Chemical Quenching of Singlet Oxygen by Carotenoids in Plants[1][C][W]

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Carotenoids are considered to be the first line of defense of plants against singlet oxygen ($^1O_2$) toxicity because of their capacity to quench $^1O_2$ as well as triplet chlorophylls through a physical mechanism involving transfer of excitation energy followed by thermal deactivation. Here, we show that leaf carotenoids are also able to quench $^1O_2$ by a chemical mechanism involving their oxidation. In vitro oxidation of β-carotene, lutein, and zeaxanthin by $^1O_2$ generated various aldehydes and endoperoxides. A search for those molecules in Arabidopsis (*Arabidopsis thaliana*) leaves revealed the presence of $^1O_2$-specific endoperoxides in low-light-grown plants, indicating chronic oxidation of carotenoids by $^1O_2$. β-Carotene endoperoxide, but not xanthophyll endoperoxide, rapidly accumulated during high-light stress, and this accumulation was correlated with the extent of photosystem (PS) II photoinhibition and the expression of various $^1O_2$ marker genes. The selective accumulation of β-carotene endoperoxide points at the PSII reaction centers, rather than the PSII chlorophyll antennae, as a major site of $^1O_2$ accumulation in plants under high-light stress. β-Carotene endoperoxide was found to have a relatively fast turnover, decaying in the dark with a half time of about 6 h. This carotenoid metabolite provides an early index of $^1O_2$ production in leaves, the occurrence of which precedes the accumulation of fatty acid oxidation products.

Reactive oxygen species (ROS) are unavoidable by-products of photosynthesis (Apel and Hirt, 2004; Li et al., 2009). On the one hand, the photosynthetic electron transport chain can transfer electrons to molecular oxygen, leading to the formation of reduced forms of oxygen, such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical. Moreover, by initiating photorespiration, oxygenation of Rubisco brings about the formation of H_2O_2 in the peroxisomes (Peterhansel and Maurino, 2011). On the other hand, when in the triplet excited state, chlorophylls can transfer excitation energy to oxygen, leading to the formation of singlet oxygen ($^1O_2$; Krieger-Liszkay, 2005; Ledford and Niyogi, 2005; Triantaphylidès and Havaux, 2009). The production of those different ROS can be strongly enhanced by various environmental stress conditions that inhibit the photosynthetic processes and hence cause absorption of light energy in excess to what can be used by the photosynthetic processes. Under those conditions, overreduction of the photosynthetic electron transport chain and accumulation of triplet chlorophylls promote leakage of electrons/energy to oxygen. Photoinduced ROS production can cause damage to macromolecules, including lipids and proteins, resulting in cellular alterations, decreased photosynthetic efficiency, and impaired growth. Using hydroxy fatty acids as specific reporters of $^1O_2$-mediated, free radical-induced, and lipoxygenase-catalyzed lipid peroxidation processes, it was shown that photooxidative damage to plant leaves is always associated with $^1O_2$-induced lipid peroxidation whatever the initial ROS production is (Triantaphylidès et al., 2008). Thus, $^1O_2$ seems to be the major ROS ultimately involved in photooxidative stress-induced cell death.

Chloroplasts contain a panoply of antioxidant mechanisms to protect themselves from photooxidative stress (Van Breusegem et al., 2008; Li et al., 2009). Carotenoids constitute the first line of defense against $^1O_2$ toxicity (Krinsky, 1979; Cogdell and Frank, 1987;

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Triantaphylidès and Havaux, 2009). They are able to quench this ROS and also directly quench triplet chlorophylls, the major source of $^1O_2$ in plant leaves. Two main mechanisms are involved in quenching $^1O_2$, which account for the protection of biological systems. The interaction between $^1O_2$ and a quencher leads to the production of an excited complex followed by competing reactions involving either energy transfer or chemical reactions. The mechanism related to energy transfer is denoted as physical quenching: The quencher molecule deactivates $^1O_2$ to the triplet unreactive ground state, gains energy to a triplet excited state, and then looses readily its energy to the environment and returns to its original state. Carotenoids are typical molecules acting according to this scheme (Edge et al., 1997; Stahl and Sies, 2003; Triantaphylidès and Havaux, 2009). The second type of mechanism is denoted as chemical quenching, or scavenging and involves a chemical reaction between the quencher and $^1O_2$. The quencher is oxidized and consumed, unless enzymatic recycling of the oxidized quencher can occur, as is the case e.g. with ascorbate (Noctor and Foyer, 1998). In many cases, however, the quencher is replaced as a result of de novo synthesis.

Chemical quenching of $^1O_2$ by carotenoids is believed to be a minor side reaction compared with the physical quenching mechanism (Edge et al., 1997; Stahl and Sies, 2003). Nevertheless, in vitro experiments have shown that carotenoids in solvent can be oxidized by $^1O_2$, thus indicating the existence of a chemical quenching capacity for those compounds (Foote and Denny, 1968; Stratton et al., 1993; Yamauchi et al., 1998; Fiedor et al., 2001; Montenegro et al., 2002). However, the occurrence of this mechanism in vivo during exposure of plants to high-light stress is not documented. Although carotenoid oxidation products can be found in plant tissues, they have been attributed so far to the action of oxidative enzymes, the carotenoid cleavage dioxygenases (CCDs; Bouvier et al., 2005; Walter and Strack, 2011). On the contrary, in animals and humans, carotenoid oxidation products resulting from reactions with ROS have been detected in various tissues. In particular, skin and eye samples have been reported to contain carotenoid metabolites resulting from $^1O_2$ oxidation (Khachik et al., 1997; Bernstein et al., 2001; Bando et al., 2004; Bhosale and Bernstein, 2005), indicating that chemical quenching of $^1O_2$ can take place in biological tissues.

In this paper, we characterize the products generated in vitro by $^1O_2$ oxidation of carotenoids, and we identify $^1O_2$-specific endoperoxide molecules for both β-carotene and xanthophylls (lutein, zeaxanthin). We show the presence of these compounds in plant leaves, indicating that carotenoids do quench $^1O_2$ by a chemical reaction. Under high-light stress, a selective accumulation of β-carotene endoperoxide was observed in Arabidopsis (*Arabidopsis thaliana*) leaves, which was well correlated with PSII photoinactivation, pointing at the β-carotene-containing PSII reaction center as a major source of $^1O_2$ under photooxidative stress.

RESULTS

In Vitro Carotenoid Oxidation

β-Carotene solubilized in toluene/methanol and bubbled with oxygen was illuminated in the presence of the $^1O_2$ generator Rose Bengal. As shown in Figure 1, A and B, several oxidation products (numbered 1–8) were detected by their light absorption at 445 nm after separation by reverse-phase HPLC. As expected, all products were more polar than β-carotene, with one major oxidation product appearing in Figure 1B as peak 6 at an elution time of 12.25 min. The chromatogram shown in Figure 1B resembles very much the chromatogram of β-carotene oxidation products previously reported in UV-irradiated skin homogenates prepared from mice fed a β-carotene-supplemented diet (Bando et al., 2004). In this previous study, the major oxidation product was identified as β-carotene endoperoxide. This compound was also reported to be a major product generated in vitro from β-carotene by (bacterio) chlorophyll-sensitized photooxidation (Yamauchi et al., 1998; Fiedor et al., 2001) and was even identified as the primary oxidation products in early stage photolyzed solution of β-carotene (Montenegro et al., 2002). We collected the compound eluted in peak 6 and measured its absorbance spectrum in methanol/hexane (Fig. 1C). The absorbance maxima (404, 424, and 450 nm) coincided with the absorption peak wavelengths of the β-carotene 5,8-endoperoxide (mass, 568) previously determined by Stratton et al. (1993) in the same solvent mix. The eluted peak 6 was also subjected to mass spectrometry (MS) analysis. Although peak 6 displayed a rather complex fragmentation pattern and appeared to contain coeluted minor products, its mass spectrum nevertheless showed the expected $[M+H]^+$ ion at a mass-to-charge ratio (m/z) of 569 (data not shown).

The xanthophylls, lutein and zeaxanthin, were also oxidized by $^1O_2$ using methylene blue as photosensitizer, and HPLC analyses showed the production of a variety of oxidation products for both xanthophylls (Supplemental Fig. S1). Similar to the oxidation of β-carotene, the products generated by $^1O_2$ oxidation of lutein and zeaxanthin were all more polar than the parent carotenoids.

A previous study (Stratton et al., 1993) identified three carotenals besides β-carotene endoperoxide as main products generated by the photosensitized oxidation of β-carotene. Those compounds resulted from the cleavage of double bonds along the polyene chain of the carotenoid to form aldehydes. As a consequence, we analyzed the carotenoid oxidation products of β-carotene and xanthophylls by HPLC-tandem MS (MS/MS) to seek for this type of oxidation products. In preliminary experiments, we analyzed the fragmentation pattern of a series of purified oxidation products of carotenoids and observed that molecular ions at m/z 119 or 175 were present in almost all fragmentation spectra, thus constituting a signature of carotenoid-derived products, as already reported in previous studies (Stratton et al., 1993;

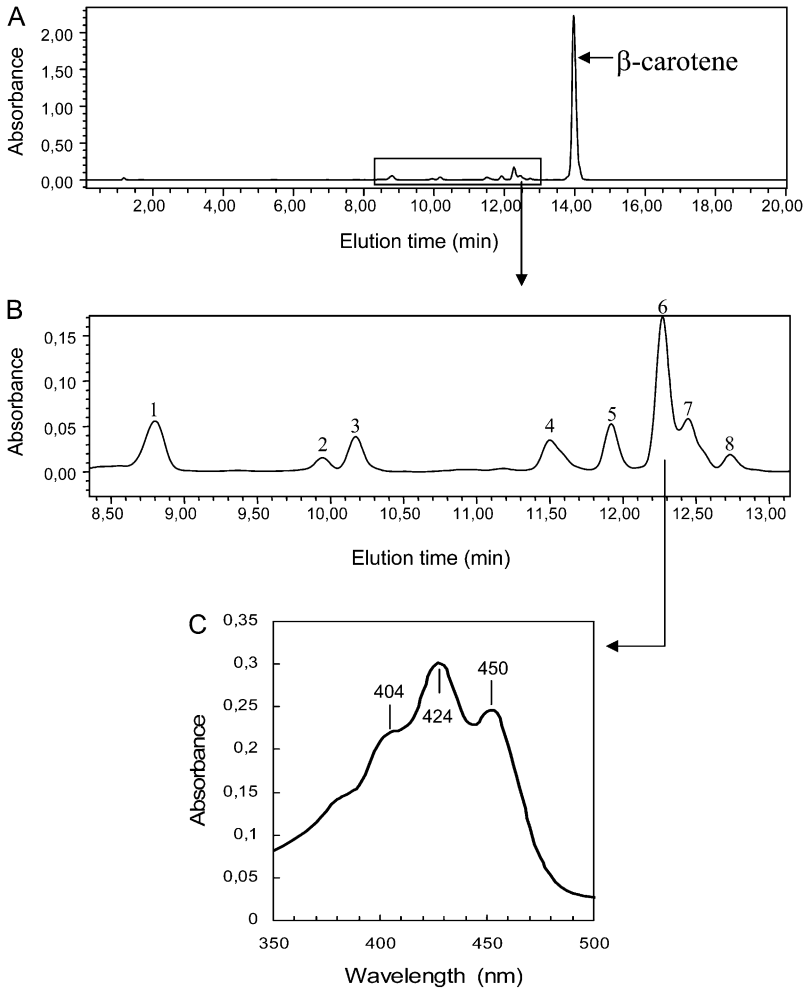

Figure 1. A, UV-visible HPLC elution profile of β-carotene oxidation products obtained from the reaction between β-carotene and $^1O_2$ generated in the light by Rose Bengal in toluene/methanol. Detection wavelength was 445 nm. B, Eight peaks, labeled 1 to 8, were detected in the HPLC chromatogram. The major peak, labeled 6, was characterized by its absorbance spectrum in toluene/methanol (C).

Prasain et al., 2005; Lakshminarayana et al., 2008). Therefore, the carotenoid oxidation products were analyzed in the so-called precursor ion scan mode to detect all the potential oxidation products whose fragmentation gave molecular ions at m/z 119 or 175. Figure 2 shows typical chromatograms obtained for m/z 119 (A) and m/z 175 (B). This analysis allowed us to identify, in addition to the endoperoxide (labeled i), a large number of aldehydic oxidation products listed in Figure 3 (a–h). We also found a parent product at m/z 272 that was tentatively identified as a possible epoxide derivative (Fig. 2j). A similar analysis performed on the oxidation products of lutein and zeaxanthin also led to the identification of an endoperoxide and different aldehydes (Supplemental Fig. S2). In a second step, the oxidation products being identified, we used the multiple reaction monitoring mode (MRM) to exclusively focus our analyses on the masses of the identified aldehydes and improve the sensitivity of detection. Figure 4B shows a chromatogram that represents the sum of all the detected products generated by the oxidation of β-carotene, together with the chromatogram obtained using absorbance detection at 450 nm (Fig. 4A). The different products were individualized with the m/z values of their corresponding $[M+H]^+$ ions in Supplemental Figure S3 (for β-carotene oxidation products) and Supplemental Figure S4 (for lutein/zeaxanthin oxidation products). For both β-carotene and xanthophylls, oxidation by $^1O_2$ generated a variety of aldehydes, with different chain lengths, in addition to an endoperoxide. The products e to g in Figures 3 and 4 correspond to β-apo-14′-carotenal, β-apo-10′-carotenal, and β-apo-8′-carotenal previously identified by Stratton et al. (1993) as major aldehydic products generated from $^1O_2$ oxidation of β-carotene. By analogy with the chromatograms shown in Bando et al. (2004), the peak (labeled 7) that appeared after the endoperoxide peak in Figures 1B and 4B can be assumed to be β-carotene 5,6-epoxide.

Using this sensitive approach, we analyzed the time course of the changes in the content of the endoperoxides and major aldehydes formed during $^1O_2$ oxidation of β-carotene, lutein, and zeaxanthin (Fig. 5). For β-carotene and lutein, the accumulation of the endoperoxide (m/z 569 and 602, respectively) occurred during the first 15 min of illumination and then declined. A similar transitory accumulation of β-carotene endoperoxide was reported for bacteriochlorophyll-photosensitized oxidation of β-carotene in vitro (Fiedor et al., 2001). In

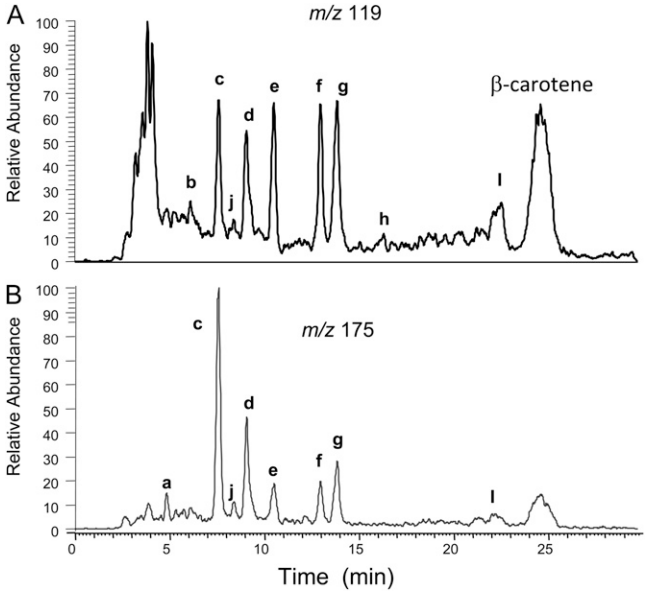

Figure 2. Analysis of β-carotene oxidation products by HPLC-MS/MS in the precursor ion scan mode. Scan of the fragment ions $[M+H]^+$ at *m/z* 119 (A) or *m/z* 175 (B). Ten peaks were detected and labeled a to j.

liposomes, it has been shown that β-carotene endoperoxide is more sensitive to oxidation than β-carotene itself (Stratton and Liebler, 1997), and this may explain the depletion of the endoperoxide observed in our experiments for long oxidation treatments. The aldehydes derived from β-carotene followed the same trend, whereas oxidation of lutein in vitro led to a very low formation of aldehydic oxidation products. Oxidation of zeaxanthin appeared to be slower than that of β-carotene and lutein, with significant accumulation of zeaxanthin endoperoxide and zeaxanthinals occurring after 30 min of illumination only.

In Vivo Carotenoid Oxidation

Knowing the variety of products that can be produced in vitro by $^1O_2$ oxidation of β-carotene and xanthophylls, we looked for those oxidation products in leaves of Arabidopsis plants exposed to photooxidative stress conditions (photon flux density of 1,400 μmol m^{-2} s^{-1} combined with air temperature of 7°C). Among the products listed in Figure 3, we were able to detect, using the sensitive MRM approach, significant levels of β-carotene endoperoxide only. Carotenals were present in very low, hardly measurable amounts. As shown in Figure 6A, the β-carotene endoperoxide levels increased rapidly to reach a plateau after about 4.5 h in high light. For long exposures to high light (>51 h), the endoperoxide levels decreased back to the initial level measured before stress. This suggests plant acclimation to light stress, leading to decreased production of $^1O_2$ or possible secondary decomposition of initially produced endoperoxide, as observed in vitro. The involvement of $^1O_2$ was checked with $^1O_2$ sensor green (SOSG), a probe that becomes fluorescent in the presence of $^1O_2$ (Flors et al., 2006). Attached leaves were infiltrated with the fluorescent probe using a syringe, and the plants were subsequently exposed for 30 min to high light. $^1O_2$ was produced in leaves during the light treatment, as monitored by the SOSG fluorescence at 525 nm (Fig. 6B). However, $^1O_2$ production was noticeably lower in plants acclimated for 99 h to high light compared with control, unacclimated plants. In contrast with the carotene endoperoxide accumulation, the lutein/zeaxanthin endoperoxide levels in Arabidopsis leaves remained low throughout the light stress treatment (Fig. 6A). Thus, β-carotene appears to be a preferential in vivo target of $^1O_2$ compared with xanthophylls. $^1O_2$ is believed not to be produced in PSI (Hideg and Vass, 1995). To check this aspect, we analyzed the β-carotene endoperoxide levels in Arabidopsis leaves after illumination with blue-green or far-red light. Exciting PSI with far-red light (70 W m^{-2}) did not induce the formation of the β-carotene endoperoxide, whereas preferential excitation of PSII with blue-green light (same irradiance) was associated with a substantial increase in this compound after 8 h (Supplemental Fig. S5). These findings are in line with the idea that PSI is not a major source of $^1O_2$ in leaves.

In parallel with carotenoid oxidation products, we measured the expression of several genes that have been identified as specific $^1O_2$ markers in the $^1O_2$-overproducing *flu* Arabidopsis mutant, such as MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE18 (MAPKKK18), DISEASE RESISTANCE PROTEIN (DRP), and BON1-associated protein1 (BAP-1; op den Camp et al., 2003). The three genes were induced by the stress treatment, confirming the production of $^1O_2$ during illumination of the plants at low temperature (Fig. 7). Interestingly, the increased expression of these genes was cancelled for

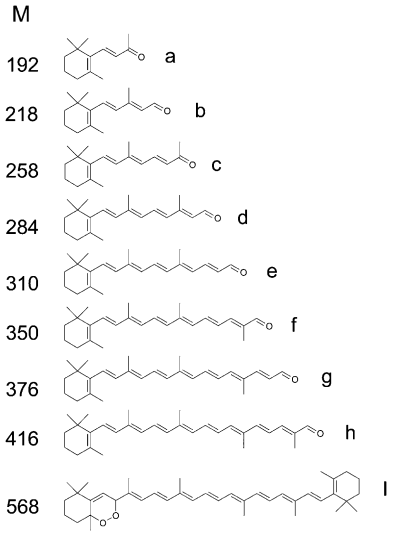

Figure 3. List of $^1O_2$ oxidation products of β-carotene identified by HPLC-MS/MS with their corresponding molecular mass (M). The letters correspond to the labels of the HPLC peaks in Figure 2.

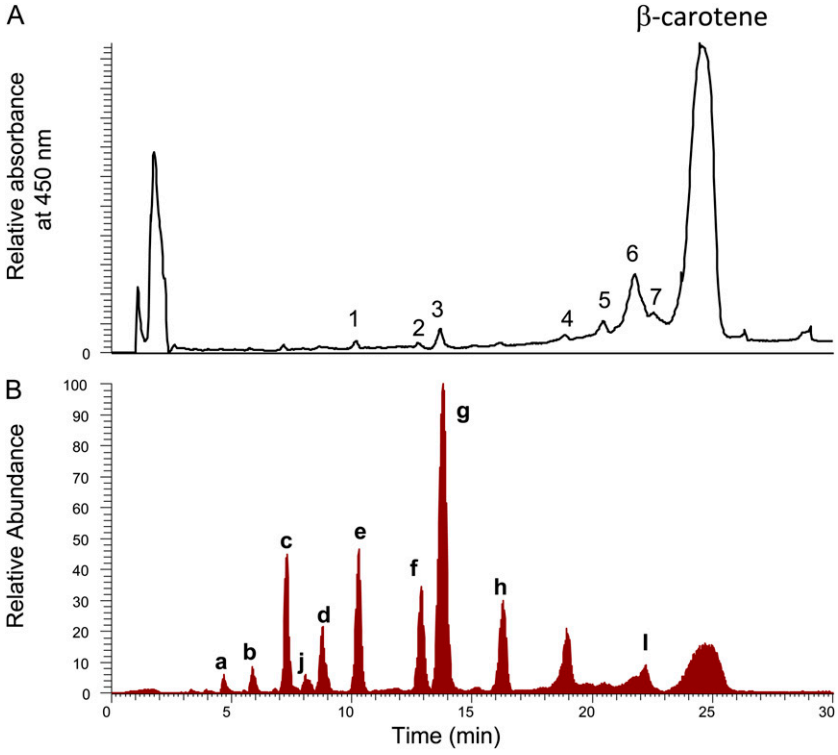

Figure 4. Chromatograms of $^1O_2$ oxidation products of β-carotene. A, UV-visible chromatogram at 450 nm showing the peaks 1 to 7 observed in Figure 1B. B, Precursor ion scan chromatogram of detected compounds (a–j, see Fig. 3) that give a common fragment ion at *m/z* 119. The peak at elution time 24.5 min is β-carotene. [See online article for color version of this figure.]

long stress treatments (>51 h), as was the accumulation of β-carotene endoperoxide, showing a correlation between the $^1O_2$ genetic markers and the β-carotene endoperoxide accumulation.

Photooxidative stress in plant cells is associated with oxidation of macromolecules such as lipids (Girotti and Kriska, 2004; Montillet et al., 2004; Triantaphylidès et al., 2008). Using HPLC and UV detection, we quantified accumulation of hydroxy fatty acids (hydroxy octadecatrienoic acid [HOTE]) in leaves (Fig. 8A). No significant increase in lipid peroxidation was observed before 2 d of light stress. Clearly, photoinduced accumulation of

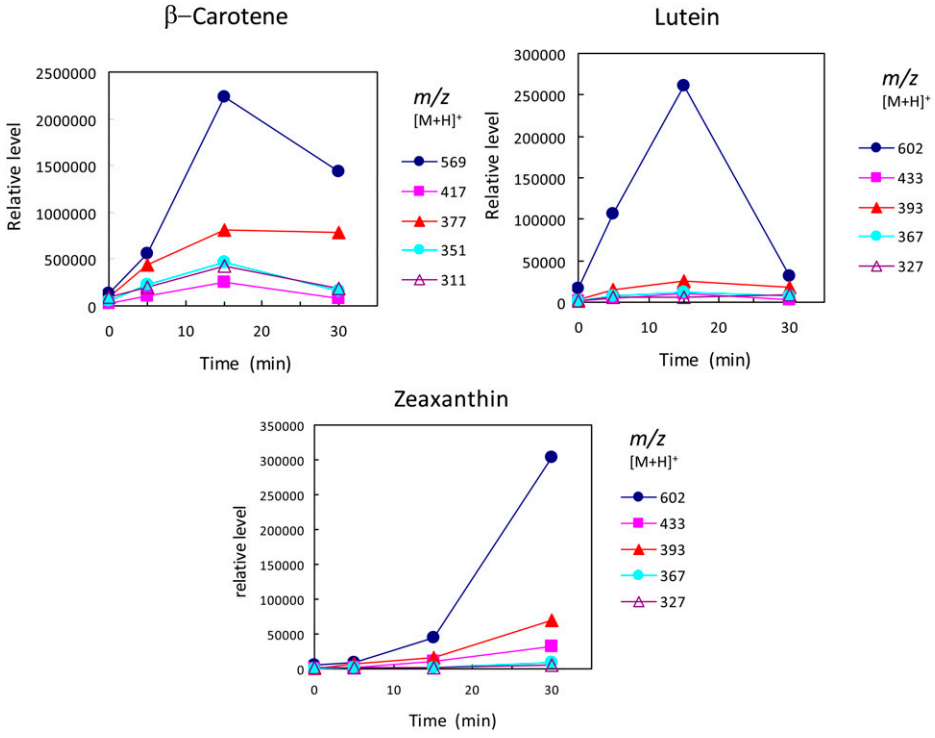

Figure 5. Time course of the changes in various β-carotene and xanthophyll oxidation products generated during illumination of the carotenoids in the presence of Rose Bengal or methylene blue in toluene/methanol, as determined by MRM analysis of the transitions leading to the *m/z* 119 ion (β-carotene) or the *m/z* 175 ion (xanthophylls); the parent ions are indicated on the right of each graph (*m/z* $[M+H]^+$).

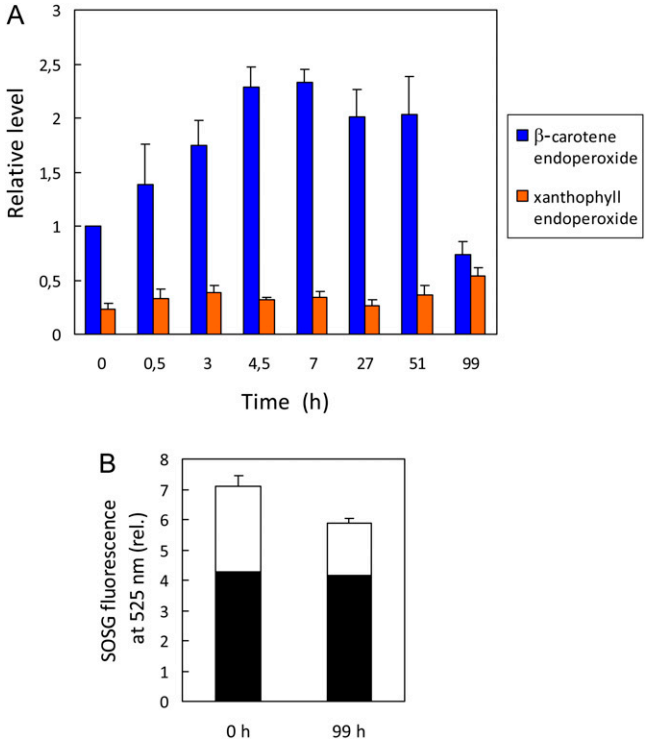

Figure 6. A, Time course of the changes in β-carotene and lutein/zeaxanthin endoperoxide levels in Arabidopsis leaves during high-light stress at low temperature (1,400 μmol photons m^{-2} s^{-1}, 7°C). Because of similar mass, lutein and zeaxanthin endoperoxides could not be distinguished. Data are mean values of four separate measurements + SD. B, Photoinduced $^1O_2$ production in Arabidopsis leaves grown in low light (0 h) or acclimated for 99 h to high-light stress at low temperature. $^1O_2$ was measured by the increase in SOSG fluorescence intensity after 30-min illumination at 1,400 μmol photons m^{-2} s^{-1} at 7°C (in white). The SOSG fluorescence level before the 30-min illumination is indicated in black. Data are mean values of five separate measurements +SD. [See online article for color version of this figure.]

β-carotene endoperoxide (Fig. 6A) was faster than HOTE accumulation, indicating that the carotenoid oxidation product is an early indicator of $^1O_2$ stress in plants.

The decrease in β-carotene endoperoxide during long stress exposures suggests that this product can be metabolized by plants or that it spontaneously decomposes. Therefore, we analyzed the changes in the β-carotene endoperoxide content of leaves following transfer of the plants from high light to darkness (Fig. 9). We observed a rather rapid decline of the endoperoxide, with a 50% loss of the product content being reached after about 6 to 7 h in the dark.

We measured the β-carotene endoperoxide levels in leaves of different plant species (Supplemental Fig. S6). The compound was found in all plant species, suggesting that it is a universal $^1O_2$ marker.

β-Carotene Endoperoxide in Arabidopsis Mutants

The changes in β-carotene endoperoxide levels were negatively correlated with PSII photoinhibition; accumulation of the endoperoxide was accompanied with a decrease in the PSII photochemical efficiency (maximum photochemical efficiency of PSII in the dark-adapted state [F_v/F_m]) and vice versa (Fig. 8B). PSII photoinhibition was also accompanied by a decrease in β-carotene content (Fig. 8C) and, for long exposure times (>7 h), by a decrease in the D1 protein (Fig. 8D).

The *npq4* mutant of Arabidopsis is deficient in photoprotective thermal energy dissipation (NPQ) in the PSII antennae, leading to increased excitation delivery to the PSII centers and hence to increased sensitivity to PSII photoinhibition (Niyogi et al., 1998). Exposing *npq4* Arabidopsis plants to the combination of high light and low temperature was observed to lead to enhanced PSII photoinhibition relative to the wild type (Havaux and Kloppstech, 2001). As shown in Figure 10, this treatment also brought about an increased accumulation of β-carotene endoperoxide relative to the wild type, Columbia-0 (Col-0). In contrast, the tocopherol-deficient *vte1* mutant did not differ from the wild type (Col-2) in terms of β-carotene endoperoxide accumulation. Consistently, α-tocopherol did not inhibit the formation of β-carotene 5,8-endoperoxide during photooxidation of β-carotene in methyllinoleate (Yamauchi et al., 1998). The double mutant *npq4 vte1* appeared to behave like the single *npq4* mutant under high-light stress, displaying increased β-carotene endoperoxide content compared to the wild type. We also examined the endoperoxide level in a catalase-deficient mutant (Fig. 10). The function of catalase is to detoxify H_2O_2 produced by photorespiration in the peroxisomes, so that the ROS initially produced in this mutant during high-light stress are free radicals. High-light stress induced an increase in the β-carotene endoperoxide levels, but this effect was not more pronounced in the *cat2* mutant compared with the wild type, confirming that this compound is specific to $^1O_2$ attack on β-carotene.

DISCUSSION

Chemical Quenching of $^1O_2$ by Carotenoids

This study has identified a rather large range of products generated during the in vitro oxidation of β-carotene and xanthophylls by $^1O_2$, thus extending previous studies (Stratton et al., 1993; Yamauchi et al., 1998; Fiedor et al., 2001; Montenegro et al., 2002). More precisely, the presented results confirm the formation of an endoperoxide that appears to be a major oxidation product for both β-carotene and xanthophylls. This study also shows that $^1O_2$ is able to cleave every double bond of the β-carotene polyene chain, resulting in a variety of aldehydes (carotenals) with different chain lengths. Xanthophylls behaved similarly, although the variety of detected aldehydes was lower. Importantly, carotenoid endoperoxide is specifically formed by cycloaddition of $^1O_2$ on the carotenoid molecule, as expected from the known reactivity of

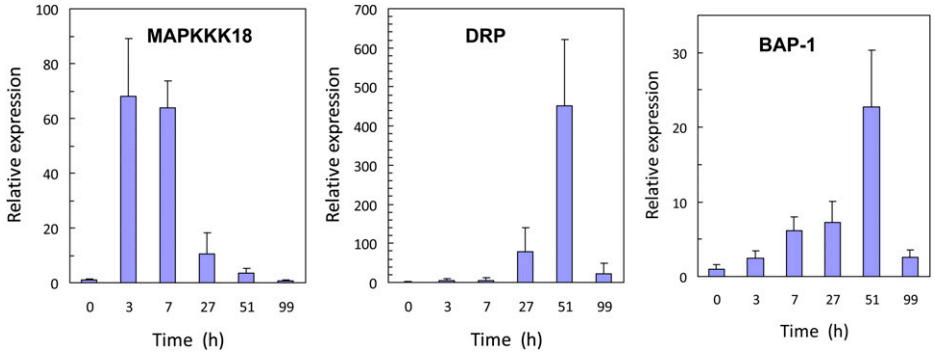

Figure 7. Time course of the changes in the transcript levels of several $^1O_2$ gene markers in Arabidopsis leaves exposed to high-light stress at low temperature. Selected genes were: MAPKKK18 (At1g05100), DRP (At1g57630), and BAP-1 (At3g61190). Data are mean values of four to five independent measurements + SD. [See online article for color version of this figure.]

$^1O_2$. Because this compound cannot be formed by enzymatic oxidation or other ROS, it can be considered as a specific $^1O_2$ marker.

The rate of formation of zeaxanthin endoperoxide in the in vitro oxidation experiments appeared to be slower than the formation of β-carotene and lutein endoperoxides. Similarly, zeaxanthinals were formed later during the in vitro $^1O_2$ treatment than luteinals and carotenals. This suggests that zeaxanthin is less rapidly oxidized by $^1O_2$ compared with the other carotenoids tested in this study. Zeaxanthin is known to play a crucial role in the photoprotection of plants (Ruban and Johnson, 2010; Jahns and Holzwarth, 2012), with zeaxanthin-deficient or -overexpressing plants exhibiting a strongly reduced or increased tolerance to oxidative stress, respectively (Havaux and Niyogi, 1999; Johnson et al., 2007). Moreover, the in vivo antioxidant capacity of zeaxanthin has been shown to be higher than that of all other xanthophylls (Havaux et al., 2007; Dall'Osto et al., 2010), although the mechanism(s) behind this enhanced antioxidant capacity is unknown. Based on our results, the increased antioxidant activity of zeaxanthin in planta relative to lutein does not seem to be related to an enhanced capacity of $^1O_2$ chemical quenching. Similarly, zeaxanthin does not seem to differ from lutein and β-carotene in terms of $^1O_2$ physical quenching efficiency; the rate constant for physical quenching of $^1O_2$ in solvents has been reported to be in the same range for those carotenoids (Conn et al., 1991; Edge et al., 1997; Triantaphylidès and Havaux, 2009). Accordingly, a previous study has shown that the antioxidant activity of zeaxanthin is empowered by interaction with antenna proteins, but this effect could not be explained by a change in $^1O_2$ scavenging activity, suggesting the involvement of an additional mechanism that remains to be identified (Dall'Osto et al., 2010).

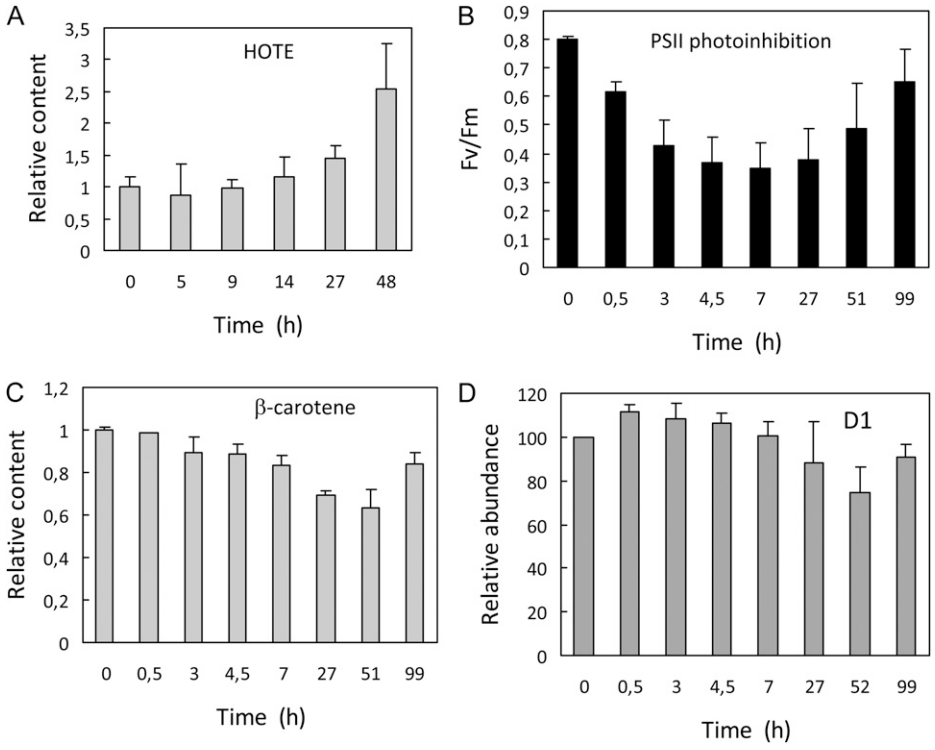

Figure 8. A, Accumulation of HOTEs in Arabidopsis leaves under high-light stress at low temperature (1,400 μmol m^{-2} s^{-1} at 7°C). Data are normalized to the initial HOTE level measured at time 0. B, PSII photoinhibition measured by the F_v/F_m chlorophyll fluorescence ratio. C, Loss of β-carotene. D, Changes in D1 protein abundance. Mean values of three to 10 separate measurements + SD.

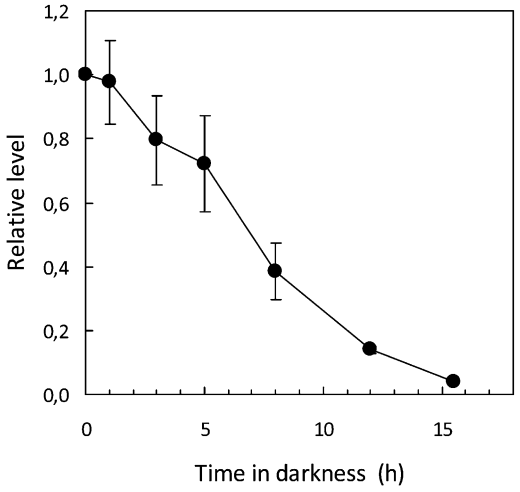

Figure 9. β-Carotene endoperoxide decay in the dark. Arabidopsis plants were pre-exposed for 3 h to high light at low temperature (1,400 μmol photons m^{-2} s^{-1}, 7°C) and then transferred to darkness at time 0. Data are normalized to the endoperoxide level at time 0 and are mean values of three separate measurements + SD.

We searched for carotenoid oxidation products in Arabidopsis leaves exposed to high-light stress, and we found carotenoid endoperoxides only; no significant level of carotenoid aldehydes was measured in vivo. The lack of detectable amounts of aldehydes could be due to the high reactivity of those compounds, which is because of the presence of reactive α,β-unsaturated carbonyl moiety. They can act as reactive electrophile species, reacting with nucleophilic targets, such as e.g. Cys residues in glutathione or proteins, and hence escaping detection (Farmer and Davoine, 2007; Mueller and Berger, 2009). Moreover, aldehyde conjugation to glutathione is a detoxification process catalyzed by glutathione *S*-transferases (Wilce and Parker, 1994). Aldehyde detoxification might also be carried out by alcohol or aldehyde dehydrogenases (Hideg et al., 2003; Sunkar et al., 2003; Kotchoni et al., 2006). Alternatively, aldehyde production during carotenoid oxidation could be lower than the formation of the endoperoxide, as suggested by the differential accumulation of carotenoid endoperoxide and aldehydes during in vitro oxidation. Accordingly, β-carotene endoperoxide is believed to be the primary product generated by $^1O_2$ oxidation of β-carotene in vitro (Montenegro et al., 2002). The presence of β-carotene endoperoxide in low- or high-light-grown plants indicates that chemical quenching of $^1O_2$ by carotenoids does occur in plants. This mechanism is generally overlooked in studies of the antioxidant functions of carotenoids because physical quenching of $^1O_2$ is assumed to be the main mode of action of those compounds (Edge et al., 1997; Stahl and Sies, 2003). However, the accumulation of β-carotene endoperoxide reported here under photooxidative stress conditions indicates the occurrence of chemical quenching activity of carotenoids as an additional photoprotective mechanism that takes place in plant tissues.

A previous in vitro study (Fiedor et al., 2005) showed that β-carotene continues to decay in the dark after previous irradiation in the presence of a photosensitizer. This phenomenon was explained by the reactivity of the carotene endoperoxides produced during $^1O_2$ oxidation, which can promote β-carotene autooxidation. Consequently, $^1O_2$-induced formation of β-carotene endoperoxides from β-carotene and the accumulation of the latter compounds in planta during high-light stress can have important physiological implications by promoting oxidation of carotenoids and possibly other molecules, such as lipids, hence propagating oxidative stress in plant cells. The decay of β-carotene endoperoxide observed in Arabidopsis leaves placed in darkness could reflect this phenomenon. Alternatively, carotenoid endoperoxides could be metabolized by some enzymatic processes such as CCDs. Cleavage of carotenoids or carotenoid-derived molecules by CCDs can generate a variety of oxidized compounds (Bouvier et al., 2005; Walter and Strack, 2011). Some of those products are involved in plant defense or architecture (Gomez-Roldan et al., 2008; Tsuchiya et al., 2010; Dor et al., 2011). In animals, ROS-induced oxidized carotenoid derivatives have been reported to be biologically active, playing a role in enzyme inhibition, changes in gene expression, transcription activation, or apoptosis (Siems et al., 2000; Sharoni et al., 2004; Kuntz et al., 2006; Lindshield et al., 2007; Kalariya et al., 2008). Although a signaling function of oxidized carotenoids has not been reported

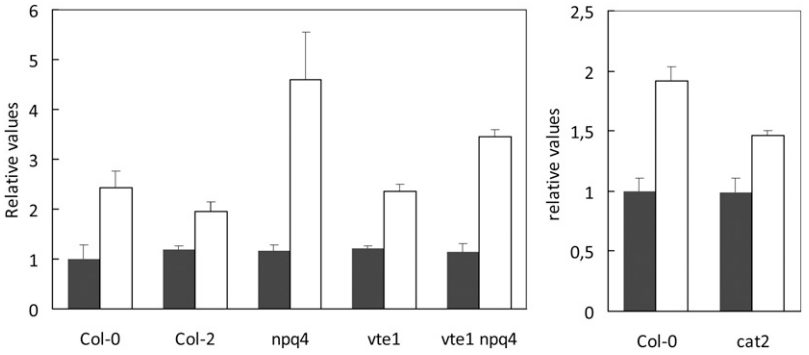

Figure 10. Changes in β-carotene endoperoxide levels in wild-type Arabidopsis plants (ecotypes Col-0 and Col-2) and mutant plants (single mutants *cat2*, *npq4*, and *vte1*, and double mutant *npq4 vte1*) after 3-h exposure to high light at low temperature (1,400 μmol photons m^{-2} s^{-1}, 7°C). Black bars, Control plants before light treatment. White bars, Light-treated plants. Data are normalized to the endoperoxide level in control Col-0 plants and are mean values of three separate measurements.

in plants exposed to photooxidative stress, the $^1O_2$ chemical quenching activity of carotenoids reported here is a potential source of such bioactive molecules.

β-Carotene Endoperoxide, an Early Marker of $^1O_2$ Stress

In plants, the half-life of β-carotene endoperoxide in darkness was about 6 h, so that this compound in leaves disappeared almost completely during the night period (16 h). Therefore, the presence of β-carotene endoperoxide in Arabidopsis plants grown in normal light (200 μmol photons m^{-2} s^{-1}) suggests chronic oxidation of β-carotene by $^1O_2$. This is in line with hydroxy fatty acid analyses previously performed on Arabidopsis leaves; significant levels of lipid oxidation products specifically produced by $^1O_2$ were found in leaves of low-light-grown leaves (Triantaphylidès et al., 2008). Upon high-light stress (1,400 μmol m^{-2} s^{-1}), β-carotene endoperoxide increased rapidly, reaching a new steady state within around 4.5 h. The increase was much faster than the accumulation of lipid oxidation products, indicating that this carotenoid oxidation product constitutes an early index of $^1O_2$ production and toxicity. This was confirmed by the correlation found between the endoperoxide levels and the expression of various $^1O_2$ marker genes and the levels of $^1O_2$ as measured by SOSG fluorescence. The accumulation of β-carotene endoperoxide during high-light stress was also associated with changes in β-carotene concentration, suggesting that a significant fraction of the β-carotene pool was oxidized and converted to the endoperoxide. For instance, after 7 h in high light, approximately 15% of the pool was lost, suggesting that chemical quenching of $^1O_2$ by carotenoids is a significant, physiologically relevant process. However, it is difficult to compare directly the β-carotene endoperoxide and β-carotene levels. Firstly, part of the β-carotene losses, especially after long exposure times, is not necessarily an exclusive effect of $^1O_2$ and could also result from reaction with free radicals. Secondly, the consumption of β-carotene by oxidative reactions can be partially compensated by de novo synthesis, possibly leading to underestimation of the extent of β-carotene oxidation. ^{14}C pulse-chase labeling experiments revealed a continuous flux of newly fixed carbon into β-carotene in photosynthesizing leaves transferred to high light (Beisel et al., 2010), suggesting rapid turnover of this pigment, in the hour time scale. Surprisingly, no evidence was found for ^{14}C incorporation into xanthophylls, and this could be related to the maintenance of a low level of lutein/zeaxanthin endoperoxide observed in the present study in high-light-exposed Arabidopsis leaves. Taken together, those findings suggest a low turnover of the xanthophylls compared to β-carotene.

The Source of $^1O_2$ in Vivo

The oxidation of β-carotene into β-carotene endoperoxide by $^1O_2$ has been reported in vitro, but the presence of this compound in plant leaves is not documented. Also, we are not aware of any previous work describing the products generated by the oxidation of lutein or zeaxanthin by $^1O_2$ and the formation of endoperoxides from those carotenoids. In the present study, both xanthophyll endoperoxide and β-carotene endoperoxide were detected in planta. However, lutein/zeaxanthin endoperoxide appeared to occur at a very low level in Arabidopsis leaves and, contrary to β-carotene endoperoxide, it did not accumulate in leaves exposed to high-light stress. In plant leaves, $^1O_2$ is supposed to be produced from triplet chlorophylls in PSII (Krieger-Liszkay, 2005; Triantaphylidès and Havaux, 2009). Until now, attempts to measure $^1O_2$ in PSI has been unsuccessful, and this photosystem is thus assumed not to produce $^1O_2$ (Hideg and Vass, 1995). This assumption is confirmed by the absence of significant levels of β-carotene endoperoxide induced by PSI excitation with high-irradiance far-red light.

In PSII, β-carotene is localized in the reaction center, whereas xanthophyll carotenoids are present in the chlorophyll antennae (Siefermann-Harms, 1985). High-resolution crystal structure of PSII revealed the presence of 11 β-carotenes (Umena et al., 2011). There are no available data on the relative production of $^1O_2$ in the PSII reaction center and its antennae; therefore, the main source of $^1O_2$ in planta is still a matter of debate (Rinalducci et al., 2004; Krieger-Liszkay, 2005; Mozzo et al., 2008). However, the increased formation of β-carotene endoperoxide in leaves exposed to light stress without concomitant accumulation of lutein/zeaxanthin endoperoxide points at the PSII reaction centers as a major site of $^1O_2$ accumulation in chloroplasts. The distance between the reaction center pigment P680 and the two β-carotene molecules bound to the D1/D2 protein complex of the PSII reaction centers is believed to be too large to enable direct quenching of triplet P680 by the carotenes (Trebst, 2003; Telfer, 2005). This suggests that PSII centers do produce $^1O_2$ and that the primary function of the carotenes must be restricted to $^1O_2$ quenching. Conversely, a close distance between xanthophylls and chlorophylls is maintained in the PSII antennae by the proteins that coordinate the pigments (Kühlbrandt et al., 1994; Liu et al., 2004). Chlorophyll-to-carotenoid triplet transfer can thus occur, limiting $^1O_2$ production in the PSII antennae (Dall'Osto et al., 2006). As a consequence, the probability of $^1O_2$ production is higher in the PSII centers compared to the light-harvesting antennae. The preferential accumulation of β-carotene endoperoxide during high-light stress is consistent with this idea.

Increased energy delivery to the PSII reaction centers in the *npq4* mutant deficient in the energy dissipation mechanism NPQ was reported to increase PSII inactivation (Niyogi et al., 1998; Havaux and Kloppstech, 2001) and, as shown here, it is also associated with an enhancement of the formation of β-carotene endoperoxide. On the contrary, mutational suppression of tocopherol, an antioxidant dissolved in thylakoid membrane lipids, in the *vte1* mutant had no impact on the endoperoxide

concentration under high-light stress. Tocopherols are known to protect PSII from photoinhibition (Havaux et al., 2005; Krieger-Liszkay et al., 2008). Our results indicate that they do not fulfill their protective function by preventing $^1O_2$ oxidation of β-carotene. This is consistent with the idea that the role of tocopherols is to protect the repair of photodamaged PSII rather than to modulate the rate of damage of PSII (Inoue et al., 2011). The β-carotene endoperoxide levels were correlated with the photoinhibition of PSII, as measured by the decrease in the F_v/F_m chlorophyll fluorescence parameter. For stress treatments longer than 7 h, the inhibition of PSII photochemical activity was also associated with a substantial loss of the D1 protein. The latter phenomenon is believed to be triggered by $^1O_2$ produced in the reaction center (Andersson and Aro, 2004) and therefore can be considered as an indirect index of $^1O_2$ production in the PSII reaction centers. The correlation found in this study between β-carotene endoperoxide levels, D1 levels, and PSII photochemical efficiency is in line with the idea that $^1O_2$ originates from PSII centers under high light and reacts with PSII reaction center components, such as β-carotene and D1 protein.

To sum up, this study has shown that carotenoids, and among them especially β-carotene, can act in vivo as chemical quenchers of $^1O_2$, with β-carotene endoperoxide being a major oxidation product generated from this activity in planta. The latter product provides a new internal probe of $^1O_2$ production, which could be used for the early detection of $^1O_2$ stress in plants. It should be stressed that other carotenoid metabolites generated by $^1O_2$ oxidation of carotenoids, such as aldehydes, can be also formed enzymatically by CCDs and are therefore not specific to $^1O_2$. The selective accumulation of β-carotene endoperoxide in leaves exposed to high-light stress, with no concomitant accumulation of the corresponding oxidation product of xanthophylls, identifies the PSII reaction centers, rather than the PSII chlorophyll antennae, have a major source of $^1O_2$ in plants.

MATERIALS AND METHODS

$^1O_2$ Oxidation of β-Carotene, Lutein, and Zeaxanthin

β-Carotene, zeaxanthin, or lutein (80 μg), supplemented with Rose Bengal or Methylene Blue, were dissolved in 7 mL of toluene:methanol (85:15, v/v). The carotenoid-photosensitizer mixes were kept at 7°C while bubbled with O_2 under illumination (450 $\mu mol \cdot m^{-2} s^{-1}$ photon flux density) provided by HQI metal halide lamps (Osram) for 0, 5, 15, and 30 min. Photooxidized samples were subsequently evaporated to dryness under an N_2 stream and kept at −80°C until analysis. Carotenoid oxidation was monitored by UV-visible HPLC of the photooxidized samples resuspended in methanol. HPLC was performed with a Waters HPLC system equipped with a Waters Nova-Pak C18 column (4 μm, 3.9 × 300 mm), as previously described (Havaux et al., 2005). The detection wavelength was 445 nm.

Plant Material

Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Columbia [Col-0] or Col-2) were grown from seeds on soil under low light (250 μmol photons $m^{-2} s^{-1}$; day/night, 8/16 h; 20°C/18°C; 55% relative humidity) for 4 to 5 weeks. For comparison purposes, the following Arabidopsis mutants were also used: the PSII protein PsbS-deficient mutant *npq4* (Li et al., 2000), the tocopherol-deficient mutant *vte1* (Porfirova et al., 2002), the double mutant *npq4 vte1* (Havaux et al., 2005), and the catalase-deficient *cat2* mutant (Vandenabeele et al., 2004). The *npq4* and *cat2* mutants were obtained in the Col-0 background, whereas the wild-type ecotype of *vte1* was Col-2. The catalase-deficient *cat2* plants were grown under nonphotorespiratory conditions by increasing CO_2 in the growth chamber to 2,000 μL L^{-1}. Subsequently, plants were subjected to photooxidative stress by being transferred in controlled growth chambers at the beginning of the day period at low temperature (day/night, 8/16 h; 7°C/10°C) and high light (1,400 μmol $m^{-2} s^{-1}$) with 380 ppm atmospheric CO_2. Leaf samples were collected at different time points of stress, weighed before freezing in liquid nitrogen, and stored at −80°C. Carotenoids were extracted from leaves in 100% acetone and analyzed by UV-visible HPLC, as previously described (Havaux et al., 2005).

HPLC-MS Characterization of $^1O_2$ Oxidation Products of β-Carotene, Lutein, and Zeaxanthin

Dry oxidized samples were redissolved in 500 μL of acetone, supplemented with 500 μL of ammonium formate (2 mM), and 10 μL of the obtained mix was injected onto the HPLC-MS/MS system that was composed by an Agilent HP1100 HPLC system connected to an API3000 triple quadrupole mass spectrometer (Applied Biosystems). Oxidation compounds of carotenoids were retained on an Uptisphere C8 (3 μm, 20 × 150 mm) obtained from Interchim maintained at 28°C. The mobile phase was a gradient of 2 mM ammonium formate and acetonitrile at a flow rate of 200 μL min^{-1}. The proportion of acetonitrile rose from 50% to 95% for 20 min, and then the conditions were maintained for 5 min to elute oxidation products of β-carotene and of xanthophylls. After each sample run, the HPLC system was flushed with acetonitrile: ammonium formate (2 mM; 98:2, v/v) for 5 min to remove strongly retained residues, followed by a 15-min equilibration period with the initial mobile phase composition before the next injection. All MS analyses were performed in positive electrospray ionization mode. First, total ion chromatograms (TICs) and UV/visible chromatograms at 450 nm were produced. The TICs were acquired by scanning from m/z 80 to 700, and corresponding mass spectra of the compounds within the TIC peaks were obtained, thereby identifying the molecular ions of interest. Subsequently, product ion scan experiments of each ion of interest were used to determine and optimize the specific transition characteristics of each compound (MS/MS). The selected transitions were used to perform MRM experiments on $^1O_2$ oxidized reference carotenoids and on photooxidized leaf samples. Each transition was monitored using a 50-ms dwell time, collision energy being 40 eV. Ionization potential was 5,500 V.

RNA Isolation and Quantitative Reverse Transcription-PCR

For each condition, fresh material was harvested on at least three different plants and immediately frozen in liquid nitrogen. Total RNA was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel) following the manufacturer's protocol and then treated with the Turbo DNA-*free* (Ambion) according to the manufacturer's instructions. Each extraction procedure was performed at least five times.

Quantitative reverse transcription (qRT)-PCR experiments were carried out with cDNA synthesized with the SuperScript III ReverseTranscriptase (Invitrogen) from 500 ng of total RNA. Resulting cDNAs were then diluted and used to determine expression profiles according to the different conditions. Specific primers for each gene selected for analysis were designed using Primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The designed primer sequences were verified by comparing them with sequences of the Arabidopsis transcript, using TAIR BLAST (http://www.arabidopsis.org/Blast/). The oligonucleotides used for qRT-PCR are: BAP-1 (At3g61190, 5′-TAAACCGGAGACCCATCAAG-3′, 3′-TCGACATTTCTCGTCGATTTT-5′), MAPKKK18 (At1g05100, 5′-TAAAATCCGCCGAGTTTCAC-3′, 3′-ATCCGATGACGTACGGAGAG-5′), and DRP (At1g57630, 5′-CAAACAGGCGATCAAAGGAT-3′, 3′-CAACACCACGAAGAAGCGTA-5′). The profiline *PRF1* (At2g19760, 5′-AGAGCGCCAAATTTCCTCAG-3′, 3′-CCTCCAGGTCCCTTCTTCC-5′) has been taken as housekeeping gene to normalize the expression of genes of interest. qRT-PCR was performed using LightCycler 480 SYBR Green I Master (Roche) in the qPCR thermal cycler (LightCycler 480 Real-Time PCR System, Roche). Each reaction was prepared using 2 μL of cDNA diluted 20-fold, 2 μL of SYBR Green I Master, and 1 μM forward and reverse primers in a total volume of 5 μL. The amplification profile consisted in 95°C for 10 min and 45 cycles of 95°C denaturation for 15 s, annealing at 62°C for 15 s, and extension at 72°C for 15 s. All reactions were performed in triplicate.

D1 Protein Quantification

After grinding the leaves in liquid N_2, powder was resuspended in 50 mM Tris-HCl, pH 8, 50 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, agitated for 30 min at 4°C, and centrifuged (10,000*g*, 4°C, 10 min). The pellet was resuspended in 50 mM Tris-HCl, pH 8, and 1% SDS, agitated for 1 h at 4°C, and centrifuged (10,000*g*, 4°C, 30 min). The membrane proteins presented in the supernatant were precipitated at −20°C by addition of 4 acetone volumes for 2 h.

Proteins, separated by SDS-PAGE, were electroblotted onto 0.45-μm nitrocellulose in 25 mM Tris, pH 6.8, 200 mM Gly, 0.1% SDS, and 10% ethanol. The antibody PsbA D1 (PsbA, D1 protein of PSII, DE-loop, Agrisera) was used at a dilution of 1:10,000 in 25 mM Tris-HCl, pH 8, 140 mM NaCl, 2.7 mM KCl, and 0.2 Tween 20. Bound antibodies were detected using the Alexa fluor 680 goat anti-rabbit IgG (Invitrogen) at the dilution 1:10,000 in 25 mM Tris-HCl, pH 8, 140 mM NaCl, 2.7 mM KCl, and 0.2 Tween 20. Immunodetection was visualized by the LI-COR Odyssey Biosciences and quantified with the software Odyssey 2.1.

Fluorescence Determination of $^1O_2$ Production

Attached leaves were slowly infiltrated with 100 μM SOSG (Invitrogen) under pressure with a syringe. A 1-mL syringe, without needle and filled with the solution to be infiltrated, was pushed against the lower surface of the leaf, and the solution (100 μL) was forced to enter the inside of the leaf under pressure. Plants with SOSG-infiltrated leaves were exposed for 30 min to white light of photon flux density 1,400 μmol photons m^{-2} s^{-1}. SOSG fluorescence was excited at 475 nm and recorded at 525 nm.

PSII Photoinhibition

The maximal quantum yield of PSII photochemistry was measured as the F_v/F_m chlorophyll fluorescence ratio using a PAM-2000 fluorometer (Walz). F_m is the maximal chlorophyll fluorescence induced by a short pulse (800 ms) of intense white light and Fv is $F_m - F_o$, where F_o is the initial fluorescence level measured with a weak, nonactinic red light modulated at 600 Hz. A 2-s pulse of far-red light was used to oxidize Q_A and to measure the true F_o level.

Lipid Peroxidation

Hydroxy fatty acids (HOTEs) were analyzed by HPLC using the method of Montillet et al. (2004) as previously described (Johnson et al., 2007; Triantaphylidès et al., 2008).

Supplemental Data

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

Supplemental Figure S1. UV-visible HPLC elution profile of xanthophyll oxidation products generated by the reaction with $^1O_2$ produced by methylene blue in the light in toluene/methanol solvent mixture.

Supplemental Figure S2. List of $^1O_2$ oxidation products of zeaxanthin identified by HPLC-MS/MS.

Supplemental Figure S3. MRM chromatograms of individualized aldehydic β-carotene oxidation products generated in vitro from the reaction with $^1O_2$.

Supplemental Figure S4. MRM chromatogram of the zeaxanthin oxidation products generated from the reaction with $^1O_2$.

Supplemental Figure S5. β-Carotene endoperoxide levels in detached Arabidopsis leaves during illumination with blue-green light (400–600 nm) or far-red light (>715 nm).

Supplemental Figure S6. β-Carotene endoperoxide levels in various plant species grown in low or high light.

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