to induce chloroplast-to-chromoplast differentiation once reaching maximum crop biomass. Furthermore, we foresee that future synthetic biology approaches could be used to couple the activity of new biosynthetic pathways with controlled chromoplast biogenesis to enhance the production capacity of chromoplast located added-value chemicals in plants.

Therefore, a first aspect of the invention refers to a method of inducing chloroplast-to-chromoplast differentiation biogenesis in cell cultures, leaf vegetables, green fruits and/or forage crops that comprises the delivery, by preferably, but not limited to, agroinfiltration or any alternative method such as microbombardment or the use of viral vectors, of a recombinant plasmid or expression cassette in the leaf vegetables, green fruits or forage crops, wherein said construct is characterized in that it comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity and/or may overexpress an endogenous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal in its N-terminus, and wherein said construct is capable of delivering an amount of phytoene in the chloroplasts of the plant tissue sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably said construct is capable of delivering at least about **0.01 µg/mg DW** of phytoene in the chloroplasts of the plant tissue.

In the context of the present invention, the stament “an amount of phytoene in the chloroplasts of the plant tissue sufficient to induce chloroplast to chromoplast differentiation in said plant tissue” can be the amount that results in the reduction of at least 50% of chlorophyll content in leaves (w/w), **or** 50% reduciton of photosynthesis-related parameters such as effective quantum yield at PSII (ϕPSII) **or** non-photochemical quenching (NPQ) when measured against the time where no phytoene is applied on the green tissues or cells of interest. Depending on the plant, tissue or cell of interest, as illustrated below with the examples of spinach, brussel sprouts, forage crops or avocado, the construct can be modified using adecuate promoters and phytoene synthases able to achieve the above mentioned reduction in the above enumerated measures of chloroplast photosynthetic capacity (including but not restricted to, chlorophyll content, effective quantum yield or non-photochemical quenching).

The skilled person will be able to determine the concentration of chlorophyll in leaves using methods as the one published by Schertz in 1928 (F.M. Schertz (1928) The quantitative deteermination of chlorophyll. Plant Physiology 1928 Jul;3 (3):323-334), or more inmediate methods as new as the one described by Cortazar (Cortazar, 2015 Cortazar B., et al. Quantification of plant chlorophyll content using google glass. Lab on Chip 2015; (15) 1708). Likewise, effective quantum yield measurement and non-photochemical quenching (NPQ) can be executed with currently available instruments (as described herein using a MAXI-PAM fluorometer) or alternatively following the teachings of Murchie et al. (2013), Murchie et. Al. Chlorophyll fluoreescene analysis: a guide to good proactice and understanding some neew applications. Journal of experimental Botany: 64 (13) 3983).

A second aspect of the invention refers to a method to increase carotenoid and tocopherol contents in cell cultures, leaf vegetables, green fruits and/or forage crops that comprises the delivery, by preferably, but not limited to, agroinfiltration or any alternative method such as microbombardment or the use of viral vectors, of a recombinant plasmid or expression cassette in the leaf vegetables, green fruits and/or forage crops, wherein said construct is characterized in that it comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity and/or may overexpress an endogenous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal and wherein said construct is capable of delivering an amount of phytoene in the chloroplasts of the plant tissue sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably said construct is capable of delivering at least about **0.01 µg/mg DW** of phytoene in the chloroplasts of the plant tissue.

A third aspect of the invention refers to a method to enhance the production capacity of lipophilic chemicals in plants, the method comprising the delivery, by preferably, but not limited

to, agroinfiltration or any alternative method such as microbombardment or the use of viral vectors, of a recombinant plasmid or expression cassette in cell cultures, leaf vegetables, green fruits and forage crops, wherein said construct is characterized in that it comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity and/or may overexpress an endogenous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal and wherein said construct is capable of delivering an amount of phytoene in the chloroplasts of the plant tissue sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably said construct is capable of delivering at least about **0.01 µg/mg DW** of phytoene in the chloroplasts of the plant tissue.

A fourth aspect of the invention refers to a modified cell culture, leaf vegetable, green fruit and/or forage crop, or to a composition comprising said modified cell culture, leaf vegetable, green fruit and/or forage crop, wherein the modified cell culture, leaf vegetable, green fruit and/or forage crop comprises a recombinant plasmid or expression cassette (construct) that results in a enhanced phytoene synthase activity and accummulation within chloroplasts either by constitutive or inducible expression, that is, either inserted in the plant genome or through a transient method of expression, wherein said recombinant plasmid or expression cassette is characterized in that it comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity and/or may overexpress an endogenous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal in its N-terminus, and wherein said construct is capable of delivering an amount of phytoene in the chloroplasts of the plant tissue sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably said construct is capable of delivering at least about **0.01 µg/mg DW** of phytoene in the chloroplasts of the plant tissue.

In the context of the present invention, plasmid construct is preferably understood as (p)crtB or crtbB or any plasmid encoding a phytoene synthase included in the list above and which is able to translocate the phytoene generating enzyme to the plant plastids of interest.

In the context of the present invention, (p)crtB is understood as 35S:(p)crtB-pGWB605 (SEQ ID NO 1) or as 35S:(p)crtB-pGWB405 (SEQ ID NO 2) two constructs driving *Pantoea ananatis* phytoene synthase expression with a functionally linked plastid-targeting signal of hydroxymethylbutenyl 4-diphosphate synthase from *Arabidopsis thaliana* able to translocate crtB to plastids and difering only in the selection marker/antibiotic and further identified as SEQ No ID 1 and 2.

>(p)crtB_stop_pGWB605

CTTTTCACGCCCTTTTAAATATCCGATTATTCTAATAAACGCTCTTTTCTCTTAGGTTTACCCGCCAATATATCCTGTC
AAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCTGATCCAAGCTCAAGCTGCTCTAGCATTCGCCATTC
AGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATG
TGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAA
GCTTGCATGCCTGCAGGTCCCCAGATTAGCCTTTTCAATTTCAGAAAGAATGCTAACCCACAGATGGTTAGAGAGG
CTTACGCAGCAGGTCTCATCAAGACGATCTACCCGAGCAATAATCTCCAGGAAATCAAATACCTTCCCAAGAAGGT
TAAAGATGCAGTCAAAAGATTCAGGACTAACTGCATCAAGAACACAGAGAAAGATATATTTCTCAAGATCAGAAG
TACTATTCCAGTATGGACGATTCAAGGCTTGCTTCACAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAG
GTAGTTCCCACTGAATCAAAGGCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACT
GGCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGAGCACGAC
ACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGG
TAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGG
TGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAA
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ATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAA
GTTCATTTCATTTGGAGAGAACACGGGGGACTCTAGAGTTATCAACAAGTTTGTACAAAAAAGCAGGCT**ATGGCG**
ACTGGAGTATTGCCAGCTCCGGTTTCTGGGATCAAGATACCGGATTCGAAAGTCGGGTTTGGTAAAAGCATGAA
TCTTGTGAGAATTTGTGATGTTAGGAGTCTAAGATCTGCTAGGAGAAGAGTTTCGGTTATCCGGAAT*ATGAATA*
ATCCGTCGTTACTCAATCATGCGGTCGAAACGATGGCAGTTGGCTCGAAAAGTTTTGCGACAGCCTCAAAGTTATT
TGATGCAAAAACCCGGCGCAGCGTACTGATGCTCTACGCCTGGTGCCGCCATTGTGACGATGTTATTGACGATCAG
ACGCTGGGCTTTCAGGCCCGGCAGCCTGCCTTACAAACGCCCGAACAACGTCTGATGCAACTTGAGATGAAAACGC
GCCAGGCCTATGCAGGATCGCAGATGCACGAACCGGCGTTTGCGGCTTTTCAGGAAGTGGCTATGGCTCATGATA
TCGCCCCGGCTTACGCGTTTGATCATCTGGAAGGCTTCGCCATGGATGTACGCGAAGCGCAATACAGCCAACTGGA
TGATACGCTGCGCTATTGCTATCACGTTGCAGGCGTTGTCGGCTTGATGATGGCGCAAATCATGGGCGTGCGGGAT
AACGCCACGCTGGACCGCGCCTGTGACCTTGGGCTGGCATTTCAGTTGACCAATATTGCTCGCGATATTGTGGACG
ATGCGCATGCGGGCCGCTGTTATCTGCCGGCAAGCTGGCTGGAGCATGAAGGTCTGAACAAAGAGAATTATGCGG
CACCTGAAAACCGTCAGGCGCTGAGCCGTATCGCCCGTCGTTTGGTGCAGGAAGCAGAACCTTACTATTTGTCTGC
CACAGCCGGCCTGGCAGGGTTGCCCCTGCGTTCCGCCTGGGCAATCGCTACGGCGAAGCAGGTTTACCGGAAAAT
AGGTGTCAAAGTTGAACAGGCCGGTCAGCAAGCCTGGGATCAGCGGCAGTCAACGACCACGCCCGAAAAATTAAC
GCTGCTGCTGGCCGCCTCTGGTCAGGCCCTTACTTCCCGGATGCGGGCTCATCCTCCCCGCCCTGCGCATCTCTGGC
*AGCGCCCGCTC*TAGACCCAGCTTTCTTGTACAAAGTGGTTCGATCTAGAGGATCCATGGTGAGCAAGGGCGAGGA
GCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTGAACGGCCACAAGTTCAGCGTGTCCG
GCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTG
CCCTGGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGC
ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTA
CAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCA
AGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGAC
AAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGA
CCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCC
GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACT
CACGGCATGGACGAGCTGTACAAGTAAAGCGGCCCGAGCTCGAATTTCCCCGATCGTTCAAACATTTGGCAATAA
AGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTA
ATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATA
CGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCG
GGAATTGGTTCCGGAACCAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAGGCTG
AATTAGGCGCGCCTATTTCTATTAATTCCCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTT
GCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAAT
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In the context of the present invention, crtbB is understood as 35S:crtB-pGWB605 (SEQ ID NO 4) or as 35S:crtB-pGWB405 (SEQ ID NO 3) two constructs driving *Pantoea ananatis* phytoene synthase expression without a functionally linked plastid-targeting signal, but where internal crtB protein localization signals are still able to translocate sufficient phytoene synthase activity to plastids and further identified as SEQ No ID 3 and 4.

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CAGAAGACGGCTGCACTGAACGTCAGAAGCCGACTGCACTATAGCAGCGGAGGGGTTGGATCAAAGTACTTTGA
TCCCGAGGGGAACCCTGTGGTTGGCATGCACATACAAATGGACGAACGGATAAAC

HDS signal peptide is shown in bold in the above sequences
crtB is shown in italics in the above sequences.
STOP codon is shown undeline in the above sequences.

In the context of the present invention, plastid-targeting signal in its N-terminus is preferably understood as the sequence of the hydroxymethylbutenyl 4-diphosphate synthase gene from *A. thaliana* cDNA, of which cloning procedures are detailed in Table 6 and in the Methods section below.

In the context of the present invention, a plasmid construct or an expression vector capable of accumulating at least about **0.01 µg/mg DW** of phytoene in the leaf vegetables and forage crops between 36 and 48 hpi, is understood as (p)crtB or crtbB, or any other plasmid encoding a phytoene synthase which is able to direct its translocation to the plant plastids of interest.

In a preferred embodiment of any of the above aspects, the polypeptide exhibiting phytoene synthase activity may be any known phytoene synthase capable of reaching the chloroplast, such as selected from known bacterial, algal or plant phytoene synthases. Preferably, the polypeptide exhibiting phytoene synthase activity is a CrtB, more preferably from *Pantoea ananatis* (GenBank accession number: P21683) also preferably any CrtB selected from any of the sequence identified in table 1). Also preferably, the CrtB gene expressed or overexpressed in the modified plant tissue of the invention may encode an endogenous phytoene synthase, a heterologous phytoene synthase or an improved variant of the endogenous phytoene synthase.

The polypeptide exhibiting phytoene synthase activity may also be any polypeptide exhibiting phytoene synthase activity and having at least 60%, preferably 65, 70, 75, 80, 85, 90, 95, 96, 97, 98 or 99%, identity to any phytoene synthase listed in table 1.

The CrtB gene used in the present invention may also encode an improved phytoene synthase, i.e. an enzyme that possesses at least one mutation in its sequence, in comparison with the amino acid sequence of the wild-type enzyme, said mutation leading to an increase of its activity, an increased specific catalytic activity, an increased specificity for the substrate, an increased protein or RNA stability and/or an increased intracellular concentration of the enzyme, or leading to a feedback inhibition resistant mutant.

In another preferred embodiment of any of the above aspects, examples of expression

cassettes useful in the present invention comprise at least a CrtB gene operably linked to a plastid-targeting signal in its N-terminus and optionally one or more control sequences, typically comprising a transcriptional promoter and a transcription terminator, that direct the expression of said gene. A control sequence may include a promoter that is recognized by the host cell. The promoter may contain transcriptional control sequences that mediate the expression of the enzyme. The promoter may be any polynucleotide that shows transcriptional activity in the leaf vegetables or forage crops. The promoter may be a native or heterologous promoter.

Thus, in a preferred embodiment of any of the above aspects of the invention, the plasmid construct or expression vector further comprises a promoter inducing a high level of expression of the gene, i.e. a strong promoter, using elements stabilizing the corresponding messenger RNA or modifying Ribosome Binding Site (RBS) sequences and sequences surrounding them. The promoters suitable to be used in the present invention are known by the skilled person and can be constitutive or inducible, and native or heterologous.

In another embodiment of any of the above aspects, the plasmid construct or expression vector further comprises a specific promoter, i.e., one that can drive the expression of crtB and increase phytoene production in the tissue of interest, such as shown in the following non-limiting examples:

1. if one intends to drive the accumulation of carotenoids in the early leaves of spinach, one would use spinach specific promoters PsaF (Wöstemeyer A., J. Plant Physiol. 160. 503 –508 (2003)) or PetH (Mohanty A., Indian Journal of Biochemistry and Biophysics (37); 447); or
2. if one intends to drive the accumulation of vitamin A in a forage crop such as rye grass, one could use the *LpRbcS or LpCAB* promoters (*Panter et al, Agronomy* **2017**, *7*(2), 36; https://doi.org/10.3390/agronomy7020036) using the teachings of Panter 2017 (Agronomy 7 (2) 36); or
3. if one intends to drive the appearance of yellowishyelowish, redish colours naturally occuring from the accummulation carotenoids in the mesocarp of avocado fruit one could use the type 3 metallothionein-like gene promoter, MT3-A and the teachings of patent US7732669, or
4. if one intends to promote the antioxidant properties properties or flavours and organoleptic characteristics of brussel sprouts by introducing new carotenoids on them, one can use the teachings of patent US20180195081 and the plastocyanin promoter of *Pisum sativum* to drive phytoene production on the tissue of interest.

Examples of suitable promoters able to drive expression in chlorophyll containing tissues further include but are not restricted to; pea rbc-3A (Gilmarin and Chua, 1990), arabidopsis CAB2

(Carre and Kay. 1995), ST-LS1 of *Solanum Tuberosum* (Dupre 1991, Plant J. 1,115) or alfalfa RAc (Potenza, 2004), or derivatives of such promoters.

Preferably, the promoter is a specific and strong constitutive promoter.

Preferably, the expression vector may be present in the leaves in 1 to 5, 20, 100 or 500 copies.

A control sequence may also comprise a transcription terminator, which is recognized by the cells of the leaf vegetables or forage crops to terminate transcription. The terminator is operably linked to the 3'-terminus of the gene. Any terminator that is functional in the cells of the leaf vegetables or forage crops may be used in the present invention.

The host cells of the leaf vegetables, green fruits or forage crops of the fourth aspect, may be transformed, transfected or transduced in a transient or stable manner. The recombinant plants of the invention may be obtained by any method known by the skilled person, such as electroporation, conjugation, transduction, competent cell transformation, protoplast transformation, protoplast fusion, biolistic "gene gun" transformation, PEG-mediated transformation, lipid-assisted transformation or transfection, chemically mediated transfection, lithium acetate -mediated transformation or liposome-mediated transformation.

The term "recombinant or modified plant" as used herein encompasses the modified leaf vegetables, green fruits or forage crops as well as any progeny that is not identical to the parent leaf vegetables or forage crops, in particular due to mutations that occur during replication.

To enhance the production of phytoene, the modified plant of the fourth aspect of the invention or of the methods of any of aspects one to three, may also be genetically modified in order to increase the production of geranylgeranyl diphosphate (GGPP). In particular, the modified leaf vegetables or forage crops of the invention may be genetically modified to increase the carbon flux to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and/or to increase the conversion of IPP and DMAPP to geranylgeranyl diphosphate (GGPP). The carbon flux to IPP and DMAPP may be increased by enhancing the 2-C- methyl-D-erythritol 4-phosphate/l-deoxy-D-xylulose 5-phosphate (MEP/DXP) pathway. As used herein, the term "MEP pathway" or "MEP/DXP pathway" refers to the biosynthetic pathway leading to the formation of IPP and DMAPP from the condensation of pyruvate and D-glyceraldehyde 3 -phosphate to 1 -deoxy-D-xylulose 5- phosphate (DXP). This pathway involves the following enzymes: 1 -deoxy-D-xylulose 5- phosphate synthase (EC 2.2.1.7), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (EC 2.7.7.60), 4- diphosphocytidyl-2-C-methyl-D-erythritol kinase (EC 2.7.1.148), 2-C-methyl-D- erythritol 2,4-cyclodiphosphate synthase (EC 4.6.1.12), 4-hydroxy-3-

methylbut-2-en-l- yl diphosphate synthase (EC 1.17.7.1), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC 1.17.1.2), and isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2).

This pathway may be enhanced by any method known by the skilled person, for example by a method described in the patent application WO 2015/189428. In particular, this pathway may be enhanced by increasing at least one enzymatic activity selected from the group consisting of DXP synthase (DXS), DXP reductoisomerase (DXR), IspD, IspE, IspF, IspG, IspH and IPP isomerase activities (IDI), preferably by increasing at least DXP synthase and IPP isomerase activities.

An enzymatic activity (e.g. DXS, DXR, IspD, IspE, IspF, IspG, IspH, IDI or FPPS activity) may be increased as detailed above for the phytoene synthase activity, i.e. by overexpression of an endogenous gene or expression of a heterologous gene, and/or expression of an improved variant of the endogenous enzyme.

The term "DXS" or "DXP synthase" refers to the enzyme 1-deoxy-D-xylulose 5- phosphate synthase (EC 2.2.1.7) encoded by the dxs gene which catalyzes the condensation of pyruvate and D-glyceraldehyde 3-phosphate to 1-deoxy-D-xylulose 5- phosphate (DXP). The names of gene product, "DXP synthase", "DXS" or "DXPS", are used interchangeably in this application. The DXP synthase activity can be determined by using a radiolabeled substrate as described by Lois et al. (1998) or any other method known by the skilled person. The term "DXP reductoisomerase" or "DXR" refers to the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267) encoded by the dxr gene. The term "IspD" refers to the enzyme 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (EC 2.7.7.60) encoded by the ispD gene. The term "IspE" refers to the enzyme 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, (EC 2.7.1.148) encoded by the ispE gene. The term "IspF" refers to the enzyme 2-C-methyl-D-erythritol 2,4- cyclodiphosphate synthase (EC 4.6.1.12) encoded by the ispF gene. The term "IspG" refers to the enzyme 4-hydroxy-3-methylbut-2-en-l-yl diphosphate synthase (EC 1.17.7.1) encoded by the ispG gene. The term "IspH" refers to the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, also named hydroxymethylbutenyl pyrophosphate reductase, (EC 1.17.1.2) encoded by the ispH gene. The term "IDI", "IPP isomerase" or "isopentenyl pyrophosphate isomerase" refers to the enzyme isopentenyl- diphosphate delta-isomerase (EC 5.3.3.2) encoded by the idi gene that catalyzes the 1,3- allylic rearrangement of the homoallylic substrate isopentenyl (IPP) to its allylic isomer, dimethylallyl diphosphate (DMAPP). According to the organism, the nomenclature of the above identified enzymes and encoding genes may vary. However, for the sake of clarity, in the present specification, these terms are used independently from the origin of the enzymes or genes.

Preferably, at least one gene selected from the group consisting of dxs, dxr, ispD, ispE, ispF, ispG, ispH and idi genes, is overexpressed, more preferably at least dxs and/or idi genes, and even more preferably at least dxs and idi genes are overexpressed.

In an embodiment, said composition of the fourth aspect of the invention or of any of its preferred embodiments, is a cosmetic or pharmaceutical composition. In another embodiment, said composition is a nutraceutical or nutricosmetic composition, or a food or feed additive.

As used herein, the term "nutraceutical composition" refers to a composition comprising nutrients isolated or purified from food and having a beneficial effect on the health of the consumer. As used herein, the term "nutricosmetic composition" refers to a composition comprising nutritional oral ingredients and which is formulated and marketed specifically for beauty purposes.

In a particular embodiment, the cosmetic or pharmaceutical composition is a skin whitening, lightening or bleaching composition or a composition to prevent aging, oxidative or photo-oxidative damages.

Phytoene was also known to show anticarcinogenic and anti-inflammatory properties. Thus, in an embodiment, the composition is a pharmaceutical composition to be used in the treatment of cancer or in the treatment of inflammatory disorders.

Preferably, the composition of the invention is to be administered by topical or oral route.

The composition of the of the fourth aspect of the invention or of any of its preferred embodiments may obviously, depending on its use, comprise also other ingredients, such as cosmetic or pharmaceutical acceptable carriers, preservatives, antioxidants such as carotenoids, as well as pharmaceutically or cosmetically active ingredients. In a preferred embodiment, the composition further comprises a hydrophobic carrier, which may be selected from oils typically used in the cosmetic, pharmaceutical or food industry, such as vegetable, mineral or synthetic oils.

Further aspects and advantages of the present invention will be described in the following examples, which should be regarded as illustrative and not limiting.

EXAMPLES

Plant material and growth conditions

N. tabacum (Xanthi nc) (Li and Carrington 1995), *N. benthamiana* (RDR6i) (Schwach et al. 2005), and *A. thaliana* (Columbia-0 and Landsberg *erecta*) plants were grown under standard conditions as described previously (Llorente et al. 2016; Majer et al. 2017). For generating dark-induced leaf senescence, detached leaves were maintained inside dark, humid chambers until visible yellowing occurred. Collected plant material was frozen in liquid nitrogen, lyophilized, and then homogenized to a fine powder using a TissueLyser system (Qiagen) for further analyses.

Gene constructs and transient expression assays

The viral vectors used in this study have been described (Majer et al. 2017), except for a plasmid with recombinant clone TEV-GFP, which was also constructed from plasmid pGTEVa (Bedoya et al. 2012). To generate the different crtB versions, we amplified by PCR the *Pantoea ananatis crtB* gene from plasmid pACCRT-EIB (Cunningham et al. 1993) and the plastid-targeting sequence of the hydroxymethylbutenyl 4-diphosphate synthase gene from *A. thaliana* cDNA (Gas et al. 2009). We also amplified the cDNA sequences encoding AtPSY from *A. thaliana* and SlPSY2 from *S. lycopersicum*. All primers used for cloning procedures are detailed in Table 2.

Table 2. Primers used for cloning procedures

Name	Sequence
PacrtB_N-tag_attB1-F	5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAATAATCCGTCGTTACTCAATC-3´
PacrtB_C-tag_attB2-R	5´-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGAGCGGGCGCTGCCAGAGATG-3´
PacrtB_STOP_attB2-R	5´-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGAGCGGGCGCTGCCAGAGATG-3´
AtHDS_N-tag_attB1-F	5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGACTGGAGTATTGCCAGCTC-3´
crtB-5'-AtHDS-3'-R	5´-GATTGAGTAACGACGGATTATTCATATTCCGGATAACCGAAACTCTTCTC-3´
AtPSY_N-tag_attB1-F	5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAAGCTTAGTAGCAAGTCCTTC-3´
AtPSY_STOP_attB2-R	5´-GGGGACCACTTTGTACAAGAAAGCTGGGTTCATATCGATAGTC

	TTGAACTTGAAG-3´
SlPSY2_N-tag_attB1-F	5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCTGTTGCTTTGTTGTGGGTTG-3´
SlPSY2_STOP_attB2-R	5´-GGGGACCACTTTGTACAAGAAAGCTGGGTTCATGTCTTTGCTAGTGGGGAAGAAG-3´
PacrtE_N-tag_attB1-F	5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGACGGTCTGCGCAAAAAAACAC-3´
PacrtE_STOP_attB2-R	5´-GGGGACCACTTTGTACAAGAAAGCTGGGTTTATACTGACGGCAGCGAGTTTTTTGTC-3´

PCR products were cloned using the Gateway system first into plasmid pDONR-207 and then into plasmid pGWB405 (Nakagawa et al. 2007) to generate 35S:crtB-pGWB405, 35S:(p)crtB-pGWB405, 35S:(p)crtB-GFP-pGWB405, 35S:AtPSY-pGWB405, and 35S:SlPSY2-pGWB405 or into plasmid pGWB506 (Nakagawa et al. 2007) to generate 35S:GFP-crtB-pGWB506.

For transient expression studies using viral vectors, leaves of 4 to 6 week-old *N. tabacum* and *A. thaliana* were mechanically inoculated with crude extracts from frozen-stored infected plant tissue and collected upon the appearance of the yellowing phenotype as described previously (Majer et al. 2017). For agroinfiltration experiments, leaves of 4 to 6 week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain GV3101 carrying plasmids of interest following the procedure described previously (Sparkes et al. 2006). Infiltration cultures were grown on LB medium at 28°C and used at an OD600 of 0.5, except for experiments performed with serial culture dilutions to express (p)crtB at different levels. For pharmacological inhibition of phytoene conversion, leaves were infiltrated with a 2 µM solution of norflurazon (NF) in water and 0.05 % Tween 20. The infiltration was performed next to leaf areas that had been agroinfiltrated with different constructs 24 h earlier. Infiltrated plants were returned to the same growth conditions after infiltration.

Transmission electron microscopy

Transmission electron microscopy of plant leaves was performed as previously described (D'Andrea et al. 2018).

Metabolite analyses

Carotenoids were analyzed as previously described (Llorente et al. 2016). Phytoene was quantified using a concentration curve with a commercial standard (Sigma). Lipids were extracted, separated by Thin Layer Chromatography (TLC), and methylated as described

(Wang and Benning 2011). Primary metabolites were extracted as described previously (Lisec et al. 2006) using approximately 20 mg of lyophilized leaf tissue. Derivatization and gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analyses were carried out as described previously (Lisec et al. 2006). Metabolites were identified manually by TagFinder software using the reference library mass spectra and retention indices from the Golm Metabolome Database, http://gmd.mpimp-golm.mpg.de. The parameters used for the peak annotation of the 52 metabolites can be found in Table 3.

Table 3. Overview of the metabolite reporting list.

A	B	C	D	E	F	G	H	I

Experiment title: Metabolite profiles of crtB infiltrated leaves
Organism/Plant species: *Nicotiana benthamiana*
Organ/tissue: Leaves
Analytical tool: GC-TOF-MS

Peak/compound no.- number referenced back to the main text
Ret. Time- Time expected, Tag Time Index and Time deviation
Putative Name- putative identification of the metabolite/derivative
Corresponding metabolite name in literature
Mol. Formula- molecular formula of the metabolite or its FA adduct;
Mass to charge ratio (m/z)
(S)- identification confirmed by a standard compound
I, II, III- different isomers
Identification level (A; B; C; D)- (A) standard or NMR; (B) MS/MS; (C) MS^n; (D) MS only

Peak/Compound no.	Time Expected	Tag Time Index	Time Deviation	Putative metabolite name (Derivative)	Corresponding Metabolite in Literature	Metabolite Class	Mol formula	Mass to charge ratio (m/z)	Species detected before	References	Identification level (A-D)
1	222650	222963	0.13	M000071_A104002-101_METB_222650_TOF_Pyruvic acid (1MEOX) (1TMS)	Pyruvic acid	Acid (Oxo)	C3H4O3	174	Arabidopsis, tomato, potato and others		A
2	271580	271909	0.13	M000030_A122001-101_METB_271580_TOF_Valine, DL- (2TMS)	Valine	Acid (Amino)	C5H11NO2	144	Arabidopsis, tomato, potato and others		A
3	291783	292028	0.07	M000053_A129003-101_METB_291783_TOF_Glycerol (3TMS)	Glycerol	Polyol (Triol)	C3H8O3	205	Arabidopsis, tomato, potato and others		A
4	319193	319264	0.05	M000017_A132002-101_METB_319193_TOF_Isoleucine, L- (2TMS)	Isoleucine	Acid (Amino)	C6H13NO2	158	Arabidopsis, tomato, potato and others		A
5	325180	325266	0.03	M000031_A133001-101_METB_325180_TOF_Glycine (3TMS)	Glycine	Acid (Amino)	C2H5NO2	174	Arabidopsis, tomato, potato and others		A
6	333520	332787	-0.19	M000075_A129001-101_METB_333520_TOF_Phosphoric acid (3TMS)	Phosphoric acid	Acid (Phosphate)	H3O4P	299	Arabidopsis, tomato, potato and others		A
7	338693	338728	0.02	M000029_A132003-101_METB_338693_TOF_Proline, L- (2TMS)	Proline	Acid (Amino)	C5H9NO2	142	Arabidopsis, tomato, potato and others		A

8	342530	342424	-0.04	M000114_A133002-101_METB_342530_TOF_Butyric acid, 4-amino- (2TMS)	Butyric acid, 4-amino-	Acid (Amino)	C4H9NO2	232	Arabidopsis, tomato, potato and others		A
9	354060	354337	0.05	M000026_A138002-101_METB_354060_TOF_Alanine, DL- (3TMS)	Alanine	Acid (Amino)	C3H7NO2	188	Arabidopsis, tomato, potato and others		A
10	357523	357554	0.01	M000015_A138001-101_METB_357523_TOF_Serine, DL- (3TMS)	Serine	Acid (Amino)	C3H7NO3	204	Arabidopsis, tomato, potato and others		A
11	365427	365276	-0.06	M000074_A134001-101_METB_365427_TOF_Succinic acid (2TMS)	Succinic acid	Acid (Dicarboxylic)	C4H6O4	247	Arabidopsis, tomato, potato and others		A
12	368365	367639	-0.21	M000016_A140001-101_METB_368365_TOF_Threonine, DL- (3TMS)	Threonine	Acid (Amino)	C4H9NO3	218	Arabidopsis, tomato, potato and others		A
13	371255	371251	-0.01	M000067_A137001-101_METB_371255_TOF_Fumaric acid (2TMS)	Fumaric acid	Acid (Dicarboxylic)	C4H4O4	245	Arabidopsis, tomato, potato and others		A
14	373395	372876	-0.15	M000076_A133003-101_CONT-METB_373395_TOF_Maleic acid (2TMS)	Maleic acid	Acid (Dicarboxylic)	C4H4O4	170	Arabidopsis, tomato, potato and others		A
15	386210	386104	-0.03	M000457_A133004-101_METB_386210_TOF_Nicotinic acid (1TMS)	Nicotinic acid	Acid (N-heterocycle)	C6H5NO2	180	Arabidopsis, tomato, potato and others		A
16	394250	394364	0.03	M000027_A144001-101_METB_394250_TOF_Alanine, beta- (3TMS)	Alanine, beta-	Acid (Amino)	C3H7NO2	174	Arabidopsis, tomato, potato and others		A
17	408455	408938	0.19	M000054_A150002-101_METB_408455_TOF_Erythritol (4TMS)	Erythritol	Polyol (Tetraol)	C4H10O4	217	Arabidopsis, tomato, potato and others		A
18	425325	425219	-0.04	M000066_A148001-101_METB_425325_TOF_Malic acid, 2-methyl-, DL- (3TMS)	Malic acid, 2-methyl-	Acid (Hydroxy)	C5H8O5	247	Arabidopsis, tomato, potato and others		A
19	457283	457546	0.08	M000033_A152002-101_METB_457283_TOF_Aspartic acid, L- (3TMS)	Aspartic acid	Acid (Amino)	C4H7NO4	232	Arabidopsis, tomato, potato and others		A
20	474325	474338	-0.02	M000018_A152001-101_METB_474325_TOF_Methionine, DL- (2TMS)	Methionine	Acid (Amino)	C5H11NO2S	176	Arabidopsis, tomato, potato and others		A
21	490485	490804	0.02	M000028_A162001-101_METB-METB_490485_TOF_Ornithine, DL- (3TMS)	Ornithine (Arginine, Citrulline)a	Acid (Amino)	C5H12N2O2	142	Arabidopsis, tomato, potato and others		A
22	492650	492781	0.04	M000032_A174008-101_MST_492650_TOF_Glutamine, DL- (4TMS)	Glutamine	Acid (Amino)	C5H10N2O3	227	Arabidopsis, tomato, potato and others		A
23	498150	498741	0.1	M000579_A166001-101_METB_498150_TOF_Xylose, D- (1MEOX) (4TMS)	Xylose	Sugar (Pentose, aldose)	C5H10O5	160	Arabidopsis, tomato, potato and others		A
24	507780	507842	0	M000036_A163001-101_METB_507780_TOF_Glutamic acid, DL- (3TMS)	Glutamic acid	Acid (Amino)	C5H9NO4	246	Arabidopsis, tomato, potato and others		A
25	515530	515522	0.01	M000590_A172002-101_METB_515530_TOF_Rhamnose, DL- (1MEOX) (4TMS)	Rhamnose	Sugar (Hexose, deoxy)	C6H12O5	117	Arabidopsis, tomato, potato and others		A
26	517180	517715	0.11	M000186_A175002-101_METB-METB_517180_TOF_Putrescine (4TMS)	Putrescine (Agmatine)a	Amine (Poly)	C4H12N2	174	Arabidopsis, tomato, potato and others		A

27	523847	523298	-0.21	M000571_A158004-101_METB_523847_TOF_Glutaric acid, 2-oxo- (1MEOX) (2TMS)	Glutaric acid, 2-oxo-	Acid (Oxo)	C5H6O5	198	Arabidopsis, tomato, potato and others		A
28	528270	528521	0.06	M000591_A175001-101_METB_528270_TOF_Fucose, DL- (1MEOX) (4TMS)	Fucose	Sugar (Hexose, deoxy)	C6H12O5	117	Arabidopsis, tomato, potato and others		A
29	531145	530790	-0.08	M000011_A164001-101_METB_531145_TOF_Phenylalanine, DL- (2TMS)	Phenylalanine	Acid (Amino)	C9H11NO2	218	Arabidopsis, tomato, potato and others		A
30	550100	549804	-0.04	M000013_A168001-101_METB_550100_TOF_Asparagine, DL- (3TMS)	Asparagine	Acid (Amino)	C4H8N2O3	116	Arabidopsis, tomato, potato and others		A
31	558110	557694	-0.1	M000477_A176004-101_MST_558110_TOF_Glucosone, 3-deoxy- (2MEOX) (3TMS)	3-deoxy-Glucosone	Sugar (Hexose)		231	Arabidopsis, tomato, potato and others		A
32	574230	574005	-0.06	M000328_A177002-101_METB_574230_TOF_Glycerol-3-phosphate, DL- (4TMS)	Glycerol-3-phosphate	Alcohol (Phosphate)	C3H9O6P	299	Arabidopsis, tomato, potato and others		A
33	615467	615798	0.05	M000014_A192003-101_METB_615467_TOF_Lysine, L- (4TMS)	Lysine	Acid (Amino)	C6H14N2O2	156	Arabidopsis, tomato, potato and others		A
34	624990	625161	0.04	M000082_A185002-101_METB-METB_624990_TOF_Dehydroascorbic acid dimer (TMS)	Dehydroascorbic acid dimer	Acid (Hydroxy)	C6H6O6	173	Arabidopsis, tomato, potato and others		A
35	638360	638763	0.11	M000081_A191004-101_METB_638360_TOF_Tyramine (3TMS)	Tyramine	Amine (Aromatic)	C8H11NO	174	Arabidopsis, tomato, potato and others		A
36	651990	650990	-0.18	M000001_A195002-101_METB_651990_TOF_Ascorbic acid, L(+)- (4TMS)	Ascorbic acid	Acid (Hydroxy)	C6H8O6	117	Arabidopsis, tomato, potato and others		A
37	653910	654566	0.2	M000060_A209002-101_METB_653910_TOF_Inositol, myo- (6TMS)	Inositol, myo-	Polyol (Inositol)	C6H12O6	305	Arabidopsis, tomato, potato and others		A
38	658337	658164	-0.02	M000035_A194002-101_METB_658337_TOF_Tyrosine, DL- (3TMS)	Tyrosine	Acid (Amino)	C9H11NO3	218	Arabidopsis, tomato, potato and others		A
39	744935	744770	-0.03	M000649_A214001-101_METB_744935_TOF_Caffeic acid, trans- (3TMS)	Caffeic acid, trans-	Acid (Phenylpropanoic)	C9H8O4	219	Arabidopsis, tomato, potato and others		A
40	776505	772923	-0.46	M000513_A233002-101_METB-METB_776505_TOF_Glucose-6-phosphate (1MEOX) (6TMS)	Glucose-6-phosphate	Sugar (Phosphate)	C6H13O9P	387	Arabidopsis, tomato, potato and others		A
41	790770	790178	-0.06	M000012_A223002-101_METB_790770_TOF_Tryptophan, L- (2TMS)	Tryptophan	Acid (Amino)	C11H12N2O2	202	Arabidopsis, tomato, potato and others		A
42	870355	870035	-0.04	M000048_A274001-101_METB_870355_TOF_Maltose, D- (1MEOX) (8TMS)	Maltose	Sugar (Disaccharide)	C12H22O11	204	Arabidopsis, tomato, potato and others		A
43	876240	875778	-0.04	M000671_A274002-101_METB_876240_TOF_Trehalose, alpha,alpha'-, D- (8TMS)	Trehalose, alpha,alpha'-	Sugar (Disaccharide)	C12H22O11	191	Arabidopsis, tomato, potato and others		A
44	345050	344940	-0.03	M000073_A135003-101_METB_345050_TOF_Glyceric acid, DL- (3TMS)	Glyceric acid	Acid (Hydroxy)	C3H6O4	189	Arabidopsis, tomato, potato and others		A
45	440995	441530	0.12	M000065_A149001-101_METB_440995_TOF_Malic acid, DL- (3TMS)	Malic acid	Acid (Hydroxy)	C4H6O5	233	Arabidopsis, tomato, potato and others		A

46	458330	458306	-0.01	M000078_A156001-101_METB_458330_TOF_Threonic acid (4TMS)	Threonic acid	Acid (Hydroxy)	C4H8O5	292	Arabidopsis, tomato, potato and others		A
47	840783	840145	-0.08	M000044_A264001-101_METB_840783_TOF_Sucrose, D- (8TMS)	Sucrose	Sugar (Disaccharide)	C12H22O11	361	Arabidopsis, tomato, potato and others		A
48	996820	995821	-0.1	M000644_A299001-101_METB_996820_TOF_Quinic acid, 3-caffeoyl-, cis- (6TMS)	Quinic acid, 3-caffeoyl-, cis-	Conjugate (Phenylpropanoyl, Quinic)	C16H18O9	255	Arabidopsis, tomato, potato and others		A
49	1025600	1024853	-0.07	M000003_A311001-101_METB_1025600_TOF_Quinic acid, 3-caffeoyl-, trans- (6TMS)	Quinic acid, 3-caffeoyl-, trans-	Conjugate (Phenylpropanoyl, Quinic)	C16H18O9	345	Arabidopsis, tomato, potato and others		A
50	579380	579380	0	M000606_A187002-101_METB_579380_TOF_Fructose, D- (1MEOX) (5TMS)	Fructose	Sugar (Hexose, ketose)	C6H12O6	217	Arabidopsis, tomato, potato and others		A
51	598880	598880	0	M000040_A191001-101_METB-METB_598880_TOF_Glucose, D- (1MEOX) (5TMS)	Glucose	Sugar (Hexose, aldose)	C6H12O6	160	Arabidopsis, tomato, potato and others		A
52	592883	592883	-0.14	M000069_A182004-101_METB-METB_592883_TOF_Citric acid (4TMS)	Citric acid	Acid (Tricarboxylic)	C6H8O7	273	Arabidopsis, tomato, potato and others		A
53	501603	501650	0	M000155_A173001-101_IS (QUAN)_501603_TOF_Ribitol, D- (5TMS)	Ribitol	Polyol (Pentitol)	C5H12O5	205	Internal standard		A

Data were normalized to the mean value of the GFP control samples (i.e. the value of all metabolites for GFP control samples was set to 1). The means and standard errors of five to six replicates at 96 hpi are presented in Table 4.

Table 4. Relative metabolite levels in *N. benthamiana* leaf sections infiltrated with control GFP and (p)crtB constructs (for details see M&M section). Data is presented as means ± SE of five to six biological replicates normalized to the mean level of the control GFP samples. Asterisks denote significant differences ($P < 0.05$) to the GFP samples.

Metabolite	GFP	crtB
Pyruvate	1±0.09	1.05±0.04
Valine	1±0.08	1.33±0.03
Glycerol	1±0.10	0.8±0.12
Isoleucine	1±0.11	1.38±0.1
Glycine	1±0.34	0.88±0.2
Phosporic acid	1±0.08	1.2±0.08
Proline	1±0.18	1.04±0.26
GABA	1±0.16	0.68±0.1
Alanine	1±0.15	1.41±0.49
Serine	1±0.14	1.01±0.11
Succinate	1±0.10	1.1±0.11
Threonine	1±0.06	1.24±0.11
Fumarate	1±0.11	0.85±0.07
Maleate	1±0.12	1.28±0.12
Nicotinate	1±0.07	1.08±0.1
beta-alanine	1±0.07	1.22±0.08
Erythritol	1±0.02	1.24±0.03
2-methyl-Malate	1±0.04	1.09±0.04
Aspartate	1±0.03	1.35±0.02
Methionine	1±0.14	1.03±0.12
Ornithine	1±0.18	0.91±0.06
Glutamine	1±0.21	1.66±0.7
Xylose	1±0.09	1.13±0.04
Glutamate	1±0.15	1.55±0.17
Rhamnose	1±0.07	1.17±0.05
Putrescine	1±0.11	1.81±0.12
2-oxo-Glutarate	1±0.13	1.42±0.11
Fucose	1±0.08	1.19±0.06
Phenyalanine	1±0.10	1.03±0.08
Asparagine	1±0.16	1.43±0.18
3-deoxy-Glucosone	1±0.08	0.87±0.04
Glycerol-3-P	1±0.10	0.89±0.08
Lysine	1±0.15	0.99±0.05
Dehydroascorbate	1±0.11	0.7±0.08
Tyramine	1±0.08	1.02±0.05
Ascorbate	1±0.02	0.77±0.07
myo-Inositol	1±0.07	0.83±0.1

Tyrosine	1±0.13	1.53±0.15
trans-Caffeate	1±0.32	1.44±0.06
Glucose-6-P	1±0.06	1.1±0.04
Tryptophan	1±0.28	2.1±0.51
Maltose	1±0.10	0.92±0.09
Trehalose	1±0.09	0.95±0.07
Glycerate	1±0.16	0.72±0.11
Malate	1±0.09	1.33±0.1
Threonate	1±0.11	1.24±0.09
Sucrose	1±0.11	1.14±0.06
Quinic acid, 3-caffeoyl-, cis-	1±0.07	1.07±0.07
Quinic acid, 3-caffeoyl-, trans-	1±0.08	1.02±0.06
Fructose	1±0.10	0.56±0.04
Glucose	1±0.06	0.74±0.07
Citrate	1±0.09	2.28±0.31

Transcript analyses

Total RNA was extracted from leaves with the Maxwell 16 LEV Plant RNA Kit (Promega) and quantified with a NanoDrop (Thermo Scientific) as described (Majer et al. 2017). For reverse transcription-quantitative PCR (RT-qPCR) analyses, the First Strand cDNA Synthesis Kit (Roche) was used to generate cDNA according to the manufacturer's instructions, with anchored oligo$(dT)_{18}$ primers and 1 µg of total RNA. Relative mRNA abundance was evaluated via quantitative PCR using LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 real-time PCR system (Roche). Gene expression analysis of *N. tabacum SAG12* gene was conducted using primers NtSAG12_qPCR-F (5'-A T T C A T G G G G C A G T A A A T G G-3') (SEQ ID NO 5) and NtSAG12_qPCR-R (5'-G A A G C G T C C A T A G C A A G T C C-3') (SEQ ID NO 6) and the L25 ribosomal protein gene for normalization (Schmidt and Delaney 2010) using primers NtRPL25_qPCR-F (5'-C A A G G C A C A G G C A G C T A A G G-3') (SEQ ID NO 7) and NtRPL25_qPCR-R (5'-A G G T C G G T G G A A T G T A A C T T T T G-3') (SEQ ID NO 8).

RNAseq service was performed by Sequentia Biotech SL (Barcelona, Spain). RNA concentration in each sample was assayed with a ND-1000 spectrophotometer (NanoDrop) and its quality assessed with the TapeStation 4200 (Agilent Technologies). Indexed libraries were prepared from 1 µg/ea purified RNA with TruSeq Stranded mRNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quantified using the TapeStation 4200 and pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled samples of 2 nM. The pooled samples were subject to cluster generation and sequencing using an Illumina NextSeq 500 System (Illumina) in a 2x75 paired end format at a final concentration of 1.8 pmol. The raw sequence files generated (.fastq files)

underwent quality control analysis using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data analysis was performed with the online platform AIR (www.transcriptomics.cloud) (Vara et al. 2019) using the SolGenomics Network (https://solgenomics.net/) *N. benthamiana* 1.01 (Niben v101) reference genome.

Protein extraction and immunoblot analyses

Protein extraction, quantification, and immunoblot analyses were performed as described (Pulido et al. 2016) using anti-fibrillin (Simkin et al. 2007) or anti-PsbA serum (Agrisera). Thylakoid membrane isolation was performed as described (Russell et al. 1995).

Photosynthetic measurements

Photosynthetic efficiencies were assessed by measuring chlorophyll *a* fluorescence with a MAXI-PAM fluorometer (Heinz Walz GmbH). Leaves previously agroinfiltrated were placed under the camera and effective quantum yield (ΔF/Fm') was measured as (Fm'−Fs)/ Fm', where Fm' and Fs are, respectively, the maximum and the minimum fluorescence of light exposed plants. The actinic light (AL) chosen was 21 PAR as the last value of light able to generate a response in the p(crtB) infiltrated part before having a null photosynthetic activity. Each value is the average result of three biological replicates and three different AOI for each replicate.

NPQ was also measured using the MAXI-PAM unit. All recordings were performed every day at the same time slot, but the order of the samples was randomized in order to reduce the bias related to the length of the NPQ protocol. Plants were dark-adapted for 30 min before measurement and then submitted to a continuous AL of 801 PAR for 10 min. After this period leaves were left recovering in darkness for 40 min. During the whole protocol, Fs and Fm' values were registered every 60 seconds. NPQ and its relative components qE, qZ and qI were calculated as described (Coate et al. 2013) with some modifications. Briefly: NPQ was calculated as (Fm-Fm0)/Fm, The relative contributions of qE, qZ and qI to NPQmax were estimated by monitoring NPQ relaxation kinetics in the dark: following the 10 min exposure to saturating light used to measure NPQ, leaves were left in darkness, and Fm0 was measured again after 10 and 40 min when the Ft value was appearing stable and not recovering anymore. The qE component of NPQ relaxes within 10 min of a leaf being placed in darkness such that NPQ persisting after 10 min in the dark consists of qZ + qI. The qZ component of NPQ relaxes within tens of minutes so that NPQ persisting after 40 min in the dark consists of qI, which is either irreversible in the dark or requires several hours to relax. Consequently, (qI+qZ) was calculated as (Fm-Fm1)/Fm1, where Fm1 is the value of Fm measured after 10 min in the dark following NPQmax measurement. qZ was calculated as (Fm-Fm2)/Fm2, where Fm2 is the value of Fm measured after 40 min in the dark following measurement of NPQmax. qE was calculated

as NPQmax–(qI+qZ) and qI was calculated as (qI+qZ)-qZ.

For the calculation of the de-epoxidation state (DES), agroinfiltrated leaf areas were exposed for 10 min to a continuous AL of 801 PAR in the MAXI-PAM unit, sampled under the same light and immediately frozen before pigment extraction and quantification. The operation of the xanthophyll cycle, comprising the sequential de-epoxidation of the pigments violaxanthin (Vx) to antheraxanthin (Ax) and zeaxanthin (Zx) was followed by calculating DES as (Zx+0.5×Ax)/(Zx+Ax+Vx), where Zx, Ax and Vx are the concentrations of the corresponding xanthophylls.

Respiration measurements

Before respiration measurements, *N. benthamiana* plants were placed in the dark for about 30 min to avoid light-enhanced dark respiration. Four leaf discs of 3.8 cm^2 each were harvested from leaf sections of two different plants infiltrated with the (p)crtB construct, weighted and placed into the respiration cuvette containing the respiration buffer (30 mM MES pH 6.2, 0.2 mM $CaCl_2$). Oxygen uptake rates were measured in darkness using a liquid-phase Clark-type oxygen electrode (Rank Brothers) at a constant temperature of 25ºC. Dry weights from leaf discs were determined after drying for 2 days at 60ºC.

Statistical analyses

Differentially expressed genes (DEGs) were identified by comparing crtB and GFP RNAseq datasets with the DESeq2 statistical method in the AIR platform. The resulting crtB/GFP list was filtered using cut-offs of FDR <0.05 and log2-transformed fold change, log2(FC), >0.585 for upregulated genes and <-0.599 for downregulated genes. Gene ontology enrichments were identified by the Parametric Analysis of Gene Set Enrichment (PAGE) function of the AgriGO v2.0 web-based tool (http://bioinfo.cau.edu.cn/agriGO/) after transforming the gene IDs to the Niben v04.4 annotation. For the comparison of different biological systems, we selected the significantly enriched gene ontologies from our *N. benthamiana* RNAseq experiment (p and q values <0.05) and compared their Z-score values with those obtained from the analysis of published RNAseq data of tomato fruit ripening and Arabidopsis leaf senescence. In particular, we used the RPM values of the Total Pericarp MG, LR and RR ripening stages (Shinozaki et al. 2018) and the FPKM values of the 16D and 30D senescence stages (Woo et al. 2016). DEGs resulting from LR/MG, RR/MG and 30D/16D comparisons were filtered as described above for *N. benthamiana* crtB/GFP.

Student's t-tests were used for statistical analyses in metabolite profiling and respiration analyses. All other statistical analyses were performed using GraphPad Prism 5.0a (GraphPad

Software).

Example 1. The bacterial crtB enzyme induces the transformation of chloroplasts into chromoplasts when delivered to leaf cells (chlorophyll containing cells).

The first step specific of the carotenoid pathway is the conversion of geranylgeranyl diphosphate (GGPP) to phytoene, catalyzed by phytoene synthase (referred to as PSY in plants and crtB in bacteria). Desaturation and isomerization reactions then transform the non-colored phytoene molecule into lycopene, a red carotenoid that is later metabolized to orange-colored beta-carotene (pro-vitamin A) and derived yellowish xanthophylls such as violaxanthin and neoxanthin in one branch of the pathway. A second branch converts lycopene into lutein, a different xanthophyll. In plants, these reactions take place only in plastids (Rodriguez-Concepcion et al. 2018). We previously found that the virus-mediated expression of a bacterial *crtB* gene in tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), *Arabidopsis thaliana* and many other plants caused leaf yellowing (Majer et al. 2017). This phenotype was deduced to result from a combination of virus-induced chlorosis and increased accumulation of colored carotenoids downstream of phytoene. Besides typical chloroplast carotenoids such as lutein, beta-carotene, violaxanthin and neoxanthin, leaves expressing *crtB* accumulated lycopene (Majer et al. 2017). Lycopene is abundant in chromoplasts-containing organs such as the ripe fruits of tomato, but it is not normally found in leaf chloroplasts, where it is readily converted into downstream carotenoids. To test whether plastid ultrastructure was changing to accommodate a new carotenoid profile, we performed transmission electron microscopy (TEM) analysis of tobacco leaves infected with crtB-producing viruses. TEM revealed plastids with remodeled internal structures of distinctive chromoplast morphology (Fig. 1A). These plastids were devoid of organized photosynthetic thylakoid membranes and grana but contained tightly packed membrane stacks and a proliferation of small electron-dense (i.e. lipid-containing) plastoglobules typically observed in chromoplasts, where they contribute to carotenoid storage and stability (Fraser et al. 2007; Egea et al. 2010; Jeffery et al. 2012; Lado et al. 2015). Very similar structures were observed in the chromoplast-like plastids that developed when the *crtB* gene was expressed in virus-infected *Arabidopsis* leaves (Fig. 1B) but also when transiently expressed in agroinfiltrated *Nicotiana benthamiana* leaves (Fig. 1C).

To further substantiate the identity of the plastids that developed in crtB-producing leaves, we analyzed the level of chloroplast and chromoplast marker proteins by immunoblot analysis. Virus-infected or Agrobacterium-mediated expression of *crtB* in *N. tabacum* or *N. benthamiana* leaves, respectively, resulted in increased levels of fibrillin (Fig. 1D), a protein associated to carotenoid accumulation and chromoplast development (Deruere et al. 1994; Singh and McNellis 2011). In contrast, the levels of D1 (also known as PsbA), a core component of photosystem II (PSII) crucial for the photosynthetic electron transport chain that is highly

downregulated during chloroplast to chromoplast differentiation (Barsan et al. 2012; Kahlau and Bock 2008), decreased in crtB-producing leaves (Fig. 1D). The main photosynthetic membrane lipid of chloroplasts, monogalactosyl diacylglycerol (MGDG), was also decreased in leaves containing chromoplast-like plastids (Fig. 9A).

Unlike that observed with viral vectors in *N. tabacum* (Majer et al. 2017), Agrobacterium-mediated expression of *crtB* in *N. benthamiana* did not reduce chlorophyll levels compared to control leaf tissues agroinfiltrated with GFP (Fig. 9B). This result indicates that the chlorosis associated with virus infection is not necessary for chloroplasts to be transformed into chromoplasts-like plastids and supports the conclusion that the crtB-triggered leaf yellowing phenotype is not a senescence process, which typically leads to chlorophyll loss. TEM examination of dark-induced senescent *N. benthamiana* leaves confirmed that the plastids found in crtB-producing cells were completely different from the gerontoplasts with very large and electron-lucent plastoglobules that develop in senescent leaf cells (Fig. 1E). Low expression of the senescence marker gene *SAG12* (Gan and Amasino 1995) in virus-infected *crtB*-expressing tobacco leaves further argued against the possibility of a senescence process (Fig. 9C). **These results together suggest that expressing the bacterial *crtB* gene in leaf cells is sufficient to differentiate chloroplasts into plastids of chromoplast phenotype.** Arabidopsis double mutants defective in OR chaperones (AtOR and AtOR-like) (Zhou et al. 2015) showed the characteristic yellow phenotype associated with carotenoid overaccumulation after infection with crtB-containing viral vectors (Fig. 9D). This result suggests that OR activity is not required for the crtB-mediated differentiation of leaf chloroplasts into chromoplasts.

Example 2. The crtB enzyme only triggers chloroplast-to-chromoplast differentiation when localized in plastids

Consistent with the lack of natural or predicted plastid-targeting signals in crtB, a C-terminal fusion to the green fluorescent protein (GFP) resulted in the localization of the crtB-GFP protein to the cytosol in *N. benthamiana* leaf cells (Majer et al. 2017). It could not be excluded, however, that part of the crtB-GFP protein could localize to chloroplasts. By using *N. benthamiana* line RDR6i, in which gene silencing is impaired (Schwach et al. 2005), we could confirm that the crtB-GFP protein was present in both cytosol and chloroplasts of agroinfiltrated cells (Fig. 2). When GFP was fused to the N-terminus of crtB, the resulting protein (GFP-crtB) was completely excluded from chloroplasts (Fig. 2). It is therefore likely that the bacterial crtB enzyme harbors a cryptic plastid-targeting signal in its N-terminus that becomes blocked and hence inactivated in the GFP-crtB protein. To unambiguously target crtB to the chloroplast, we next added the plastid-targeting sequence of the Arabidopsis MEP pathway enzyme HDS (Gas et al. 2009) to the crtB-GFP reporter. As expected, the resulting (p)crtB-GFP protein was only found in chloroplasts (Fig. 2). Agroinfiltrated leaf tissues either expressing crtB-GFP or the

plastidic (p)crtB-GFP version developed the characteristic yellow phenotype, whereas tissues expressing the cytosolic GFP-crtB version (renamed as (c)crtB) remained as green as the control expressing GFP (Fig. 3A) or a plastid-targeted version of GFP (Fig. 10). Analysis of carotenoid and chlorophyll contents showed identical profiles for leaf sections agroinfiltrated with GFP and (c)crtB and confirmed that, similary to crtB, (p)crtB was able to trigger carotenoid overaccumulation without changing chlorophyll levels (Fig. 3B). Despite unchanged chlorophyll contents, estimation of photosynthesis-related parameters such as effective quantum yield at PSII (ϕPSII) and non-photochemical quenching (NPQ) showed that both crtB and (p)crtB, but not (c)crtB, had a dramatic impact on chloroplast function (Fig. 3C). The fact that plastid targeting of GFP did not cause any ϕPSII defect (Fig. 10) confirms that disturbance of chloroplast photosynthesis is not caused by the accumulation of a foreign protein but specifically by crtB. TEM analyses confirmed that (p)crtB induced the differentiation of chromoplast-like plastids very similar to those found in leaf tissues expressing the untargeted crtB enzyme, whereas only chloroplasts were present in leaves producing either (c)crtB or GFP (Fig. 1D). **These results confirm that crtB activity inside chloroplasts elicits a non-natural (i.e. artificial) differentiation of chromoplasts.**

Example 3. Artificial chromoplast biogenesis induces profound changes in nuclear gene expression and primary cell metabolism

The vast majority of plastidial proteins are encoded by chromosomal genes (Jarvis and Lopez-Juez 2013). We therefore reasoned that the dramatic remodeling of plastidial ultrastructure associated with crtB-triggered chromoplast differentiation would require changes in nuclear gene expression. Transcriptomic (i.e., RNA-seq) analyses of *N. benthamiana* leaf sections at 5 days post-infiltration (dpi) with Agrobacterium cultures to produce (p)crtB or GFP showed that about 5.000 genes were differentially expressed in the two samples.

Such a massive reprogramming of gene expression included the upregulation of 3.183 genes as well as the downregulation of 1,803 genes. Gene Ontology (GO) term enrichment analyses (Table 7) showed overrepresentation of genes involved in protein folding and binding to RNA and ribosomes among those induced by (p)crtB (Fig. 5).

Table 5. Parametric Analysis of Gene Set Enrichment (PAGE) by AgriGO. p value ≤ 0,05 ; q value ≤ 0,05

Cellular Component

Nicotiana benthamiana						Tomato Fruit Ripening*		Senescence**
crtB vs GFP						OR vs MG	RR vs MG	30D vs 16D
GO Accession	Description	number of genes	Z-score	p value	q value	Z-score	Z-score	Z-score
GO:0005623	cell	349	3.5	0.00054	0.0013	-1.2	-0.25	0.22
GO:0044464	cell part	349	3.5	0.00054	0.0013	-1.2	-0.25	0.22
GO:0005737	cytoplasm	138	5.9	4.7E-09	0.00000015	1.3	2.2	-1
GO:0044444	cytoplasmic part	110	5.3	0.0000001	0.0000017	1.8	3.1	-1.9
GO:0019898	extrinsic to membrane	13	3.2	0.0012	0.0027	#N/A	#N/A	-0.41
GO:0005622	intracellular	222	4.4	0.00001	0.000048	-0.18	0.66	-0.19
GO:0043232	intracellular non-membrane-bounded organelle	86	4.8	0.0000013	0.000007	0.72	1.1	-1.5
GO:0043229	intracellular organelle	150	3.6	0.00033	0.00095	0.93	2.2	-0.7
GO:0044424	intracellular part	191	4.1	0.000045	0.00016	0.13	1.3	-0.19
GO:0032991	macromolecular complex	106	4.2	0.000026	0.0001	1	1.6	-0.74
GO:0043228	non-membrane-bounded organelle	86	4.8	0.0000013	0.000007	0.72	1.1	-1.5
GO:0043226	organelle	150	3.6	0.00033	0.00095	0.93	2.2	-0.7
GO:0030529	ribonucleoprotein complex	81	5.2	0.00000026	0.0000028	2.1	2.3	-2.2
GO:0005840	ribosome	76	5.1	0.00000039	0.0000032	2.1	2.2	-3.6

An enrichment of genes with roles in transmembrane transport, cell signaling (protein phosphorylation, calcium binding), and nuclear gene expression (transcription factors) was observed among those repressed when chromoplast biogenesis was artificially induced in *N. benthamiana* leaves (Fig. 4). Consistent with our previous conclusions, this profile was strikingly similar to that of ripening tomato fruits (where chromoplasts naturally differentiate from

chloroplasts) but very different from that of senescent Arabidopsis leaves (Fig. 4). An important difference, however, is that crtB-triggered chromoplastogenesis hardly involved changes in the expression of genes with roles in carotenoid biosynthesis, degradation, or storage (Fig. 11 and Table 8).

Table 6. Expression values (log2FC) of carotenoid-related genes.

Nicotiana benthamiana			Tomato Fruit Ripening*			Senescence**	
Annotation	Gene ID	crtB vs GFP	Gene ID	OR vs MG	RR vs MG	Gene ID	30D vs 16D
DXS1-1	Niben101Scf00246g04005	-0.23021538	Solyc01g067890	2.14514506	2.45304771	AT4G15560.1	-3.94190625
DXS1-2	Niben101Scf01386g02004	-0.30635574	Solyc01g067890	2.14514506	2.45304771	AT4G15560.1	-3.94190625
DXS2-2	Niben101Scf02399g01011	-0.4195865	Solyc11g010850	-5.81021458	-6.22525208	AT4G15560.1	-3.94190625
DXS3-1	Niben101Scf09310g02012	0.02524454	Solyc01g028900	0.26531617	0.57522891	AT4G15560.1	-3.94190625
DXS3-2	Niben101Scf00155g00008	-0.03477633	Solyc01g028900	0.26531617	0.57522891	AT4G15560.1	-3.94190625
DXS4-1	Niben101Scf01396g03001	0.33727492	Solyc08g066950	#N/A	#N/A	AT4G15560.1	-3.94190625
DXS4-2	Niben101Scf06579g01017	0.40550005	Solyc08g066950	#N/A	#N/A	AT4G15560.1	-3.94190625
DXR1	Niben101Scf10366g00014	0.08890313	Solyc03g114340	1.03311723	1.26597076	AT5G62790.2	0.27681825
DXR2	Niben101Scf01764g03022	-0.17568333	Solyc03g114340	1.03311723	1.26597076	AT5G62790.2	0.27681825
MCT1	Niben101Scf06862g01003	0.50133999	Solyc01g102820	-0.61501356	-0.17744245	AT2G02500.1	-2.92543533
MCT2	Niben101Scf00705g01003	1.17192242	Solyc01g102820	-0.61501356	-0.17744245	AT2G02500.1	-2.92543533
CMK	Niben101Scf04558g02010	0.94156008	Solyc01g009010	2.46427022	2.50722381	AT2G26930.1	-2.7855631
MDS1	Niben101Scf06180g00014	0.17336602	Solyc08g081570	0.51629798	0.63762252	AT1G63970.1	-1.08589359
MDS2	Niben101Scf02334g01022	0.70644162	Solyc08g081570	0.51629798	0.63762252	AT1G63970.1	-1.08589359
HDS1	Niben101Scf09264g00009	-0.2053444	Solyc11g069380	0.64105656	0.76915373	AT5G60600.1	-1.02968391

HDS2	Niben101Scf03422g02008	-0.28982467	Solyc11g069380	0.64105656	0.76915373	AT5G60600.1	-1.02968391
HDS3	Niben101Scf11366g02020	0.09747509	Solyc11g069380	0.64105656	0.76915373	AT5G60600.1	-1.02968391
HDS4	Niben101Scf00555g00015	-0.134304	Solyc11g069380	0.64105656	0.76915373	AT5G60600.1	-1.02968391
HDR1	Niben101Scf33689g00006	0.19495312	Solyc01g109300	1.09431454	1.58033083	AT4G34350.1	-0.00177132
HDR2	Niben101Scf10575g00008	-0.06462816	Solyc01g109300	1.09431454	1.58033083	AT4G34350.1	-0.00177132
IDI1	Niben101Scf01688g02006	0.29559168	Solyc08g075390	-0.34081797	-0.34233585	AT1G79690.2	#N/A
IDI2-1	Niben101Scf05848g05012	-0.01053487	Solyc05g055760	-2.74481604	-3.11698689	AT5G16440.1	-0.69597184
IDI2-2	Niben101Scf02499g03007	0.08025426	Solyc05g055760	-2.74481604	-3.11698689	AT5G16440.1	-0.69597184
IDI2-3	Niben101Scf17839g02005	-0.05378046	Solyc05g055760	-2.74481604	-3.11698689	AT5G16440.1	-0.69597184
IDI3	Niben101Scf01514g04018	0.42803591	Solyc04g056390	1.64634	1.81320373	AT3G02780.1	0.0718326
GGDS1	Niben101Scf10468g00010	-1.15307836	Solyc11g011240	-1.48828648	-1.90332398	AT1G49530.1	1.756533
GGDS2-1	Niben101Scf00929g03016	0.90603743	Solyc04g079960	5.64040541	5.33589199	AT2G18620.1	-1.28265092
GGDS2-2	Niben101Scf03110g02006	0.42144244	Solyc04g079960	5.64040541	5.33589199	AT2G18620.1	-1.28265092
GGDS3-1	Niben101Scf13710g03002	-0.04545295	Solyc02g085700	1.8857085	1.96455837	AT2G18640.1	0
GGDS3-2	Niben101Scf02772g01013	-0.31942066	Solyc02g085700	1.8857085	1.96455837	AT2G18640.1	0
GGDS4	Niben101Scf13710g03011	-0.10963643	Solyc02g085710	1.19136217	1.31708219	AT2G18640.1	0
GGDS6-1	Niben101Scf02814g03006	0.12431639	Solyc09g008920	0.1189468	0.11128846	AT3G14530.1	0
GGDS6-2	Niben101Scf13146g00006	0.06426651	Solyc09g008920	0.1189468	0.11128846	AT3G14530.1	0
GGDS7	Niben101Scf04945g00007	0.37429326	Solyc07g061990	1.63391701	1.68319975	AT3G14530.1	0
GGDS8-1	Niben101Scf02387g03017	-0.69205585	Solyc07g064660	5	4.7725895	AT3G14530.1	0
GGDS8-2	Niben101Scf02387g03005	-0.01732	Solyc07g064660	5	4.7725895	AT3G14530.1	0

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GGDS8-3	Niben101Scf01269g12011	-1.34150869	Solyc07g064660	5	4.7725895	AT3G14530.1	0
GGDS9-1	Niben101Scf17922g01005	0.11277801	Solyc08g023470	0.4882885	0.6382634	AT3G14530.1	0
GGDS9-2	Niben101Scf00885g19001	-0.048676	Solyc08g023470	0.4882885	0.6382634	AT3G14530.1	0
GGDS9-3	Niben101Scf04212g00013	0.00425366	Solyc08g023470	0.4882885	0.6382634	AT3G14530.1	0
Or1-1	Niben101Scf00163g13002	0.18243089	Solyc03g093830	0.9527784	1.3078861	AT5G61670.1	0.65733593
Or1-2	Niben101Scf09760g01005	-0.0425342	Solyc03g093830	0.9527784	1.3078861	AT5G61670.1	0.65733593
Or2	Niben101Scf09089g01027	1.0458003	Solyc09g010110	1.8621163	2.1146218	AT5G06130.2	0.17966523
PSY1-1	Niben101Scf01959g00004	0.30620938	Solyc03g031860	6.6487129	6.7784343	AT5G17230.3	-0.70162282
PSY1-2	Niben101Scf04020g00002	-0.00153691	Solyc03g031860	6.6487129	6.7784343	AT5G17230.3	-0.70162282
PSY2	Niben101Scf07253g01008	0.04575261	Solyc02g081330	0.2622186	0.7672659	AT5G17230.3	-0.70162282
PDS1	Niben101Scf01283g02002	0.62511822	Solyc03g123760	1.4434546	1.6153803	AT4G14210.1	-0.57042869
PDS2	Niben101Scf14708g00023	0.97221568	Solyc03g123760	1.4434546	1.6153803	AT4G14210.1	-0.57042869
ZDS1	Niben101Scf02804g00014	0.65362041	Solyc01g097810	1.6691961	1.9212584	AT3G04870.1	0.40336004
ZDS2	Niben101Scf11383g02015	0.6292887	Solyc01g097810	1.6691961	1.9212584	AT3G04870.1	0.40336004
ZDS3	Niben101Ctg11118g00001	-1.38562855	Solyc01g097810	1.6691961	1.9212584	AT3G04870.1	0.40336004
Z-ISO1	Niben101Scf12372g00005	0.66317765	Solyc12g098710	7.4877246	7.4582065	AT1G10830.1	-0.17892053
Z-ISO2	Niben101Scf05548g02009	0.5984954	Solyc12g098710	7.4877246	7.4582065	AT1G10830.1	-0.17892053
CRTISO1-1	Niben101Scf12589g00002	0.42545455	Solyc10g081650	2.2803101	2.4440048	AT1G06820.1	-0.5888728
CRTISO1-2	Niben101Scf00911g02014	0.39973573	Solyc10g081650	2.2803101	2.4440048	AT1G06820.1	-0.5888728
CRTISO1-3	Niben101Scf02268g02003	0.0812683	Solyc10g081650	2.2803101	2.4440048	AT1G06820.1	-0.5888728
CRTISO2-1	Niben101Scf07508g08001	1.38828085	Solyc05g010180	0.0095057	0.0779355	AT1G57770.1	-2.7667

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CRTISO2-2	Niben101Scf07231g07005	1.11313276	Solyc05g010180	0.00950257	0.07793255	AT1G57770.1	-2.76673334
CRTISO2-3	Niben101Scf07231g07006	0.9128608	Solyc05g010180	0.00950257	0.07793255	AT1G57770.1	-2.76673334
LCYB-B1-1	Niben101Scf06266g00002	0.60626776	Solyc04g040190	-1.23501359	-0.66965626	AT3G10230.1	-1.31746519
LCYB-B1-2	Niben101Scf01750g14007	0.20927216	Solyc04g040190	-1.23501359	-0.66965626	AT3G10230.1	-1.31746519
LCYB-B1-3	Niben101Scf08515g00010	-0.45273986	Solyc04g040190	-1.23501359	-0.66965626	AT3G10230.1	-1.31746519
LCYB-B1-4	Niben101Scf00980g07019	0.52525296	Solyc04g040190	-1.23501359	-0.66965626	AT3G10230.1	-1.31746519
LCYB-B1-5	Niben101Scf00980g07018	0.64179701	Solyc04g040190	-1.23501359	-0.66965626	AT3G10230.1	-1.31746519
LCY-B2-1 (CYC-B)	Niben101Scf06266g00002	0.60626776	Solyc10g079480	-3.11873755	-2.74676877	AT2G32640.1	-5.16336401
LCY-B2-2(CYC-B)	Niben101Scf00980g07019	0.52525296	Solyc10g079480	-3.11873755	-2.74676877	AT2G32640.1	-5.16336401
LCY-B2-3 (CYC-B)	Niben101Scf01750g14007	0.20927216	Solyc10g079480	-3.11873755	-2.74676877	AT2G32640.1	-5.16336401
LCY-B2-4 (CYC-B)	Niben101Scf08515g00010	-0.45273986	Solyc10g079480	-3.11873755	-2.74676877	AT2G32640.1	-5.16336401
LCY-B2-5 (CYC-B)	Niben101Scf00980g07018	0.64179701	Solyc10g079480	-3.11873755	-2.74676877	AT2G32640.1	-5.16336401
LCYE1	Niben101Scf18343g00013	0.2255943	Solyc12g008980	-4.427789	-4.91759427	AT5G57030.1	-3.32977743
LCYE2	Niben101Ctg15093g00004	0.50004656	Solyc12g008980	-4.427789	-4.91759427	AT5G57030.1	-3.32977743
BCH1	Niben101Scf01232g03010	0.94805179	Solyc06g036260	0.5800040 8	1.15053156	AT4G25700.1	3.26247837
BCH2-1	Niben101Scf02285g01028	-0.97701909	Solyc03g007960	2.0478944 6	2.3662974 8	AT5G52570.1	-1.14605592
BCH2-2	Niben101Scf03114g06003	-0.47216736	Solyc03g007960	2.0478944 6	2.3662974 8	AT5G52570.1	-1.14605592
CYP97A3-1	Niben101Scf05316g03010	0.57663486	Solyc04g051190	0.3489785 3	0.5331855 2	AT1G31800.1	-3.06951393
CYP97A3-2	Niben101Scf00177g11013	0.72349265	Solyc04g051190	0.3489785 3	0.5331855 2	AT1G31800.1	-3.06951393

CYP97B3-1	Niben101Scf05191g00014	0.95949142	Solyc05g016330	-0.46287506	-0.6686652	AT4G15110.1	-2.01738187
CYP97B3-2	Niben101Scf01559g03018	0.79789129	Solyc05g016330	-0.46287506	-0.6686652	AT4G15110.1	-2.01738187
CYP97C1	Niben101Scf02502g11005	1.23297677	Solyc10g083790	-0.70454412	-1.03679857	AT3G53130.1	-3.88688293
ZEP1-1	Niben101Scf09456g00020	1.11206701	Solyc06g060880	-3.00375214	-3.56634682	AT5G67030.1	-0.29193
ZEP1-2	Niben101Scf09317g00001	0.98085946	Solyc06g060880	-3.00375214	-3.56634682	AT5G67030.1	-0.29193
ZEP1-3	Niben101Scf00428g12019	0.52628279	Solyc06g060880	-3.00375214	-3.56634682	AT5G67030.1	-0.29193
ZEP1-4	Niben101Scf11484g00007	0.54747099	Solyc06g060880	-3.00375214	-3.56634682	AT5G67030.1	-0.29193
ZEP1-5	Niben101Scf10336g03007	1.5820713	Solyc06g060880	-3.00375214	-3.56634682	AT5G67030.1	-0.29193
ZEP2-1	Niben101Scf01553g01001	-0.3038517	Solyc02g090890	-1.40159506	0.11398218	AT5G67030.1	-0.29193
ZEP2-2	Niben101Scf16082g00007	-0.58673598	Solyc02g090890	-1.40159506	0.11398218	AT5G67030.1	-0.29193
VDE1	Niben101Scf07893g00003	1.21913015	Solyc04g050930	-0.12353046	-0.07524187	AT1G08550.1	-2.91577978
VDE2	Niben101Scf00177g07008	1.34776205	Solyc04g050930	-0.12353046	-0.07524187	AT1G08550.1	-2.91577978
NSY1-1	Niben101Scf00757g05003	-0.53788271	Solyc02g089050	0	-2.80735492	AT1G67080.1	-1.85469373
NSY1-2	Niben101Scf05761g01007	-0.45767738	Solyc02g089050	0	-2.80735492	AT1G67080.1	-1.85469373
NSY1-3	Niben101Scf12735g00009	1.66129276	Solyc02g089050	0	-2.80735492	AT1G67080.1	-1.85469373
NSY1-6	Niben101Scf06008g01017	-0.07363715	Solyc02g089050	0	-2.80735492	AT1G67080.1	-1.85469373
NSY2-1	Niben101Scf10375g01002	-0.27144807	Solyc02g063170	-0.58772364	-0.59770773	AT1G67080.1	-1.85469373
NSY2-2	Niben101Scf00894g03001	-0.17338294	Solyc02g063170	-0.58772364	-0.59770773	AT1G67080.1	-1.85469373
NSY2-3	Niben101Scf02381g04020	0.47495217	Solyc02g063170	-0.58772364	-0.59770773	AT1G67080.1	-1.85469373

NSY2-4	Niben101Scf01991g03009	-0.47944282	Solyc02g063170	-0.58772364	-0.59770773	AT1G67080.1	-1.85469373
CCD1-1	Niben101Scf03264g05010	0.62325817	Solyc01g087250	-1.537539	-1.26964221	AT3G63520.1	-0.79643162
CCD1-2	Niben101Scf10058g00024	0.36730614	Solyc01g087250	-1.537539	-1.26964221	AT3G63520.1	-0.79643162
CCD1-3	Niben101Scf10058g00019	0.08776269	Solyc01g087250	-1.537539	-1.26964221	AT3G63520.1	-0.79643162
CCD4-1	Niben101Scf08613g02006	-0.66617881	Solyc08g075490	-5.62447585	-6.42351869	AT4G19170.1	-3.18367838
CCD4-2	Niben101Scf18822g02016	-1.2901422	Solyc08g075480	-1.28950662	-1.28950662	AT4G19170.1	-3.18367838
CCD4-3	Niben101Scf18822g02009	-1.07864674	Solyc08g075480	-1.28950662	-1.28950662	AT4G19170.1	-3.18367838

Energy and carbon required for carotenoid biosynthesis rely on photosynthesis (i.e. Calvin-Benson cycle) in chloroplasts. Because chromoplast differentiation in crtB-producing *N. benthamiana* leaves was associated with impairment of photosynthesis (Fig. 3C) and changes in the expression of genes potentially controlling the activity of many enzymes (Fig. 4), we asked whether primary cell metabolism might also be reprogrammed. To test this possibility, we conducted a GC-TOF-MS analyses of primary metabolites, including sugars, amino acids, and organic acids (Fig. 5). A total of 52 metabolites were annotated and their relative levels in (p)crtB leaf sections were normalized to the mean levels of GFP control sections. From these, 13 showed statistically significant changes: 3 were decreased and 10 were increased in (p)crtB samples (Fig. 5). We observed reductions in the levels of ascorbate but also of glucose and fructose, which are the main soluble carbohydrate stores and transport sugars as well as the main substrates for respiration. This, together with the increments in several intermediates of the tricarboxylic acid (TCA) cycle (citrate, 2-oxoglutarate and malate) and amino acids such as valine, isoleucine, aspartate and glutamate derived metabolites (Fig. 5), suggested that sugars were used to produce ATP through the TCA cycle to sustain amino acid synthesis and likely other cellular functions. Indeed, respiration rate determined as total oxygen consumption in the dark was found to be upregulated in chromoplast-containing leaf tissues (Fig. 5). We concluded that (p)crtB induced glycolytic and oxidative energy metabolism likely to compensate for photosynthesis shut down and subsequent reduction in the production of ATP and carotenoid substrates by the Calvin-Benson cycle.

Example 4. Reaching a phytoene threshold is required to promote carotenoid overaccumulation in leaves.

In contrast with our results using a bacterial phytoene synthase (crtB), it has been previously shown that overproducing plant PSY enzymes does not increase carotenoid contents of leaves (Fraser et al. 2007; Maass et al. 2009; Busch et al. 2002). Consistent with these reports, when we transiently expressed PSY-encoding genes from Arabidopsis and tomato in *N. benthamiana* leaves, none of them induced the chromoplast-associated yellow phenotype (Fig. 12). Interestingly, the plant enzymes yielded significantly lower levels of phytoene than the plastid-targeted (p)crtB protein (Fig. 12).

The described results led us to hypothesize that a certain threshold level of phytoene or a downstream derivative might be needed to elicit chloroplast to chromoplast transformation. To test this hypothesis, we performed two experiments. In the first one, leaves of *N. benthamiana* plants were agroinfiltrated with serial dilutions of Agrobacterium cultures to express the (p)crtB construct at different levels. Samples infiltrated with low Agrobacterium densities showed no changes in total leaf carotenoid contents despite progressive increases in phytoene levels (Fig. 6A). When phytoene accumulated at levels higher than 20 ng/mg DW, however, a concomitant increase in total carotenoids was observed (Fig. 6A). The second experiment followed the time course of chromoplast differentiation after agroinfiltration of *N. benthamiana* leaves with the construct to express (p)crtB. Several leaves were agroinfiltrated with the construct and then pigment contents were measured at different time points. Phytoene became detectable at 36 hours post-infiltration (hpi), suddenly increased at 48 hpi, and then kept accumulating until 84 hpi (Fig. 5B). Downstream carotenoids started to increase at 48 dpi (Fig. 5B), again supporting the conclusion that carotenoid contents only increase once phytoene supply exceeds a certain threshold.

Example 5. The supply of phytoene in plastids functions as a metabolic threshold switch that regulates chloroplast-to-chromoplast differentiation

Based on the described results, two mechanisms could be envisioned to explain how phytoene overproduction eventually led to chromoplast differentiation in leaves: (1) phytoene might readily feed the carotenoid pathway and promote carotenoid biosynthesis to reach levels so high that eventually changed plastid ultrastructure to accommodate these lipophilic compounds, or (2) phytoene per se might trigger the plastidial membrane remodeling process either directly (in the plastid) or indirectly (via signaling to the nucleus). Membrane reorganization would influence carotenoid biosynthesis, storage and/or degradation to achieve high carotenoid levels.

As a proxy of membrane remodeling dynamics, we monitored ϕPSII, NPQ and D1 protein levels

during the crtB-mediated chloroplast to chromoplast transition (Fig. 7). These parameters are estimators of photosynthesis, photoprotection, and photodamage and hence of chloroplast membrane system (thylakoids and grana) functionality. In leaves agroinfiltrated with (p)crtB, both ϕPSII (Fig. 7A) and NPQ (Fig. 7B) remained unchanged up to 36 hpi, and then started to decrease as the levels of both phytoene and carotenoids increased (Fig. 6B). In the case of NPQ, the drop was mainly due to a decrease in qE, consistent with impairment of fast photoprotective responses such as the carotenoid-dependent xanthophyll cycle (Fig. S5). The levels of D1 started to decrease later, between 48 and 60 hpi (Fig. 7C), likely as a result of photodamage. These results together indicate that crtB-mediated upregulation of the carotenoid pathway rapidly disrupts the chloroplast photosynthetic membrane functionality.

To next test whether phytoene accumulation by itself could trigger the initial remodeling of plastidial membranes, we used norflurazon (NF) to block phytoene conversion into downstream carotenoids (Ortiz-Alcaide et al. 2019). *N. benthamiana* leaves were agroinfiltrated with different constructs and at 24 hpi some of them were also infiltrated with NF. The presence of NF led to the accumulation of some phytoene and slightly reduced carotenoid levels in GFP-producing leaves at 96 hpi (Fig. 8). NF-treated (p)crtB samples accumulated much more phytoene than GFP controls but showed similar levels of downstream carotenoids. Analysis of ϕPSII at different time points after agroinfiltration and NF treatment of these samples showed a negative correlation with phytoene rather than downstream or total carotenoid levels (Fig. 8). When the levels of total carotenoids (but not phytoene) were increased in leaves transiently expressing the Arabidopsis gene encoding PAR1, a transcription cofactor that promotes total carotenoid biosynthesis in photosynthetic tissues (Roig-Villanova et al. 2007; Bou-Torrent et al. 2015), no changes in ϕPSII were observed compared to GFP-producing controls (Fig. 8). We therefore conclude that phytoene accumulation by itself can disrupt the functionality (i.e. the identity) of the chloroplast. The observation that ϕPSII shows a much stronger and progressive reduction in untreated (p)crtB samples as carotenoids accumulate (Fig. 8) further suggests that exceeding a phytoene threshold might start the initial steps of the chromoplast differentiation program but production of downstream carotenoids is required to complete it.

In summary, our results are consistent with the existence of a two-step process responsible for the transformation of crtB-producing chloroplasts into chromoplasts. First, a metabolic switch mechanism elicits the initial changes of the process when the supply of phytoene exceeds a threshold, and second, increased accumulation of carotenoids due to enhanced phytoene availability but also to phytoene-triggered changes in plastid membranes completes the differentiation of chloroplasts into chromoplasts.

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CLAIMS

1. A method of inducing chloroplast-to-chromoplast differentiation biogenesis in leaf vegetables and/or forage crops that comprises the induction of a constitutive, inducible or transient expression of a plasmid construct or expression vector in the leaf vegetables and/or forage crops, wherein said plasmid construct is characterized in that the plasmid construct comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal in its N-terminus, and wherein said plasmid construct or expression vector is capable of accumulating in the chloroplasts of the plant tissue an amount of phtyoene sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably at least about **0.01 µg/mg DW** of phytoene in the leaf vegetables or forage crops between 36 and 48 hpi.

2. A method to increase carotenoid and tocopherol contents in leaf vegetables and forage crops that comprises the induction of a constitutive, inducible or transient expression of a plasmid construct or expression vector in the leaf vegetables and forage crops, wherein said plasmid construct is characterized in that the plasmid construct comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal in its N-terminus, and wherein said plasmid construct or expression vector is capable of accumulating in the chloroplasts of the plant tissue an amount of phtyoene sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably at least about **0.01 µg/mg DW** of phytoene in the leaf vegetables or forage crops between 36 and 48 hpi.

3. A method to enhance the production and storage capacity of lipophilic chemicals in plants, the method comprising the the induction of a constitutive, inducible or transient expression of a plasmid construct or expression vector in the leaf vegetables and forage crops, wherein said plasmid construct is characterized in that the plasmid construct comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity and/or may overexpress an endogenous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal in its N-terminus, and wherein said plasmid construct or expression vector is capable of accumulating in the chloroplasts of the plant tissue an amount of phtyoene sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably at least about **0.01 µg/mg DW** of phytoene in the leaf vegetables or forage crops between 36 and 48 hpi.

4. A modified leaf vegetable or forage crop comprising the constitutive, inducible or transient expression of a plasmid construct or expression vector, wherein said plasmid construct is characterized in that the plasmid construct comprises a heterologous nucleic acid encoding a polypeptide exhibiting phytoene synthase activity, wherein said polypeptide exhibiting phytoene synthase activity further comprises a plastid-targeting signal, and wherein said plasmid construct or expression vector is capable of accumulating in the chloroplasts of the plant tissue an amount of phtyoene sufficient to induce chloroplast to chromoplast differentiation in said plant tissue, preferably at least about **0.01 µg/mg DW** of phytoene in the leaf vegetables or forage crops between 36 and 48 hpi.

5. The method of any of claims 1 to 3 or the modified leaf vegetable or forage crop of claim 4, wherein the polypeptide exhibiting phytoene synthase activity is CrtB.

6. The method of any of claims 1 to 3 or the modified leaf vegetable or forage crop of claim 4, wherein the polypeptide exhibiting phytoene synthase activity *Pantoea ananatis* crtB (GenBank accession number: P21683).

7. The method of claim 5 or 6 or the modified leaf vegetable or forage crop of claim 5 or 6, wherein plastid-targeting signal is the sequence of the hydroxymethylbutenyl 4-diphosphate synthase gene from *A. thaliana* cDNA.

8. The method or the modified leaf vegetable or forage crop of any of the precedent claims, wherein the expression cassette comprise at least a CrtB gene operably linked to a plastid-targeting signal, preferably in its N-terminus and optionally one or more control sequences, typically comprising a transcriptional promoter and a transcription terminator, that direct the expression of said phytoene synthase in the species and tissue/organ of interest.

9. The method or the modified leaf vegetable or forage crop of claim 8, wherein the control sequence includes a promoter that is recognized by the host cell.

10. The method or the modified leaf vegetable or forage crop of any of the precedent claims, wherein the production of phytoene is enhanced by genetically modified the leaf vegetables and forage crops to increase the carbon flux to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and/or to increase the conversion of IPP and DMAPP to geranylgeranyl diphosphate (GGPP).

11. A cosmetic or pharmaceutical composition comprising the leaf vegetable or forage crop of any of the precedent claims, or an extract therefrom.

12. A nutraceutical or nutricosmetic composition, or a food or feed additive comprising the leaf vegetable or forage crop of any of the precedent claims.

13. The cosmetic or pharmaceutical composition of claim 11, wherein the cosmetic or pharmaceutical composition is a skin whitening, lightening or bleaching composition or a composition to prevent aging, oxidative or photo-oxidative damages.

14. The pharmaceutical composition of claim 11, for use in the treatment of cancer or in the treatment of inflammatory disorders. (cardiovascular disease)

Figures

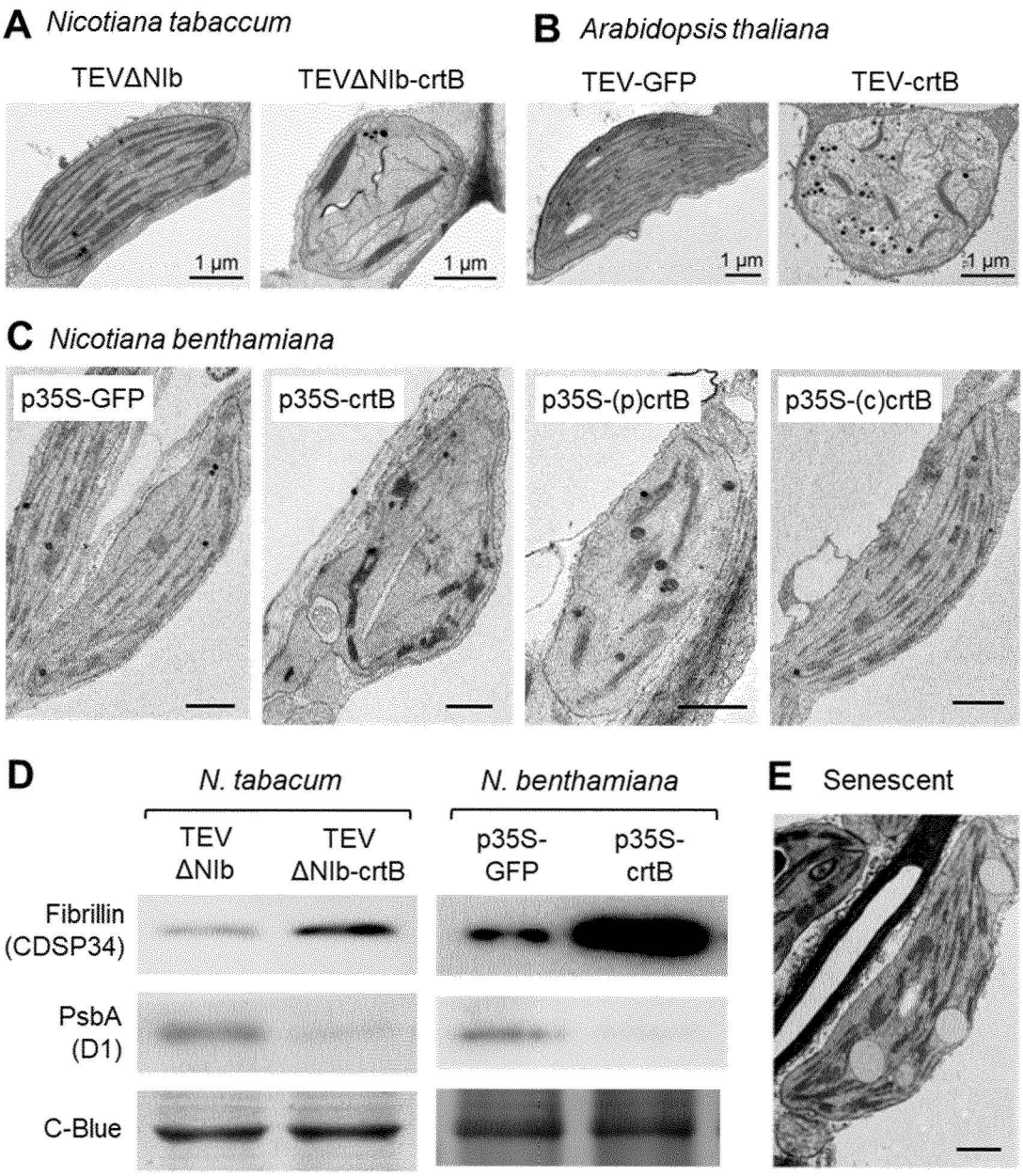

Fig. 1

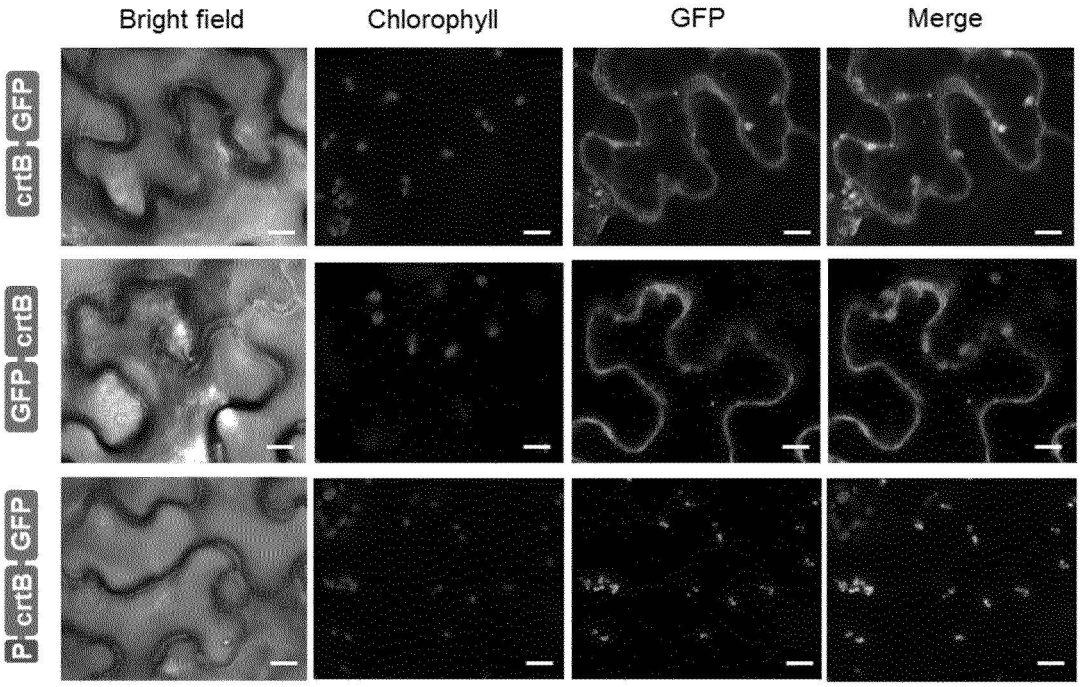

Figure 2.

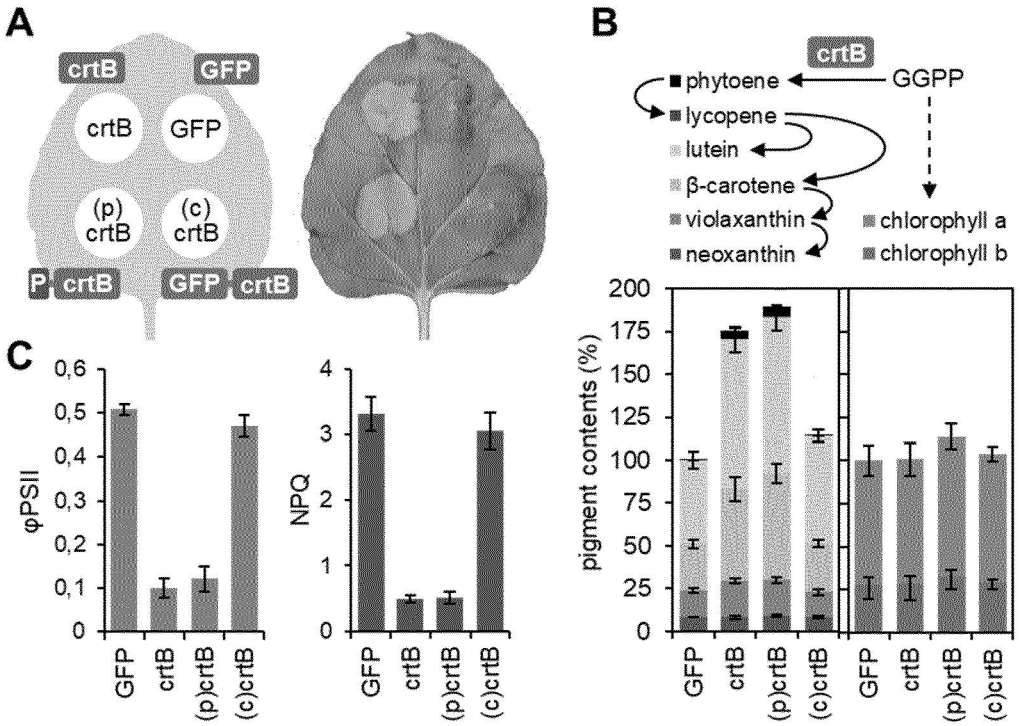

Figure 3.

Figures

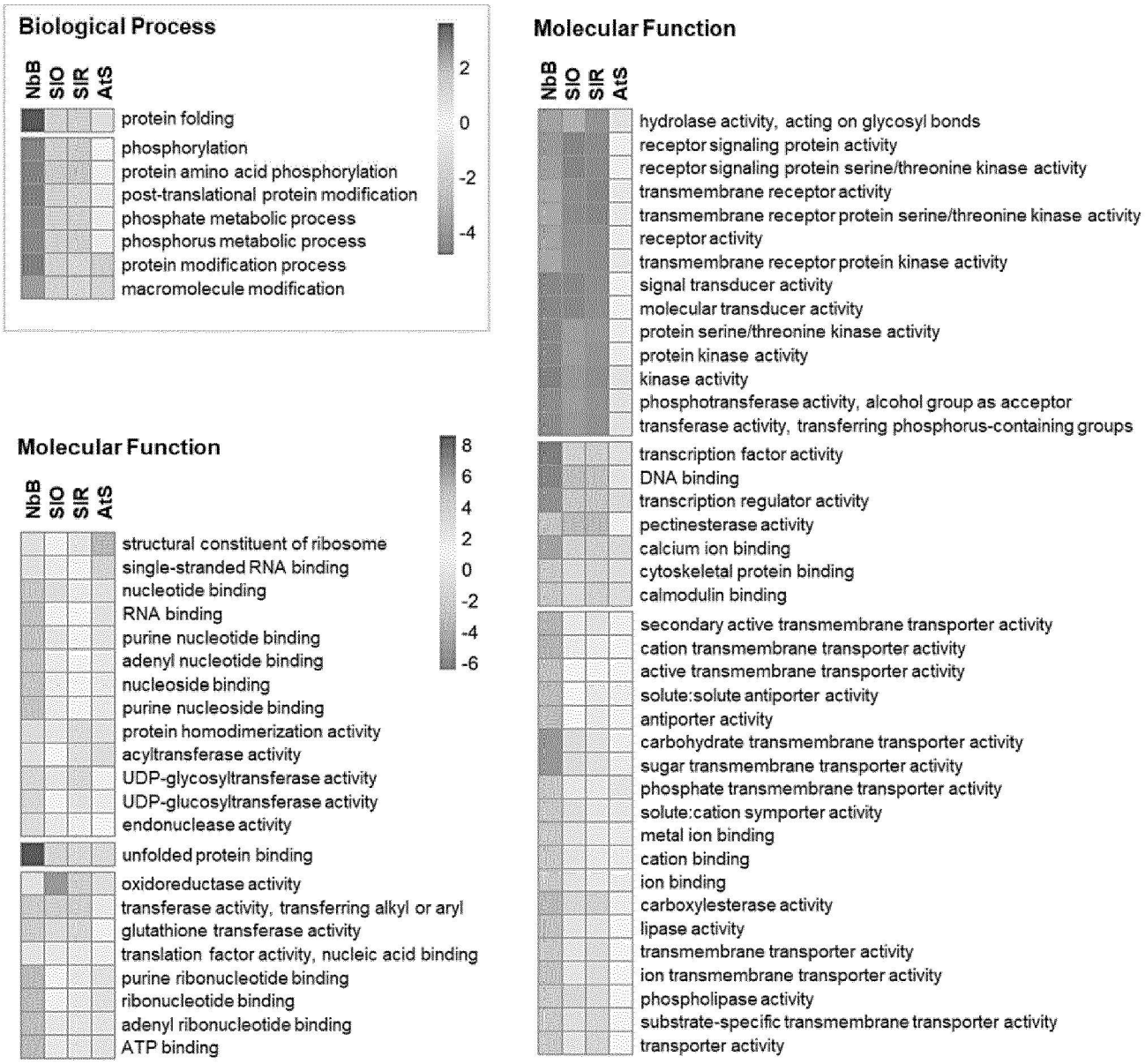

Figure 4.

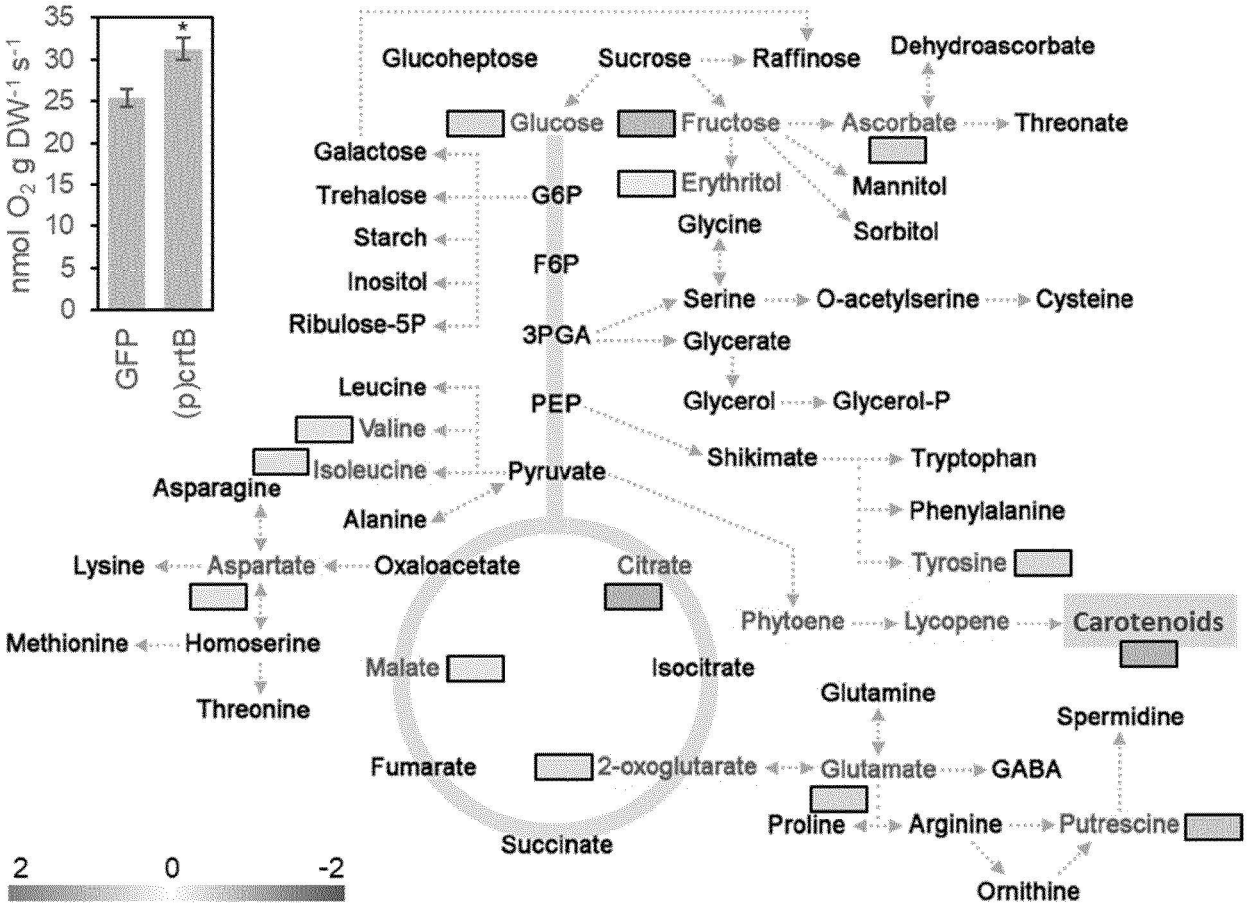
nmol O_2 g DW^{-1} s^{-1}
35
30
25
20
15
10
5
0
GFP
(p)crtB
*
Glucoheptose
Sucrose
Raffinose
Dehydroascorbate
Glucose
Fructose
Ascorbate
Threonate
Galactose
Trehalose
Starch
Inositol
Ribulose-5P
G6P
F6P
3PGA
PEP
Pyruvate
Erythritol
Mannitol
Sorbitol
Glycine
Serine
O-acetylserine
Cysteine
Glycerate
Glycerol
Glycerol-P
Shikimate
Tryptophan
Phenylalanine
Tyrosine
Leucine
Valine
Isoleucine
Asparagine
Alanine
Lysine
Aspartate
Oxaloacetate
Citrate
Methionine
Homoserine
Threonine
Malate
Isocitrate
Phytoene
Lycopene
Carotenoids
Glutamine
Spermidine
Fumarate
2-oxoglutarate
Glutamate
GABA
Proline
Arginine
Putrescine
Succinate
Ornithine
2
0
-2

Figure 5.

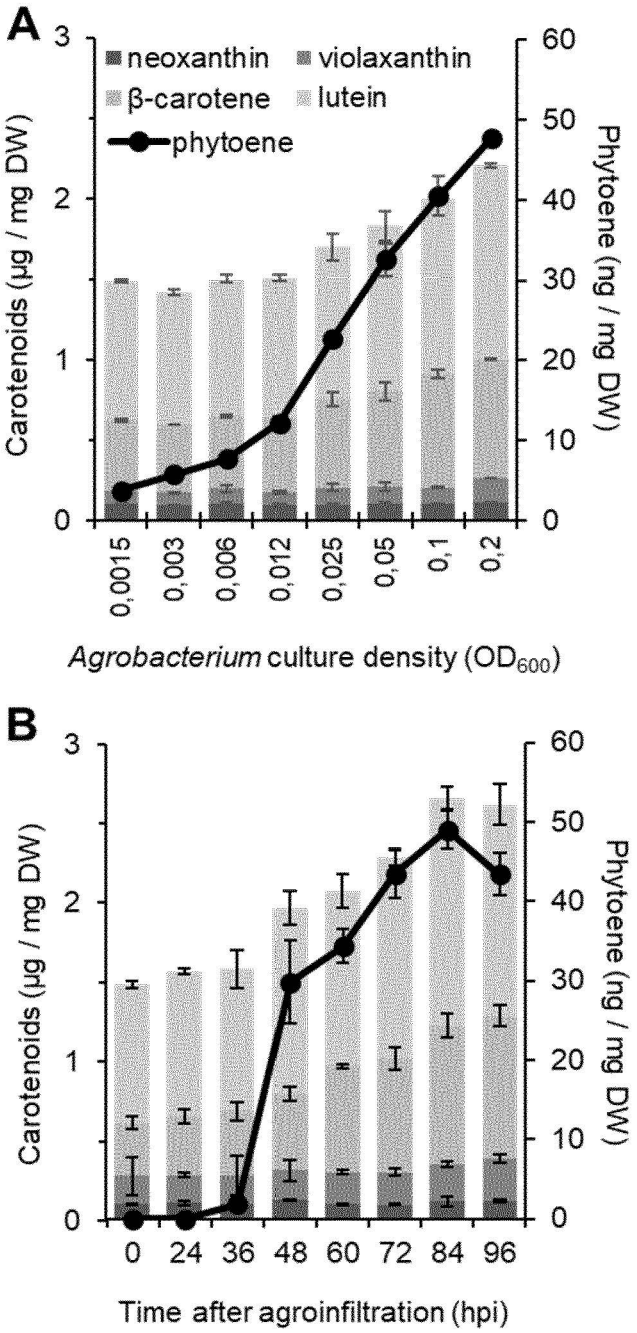
A
neoxanthin
violaxanthin
β-carotene
lutein
phytoene
Carotenoids (µg / mg DW)
Phytoene (ng / mg DW)
0,0015
0,003
0,006
0,012
0,025
0,05
0,1
0,2
Agrobacterium culture density (OD600)
B
Carotenoids (µg / mg DW)
Phytoene (ng / mg DW)
0
24
36
48
60
72
84
96
Time after agroinfiltration (hpi)

Figure 6.

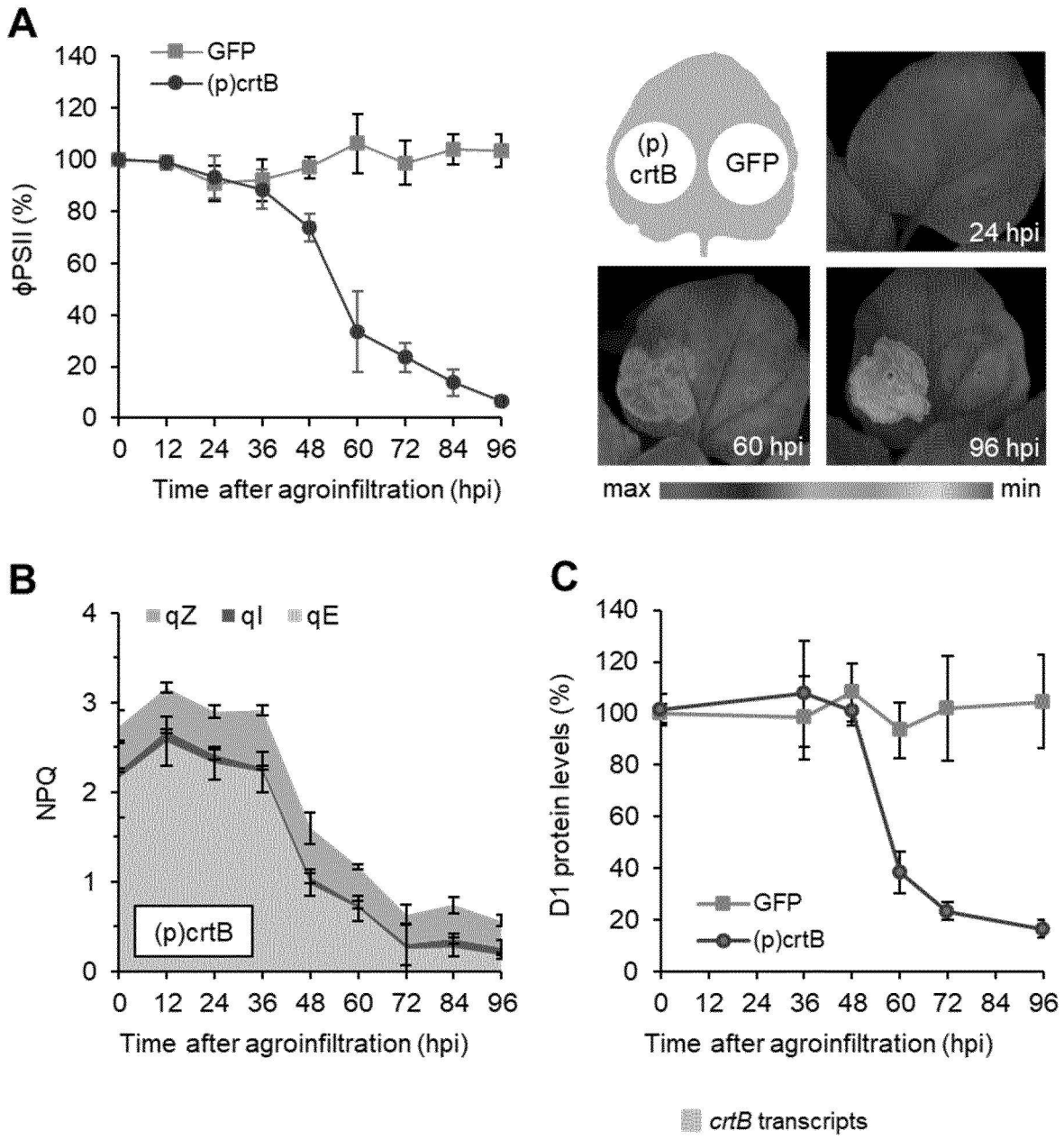
A
GFP
(p)crtB
ΦPSII (%)
Time after agroinfiltration (hpi)
(p) crtB
GFP
24 hpi
60 hpi
96 hpi
max
min
B
qZ
qI
qE
NPQ
(p)crtB
Time after agroinfiltration (hpi)
C
D1 protein levels (%)
GFP
(p)crtB
Time after agroinfiltration (hpi)
crtB transcripts

Figure 7.

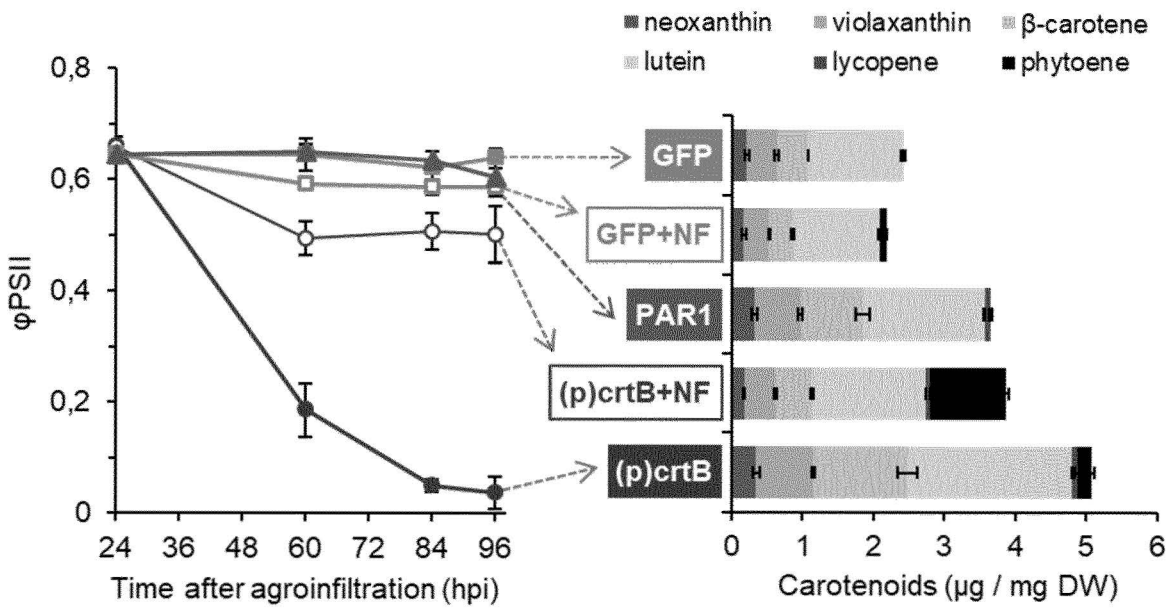
neoxanthin
violaxanthin
β-carotene
lutein
lycopene
phytoene
0,8
0,6
0,4
0,2
0
φPSII
24 36 48 60 72 84 96
Time after agroinfiltration (hpi)
GFP
GFP+NF
PAR1
(p)crtB+NF
(p)crtB
0 1 2 3 4 5 6
Carotenoids (µg / mg DW)

Figure 8.

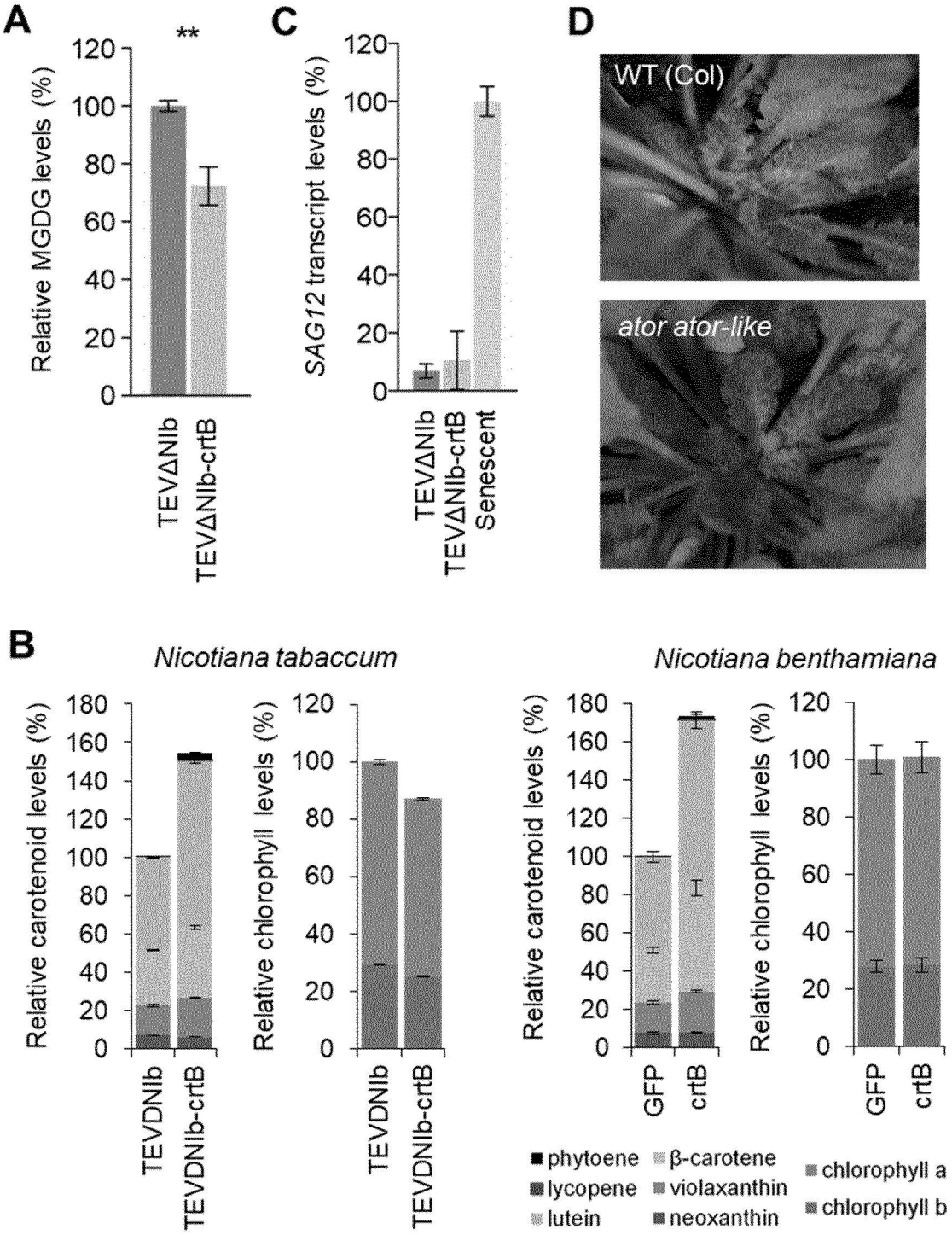
A
**
Relative MGDG levels (%)
120
100
80
60
40
20
0
TEVΔNIb
TEVΔNIb-crtB
C
SAG12 transcript levels (%)
120
100
80
60
40
20
0
TEVΔNIb
TEVΔNIb-crtB
Senescent
D
WT (Col)
ator ator-like
B
Nicotiana tabaccum
Relative carotenoid levels (%)
180
160
140
120
100
80
60
40
20
0
TEVDNIb
TEVDNIb-crtB
Relative chlorophyll levels (%)
120
100
80
60
40
20
0
TEVDNIb
TEVDNIb-crtB
Nicotiana benthamiana
Relative carotenoid levels (%)
180
160
140
120
100
80
60
40
20
0
GFP
crtB
Relative chlorophyll levels (%)
120
100
80
60
40
20
0
GFP
crtB
phytoene
β-carotene
lycopene
violaxanthin
lutein
neoxanthin
chlorophyll a
chlorophyll b

Figure 9.

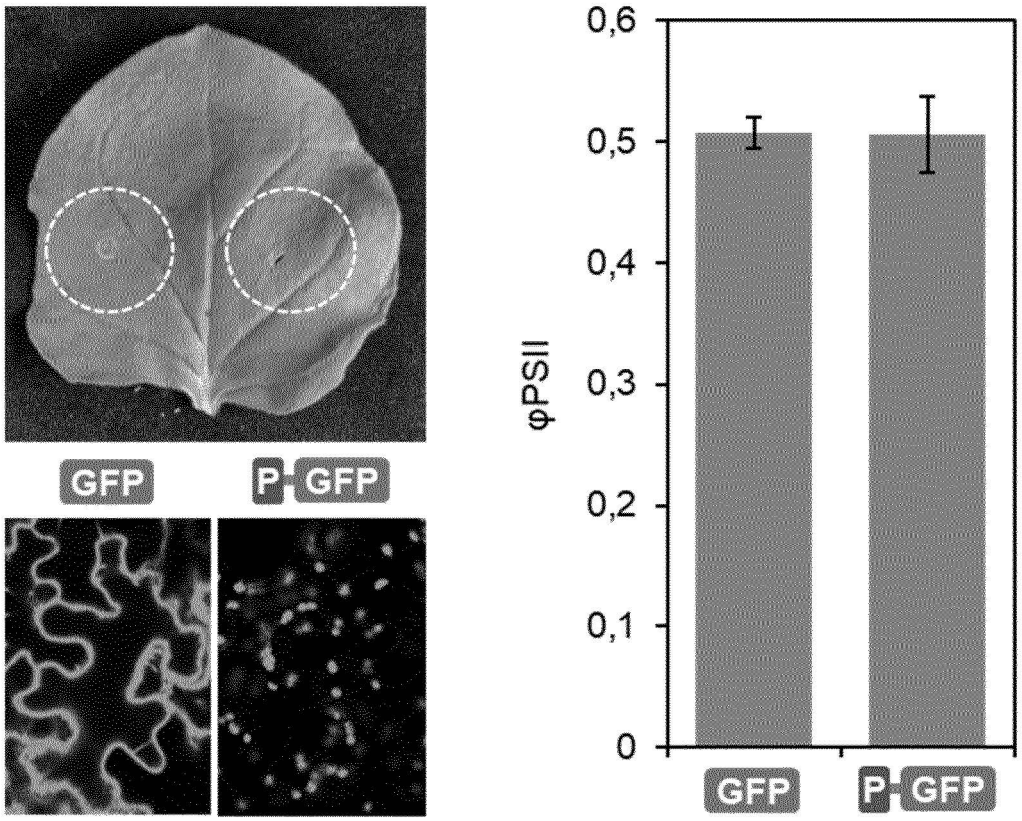

Figure 10.

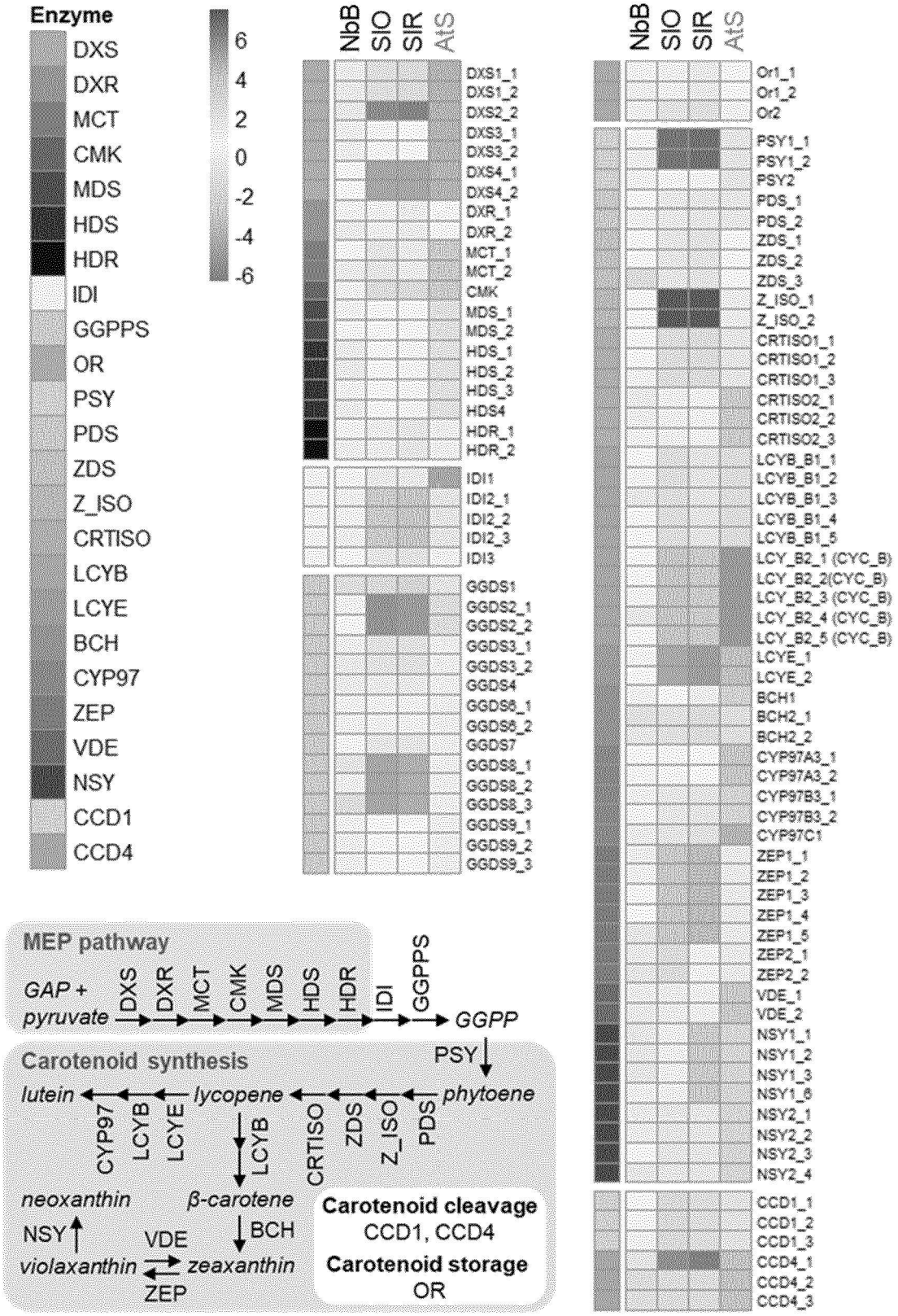
Enzyme
DXS
DXR
MCT
CMK
MDS
HDS
HDR
IDI
GGPPS
OR
PSY
PDS
ZDS
Z_ISO
CRTISO
LCYB
LCYE
BCH
CYP97
ZEP
VDE
NSY
CCD1
CCD4
6
4
2
0
-2
-4
-6
NbB
SlO
SlR
AtS
DXS1_1
DXS1_2
DXS2_2
DXS3_1
DXS3_2
DXS4_1
DXS4_2
DXR_1
DXR_2
MCT_1
MCT_2
CMK
MDS_1
MDS_2
HDS_1
HDS_2
HDS_3
HDS4
HDR_1
HDR_2
IDI1
IDI2_1
IDI2_2
IDI2_3
IDI3
GGDS1
GGDS2_1
GGDS2_2
GGDS3_1
GGDS3_2
GGDS4
GGDS6_1
GGDS6_2
GGDS7
GGDS8_1
GGDS8_2
GGDS8_3
GGDS9_1
GGDS9_2
GGDS9_3
Or1_1
Or1_2
Or2
PSY1_1
PSY1_2
PSY2
PDS_1
PDS_2
ZDS_1
ZDS_2
ZDS_3
Z_ISO_1
Z_ISO_2
CRTISO1_1
CRTISO1_2
CRTISO1_3
CRTISO2_1
CRTISO2_2
CRTISO2_3
LCYB_B1_1
LCYB_B1_2
LCYB_B1_3
LCYB_B1_4
LCYB_B1_5
LCY_B2_1 (CYC_B)
LCY_B2_2(CYC_B)
LCY_B2_3 (CYC_B)
LCY_B2_4 (CYC_B)
LCY_B2_5 (CYC_B)
LCYE_1
LCYE_2
BCH1
BCH2_1
BCH2_2
CYP97A3_1
CYP97A3_2
CYP97B3_1
CYP97B3_2
CYP97C1
ZEP1_1
ZEP1_2
ZEP1_3
ZEP1_4
ZEP1_5
ZEP2_1
ZEP2_2
VDE_1
VDE_2
NSY1_1
NSY1_2
NSY1_3
NSY1_6
NSY2_1
NSY2_2
NSY2_3
NSY2_4
CCD1_1
CCD1_2
CCD1_3
CCD4_1
CCD4_2
CCD4_3
MEP pathway
GAP + pyruvate
DXS
DXR
MCT
CMK
MDS
HDS
HDR
IDI
GGPPS
GGPP
Carotenoid synthesis
PSY
lutein
lycopene
phytoene
CYP97
LCYB
LCYE
LCYB
CRTISO
ZDS
Z_ISO
PDS
neoxanthin
β-carotene
NSY
VDE
BCH
violaxanthin
zeaxanthin
ZEP
Carotenoid cleavage
CCD1, CCD4
Carotenoid storage
OR

Figure 11.

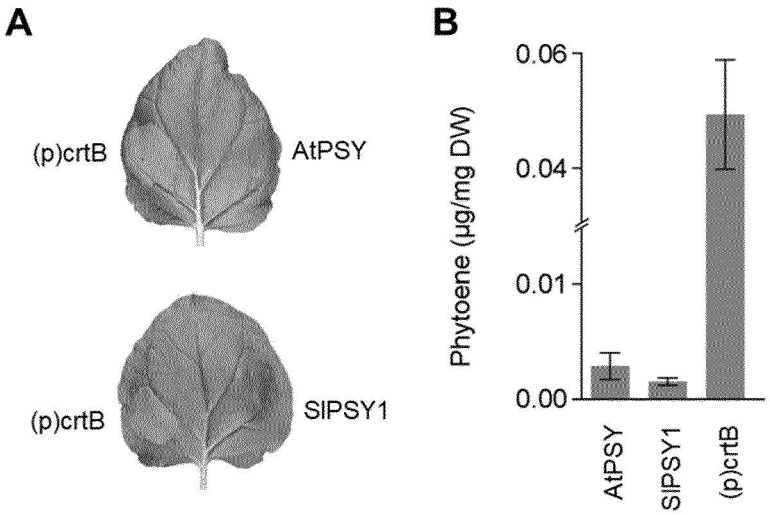
A
(p)crtB
AtPSY
(p)crtB
SlPSY1
B
Phytoene (µg/mg DW)
0.06
0.04
0.01
0.00
AtPSY
SlPSY1
(p)crtB

Figure 12.

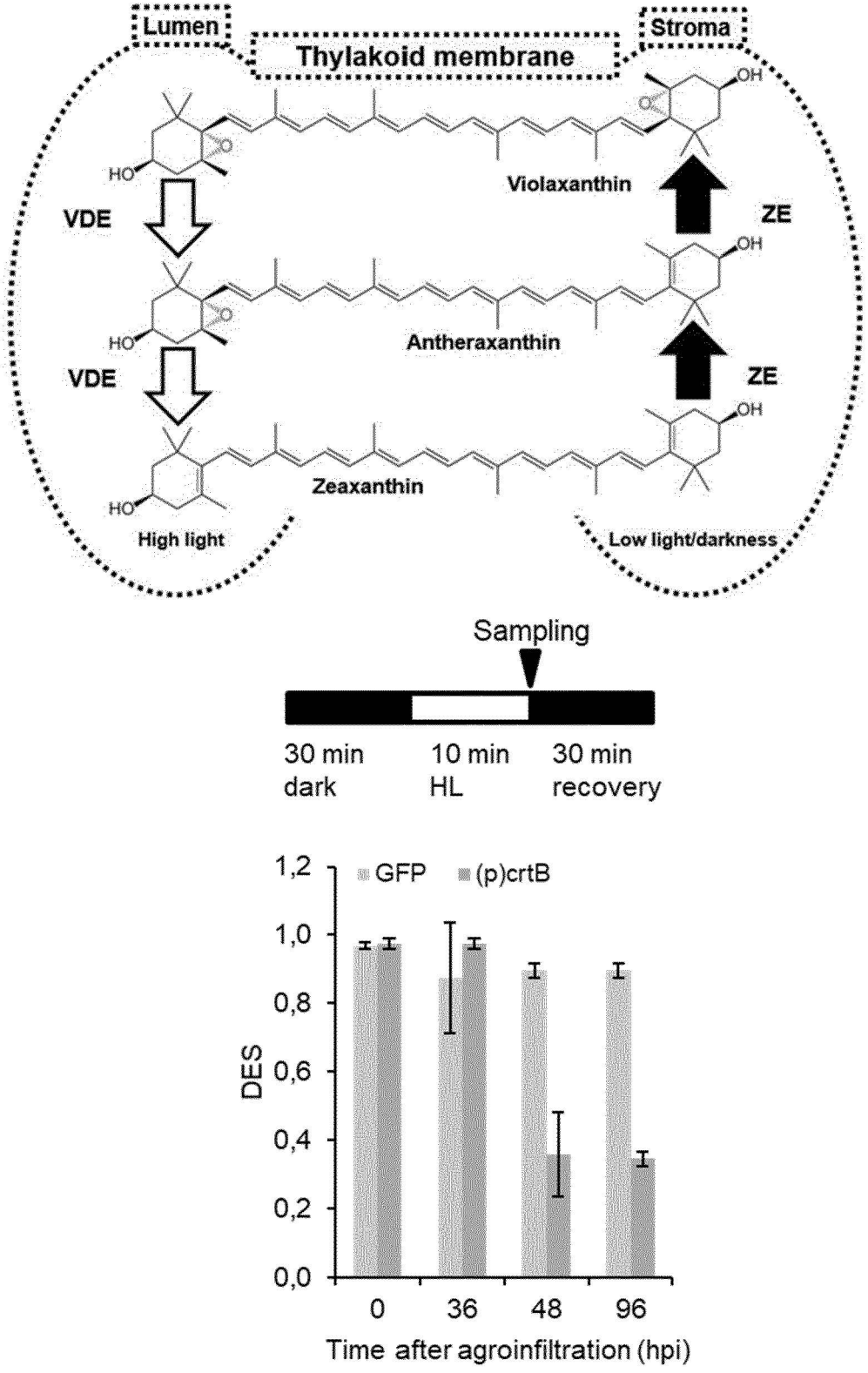
Lumen
Thylakoid membrane
Stroma
Violaxanthin
VDE
ZE
Antheraxanthin
VDE
ZE
Zeaxanthin
High light
Low light/darkness
Sampling
30 min dark
10 min HL
30 min recovery
GFP
(p)crtB
DES
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
36
48
96
Time after agroinfiltration (hpi)

Figure 13.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/075525

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/10 C12N15/82
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/085656 A2 (SYNGENTA LTD [GB]; DRAKE CAROLINE RACHEL [GB] ET AL.) 7 October 2004 (2004-10-07) see the whole document; page 19, line 14 - line 23	2-14
X	WO 96/13149 A1 (AMOCO CORP [US]) 9 May 1996 (1996-05-09) see the whole document; claim 3	2-14
X	WO 98/06862 A1 (CALGENE INC [US]; SHEWMAKER CHRISTINE K [US]) 19 February 1998 (1998-02-19) see the whole document; claim 80	2-14

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 October 2020	30/10/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kania, Thomas

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/88169 A2 (MONSANTO TECHNOLOGY LLC [US]) 22 November 2001 (2001-11-22) the whole document	2-14
A	DIRK MAASS ET AL: "Carotenoid Crystal Formation in Arabidopsis and Carrot Roots Caused by Increased Phytoene Synthase Protein Levels", PLOS ONE, vol. 4, no. 7, 28 July 2009 (2009-07-28), page e6373, XP055669962, DOI: 10.1371/journal.pone.0006373 cited in the application	1-14
A	PAUL D. FRASER ET AL: "Manipulation of Phytoene Levels in Tomato Fruit: Effects on Isoprenoids, Plastids, and Intermediary Metabolism", THE PLANT CELL, vol. 19, no. 10, 1 October 2007 (2007-10-01), pages 3194-3211, XP055669960, US ISSN: 1040-4651, DOI: 10.1105/tpc.106.049817 cited in the application	1-14
A	ESZTER MAJER ET AL: "Rewiring carotenoid biosynthesis in plants using a viral vector", SCIENTIFIC REPORTS, vol. 7, no. 1, 31 January 2017 (2017-01-31), XP055669910, DOI: 10.1038/srep41645	1-14
A	XIANGJUN ZHOU ET AL: "Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 112, no. 11, 9 February 2015 (2015-02-09), pages 3558-3563, XP055669966, US ISSN: 0027-8424, DOI: 10.1073/pnas.1420831112	1-14
A	DEAN DELLAPENNA ET AL: "VITAMIN SYNTHESIS IN PLANTS: Tocopherols and Carotenoids", ANNUAL REVIEW OF PLANT BIOLOGY, vol. 57, no. 1, 1 June 2006 (2006-06-01), pages 711-738, XP055096329, ISSN: 1543-5008, DOI: 10.1146/annurev.arplant.56.032604.144301	1-14

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI LI ET AL: "Chromoplast biogenesis and carotenoid accumulation", ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, ACADEMIC PRESS, US, vol. 539, no. 2, 12 July 2013 (2013-07-12) , pages 102-109, XP028761600, ISSN: 0003-9861, DOI: 10.1016/J.ABB.2013.07.002 -----	1-14
A	GIULIANO GIOVANNI ED - JIN YONG-SU ET AL: "Provitamin A biofortification of crop plants: a gold rush with many miners", CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 44, 3 March 2017 (2017-03-03), pages 169-180, XP029970634, ISSN: 0958-1669, DOI: 10.1016/J.COPBIO.2017.02.001 -----	1-14
A	RODRIGUEZ-CONCEPCION MANUEL ET AL: "A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health", PROGRESS IN LIPID RESEARCH, PERGAMON PRESS, PARIS, FR, vol. 70, 19 April 2018 (2018-04-19), pages 62-93, XP085397462, ISSN: 0163-7827, DOI: 10.1016/J.PLIPRES.2018.04.004 -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/075525

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004085656 A2	07-10-2004	AT 382702 T	15-01-2008
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		WO 0188169 A2	22-11-2001