Journal Of Experimental Botany, Vol. 63, No. 14, pp. 5189–5202, 2012
doi:10.1093/jxb/ers179 Advance Access publication 12 July, 2012
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Arabidopsis cpSRP54 regulates carotenoid accumulation in *Arabidopsis* and *Brassica napus*

Bianyun Yu[1,2], Margaret Y. Gruber[1,*], George G. Khachatourians[2], Rong Zhou[1], Delwin J. Epp[1], Dwayne D. Hegedus[1], Isobel A. P. Parkin[1], Ralf Welsch[3] and Abdelali Hannoufa[4,*]

[1] Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK, S7N 0X2, Canada
[2] Department of Food and Bioproduct Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, S7N 5A8, Canada
[3] Institute for Biology II, Cell Biology, Schaenzlestr. 1, 79104 Freiburg, Germany
[4] Agriculture and Agri-Food Canada, 1391 Sandford Street, London, ON, N5V 4T3, Canada
* To whom correspondence should be addressed: E-mail: abdelali.hannoufa@agr.gc.ca and margie.gruber@agr.gc.ca

Received 4 May 2012; Revised 4 May 2012; Accepted 18 May 2012

Abstract

An *Arabidopsis thaliana* mutant, *cbd* (*carotenoid biosynthesis deficient*), was recovered from a mutant population based on its yellow cotyledons, yellow-first true leaves, and stunted growth. Seven-day-old seedlings and mature seeds of this mutant had lower chlorophyll and total carotenoids than the wild type (WT). Genetic and molecular characterization revealed that *cbd* was a recessive mutant caused by a T-DNA insertion in the gene *cpSRP54* encoding the 54 kDa subunit of the chloroplast signal recognition particle. Transcript levels of most of the main carotenoid biosynthetic genes in *cbd* were unchanged relative to WT, but expression increased in carotenoid and abscisic acid catabolic genes. The chloroplasts of *cbd* also had developmental defects that contributed to decreased carotenoid and chlorophyll contents. Transcription of *AtGLK1* (Golden 2-like 1), *AtGLK2*, and *GUN4* appeared to be disrupted in the *cbd* mutant suggesting that the plastid-to-nucleus retrograde signal may be affected, regulating the changes in chloroplast functional and developmental states and carotenoid content flux. Transformation of *A. thaliana* and *Brassica napus* with a gDNA encoding the *Arabidopsis cpSRP54* showed the utility of this gene in enhancing levels of seed carotenoids without affecting growth or seed yield.

Key words: Accumulation, *Arabidopsis thaliana*, biosynthesis, *Brassica napus*, carotenoids, gene expression.

Introduction

Carotenoids are essential components of the photosynthetic machinery and play a critical role in preventing photooxidative damage (Howitt and Pogson, 2006). Carotenoids are precursors of the plant hormone abscisic acid (ABA) (McCarty, 1995). They are also precursors of vitamin A, and some carotenoids protect against human age-related macular degeneration (Beatty *et al*., 2004). A few carotenoids are used as colourants in the food and cosmetics industries and some are important supplements in fish feed formulations (Umeno *et al*., 2005).

Carotenoids are derived from the isoprenoid pathway and are synthesized in almost all types of plant plastids (Howitt and Pogson, 2006). They co-accumulate with chlorophyll in pigment-binding protein complexes embedded in thylakoid membranes (Botella-Pavía and Rodríguez-Concepción, 2006). Generally, the amounts of β-carotene and lutein are proportional to the content of chlorophyll a and b, respectively (Pogson *et al*., 1996). Carotenoid-deficient mutants usually show impaired photosynthesis (reviewed by DellaPenna, 1999; Dong *et al*., 2007)

Three independent plastid-to-nucleus retrograde signalling pathways have been described (Nott *et al*., 2006). The best-studied retrograde pathway is triggered by the accumulation of the chlorophyll precursor, Mg-Protoporphyrin IX (Mg-ProtoIX), in the tretrapyrrole pathway (Larkin *et al*., 2003; Strand *et al*., 2003). *GUN4* (*GENOMES UNCOUPLED 4*) is a chloroplast-encoded Mg-ProtoIX-binding protein and can stimulate Mg-chelatase activity (Larkin *et al*., 2003). *GUN1* encodes a chloroplast-localized pentatricopeptide repeat protein, and the three known chloroplast retrograde signal pathways intersect upstream of *GUN1* (Koussevitzky *et al*., 2007). *AtGLK1* and *AtGLK2* (Golden 2-Like) are redundant members of the GARP superfamily of transcription factors (Fitter *et al*., 2002). *AtGLK1* is downregulated by GUN1 when plastids are dysfunctional and has been reported as a positive regulator of photosynthesis-related nuclear gene expression in a plastid-to-nucleus signalling pathway (Kakizaki *et al*., 2009). ABI4 acts as a negative regulator of photosynthesis-related nuclear gene expression and part of the GUN4/GUN1 signalling pathway (Koussevitzky *et al*., 2007).

Although carotenoid biosynthesis pathways in plants have been well documented (Howitt and Pogson, 2006), the mechanisms that regulate carotenoid biosynthesis and accumulation in plants are less understood. A combination of factors are known to regulate carotenoid accumulation in plants. These include metabolic controls that determine the rate of anabolism and catabolism, availability of storage compartments, and transcriptional and epigenetic regulation (Hannoufa and Hossain, 2012). To gain insight into additional mechanisms, an activation tagged population of *Arabidopsis thaliana* was screened, and this led to the identification of a new T-DNA insertion mutant named *cbd* (*carotenoid biosynthesis deficiency*). The phenotype of the *cbd* mutant was shown to be due to a mutation in the chloroplast signal recognition particle 54 kDa subunit gene (*cpSRP54*). The mutation reduced chlorophyll and carotenoid content, impaired chloroplast development, and caused an ABA deficiency in seedlings of *cbd*. These data showed that over-expression of *cpSRP54* in *Arabidopsis* and *Brassica napus* raised carotenoid levels in seeds without affecting growth or seed yield and suggested that *cpSRP54* can be used as a tool for biofortification of carotenoids in oilseed crops and other crop plants.

Materials and methods

Arabidopsis *plant materials and identification of carotenoid-deficient mutants*

A. thaliana (ecotype Columbia) T-DNA activation tagged SK mutant population was generated using the pSKI015 vector at Agriculture and Agri-Food Canada (Robinson *et al*., 2009; www.brassica.ca). The *cpSRP54* mutants, CS850421 and SALK_079668 (Alonso *et al*., 2003), were obtained from the *Arabidopsis* Biological Resource Center (ABRC, www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). The mutants were analysed by PCR amplification using one primer specific to the T-DNA (LBb1, Table 1) and two primers specific to the flanking genomic DNA sequence (P1 and P2 for CS850421; P3 and P4 for SALK_079668). Homozygous *Arabidopsis* mutant seeds were used for further studies.

SK mutants with defects in carotenoid accumulation were identified by sowing approximately 1000 seeds from each T_2 super pool (37 pools, 100 lines per pool) on RediEarth soil (WR Grace, Ajax, Canada) and growing the lines in a controlled greenhouse environment (light intensity 230 µmol m^{-2} s^{-1}; 16/8 light/dark; 20/17 °C). *Arabidopsis* seedlings with light green or pale yellowish leaves were selected as putative carotenoid mutants. For subsequent analysis, sterilized homozygous mutant seeds were incubated in 0.05% agarose at 4 °C for 2 days, then placed on Petri dishes containing 1/2 Murashige and Skoog basal medium (MS), 1% sucrose, and 0.8% agar and incubated in a growth cabinet at 60–80 $\mu mol.m^{-2}.s^{-1}$ under 16/8 light/dark at 22 °C. Seven-day-old *Arabidopsis* seedlings were used for carotenoid analysis, semi-quantitative reverse transcription PCR (RT-PCR), quantitative real-time PCR (qRT-PCR), and ABA quantification. For carotenoid analysis and qRT-PCR analysis of *cpSRP54* gene expression in transgenic *Arabidopsis* plants, 8-day-old seedlings grown under the same growth cabinet conditions as above were used.

Molecular characterization of the Arabidopsis cbd *mutant,* cbd *complementation lines, and wild-type over-expression lines*

Genomic DNA was isolated using the method of Michiels *et al*. (2003), and used for thermal asymmetric interlaced (TAIL)-PCR (Liu *et al*., 1995) and genome walking (BD GenomeWalker Universal Kit, BD Biosciences Clontech, Mississauga, ON, Canada) to identify T-DNA insertion sites in the mutant line. The results were confirmed by PCR with one T-DNA-specific primer, pSKTAIL-L1, and two primers, P5 and P6, specific to the flanking genomic DNA sequence (Table 1).

For molecular complementation of mutant line *cbd* and development of *cpSRP54* over-expression lines in a wild-type (WT) background, a 6.2-kb region of genomic DNA (gDNA) including the native promoter, 5′-untranscribed region (UTR), coding region, 3′-UTR, and 3′ flanking sequence of *cpSRP54* (*At5g03940*), was amplified by PCR with primers P9 and P10 (Table 1), cloned into the pCR8/GW/TOPO TA cloning vector (Invitrogen, Burlington, ON, Canada), and then subsequently subcloned into the binary vector pMDC99 (Curtis and Grossniklaus, 2003). Primers P7 and P8 (Table 1) were used to amplify the full-length *cpSRP54* coding region (CDS), which was cloned in an identical cloning vector and subsequently subcloned into the binary vector pMDC83 (Curtis and Grossniklaus, 2003). Binary plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101pMP90, and *A. thaliana* transformation was conducted according to the floral dip procedure (Clough and Bent, 1998).

RNA isolation, semi-quantitative RT-PCR, and qRT-PCR of Arabidopsis *tissues*

Total RNA was isolated from mutant, WT, and complemented/over-expression transgenic *Arabidopsis* plants using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) and used for RT-PCR analysis of *cpSRP54* gene expression and co-amplification of the internal control gene, *AtACT2* (*AT3G18780*) (primers listed in Table 1). cDNA synthesis was conducted using 1 µg of total RNA and SuperScriptII reverse transcriptase (Invitrogen). PCR amplification was performed using an initial denaturation at 94 °C for 4 min, followed by 26 cycles at 94 °C for 25 s, 55 °C for 30 s, and 72 °C for 1 min 45 s, and a final extension at 72 °C for 7 min. The amplicon was confirmed by sequencing.

For qRT-PCR, total RNA and cDNA preparations were made as described above. cDNA samples were diluted 10-fold and 2 µl diluted cDNA was used in each PCR reaction. qRT-PCR reactions were performed on three cDNA batches with a Bio-Rad CFX Manager System (Bio-Rad Laboratories, Mississauga, ON, Canada) using 12.5 µl of 2× SYBR Green qPCR Master Mix (Invitrogen) and gene-specific primers

Table 1. Primers used to characterize *Arabidopsis cbd* mutant

Primers used for genotyping and molecular complementation

Primer	*AGI code*	*Sequence (from 5′ to 3′)*	Purpose
pSKTAIL-L1	N/A	TTCTCATCTAAGCCCCCATTTGG	Confirmation of T-DNA insertion site in *cbd* mutant
LBb1	N/A	GCGTGGACCGCTTGCTGCA	Confirmation of T-DNA insertion site in SALK lines
P1	AT5G03940	ACCGGTTCTTGTTTTTGTTTCATTAGCA	Confirmation of T-DNA insertion site in CS850421
P2	AT5G03940	CATTCGCTCTCCACGTCCTACCAGTTTA	Confirmation of T-DNA insertion site in CS850421
P3	AT5G03940	AGCCCCATTGTCAGGTTATTTCGCA	Confirmation of T-DNA insertion site in SALK_079668
P4	AT5G03940	GAGCTCACGGGCAACGAAATAAGAAAGA	Confirmation of T-DNA insertion site in SALK_079668
P5	AT5G03940	TATTATGGATACTGCAGGGAGGCTTCA	Confirmation of T-DNA insertion site in *cbd*
P6	AT5G03940	TTGTTCTGCTTTCAATGCGTCCTCG	Confirmation of T-DNA insertion site in *cbd*
P7	AT5G03940	ATGGAGGCTCTTCAATTTTCCAGCGT	Forward primer for amplification of *cpSRP54* coding region
P8	AT5G03940	GTTACCAGAGCCGAAGCCACGAGGA	Reverse primer for amplification of *cpSRP54* coding region
P9	AT5G03940	GTTTTCCTTTGGTTCAAATTTCCATTTA	Forward primer for amplification of *cpSRP54* genomic sequence
P10	AT5G03940	GTATTTTAGTTATGCAAAGCCTATGAA	Reverse primer for amplification of *cpSRP54* genomic sequence
AtACT2-F	AT3G18780	GATATGGAAAAGATCTGGCATCAC	Forward primer for amplification of *AtACT2*
AtACT2-R	AT3G18780	TCATACTCGGCCTTGGAGATCCAC	Reverse primer for amplification of AtACT2

Gene-specific primers used in quantitative real-time PCR

Gene	AGI code	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Reference
PSY	AT5G17230	TGCGGTGAAGTTTGCGCTGA	TGAAGCATTTGGCCCATCCA	
PDS	AT4G14210	GTCGGTCACGCGCTCAGGTA	CGAGATGCTGACATGGCCAGA	
ZDS	AT3G04870	CCATCGTCACGAGGCCTAGAA	TGTGTATGAACCGGCGAGGA	
bLYC	AT3G10230	TGGTAGCGCTGCTCTTTTGGA	ACCAGCAGGACCACCACCAA	
BCH1	AT4G25700	GGCACGCTTCTCTATGGAATATGCATGA	GAATCCATAAGAGAGGAGACCAATCGCT	
BCH2	AT5G52570	ATGGAGTTTTGGGCAAGATGGGCTCAT	CAGGAACCGCGTTTGTTATAGCAAACACA	
LUT1	AT3G53130	CGAAATCCCAATCATGGGTCA	GCACCTCCGAGGAGATCAGC	
ZEP	AT5G67030	ATGACCGGCTTCGAGAGTGG	TTCCGACGATGCAAGGTTGA	
VDE	AT1G08550	ACCGCTCCGCTGTTGCTAAA	TGGCAATGCACTTTGCGAGT	
CCD1	AT3G63520	TGTTCCGCGTGAGACAGCAG	CCACCACTGCCACCGGTTC	
CCD4	AT4G19170	AAGATCTCCGGTGTGGTGAAGC	CCGGATTACCAGGATCCCTAGC	
CCD7	AT2G44990	CTAAACCGTGGCGACGACAA	CCGGAAAATCTGACGGCTTG	
CCD8	AT4G32810	CGTTTATGCATGCGGTGCTC	GGTCGAGGCACGAAGAATGG	
CAO	AT1G44446	CCGGTGGAACACGGTTTACTTCTAGATA	AGTATCCTTGGAGACCCGAGGTAGGTGT	
PORA	At1g03630	CTCGGTGTTTCAACCTTTGG	CAGATAGAATCAGCCAAAACACAAC	Kakizaki et al. (2009)
PORB	AT4G27440	TTCCGAGAGCACATTCCTCTCTTCCGT	CCCTGATTTCGTCAAGCTTGGATCACT	
Lhca2	AT3G61470	ACATCTACACTGGCACTGGTCCTATTGA	ACACACAAACGCATTCACCTCCCCATAA	
Lhcb1	AT1g29930	AGCTCAAGAACGGAAGATTGG	GCCAAATGGTCAGCAAGGTT	Kakizaki et al. (2009)
Lhcb2.1	AT2g05100	AAGTCGTGAATGTACTTATTGGTG	GGTGGTGTGGTTCATTAAAGGT	Kakizaki et al. (2009)
LSU	ATCg00490	TTGCCGAGATAATGGCCTACTT	AACAAAGCCCAAAGTTGACTCC	Kakizaki et al. (2009)
OE33	AT5G66570	ACCGTCAAGGCAGACAGTGTAAGCAAGA	CTTGAAATTGACGCTTCCGTCTGAAGCA	
PSBA	ATCG00020	GTGTATTCGGCGGCTCCCTTTTTAGT	CTTCTTCTTGCCCGAATCTGTAACCTTCA	
PSBS	AT1G44575	CATTGGAGCTCTCGGAGACAGAGGAA	CTCGTTCGCCTTCGTGAACCCAAACAAT	
NCED3	AT3g14440	TAACGCCGTTAGCTTAGAGGTTGAAGCA	AGAACTCACACGACCTGCTTCGCCAAAT	
NCED5	AT1G30100	TGTTCACGACGAGGAGAGTTGG	TATCCGCCGAATTCACGAAAGT	
NCED6	AT3G24220	GAGCTGGGATCGGTCTAGTGGA	TTGACCGTCGATCTTCACTTGG	
NCED9	AT1G78390	TCGACGGAGACGGTATGGTACA	TCTCCAATTGCTTTGGGGAAAA	
ABA2	AT1G52340	ACGGTTGATGATGTAGCGAACGCTGTT	CATCTGAAGACTTTAAAGGAGTGGTTAG	
AAO3	AT2G27150	TGGACTGCTCCTTCTGGTGATG	CTTGATGCTTCTTGGCGAGACA	
CYP707A1	AT4G19230	ATTTGATCCATCAAGATTCGAGGTGGCT	ACCAAACCTGTACTTGGTGGTGAGATGA	
CYP707A2	AT2G29090	GCGAATCCATCACTCCTCCGAATTCTT	TCACTTCCTGGACATGAGTGCACTCCAT	
CYP707A3	AT5G45340	CGAGATTCGAAGTTGCGCCGAAACCGA	GATTGACCATCTGTACTTAGTGGTGAGA	
CYP707A4	AT3G19270	GTGTGCTAACCCAAGAACAGATTGCAGA	CAGCCTTAACAGCTTCTAGAAGTTTCTGA	
At5g12240	At5g12240	TGGCACGATGCACCGACTGTTGCT	CTAGTCAATCTAACA AGCCAGTAAGCTA	Czechowski et al. (2005)
UBC	At5g25760	TGCTTGGAGTCCTGCTTGGA	TGTGCCATTGAATTGAACCCTCT	Czechowski et al. (2005)

Table 2. Primers used in this study to generate and characterize transgenic *cpSRP54*$^{+}$ *B. napus* lines

Primers used for vector construction and confirmation of transgenic lines			
Primer name	**Sequence (from 5′ to 3′)**	**Purpose**	
FFC-F31	ggatccGTACCAAGAA GATTGCGGAG	Cloning of *cpSRP54* (*FFC*) genomic sequence, add *Bam*HI site (in lower case)	
FFC-R34	gtcgacGTATTTTAGTTATGCAAAGCCTATGAA	Cloning of *cpSRP54* (*FFC*) genomic sequence, add *Sal*I site (in lower case)	
napin-F5	AGCTCCCAATTTATATTCCCAACGGCAC	Confirmation of transgenic lines	
FFC-R6	GAGCTCACGGGCAACGAAATAAGAAAGA	Confirmation of transgenic lines	
Gene-specific primers used in quantitative real-time PCR			
Gene name	**Forward primer (from 5′ to 3′)**	**Reverse primer (from 5′ to 3′)**	**Reference**
cpSRP54	ACAAAAGGCTCCACCTGGAACTGC	AGCCGAAGCCACGAGGACCA	Fujisawa et al. (2009)
BnbLYC	TCCACTGTTGTCTGCAGTGACG	CATCGACCTCAGCAACGATACC	Fujisawa et al. (2009)
BneLYC	TGAGGAGGTGTGTGGAGTCAGG	GAAGCTGCTCCAGAAGCAACAG	Fujisawa et al. (2009)
BnBCH	CAGAGGCTTCTCGGTCTGCTAC	CCTCTCGGACTTCTTCCTCTCC	Fujisawa et al. (2009)
BnCRTISO	GAGGTGGCAGCTGGAATCATAC	TCCTCTTGGCATTGGTCCATAC	Fujisawa et al. (2009)
BnPDS	GGCTGCAGTGGAAGGAACACTC	TCTCTGGCCATGTCAGCATCTC	Fujisawa et al. (2009)
BnPSY	CCAAAGCAACGACCGAGAGTG	CATCTGAGAGACCAGCCTGAGC	Fujisawa et al. (2009)
BnVDE	TCACGACCGTACGAGATTCTTC	AATCCAGATAAGGGTCGTGAGG	Fujisawa et al. (2009)
BnZDS	GCAATGAAAGACATTCGCAACC	TCTCGCACTCATGTTGTCACAG	Fujisawa et al. (2009)
BnZEP	TGCTGAAGAAGTCATGGAAGCTG	CTGCTAATCACCCGAGTCACAGG	Fujisawa et al. (2009)
BnACT3	GCATCCCTCAGCACTTTCCAACAGA	ACCACGAACCAGAAGGCAGAAACACT	

listed in Table 1. Amplifications of the ubiquitin-conjugating enzyme gene (*UBC*) and *At5g12240* (Czechowski *et al*., 2005) were used as internal controls for normalization of expression levels. Amplification efficiency for internal controls and all target genes were 100%. Triplicate seedling samples were used and analysed statistically using ANOVA.

B. napus *transformation and plant growth conditions*

Arabidopsis cpSRP54 genomic sequence including 5′- and 3′-UTR was amplified by PCR using primers FFC-F31 and FFC-R34 (Table 2) with added *Bam*HI and *Sal*I sites, respectively, and TA-cloned into vector pCR2.1-TOPO for sequencing. Subsequently the *cpSRP54* fragment was subcloned into the *Bam*HI and *Sal*I sites of the modified binary vector pBI121 in which the CaMV 35S promoter was replaced with the seed-specific *B. napus* napin promoter (Rask *et al*., 1998) at the *Bam*HI and *Hind*III sites. Cotyledons with cotyledonary petioles from 5-day-old seedlings of *B. napus* doubled haploid line DH12075 were used as explants for transformation with *A. tumefaciens* GV3101 pMP90 harbouring the *cpSRP54*$^{+}$ construct. One ml of the overnight *A. tumfaciens* culture was added to liquid MS media containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 g l^{-1} MES, 1% DMSO, and 0.04 g l^{-1} acetosyringone buffered at a pH 5.6. *B. napus* explants were floated in this *A. tumfaciens* suspension for 2 hours after which the explants were removed, placed on filter paper moistened with MS media, incubated in the dark at 15 °C for 5 days, and transferred to selection media containing 0.7% Phytablend agar (Caisson Laboratories, North Logan, UT, USA), 3% sucrose, 4.5 mg l^{-1} benzylaminopurine, 450 mg l^{-1} timentin, and 20 mg l^{-1} kanamycin. After 4–6 weeks, regenerated green shoots were transferred every 2 weeks to shoot selection media with BA at 0.5 mg l^{-1} until normalized, then placed in 100 ml rooting jars containing MS medium with 3% sucrose, 0.1 mg l^{-1} NAA, 0.7% Phytablend agar, 450 mg l^{-1} timentin, and buffered to pH 5.8. Rooted shoots were transferred to pots in a controlled environment greenhouse 16/8 light/dark at 20/17 °C. Only those plants with a confirmed transgene as determined by PCR using napin-F5 and FFC-R6 primers (Table 2) were subjected to further analysis.

RNA extraction and qRT-PCR analysis of developing B. napus *seeds*

Total RNA was isolated from developing *B. napus* seeds at 34 days post anthesis (DPA) as described by Suzuki *et al*. (2004) with some modifications. Briefly, the seeds were ground into fine powder in liquid nitrogen. Approximately 50 mg seeds were used for RNA extraction with 700 µl of extraction buffer. After extraction with chloroform/isoamylalcohol (CIA, 24:1) and a mixture of water-saturated phenol containing 35% (w/v) guanidium thiocyanate and 1/10 (v/v) 2 M sodium acetate (pH 4.0), the supernatant was transferred to a new tube and a half volume of 100% ethanol was added to precipitate RNA. Subsequent procedures were performed according to the manufacturer's instructions for the RNeasy Plant Mini Kit (Qiagen). cDNA synthesis and qRT-PCR reactions were conducted as described for *Arabidopsis* tissue except that 1 µl of 10-fold diluted cDNA was used in each PCR reaction. Amplification of *B. napus BnACT3* was used as an internal control for the normalization of expression levels. For each independent transgenic line, triplicate seed samples were tested for expression and analysed statistically using ANOVA.

Extraction and analysis of carotenoids from seeds and seedlings

A. thaliana and *B. napus* seeds were harvested immediately upon maturation of soil-grown plants and stored at –20 °C. Approximately, 150 mg seeds or 50 mg agar-grown seedlings was used for carotenoid analysis. Seed carotenoid extraction and HPLC analysis were conducted as described previously (Yu *et al*., 2007). For carotenoid analysis of seedlings, saponification was conducted at room temperature for 30 min. Procedures of extraction and HPLC analysis following saponification were the same for seedlings and seeds. Triplicate samples were used for carotenoid analysis and analysed statistically using ANOVA.

Chlorophyll extraction and quantification in Arabidopsis

Seven-day-old *Arabidopsis* seedlings (20–40 mg freshweight) were ground into fine powder with liquid nitrogen. The sample powder was suspended in 1 ml of ice-cold 80% acetone and centrifuged at 13,000 *g* for 10 min at 4 °C. The chlorophyll content of the supernatant was measured at 647 nm and 664 nm, and calculated using the formula described by Inskeep and Bloom (1985).

Detection of cell death and histochemical detection of O_2^- *in* Arabidopsis

Arabidopsis WT and mutant seeds were sterilized and grown on Petri dishes under 16/8 light/dark at 22 °C (standard condition, SD) for

10 days. Subsequently, one group of WT and mutant plants were maintained under the same conditions for another 10 days, while another group was transferred to continuous white light (WL) at room temperature to grow for another 10 days. The fifth and sixth leaves were harvested and used for detection of cell death and histochemical detection of O_2^- at the end of these treatments. Cell death was examined by Evan's Blue staining as described in Dong *et al*. (2007). O_2^- accumulation in leaves was detected according to Fitzgerald *et al*. (2004).

Transmission electron microscopy of Arabidopsis *chloroplasts*

The first true leaf (yellow in *cbd*) from 10-day-old seedlings (at least 10 seedlings for each) was grown on 1/2 MS + 1% sucrose agar, fixed with 2.5% glutaraldehyde and 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) and embedded with resin Eponate 12 after serial dehydration with ethanol to observe chloroplast structure. Ultrathin sections were floated onto 200-mesh copper grids (Ted Pella, Redding, CA, USA) which had been coated with Formvar. Grids were stained for approximately 30 minutes with a 2% solution of uranyl acetate, and then rinsed with distilled water before staining for 10 minutes in Reynolds lead citrate (Reynolds, 1963). Grids were left to dry at least 24 hours before observation in a Philips CM 10 transmission electron microscope (TEM).

ABA quantification

To quantify the ABA content of *cbd*, CA850421, SALK_079668, and WT plants, approximately 20 mg of respective 7-day-old seedlings was extracted with 80% methanol as described by Galpaz *et al*. (2008). ABA was quantified by enzyme immunoassay using a Phytodetek ABA test kit (Agdia, Elkhart, IN, USA) following the manufacturer's instructions. Triplicate samples were used and analysed statistically using ANOVA.

Results

Identification of the cbd *mutant*

The *cbd* mutant was identified from the SK *A. thaliana* T-DNA activation tagged mutant population (Robinson *et al*., 2009). Seven-day-old seedlings of the *cbd* mutant had yellow cotyledons (Fig. 1A), which became greener with time. All newly emerged true leaves were yellow and became increasingly green as they matured, but not to the same extent as WT leaves (Fig. 1A, 1B). Stems, inflorescences, flower bud sepals, and siliques were also pale yellow, and all of these organs became light green later in development. Mature *cbd* mutant plants grew slower and were smaller than WT (Fig. 1C).

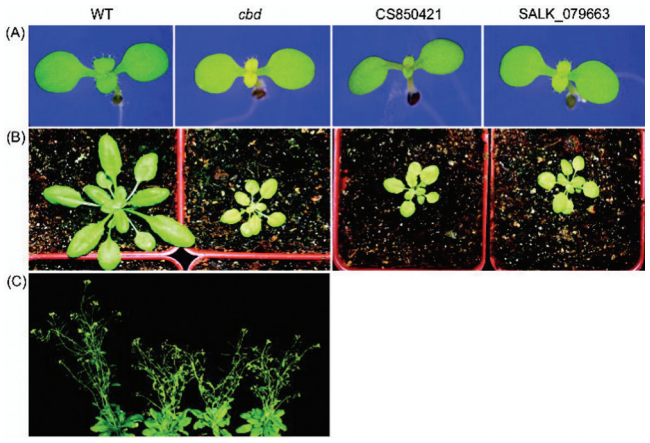

Fig. 1. Phenotypes of *cbd*, SC850421 and SALK_079668 mutants. (A) 7-day-old mutant WT seedlings grown on 1/2 MS media. (B) 25-day-old mutant and WT plants grown in a co-co soil-less mix in the greenhouse. (C) Mature plants of mutants and WT, respectively.

Total chlorophyll was reduced by more than 50% in the 7-day-old seedling of *cbd*, with both chlorophyll a and b significantly decreased (Fig. 2A). Seedlings of the mutant had approximately 40% of total carotenoid compared to WT. All individual carotenoid compounds were reduced in *cbd* seedlings except for the minor carotenoid, zeaxanthin, which was not affected (Fig. 2B). The total and individual carotenoid contents of *cbd* mature seeds were also lower than that of WT seeds, with the exception of zeaxanthin, which was somewhat higher in the mutant (Fig. 2C). To examine if the phenotype in the *cbd* mutant was caused by a single gene mutation, the mutant line was backcrossed to WT. All F_1 progeny ($n = 27$) had a WT appearance. The segregation ratio of F_2 progeny phenotypes (302:107, WT/mutant) indicated that the *cbd* phenotype is due to a single recessive mutation.

Cell death and superoxide accumulation in cbd *leaves*

Evan's Blue stain was used to investigate the extent of cell death in young yellow leaves of the *cbd* mutant. Under both SD and WL conditions, large blue-green patches of dead cells were observed in *cbd* leaves (Fig. 2D). More severe cell death occurred under WL in *cbd*, while little obvious staining was found in WT. The accumulation of superoxide (O_2^-), a major species of ROS, was detected by staining the young leaves with nitroblue tetrazolium and observing the purple-blue precipitate. Under SD, superoxide was not detected in WT but it could be easily seen in *cbd* (Fig. 2E). Furthermore, *cbd* accumulated abundant superoxide under WL.

Defective chloroplast development in the cbd *mutant*

Reduced chlorophyll and carotenoid levels and yellow phenotypes in true leaves of the *cbd* mutant suggested possible defects in chloroplast development. To analyse the effect of the *cpSRP54* mutation on chloroplast development, the chloroplast ultra-structure of the first true leaf from 10 individual 10-day-old seedlings of WT and *cbd* was examined by TEM. Chloroplasts were well developed and organized in the WT mesophyll cells. However, the chloroplasts of *cbd* had reduced grana stacking with fewer linkages between grana (Fig 2F).

Molecular and genetic characterization of the cbd *mutant*

TAIL-PCR revealed that a single T-DNA was inserted into the 11th exon of the *At5g03940* locus (Fig. 2G). This encodes the 54 kDa subunit of the chloroplast signal recognition particle (cpSRP54), also referred to as Fifty Four Chloroplast homologue (FFC). Two mutants, two allelic salk line mutants, SALK_079668 (Alonso *et al*., 2003) and CS850421, showed morphological phenotypes similar to those of *cbd*, although not as extreme (Fig. 1). In addition, SALK_079668 and CS850421 had similar chlorophyll and carotenoid patterns in both 7-day-old seedlings and carotenoid patterns in mature seeds (Fig. 2B, 2C), although SALK_079668 was not as affected as CS850421 and *cbd*.

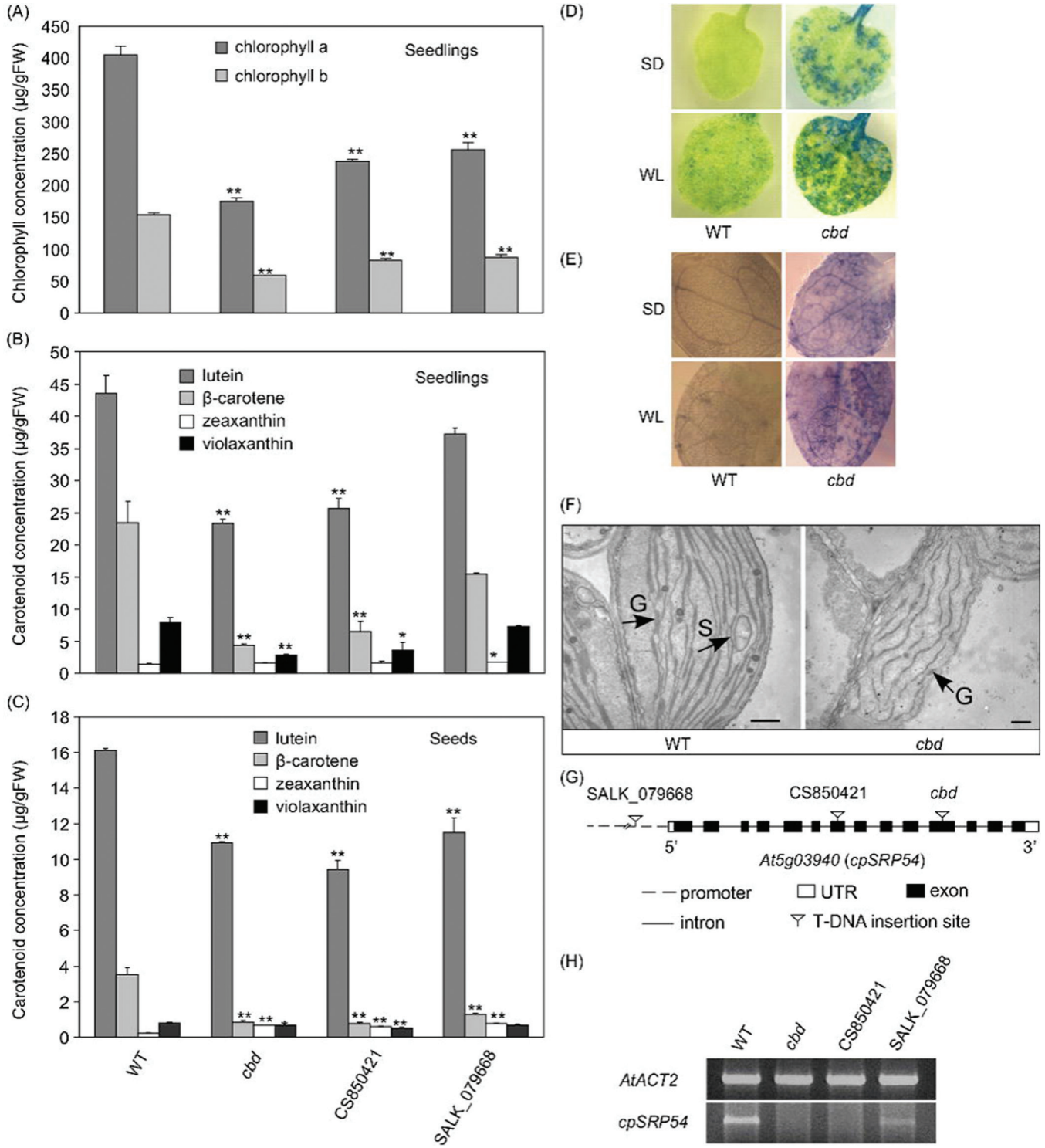

Fig. 2. Characterization of chloroplasts and *cpSRP54* transcript abundance in *cpSRP54*-deficient mutant lines and WT *Arabidopsis*. (A) Chlorophyll content of 7-day-old seedlings of mutant lines and WT. (B) Carotenoid content of 7-day-old seedlings of mutant lines and WT. (C) Carotenoid content of mature seeds of mutant lines and WT. Values are mean ± standard error of three extractions from independent seedling plates or seed batches. Significant difference was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) relative to WT *Arabidopsis*. (D, E) Representative staining of leaves of WT and *cbd* plants grown under standard light (SD, 16/8 light/dark) and continuous white light (WL): (D) Evan's Blue, where dead cells show as blue-green patches; (E) nitroblue tetrazolium, where the presence of superoxide results in a purple-blue precipitate. (F) Representative TEM of chloroplasts of WT and *cbd*. G, grana; S, starch granule. Bar, 500 nm. (G) Schematic diagram of T-DNA insertion sites in *cpSRP54* mutant lines *cbd*, CS850421, and SALK_079668. (H) Semi-quantitative RT-PCR analysis of *cpSRP54* in mutant lines and WT.

Gene expression profile in mutants

Semi-quantitative RT-PCR was conducted to analyse *cpSRP54* gene expression in *cbd* mutant and two allelic salk line mutants. SALK_079668 had one T-DNA insertion in the promoter region (–326 bp) and CS850421 had one T-DNA in the 7th exon of the *At5g03940* locus (Fig. 2G). To show that these two recessive mutants were allelic to *cbd*, reciprocal crosses between these mutants and *cbd* were conducted. All the F_1 progeny (n = 156 for *cbd* {female} × CS850421 {male}; n = 123 for CS850421{female} × *cbd* {male}; n = 47 for *cbd* {female} × SALK_079668 {male}; n = 14 for SALK_079668 {female} × *cbd* {male}) showed the same phenotype as *cbd*. These genetic data confirmed the recessive nature of *cbd* and that CS850421 and SALK_079668 were allelic to it.

Semi-quantitative RT-PCR was conducted to examine expression of *cpSRP54* gene in 7-day-old seedlings of the three mutant plants. Analysis showed that the transcript of the full-length *cpSRP54* coding region (CDS) was undetectable in *cbd* and CS850421 mutants, while SALK_079668 had a much lower level of full-length *cpSRP54* transcript compared to WT (Fig. 2H). This is consistent with the observed total carotenoid level for SALK_079668, which although reduced in comparison to WT, was higher than in c*bd* and CS850421 (Fig. 2B, 2C).

A molecular complementation experiment was conducted to confirm whether the *cbd* phenotype was caused by a mutation in *cpSRP54*. Constructs containing *cpSRP54* gDNA and CDS were introduced individually into *cbd*. T_2 transgenic plants resulting from both *cbd* complementation strategies (20 lines per construct) showed restoration of the WT growth and colour phenotype (Fig. 3A). WT levels of carotenoids were shown in seedlings of *cbd* complementation lines constructed with CDS, but levels were 15% higher in complementation lines constructed with the gDNA relative to WT (Fig. 3B). This carotenoid pattern resulted from *cpSRP54* transcript levels (qRT-PCR analysis) which were only slightly higher than WT in *cbd* plants transformed with the *cpSRP54* gDNA, but much stronger in mutant plants transformed with *cpSRP54* CDS (potentially due to the strong constitutive promoter, CaMV35S) (Fig. 3C). Both constructs were also used to overexpress *cpSRP54* in WT background. Visible phenotype differences were not observed in these over-expression transgenic lines (20 lines per construct) (data not shown). However, the total carotenoid level (lutein and β-carotene) of 8-day-old seedlings transformed with *cpSRP54* gDNA was 15% higher than in WT or over-expression lines transformed with CDS, even though transcript levels of the two constructs were relatively similar in this background (Fig. 3B, 3C). Taken together, these data clearly demonstrate that disruption of *cpSRP54* was the cause of the altered leaf and cotyledon colour and altered carotenoid profile in the *cbd* mutant.

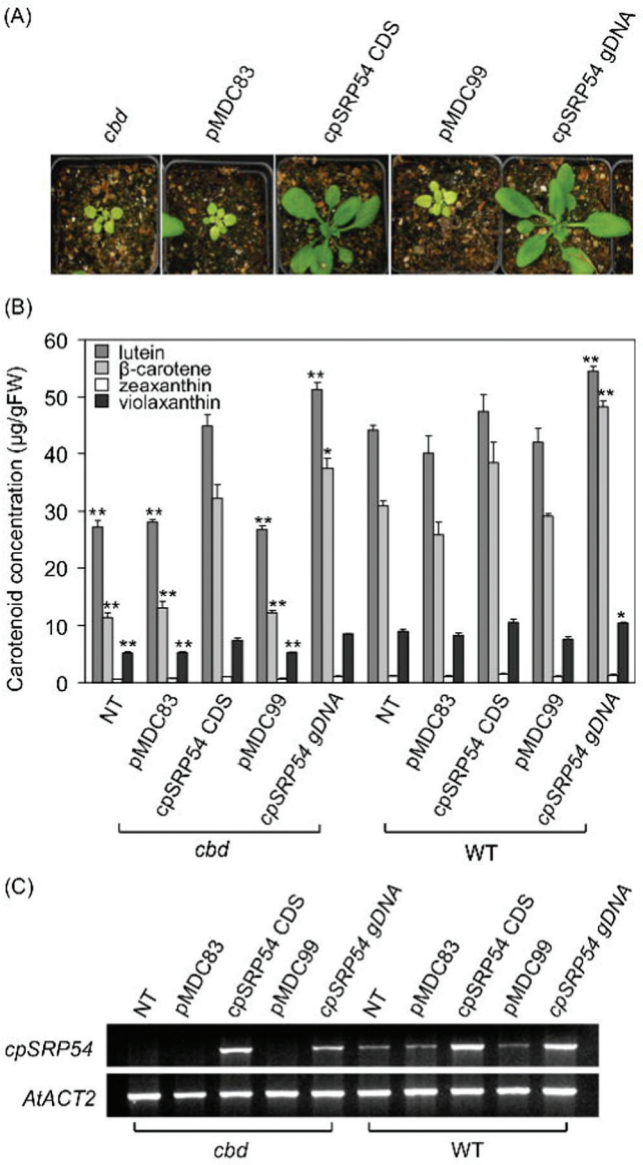

Fig. 3. Complementation of *cbd* and over-expression of *Arabidopsis cpSRP54* in WT *Arabidopsis*. (A) Representative phenotypes of *Arabidopsis cbd* lines complemented with an *Arabidopsis cpSRP54* gDNA or CDS. Three-week-old seedlings are shown. From left to right: non-transformed *cbd*, *cbd* transformed with empty vector pMDC83, *cbd* with pMDC83-*cpSRP54* CDS, *cbd* with empty vector pMDC99, and *cbd* with pMDC99-*cpSRP54* gDNA. (B) Representative carotenoid content of 8-day-old seedlings. Value are mean ± standard error of three *cbd*-complemented lines per construct and three over-expression lines per construct. Significant difference was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) relative to WT *Arabidopsis*. (C) Semi-quantitative RT-PCR confirmation of *cpSRP54* expression in *cbd*-complemented and over-expression transgenic lines as indicated. The CDS of *cpSRP54* was amplified and the *AtACT2* gene was used as an internal control. NT, non-transformed control.

Impact of cpSRP54 *expression changes on the transcription of carotenoid and photosynthesis-related genes*

Given the reduced levels of carotenoids and chlorophylls in seedlings of the *cbd* mutant, qRT-PCR was carried out on 7-day-old seedlings of *cbd* and WT to examine whether the *cpSRP54* mutation had an impact on the expression of carotenoid and chlorophyll biosynthetic genes. Expression of

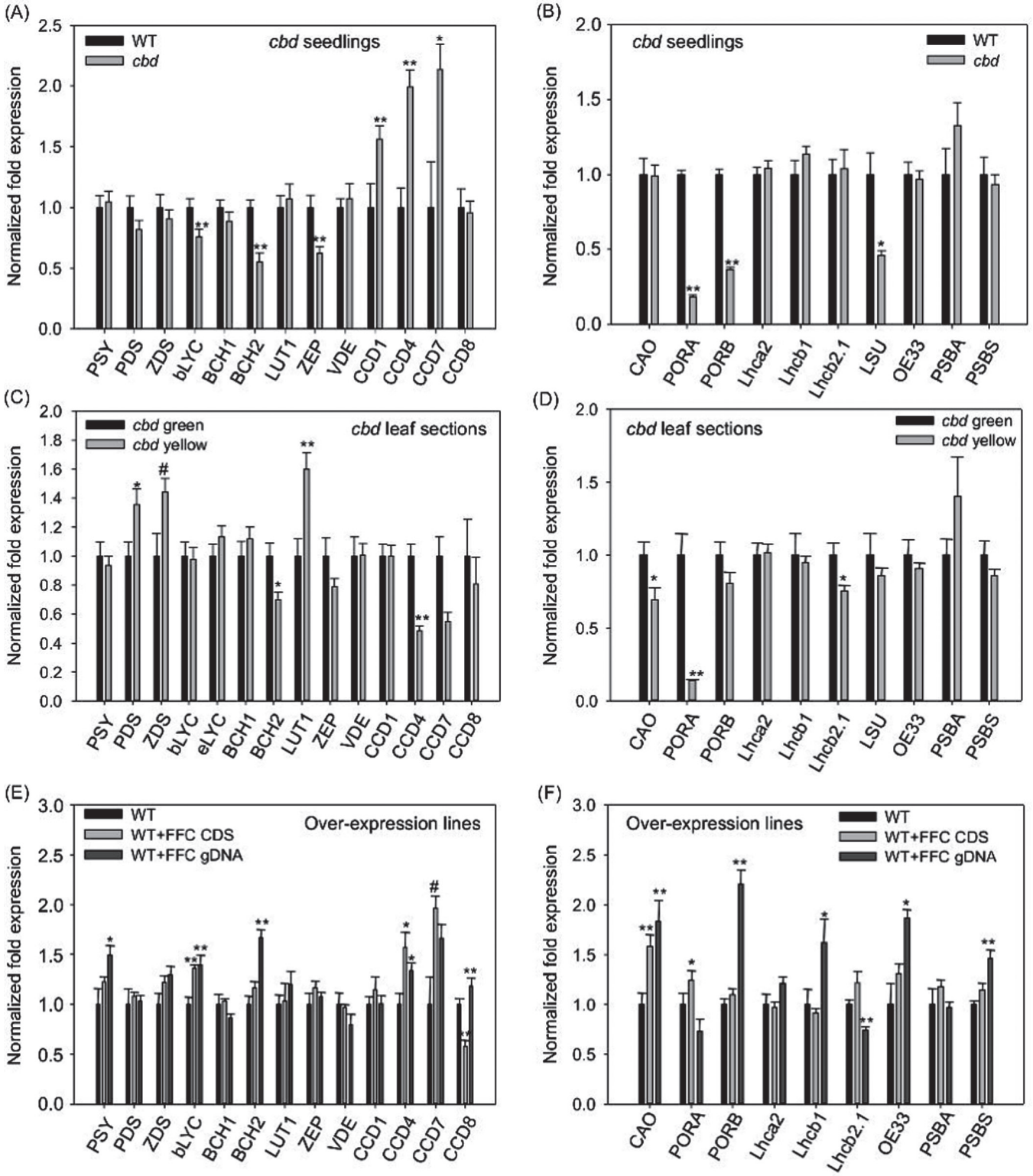

Fig. 4. Quantitative real-time PCR analysis of carotenoid and photosynthesis gene transcripts in the *cbd* mutant and *cpSRP54*+ over-expression *Arabidopsis* lines. (A) Carotenoid gene profiles and (B) chlorophyll and photosynthesis gene profiles in *cbd* 7-day-old seedlings. (C) Carotenoid gene profiles and (D) chlorophyll and photosynthesis gene profiles in yellow and green sections of *cbd* rosette leaves. (E) Carotenoid gene profiles and (F) chlorophyll and photosynthesis gene profiles in 8-day-old seedlings of three *Arabidopsis cpSRP54* over-expression lines per construct. Values are mean ± standard error of three replicates. Significant difference was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) relative to WT *Arabidopsis* (A, B, E, F) and green sections of *cbd* (C, D).

lycopene β-cyclase (bLYC), and especially β-carotene hydroxylase 2 (*BCH2*) and zeaxanthin epoxidase (*ZEP*), was significantly decreased in *cbd* compared to WT (Fig. 4A). Phytoene synthase (*PSY*), the key enzyme in the carotenoid biosynthesis pathway, and other biosynthetic enzymes did not show transcript differences between *cbd* and WT; neither did PSY protein levels show changes (Supplementary Fig. S1, available at *JXB* online). In contrast, carotenoid cleavage dioxygenases (*CCD*) were oppositely affected such that higher transcript levels were observed for *CCD1*, *CCD4*, and *CCD7* in *cbd* seedlings (Fig 4A). Transcripts of both protochlorophyllide oxidoreductase a and b (*PORA* and *PORB*) and the chloroplast-encoded large subunit of Rubisco (*LSU*) were significantly decreased in the leaves of *cbd* mutant (Fig. 4B). In contrast, transcription of *CAO* (chlorophyllide a oxygenase), which converts chlorophyllide a to chlorophyllide b, was not significantly affected in the mutant (Fig. 4B), even though chlorophyll b content was decreased. Expression of light-harvesting antenna protein genes for photosystems (PS) I and II (*Lhca* and *Lhcb*, respectively), proteins that utilize other thylakoid targeting pathways such as *PSBS* (spontaneous pathway), *OE33* (Sec pathway) and the chloroplast-encoded gene *PSBA* (*D1*), which requires cpSRP54 to translocate to its thylakoid location (Amin *et al*., 1999), were also not affected.

Gene expression was examined in more detail in green sections relative to yellow sections in *cbd* mutant leaves (the third and fourth rosette leaf from nine 30-day-old plants). Expression of the *BCH2* and *CCD4* genes was significantly lower in yellow sections relative to green ones (Fig. 4C). Surprisingly, the expression of *PDS* and *LUT1* genes was significantly higher in the yellow sections and there was a trend towards higher *ZDS* expression. The chlorophyll biosynthetic genes, *CAO* and *PORA*, and light-harvesting gene *Lhcb2.1* also had significantly lower expression in yellow sections (Fig. 4D), even though the pattern in whole seedlings (containing a mixture of green and yellow tissue) was less extreme (Fig. 4B).

Finally, expression of genes related to carotenoids and chlorophylls in *Arabidopsis* plants overexpressing *cpSRP54* was analysed. *bLYC* and *CCD4* had higher expression in the *cpSRP54* CDS and gDNA over-expression lines relative to WT (Fig. 4E), as did the chlorophyll biosynthetic gene *CAO* (Fig. 4F). In some cases, enhanced expression occurred only with gDNA over-expression, e.g. transcripts of *PSY*, *BCH2*, and *CCD8* were higher only in the *cpSRP54* gDNA lines (Fig. 4E). Oddly, *CCD8* was reduced in the CDS lines. Higher *Lhcb1* and *PORB* and lower *Lhcb2.1* transcripts accumulated in the gDNA over-expression lines (Fig. 4E, 4F) compared to WT. Moreover, transcripts encoding chloroplast proteins utilizing the non-SRP thylakoid targeting pathways, e.g. *PSBS* and *OE33*, were significantly higher in the over-expression lines transformed with the gDNA relative to WT and relative to lines overexpressing the *cpSRP54* CDS (Fig. 4F).

Plastid-to-nucleus retrograde signalling may regulate nuclear gene expression in cbd

The downregulation of chlorophyll and carotenoid biosynthetic genes in the *cbd* mutant could be an indirect response to defects in chloroplast development and suggested the potential involvement of a plastid-to-nucleus retrograde signalling mechanism. qRT-PCR showed that the expression of *GUN4*, but not *GUN1*, was approximately 30% lower in the yellow sections of *cbd* than in green sections (Fig. 5). This result suggested that Mg-ProtoIX synthesis could be reduced in the *cbd* yellow sections. Therefore, it seemed unlikely that downregulation of chlorophyll and carotenoid biosynthetic genes in the *cbd* yellow sections resulted from the build-up of Mg-ProtoIX. However, *AtGLK1* and *AtGLK2*, transcripts coding for functionally redundant proteins that positively regulate photosynthesis-related nuclear gene expression (PRNGE) in plastid-to-nuclear signalling (Fitter *et al*., 2002), were decreased 2-fold in yellow sections relative to green sections of the *cbd* mutant (Fig. 5). Coincidently, the expression of *ABI4*, which acts as a negative regulator of PRNGE and is part of the GUN1/GUN4 pathway (Koussevitzky *et al*., 2007), was not changed in yellow leaf sections compared to green sections of the *cbd* mutant (Fig. 5).

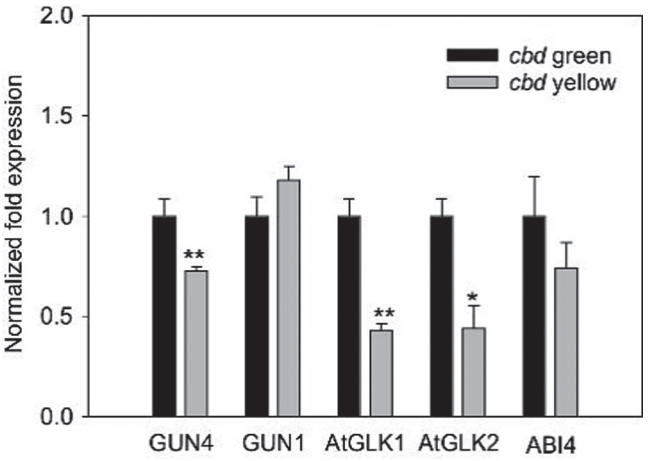

Fig. 5. Expression patterns of genes involved in the plastid-to-nucleus retrograde signalling in yellow and green sections of the *cbd* mutant. Significant difference was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) relative to green sections of *cbd* mutant.

ABA biosynthesis is altered in the cbd *mutant*

Since carotenoids are precursors of ABA and an increase in *CCD* transcripts and a reduction of carotenoids in *cbd* were observed, changes to levels of ABA were anticipated. To test this hypothesis, the ABA content of 7-day-old seedlings of the mutants and WT were measured. The results showed that *cbd* and the two allelic lines CS850421 and SALK_079668 had 2–3-fold lower ABA levels (Fig. 6A). To investigate whether the *cbd* mutant has an altered response to ABA, the effect of exogenous ABA on root growth was tested. *cbd*, the two allelic mutant lines, and WT plants had similar growth kinetics when treated with ABA (data not shown), suggesting that these mutants have a normal response to ABA. Since ABA level was reduced in *cbd*, the expression of genes involved in ABA metabolism was examined by qRT-PCR. Nine-*cis*-expoxycarotenoid dioxygenases (NCEDs), the first family of enzymes committed specifically to ABA synthesis, cleave the epoxycarotenoid precursor to form xanthoxin (Nambara and Marion-Poll, 2005). The transcript

levels of three of these genes, *NCED3*, *NCED6*, and *NCED9*, were upregulated in *cbd* (Fig. 6B), whereas those of *NCED5*, *ABA2* (short-chain alcohol dehydrogenase), and AAO3 (abscisic aldehyde oxidase, catalysing the final step in ABA biosynthesis) were not significantly affected in *cbd* (Fig. 6B). Hence altered ABA biosynthesis was not the cause of the lower ABA level in *cbd*. The major catabolic route leading to 8'-hydroxy ABA is catalysed by the cytochrome P450 enzyme, ABA 8'-hydroxylase, encoded by members of the CYP707A family, *CYP707A1–CYP707A4* (Kushiro *et al*., 2004). The expression of CYP707A1 and CYP707A2 was significantly increased in the *cbd* mutant (Fig. 6B), which was consistent with the decreased ABA content.

Expression of Arabidopsis cpSRP54 *resulted in higher carotenoid accumulation in* B. napus *seeds*

Since over-expression of *cpSRP54* elevated carotenoids in *Arabidopsis* seedlings, a decision was made to determine if *cpSRP54* could be used to enhance the carotenoid levels in seeds of the oilseed crop, *B. napus*. T_1 *cpSRP54*$^+$ developing seeds in a DH12075 background showed varied transcript abundance for *cpSRP54* at 34 DPA (Fig. 7A). Growth was not affected in these transgenic *B. napus* lines; neither was the seed yield changed at all (Supplementary Fig. S2). A range of T_0 and T_1 transgenic seeds accumulated substantially greater levels of the major *B. napus* seed carotenoids, lutein and β-carotene, ranging 1.3–10.9-fold higher compared with levels observed in WT DH12075 and empty vector control plants (Fig. 7B for seed from T_1 lines; Supplementary Fig. S3 for T_0 parental plants). Violaxanthin and zeaxanthin were at trace levels in T_1 lines. The T_0 plant BY1179 accumulated the highest amount of lutein (9-fold higher than control plants), and 41-fold higher β-carotene occurred in T_0 plants BY1179 and BY1198 (Supplementary Fig. S3). Six independent events (plants) with significantly higher total carotenoid, along with one empty vector control line and a WT DH12075 control line, were selected for further analysis. T_1 seeds derived from plant BY1171 had the highest amount of lutein (5-fold higher) and β-carotene (10-fold higher) compared with control seed, while levels in T_1 plants derived from BY1179 were only 2-fold higher (Fig. 7B). Except for T_1 lines derived from BY1208 (BY1251–BY1254), seeds of all other T_1 lines had significantly higher (or a trend towards higher) β-carotene and/or lutein. However, transcript levels of *cpSRP54* did not correlate well with carotenoid content in the transgenic *B. napus* seeds.

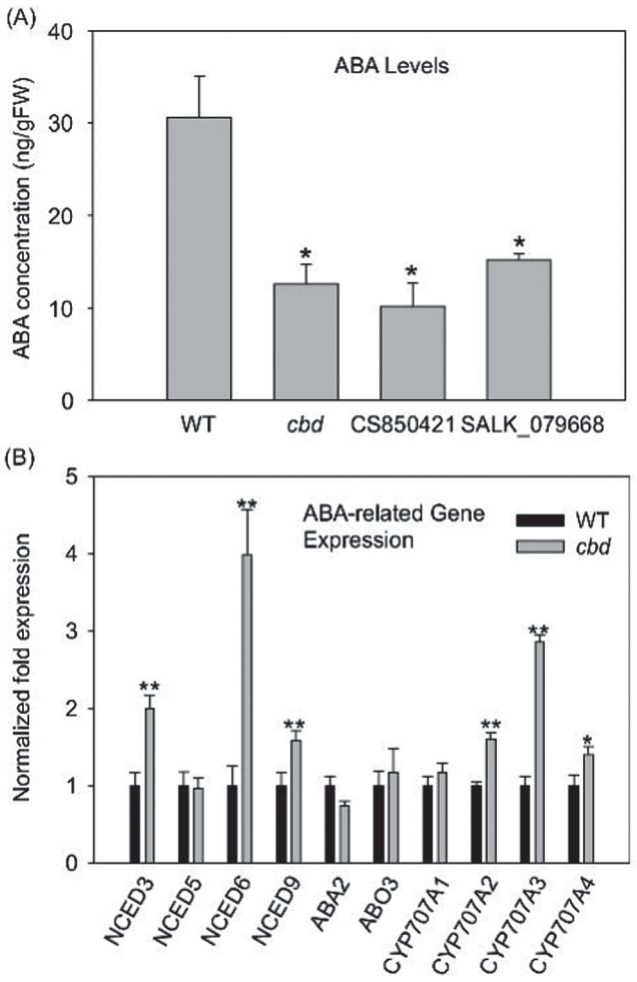

Fig. 6. ABA content and expression of ABA metabolic genes in the *cbd* mutant and WT. (A) ABA content in 7-day-old seedlings. (B) Expression patterns of ABA biosynthetic and catabolic genes in 7-day-old seedlings. Significant difference was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) relative to WT *Arabidopsis.*

Next, a wide range of carotenoid biosynthetic genes in developing seeds of T_1 lines were tested at 34 DPA for transcript levels in the *cpSRP54*$^+$ transgenic *B. napus*. Gene expression for many of these genes was enhanced relative to control (Fig. 7C) and was consistent with carotenoid content, except for line BY1234. Expression of carotenoid biosynthetic genes was especially enhanced in T_1 line BY1236, which is consistent with its highest carotenoid content. However, the transcript level of *carotenoid isomerase* (*CRTISO*) was not significantly changed in any lines tested.

To investigate whether altered carotenoid profiles affected fatty acid biosynthesis in the seeds of transgenic lines, oil content and fatty acid (FA) profiles were measured in T_0 and T_1 seeds (Supplementary Table S1). Of the 22 T_0 independent transgenic plants tested, the majority (14 lines) had FA profiles and oil content identical to DH12075 control seed. Only eight had modified FA profiles and lower oil content, ranging from 26.13% fresh-weight (FW) in BY1179 seeds to 39.33% in line BY1180. Five lines with reduced oil content were detected in the T_1 generation as well, and the lines with reduced oil content included those with increased carotenoid levels. Increased unsaturated fatty acids $C_{18:2}$ and $C_{18:3}$ and decreased $C_{18:1}$ were also shown in some lines over both generations compared to DH12075 and empty vector control plants, for example T_0 plants BY1170 and BY1202 and T_1 lines BY1241 and BY1260. Overall, however, the magnitude of these changes to fatty acids was small or non-existent.

Discussion

In addition to their many benefits to human health and nutrition, carotenoids are essential components of the plant light-harvesting system and play significant roles in plant biology. These roles include photosynthesis, protection against photooxidative

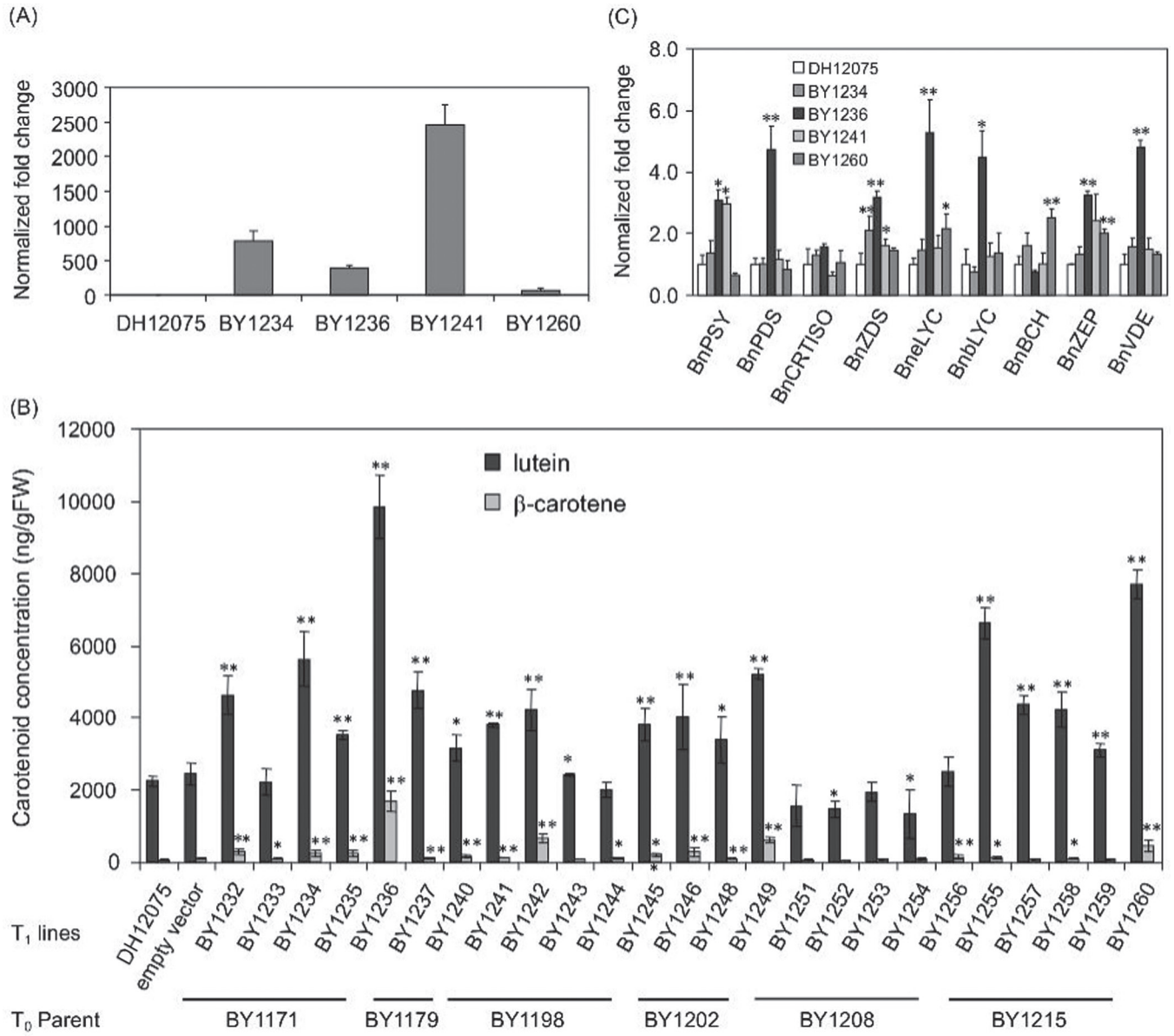

Fig. 7. *B. napus* expressing the *Arabidopsis cpSRP54* gene. (A) Semi-quantitative RT-PCR analysis of *cpSRP54* transgene expression in T_1 developing seeds at 34 days post anthesis (DPA). (B) Carotenoid profiles of *B. napus* seeds from *cpSRP54*$^+$ expression lines (T_1), an empty vector control plant, and WT DH12075. [Violaxanthin and zeaxanthin were at trace levels.] (C) Carotenoid gene expression profiles in T_1 transgenic *cpSRP54*$^+$ *B. napus* developing seeds at 34 DPA. Values are mean ± standard error of three replicates. Significant difference was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) relative to DH12075.

damage, and membrane stabilization (Botella-Pavía and Rodríguez-Concepción, 2006). Therefore, a major deficiency in carotenoid biosynthesis or accumulation, such as occurred with the current *Arabidopsis cbd* mutant, would be expected to affect plant development and chloroplast assembly. Dong *et al*. (2007) reported that *A. thaliana* carotenoid deficient mutants *spc1-1* (*spontaneous cell death1-1*) and *spc1*-2, which have mutations in the gene encoding a putative ζ-carotene desaturase (*ZDS*), had bleached leaves, excessive production of superoxide radicals, abnormal chloroplast development, and arrest of growth leading to seedling lethality. The current *cbd* mutant, with mutation in *cpSRP54*, manifested several similar phenotypes similar to those of *spc* mutants: reduced carotenoid and chlorophyll content in the seedlings, yellow cotyledons and first true leaves, as well as reduced growth. Molecular complementation of *cbd*, using both genomic and cDNA sequences, confirmed that the phenotypic changes were due to the mutational disruption of *cpSRP54*.

Over-expression of a genomic copy of *cpSRP54* in WT *Arabidopsis* led to an increase in total carotenoid content without visible morphological or developmental effects. However, lines transformed with the *cpSRP54* CDS, in spite of much higher levels of *cpSRP54* transcript than WT, did not show a significantly altered total carotenoid content. This suggests that more complex regulatory mechanisms may impact *cpSRP*54 expression, such as post-transcriptional gene silencing (Stam *et al*., 1997) or RNA splicing, which could result in altered and non-functional enzymes or post-translational modification. A similar finding was reported for the N plant resistance gene where transgenic plants

expressing the N cDNA failed to exhibit complete resistance to tobacco mosaic virus, whereas transgenic plants harbouring a cDNA bearing an intron and containing 3'-N genomic sequences were resistant (Dinesh-Kumar and Baker, 2000). This indicates that for some genes, expression of genomic sequences may be preferable to cDNA to achieve complete protein functionality.

Carotenoids are essential for quenching excessive free radicals and reactive oxygen species (ROS) and providing protection against photooxidation (Botella-Pavía and Rodríguez-Concepción, 2006). Plants with reduced carotenoids are known to generate photooxidative species such as ROS (Dong *et al*., 2007). Due to the substantial reduction of carotenoids in the *Arabidopsis cbd* mutant, an enhancement of superoxide could be detected in this mutant. This and potentially other ROS molecules could be the reason for the slow growth, small stature, and leaf cell death phenotype in this mutant. The carotenoid deficiency mutant *spc1*, encoding a ZDS essential for carotenoid biosynthesis, also showed defects in chloroplast development (Dong *et al*., 2007). The *cbd* chloroplast had reduced grana thylakoids, indicating the importance of cpSRP54 for plastid development and plant growth. This is consistent with a study on over-expression of dominant negative forms of cpSRP54, in which the alteration of a single amino acid in *Arabidopsis* resulted in delayed chloroplast development in the first true leaf with fewer thylakoid membranes and grana stacks than in control counterparts (Pilgrim *et al*., 1998).

The *cbd* mutant showed reduced amounts of total carotenoids with very few changes in the transcription of genes encoding carotenoid biosynthetic enzymes. Although *BCH2* expression was decreased in *cbd*, its related minor carotenoid, zeaxanthin, showed no substantial change in level. This inconsistency could be due to redundant function of *BCH1* and/or lower expression of *ZEP*. The *cbd* mutant and allelic SALK lines also had similar levels of PSY protein relative to WT. This suggests that mutation of *cpSRP54* did not substantially affect the expression or translation of carotenoid biosynthetic genes. Nevertheless, the expression of several *CCD*, *NCEDs* and ABA-related *CYP450* genes were enhanced in *cbd*. The CCD1 enzyme cleaves a variety of carotenoid substrates at the $C_{9–10}$ and $C_{9'-10'}$ double bonds (Schwartz *et al*., 2001). CCD4 can participate in dark-induced breakdown of carotenoids (Ytterberg *et al*., 2006) and CCD7 coordinately synthesizes strigolactones with CCD8 (Vogel *et al*., 2010). This upregulation of the *CCD* and *NCED* genes could lead to increased catabolism of carotenoids and hence to reduced carotenoids in *cbd*. Strangely, expression of *NCEDs*, encoding enzymes committed specifically to ABA synthesis, was upregulated in *cbd* (Fig. 6B). However, the increase in expression of ABA catabolic genes, CYP707A family members, is consistent with the decreased ABA content found in the *cbd* mutant.

A plastid-to-nucleus retrograde signalling mechanism has been reported to regulate the expression of photosynthetic genes in the nucleus, and *Lhcb* is perhaps most responsive to this signal (Strand *et al*., 2003). In the current study, there was evidence suggesting disruption of this chloroplast retrograde signalling in the *cbd* mutant by a decrease in transcripts of *AtGLK1*, *AtGLK2*, and the retrograde signalling gene, *GUN4* in yellow sections of the *cbd* mutant. Reduced gene expression of *Lhcb2.1* and *PORA* in yellow leaf sections and the lower expression of the chlorophyll biosynthetic genes *PORA*, *PORB*, and *LSU* in whole seedlings could be a response to this change in plastid-to-nucleus signalling pathway. The increase in expression of a few carotenoid biosynthetic genes and the decrease in expression of carotenoid degradation genes in yellow leaf sections of the *cbd* mutant suggest that the *cbd* mutant is attempting to increase the antioxidation capacity in the leaf. Thus *AtGLK1* and *AtGLK2* may be involved in fine-tuning carotenogenesis and degradation in response to the functional state of the plastid. However, this does not preclude the possibility that plastid retrograde signalling may be disrupted by enhanced levels of ROS resulting from reduced carotenoid accumulation (Foudree *et al*., 2010).

The storage and sequestration of carotenoids within various plastid types is an important regulatory mechanism for carotenoid accumulation. Organelle biogenesis partly determines the size of the carotenoid storage compartment of plastids (Lopez *et al*., 2008; Cazzonelli and Pogson, 2010) and carotenoids co-accumulate with chlorophyll in a pigment-binding protein complex embedded in thylakoid membranes in leaf tissues (Botella-Pavía and Rodríguez-Concepción, 2006). Both *hp-2* and *hp-3* mutants had enlarged plastid compartment sizes, which were coupled with enhanced levels of carotenoid (Kolotilin *et al*., 2007; Galpaz *et al*., 2008). It also has been reported that the carotenogenic pathway is appreciably stimulated in the presence of sequestering structures (Rabbani *et al*., 1998). Consequently, the defects of chloroplast development in *cbd* and, hence, the reduced storage compartment size for carotenoid accumulation in this mutant, could contribute to the decreased carotenoid content in *cbd*. Impaired chloroplast targeting could also feedback via the plastid-to-nucleus retrograde signalling to reduce chlorophyll and carotenoid content in *cbd* seedlings.

All true leaves of the *cbd* mutant were yellow during early stages of development but became greener as they matured, Moreover, *cbd* could survive in the homozygous state, suggesting that the mutation is not lethal and that alternate thylakoid protein targeting systems can compensate for the loss of the cpSRP pathway, depending on the developmental stage. This possibility is supported by the work of Tzvetkova- Chevolleau *et al*. (2007), who showed that cpSRP43 functions independently of cpSRP54/cpFtsY when targeting LHCPs to the thylakoid membrane. These observations are consistent with the idea that cpSRP54 is non-essential and that plants can compensate for its loss by employing alternate thylakoid targeting pathways.

Suppression of *cpSRP54* expression appears to unleash a cascade of molecular and physiological events with consequences on plant growth and development in the *Arabidopsis cbd* mutant. Disruption of the plastid-to-nucleus retrograde signalling and deformed plastids may be responsible in part for the reduced carotenoid content in this line. Reduced carotenoid content impacts the antioxidative capacity of the cell, thus allowing for the accumulation of ROS, which affects plant performance as manifested by reduced growth, stunted appearance, and enhanced cell death. On the other hand, over-expression of *cpSRP54* in *Arabidopsis* and expression in *B. napus* enhances seed carotenoid content, with no significant impact on growth, seed oil, or seed yield. These experiments point to the *Arabidopsis cpSRP54* gene as a new tool/mechanism for enhancing carotenoids and improving the nutritional

value of crop plants. Increased carotenoid levels have also been enhanced in another important crop (potato tubers) by inducing the formation of chromoplasts containing carotenoid-sequestering structures using the *Or* transgene (Lopez *et al*., 2008).

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Western blot analysis of PSY extracted from 7-day-old seedlings of *cbd* mutant and wild-type *Arabidopsis*

Supplementary Fig. S2. Seed yield of T_0 and T_1 *B. napus* plants expressing *Arabidopsis cpSRP54*

Supplementary Fig. S3. Carotenoid profiles of *B. napus* seeds from *cpSRP54*$^+$ expression lines (T_0), an empty vector control plant, and wild-type DH12075

Supplementary Table S1. Oil content and fatty acid profiles of T_0 and T_1 *B. napus* seed expressing *Arabidopsis cpSRP54*

Acknowledgements

The authors thank Dr. Guosheng Liu for technical assistance with TEM and the ABRC for distributing *Arabidopasis* mutant seeds SALK_079668 and CS850421. Funding was provided by Genome Alberta/Genome Canada as part of the 'Designing Oilseeds for Tomorrow's Markets' project.

References

Alonso JM, Stepanova AN, Leisse TJ, *et al***.** 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301,** 653–657.

Amin P, Sy DAC, Pilgrim ML, Parry DH, Nussaume L, Hoffman NE. 1999. *Arabidopsis* mutants lacking the 43- and 54-kilodalton subunits of the chloroplast signal recognition particle have distinct phenotypes. *Plant physiology* **121,** 61–70.

Beatty S, Nolan J, Kavanagh H and O'Donovan O. 2004. Macular pigment optical density and its relationship with serum and dietary levels of lutein and zeaxanthin. *Archives of Biochemistry and Biophysics* **430,** 70–76.

Botella-Pavía P, Rodríguez-Concepción M. 2006. Carotenoid biotechnology in plants for nutritionally improved foods. *Physiologia Plantarum* **126,** 369–381.

Cazzonelli CI, Pogson BJ. 2010. Source to sink: regulation of carotenoid biosynthesis in plants. *Trends in Plant Science* **15,** 266–274.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiology* **133,** 462–469.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiology* **139,** 5–17.

DellaPenna D. 1999. Carotenoid synthesis and function in plants: insights from mutant studies in *Arabidopsis*. *Pure and Applied Chemistry* **71,** 2205–2212.

Dinesh-Kumar SP, Baker BJ. 2000. Alternatively spliced *N* resistance gene transcripts: their possible role in tobacco mosaic virus resistance. *Proceedings of the National Academy of Sciences, USA*, **97,** 1908–1913.

Dong H, Deng Y, Mu J, Lu Q, Wang Y, Xu Y, Chu C, Chong K, Lu C, Zuo J. 2007. The *Arabidopsis Spontaneous Cell Death1* gene, encoding a ζ-carotene desaturase essential for carotenoid biosynthesis, is involved in chloroplast development, photoprotection and retrograde signalling. *Cell Research* **17,** 458–470.

Fitter DW, Martin DJ, Copley MJ, Scotland RW, Langdale JA. 2002. GLK gene pairs regulate chloroplast development in diverse plant species. *The Plant Journal* **31,** 713–727.

Fitzgerald HA, Chern M-S, Navarre R, Ronald PC. 2004. Overexpression of (*At*)*NPR1* in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. *Molecular Plant–Microbe Interactions* **17,** 140–151.

Foudree A, Aluru M, Rodermel S. 2010. PDS activity acts as a rheostat of retrograde signaling during early chloroplast biogenesis. *Plant Signaling & Behavior* **5,** 1619–1622.

Fujisawa M, Takita E, Harada H, Sakurai N, Suzuki H, Ohyama K, Shibata D, Misawa N. 2009. Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. *Journal of Experimental Botany* **60,** 1319–1332.

Galpaz N, Wang Q, Menda N, Zamir D, Hirschberg J. 2008. Abscisic acid deficiency in the tomato mutant *high-pigment 3* leading to increased plastid number and higher fruit lycopene content. *The Plant Journal* **53,** 717–730.

Hannoufa A, Hossain Z. 2012. Regulation of carotenoid accumulation in plants. *Biocatalysis and Agricultural Biotechnology* **1,** 198–202.

Howitt C, Pogson BJ. 2006. Carotenoid accumulation and function in seeds and non-green tissues. *Plant, Cell and Environment* **29,** 435–445.

Inskeep WP, Bloom PR. 1985. Extinction coefficients of chlorophyll a and b in *N*,*N*-dimethylformamide and 80% acetone. *Plant Physiology* **77,** 483–485.

Kakizaki T, Matsumura H, Nakayama K, Che F-S, Terauchi R, Inaba T. 2009. Coordination of plastid protein import and nuclear gene expression by plastid-to-nucleus retrograde signalling. *Plant Physiology* **151,** 1339–1353.

Kolotilin I, Koltai H, Tadmor Y, Bar-Or C, Reuveni M, Meir A, Nahon S, Shlomo H, Chen L, Levin I. 2007. Transcriptional profiling of *high pigment-2dg* tomato mutant links early fruit plastid biogenesis with its overproduction of phytonutrients. *Plant Physiology* **145,** 389–401.

Koussevitzky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, Surpin M, Lim J, Mittler R, Chory J. 2007. Signals from chloroplasts converge to regulate nuclear gene expression. *Science* **316,** 715–719.

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y Nambara E. 2004. The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. *The EMBO Journal* **23,** 1647–1656.

Larkin RM, Alonso JM, Ecker JR, Chory J. 2003. GUN4, a regulator of chlorophyll synthesis and intracellular signalling. *Science* **299,** 902–906.

Liu YG, Mitsukawa N, Oosumi T, Whittier RF. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *The Plant Journal* **8,** 457–463.

Lopez AB, Eck JV, Conlin BJ, Paolillo DJ, O'Neill J, Li L. 2008. Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *Journal of Experimental Botany* **59,** 213–223.

McCarty DR. 1995. Genetic control and integration of maturation and germination pathways in seed development. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 71–93.

Michiels A, Van den Ende W, Tucker M, Van Riet L, Van Laere A. 2003. Extraction of high-quality genomic DNA from latex-containing plants. *Analatytical Biochemistry* **315,** 85–89.

Nambara E, Marion-Poll A. 2005. Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56,** 165–185.

Nott A, Jung H-S, Koussevitzky S, Chory J. 2006. Plastid-to-nucleus retrograde signalling. *Annual Review of Plant Biology* **57,** 739–759.

Pilgrim ML, van Wijk K-J, Parry DH, Sy DAC, Hoffman NE. 1998. Expression of a dominant negative form of cpSRP54 inhibits chloroplast biogenesis in *Arabidopsis*. *The Plant Journal* **13,** 177–786.

Pogson BJ, McDonald K, Truong M, Britton G, DellaPenna D. 1996. *Arabidopsis* carotenoid mutants demonstrate lutein is not essential for photosynthesis in higher plants. *The Plant Cell* **8,** 1627–1639.

Rabbani S, Beyer P, Lintig JV, Hugueney P, Kleinig H. 1998. Induced β-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant Physiology* **116,** 1239–1248.

Rask L, Ellerstrom M, Ezcurra I, Stalberg K, Wycliffe P. 1998. Seed-specific regulation of the napin promoter in *Brassica napus*. *Journal of Plant Physiology* **152,** 595–599.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17,** 208–212.

Robinson SJ, Tang LH, Mooney BA, *et al***.** 2009. An archived activation tagged population of *Arabidopsis thaliana* to facilitate forward genetics approaches. *BMC Plant Biology* **9,** 101.

Schwartz SH, Qin X, Zeevaart JAD. 2001. Characterization of a novel carotenoid cleavage dioxygenase from plants. *Journal of Biological Chemistry* **267,** 25208–25211.

Stam M, Mol J, Kooter J. 1997. The silence of genes in transgenic plants. *Annals of Botany* **79,** 3–12.

Strand A, Asami T, Alonso J, Ecker JR, Chory J. 2003. Chloroplast to nucleus communication trigged by accumulation of Mg-protoporphyrinIX. *Nature* **421,** 79–83.

Suzuki Y, Kawazu T, Koyama H. 2004. RNA isolation from siliques, dry seeds, and other tissues of *Arabidopsis thaliana*. *BioTechniques* **37,** 542–544.

Tzvetkova-Chevolleau T, Hutin C, Noël LD, *et al***.** 2007. Canonical signal recognition particle components can be bypassed for posttranslational protein targeting in chloroplasts. *The Plant Cell* **19**,1635–1648.

Umeno D, Tobias AV, Arnold FH. 2005. Diversifying carotenoid biosynthetic pathway by directed evolution. *Microbiology and Molecular Biology Reviews* **69,** 51–78.

Vogel J., Walter MH, Giavalisco P, *et al*. 2010. SlCCD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. *The Plant Journal* **61,** 300–311.

Ytterberg AJ, Peltier J-B, van WiJk KJ. 2006. Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. *Plant Physiology* **140,** 984–999.

Yu B, Lydiate DJ, Schäfer UA, Hannoufa A. 2007. Characterization of a β-carotene hydroxylase of *Adonis aestivalis* and its expression in *Arabidopsis thaliana*. *Planta* **226,** 181–192.