The differentiation of chloroplasts into chromoplasts involves a series of biochemical changes that culminate with the intense accumulation of long chain chromophore carotenoids such as lycopene, rhodoxanthin, astaxanthin, anhydroescloltzxanthin, capsanthin, and capsorubin. The signal pathways mediating these transformations are unknown. Chromoplast carotenoids are known to accumulate in green tissues experiencing stress conditions, and studies indicate that they provide efficient protection against oxidative stress. We tested the role of reactive oxygen species (ROS) as regulators of chromoplast carotenoid biosynthesis in vivo. The addition of ROS progenitors, such as menadione, tert-butyldihydroperoxide, or parapat and proxidants such as diamide or buthionine sulfoximine to green pericarp discs of pepper fruits rapidly and dramatically induce the simultaneous expression of multiple carotenogenic gene mRNAs that give rise to capsanthin. Similarly, down-regulation of catalase by amitrole induces expression of carotenogenic gene mRNAs leading to the synthesis of capsanthin in excised green pericarp discs. ROS signals from plastids and mitochondria also contribute significantly to this process. Analysis of the capsanthin-capsorubin synthase promoter in combination with a β-glucuronidase reporter gene reveals strong activation in transformed pepper protoplasts challenged with the above ROS. Collectively these data demonstrate that ROS act as a novel class of second messengers that mediate intense carotenoid synthesis during chromoplast differentiation.

Plastids are plant organelles whose diverse functions include photosynthesis, gravity perception, and biogenesis of micro- and macromolecules. These functions do not occur in all plastids but are associated with structurally distinct plastid types. Plastid differentiation is a highly coordinated process involving programmed, multi-phase events that are transduced by a variety of stimuli. These transducers activate numerous morphological and biochemical changes that ultimately affect plastid compartmentalization. This is particularly evident in chromoplasts undergoing the transformation to chromoplasts, an event characterized by the synthesis and accumulation of carotenoids into unique plastid substructures. These changes are accompanied by the yellow to red color shift in flowers, fruits, and roots of certain plants (1, 2). Although the molecular events controlling these alterations are largely unknown, emerging evidence suggests that reactive oxygen species (ROS) may play a vital role in this process (3). In this paper we present data showing how oxidative stress affects carotenogenesis in plants.

Data from several model systems indicate that ROS operate at the molecular level. Homeostatic variations in ROS levels are known to activate normal and pathological events during animal cell development (4, 5). For example, it has been shown that hydrogen peroxide acts as a potent activator of NF-kB, a transcription factor that is involved in inflammation, immune responses, and acute-phase responses in animal cells (4).

Plants are also subject to oxidative stresses (6, 7). Being immobile and because of oxygenic photosynthesis, they have the highest internal oxygen concentrations of any other organism. The concentration of molecular oxygen in plant leaf cells is 250 μM, which exceeds the concentration of 0.1 μM found in mammalian mitochondria (8). It has been estimated that 1% of the oxygen consumed by plants is diverted into active oxygen (9). As a consequence, plant cells have developed an array of nonenzymatic and enzymatic mechanisms for scavenging this toxic component. Neutralization by isoprenoids such as carotenoids and tocopherols (10, 11) are examples of nonenzymatic mechanisms. Plants and other organisms also rely on the enzymatic scavenging of ROS by catalase, glutathion peroxidase, ascorbate peroxidase, and superoxide dismutase (6).

The damaging effects of ROS have been examined during plant stress (12) and senescence (7). More recently, they have also been implicated in plant-pathogen interactions (13–15). Multiple observations also suggest that oxidative stress regulates carotenoid biosynthesis during the chloroplast to chromoplast transition in plants. First, in Reseda odorata leaves, rodoxanthin, a chromoplast-specific carotenoid, shows a de novo accumulation following stembark removal (16). Apparently this procedure causes a transient disruption of turgor pressure, which leads to oxidative stress (17). Second, in Aloe vera (18) and Cryptomeria (19), stress from drought and high irradiance or low temperatures induces the de novo accumulation of rhodoxanthin in chloroplasts, which subsequently undergoes a transformation into photosynthetically active chloro-chromoplasts. Similarly, it is known that drought and low or high temperatures can induce oxidative stress (12). These conditions lead to the de novo accumulation of anhydroescloltzxanthin, a chromoplast carotenoid in Buxus sempervirens leaves (20). One explanation is that the extended chromophores of rhodoxanthin and anhydroescloltzxanthin provide better adverse effects on ROS scavenging pathways.

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** The nucleotide sequence(s) reported in this paper has been deposited to the GenBank™/EBI Data Bank with accession number(s) Y14165.

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The abbreviations used are: ROS, reactive oxygen species; HPLC, high performance liquid chromatography; DPI, diphenylene iodonium; AT, aristolochic acid.
photodynamic screens for protection from the oxidative stresses generated under these conditions than do normal leaf carotenoids, in good agreement with data based on model studies (21–23). This mechanism is also observed in nonplants, such as the yeast Phaffia rhodozyma, where singlet oxygen and peroxyl radicals control the biosynthesis of astaxanthin, another carotenoid with an extended chromophore (24).

These data point to oxidative stress as a potent driving force for the expression of carotenoid biosynthetic genes during the chloroplast to chromoplast transition in plants. To test this, we made use of pepper (Capsicum annum) fruit system (3). As pepper fruits ripen and chloroplasts differentiate into chromoplasts, they accumulate large quantities of carotenoids that include capsanthin and capsorubin, compounds responsible for the red color change. Their pathway is shown in Fig. 1. Several cDNAs for enzymes of this pathway have been characterized (for a review, see Ref. 2). Using these as molecular probes and the promoter directing the biosynthesis of the chromoplast-specific carotenoid, capsanthin, we show that ROS are potent inducers of carotenogenic gene mRNAs expression during chromoplast development.

EXPERIMENTAL PROCEDURES

Plant Material—Bell pepper (C. annum cv Yolo Wonder) plants were grown under controlled greenhouse conditions, and fruits were taken at the mature green stage.

Pericarp Disc Incubation—After sterilization with 2% sodium hypochlorite, pericarp discs (1.5 cm in diameter) were excised with a cork borer. The discs were immediately treated with 20 μM diphenylene iodonium and 200 μM aristolochic acid, unless otherwise stated, to avoid any wound-induced oxidative stress from challenged cells. Test solutions of 50 μl containing different compounds were deposited in the center of the discs. Generally, six discs were incubated in the presence of light in 12-well titration plates (Corning Glass) at 25 °C as described previously (25).

Polysome Isolation—Free and membrane-bound polysomal fractions were isolated from frozen pericarp discs using previously described procedures (26).

Molecular Analysis—Total RNAs were isolated from frozen polysomal fractions or pericarp discs as described previously (30). The carotenogenic gene probes were available from previous studies. These include geranylgeranyl pyrophosphate synthase (Ggpp) (31), phytoene synthase (Psy) (32), phytoene desaturase (Pds) (33), lycopene cyclase (34) (Lcy), zeaxanthin epoxidase (35) (Zepd), and capsanthin capsorubin synthase (Ccs) (36). The heat shock probe (Hsp 70 cDNA) was partially characterized from pepper and is available upon request. Total RNA (20 μg) were blotted, and a pepper cDNA encoding 25 S ribosomal RNA was used to ensure that equivalent amounts of RNA were blotted. The purified probes were used for hybridization according to standard procedures (37).

The capsanthin-capsorubin synthase promoter was isolated from genomic pepper DNA according to the polymerase chain reaction method described previously (38). To this end 15 μg of pepper DNA was digested with SacI, DraI, StuI, and PstII yielding blunt fragments that were ligated to the adaptor primer. After amplification of the StuI fragment using the adaptor primer and the gene-specific probe: CAT- TAGAGAAAAATGCAGAGTTAT (36), a 650 base pair was amplified by polymerase chain reaction (38) and sequenced to give capsanthin-cap- sorubin promoter-1 (Ccs 1) (accession number Y14165). Two restriction sites, PstI (5‘ end) and BamHI (3‘ end), were introduced to subclone the promoter fragment in frame of Escherichia coli β-glucuronidase gene using pBI101 plasmid (39). The promoter activity was tested by transient expression using protoplasts prepared from mature green pepper fruit pericarp according to the polyethylene glycol procedure (40). Before transformation, the plasmid vector was linearized, and a sample (40 μg) of purified plasmid DNA was administered to 2.5 × 10⁴ protoplasts. β-Glucuronidase activity was monitored after a 12-h transfection as described previously (39).

Other Analytical Methods—Aconitase activity was determined as described previously (41). Analysis of methanesulfonic acid after dimethyl sulfoxide (Me₂SO) treatment was carried out as described before (42). Protein concentration was determined by the Bio-Rad assay.

RESULTS AND DISCUSSION

Wound-induced Expression of Carotenogenic Gene mRNAs—Chromoplasts in pepper fruits begin differentiating when the fruit reaches its mature green stage, and full conversion requires approximately 14 days. Chloroplast to chromoplast differentiation can be conveniently assessed using green pericarp discs isolated from pepper fruits. This in vitro system offers advantages because the discs mimic the developmental changes that occur in whole fruit, and the time course for ripening is more uniform and rapid (25). When discs were incubated alone, in the absence of other treatments, the time-dependent accumulation of two carotenogenic gene transcripts encoding geranylgeranyl pyrophosphate synthase (Ggpp) and capsanthin-capsorubin synthase (Ccs) (Fig. 2A) was observed, in good agreement with previous work (43). However, when discs were incubated in the presence of diphenylene iodonium and aristolochic acid (Fig. 2A), accumulation of these two gene transcripts was blocked. Diphenylene iodonium (DPI) inhibits plasmalemna superoxide synthase in plant cells (14), whereas aristolochic acid (AT) is a specific inhibitor of phospholipase A2 activity (44). Wounding is known to induce heat formation in fruits (45), and to drastically affect their pigmentation (46) and...
metabolism (47, 48). Mechanical destruction of cell walls (49) and associated osmotic perturbations (17) lead to oxidative bursts, which generate ROS by a superoxide synthase located in the plasmalemma of challenged cells. Wounding can also induce lipolytic activity (50), yielding fatty acid precursors for jasmonic acid synthesis, which could also potentially activate the expression of Ggps and Ccs (51). Under our conditions, DPI and AT inhibit these responses. Because of its electrophilicity, DPI probably does not permeate cells and reacts only at the periphery of the cut discs. When DPI and AT are combined, they appear to abolish any signals that are transduced by the oxidative burst or jasmonate pathway of challenged peripheral cells.

To test whether ROS might induce carotenogenic genes in developing chromoplasts, DPI- and AT-treated pericarp discs were used to manipulate the in vivo formation of ROS in a predictable way to evaluate their effect on carotenogenesis.

**Induction of the Expression of Carotenogenic Gene mRNAs Following Inhibition of Carotenogenesis**—Inhibition of plant carotenoids during the early steps of their biosynthesis leads to elevated photooxidation. Using the desaturase inhibitor metflurazon in our pericarp disc system, we observed a significant accumulation of phytoene in treated versus untreated discs (Fig. 2B). Similar data were observed with other desaturase and cyclase inhibitors, namely, diflufenican, LS 80707, and 2-(4-chlorophenylthio)-triethylamine (CPTA). Thus, blocking carotenogenesis appears to activate the expression of genes for carotenoid biosynthesis. This agrees with previous data where the expression of Pds mRNAs (52, 53) were examined in tomato seedlings grown in the dark or light.

The fact that oxidative stress serves as a driving force behind carotenogenesis can be inferred from the effect of herbicides, which inhibit phytoene desaturase (fluridone and norflurazon, SAN 6706) or ζ-carotene desaturase (J852) on barley seedlings (54). Here, phytoene, phytofluene, and ζ-carotene accumulation is elevated 2-fold higher on a molar basis than the levels of carotenoid end products (β-carotene and xanthophylls) produced by control seedlings (54). Similar results were observed in wheat seedlings treated with SAN 9789 (55) and in Phycomyces whose phytoene desaturase was blocked with several inhibitors (56).

The induction of these genes could be explained by a number of ways. Although feedback regulation may be responsible, two other possibilities could explain this induction. The first is photooxidative stress, caused by the absence of biologically active carotenoids that protect excited chlorophylls. However, under our conditions, xanthophylls are virtually unaltered, suggesting that this mechanism is not likely to be operative.

Inhibition of carotenoid biosynthesis by metflurazon and related substituted pyridazinones offers a second possible mechanism. These compounds inhibit photosynthetic electron transport at the plasmalemma level (54, 57). Also, aryloxyalkylamine derivatives, structurally similar to 2-(4-chlorophenylthio)-triethylamine, act as uncouplers of photophosphorylation by dissipating the proton gradient (58). These photosynthetic perturbations trigger futile electron cycling and generate ROS (12), which could ultimately act to induce carotenogenic gene mRNAs expression. This is supported by work showing that superoxide anions generated by the Mehler reaction lead to induction of carotenoid biosynthesis in Dunaliella salina (59).
butylhydroperoxide (1 mM).

A

Menadione

|       | Ggps | Psy | Pds |
|-------|------|-----|-----|
| 0     | 6    | 12  | 24  |
| 0     | 6    | 12  | 24  |

25 S

B

tert-Butylhydroperoxide

|       | Ggps | Psy | Pds |
|-------|------|-----|-----|
| 0     | 6    | 12  | 24  |
| 0     | 6    | 12  | 24  |

25 S

C

Aminotriazole

|       | Ggps | Psy | Pds |
|-------|------|-----|-----|
| 0     | 6    | 12  | 24  |
| 0     | 6    | 12  | 24  |

25 S

This was further examined in our system by evaluating the effects of different ROS progenitors.

**Induction of the Expression of Carotenogenic Gene mRNAs by Artificial, Exogenous ROS Progenitors**—To test the involvement of superoxide anion, we used menadione, which is partially reduced in vivo to yield a semiquinone radical that reduces molecular oxygen into a superoxide anion (60). When pericarp discs were treated with this cyclic redox system, a strong accumulation of Ggps and Cs mRNAs was observed (Fig. 3A). Because superoxides are usually metabolized via hydroperoxides, we decided to test their effects.

Organic hydroperoxides, widely produced during plant metabolism, participate in the synthesis of jasmionic acid and traumatin. Studies on animal cells reveal that a $10^{-9}$ to $10^{-7}$ M concentration of lipid hydroperoxides exerts regulatory roles (61). We observed that tert-butylhydroperoxide causes the pronounced induction of different carotenogenic gene mRNA expression except for lycopene cyclase (Fig. 3B) and that the concentration optimum was extremely low. Levels above 5 mM resulted in a strong bleaching effect, exhibiting a 95% decrease in chlorophyll and carotenoid content. This toxic effect could have been due to excess free radical formation, which can greatly enhance the autoxidation of polyunsaturated molecules in an ambient oxygen atmosphere. This has been observed with β-carotene (62, 63) and xanthophylls (64). Because the hydrophobicity of organic peroxides allows them to react with membrane components, we also examined the pattern of α-tocopherol evolution as a consequence of peroxide treatment. Data revealed that after 24 h, tocopherol content decreased to 50% following peroxide treatment, suggesting that α-tocopherol participates in peroxyl radical quenching. Tocopherols appear to control the extent of peroxyl radical formation more effectively than carotenoids because they trap the lipid peroxyl radical (11). The quench rate of peroxyl radicals by α-tocopherol ranges between $10^5$ M$^{-1}$ s$^{-1}$ and is far more rapid than the autopropyagation of peroxyl radicals, which ranges between $10^{-1}$ to $5 \times 10^2$ M$^{-1}$ s$^{-1}$ (65). Our results with artificial compounds extend previous observations showing that oxidative stress induced the accumulation of the long-chromophore carotenoid, astaxanthin, in the yeast P. rhodozyma (24, 66) and the alga Hematoecoccus pluvialis (67). This argues that under physiological conditions, carotenogenic genes might be subject to regulation by endogenously generated ROS. This was further examined in our study.

**Down-regulation of ROS-scavenging Enzymes Induces the Expression of Carotenogenic Gene mRNAs**—Hydrogen peroxide produced during normal plant metabolism diffuses freely across biological membranes. As hydrogen peroxide is a potent signal molecule in plants (14, 15), we attempted to downregulate the activity of catalase, ascorbate peroxidase, and glutathione peroxidase, which are prominent enzymatic peroxide scavengers. We reasoned that intracellular increases in hydrogen peroxide would induce an effect similar to that observed when exogenous artificial peroxides are added. To this end we used amitrole (3-amino-1,2,4-triazole) to inhibit catalase activity (68). The specificity of amitrole is well documented in plants where it does not block histidine biosynthesis, as in bacteria (68). We also observed that under in vivo, physiological concentrations, amitrole did not inhibit lycopene cyclase (69), contrary to previous reports (for a review, see Ref. 54). The amitrole concentration used (20 mM) was optimum for inhibiting different plant catalase isozymes (70) and resulted in a massive increase in expression of different carotenogenic gene transcripts (Fig. 3C). This was paralleled by a de novo synthesis of the chromoplast-specific carotenoid, capsanthin, which was not detected in control discs (Fig. 4).

The potential involvement of hydrogen peroxide is reinforced by the fact that aconitase activity of treated pepper discs was progressively down-regulated to 50% after 24 h of treatment. In its catalytic form, aconitase has a cubane cluster [4Fe$^+\cdot4S]^{2+}$, which after loss of the labile iron atom due to in vitro oxidation by superoxides or peroxides, is converted to an inactive enzyme containing a paramagnetic cluster [2Fe$^+\cdot4S]^{1+}$ (71–73). In this context, it is noteworthy that in animal cells, aconitase is involved in signaling induced by nitric oxide (74).

Overall, these data suggest cross-talk between ROS-scavenging enzymes and plastid carotenogenesis, which might be inferred by the link between catalase and carotenoid biogenesis as shown by earlier studies with fungi and plants. In Fusarium aquaeductuum, carotenogenesis is photoinductible, and the effect of light can be mimicked by hydrogen peroxide (75). Studies of chloroplast pigment-deficient mutants in plants indicate that catalase activity is strongly reduced (76). Also, barley mutants deficient in catalase show an alteration in plastid ultrastructure (77). In Dunaliella bardawil, carotenoid overaccumulation is paralleled by an increase in catalase activity...
(78). A similar increase is observed during the ripening of Carica papaya fruits (79), where chromoplast differentiation and increases in carotenoid accumulation are observed. In this regard, it is notable that pear fruit ripening can be induced by inhibition of catalase, which causes the build-up of peroxides (80). Similarly, the ripening of saskatoon fruit is paralleled by increased oxidative stress (81).

The link between catalase and plastid carotenogenesis is further strengthened by studies involving the catalase inhibitor amitrole. It is interesting to note that treatments of maize (82), wheat (83), radish (84), and barley (85) seedlings with amitrole lead to the de novo accumulation of lycopene, a chromoplast-specific carotenoid. Because amitrole is not a carotenoid cyclase inhibitor (69), we propose that elevated hydrogen peroxide, due to catalase inhibition, initiates the production of ROS, which subsequently elicits lycopene synthesis as a defense or adaptation response. Indeed, chromoplast carotenoids possessing extended chromophores such as lycopene, capsanthin, or capsorubin are particularly well suited for quenching ROS compared with normal leaf carotenoids (21, 22). In this respect, it is noteworthy that several maize mutants, when grown autotrophically at high light intensity, become bleached and accumulate lycopene (86). Similarly, cupric ions, which act as strong oxidants by inducing the formation of hydroxyl radicals in plastids via the Haber-Weiss mechanism (87), activate the synthesis of lycopene in tomato fruits (88).

Glutathion peroxidase and ascorbate peroxidase can replace catalase as scavengers of hydrogen peroxides, but they have much broader substrate specificities. This necessitated an evaluation of their down-regulation. To this end, p-aminophenol, an irreversible inhibitor of ascorbate peroxidase (89), diamide, a potent oxidant of reduced glutathion (GSH) (90), and bathionine sulfoximine, an irreversible inhibitor of \( \gamma \)-glutamylcysteine synthetase (91), were used. Results showed that treating pericarp discs with these inhibitors to block ascorbate peroxidase and to limit the availability of GSH strongly activated the expression of carotenogenic genes (Fig. 5A).

In assessing the effect of oxidative stress caused by GSH depletion, it was noted that thiol reagents capable of operating in the dark, such as p-chloromercuribenzoate or p-hydroxymercuribenzoate, can substitute for light to photoinduce carotenogenesis in F. aquaeductuum (92). This specificity was reversed by cysteine or 2-mercaptopropanol (93). This oxidative mechanism has also been observed in Brevibacterium sp. KY-4313, which forms the long carotenoid chromophore, canthaxanthin. This was inhibited when GSH is present in the culture medium (94).

Our demonstration that endogenously generated ROS inactivate the expression of carotenogenic gene mRNAs in plants raises the question of the role played by plastids and mitochondria in this process, since these organelles represent two main sites of oxygen production in plant cells.

**ROS Signals Generated From Plastids and Mitochondria Induce the Expression of Carotenogenic Gene mRNAs**—The herbicide paraquat (1,1’-dimethyl-4,4-bipyridinium ion) has a strong negative redox potential (\( E^o = -0.446 \) v) and diverts electron transport from photosystem I (57). In the presence of paraquat, photosynthetic electrons are transferred to molecular oxygen, yielding superoxide anions. Although paraquat has secondary effects, such as the disruption of membrane integrity, plastids are its main target. This is reinforced by the fact that paraquat-resistant plants possess up to a 300% increase in plastid superoxide dismutase content (95). Pericarp discs treated with paraquat showed a strong induction of capsanthin-capsorubin synthase mRNA accumulation (Fig. 5B). The effect was strongly reduced in the presence of 10% dimethyl sulfoxide (Me\(_2\)SO). This suggests the involvement of a free radical mechanism, as Me\(_2\)SO is generally nontoxic and is stoichiometrically oxidized by hydroxyl radical, HO’, to yield methanesulfonic acid (42). The scavenging reaction afforded by Me\(_2\)SO thus explains this reduced expression.

In mitochondria it has been shown that hydrogen peroxide is produced during normal electron transfer reactions and that this process accounts for 1–2% of the oxygen taken up (96). This activity is enhanced by antimycin A treatment (96). It is also known that mitochondrial electron transfer participates in signal transduction of the tumor necrosis factor (97). When molecular oxygen is reduced to the superoxide anion, it is then converted to hydrogen peroxide by mitochondrial superoxide dismutase. Based on this, we used rotenone, thenoxyltrifluoroacetone, and antimycin A, to block cytochrome electron transport chain (Fig. 6A). One might expect that this would exacerbate the formation of superoxide anion and hydrogen peroxide from mitochondria. We noted that antimycin A markedly induced the expression of \( \text{Gggs} \) and \( \text{Ccs} \) mRNAs (Fig. 6B) compared with rotenone and thenoxyltrifluoroacetone, which prematurely abort the electron transfer pathway.

Our data suggest that plant mitochondria participate in signal mechanisms involving ROS. Similarly, in the dark, antimycin A, an electron transport inhibitor between cytochrome \( b \) and \( c_1 \), replaces the light-induced accumulation of carotenoid in Mycobacterium marinum (98) and Leptosphaeria michotii, a fungus (99). Relevant to this is the yeast, P. rhodozyma, which shows a maximum accumulation of carotenoids in the later phases of culture, when respiration becomes insensitive to cyanide (24). This effect is mimicked by antimycin. A likely explanation is that electron transport is short-circuited, leading to the formation of superoxide anions and hydrogen peroxide, which trigger carotenoid biogenesis.

**Run-on Transcription and Specific Integration of Carotenogenic Gene mRNAs into Polysomal Fractions**—To better under-
stand the role of ROS, pericarp discs treated with different ROS-inducing compounds were used to analyze transcription and integration of Ggps and Ccs mRNAs into polysomal fractions. Run-on analysis showed that Ggps was barely transcribed in control tissues, whereas Ccs was not transcribed (Fig. 7). Conversely, the transcription of Ggps and Ccs was stimulated in amitrole, tert-butylhydroperoxide, and diamide-treated discs. Expression of a pepper heat shock protein 70 (Hsp 70) mRNA, used as a control, was identical in all treatments, thus demonstrating the specificity of the observed effects (Fig. 7). Analysis of polysomal fractions revealed that amitrole specifically induced the recruitment of Ggps and Ccs mRNAs into the high molecular mass polysomal fraction of treated discs (Fig. 8).
ROS Drive the Functioning of Capsanthin-Capsorubin Synthase Promoter—The partial Ccs-1 promoter was characterized and displayed homologies to several cis-acting elements previously characterized (Fig. 9A). The EcoRI-ScaI fragment of Ccs cDNA (36) hybridized specifically to a pepper 3.2-kilobase pair ScaI genomic fragment containing the coding domain and the 5′-untranslated region of Ccs including the partial promoter (Ccs-1) (Fig. 9B). Transient assays were used to analyze the function of the Ccs-1 promoter under different oxidative stress conditions using protoplasts from mature green fruits pericarp displaying at least 90% viability as judged by fluorescein diacetate staining. Linear plasmids containing a β-glucuronidase reporter gene driven by the Ccs-1 promoter were introduced into green fruit protoplasts and incubated for 12 h in the presence of prooxidants. Data obtained are the mean from three different protoplasts preparations. S.E. normalized as a proportion of the mean is shown.

ROS Scavenging Delays Carotenoid Overaccumulation and Chromoplast Differentiation—Based on the results, we deter-
measured whether ROS could mediate the differentiation of chromoplasts to chromoplasts. We reasoned that if endogenously produced ROS were quenched, we would expect to be able to demonstrate their regulatory role. To this end, intact pepper fruits were dipped in 10% Me2SO, a radical quencher (42), and allowed to ripen for 12 days at room temperature. Data obtained (Fig. 10) reveal a significant reduction of the expression of Ggps and Ccs mRNAs and reduced accumulation of chromoplast ketocarotenoids capsanthin and capsorubin in treated fruits compared with controls (Fig. 10). Moreover, this was allowed to ripen for 12 days at room temperature. Data obtained (Fig. 10) reveal a significant reduction of the expression of Ggps and Ccs mRNAs and reduced accumulation of chromoplast ketocarotenoids capsanthin and capsorubin in treated fruits compared with controls (Fig. 10). Moreover, this was allowed to ripen for 12 days at room temperature. Fig. 10, demonstrating that OH radicals formed during the ripening period were quenched. This may explain why Me2SO inhibits chromoplast carotenoid accumulation in ripening tomato fruit (100).

It appears that ROS, produced as a consequence of aerobic life, are widely used for signal transduction because they are ubiquitous, diffusible, and uncharged (i.e. hydrogen peroxide) molecules. Virtually all cellular compartments have the ability to neutralize the toxic effects of ROS, which could otherwise lead to cell death at high concentrations (101). This secondary messenger role of ROS is further supported by the fact that oxidative stress induces a transient movement of cytosolic calcium in plants (102). Taken together, our results suggest that in vivo, transient, oxidative stress plays a key role in the induction of chromoplast carotenoid biosynthesis and in the transformation of chloroplasts into chromoplasts (3). In a similar vein, although the mechanism has not been explored, exposure to O3 leads to increased carotenogenesis in poplar (103) and Norway spruce (104). The specific activation of carotenogenic genes under our experimental conditions is further supported by the fact that none of the ROS species used in this work induced the accumulation of capsidial, a typical isoprene stress metabolite produced in elicited pepper cells (43). Future work is needed to scrutinize the signal transduction pathways involved in the massive biosynthesis of carotenoids during chromoplast differentiation.

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