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Impact and interaction of lipophilic antioxidants in mutants and transgenic plants

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Summary

Carotenoids and tocopherols are lipophilic antioxidants with important functions in plants and humans. Due to their nutritional value and putative health benefits, they have become a focus of intensive research. The identification of all genes of the carotenoid and tocopherol biosynthesis has enabled the manipulation of their biosynthetic pathways, aiming for quantitative and qualitative improvement. In plants, carotenoids and tocopherols are of crucial importance because of their protective abilities, which help to keep them alive even under light stress conditions. A wealth of information has accumulated concerning the responses of plants to various environmental stress factors. Here, we summarize some of the recent data concentrating on the impact and possible interaction of lipophilic antioxidants in mutants and transgenic plants with altered status of lipophilic antioxidants.

Introduction

In recent years, the importance of carotenoids and tocopherols has been increasingly recognized due to the emerging knowledge of their health benefits. Because humans can synthesize neither carotenoids nor tocopherols, they rely on their uptake through diet for the production of vitamin A and the supply with vitamin E (Bramley et al., 2000; Fraser and Bramley, 2004). Carotenoids are thought to exercise protective functions against certain cancers (Giovannucci, 2002) and prevent or ameliorate some chronic disease states (Mayne, 1996; Mares-Perlman et al., 2002). Similarly, a role of tocopherol in health protection and improvement of age-related ailments has been suggested (Nakagawa et al., 2004; Pfluger et al., 2004). However, the protective function of carotenoids

Abbreviations: LHC, light harvesting complex; NPQ, non-photochemical quenching; PS II, photosystem II; ROS, reactive oxygen species

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and tocopherols is not restricted to aspects of human health and nutrition, since both antioxidants are also involved in plant stress protection (Demmig-Adams and Adams, 2002).

In the following, we will focus on the impact and role of carotenoids and tocopherols as lipophilic antioxidants in relevant mutants and transgenic plants starting with a short overview of the carotenoid and tocopherol biosynthesis.

Biosynthesis of carotenoids

Carotenoids are tetraterpenoids derived from a C_5 isoprene unit (isopentenyl diphosphate, IPP) and are therefore biosynthetically linked with the synthesis of other isoprenoid compounds such as tocopherols, chlorophylls and plastoquinones. Detailed investigations of the isoprenoid biosynthesis and functional genomics have shown that the IPP

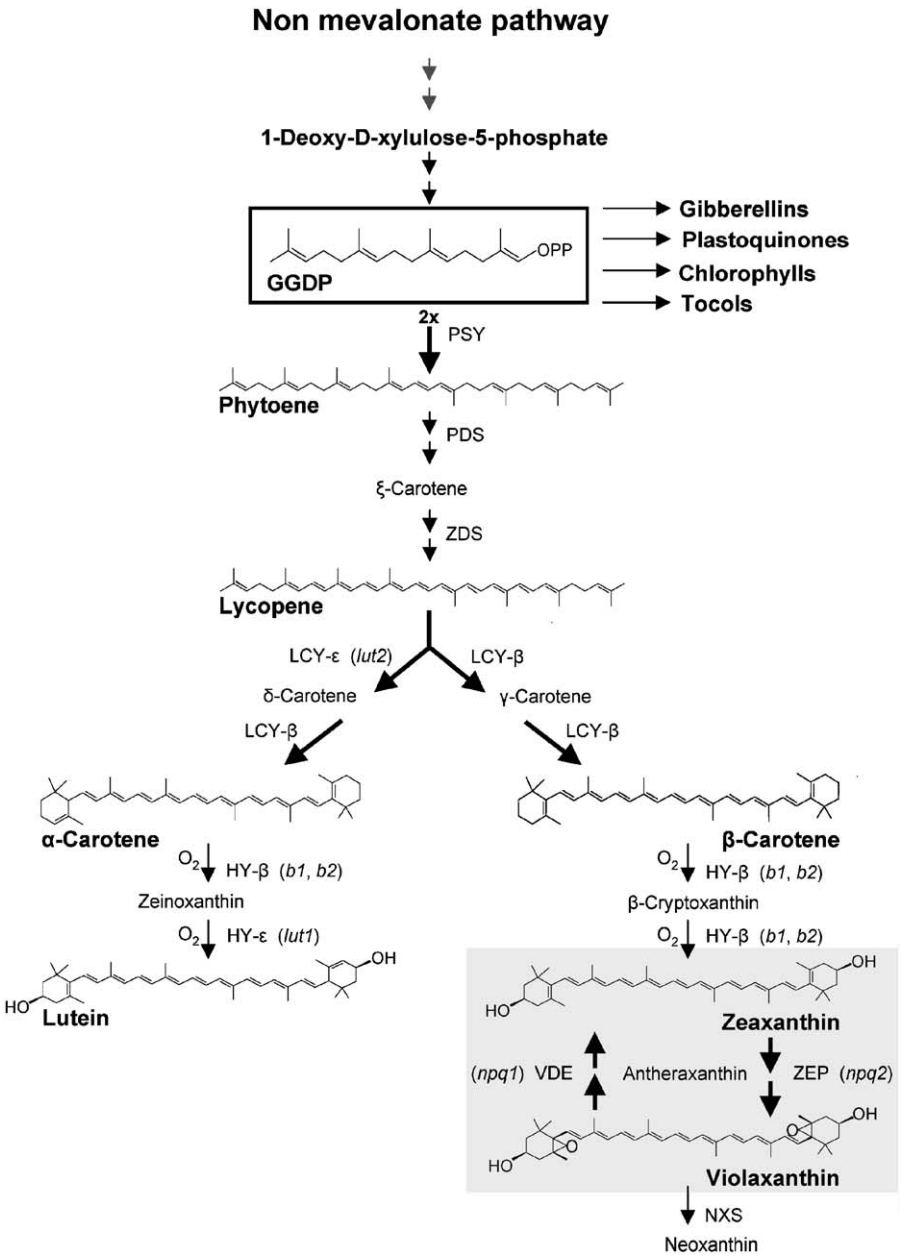

Figure 1. Biosynthesis of carotenes and xanthophylls. The xanthophyll cycle is highlighted in light gray. Enzymes are presented in gray, corresponding mutants are shown in brackets ( ). PSY: phytoene synthase, PDS: phytoene desaturase, ZDS: ξ-carotene desaturase, LCY-β: β-lycopene cyclase, LCY-ε: ε-lycopene cyclase, HY-β: β-carotene hydroxylase, HY-ε: ε-carotene hydroxylase, ZEP: zeaxanthin epoxidase, VDE: violaxanthin de-epoxidase, NXS: neoxanthin synthase.

used in the formation of carotenoids originates from the plastid localized mevalonic acid (MVA) independent pathway (Rodríguez-Concepción and Boronat, 2002). The carotenoid biosynthetic pathway has been recently reviewed (Fraser and Bramley, 2004), and a schematic outline is given in Fig. 1. The first step of carotenogenesis is the formation of phytoene from two molecules of geranylgeranyl diphosphate (GGDP) via the enzyme phytoene synthase (PSY). In plants, four desaturation steps yield the red colored lycopene that serves as a substrate of cyclization reactions leading to α- and β-carotene, respectively. Introduction of oxygen groups due to hydroxylation and epoxidation gives rise to the β-carotene derived xanthophylls (e.g. zeaxanthin, antheraxanthin and violaxanthin) and the α-carotene derivative lutein, the main xanthophyll in leaves. The genes encoding carotenoid biosynthetic enzymes in higher plants have been isolated by different approaches (Cunningham and Gantt, 1998), and map-based cloning has finally disclosed the identity of the α-carotene hydroxylase gene as a P450 type monooxygenase filling the final gap in the pathway (Tian et al., 2004).

Tocopherol biosynthesis

Vitamin E or tocochromanols (= tocols) comprise tocopherols and tocotrienols (Munné-Bosch and Falk, 2004). They are low molecular weight molecules of amphipathic nature. Their chemical properties are favorable for their membrane-bound location and enable an orientated position within the membrane: embedding of the hydrophobic tail in the membrane and exposure of the redox-active head group to the membrane surface. A summary of tocopherol and tocotrienol biosynthesis is depicted in Fig. 2. Tocopherol biosynthesis requires hydroxyphenylpyruvate provided by the shikimate pathway as a substrate for the production of the

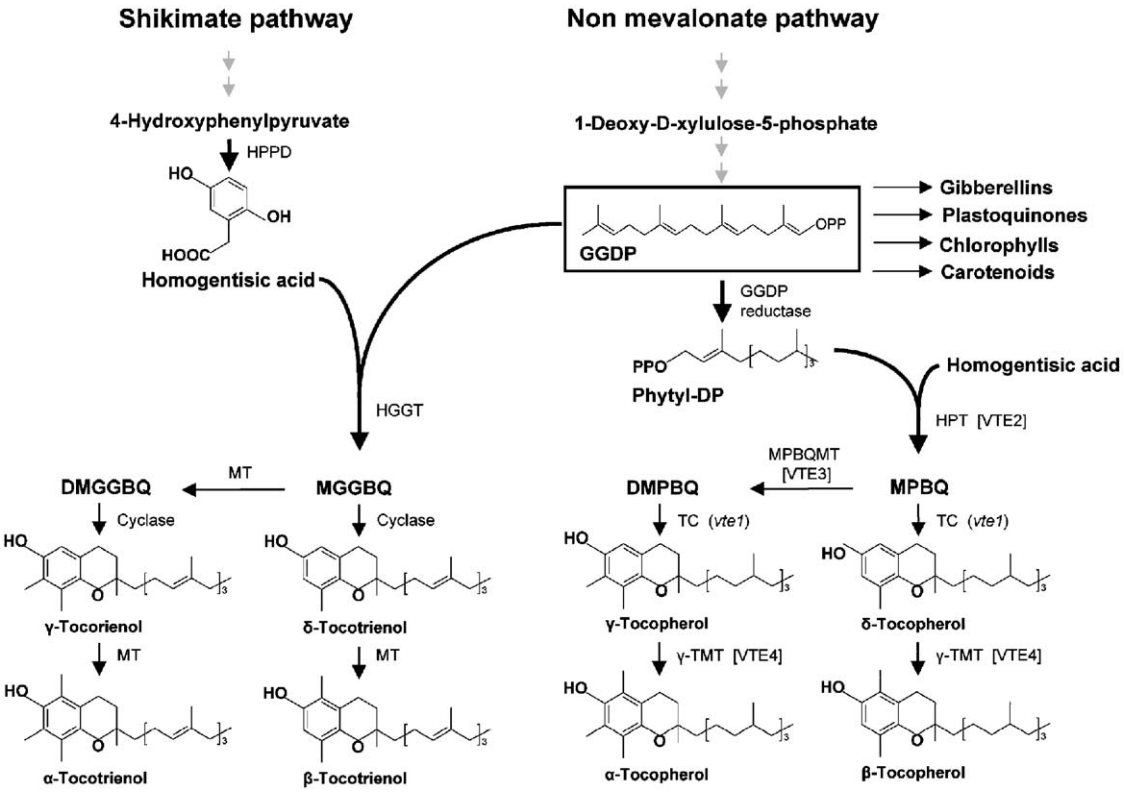

Figure 2. Biosynthesis of tocopherols and tocotrienols. Tocopherol synthesis starting from geranylgeranyl diphosphate (GGDP) is shown on the right-hand side of the figure, the conversion of GGDP to tocotrienols is presented on the left. Enzymes are illustrated in gray, corresponding names are shown in brackets [ ]. Mutants are shown in brackets ( ). HPT: homogentisate phytyltransferase, TC: tocopherol cyclase, γ-TMT: γ-tocopherol methyltransferase, MPBQ: methylphytylbenzoquinone, MPBQMT: methylphytylbenzoquinone methyltransferase, DMPBQ: dimethylphytylbenzoquinone, HGGT: homogentisic acid geranylgeranyl transferase, MGGBQ: methylgeranylgeranyl benzoquinone, MT: methyltransferase, DMGGBQ: dimethylgeranylgeranyl benzoquinol.

aromatic precursor homogentisate catalyzed by hydroxyphenylpyruvate dioxygenase (HPPD; Norris et al., 1998). The first step of tocopherol synthesis is the condensation of homogentisic acid (HGA) with phytyl diphosphate (PDP) to yield 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) by the membrane-bound enzyme homogentisate phytyltransferase (HPT1/VTE2). PDP is either derived via the non-mevalonic acid pathway from geranylgeranyl diphosphate or generated from phytol. MPBQ can then be further methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ). Both MPBQ and DMPBQ are subsequently subjected to cyclization by tocopherol cyclase (TC/VTE1) to yield either δ- or γ-tocopherol (Porfirova et al., 2002). Methylation of δ- and γ-tocopherol carried out by γ-tocopherol methyltransferase (γ-TMT/VTE4) leads to the formation of the end products β- and α- tocopherol, respectively.

In contrast to tocopherol biosynthesis, the formation of tocotrienols makes direct use of GGDP without prior modification. The condensation with homogentisic acid leads to the formation of 2-methyl-6-geranylgeranyl benzoquinone. This step in tocotrienol biosynthesis is catalyzed by the enzyme homogentisic acid geranygeranyl transferase (HGGT), whose gene sequence shows considerable sequence homology to *hpt* genes (Cahoon et al., 2003). The consecutive reactions are similar to the tocopherol synthesis including methylations and cyclization. Interestingly, both pathways indicate a role of GGDP as one of the key substrate necessary for the production of tocopherols and tocotrienols.

Protective function of carotenoids and tocopherols in plant stress protection

In excess light, high levels of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) accumulate in plants (Niyogi, 1999). This accumulation can lead to severe damage or even cell death, and represents a major threat to plant viability and productivity. Photoprotection is therefore an absolute necessity. Besides carotenoids and tocopherols plant protection involves the water-soluble low-molecular weight molecules ascorbate (vitamin C; Smirnoff, 2000a) and glutathione (Noctor and Foyer, 1998), the main antioxidants in chloroplasts. Ascorbate and glutathione are connected via the ascorbate–glutathione cycle. There is also a functional relationship between these water-soluble and lipophilic antioxidants. Ascorbate and glutathione are required for the regeneration of tocopherol, whereas ascorbate is a cofactor for the enzyme violaxanthin de-epoxidase (Eskling et al., 1997; Smirnoff, 2000b). Additionally, ROS are detoxified by a network of scavenging enzymes. These include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) (Mittler, 2002).

The protective function of carotenoids and tocopherols is rooted in their high antioxidative potential. Carotenoids (e.g. carotenes and xanthophylls) are indispensable for plant photoprotection and their role has been comprehensively reviewed (Niyogi, 1999; Baroli and Niyogi, 2000; Demmig-Adams and Adams, 1996, 2002). They are very efficient quenchers and scavengers of reactive oxygen species (ROS), and, in particular, of $^1O_2$. In higher plants, a dynamic protection mechanism, the xanthophyll cycle, enhances plant photoprotection (Demmig et al., 1987) by providing a way to safely dissipate excess light energy by non-photochemical quenching (NPQ). The xanthophyll cycle comprises the three xanthophylls zeaxanthin, antheraxanthin and violaxanthin and their reversible interconversion. Non-photochemical quenching is correlated with the high light induced accumulation of zeaxanthin as a consequence of violaxanthin de-epoxidation at low pH and requires the presence of the PsbS protein of photosystem II (Li et al., 2000; Demmig-Adams and Adams, 2002). Besides its functional role in thermal energy dissipation (Holt et al., 2005), an involvement of zeaxanthin in stabilization of the thylakoid membrane and protection against lipid peroxidation has been proposed (Havaux, 1998). In the thylakoid membrane, carotenoids are essential structural components of the light harvesting complexes where they serve as accessory pigments (Horn and Paulsen, 2002).

Similar to carotenoids, tocopherols are also involved in the quenching and scavenging of $^1O_2$ (Neely et al., 1988) and act as highly efficient recyclable chain reaction terminators for the removal of polyunsaturated fatty acid (PUFA) radical species generated during lipid oxidation (Munné-Bosch and Alegre, 2002; Munné-Bosch and Falk, 2004). Furthermore, tocopherols contribute to membrane stability by influencing its fluidity and permeability (Fryer, 1992) and might participate in protection of the D1 protein against high light (Trebst et al., 2002). The major tocochromanol in leaves is α-tocopherol, whereas seeds accumulate higher levels of tocotrienols (Grusak and DellaPenna, 1999).

Table 1. Characteristics of carotenoid biosynthesis mutants and transgenics of higher plants

Carotenoids	Mutants/ transgenics	Growth/ stress	Car	VAZ	NPQ	F_v/F_m	Toco	Hydrophilic antioxidants	Stress tolerance	Ref.
Early steps	*dxs* OE	normal	Car▲	n.d.	n.d.	n.d.	$▲_{\alpha-Toco}$	n.d.	n.d.	1
	psy OE	normal	Car▲ P▲	n.d.	n.d.	n.d.	n.d.	n.d.	dwarfism ▼	2
	psy AS	normal	Car▼	n.d.	n.d.	n.d.	n.d.	n.d.	lethal	2
	pds OE/ (*crtI* OE)	normal	Car±	▲	n.d.	n.d.	n.d.	n.d.	norfl. resist.	2,3,4
	pds AS	normal	Car(▼) P▲ C_β▼	n.d.	n.d.	n.d.	n.d.	n.d.	lethal	2
Cyclization/ isomerization	*crtISO*/ (*ccr1*/2)	etiolated	Lyc_{cis}▲		n.d.	n.d.	n.d.	n.d.	(▼)	5
Hydroxylation	*hy-β* OE	HL/heat/UV-B	Car(▲)	▲	±	±	n.d.	n.d.	▲	6,7
	hy-β AS	normal	Car▼ C_β▲ V N▼	▼	▼	n.d.	n.d.	n.d.	(▼)	8,9
	b1	normal	Car(±) L▲ V N▼	(▼)	(▼)	±	n.d.	n.d.	±	10
	b2	normal	Car(±) N▼	±	±	±	n.d.	n.d.	±	10
	b1 b2	normal	Car(▼) L▲ V N▼	▼	▼	(▼)	n.d.	n.d.	(▼)	10
	lut1	normal	Car(±) Z_{eino}V▲ L▼	▲	▼	±	n.d.	n.d.	(±)	11,12
	lut2	norm./HL	Car▼ C_β A▲ L▼	▲	▼	±	n.d.	n.d.		8,16
	lut1 b1	normal	*C_β Z_{eino}V▲ L N▼	▲	▼	(▼)	n.d.	n.d.	(▼)	10
	lut1 b2	normal	*C_β Z_{eino}V▲ L N▼	▲	▼	(▼)	n.d.	n.d.	(▼)	10
	lut1 b1 b2	normal	*C_β Z_{eino}▲ L V N▼	▼	▼	(▼)	n.d.	n.d.	(▼)	10,13
Epoxidation	*npq1*	HL/+cold	Car± C_β Z▼	(▲)	▼	▼/±	$▲_{\alpha-Toco}$ YL	n.d.	PI▼ ▼	14,15,16
	npq1 lut2	HL	Car▼ A▲ Z L▼	▲	▼	±	n.d.	n.d.	bleaching ▼	16
	npq4 npq1	HL	Car(±) C_β Z▼	±	▼	▼	n.d.	n.d.	▼	18
	vtc2 npq1	HL	Car▼	▼	▼	▼	$(▼)_{\alpha-Toco}$	$▼_{ASA}$ $▲_{GSH}$	▼	19,20
	npq2/(*aba1*)	HL/+cold	Car(±) Z▲ V (N)▼	▲	±	(▼)	n.d.	n.d.	PI▼	14,17
	lut1 aba1	seedlings	Car± C_βZZ$_{eino}$▲L▼		▼		n.d.	n.d.	vir.gr. (▼)	12
	lut2 aba1	seedlings HL/ +cold	Car▼ C_β Z▲ L▼	n.d.	▼	±	n.d.	n.d.	vir.gr. ▼	12,21
	vde OE	HL	Car(±) Z▲	±	▲	n.d.	n.d.	n.d.	n.d.	22
	vde AS	HL/HL+W	Car(±) Z▼	±	▼	±/▼	n.d.	n.d.	PI▲	23,24,25

Mutants and transgenic plants were grouped according to the step of the carotenoid biosynthetic pathway in which they are affected. AS: anti-sense, OE: over-expression. The following parameters were considered: carotenoid content and composition (Car), pool of violaxanthin+antheraxanthin+zeaxanthin (VAZ), non-photochemical quenching (NPQ), PSII efficiency (F_v/F_m), tocopherol content and composition (Toco) as well as hydrophilic antioxidants and the observed stress tolerance. ▲: increase, (▲): slight increase, ▼: reduction, (▼): slight reduction, ±: similar to wild type control, n.d.: not determined, normal: growth conditions (ranging from 70 to 150 $\mu mol\,m^{-2}\,s^{-1}$), HL: high light conditions (ranging from 700 to 2000 $\mu mol\,m^{-2}\,s^{-1}$), heat: heat stress (40 °C), UV-B: UV-B treatment, cold: cold stress (10 °C), W: water stress, *:carotenoid content was based on various parameters (compare references). P: phytoene, Lyc_{cis}: *cis*-lycopene, C_β: β-carotene, Z_{eino}: zeinoxanthin, L: lutein, Z: zeaxanthin, A: antheraxanthin, V: violaxanthin, N: neoxanthin, ASA: total ascorbate, GSH: glutathione, YL: young leaves, norfl. resist.: herbicide resistance to norflurazone, PI: photoinhibition, vir. gr.: virescent greening. References (Ref.): (1) Estévez et al., 2001; (2) Busch et al., 2002; (3) Wagner et al., 2002; (4) Misawa et al., 1994; (5) Park et al., 2002; (6) Davison et al., 2002; (7) Götz et al., 2002; (8) Pogson and Rissler, 2000; (9) Rissler and Pogson, 2001; (10) Tian et al., 2003; (11) Pogson et al., 1996; (12) Pogson et al., 1998; (13) Tian et al., 2004; (14) Niyogi et al., 1998; (15) Havaux et al., 2000; (16) Niyogi et al., 2001; (17) Leon-Klosterziel et al. (1996); (18) Havaux and Niyogi, 1999; (19) Müller-Moulé et al., 2003; (20) Müller-Moulé et al., 2004; (21) Havaux et al., 2004; (22) Hieber et al., 2002; (23) Chang et al., 2000; (24) Verhoeven et al., 2001; (25) Sun et al., 2001.

Table 2. Characteristics of mutants and transgenic plants modified in tocopherol biosynthesis

Tocopherols	Mutants/ transgenics	Growth/ stress	Car	VAZ	NPQ	F_v/F_m	Toco	Hydrophilic antioxidants	Stress tolerance	Ref.
Condensation	*hppd* OE	Normal	n.d.	n.d.	n.d.	n.d.	▲$_{seeds}$	n.d.	n.d.	1
	hpt1 /(*vte2*) OE	HL	Car±	n.d.	n.d.	n.d.	▲$_{seeds}$	n.d.	n.d.	2,3
	hpt1y-tmt OE	normal	n.d.	n.d.	n.d.	n.d.	▲$_{seeds+leaves}$	n.d.	n.d.	2
Methylation	*mpbqmt* /(*vte3*) OE	normal	n.d.	n.d.	n.d.	n.d.	▲$_{\alpha-Toco}$	n.d.	n.d.	4
	y-tmt /(*vte4*) OE	normal	n.d	n.d.	n.d.	n.d	shift $_{\gamma\rightarrow\alpha}$	n.d.	n.d.	4
Cyclization	*vte1*	HL	Car±	±	▲	n.d.	±/▼	▲$_{ASA}$ ▲$_{GSH}$	n.d.	5,6
	tc /(*vte1*) OE	HL	n.d.	n.d.	n.d.	n.d.	▲	▼$_{ASA}$ ▼$_{GSH}$	n.d.	6
	vte1 vtc1	HL	Car±	±	±	n.d.	n.d.	▼$_{ASA}$ ▲$_{GSH}$	±	6
	vte1 cad2	HL	Car▼	▼	±	n.d.	n.d.	▲$_{ASA}$ ▼$_{GSH}$	▼	6
	sxd1	HL	n.d.	n.d.	n.d.	n.d.	▼$_{\alpha+\gamma-Toco}$ ▼$_{Tocotrienol}$	n.d.	▼	7

Mutants and transgenic plants were grouped according to the step of the tocopherol biosynthetic pathway in which they are affected. As: anti-sense, OE: over-expression. The following parameters were considered: carotenoid content and composition (Car), pool of violaxanthin+antheraxanthin+zeaxanthin (VAZ), non-photochemical quenching (NPQ), PSII efficiency (F_v/F_m), tocopherol content and composition (Toco) as well as hydrophilic antioxidants and the observed stress tolerance. ▲: increase, (▲): slight increase, ▼: reduction, (▼): slight reduction; ±: similar to wild type control, n.d.: not determined, normal: growth conditions (ranging from 70 to 150 $\mu mol\, m^{-2}\, s^{-1}$), HL: high light conditions (ranging from 700 to 2000 $\mu mol\, m^{-2}$ s-1), ASA: total ascorbate, GSH: glutathione. References (Ref.): (1) Falk et al., 2003; (2) Collakova and DellaPenna, 2003a; (3) Collakova and DellaPenna, 2003b; (4) van Eenennaam et al., 2003; (5) Porfirova et al., 2002; (6) Kanwischer et al., 2005; (7) Hofius et al., (2004).

Interactions of antioxidants and putative compensation mechanisms

The assumption that carotenoids and tocopherols are indispensable for protection against high light and antioxidative stress, and may functionally replace each other is far too simplistic. A good comparison of the impact and interdependence of these lipophilic antioxidants is often hampered by the availability of relevant data, differences in stress conditions and variations in experimental design. The complexity and variety of plant stress protective systems adds to complicate the picture. Tables 1 and 2 summarize some current results focusing on the effects of altered carotenoid or tocopherol contents on plant stress tolerance and putative compensatory mechanisms.

For the purpose of increasing the amount of specific antioxidants, over-expression by genetic manipulation of the pathway is most desirable. Readers are referred to several excellent reviews considering recent achievements and developments (Ajjawi and Shintani, 2004; Fraser and Bramley, 2004; Sattler et al., 2004). However, this does not diminish the importance of mutants defective in certain steps of the biosynthetic pathways and the usefulness of transgenic antisense/RNAi plants with altered sets of lipophilic antioxidants to obtain a more thorough insight in function and interaction of these components in plants (cf. Baroli and Niyogi, 2000; Demmig-Adams and Adams, 2002).

Mutants and transgenic plants with alterations in early steps of carotenoid biosynthesis

Mutants and transgenic plants impaired in early steps of carotenoid biosynthesis (e.g. phytoene synthase and phytoene desaturase, and carotenoid isomerase) are devoid of colored carotenoids or accumulate only limited amounts of precursors (Sandmann, 1991; Römer, 1999; Park et al., 2002). They are unable to survive under normal growth and high light conditions and suffer severe photooxidation. Down-regulation of phytoene synthase (*psy*) or phytoene desaturase (*pds*) in antisense tobacco plants had a lethal effect (Busch et al., 2002). These findings illustrate the absolute necessity of carotenoids (Table 1). On the contrary, transgenic plants over-expressing *psy* (Busch et al., 2002) or *pds* (Misawa et al., 1994; Wagner et al., 2002) were viable. As shown previously, constitutive over-expression of *psy* caused a dwarf phenotype in plants with high expression levels of the transgene due to shortage of precursor supply for interconnected isoprenoid pathways such as gibberellic acid and chlorophyll biosynthesis (Fray et al., 1995). Interestingly, the over-expression of 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) in *Arabidopsis* resulted in an enhanced flux through the pathway and led to an increase in carotenoids and α-tocopherol (Estévez et al., 2001).

Mutants and transgenic plants with alterations in cyclization and isomerization

Cyclization of lycopene seems also to be crucial for the survival of photosynthetic organisms. This step is a key regulatory point of carotenoid biosynthesis, channeling metabolites either towards the α- or the β-branch of the pathway (Cunningham et al., 1996). To date, no photoautotrophic mutants could be isolated which are completely devoid of ε- and β-lycopene cyclase (LCY) activity demonstrating the enormous importance of carotenes with ionone ring structures (e.g. β-carotene) for photoprotection and plant viability. Co-suppression of β-*lcy* in tobacco decreased the amount of β-carotene and its derivatives and led to an augmentation of α-tocopherol, putatively as a compensation for the loss of carotenoids (Römer, personal communication). Plants over-expressing lycopene cyclases in a fruit-specific manner were outside the scope of this article and not considered. A defect in the isomerization blocked the formation of *all-trans*-lycopene, whereupon *cis*-lycopene accumulated in etiolated *Arabidopsis* plants (Park et al., 2002, Table 1).

Mutants and transgenic plants with altered carotene hydroxylation

Hydroxylation reactions are responsible for the formation of the xanthophylls lutein and zeaxanthin. Deficiencies in the carotene hydroxylation steps can be partially overcome (Table 1). This was elegantly shown using specific mutants of *Arabidopsis* termed *lut1* and *lut2* (Pogson et al., 1996, 1998). The *lut1* mutation disrupts ε-ring hydroxylation without affecting β-hydroxylation, and provides first genetic evidence for the existence of a distinct ε-ring specific hydroxylase (Pogson et al., 1996,1998) whose gene was recently cloned (Tian et al., 2004). As a consequence, the mutants were unable to synthesize the main leaf xanthophyll lutein (cf. Table 1). However, plant viability and photosynthetic capacity were not drastically affected. The absence of lutein can be clearly compensated for by the accumulation of nearly

equimolar levels of β-carotene derived xanthophylls (Pogson et al., 1998), thus structurally and functionally replacing this pigment. The capability to shift between the different branches of xanthophyll formation seems to be a general phenomenon in higher plants emphasizing the flexibility and adaptive ability of the photosynthetic apparatus. This assumption is corroborated by results of reconstitution experiments revealing that some of the binding sites in reconstituted light harvesting complexes (LHC) could be occupied by various xanthophylls (Bassi and Caffari, 2000). Whether the tocopherol content of these mutants is changed remains to be examined.

The impact of alterations in the synthesis of β-carotene derived xanthophylls is more complex. Hydroxylation of β-carotene leads to the production of zeaxanthin by β-carotene hydroxylase. Several structural β-carotene hydroxylase genes have been isolated (Tian and DellaPenna, 2004). Complementing previous investigations using various carotenoid mutants, the generation of T-DNA knockout mutants of *Arabidopsis* for β-carotene hydroxylases (*b1*, *b2*) and the availability of the *lut1* mutant together with the production of β-carotene hydroxylase antisense plants (Rissler and Pogson, 2001) have enabled a detailed compositional carotenoid analysis (Tian et al., 2003; Tian and DellaPenna, 2004). Surprisingly, the *b1, b2* single mutants, as well as the corresponding double mutants, were viable and little affected in plant growth under normal conditions, a fact that illustrates a certain degree of functional redundancy within the β-carotene hydroxylases. Additionally, the *hy-β* antisense plants exhibited only a small reduction of NPQ, although they contained less zeaxanthin after high light illumination (Rissler and Pogson, 2001). The relative stress tolerance could also be ascribed to a compensatory increase of other antioxidants with related functions. Supporting this hypothesis, analysis of antioxidant levels in tobacco *hy-β* AS plants revealed an elevation of tocopherol content (Woitsch and Römer, personal communication). The *b1 b2* double mutant showed a decrease in β-carotene derived xanthophylls containing two hydroxylated β-rings which was compensated by an increase in the α-carotene-derived xanthophyll lutein with only one hydroxylated β-ring. Therefore, just one hydroxylated β-ring seems to be necessary for the assembly of a functional light harvesting complex (LHC II). In accordance, Phillip et al. (2002) showed that the binding of xanthophylls to LHC II requires 3-hydroxy-β-ring groups, but not necessarily specific β-carotene derivatives. The lack of lutein in the *lut1* mutants and double/triple mutants (*lut1 b1, lut1 b2, lut1 b1 b2*) was partially counteracted by a shift to higher levels of β-carotene derived xanthophylls.

Over-expression of *hy-β* of plant or bacterial origin resulted in an improvement of high light stress resistance and UV-B tolerance in *Arabidopsis* and tobacco, respectively (Davison et al., 2002; Götz et al., 2002). These results provide further evidence for the importance of zeaxanthin in plant protection. Unfortunately, tocopherol levels of these transgenic plants were not determined.

Mutants and transgenic plants with alterations in carotenoid epoxidation and de-epoxidation

Epoxidation reactions are responsible for the production of violaxanthin from zeaxanthin under low light conditions whereas de-epoxidation reactions are converting violaxanthin back to zeaxanthin upon high light exposure. The reversible interconversion of these xanthophyll pigments via the intermediate antheraxanthin is caused by xanthophyll cycle activity (Demmig-Adams and Adams, 1996).

Several mutants defective in the epoxidation of zeaxanthin (*npq2*, *aba1/2*) and de-epoxidation of violaxanthin (*npq1*) have been isolated in *Arabidopsis* (see Table 1). The inability to synthesize violaxanthin and neoxanthin due to defects in the structural gene encoding zeaxanthin epoxidase (Marin et al., 1996; Niyogi et al., 1998) resulted in the accumulation of zeaxanthin as the only β-carotene derived xanthophyll in *npq2* and *aba1* mutants of *Arabidopsis*. The high level of zeaxanthin in *npq2* affected the kinetics of induction and relaxation but not the extent of NPQ. Despite their high zeaxanthin contents, the stress tolerance of these mutants was not significantly ameliorated (Hurry et al., 1997; Niyogi et al., 1998).

A defect in violaxanthin de-epoxidation as described in the *npq1* mutant of *Arabidopsis* inhibited the high light induced zeaxanthin formation and caused a remarkable decrease in NPQ. Photodamage of the *npq1* mutant was dependent on the developmental leaf stage. Younger leaves of the *npq1* mutant were relatively unaffected by high light stress and showed significantly higher α-tocopherol contents and VAZ (total of violaxanthin, antheraxanthin and zeaxanthin) levels than did the wild type (Havaux et al., 2000) putatively compensating for the lack of zeaxanthin. No increase of the water-soluble antioxidants glutathione and ascorbate was detected in the mutant compared to the wild type. Acclimation of

the *npq1* mutant at 1500 μmol m^{-2} s^{-1} for 3 days resulted in similar values to those of the wild type (Havaux et al., 2000). Short-term exposure to combinatorial light and cold stress caused a more pronounced photoinhibition in the *npq1* mutant. Stress was counteracted by the accumulation of carotenoids, tocopherols and flavonoids during photoacclimation (Havaux and Kloppstech, 2001). Moreover, various double mutants were generated, such as *npq1 lut2* (Niyogi et al., 2001) or *lut2 aba1* (Pogson et al., 1998), that are impaired in the synthesis of lutein as well as certain β-carotene derived xanthophylls (zeaxanthin or antheraxanthin and violaxanthin, respectively). After transfer to high light, the *npq1 lut 2* mutant suffered more photobleaching than the single mutant *npq1* (Havaux and Niyogi, 1999). Despite the deficiency in lutein and zeaxanthin this double mutant displayed an astonishing acclimation to high light conditions (Niyogi et al., 2001). The *lut2 npq2* double mutant contains no other xanthophyll but zeaxanthin and is unable to form trimeric light harvesting complexes thus compromising the structural integrity of the LHC complex. Although photoinhibition was similar in wild type and mutant, the double mutant exhibited a greater resistance to photooxidation and was less prone to lipid peroxidation (Havaux et al., 2004).

In summary, the results obtained from experiments using mutants impaired in carotenoid epoxidation or de-epoxidation reactions showed the flexibility of the light harvesting system with respect to its xanthophyll composition. Only under specific experimental or environmental conditions were the disadvantages of the alteration of the normal full set of xanthophylls clearly visible. Whereas a defect in violaxanthin de-epoxidation negatively affected NPQ and stress tolerance under high light conditions, the substitution of all xanthophylls by zeaxanthin significantly reduced the size of the light harvesting antenna of PS II and diminished the photosynthetic capacity under low light. These findings demonstrate that the selective gain of a higher proportion of one xanthophyll with certain properties beneficial under a specific condition can be accompanied by a partial loss of plant performance under a different situation. Optimal use and balance of light energy in fluctuating environmental conditions seems therefore to require the whole range of α- and β-carotene derived xanthophylls and the presence of a functional xanthophyll cycle as a dynamic protection mechanism.

To unravel the networking of antioxidative defense components, additional mutants were studied including double mutants impaired in different types of antioxidants. Previously, a link between the formation of zeaxanthin due to xanthophyll cycle activity, non-photochemical quenching and the availability of ascorbate has been postulated based on the finding that ascorbate deficiency in the single ascorbate mutant *vtc2* is limiting violaxanthin de-epoxidation (Müller-Moulé et al., 2002). Analysis of mutants with lower ascorbate contents, in combination with either a lack in non-photochemical quenching (*vtc2 npq4*) due to the absence of the PsbS protein or a lack in zeaxanthin formation (*vtc2 npq1*), revealed signs of persistent photooxidative stress. Accumulation of significantly more glutathione than the wild type implied a compensation of the reduced ascorbate levels (Müller-Moulé et al., 2004).

Mutants and transgenic plants with altered tocochromanol synthesis

Unlike the lack of carotenoids, the complete absence of tocopherols does not seem to be deleterious to plants. Mutants of the cyanobacteria *Synechocystis* as well as the *vte1* mutant of *Arabidopsis thaliana* are able to survive under normal and high light conditions (Collakova and DellaPenna, 2001; Dähnhardt et al., 2002). However, tocopherol levels are strongly increased upon unfavorable environmental conditions, e.g. high light stress and drought or due to senescence (Chrost et al., 1999; Havaux et al., 2000; Munné-Bosch and Alegre, 2000, 2003; Collakova and DellaPenna, 2003b) indicating a physiologically significant role. A growing body of evidence suggests that tocopherols can be functionally replaced by other antioxidants. The antioxidative defense system in younger leaves seems to be especially adaptable and flexible in this respect. For example, the reduced amount of tocopherols due to down-regulation of geranylgeranyl reductase (Graßes et al., 2001) was counteracted by an increase in xanthophyll cycle pigments in transgenic tobacco plants (Havaux et al., 2003). Vice versa, a reduction in xanthophyll cycle pigments in the *npq1* mutant of *A. thaliana* caused an elevation of α-tocopherol during long-term high light illumination (Havaux et al., 2000). Although the tocopherol and tocotrienol content was successfully enhanced by genetic engineering of different steps of the biosynthetic pathway, the majority of over-expressing lines were not tested for the level of other antioxidants or alterations of plant stress tolerance. So far, the most comprehensive analysis was performed by Kanwischer et al. (2005). The consequences of antioxidant deficiencies in

mutants with drastically reduced amounts of glutathione (*cad2*), ascorbate (*vtc1*) and tocopherols (*vte1*), as well in transgenic plants with elevated tocopherol levels, were investigated. Tocopherol deficiency in the *vte1* mutant resulted in an increase of total ascorbate and glutathione content, whereas wild-type plants over-expressing *vte1* exhibited lower levels of these water-soluble antioxidants relative to non-transformed wild-type controls (Table 2). In the *vte1 vtc1* double mutant the elevation of glutathione was even more pronounced than in the *vte1* mutant. Only in the *vte1 cad2* double mutant (devoid in tocopherol and glutathione) a marked decrease in photosynthetic pigments was observed. RNAi-mediated silencing of the potato *SXDS1* gene caused a drastic reduction of tocopherol and tocotrienols in source leaves due to a disruption of tocopherol cyclization and led to a large decrease in photosynthetic capacity (Hofius et al., 2004).

Taken together, the data obtained from various carotenoid and tocopherol mutants and transgenic plants emphasize the complexity of the plant defense system and the intricate relation and delicate balance between various components of the antioxidative defense system. The detailed dissection of plant stress responses in wild type, mutants and transgenics bringing together expertise from different areas of research and the use of more standardized experimental conditions will certainly further our understanding of the fascinating ways in which plants are able to protect themselves from biotic or abiotic stresses.

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