The Metabolic and Developmental Roles of Carotenoid Cleavage Dioxygenase4 from Potato[1[W]]

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The factors that regulate storage organ carotenoid content remain to be fully elucidated, despite the nutritional and economic importance of this class of compound. Recent findings suggest that carotenoid pool size is determined, at least in part, by the activity of carotenoid cleavage dioxygenases. The aim of this study was to investigate whether Carotenoid Cleavage Dioxygenase4 (CCD4) activity affects potato (*Solanum tuberosum*) tuber carotenoid content. Microarray analysis revealed elevated expression of the potato *CCD4* gene in mature tubers from white-fleshed cultivars compared with higher carotenoid yellow-fleshed tubers. The expression level of the potato *CCD4* gene was down-regulated using an RNA interference (RNAi) approach in stable transgenic lines. Down-regulation in tubers resulted in an increased carotenoid content, 2- to 5-fold higher than in control plants. The increase in carotenoid content was mainly due to elevated violaxanthin content, implying that this carotenoid may act as the in vivo substrate. Although transcript level was also reduced in plant organs other than tubers, such as leaves, stems, and roots , there was no change in carotenoid content in these organs. However, carotenoid levels were elevated in flower petals from RNAi lines. As well as changes in tuber carotenoid content, tubers from RNAi lines exhibited phenotypes such as heat sprouting, formation of chain tubers, and an elongated shape. These results suggest that the product of the CCD4 reaction may be an important factor in tuber heat responses.

The oxidative cleavage of carotenoids, catalyzed by carotenoid cleavage dioxygenases (CCDs), gives rise to a wide range of apocarotenoid products depending on the carotenoid substrate and the site of cleavage (for review, see Auldridge et al., 2006a). The apocarotenoid products fulfill many roles. Some are further metabolized to form plant hormones such as abscisic acid (ABA; Zeevaart and Creelman, 1988) or strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008). Other apocarotenoids accumulate in fruit and flowers, where they give rise to strong aromas such as β-ionone (Dudareva et al., 2006) or colors such as α-crocin, a component of the spice saffron (*Crocus sativus*; Bouvier et al., 2003). The signaling molecule mycorradicin, produced by plant roots during symbiosis with arbuscular mycorrhyza, is also an apocarotenoid (Fester et al., 2002). Due to the diverse nature of this class of metabolites, it is likely that further apocarotenoids with new functions, and their associated *CCD* genes, remain to be discovered.

Carotenoid cleavage dioxygenases are found in animals, plants, and bacteria. In plants, CCDs are encoded by multigene families, and recent studies have started to address the function of individual family members (Auldridge et al., 2006b). The Arabidopsis (*Arabidopsis thaliana*) genome contains nine members of the CCD family (CCD1, -4, -7, and -8 and the 9-cis-epoxycarotenoid dioxygenases [NCEDs] 2, 3, 5, 6, and 9). The NCED subfamily are a group of enzymes that are specific for 9-cis-epoxycarotenoids, such as 9′-cis-neoxanthin and 9-cis-violaxanthin, and includes Viviparous14, an enzyme required for ABA biosynthesis (Schwartz et al., 1997). All NCEDs cleave epoxycarotenoids at the 11,12-position to produce xanthoxin, a precursor of ABA (Schwartz et al., 1997; Tan et al., 1997; Taylor et al., 2000).

An interesting recent finding arose from studies of a *CCD4* gene (*CmCCD4a*) from Chrysanthemum (*Chrysanthemum morifolium*; Ohmiya et al., 2006). Silencing the *CCD4a* gene by RNA interference (RNAi) resulted in a change of petal color from white to yellow, demonstrating that the accumulation of carotenoid in Chrysanthemum petals occurred only in the absence of CCD4a activity. Further studies aimed to address the function of *CCD4* genes by analyzing the products of the CCD4 reaction (Huang et al., 2009). The *CCD4* genes from five different species were expressed in

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Escherichia coli engineered to accumulate the carotenoid substrate cis-ζ-carotene, lycopene, or β-carotene. Based on these studies, it was demonstrated that all five CCD4 enzymes could use β-carotene but not cis-ζ-carotene or lycopene as substrate. The product of this reaction in the *E. coli* assay was β-ionone, and while it was concluded that the CCD enzymes cleave their substrates at the same position (9,10 and 9′,10′), they are likely to have different biochemical functions, as they accept different substrates and are expressed in contrasting patterns. It is apparent that additional experimental tools, such as the use of transgenic approaches, are needed to investigate more fully CCD functions in their native environments.

The regulation of carotenoid accumulation in different storage organs has been a much studied research area due to the nutritional and economic importance of these compounds (Giuliano et al., 2008; Cazzonelli and Pogson, 2010). In general, it is clear that biosynthetic processes are important factors, and while the basic biosynthetic pathway has been elucidated, further details continue to emerge. For example, factors that control the expression levels of the biosynthetic genes remain, with a few recent exceptions (Welsch et al., 2008; Cazzonelli et al., 2009), to be determined, a situation that contrasts with other biosynthetic pathways such as those involved in flavonoid biosynthesis (Tian et al., 2008). The mechanism of carotenoid storage in organelles is also important, as demonstrated by the effect of the *Or* mutation in cauliflower (*Brassica oleracea*; Li et al., 2001). In this case, a gain-of-function mutation in a DNA-J molecular chaperonin was associated with a large increase in carotenoid content in cauliflower pith tissues. Thus, it is apparent that multiple factors are involved in the determination of the types and extent of carotenoid accumulation in storage and other organs. In view of the Chrysanthemum findings, it is clear that the actions of CCDs also can be of considerable importance in the level of carotenoid accumulation in a particular tissue type. Further examples from grape (*Vitis vinifera*; Mathieu et al., 2005) and strawberry (*Fragaria* spp.; Garcia-Limones et al., 2008) reinforce the possibility that carotenoid pool size is determined, at least in part, by the activity of CCDs, so there is an urgent need to test the effect of *CCD* expression in important plant storage organs. Recently, CCDs have also been implicated in the control of carotenoid accumulation in chickens, as yellow skin is caused by regulatory mutation(s) that inhibit expression of the β-carotene dioxygenase 2 enzyme in skin (Eriksson et al., 2008). In potato (*Solanum tuberosum*) tubers, there is wide variation in tuber carotenoid content between cultivars (Nesterenko and Sink, 2003; Morris et al., 2004). Genetic studies have attributed the high tuber carotenoid content to the presence of an allele at the *Y* locus, which controls yellow and white flesh (Brown et al., 1993). The *Y* gene has been mapped to chromosome 3 (Bonierbale et al., 1988), and it has been demonstrated that an allele of β-carotene hydroxylase (*bch*) was likely to correspond to the *Y* locus. The genotype at *bch* accounted for a considerable proportion of the variation in total tuber carotenoid in a range of potato germplasm; however, it is apparent that other genes also contribute to this variation (Brown et al., 2006).

The aim of this study was to investigate the effects of reducing native potato *CCD4* expression on the accumulation of carotenoids in plant storage organs in order to develop new tools for nutritional enhancement and contribute toward the development of a "green factory" for isoprenoid production.

RESULTS

Identification of the Potato *CCD4* Gene

In order to identify the potato *CCD4* gene homolog, a tBLASTx search (Altschul et al., 1990) of the Dana-Farber Cancer Institute potato gene index database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=potato) was carried out using the Chrysanthemum *CCD4*a sequence (ABY60885). The best hit (83% identity at the derived polypeptide level) was with TC165427, which was identical to the MICRO.13662.C1 sequence in the Potato Oligo Chip Initiative (POCI) microarray database (sequence available at http://pgrc-35.ipk-gatersleben.de/pls/htmldb_pgrc/f?p=194:1:180221829113542). Similarly, a tBLASTx screen of the Dana-Farber Cancer Institute potato gene index with the Arabidopsis *CCD4* gene sequence (At4g19170) also gave the TC165427 sequence as the best hit (71% identity). An alignment of *CCD4* sequences including that encoded by the TC165427 sequence is shown in Supplemental Figure S1. Analysis of the draft potato genome sequence (*Solanum phureja* version 3, updated September 2009 [http://potatogenomics.plantbiology.msu.edu/]) demonstrated that the TC165427-specific sequence is contained in scaffold 001573. Genomic localization of the *CCD4* gene was performed using a cleaved-amplified polymorphic sequence marker assay (Konieczny and Ausubel, 1993; data not shown), indicating that the *CCD4* marker maps to the telomeric region of potato chromosome 8. Using the ChloroP tool (Emanuelsson et al., 1999), the potato CCD4 is predicted to have an N-terminal plastid-targeting sequence of 62 amino acids.

Low Levels of Tuber Expression of the Potato *CCD4* Gene in Yellow-Fleshed Tubers

Previously, gene expression in tubers from *S. tuberosum* group Tuberosum was compared with that in tubers from *S. tuberosum* group Phureja by microarray analysis using the POCI 44-K element Agilent microarray (Ducreux et al., 2008). The Phureja tuber type is differentiated from Tuberosum on the basis of several traits, and the higher tuber carotenoid content in Phureja is well documented (Morris et al., 2004; Burgos et al., 2009). Thus, it was of interest to inves-

tigate the relative expression levels of the *CCD4* transcript in the yellow-fleshed Phureja tubers compared with the white-fleshed Tuberosum. The expression pattern of the MICRO.13662.C1 transcript was further investigated in the previously obtained microarray data set, curated at ArrayExpress (accession nos. E-TABM-452 and E-TABM-451; http://www.ebi.ac.uk/microarray-as/aer/?#ae-main[0]). Compared with Tuberosum types (cv Desiree and cv Maris Piper), the two Phureja genotypes examined (cv 333-16 and cv Mayan Gold) expressed the *CCD4* gene at much (approximately 5-fold) lower levels in mature tubers (Fig. 1), although in earlier developmental stages there was little consistent difference between the Phureja and Tuberosum genotypes. A quantitative reverse transcription (RT)-PCR assay was designed to expand this observation to measuring the expression levels of the *CCD4* gene in other tissues of the potato plant. As with other plant *CCD4* genes, high levels of expression were measured in leaf and flower tissues (Huang et al., 2009) with much lower relative expression levels in stems, tubers, and roots (Fig. 2).

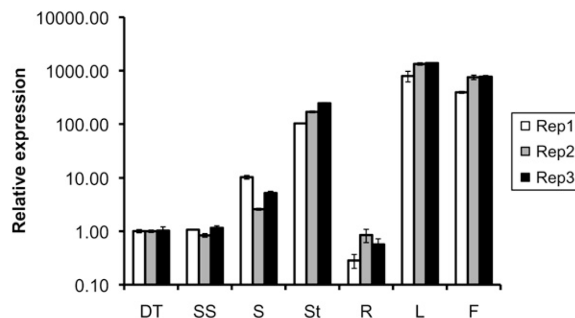

Figure 2. *CCD4* relative expression in different organs of the potato plant (cv Desiree). Values are means of triplicate assays ± SE for tissues sampled from three replicate plants. DT, Developing tuber; SS, swelling stolon; S, stolon; St, stem; R, root; L, leaf; F, flower. Values are means of triplicate assays ± SE for tissues sampled from three replicate plants.

Transgenic Down-Regulation of *CCD4* Expression Results in Enhanced Tuber Carotenoid Levels

In view of the apparent inverse relationship between *CCD4* expression and tuber carotenoid content in mature tubers, an aim of this study was to investigate the effects of *CCD4* down-regulation on tuber carotenoid content. An RNAi construct using the vector pHellsgate8 (Helliwell et al., 2002) was assembled (Supplemental Fig. S2). The construct contained a 324-bp portion of the potato *CCD4* cDNA (see "Materials and Methods") under the control of a cauliflower mosaic virus (CaMV) 35S promoter and was introduced into the white-fleshed cv Desiree by *Agrobacterium tumefaciens*-mediated transformation. Visual inspection of tuber color from regenerated plants indicated that in many (21 of 33) of the independent transformants there appeared to be enhanced tuber carotenoid content. Four of the obviously affected lines (lines 2, 4, 15, and 38) were grown from the tubers produced by the plants regenerated from tissue culture. The total carotenoid content was determined spectrophotometrically, and the carotenoid composition of mature tuber extracts was determined by HPLC in saponified and nonsaponified samples to measure the carotenoid ester content (Table I). In the four lines selected for HPLC analysis, total carotenoid levels were elevated significantly, with line 15 showing the largest increase (5.6-fold greater than levels in empty vector control tubers). HPLC analysis revealed that the main change in tuber carotenoid content was due to a large (approximately 4-fold) increase in violaxanthin (Table I), present as the major carotenoid in all samples analyzed.

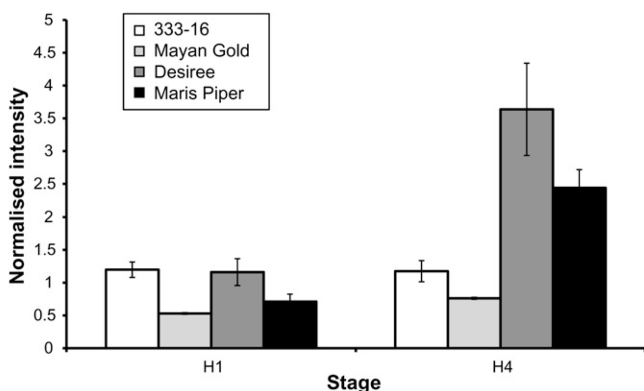

Figure 1. *CCD4* (MICRO.13362.C1) gene expression profile in tubers from Phureja (333-16, Mayan Gold) and Tuberosum (Desiree, Maris Piper) cultivars at two stages of tuber development (H1, developing tubers of 10–30 g fresh weight; H4, mature tubers at harvest) as determined by microarray analysis. Values are means of two biological replicates, and error bars represent SE.

Expression Analysis of Transgenic Lines

Microarray analysis of the *CCD4* RNAi tubers was carried out to ensure that there was no off-target silencing of other members of the *CCD* family due to transformation with the RNAi construct and to determine global effects of *CCD4* perturbation on the transcriptome, particularly with respect to known genes encoding the enzymes of the carotenoid biosynthetic pathway. Previously, a list of probes on the POCI microarray that represent carotenoid biosynthetic genes was compiled (Kloosterman et al., 2008). Microarray data were analyzed using standard statistical approaches in GeneSpring software (see "Materials and Methods"). Figure 3 shows the expression levels of the annotated *CCD* and *NCED* genes on the microarray and demonstrates that it is only the *CCD4*-specific transcript, detected by the MICRO.13662.C1 probe, that exhibits significantly lowered accumulation levels in tubers from the transgenic lines. The putative *CCD* sequences were compared with the potato genome sequence (*S. phureja* version 3; http://potatogenomics.

Table I. *Carotenoid content of CCD4 RNAi tubers*

Values shown are for mature tubers and are expressed as $\mu g\ g^{-1}$ dry weight. Values are means of three replicates ± SE. Statistical analysis of the differences in transgenic lines with respect to the wild type was done using Student's *t* test, and the significance of differences is indicated ([a]$P < 0.05$, [b]$P < 0.01$). Neo, Neoxanthin; Vio, violaxanthin; Unkn, unidentified carotenoid, Ant, antheraxanthin; Lut, lutein; Zea, zeaxanthin; Ester, total esterified carotenoid.

Line	Neo	Vio	Unkn	Ant	Lut	Zea	Ester	Total
Wild typeT	0.33 ± 0.01	2.05 ± 0.04	0.15 ± 0.02	0.11 ± 0.01	0.48 ± 0.02	0.01 ± 0.003	0.93 ± 0.05	4.06 ± 0.1
RNAi 2	0.63 ± 0.06[b]	3.74 ± 0.29[b]	0.47 ± 0.14	0.35 ± 0.05[a]	0.85 ± 0.06[b]	0.07 ± 0.01[b]	1.68 ± 0.20[a]	7.79 ± 0.4[b]
RNAi 4	0.66 ± 0.1[a]	3.46 ± 0.34[a]	0.37 ± 0.05[a]	0.40 ± 0.1[a]	1.04 ± 0.15[a]	0.12 ± 0.05	2.77 ± 0.68	8.82 ± 1.0[b]
RNAi 15	1.07 ± 0.12[b]	8.88 ± 1.11[b]	0.60 ± 0.22	1.09 ± 0.08[b]	4.59 ± 1.04[a]	0.16 ± 0.02[b]	6.52 ± 0.71[b]	22.9 ± 2.87[b]
RNAi 38	0.56 ± 0.06[b]	4.17 ± 0.18[b]	0.20 ± 0.03	0.42 ± 0.04[b]	0.71 ± 0.02[b]	0.04 ± 0.001[b]	2.10 ± 0.06[b]	8.16 ± 0.3[b]

plantbiology.msu.edu/) using the BLASTn search tool (Altschul et al., 1990). The six putative *CCD* genes analyzed gave best hits on different genomic scaffolds, demonstrating that these probes were nonredundant under these sequence assembly parameters (data not shown). The best BLASTn hits for the sequences annotated as encoding CCD and NCEDs were obtained by searching the EMBL database (available at www.ebi.ac.uk) and are shown in Supplemental Table S1. The effect of transformation with the RNAi construct on gene expression was determined by quantitative RT-PCR in other organs. As the transgene expression was driven by a constitutive 35S CaMV promoter, the reduced accumulation of the *CCD4*-specific transcript in all tissues examined was to be expected (Fig. 4). The expression levels of the known carotenoid pathway genes were compared in tubers from four RNAi lines and controls (from wild-type and empty vector lines; Supplemental Table S2). There were no significant differences in the expression levels of these genes between the two groups, indicating that carotenoid biosynthetic capacity was not affected by the transformation. Additionally, gene expression changes associated with other aspects of the *CCD4* RNAi phenotype are of interest and were revealed by the microarray analysis. Using cutoff criteria of 2-fold expression difference and a *P* value of less than 0.05, 14 genes were expressed at higher levels in the wild-type tubers (nontransformed and empty vector-transformed Desiree) than in those from the transgenic lines that were tested (lines 2, 4, 15, and 38); conversely, 43 genes were expressed at higher levels in the RNAi line tubers than in the Desiree control tubers (Supplemental Table S3). Considering that the microarrays contained 42,034 probes, there were few differentially expressed genes.

Carotenoid Levels in Other Organs of RNAi Lines

In view of the down-regulated levels of *CCD4* expression in leaves, whole flowers (including sepals, petals, carpels, and stamens), stems, and roots of RNAi transformants (Fig. 4), the level of carotenoids in these organs was also assessed. Comparing the total carotenoid content in leaves, stems, and roots of the wild type and empty vector transformants with the levels in the RNAi lines showed no significant variation in mean values (Table II). However, petals from RNAi lines were noticeably yellow compared with white controls and, as with tubers, contained significantly higher carotenoid levels.

Levels of ABA in the *CCD4* RNAi Lines

The large increases in violaxanthin and neoxanthin measured in tubers from *CCD4* RNAi lines imply that these compounds may be in vivo substrates for CCD4. Violaxanthin is also the main substrate for the NCED-type CCD enzymes that give rise to ABA (Schwartz et al., 1997). In view of this finding and also the effects on tuber development, where ABA is known to have an effect (Destefano Beltran et al., 2006), the level of ABA was determined in microtubers from the *CCD4*

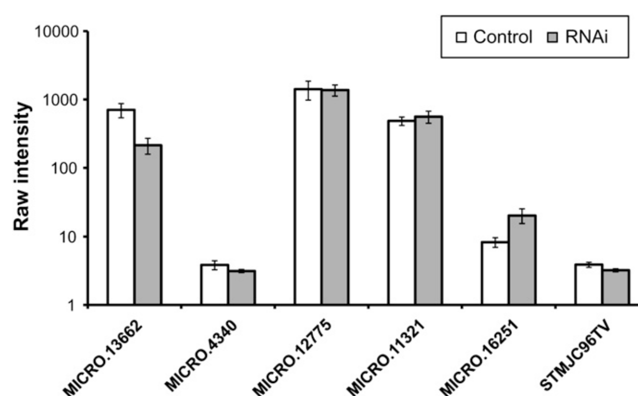

Figure 3. Expression levels (intensity values) of the annotated *CCDs* present on the POCI 44-K element Agilent microarray in *CCD4* RNAi transgenic lines compared with Desiree controls as determined by microarray analysis. Sequences are named according to their unigene identifiers available at the POCI Web site (http://pgrc-35.ipk-gatersleben.de/pls/htmldb_pgrc/f?p=194:1:1942898893016362). For control lines, microarray data are mean values from two biological replicates from two wild-type and two empty vector plants. For the RNAi lines, data are mean values from one plant from each of the four RNAi lines that show the tuber color phenotype (lines 2, 4, 15, and 38). Error bars represent SE. Gene names of the best BLAST hits have been included in Supplemental Table S1.

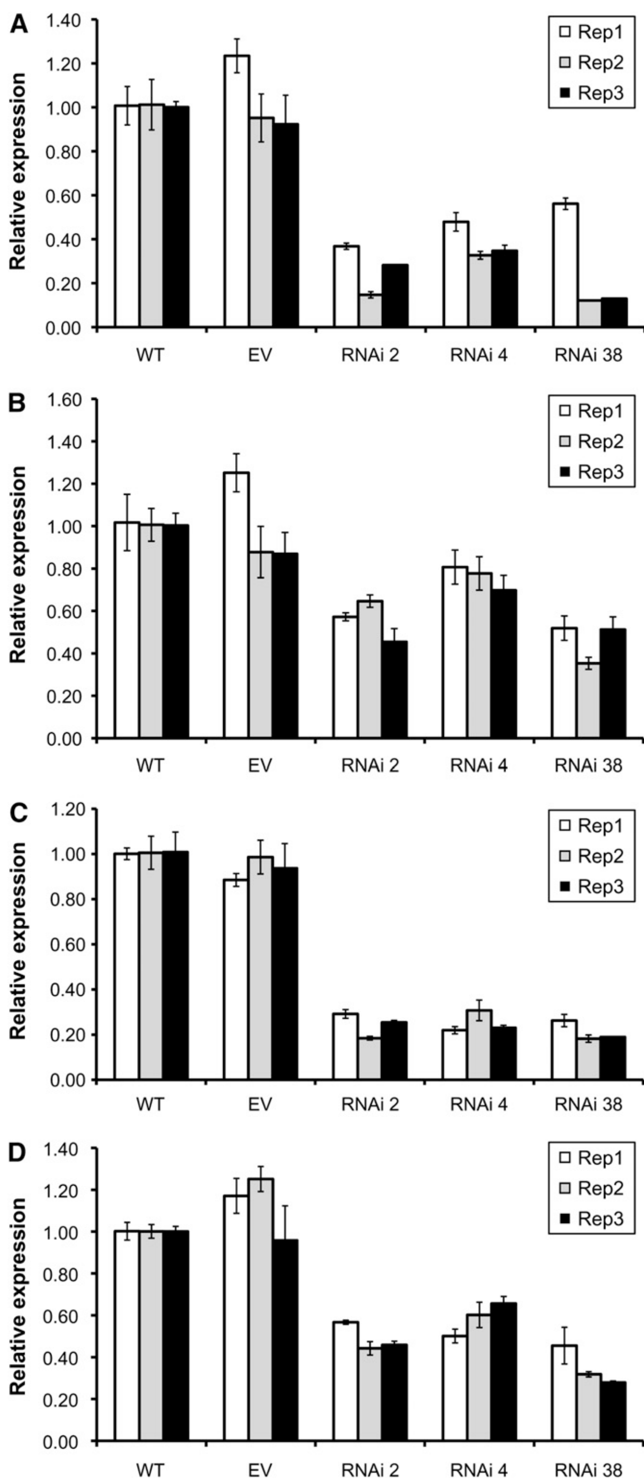

Figure 4. *CCD4* expression level in flower (A), stem (B), leaf (C), and root (D) in *CCD4* RNAi lines, empty vector (EV) controls, and wild-type (WT) Desiree. Values are means of triplicate assays ± SE for tissues sampled from three replicate plants and are expressed relative to the value in wild-type samples.

RNAi lines. Supplemental Table S4 shows that there was no significant difference in the ABA content in the *CCD4* RNAi microtubers compared with those from the wild type.

Developmental Changes Associated with *CCD4* Down-Regulation

As well as changes in tuber carotenoid content, other phenotypic changes were observed in the *CCD4* RNAi lines. In the first generation plants, of the 33 independent *CCD4* RNAi lines generated, 17 lines exhibited a chain tuber (i.e. secondary or even tertiary tubers connected by stolons to the primary tuber) phenotype (Fig. 5B), 20 exhibited one or more tubers with premature sprouting that had already occurred at harvest (Fig. 5C), and 12 contained both the chain tuber and sprouting phenotypes. None of the seven wild-type and empty vector control lines showed these phenotypes (Fig. 5A). Microtubers from the RNAi lines were generated to investigate the phenotype under controlled temperature and light conditions. The RNAi lines produced elongated, dumbbell-shaped or chain microtubers, many of which also appeared to produce sprouts as the tubers developed (Fig. 5D). None of these features were observed in control microtubers (Fig. 5E). Selected transgenic lines were grown from the first

Table II. *Total carotenoid content of flowers, stems, and leaves of control Desiree lines (wild type and empty vector transformants) compared with tissue from CCD4 RNAi lines 2, 4, 15, and 38*

Values are means of three replicates ± SE and are expressed as μg g^{-1} dry weight. Statistical analysis of the differences in transgenic lines with respect to the wild type was done using Student's *t* test, and the significance of differences is indicated ($^{a}P < 0.05$, $^{b}P < 0.001$).

Line	Tissue	Total Carotenoid Content
Wild type	Leaf	29,113.5 ± 349.5
Empty vector	Leaf	29,291.1 ± 48.1
RNAi 2	Leaf	29,509.3 ± 237.9
RNAi 4	Leaf	29,900 ± 255.4
RNAi 15	Leaf	29,486 ± 205.3
RNAi 38	Leaf	29,156.8 ± 265
Wild type	Flower petal	78.4 ± 6.8
Empty vector	Flower petal	85.4 ± 5.3
RNAi 2	Flower petal	335.5 ± 7.0[b]
RNAi 4	Flower petal	106.5 ± 2.7[a]
RNAi 15	Flower petal	452.8 ± 5.0[b]
RNAi 38	Flower petal	378.8 ± 3.9[b]
Wild type	Stem	17,091.1 ± 268.9
Empty vector	Stem	15,514.5 ± 123.2
RNAi 2	Stem	16,532.3 ± 453.3
RNAi 4	Stem	16,661.5 ± 722.8
RNAi 15	Stem	17,605.1 ± 424.9
RNAi 38	Stem	17,046.4 ± 624.4
Wild type	Root	14.4 ± 0.8
Empty vector	Root	14.6 ± 0.2
RNAi 2	Root	13.9 ± 0.8
RNAi 4	Root	13.3 ± 0.2
RNAi 15	Root	14.0 ± 0.3
RNAi 38	Root	15.0 ± 0.5

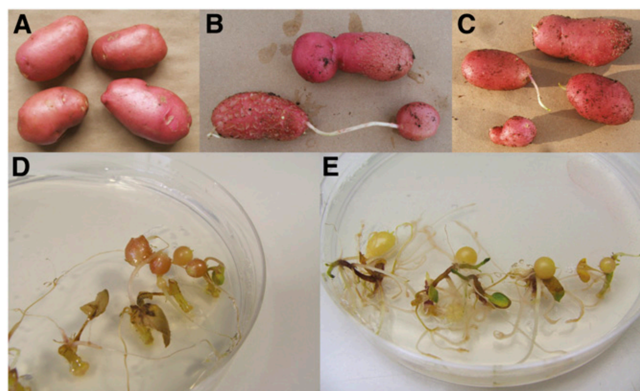

Figure 5. Phenotypes of *CCD4* RNAi tubers. A, Control tubers from wild-type Desiree grown under identical conditions to RNAi lines. B, Tubers from RNAi line 38 exhibiting dumbbell shape or chain tubers. C, Developing tubers from RNAi line 4 exhibiting a sprouting phenotype evident at harvest. D, Microtubers from RNAi line 15 exhibiting dumbbell shape, chain tubers, and sprouting during tuber development. E, Control microtubers grown under identical conditions to the RNAi microtubers.

generation tubers, and chain tubers were again apparent in these transgenic lines but were completely absent from control lines. A comparison of the length-to-width ratio in second generation tubers from the RNAi lines revealed significantly higher values for the tubers from the RNAi lines (Fig. 6).

DISCUSSION

The effect of CCD4A activity on carotenoid accumulation has been clearly demonstrated in Chrysanthemum petals (Ohmiya et al., 2006). The primary objective of this study was to investigate whether a similar mechanism is a factor in carotenoid accumulation in plant storage organs using potato tubers as an example. As potato is the third most important global food crop plant, its nutritional value is of great significance, particularly in view of the growing body of evidence regarding the importance of carotenoids in the diet (Britton et al., 2009). Based on sequence similarity with the chrysanthemum *CCD4a* gene and *CCD4* genes from other plant species, the potato *CCD4* gene was identified. Its expression level was elevated in mature tubers from two white-fleshed cultivars compared with the level in two yellow-fleshed Phureja cultivars. Although not conclusive, this observation provided the impetus to pursue a transgenic approach to investigate further the role of the potato *CCD4* gene. Constitutive down-regulation of this gene by RNAi resulted in increases in total tuber carotenoid content of up to 5.6-fold in the most strongly affected line. The enhanced tuber carotenoid effect was observed in tubers from a high proportion of the transgenic lines generated, and a similar fold increase in carotenoid content was observed in subsequent generations of tubers. Interestingly, the effect on carotenoid content was only observed in tubers and flower petals. In other potato organs, such as leaves, stems, and roots, down-regulation of *CCD4* expression was evident, but there was no significant effect on carotenoid accumulation. It may be that insufficient levels of silencing were achieved to have a significant effect on enzyme activity. Additionally or alternatively, the observation may reflect the different mode of carotenoid accumulation in green tissues (chloroplasts) compared with tubers and petals. There is little information available on the plastids in which potato tuber carotenoids accumulate. Interestingly, the expression level of *CCD4* was highest in tissues that accumulate high levels of carotenoid, suggesting that the expression level is finely tuned to the availability of substrate, as has been suggested in *C. sativus* (Rubio et al., 2008). The increase in carotenoid levels in petals indicates that the carotenoid biosynthesis pathway is active in this tissue, and in the wild type, the carotenoid is consumed by the production of colorless apocarotenoids. A similar phenomenon was observed in Chrysanthemum petals (Ohmiya et al., 2006). Microarray analysis clearly demonstrated that the *CCD4* gene was down-regulated in the RNAi lines (by up to 70%) and that there was no significant effect on the expression levels of other *CCD* genes that could be determined using the POCI array. Additionally, the expression levels of the carotenoid biosynthetic genes remained unchanged in the transgenic lines, implying that there is no change in biosynthetic capacity, and the increased tuber carotenoid levels were due to reduced carotenoid oxidation that is catalyzed by CCD4 in the wild-type tubers.

Previously, other transgenic approaches to boost potato tuber carotenoid levels have also been effective. One approach has been to manipulate the expression levels of the genes from the carotenoid biosynthetic pathway. Examples include down-regulation of zeaxanthin epoxidase (Römer et al., 2002), overexpression of the *Erwinia crtB* gene encoding phytoene synthase (Ducreux et al., 2005), and stacking of the *Erwinia crtB*,

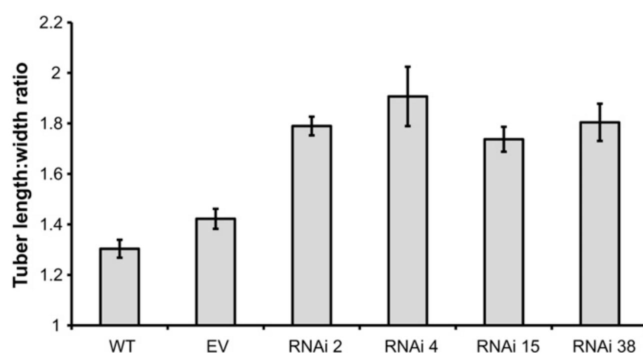

Figure 6. Tuber length-to-width ratios of *CCD4* RNAi tubers compared with controls. Values shown are for mature tubers. Values are means of nine replicates pooled from three independent plants (SE is shown for tuber length-to-width ratios). WT, Wild-type Desiree; EV, empty vector.

crtI, and *crtY* genes (Diretto et al., 2007). The latter approach resulted in up to 20-fold higher carotenoid levels than in control tubers, with a significant proportion (approximately 50%) present as provitamin A carotenoid. An alternative approach to boosting tuber carotenoid levels was to express the cauliflower *Or* gene in potato tubers, resulting in up to 10-fold higher tuber carotenoid values than for controls (Lopez et al., 2008). It is thought that the *Or* gene product is involved in generating a sink for carotenoid accumulation and thus acts by stabilizing the product of the pathway, rather than by stimulating biosynthetic capacity. However, the molecular details of how this mutation affects carotenoid storage capacity remain to be elucidated. Genetic evidence has identified a major quantitative trait locus (QTL) for tuber carotenoid content on chromosome 3, and there is good evidence that the gene underpinning this QTL is *crtRB-2*, encoding β-carotene hydroxylase (Brown et al., 2006). Equally clear from the genetic evidence is that other genes are also involved in determining tuber carotenoid content, and it will be of interest to determine whether a tuber carotenoid QTL colocates with the map position of the potato *CCD4* gene on chromosome 8. As the down-regulation of the potato *CCD4* gene is assumed to enhance carotenoid levels by reducing the levels of enzymic degradation of carotenoid, it will be interesting to investigate whether the effect of *CCD4* down-regulation can be combined with the approaches of elevating the carotenoid biosynthetic and storage capacities. Such an approach may be the way forward in terms of nutritional enhancement and developing tubers as a green factory for high economic value carotenoids such as the ketocarotenoid astaxanthin. A role for carotenoid cleavage dioxygenases in regulating carotenoid accumulation in other plant storage organs such as fruits and grains is also possible.

A further challenge in determining the function of the carotenoid cleavage enzymes is the identification of the in vivo substrates and the products of the reactions. As tubers from the transgenic lines show elevated levels of violaxanthin, the simplest interpretation is that this carotenoid is the CCD4 substrate in potato. A more complex hypothesis is that the metabolic flux from carotenoid precursors that would normally be degraded by CCD4 activity proceeds to violaxanthin, which then accumulates. The NCED subfamily of CCDs catalyze the cleavage of the 11,12 double bond of 9′-cis-neoxanthin and 9-cis-violaxanthin to form xanthoxin, an ABA precursor (Schwartz et al., 1997), whereas all CCD4 enzymes studied to date cleave at the 9,10 (9′,10′) double bonds of carotenoid substrates in vitro (Huang et al., 2009). In view of the elevated levels of violaxanthin in the RNAi lines, the levels of tuber ABA were determined, as increased violaxanthin level may result in enhanced ABA synthesis and thus account for the phenotypic effects. However, in microtubers, there was no significant difference in the ABA levels, indicating that the phenotype was not an ABA effect. Other apocarotenoids produced via a 9,10 cleavage of carotenoids include the strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008). In Arabidopsis, CCD7 and CCD8 have been shown to act in concert in the oxidation of carotenoids, leading to strigolactone biosynthesis. Mutations in *CCD7* and *CCD8* lead to reduced levels of strigolactone accumulation and enhanced shoot branching, a phenotype observed in Arabidopsis, pea (*Pisum sativum*), petunia (*Petunia hybrida*), and rice (*Oryza sativa*; for review, see Leyser, 2008). As CCD4 also catalyzes a 9,10 cleavage, it is conceivable that it is also involved in strigolactone biosynthesis. However, as single mutations in either *CCD7* or *CCD8* result in the shoot-branching phenotype, such a hypothesis would require developmental regulation of *CCD4* expression. In Arabidopsis, the *CCD4* gene (At4g19170) is expressed at very high levels in dry seed and is strongly circadian regulated (data available from the Arabidopsis eFP browser; http://www.bar.utoronto.ca/efp/development). Studies of Arabidopsis *CCD4* mutants may enable the identification of products of the CCD4 reaction and further insights into its developmental role. Constitutive overexpression of *CCD4* in a *CCD7/8* mutant background would also be of interest to see if complementation was possible.

In addition to changes in tuber carotenoid content, the RNAi transgenic lines also showed developmental phenotypes similar to that observed when potatoes are grown at cool temperatures interrupted by periods of high temperatures (Jefferies and Mackerron, 1987). The main features are heat sprouting, chain tubers, and secondary growth of tubers. The tubers from the RNAi lines were also elongated, exhibiting a significant increase in length-to-width ratio. It has been suggested that these effects are due to a fluctuation in the level of tuberization stimulus that causes tuber formation to alternate with more stolon-like growth (Ewing, 1981). Based on these observations, it is reasonable to speculate that the CCD4 reaction product has a role in temperature sensing in the plant or that reduced levels of the CCD4 product result in an affected response to heat stress, so presumably the reduced level in the RNAi lines induces heat stress-like phenotypes. These observations are consistent with the findings in leaves from *C. sativus*, where CCD4 expression was shown to be induced by heat stress (Rubio et al., 2008). Thus, determination of the in vivo product of the potato CCD4 reaction may have major implications for heat responses in plants and will be a target for future studies. Further transgenic work will be necessary to determine whether overexpression of *CCD4* can protect against heat stress.

MATERIALS AND METHODS

Binary Vector Construction and Transformation of Potato

To generate the CCD4 RNAi lines, the Ti vector pHellsgate8 (Helliwell et al., 2002) with a CaMV 35S promoter was used. A 324-bp cDNA fragment derived from the potato (*Solanum tuberosum*) *CCD4* gene was amplified from mature potato tuber cDNA (cv Maris Piper) by PCR using a forward primer (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTCAAGACTTACAA-

GTACAACATCG-3′) and a reverse primer (5′-GGGGACCACTTTGTACAA-GAAAGCTGGGTACGATTTTCGGATGTGCCGTCATGC-3′. The 324-bp fragment was subcloned into pDONR221 and then transferred into pHellsgate8 by using Gateway technology according to a protocol provided by the manufacturer (Invitrogen). The resulting binary vector (Supplemental Fig. S2) was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transformed *Agrobacterium* cells were selected by their resistance to kanamycin (100 μg mL^{-1}) and rifampicin (100 μg mL^{-1}). Potato transformation (cv Desiree) was carried out as described previously (Ducreux et al., 2005). Plants were raised in a glasshouse maintained at a daytime temperature of 20°C and a nighttime temperature of 15°C. The light intensity (photosynthetic photon flux density) ranged from 400 to 1,000 μmol m^{-2} s^{-1}, and the mean daylength was 16 h.

Total RNA Extraction from Potato Tissues

Total RNA was extracted from freeze-dried potato tissues as described previously (Ducreux et al., 2008). The method was effective for all tissues tested including tubers, stems, leaves, and flowers, providing good-quality RNA as assessed using an RNA 6000 Nano Chip on an Agilent 2100 Bioanalyzer (www.chem.agilent.com).

Quantitative RT-PCR Using the Universal Probe Library

RT of 10 μg of total RNA was performed using Invitrogen SuperScript III reverse transcriptase (www.invitrogen.com) using random hexamers for priming. cDNA (25 ng) was used as template for real-time PCR using the Universal Probe Library (UPL) system (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). Reactions were performed in 25 μL containing 1× FastStart TaqMan Probe Master (supplemented with ROX reference dye). Gene-specific primers and probe were used at concentrations of 0.2 μM and 0.1 μM, respectively. Thermal cycling conditions were as follows: 10 min of denaturation at 95°C, followed by 40 cycles of 15 s at 94°C and 60 s at 60°C. The reactions were repeated in triplicate with independent cDNAs. Relative expression levels were calculated using the method of Pfaffl (2001) using data obtained with the elongation factor-1α-specific primers as an internal reference control (Nicot et al., 2005). UPL primer and probe sequences were as follows: StEF1alpha_fwd, 5′-CTTGACGCTCTTGACCAGATT-3′; StEF1alpha_rev, 5′-GAAGACGGAGGGGTTTGTCT-3′; UPL probe number 117 (5′-AGCCC-AAG-3′); CCD4poci_fwd, 5′-GATGGTTTGATGTACCAGGATTT-3′; CCD4-poci_rev, 5′-ATATTCGGCGCAATCAACA-3′; UPL probe number 58 (5′-GGATGGAG-3′).

Analysis of Carotenoids

Peeled whole tuber samples were freeze dried and stored at −80°C prior to analysis. Total potato tuber carotenoids were extracted and analyzed by reverse-phase HPLC as detailed by Morris et al. (2004).

In Vitro Tuberization

After 28 d of culture, potato plants were excised into single nodes (10 per plantlet) and transferred to 90-mm petri dishes containing Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.8% (w/v) bacto microagar (Difco), 8% (w/v) Suc, and, where included, 9.8 μM *N*-6-benzyladenine. All dishes were sealed with Nescofilm and placed in a 16°C incubator with an 8-h photoperiod at 80 μmol m^{-2} s^{-1} for 7 d prior to incubation at 16°C, in total darkness, for a further 28 d.

Analysis of Tuber ABA Content

Freeze-dried microtuber samples (approximately 50 mg dry weight) were mechanically homogenized in 5 mL of 80% (v/v) aqueous methanol (4°C). The extracts were clarified by centrifugation (10,000*g* for 15 min), the supernatants were decanted, and the pellet was reextracted with fresh 80% aqueous acetone. After standing for 3 h (4°C), the pellet was recentrifuged and the supernatants were combined. A total of 50 ng of [$^2H_6$](+)-ABA (OlchemIm, Ltd.) was added as an internal standard. The supernatants were dried under a stream of nitrogen (35°C) and redissolved in 5 mL of 1 N formic acid. The acidified extracts were applied to a preequilibrated Oasis MCX column (Waters Associates) that was washed with 5 mL of 1 N formic acid, and the ABA-containing fraction was eluted with 5 mL of methanol. The methanol eluates were dried under nitrogen (35°C) and redissolved in 10 mM ammonium acetate (pH 5.6).

ABA was quantified by HPLC-mass spectrometry-selected ion monitoring using a Thermo Electron Surveyor MSQ system and a 2.1-mm × 150-mm, 5-μm Hypersil Gold Column (Thermo-Finnigan). HPLC solvents were as follows: A, 10 mM ammonium acetate (pH 5.6); B, methanol. HPLC operating conditions were as follows: initially 40% B, linear gradient to 80% B in 5 min, followed by a linear gradient to 100% B in 5 min (0.2 mL min^{-1}). The MSQ was operated in the negative ion electrospray mode with a probe temperature of 460°C, 30-V cone voltage, and 4.5-kV needle voltage. Ions monitored were 263 (ABA) and 269 ([$^2H_6$](+)-ABA). Each sample was injected three times (technical replicates), and there were three biological replicates per sample.

Microarray Processing

Array processing and analysis followed MIAME guidelines (http://www.mged.org/Workgroups/MIAME/miame.html). The design of the microarray experiment and the data derived from it are detailed in the public database ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/; accession no. E-TABM-961). Total RNA samples were labeled as single-color (Cy3) cDNA and purified using the Quick Amp Labeling Kit (Agilent Technologies) as recommended, using 1 μg of total RNA per sample and half reaction volumes throughout. Microarray hybridization and washing followed the manufacturer's protocols for gene expression arrays (Agilent One-Color Microarray-Based Gene Expression Analysis, version 5.7). For each array, 20 μL of combined purified labeled samples was mixed with 5 μL of 10× Blocking Agent, heat denatured at 98°C for 3 min, and cooled to room temperature. GE Hybridization buffer HI-RPM (25 μL) was added and mixed prior to hybridization (65°C for 17 h at 10 rpm). Slides were dismantled in Agilent Wash Buffer 1 and washed sequentially in Wash Buffer 1 for 1 min and Agilent Wash Buffer 2 for 1 min. Hybridized arrays were scanned at 5-μm resolution using an Agilent G2505B scanner at 532-nm (Cy3) and 633-nm (Cy5) wavelengths.

Microarray Data Analysis

For data extraction, microarray images were imported into Agilent Feature Extraction (FE version 10.5.1.1) software and aligned with the array grid template (015425_D_F_20061105). Intensity data for each spot were extracted using a defined FE protocol (GE1-v5_95_Feb07), and following standard quality control checks, data from each array were subsequently loaded into GeneSpring software (version 7.3.1; Agilent Technologies) for analysis. Default normalization and scaling were applied (Agilent FE, one-color), and data with consistently low probe intensity levels, flagged as absent in all samples, were discarded. Comparisons were made between the control (wild-type) lines and each transgenic line (RNAi lines and empty vector lines) independently using volcano plots, with thresholds of greater than 2-fold change and a Student's *t* test value of $P < 0.05$ applied to identify significantly regulated genes. Unique and overlapping genes in the RNAi lines, not present in the empty vector lists, were selected using Venn diagrams.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of *CCD4* polypeptide sequences from various plant species.

Supplemental Figure S2. Diagrammatic representation of the *CCD4* RNAi construct in pHellsgate8.

Supplemental Table S1. Sequence similarity of POCI microarray sequences annotated as encoding CCDs or NCEDs.

Supplemental Table S2. Comparison of carotenoid biosynthetic gene expression between *CCD4* RNAi and control lines as determined by microarray analysis.

Supplemental Table S3. List of genes differentially expressed between *CCD4* RNAi and control lines as determined by microarray analysis.

Supplemental Table S4. Levels of ABA (ng g^{-1} dry weight) in microtubers from wild-type Desiree and *CCD4* RNAi lines.

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