Synthetic conversion of leaf chloroplasts into carotenoid-rich plastids reveals mechanistic basis of natural chromoplast development

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Plastids, the defining organelles of plant cells, undergo physiological and morphological changes to fulfill distinct biological functions. In particular, the differentiation of chloroplasts into chromoplasts results in an enhanced storage capacity for carotenoids with industrial and nutritional value such as beta-carotene (provitamin A). Here, we show that synthetically inducing a burst in the production of phytoene, the first committed intermediate of the carotenoid pathway, elicits an artificial chloroplast-to-chromoplast differentiation in leaves. Phytoene overproduction initially interferes with photosynthesis, acting as a metabolic threshold switch mechanism that weakens chloroplast identity. In a second stage, phytoene conversion into downstream carotenoids is required for the differentiation of chromoplasts, a process that involves a concurrent reprogramming of nuclear gene expression and plastid morphology for improved carotenoid storage. We hence demonstrate that loss of photosynthetic competence and enhanced production of carotenoids are not just consequences but requirements for chloroplasts to differentiate into chromoplasts.

carotenoid | chromoplast | differentiation | phytoene | synthetic

Plastids comprise a group of morphologically and functionally diverse plant organelles capable of differentiating from one plastid type to another in response to developmental and environmental stimuli (1, 2). Such plastidial conversions are essential to sustain many fundamental biological processes and largely contribute to cell specialization in the different plant tissues. Among the different plastid types, chromoplasts are of great importance in nature and agriculture because of their capacity to accumulate high levels of carotenoids, plant pigments of isoprenoid nature that provide color in the yellow to red range (3–5). Carotenoids such as beta-carotene (provitamin A) are health-promoting nutrients that animals cannot synthesize but take up in their diets. They are also added-value compounds widely used in cosmetics, pharma, food, and feed industries as natural pigments and phytonutrients (4, 6).

Chromoplasts differentiate from preexisting plastids such as proplastids (i.e., undifferentiated plastids), leucoplasts (i.e., uncolored plastids in nonphotosynthetic tissues), and chloroplasts (i.e., photosynthetic plastids). Chloroplasts transform into chromoplasts during the development of many flowers and fruits, but only a few plant species differentiate chromoplasts in leaves (1, 5). The yellow to red colors that some leaves acquire as they senesce (e.g., in the autumn or when they are exposed to continuous darkness) are due to chloroplast carotenoids becoming visible when the chlorophylls degrade. This senescence process, however, does not involve the transformation of chloroplasts into chromoplasts but into a completely different type of plastids named gerontoplasts (1, 2).

The most prominent changes during chloroplast-to-chromoplast differentiation are the reorganization of the internal plastid structures, together with a concurrent loss of photosynthetic competence and overaccumulation of carotenoid pigments (1–3, 5, 7, 8). The remodeling of the internal plastid structures generates an increased metabolic sink capacity but it also promotes carotenoid biosynthesis. The control of chromoplast differentiation appears as a very promising strategy for improving the nutritional and health benefits of crops (5–9). The overall process is known to involve changes in gene expression (e.g., via retrograde signaling from plastids to the nucleus), hormonal regulation, protein quality control, and plastid protein import (1, 3, 5). However, very few inducers of chromoplast development have been identified to date. Orange (OR) chaperones are among the best characterized,

Significance

Carotenoids are natural pigments whose properties as provitamin A and health-promoting phytonutrients make them ideal targets for biofortification. Here, we show that plastids specialized in carotenoid overaccumulation named chromoplasts can be synthetically produced in plant tissues that do not naturally develop them. We further demonstrate that differentiation of chromoplasts from leaf chloroplasts not just causes but requires both a reduction in photosynthetic activity and a stimulation of carotenoid biosynthesis in a process hardwired to a major reprogramming of global gene expression and cell metabolism. The synthetic system that we report here should allow to boost the nutritional quality of green vegetables and forage crops once their photosynthetic activity is dispensable (e.g., just before harvesting).

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but they only work in some tissues, and the specific mechanism by which they promote chromoplast differentiation remains unclear (5). The experimental manipulation of chromoplast differentiation for fundamental studies and biotechnological applications therefore requests a much better understanding of the mechanisms regulating this process.

Results

The Bacterial crtB Enzyme Induces the Transformation of Leaf Chloroplasts into Plastids of Chromoplast Features. The first committed step of the carotenoid pathway is the conversion of geranylgeranyl diphosphate (GGPP) to phytoene, catalyzed by phytoene synthase (referred to as PSY in plants and crtB in bacteria). We previously found that the virus-mediated expression of a bacterial *crtB* gene in tobacco (*Nicotiana tabacum* and *Nicotiana benthamiana*), tomato (*Solanum lycopersicum*), *Arabidopsis thaliana*, and several other plants caused leaf yellowing due to increased accumulation of colored endogenous carotenoids downstream of phytoene (10). When the production of crtB was optimized using appropriate viral vectors for specific target hosts, an intense and widespread yellow phenotype was achieved in edible leaves such as those of lettuce (*Lactuca sativa*) (Fig. 1*A*) and green vegetables such as zucchini (*Cucurbita pepo*) (Fig. 1*B*). These results illustrate the potential of this approach to boost the nutritional value of green (i.e., chloroplast-containing) plant tissues, which are particularly recalcitrant to carotenoid enrichment. To further investigate the mechanism underlying the characteristic yellow phenotype of *crtB*-expressing tissues, we used transmission electron microscopy (TEM) to analyze plastid ultrastructure in leaves from *N. tabacum* and *Arabidopsis* plants treated with crtB-harboring viral vectors. Yellow sectors in infected leaves contained plastids with a distinctive ultrastructure that were absent in empty vector controls (Fig. 2 *A* and *B*). Very similar plastids were observed when the *crtB* gene was transiently expressed from *Agrobacterium tumefaciens*-delivered vectors in agroinfiltrated *N. benthamiana* leaves (Fig. 2*C*). These plastids were devoid of the organized photosynthetic thylakoids and grana found in typical chloroplasts but contained electron-dense (i.e., lipid-containing) membrane stacks much more tightly appressed than grana (Fig. 2*D*). The stacks were connected by what appeared to be remnants of thylakoid membranes. Plastids of crtB sectors also showed a proliferation of small electron-dense round vesicles identified as plastoglobules (Fig. 2). Loss of thylakoid and grana integrity as well as proliferation of plastoglobules and new membrane systems are features typically observed when chloroplasts differentiate into chromoplasts (3, 8, 11). TEM examination of dark-incubated senescent *N. benthamiana* leaves showed that the plastids found in crtB-producing cells were completely different from gerontoplasts (Fig. 2*E*).

To further substantiate the identity of the chromoplast-like plastids that developed in crtB-producing leaves, we analyzed chloroplast and chromoplast marker proteins by immunoblot analysis (Fig. 2*F*). Virus- or *A. tumefaciens*-mediated expression of *crtB* in *N. tabacum* or *N. benthamiana* leaves, respectively, resulted in increased levels of fibrillin, a protein associated with chromoplast development (12, 13). In contrast, the levels of D1 (also known as PsbA), a core component of photosystem II (PSII) that is highly down-regulated during chloroplast-to-chromoplast differentiation (14, 15), decreased in crtB-producing leaves (Fig. 2*F*). These results together suggest that expressing the bacterial *crtB* gene in leaf cells is sufficient to differentiate chloroplasts into chromoplast-like plastids.

We next used *Arabidopsis* double mutants defective in OR chaperones (AtOR and AtOR-LIKE) to test whether the differentiation process triggered by crtB involved pathways depending on these well-characterized promoters of chromoplast development (5, 16). Similarly, double mutants lacking cytosolic and plastidial carotenoid cleavage dioxygenases (CCD1 and CCD4, respectively) were used to investigate the possible contribution of

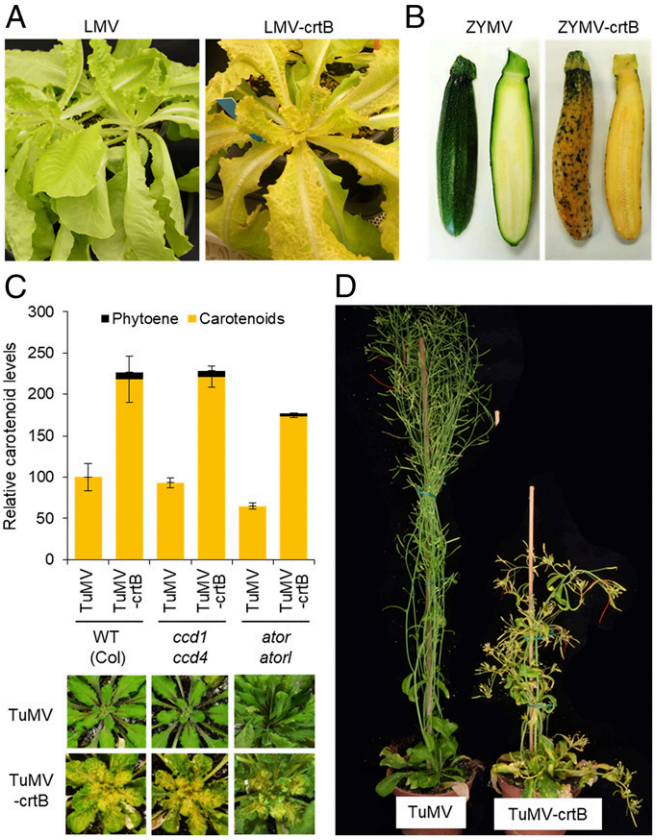

Fig. 1. Virus-mediated production of crtB causes leaf yellowing due to carotenoid overaccumulation. (*A*) Lettuce at 12 d postinoculation (dpi) with a *crtB*-expressing Lettuce mosaic virus (LMV)-derived vector or an empty control. (*B*) Zucchini from plants at 14 dpi with a *crtB*-expressing Zucchini yellow mosaic virus (ZYMV)-derived vector or an empty control. (*C*) Carotenoid analysis and representative images at 14 dpi of *Arabidopsis* (Col) WT and double mutant plants grown under short day conditions (8 h of low light and 16 h of darkness) for 2 wk (WT and *ccd1 ccd4*) or 5 wk (*ator atorl*) and then inoculated with the indicated viral vectors. Plot shows the mean and SD of $n = 3$ independent samples. Carotenoid levels are represented relative to those in WT samples inoculated with the empty vector control (TuMV). (*D*) Representative *Arabidopsis* WT plants at 38 dpi.

signaling molecules derived from enzymatic degradation of carotenoids in the differentiation mechanism (17–19). Virus-mediated expression of *crtB* in these mutants resulted in leaves showing the characteristic yellow phenotype and carotenoid overaccumulation observed in the WT (Fig. 1*C*), suggesting that OR activity or enzymatic cleavage of carotenoids are not required for the process. Besides leaves, yellowing was widespread in all other green tissues, including cauline leaves, stems, sepals, and siliques, where it remained stable until plants died (Fig. 1*D*).

The crtB Enzyme Only Triggers Chloroplast-to-Chromoplast Differentiation When Localized in Plastids. Low scores for plastidial targeting were obtained for crtB with TargetP and ChloroP servers, indicating a nonreliable prediction. However, the 12-aa-long noncatalytic N-terminal region of this protein contains some features found in plastid-targeting peptides, including five hydrophobic and two hydroxylated residues. A C-terminal fusion of the full-length crtB enzyme to the green fluorescent protein (crtB-GFP) mainly localized to the cytosol in *N. benthamiana* leaf cells, but some fluorescence was also detected in chloroplasts (10). By optimizing the transient expression conditions (using only young leaves and adding the helper component protease of the

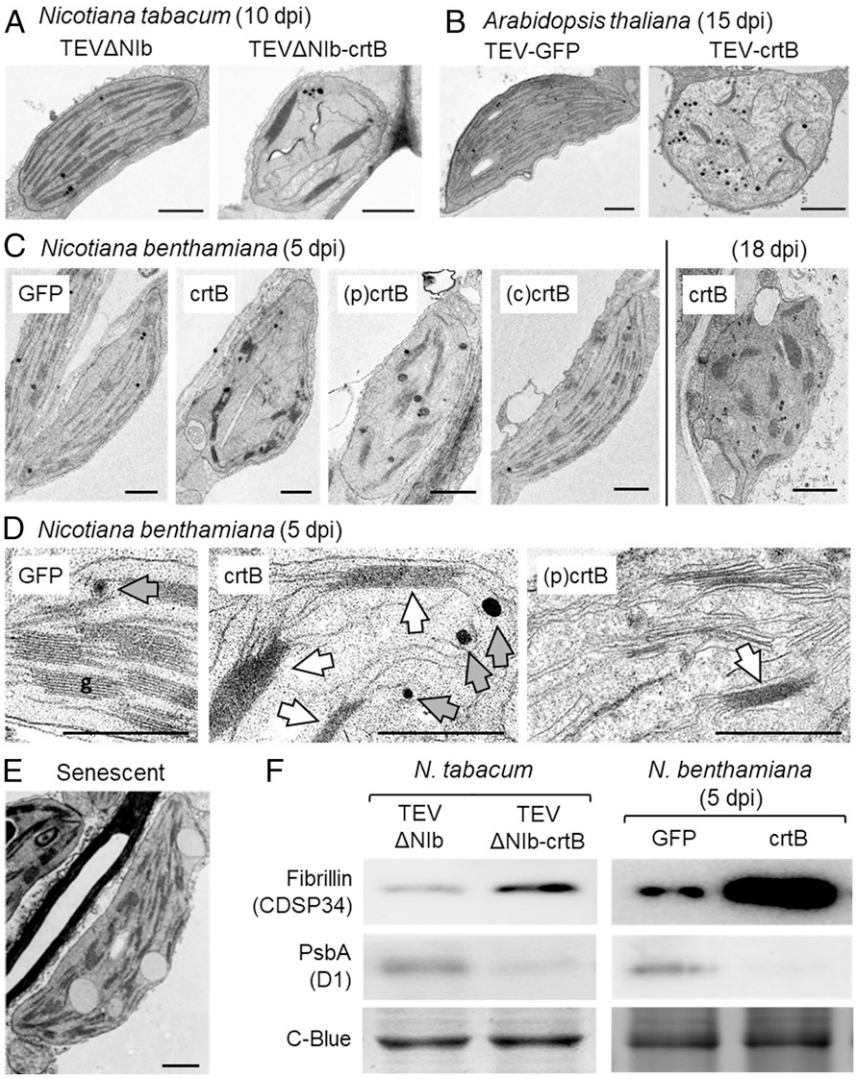

Fig. 2. Chromoplast-like plastids develop from chloroplasts in leaves producing crtB or a plastid-targeted version of the enzyme. TEM images of representative plastids from the indicated species and treatments are shown. (*A*) Plastids from *N. tabacum* leaves collected 10 dpi with TEVΔNIb (empty vector) or TEVΔNIb-crtB. (*B*) Plastids from *A. thaliana* (Ler) leaves inoculated with TEV (empty vector) or TEV-crtB at 15 dpi. (*C*) Plastids from *N. benthamiana* leaves agroinfiltrated with the indicated constructs and collected at 5 dpi (first four images) or 18 dpi (*Right*). (*D*) Magnification of plastids from *N. benthamiana* leaves agroinfiltrated with the indicated constructs and collected at 5 dpi. Grana are marked as "g," membrane stacks with white arrows, and plastoglobules with gray arrows. (*E*) Gerontoplast from a *N. benthamiana* leaf harvested from the plant and kept in the dark for 10 d (senescent). (*F*) Immunoblot analysis of plastidial proteins in leaves treated as described in *A* and *C*. Coomassie blue (C-Blue) staining is shown as a loading control. (Scale bars, 1 μm.)

Watermelon mosaic virus to prevent gene silencing), crtB-GFP fluorescence was detected at a much higher intensity. This allowed to confirm that the crtB-GFP protein was present in both cytosol and chloroplasts of agroinfiltrated cells (*SI Appendix*, Fig. S1*A*). When GFP was fused to the N terminus of crtB, the resulting protein (GFP-crtB) was completely excluded from chloroplasts (*SI Appendix*, Fig. S1*A*). It is therefore likely that the bacterial crtB enzyme harbors a cryptic plastid-targeting signal in its N terminus that becomes blocked and, hence, inactivated in the GFP-crtB protein. To unambiguously target crtB to the chloroplast, we next added the plastid-targeting sequence of the *Arabidopsis* enzyme HDS (20) to the crtB-GFP reporter. As expected, the resulting (p)crtB-GFP protein was only found in chloroplasts (*SI Appendix*, Fig. S1*A*). Agroinfiltrated leaf tissues expressing either crtB or (p)crtB developed the characteristic yellow phenotype at 4–5 d postinoculation (dpi), whereas tissues expressing the cytosolic GFP-crtB version—renamed as (c)crtB—remained green as the controls expressing GFP (Fig. 3*A*). Analysis of carotenoid contents showed identical profiles for leaf sections agroinfiltrated with GFP and (c)crtB and confirmed that, similarly to crtB, (p)crtB triggered carotenoid overaccumulation (Fig. 3*B*). Agroinfiltration of *N. benthamiana* leaves with crtB or (p)crtB constructs did not initially reduce chlorophyll levels compared to leaf tissues agroinfiltrated with GFP or (c)crtB (Fig. 3*B*). However, estimation of photosynthesis-related parameters such as effective quantum yield of PSII (ϕPSII) and nonphotochemical quenching (NPQ) showed that both crtB and (p)crtB, but not (c)crtB or GFP, had a dramatic impact on chloroplast function (Fig. 3*C*). A plastid-targeted version of GFP did not cause any yellowing or ϕPSII defect (*SI Appendix*, Fig. S1*B*), confirming that disturbance of chloroplast photosynthesis is not caused by the accumulation of a foreign protein in chloroplasts but specifically by crtB. TEM analyses confirmed that (p)crtB induced the differentiation of chromoplast-like plastids very similar to those found in leaf tissues expressing the untargeted crtB enzyme, whereas only chloroplasts were present in leaves producing either (c)crtB or GFP (Fig. 2 *C* and *D*). These results confirm that crtB elicits a synthetic

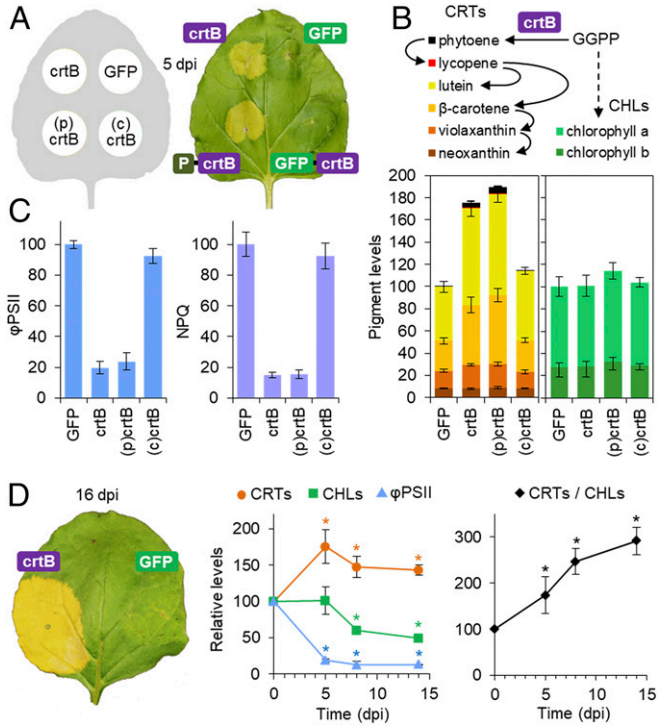

Fig. 3. Leaf tissues producing crtB show a stable phenotype of high carotenoid levels and impaired photosynthesis. (*A*) *N. benthamiana* leaf 5 d after agroinfiltration (dpi) with the indicated constructs in different sections. (*B*) Levels of carotenoids (CRTs) and chlorophylls (CHLs) in leaf sections like those shown in *A*. (*C*) ϕPSII and NPQ in leaf sections like those shown in *A*. (*D*) Changes in CRTs, CHLs, ϕPSII, and carotenoid-to-chlorophyll ratio (CRTs/CHLs) in leaf sections at different time points after agroinfiltration with crtB. A representative leaf at 16 dpi is shown at *Left*. Plots show the mean and SD of $n = 3$ independent samples. Values are represented relative to those in GFP controls. Asterisks in *D* plots mark statistically significant changes relative to 0 dpi (*t* test, $P < 0.05$).

(i.e., nonnatural) differentiation of chromoplasts only when localized in plastids, where carotenoids are made.

Differentiated leaf chromoplasts (Fig. 2*C*) and associated features such as high carotenoid levels and low ϕPSII (Fig. 3*D*) were maintained for weeks after agroinfiltration, and no reversions to chloroplasts or green color were ever observed. About a week after agroinfiltration with crtB, chlorophylls started to decrease. Carotenoids also decreased but not as much as chlorophylls, eventually resulting in a higher carotenoid-to-chlorophyll ratio and, as a consequence, a stronger yellow color as leaves became older (Fig. 3*D*). The strong yellow phenotype associated with chromoplast differentiation and carotenoid enrichment was also stably maintained when the *crtB* gene was expressed from a viral vector in *Arabidopsis*, lettuce, and zucchini (Fig. 1), strongly suggesting that the mechanism by which crtB overexpression eventually results in chromoplastogenesis is conserved in plants.

Synthetic Chromoplast Biogenesis Induces Profound Changes in Nuclear Gene Expression and Primary Cell Metabolism. The vast majority of plastidial proteins are encoded by nuclear genes (2). We therefore reasoned that the drastic remodeling of plastidial ultrastructure associated with crtB-triggered chromoplast differentiation would require changes in nuclear gene expression. RNA-sequencing (seq) analyses of *N. benthamiana* leaf samples at 96 h postinfiltration (hpi) showed that about 5,000 genes were differentially expressed in yellow (p)crtB sections compared to green GFP controls (Dataset S1). Such a massive reprogramming of gene expression included the up-regulation of 3,183 genes and the down-regulation of 1,803 genes in chromoplast-containing samples. Gene Ontology (GO) term enrichment analyses (Dataset S2) showed overrepresentation of genes involved in protein folding and binding to RNA and ribosomes among those induced by (p)crtB (*SI Appendix*, Fig. S2). Enrichment of genes with roles in transmembrane transport, cell signaling (protein phosphorylation, calcium binding), and nuclear gene expression (transcription factors) was observed among those repressed when chromoplast biogenesis was induced (*SI Appendix*, Fig. S2). This profile was strikingly similar to that of ripening tomato fruits (where chromoplasts naturally differentiate from chloroplasts) but very different from that of senescent *Arabidopsis* leaves (*SI Appendix*, Fig. S2). Many of the genes potentially involved in carotenoid biosynthesis were up-regulated during crtB-triggered transformation of leaf chloroplasts into chromoplasts (*SI Appendix*, Fig. S3). However, the changes in carotenoid-related gene expression detected during chromoplastogenesis in *N. benthamiana* leaves were modest compared to those taking place in tomato ripening fruit (*SI Appendix*, Fig. S3 and Dataset S3).

Energy and carbon required for carotenoid biosynthesis rely on photosynthesis (i.e., the Calvin–Benson cycle) in chloroplasts. Given that leaf chromoplast differentiation was associated with impairment of photosynthesis (Fig. 3), we asked whether primary cell metabolism might also be reprogrammed. Of 52 metabolites detected by gas chromatography/time-of-flight/mass spectrometry analysis in *N. benthamiana* leaves (Dataset S4), 13 displayed statistically significant changes in (p)crtB sections compared to GFP controls (*SI Appendix*, Fig. S4 and Table S1). We observed reductions in the levels of ascorbate and hexoses (glucose and fructose, the main soluble carbohydrate stores and respiration substrates). This, together with increments in tricarboxylic acid (TCA) cycle intermediates (citrate, 2-oxoglutarate, and malate) and amino acids (valine, isoleucine, aspartate, and glutamate), suggested that sugars were used to produce ATP through the TCA cycle to sustain amino acid and carotenoid biosynthesis (and likely other cellular functions). Indeed, respiration rate—determined as total oxygen consumption in the dark—was higher in chromoplast-containing leaf tissues (*SI Appendix*, Fig. S4). While an increased respiration is also associated with the onset of carotenoid overproduction in tomato and other climacteric fruits, the metabolic changes that we observed in (p)crtB-producing *N. benthamiana* leaves are often opposite to those occurring during chromoplast differentiation in tomato (11, 21). In particular, hexoses and ascorbate do not decrease but increase. We speculate that this might be because leaf metabolism is devoted to produce and export photoassimilates, whereas tomatoes are sink organs that have been selected to accumulate sugars and acids as positive taste attributes. In any case, our data together show that the activity of crtB in leaf chloroplasts is sufficient to trigger a deep reprogramming of nuclear gene expression and whole-cell metabolism associated with the differentiation of chromoplasts, a plastid type that is not naturally found in tobacco or *Arabidopsis* leaves.

Enhanced Supply of Phytoene in Chloroplasts Can Interfere with Photosynthesis. To investigate the dynamics of the crtB-dependent chromoplast differentiation process, we next followed the time course of *crtB* expression, phytoene production, and downstream carotenoid accumulation after agroinfiltration of *N. benthamiana* leaves with the (p)crtB construct. Transcripts encoding (p)crtB were reliably detected at 24 hpi and peaked at 48 hpi (Fig. 4*A*), whereas phytoene started to accumulate between 24 and 36 hpi and suddenly increased at 48 hpi (Fig. 4*B*). Downstream carotenoids began to increase at 48 hpi (Fig. 4*B*). To follow chloroplast membrane remodeling dynamics, we also monitored ϕPSII, NPQ, and D1 protein levels as estimators of photosynthesis, photoprotection, and photodamage, respectively. Both ϕPSII (Fig. 4*C*)

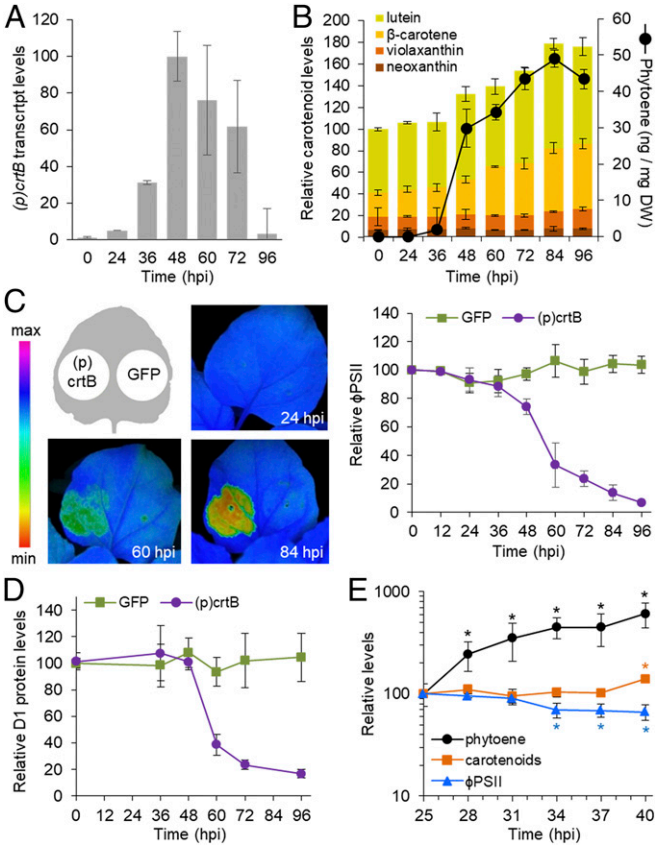

Fig. 4. Time-course of chloroplast-to-chromoplast differentiation in leaves. *N. benthamiana* leaves were agroinfiltrated with the indicated constructs, and samples were collected at the indicated time points (hours after agroinfiltration). (*A*) Levels of (p)crtB-encoding transcripts relative to the maximum in (p)crtB samples. (*B*) Absolute phytoene levels and relative downstream carotenoid contents in (p)crtB samples. (*C*) Representative chlorophyll fluorescence images and ϕPSII values. (*D*) D1 (PsbA) protein contents. (*E*) Levels of phytoene, downstream carotenoids, and ϕPSII in (p)crtB samples relative to those at 25 hpi. Asterisks mark significant changes relative to the 25 hpi values (*t* test, $P < 0.05$). Note the logarithmic scale. In all of the plots, values correspond to mean and SD values of $n = 3$ independent samples.

and NPQ (*SI Appendix*, Fig. S5) remained unchanged up to 36 hpi, and then decreased as the levels of both phytoene and downstream carotenoids increased (Fig. 4). The levels of D1 started to decrease later, between 48 and 60 hpi (Fig. 4*D*), likely as a result of photodamage. A higher temporal resolution analysis of both ϕPSII and carotenoid levels between 25 and 40 hpi showed that phytoene levels increased before ϕPSII decreased, whereas downstream carotenoids took a bit longer to accumulate (Fig. 4*E*). Taken together, these results suggest that the crtB-mediated production of phytoene causes a disruption of the chloroplast photosynthetic functionality before carotenoids start to overaccumulate.

To next confirm whether impairment of chloroplast functionality was due to phytoene overaccumulation, we used norflurazon (NF) to prevent phytoene conversion into downstream carotenoids (22). *N. benthamiana* leaves were agroinfiltrated with constructs to produce GFP, (p)crtB, and PAR1, an *Arabidopsis* transcription cofactor that promotes total carotenoid biosynthesis but not phytoene accumulation in photosynthetic tissues (23). At 24 hpi, some agroinfiltrated leaves were treated with NF (Fig. 5). Untreated leaves transiently expressing the *Arabidopsis PAR1* gene accumulated higher levels of carotenoids downstream of phytoene but did not exhibit changes in ϕPSII compared to GFP controls. NF treatment resulted in phytoene accumulation, reduced levels of downstream carotenoids, and decreased ϕPSII in all of the samples (Fig. 5). The reduction in ϕPSII in these NF-treated samples correlated with the accumulation of phytoene (the higher the levels of phytoene, the stronger the reduction of ϕPSII). However, the reduction in ϕPSII was modest compared to that observed in (p)crtB samples lacking NF (Fig. 5*A*), in which phytoene conversion into downstream carotenoids led to the differentiation of chromoplasts. We concluded that phytoene overaccumulation by itself disrupts photosynthesis but does not abolish chloroplast identity, resulting in a relatively modest reduction in ϕPSII. The much stronger decrease in ϕPSII that takes place in (p)crtB samples would not be directly due to the effect of phytoene on photosynthetic activity but resulted from the dismantling of the photosynthetic apparatus as chloroplasts accumulate downstream carotenoids and differentiate into chromoplasts. Interestingly, PAR1-mediated accumulation of carotenoids but not phytoene (Fig. 5*A*) was unable to trigger the massive drop in ϕPSII and the yellow leaf phenotype characteristic of chromoplast differentiation (Fig. 5*B*). We therefore conclude that the accumulation of phytoene causes a concentration-dependent disruption of the photosynthetic identity of chloroplasts, a condition that might be necessary for them to become chromoplasts upon the subsequent production and accumulation of downstream carotenoids.

Pharmacological Inhibition of Photosynthetic Activity Reduces the Phytoene Threshold to Initiate Chloroplast-to-Chromoplast Transition in Leaves. In contrast with the results using crtB but similar to those with PAR1, overexpression of PSY-encoding genes from *Arabidopsis* and tomato could not elicit the characteristic yellow leaf phenotype associated with chromoplast differentiation (Fig. 5 and *SI Appendix*, Fig. S6) (11, 24, 25). Interestingly, the plant enzymes

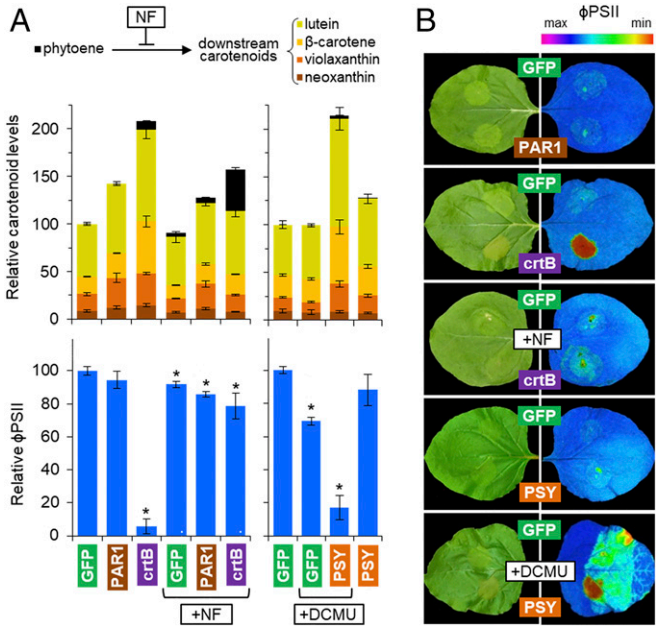

Fig. 5. Transformation of leaf chloroplasts into chromoplasts requires a reduction of photosynthetic capacity and production of carotenoids downstream of phytoene. (*A*) Carotenoid levels and ϕPSII in leaves 96 h after agroinfiltration with the indicated constructs. In all cases, plot values correspond to mean and SD values of $n = 3$ independent samples. Asterisks in ϕPSII plots mark statistically significant changes relative to untreated GFP controls (*t* test, $P < 0.05$). Samples infiltrated with NF at 24 hpi or treated with DCMU 24 h before agroinfiltration are indicated. (*B*) Representative images of agroinfiltrated leaves at 96 hpi and their corresponding chlorophyll fluorescence for ϕPSII.

yielded significantly lower levels of phytoene compared to crtB and did not substantially impact photosynthesis as deduced from ϕPSII values (Fig. 5 and *SI Appendix*, Fig. S6). We hence speculated that phytoene might act as a metabolic threshold switch that only alters the photosynthetic performance of chloroplasts when exceeding a certain level. To overcome this putative threshold, we treated *N. benthamiana* leaves with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, diuron), a widely used inhibitor of photosynthesis that interrupts the photosynthetic electron transport chain. The next day, treated and untreated control leaves were agroinfiltrated with constructs encoding either GFP or the *Arabidopsis* PSY enzyme. At 96 hpi, DCMU-treated GFP leaf sections showed a decrease in ϕPSII but unchanged carotenoid levels compared to untreated samples (Fig. 5). By contrast, DCMU-treated PSY sections showed a more dramatic drop in ϕPSII and much higher levels of carotenoids than untreated PSY or GFP controls (Fig. 5*A*). As a consequence, PSY leaf sections treated with DCMU turned yellow (Fig. 5*B*), similar to that observed when chloroplast-to-chromoplast differentiation was triggered by crtB in the absence of inhibitor. In summary, our results are consistent with the existence of a two-step process responsible for the crtB-mediated transformation of leaf chloroplasts into chromoplasts (Fig. 6). First, chloroplast identity is weakened by overaccumulation of phytoene and, second, increased production of carotenoids in preconditioned chloroplasts allows the differentiation of chromoplasts. Without preconditioning, carotenoid levels can increase but chromoplasts do not differentiate, as shown in untreated leaves producing PAR1 or PSY. Moreover, if carotenoids downstream of phytoene are not produced, preconditioned chloroplasts remain unchanged, as shown in NF-treated (p) crtB leaves (Fig. 5).

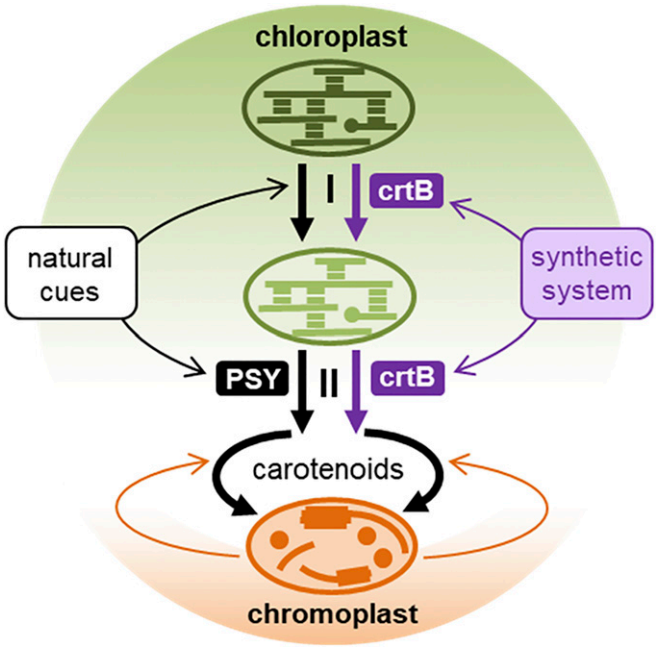

Fig. 6. Model of the chloroplast-to-chromoplast differentiation process. Plant developmental programs create organs with different degrees of photosynthetic capacity and, hence, chloroplast identity, from strong (e.g., leaves) to weak (e.g., green fruits) or absent (e.g., roots). Weakening of chloroplast identity appears to be the first phase (I) in chromoplast differentiation. In a second phase (II), developmental cues promote the expression of genes encoding PSY and other carotenoid biosynthetic enzymes. Enhanced production of carotenoids then reprograms plastid-to-nucleus communication, changes plastidial ultrastructure, and results in the differentiation of chromoplasts, which, in turn, promote biosynthesis and improve storage of carotenoids. The two phases can be synthetically engineered in leaves by overproducing phytoene using crtB. When phytoene exceeds a certain level, it interferes with the photosynthetic capacity of leaf chloroplasts. This acts as a metabolic switch that allows the formation of chromoplasts after phytoene is converted into downstream carotenoids by endogenous enzymes.

Discussion

The regulation of plastid identity is a core process in plants that remains poorly defined. Our work shows that chromoplasts can be synthetically differentiated from leaf chloroplasts in all plants tested, despite this is something that only a few species can do in nature. This synthetic system has allowed us to propose a model for chromoplast differentiation that also applies to natural systems (Fig. 6). In a first phase (I), chloroplasts must become competent (i.e., preconditioned) by lowering their photosynthetic capacity, whereas increased production of carotenoids completes the differentiation of chromoplasts in a second phase (II).

In our synthetic system, phase I was very fast (hours) and required a sufficient amount of phytoene to weaken chloroplast identity in leaves. In chloroplasts, carotenoids such as lutein, beta-carotene, violaxanthin, and neoxanthin are required to maintain the properties of photosynthetic membranes and pigment–protein complexes responsible for harvesting sunlight and transferring excitation energy to the photosystems (4, 26, 27). Phytoene is not normally detected in leaf chloroplasts as it is readily converted into downstream (photosynthesis-related) carotenoids. Overaccumulation of phytoene (or a phytoene derivative) might somehow compete with endogenous carotenoids for their binding to photosynthetic protein complexes and membranes, interfere with their functions, and eventually cause the changes that we observed in photosynthetic competence. Consistent with this hypothesis, engineered accumulation of nonchloroplast carotenoids such as astaxanthin in plants alters the properties of thylakoids and grana and interferes with the photosynthetic machinery at several other levels (27, 28). Production of astaxanthin and other ketocarotenoids in tobacco actually resulted in leaf plastids that lost their chloroplast features and exhibited a proliferation of disordered membrane systems and plastoglobules (28, 29), similar to our results (Fig. 2).

In nature, chloroplasts might become competent for differentiation into chromoplasts without the need of a phytoene boost. In tomato, the chloroplasts of green fruit are much less photosynthetically active than those of leaves (8, 30). Thus, tomato green fruit and other organs, tissues, or/and developmental stages in which chloroplast identity is weak or nonexistent (e.g., dark-grown calli, tubers, or roots) might be considered as "naturally competent" to differentiate chromoplasts when carotenoid biosynthesis is up-regulated. In agreement, overproduction of plant PSY enzymes resulted in chromoplast-like structures arising in green tomato fruit and nonphotosynthetic *Arabidopsis* tissues but had no effect on leaves (11, 24, 25) unless previously conditioned by shutting down their photosynthetic capacity (Fig. 5). Similarly, up-regulation of OR triggers carotenoid overaccumulation and chromoplast differentiation in tomato fruit, potato tubers, cauliflower curds, or *Arabidopsis* calli, but not in the leaves of any of these plants (31–34). Interestingly, OR does not appear to be required for crtB-mediated differentiation of leaf chloroplasts into chromoplasts (Fig. 1*C*). OR has been shown to promote PSY activity and stability, and some OR mutants prevent carotenoid (particularly beta-carotene) metabolism (5, 16, 35, 36). Our results suggest that no extra function would be required for OR up-regulation to cause chromoplast development from plastids with weak or no chloroplast identity. Interestingly, a role for OR in repressing chloroplast development in etiolated seedlings has been recently proposed (37).

In phase II, which likely overlaps with phase I, enhanced expression of carotenoid-related genes (*SI Appendix*, Fig. S3) likely contributes to activate the endogenous biosynthetic pathway. During this phase, carotenoid accumulation occurs concomitantly

with the remodeling of the internal plastid structures, with both factors synergistically activating each other (Fig. 6). Strikingly, in agroinfiltrated *N. benthamiana* leaves, this process takes place before chlorophylls start to degrade (Fig. 3). These results confirm that chlorophyll breakdown and chromoplast differentiation are independent processes, as already shown in mutants such as tomato *green flesh* (*gf*), in which impairment of chlorophyll degradation during fruit ripening has no effect on the formation of chromoplast membranes and the accumulation of carotenoids (38). The new structures created following the disassembly of photosynthetic grana and thylakoids (Fig. 2) likely contribute to reaching high carotenoid levels by accommodating increasing amounts of carotenoids and by preventing their degradation (1–3, 5, 7, 8). They might additionally enhance carotenoid production by stimulating the activity of endogenous carotenoid biosynthetic enzymes (including PSY), many of which are membrane-associated (39).

The buildup of carotenoids and the structural changes associated with the chloroplast-to-chromoplast transformation involve reorganization of the plastidial protein content (Fig. 2*F*) but also a global reprogramming of nuclear gene expression (*SI Appendix*, Fig. S2) and primary metabolism (*SI Appendix*, Fig. S4). It is likely that implementing these changes (as well as others required to readapt plastid protein import and quality control mechanisms to chromoplast differentiation) relies on retrograde signals produced by differentiating plastids. Carotenoid degradation can generate signaling molecules that regulate many developmental processes in plants, including plastid development (18, 19). The observation that *Arabidopsis ccd1 ccd4* mutants defective in carotenoid cleavage dioxygenase activity in the cytosol (via CCD1) and the plastids (via CCD4) were not affected in the crtB-dependent leaf phenotype (Fig. 1*C*) suggests that signals independent of CCDs or carotenoids are responsible for eliciting the changes in nuclear gene expression and cell metabolism supporting chromoplast biogenesis. The specific nature of such signals remains to be discovered.

In summary, we show that chromoplast differentiation only requires metabolic cues (i.e., enough phytoene and downstream carotenoid production). While our conclusions are based on a synthetic system (i.e., the expression of a bacterial gene in leaf cells), the similarity of the transcriptomic profiles between this process and fruit ripening (*SI Appendix*, Fig. S2) strongly supports that this is a basic general mechanism for chloroplasts to become chromoplasts. In nature, however, developmental cues play a fundamental role by making chloroplasts competent (phase I) and by regulating the expression of carotenoid biosynthetic genes (phase II). Signals produced by differentiating plastids are also hardwired to the process as they support the organellar transformation by reprogramming nuclear gene expression and whole-cell metabolism. Besides serving to successfully address a long-standing question in plant biology (i.e., plastid identity), the very simple and straightforward system that we describe here to induce chloroplast-to-chromoplast differentiation on demand is a powerful biotechnological tool that appears to work in every plant tested so far (Fig. 1) (10). Thus, creating an organellar sink to improve both the production and the storage of carotenoids and other plastidial phytonutrients in chloroplast-containing tissues once their photosynthetic activity is dispensable (e.g., just before harvesting) should allow to boost the nutritional quality of green vegetables and forage crops.

Materials and Methods

Plant Material and Growth Conditions. *N. tabacum* Xanthi nc, *N. benthamiana* RDR6i, and *A. thaliana* Columbia-0 (Col) and Landsberg *erecta* (Ler) plants were grown under standard conditions as described previously (10, 40). Growth of double mutants *ccd1 ccd4* (16) and *ator atorl* (17), both in the Col background, was facilitated by transferring them to low light (40 μmol photons·m^{-2}·s^{-1}) days after germination. For generating dark-induced leaf senescence, detached leaves were maintained inside dark, humid chambers until visible yellowing occurred. Collected plant material was frozen in liquid nitrogen, lyophilized, and then homogenized to a fine powder using a TissueLyser system (Qiagen) for further analyses.

Transmission Electron Microscopy. Transmission electron microscopy of plant leaves was performed as previously described (41).

Gene Constructs. See *SI Appendix, Materials and Methods* for details.

Transient Expression Assays. For transient expression studies using viral vectors, leaves of 4- to 6-wk-old *N. tabacum* and *Arabidopsis* plants were mechanically inoculated with crude extracts from frozen-stored infected plant tissue and collected upon the appearance of the yellowing phenotype as described previously (10). For agroinfiltration experiments, the second or third youngest leaves of 4- to 6-wk-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain GV3101 carrying vectors of interest following the procedure described previously (42). Gene silencing was prevented by coagroinfiltration with a strain carrying the helper component protease (HC-Pro) of the *Watermelon mosaic virus* (WMV) in vector HcProWMV-pGWB702 (kindly provided by Juan José López-Moya and Maria Luisa Domingo-Calap, CRAG, Barcelona, Spain). Infiltration cultures were grown on Luria–Bertani medium at 28 °C and used at optical density at 600 nm (OD_{600}) of 0.5. For pharmacological treatments, NF or diuron (DCMU) were diluted in water and 0.05% Tween 20. The treatments with NF (2 μM) were performed by infiltration with a syringe of leaf areas that had been agroinfiltrated with different constructs 24 h earlier. DCMU (10 μM) was applied on the leaf surface with a fine paintbrush 24 h before agroinfiltration.

Transcript Analyses. See *SI Appendix, Materials and Methods* for details.

Protein Extraction and Immunoblot Analyses. Protein extraction, quantification, and immunoblot analyses were performed as described (43) using anti-fibrillin (44) or anti-PsbA serum (Agrisera).

Metabolite Analyses. Carotenoids were analyzed as previously described (40). Phytoene was quantified using a concentration curve with a commercial standard (Sigma). Primary metabolites were extracted and analyzed as described in *SI Appendix, Materials and Methods*.

Photosynthetic and Respiration Measurements. See *SI Appendix, Materials and Methods* for details.

Statistical Analyses. Differentially expressed genes (DEGs) were identified by comparing crtB and GFP RNA-seq datasets with the DESeq2 statistical method in the AIR platform. The resulting crtB/GFP list was filtered using cut-offs of false discovery rate <0.05 and $\log_2$-transformed fold change ($\log_2$FC) >0.585 for up-regulated genes and <−0.599 for down-regulated genes. GO enrichments were identified by the Parametric Analysis of Gene Set Enrichment (PAGE) function of the AgriGO v2.0 web-based tool (bioinfo.cau.edu.cn/agriGO/) after transforming the gene IDs to the Niben v04.4 annotation. For the comparison of different biological systems, we selected the significantly enriched gene ontologies from our *N. benthamiana* RNA-seq experiment (*P* and *q* values <0.05) and compared their Z-score values with those obtained from the analysis of published RNA-seq data of tomato fruit ripening and *Arabidopsis* leaf senescence. In particular, we used the reads per million mapped reads values of the total pericarp at mature green (MG), light ripe (LR), and red ripe (RR) stages (45) and the fragments per kilobase million mapped reads values of the 16D and 30D senescence stages (46). DEGs resulting from LR/MG, RR/MG, and 30D/16D comparisons were filtered as described above for *N. benthamiana* crtB/GFP. Student's *t* tests were used for the rest of statistical analyses using GraphPad Prism 5.0a (GraphPad Software). Carotenoid-related genes in *N. benthamiana* were identified by BLASTp using tomato sequences. Heatmaps were made using the pheatmap package in R (https://cran.r-project.org/web/packages/pheatmap/index.html).

Data Availability. RNA-seq data were deposited in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the accession SRP238752 (BioProject ID PRJNA597608). The rest of the data presented in the paper are available in the main text and *SI Appendix*. Biological materials are available from the corresponding authors upon request.

All study data are included in the article and *SI Appendix*.

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