Nonenzymic carotenoid oxidation and photooxidative stress signalling in plants

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Abstract

Carotenoids play a crucial protective role in photosynthetic organisms as quenchers of singlet oxygen (1O2). This function occurs either via a physical mechanism involving thermal energy dissipation or via a chemical mechanism involving direct oxidation of the carotenoid molecule. The latter mechanism can produce a variety of aldehydic or ketonic cleavage products containing a reactive carbonyl group. One such molecule, the volatile β-carotene derivative β-cyclocitrinal, triggers changes in the expression of 1O2-responsive genes and leads to an enhancement of photooxidative stress tolerance. Thus, besides their well-known functions in light harvesting and photoprotection, carotenoids can also play a role through their nonenzymic oxidation in the sensing and signalling of reactive oxygen species and photooxidative stress in photosynthetic organisms. Enzymic carotenoid oxidation does not seem to play a significant role in this phenomenon. Elucidation of the carotenoid-mediated 1O2 signalling pathway could provide new targets for improving photooxidative stress tolerance of plants.

Key words: Carotenoid, carotenoid cleavage dioxygenase, photooxidative stress, reactive oxygen species, signalling, singlet oxygen.

Introduction

Carotenoids are tetraterpenoids containing 40 carbon atoms and 3–13 conjugated double bonds in their skeleton (Fig. 1). They play two key roles in photosynthetic organisms: they serve as accessory pigments in the photosystems, increasing light absorption in the blue spectral domain (420–500 nm), and they protect the photosynthetic apparatus against toxic reactive oxygen species (ROS), especially singlet oxygen (1O2) produced from triplet excited chlorophylls (3Chl*). The latter function relies on the capacity of carotenoids to quench both 3Chl* and 1O2 with a high efficiency by a physical mechanism involving excitation transfer followed by harmless thermal energy dissipation (Edge et al., 1997; Triantaphylidès and Havaux, 2009). Additional protective functions of carotenoids include stabilization of membrane lipid bilayers (Havaux, 1998), scavenging of free radicals and protection against membrane lipid peroxidation (Lim et al., 1992; Johnson et al., 2007), and regulation of light harvesting by quenching singlet excited chlorophylls (3Chl*) in the PSII chlorophyll antennae (Horton and Ruban, 2005; Jahns and Holzwarth, 2012). 1O2 is believed to be the main ROS produced in chlorophyll-containing cells during strong illumination (González-Perez et al., 2011), and it is instrumental in the development of photooxidative damage to leaves (Triantaphylidès et al., 2008). Accordingly, carotenoid-less mutants of photosynthetic organisms are highly photosensitive, suffering extensive oxidative damage and displaying high frequency mutagenesis due to 1O2 overproduction in the light (Ouchane et al., 1997). Two types of carotenoids are found in vascular plants: the unoxygenated carotenoids (e.g. β-carotene and lycopene) and the O2-containing
Endoperoxides, epoxides, and lactones were also reported during oxidation by 1O2 (Ramel et al. 1998). Similarly to β-carotene, xanthophylls can also produce endoperoxides (Stratton et al., 1998). The 1O2 backbone can be oxidized by 1O2, leading to a variety of aldehydes and ketones (Stratton et al., 1998). In vitro oxidation of β-carotene by 1O2 showed that carotenoids can be directly oxidized by 1O2, producing a variety of oxidized derivatives. In fact, each double bond in the carotenoid backbone can be oxidized by 1O2, leading to a variety of aldehydes and ketones (Stratton et al., 1993; Ramel et al., 2012a). Endoperoxides, epoxides, and lactones were also reported during 1O2 oxidation of β-carotene (Yamauchi et al., 1998; Fidor et al., 2001, 2005; Bando et al., 2004). Among those products, β-carotene-5,8-endoperoxide (Fig. 1) is believed to be the primary and the most abundant compound formed during photosensitized oxidation of β-carotene by 1O2 (Montenegro et al., 2002). Similarly to β-carotene, xanthophylls can also produce endoperoxides upon in vitro oxidation by 1O2 (Ramel et al., 2012a). Carotenoid peroxides can promote carotenoid autooxidation and possibly oxidations of other species, and therefore they can propagate oxidative stress in cells (Fidor et al., 2005). Interestingly, carotenoid endoperoxides cannot be formed enzymically or by free radical oxidation and consequently they can be considered as specific markers of 1O2 production and 1O2 attack on carotenoids.

Carotenoid metabolites, including the 1O2-specific β-carotene-5,8-endoperoxide, were found in vivo in different animal and human tissues, such as skin and eye tissues (Bernstein et al., 2001; Bando et al., 2004; Bhosale and Bernstein, 2005). Recently, some of the products identified during in vitro 1O2 oxidation of carotenoids were also detected in plants (Ramel et al., 2012a). In particular, β-carotene-5,8-endoperoxide was detected in leaves of low-light-grown plants, suggesting chronic oxidation of β-carotene by 1O2. Moreover, high-light stress was found to induce a rapid accumulation of this compound in leaves, and this accumulation was correlated with the extent of PSII photooxygenation and the loss of β-carotene. In contrast, xanthophyll endoperoxide levels were comparatively low and did not change with high-light stress. This difference cannot be explained by a differential reactivity of β-carotene and xanthophylls with 1O2; the accumulation rate of the endoperoxide during in vitro oxidation by 1O2 was found to be similar for β-carotene and lutein (Ramel et al., 2012a). The selective accumulation of β-carotene endoperoxide in high-light-exposed leaves suggests that the β-carotene-containing PSII reaction centre, rather than the xanthophyll-containing PSII chlorophyll antennae, is a major site of 1O2 accumulation in photosynthetic organisms. Likely, this is due to the fact that xanthophylls and chlorophylls are in very close proximity in the PSII antennae, allowing chlorophyll-carotenoid triplet transfer and efficient quenching of 3Chl* (Mozzo et al., 2008). In contrast, β-carotene molecules are located relatively far from chlorophylls in the PSII reaction centre, and therefore they cannot easily quench 3Chl*. The function of β-carotene in PSII is then supposed to be restricted to 1O2 quenching (Telfer et al., 2005). Photooxidative stress conditions can also induce the formation of a variety of volatile short-chain aldehydic or ketonic derivatives of β-carotene, such as β-ionone or β-cyclocitrinal (Fig. 1), in photosynthetic microorganisms (Walsh et al., 1998) and in vascular plants (Ramel et al., 2012b). In Arabidopsis, the accumulation of β-carotene oxidation products is fast, occurring within minutes during high-light stress and preceding the accumulation of fatty acid oxidation products (Ramel et al., 2012a,b). There is no report of equivalent effects on volatile compounds derived from xanthophylls. Nevertheless, 3-hydroxy-β-ionone, a xanthophyll cleavage product, was measured in the hypocotyls of dark-grown bean seedlings and exhibited some changes in concentration upon transfer to light (Kato-Noguchi, 1994).

**Oxidation of carotenoids by 1O2**

In vitro experiments with carotenoid solutions supplemented with a 1O2 generator (such as rose bengal or methylene blue) showed that carotenoids can be directly oxidized by 1O2, producing a variety of oxidized derivatives. In fact, each double bond in the carotenoid backbone can be oxidized by 1O2, leading to a variety of aldehydes and ketones (Stratton et al., 1993; Ramel et al., 2012a). Endoperoxides, epoxides, and lactones were also reported during 1O2 oxidation of β-carotene (Yamauchi et al., 1998; Fidor et al., 2001, 2005; Bando et al., 2004). Among those products, β-carotene-5,8-endoperoxide (Fig. 1) is believed to be the primary and the most abundant compound formed during photosensitized oxidation of β-carotene by 1O2 (Montenegro et al., 2002). Similarly to β-carotene, xanthophylls can also produce endoperoxides upon in vitro oxidation by 1O2 (Ramel et al., 2012a). Carotenoid peroxides can promote carotenoid autooxidation and possibly oxidations of other species, and therefore they can propagate oxidative stress in cells (Fidor et al., 2005). Interestingly, carotenoid endoperoxides cannot be formed enzymically or by free radical oxidation and consequently they can be considered as specific markers of 1O2 production and 1O2 attack on carotenoids.

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**Enzymic oxidation of carotenoids**

It should be emphasized that carotenoid oxidation is not mediated exclusively by ROS, but it can also occur in vivo through the action of specialized enzymes, the carotenoid cleavage dioxygenases (CCDs). The latter enzymes can cleave carotenoid molecules at particular positions, generating specific apocarotenoids (Bouvier et al., 2005; Floss and Walter, 2009). For instance, in Arabidopsis, AtCCD7 cleaves carotenoids at the 9,10 and/or 9',10' double bonds of the chromophore, producing β-ionone and β-apo-10'-carotenol from β-carotene. Subsequently, AtCCD8 can convert β-apo-10'-carotenol into β-apo-13-carotenol and a dialdehyde. AtCCD1 produces two β-ionone molecules and a C13 dialdehyde. CCD and CCD-related enzymes are involved in the synthesis of the hormones abscisic acid and strigolactones in plants, and also they catalyse the synthesis of aroma and flavour volatiles.
of flowers and a variety of foods (Auldridge et al., 2006). CCD are ancestral enzymes found in bacteria, plants, and animals. In animals, the CCD enzyme BCDO1 is involved in retinoid synthesis while BCDO2 is a mitochondrial CCD playing a crucial role in carotenoid homeostasis and protecting against mitochondrial dysfunction and oxidative stress (Amengual et al., 2011).

The regulation of CCD genes by abiotic or biotic stresses is largely unknown. An induction of NosCCD by high light was reported in the cyanobacterium Nostoc (Scherzinger and Al-Babili, 2008), but similar effects are not documented in vascular plants. We analysed by quantitative reverse-transcription PCR the transcript levels of the 4 AtCCD genes (AtCCD1, AtCCD4, AtCCD7, and AtCCD8) of Arabidopsis during high-light stress (Fig. 2A–D). For comparison purposes, we analysed also the expression levels of ELIP2 (Fig. 2E), a typical light-responsive gene (Heddad and Adamska, 2002). In contrast with ELIP2 whose expression strongly increased (by a factor of about 250) in response to the light treatment, none of the AtCCD genes exhibited a substantial induction. Expression levels of AtCCD1, AtCCD7, and AtCCD8 remain virtually unchanged throughout the high-light treatment (Fig. 2A,2C,2D), as was the case for the four CCD genes in control samples kept in low light (data not shown). On the contrary, a strong downregulation was observed for AtCCD4 during high-light stress. This is consistent with microarray-based transcriptomic analyses of Arabidopsis plants which showed similar effects of high-light stress on CCD gene expression (http://urgv.evry.inra.fr/CATdb; project CEA-02_Light). The synthesis of β-cyclocitral and β-ionone in knockout mutants for each of the four AtCCD genes was also analysed (Fig. 2F). The levels of those carotenoid metabolites in the AtCCD mutants were not reduced compared to the wild-type level. In fact, an increase in β-cyclocitral was even observed in the ccd1 mutant compared to wild type. Taken together, those results do not support the idea that enzymic oxidation is a major phenomenon in the production of oxidized carotenoid metabolites during photooxidative stress. Although the cleavage of carotenoids by the Arabidopsis CCDs is rather specific, we cannot exclude some overlapping activities between the different CCDs which could partially mask the effects of the mutations in the ccd mutants.

**Biological functions of carotenoid oxidation products in animals and microorganisms**

Many compounds derived from the oxidation of carotenoids, including the volatile carotenoid cleavage products shown in Fig. 1, contain a carbonyl function adjacent to a double bond. These α,β-unsaturated carbonyl groups are electrophilic: they are electron acceptors which can react with nucleophilic (electron donor) atoms common to many biological molecules (Farmer and Davoine, 2007; Mueller and Berger, 2009). In particular, reactive electrophile species (RES) are known to have a
high thioli-reactivity and, consequently, they are able to modify proteins in vivo. The reactivity of carotenoid-derived RES may, however, depend on structural factors, such as the relative position of the methyl group to the terminal aldehyde (Linnwiel et al., 2009). In animal cells, oxidized carotenoid derivatives have been reported to have various effects, including cytotoxicity (Lakshminarayana et al., 2010), inhibition of mitochondrial respiration (Siems et al., 2002), induction of P450 cytochrome (Jeong et al., 1998), enzyme inactivation (Siems et al., 2000; Hurst et al., 2005), DNA damage (Yeh and Wu, 2006; Kalariya et al., 2009), retinoid signalling antagonism or promotion (Kuntz et al., 2006; Eroglu et al., 2012), and induction of apoptosis (Janakiram et al., 2008; Kalariya et al., 2008; Liu et al., 2008). In a variety of insects, β-carotene-oxidized derivatives are components of pheromones (Rocca et al., 1983). Thioli modification by electrophilic lipids has been shown to activate transcription factors, hence inducing gene responses (Levonen et al., 2004). Similarly, lycopene-derived oxidized metabolites are active is the regulation of gene expression with some overlaps with the gene expression profile of retinoic acid (Gouranton et al., 2011; Reynaud et al., 2011).

High concentration of carotenoid RES can have inhibitory effects on photosynthesis in cyanobacteria (Shao et al., 2011). However, some beneficial effects have also been identified for the production of β-cyclocitrinal in those organisms, such as grazer repelling (Jüttner et al., 2010). By producing the carotenoid metabolite 3-hydroxy-β-ionone, some mosses inhibit growth of neighbouring plants, thus maintaining pure colonies. β-ionone and related compounds have been reported to have antifungal activity (Kato-Noguchi and Seki, 2010). The adduct compound can then be actively transported across membranes (Klein et al., 2006). Consistently, a number of glutathione-S-transferase genes were shown to be strongly induced by 1O2 oxidation of a carotenoid acts as a chloroplastic messenger in the 1O2 signalling pathway, pointing at a new function for carotenoids in stressed plants (Fig. 3). β-Cyclocitrinal is a volatile, lipid-soluble compound that should be able to cross lipid membranes and therefore it is a potential candidate for transfer of information out of the chloroplast. Alternatively, RES can conjugate reversibly with reduced glutathione, in particular through glutathione-S-transferase catalysis (Davoine et al., 2005). The adduct compound can then be actively transported across membranes (Klein et al., 2006).

Carotenoid oxidation products in photooxidative stress signalling in plants

It is known that carotenoid cleavage products can have signalling functions in plants. In particular, the carotenoid-derived strigolactones are terpenoid lactones that constitute a class of hormones newly discovered to be involved in the control of shoot branching (Gomez-Roldan et al., 2008). Apocarotenoids are also believed to serve as a source of signalling molecules for the control of root colonization by arbuscular mycorrhizal fungi (Strack and Fester, 2006). As RES, carotenoid derivatives, such as β-cyclocitrinal and β-ionone, are potential signal molecules that accumulate in plants under photooxidative stress (Ramel et al., 2012b). The signalling function of RES has been studied mainly on fatty-acyclic-derived RES, such as malondialdehyde, hexenal, or cyclopentenone phytoprostanes. Low levels of exogenous oxylipin RES have been shown to affect gene transcription (Alméras et al., 2003; Weber et al., 2004; Loeffler et al., 2005; Mueller et al., 2008), with the most pronounced effects being observed for the transcript levels of cell survival genes and stress-related genes. This is accompanied by changes in protein abundances (Dueckershoff et al., 2008).

In a recent study, exposure of Arabidopsis plants to low levels of volatile β-cyclocitrinal, leading to internal concentrations close to the levels reached in high-light-treated plants, were found to change the expression of a large set of genes (Ramel et al., 2012b). Many genes induced by this carotenoid derivative belonged to categories related to cellular defence against stress and metabolism while repression concerned many genes related to development and biogenesis. Interestingly, most of the genes affected by β-cyclocitrinal (more than 80%) were identified as O2-responsive genes, and a rather large subset of the induced genes were classified as specific to 1O2, suggesting that β-cyclocitrinal is an intermediate in the signalling of this ROS in Arabidopsis. Moreover, these effects appeared to be specific to this compound since they were not observed with the related molecule β-ionone, and the gene expression profile induced by β-cyclocitrinal overlapped only very partially with the transcription changes induced by lipidic RES. Importantly, β-cyclocitrinal-induced changes in gene expression were associated with a substantial increase in the photore sistance of the treated plants. Taken together, those results lead to the conclusion that a molecule generated by 1O2 oxidation of a carotenoid acts as a chloroplastic messenger in the 1O2 signalling pathway, pointing at a new function for carotenoids in stressed plants (Fig. 3).

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β-Cyclocitrinal is a volatile, lipid-soluble compound that should be able to cross lipid membranes and therefore it is a potential candidate for transfer of information out of the chloroplast. Alternatively, RES can conjugate reversibly with reduced glutathione, in particular through glutathione-S-transferase catalysis (Davoine et al., 2005). The adduct compound can then be actively transported across membranes (Klein et al., 2006). Consistently, a number of glutathione-S-transferase genes were shown to be strongly induced by 1O2 (Ledford et al., 2007) and also by β-cyclocitrinal (Ramel et al., 2012b).

Intriguingly, gene expression reprogramming by carotenoid RES was found to be independent of the EXECUTER 1 protein and to lead to increased resistance to photooxidation (Ramel et al., 2012b). In contrast, the EXECUTER 1-dependent 1O2 signalling pathway characterized in the flu Arabidopsis mutant was shown to lead to cell death (Wagner et al., 2004). This difference may rely on the intensity of the gene responses, which was much lower with β-cyclocitrinal relative to the gene responses in the flu mutant. In other words, different 1O2 levels must be distinguished when considering the effects on gene expression: programmed cell death could be a response to a massive 1O2 production, such as that occurring in flu mutant leaves after a dark-to-light transition, while low 1O2 levels and/or modest changes in gene expression, such as those induced by β-cyclocitrinal, are associated with acclimation to 1O2 toxicity. In a xanthophyll mutant of Arabidopsis, 1O2 stress was also observed to induce changes in gene expression without causing cell death (Alboresi et al., 2011). Acclimation to 1O2 stress by chronic exposure to low 1O2 concentrations was previously reported in the green alga Chlamydomonas reinhardtii (Ledford et al., 2007), and this phenomenon was recently shown to involve activation of a RES-induced defence response (Fischer et al., 2012). An electrophile-response element was identified in the promoter region of many genes induced during 1O2 acclimation in this algal species. In vascular plants, a different mechanism was discovered: The promoters of a large fraction of the genes induced
by cyclopentenone oxylipins were shown to contain TGA motifs which constitute putative binding sites for TGA transcription factors (Mueller et al., 2008). Moreover, using mutants affected in the expression of TGA transcription factors, it was found that indeed those transcription factors are involved in mediating gene regulation of RES oxylipins. It is worth noting that electrophile-response elements do not bind to TGAs.

The finding that plastid carotenoid metabolites can trigger changes in the expression of nuclear genes points at a new function for carotenoids, besides their well-known light-harvesting and antioxidant roles. Because β-carotene oxidation appears to be an early event during photostress, β-carotene metabolites may constitute primary sensors of high-light stress in plants. The identification of carotenoid oxidation products as components of photooxidative stress signalling pathway opens new exciting avenues for future work. In particular, elucidation of the mode of action of electrophilic carotenoid cleavage products in the acclimatory response to 1O2 could provide new approaches to manipulate the signalling pathway and hence to improve photooxidative stress tolerance in plants. Because ROS production is an intrinsic property of photosynthesis (Li et al., 2009), resistance to ROS toxicity is a factor that must be taken into account in future attempts to improve photosynthesis under natural conditions.

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