

# Tocopherol is the scavenger of singlet oxygen produced by the triplet states of chlorophyll in the PSII reaction centre

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## Abstract

Recent developments on the role of tocopherol in the antioxidant network of the chloroplast and, in particular, in the protection of PSII in high light are summarized. The origin and conditions for singlet oxygen production in the reaction centre via P680 triplet formation are discussed, as well as the scavenging of this singlet oxygen by tocopherol. This is probably the obligatory function of tocopherol in the plant in high light acclimation. Furthermore, tocopherol is part of the modulation system of ROS in stress signalling.

Key words: Photosystem II, ROS, singlet oxygen, tocopherol.

## Introduction

The occurrence of tocopherols in plant tissue, and in the chloroplast in particular, is well documented. From the many reviews since an early report by Lichtenthaler (1968), it was Munné-Bosch and Falk (2004) who recently summarized the state of tocopherol physiology and molecular biology and quoted earlier reviews. Accordingly  $\alpha$ -tocopherol is found predominantly in leaves and  $\gamma$ -tocopherol in seeds, where overexpressed tocopherol biosynthesis enzymes in plants also deposit the product (Falk *et al.*, 2003; Sattler *et al.*, 2004). Tocopherol appears to be present in all phototrophic organisms, except for some cyanobacteria.

The antioxidant role of tocopherols in plants, like vitamin E in humans, was soon recognized after its discovery (for reviews see Kaiser *et al.*, 1990; Fryer, 1992), but a precise

and detailed functional description for tocopherol in plants was possible only very recently. Both investigations of mutants and biochemical studies specify its role in the antioxidant network of the chloroplast and as a singlet oxygen scavenger in the photosystem II (PSII) reaction centre.

Tocopherols have two principal oxidation mechanisms (Neely *et al.*, 1988). Firstly, they may be oxidized in a one-electron-transfer reaction to a tocopheryl-radical. Secondly, they may react with singlet oxygen to a hydroperoxide, equivalent to a two-electron-transfer reaction (Fig. 1). Both reactions can be reversed, as both the radical as well as the hydroperoxide can be re-reduced to tocopherol by ascorbate. However, the hydroperoxide is also easily split by mild acidic conditions (as in the chloroplast lumen) to tocopherylquinone and this reaction competes with re-reduction. The cleavage to tocopherylquinone makes the second oxidation mechanism irreversible.

## Tocopherol/tocopheryl-radical in the antioxidant network of the chloroplast

In the chloroplast, both photosystems of the photosynthetic electron transport system produce a number of reactive oxygen species (ROS) in the light (Foyer and Noctor, 2003, 2005; Apel and Hirt, 2004). Their amount is increased in high light and in stress situations, for example, extreme temperature or drought (see special issue 411 of Vol. 56 of the *Journal of Experimental Botany* 2005). Under these conditions, the potential of the electron transport system exceeds the demand for reducing equivalents and ATP in the carbon assimilation cycle and/or possibly limiting subsequent reactions in the distribution of reduced carbon

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Abbreviations: PSII, photosystem II; ROS, reactive oxygen species.

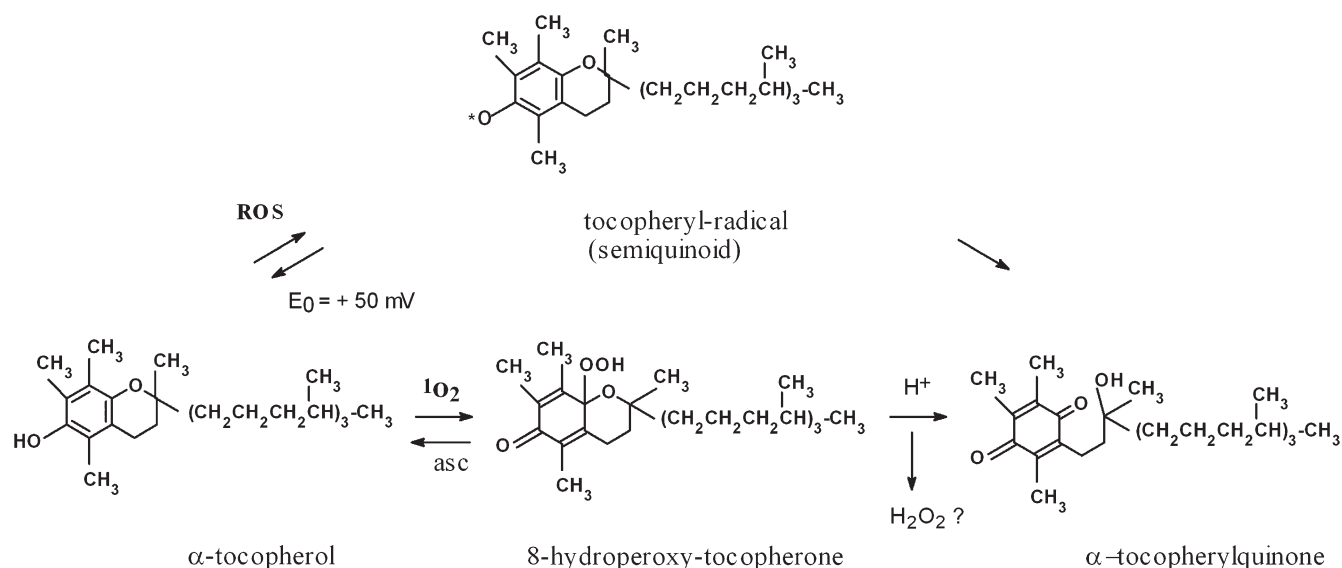


Fig. 1. Oxidation reactions in tocopherol biochemistry.

from the chloroplast to the cell. The electron flow system is saturated and the plastoquinone pool is fully reduced (see below). Before acclimation readjusts the photosynthetic system to the new situation ROS are produced as a consequence of electron transport (Ort and Baker, 2002). In the so-called Mehler reaction the primary acceptors of PSI react with oxygen, when they find the ferredoxin-NADP system already reduced. Superoxide radical anion and  $\text{H}_2\text{O}_2$  are formed. These ROS are released into the stroma compartment. There they encounter an efficient antioxidant system, extending over a large redox potential span from ferredoxin, NADP, thioredoxin, glutathione, ascorbate, and others (Foyer *et al.*, 2005). This antioxidant system was originally called the Halliwell and is nowadays referred to as the Asada and Foyer water-cycle protection system. The scavenging of reactive oxygen species and dissipation of excess energy is well reviewed, see Munné-Bosch and Falk (2004), Asada (1999), and Foyer and Nocter (2003, 2005). This established antioxidant network can now be extended by tocopherol. The concept of including tocopherol as an important compound to the antioxidant network has two advantages: (i) a more electropositive range beyond ascorbate is covered; and (ii) the antioxidant protection is extended from the hydrophilic into the lipophilic thylakoid space (Foyer *et al.*, 2005). In this antioxidant system, tocopherol may be oxidized to the radical which is then rereduced by ascorbate. In the network, tocopherol participates but it is not essential, as was shown by mutational studies where enzymatic steps in the biosynthesis of tocopherol have been deleted. There are deletions of the geranylgeranyl reductase by antisense RNA in tobacco (Graßes *et al.*, 2001; Havaux *et al.*, 2003), of the phytoltransferase step (Collakova and DellaPenna 2001; Schledz *et al.*, 2001), of the cyclase that forms the

chromane ring in *Arabidopsis* (Porfirova *et al.*, 2002; Havaux *et al.*, 2005; Kanwischer *et al.*, 2005), or in the final methylation step (Bergmüller *et al.*, 2003) in higher plants and cyanobacteria. There are also deletions in the earlier steps in tocopherol biosynthesis, as in the HPP-dioxygenase (Lee *et al.*, 1997; Dähnhardt *et al.*, 2002), but this step is before the branch of the biosynthetic pathway to plastoquinone and tocopherol. Plastoquinone deficiency is much less tolerated than that of tocopherol as plastoquinone is obligatory for both photosynthetic electron flow and the phytoene desaturase in carotenoid biosynthesis. Whereas the tobacco tocopherol deficient mutant was very light sensitive (Graßes *et al.*, 2001; Havaux *et al.*, 2003), the cyclase mutant in *Arabidopsis* by Dörmann and colleagues, although quite tocopherol deficient, seemed to have no phenotype under reasonable physiological conditions and showed only a slight light sensitivity (Porfirova *et al.*, 2002). This changed recently when double mutants in the ROS defence system were studied (Havaux *et al.*, 2005; Kanwischer *et al.*, 2005). A double mutant with both glutathione and tocopherol deficiency (Caretti *et al.*, 2002), and also a double mutant in the cyclase and the xanthophyll cycle, were now very light sensitive. The xanthophyll cycle is part of an early response to increased light intensity by converting violaxanthin to zeaxanthin which dissipates excess energy as heat. A double mutant which is deficient in both ascorbate and tocopherol, is not yet available. In a mutant with a lower ascorbate concentration in the plant, the tocopherol pool is increased (Müller-Moulé *et al.*, 2003, 2004), as it is also in a mutant deficient in violaxanthin de-epoxidase (Havaux *et al.*, 2000). It indicates that tocopherol participates in co-operation with ascorbate and the xanthophyll cycle and will respond if these are manipulated. These double mutant experiments show that the

antioxidant network in the chloroplast including tocopherol may tolerate a deficiency in one component but not in two (Munné-Bosch and Falk, 2004). There is also supporting work carried out on tocopherol-deficient *Synechocystis* mutants which show a high susceptibility to light stress when they were grown in the presence of norflurazon, an inhibitor of the phytoene desaturase and therefore carotenoid synthesis, while they showed no marked phenotype in the absence of this herbicide (Maeda *et al.*, 2005).

### Tocopherol in singlet oxygen scavenging in PSII

If, as just discussed the tocopherol/tocopheryl-radical redox system is not obligatory for the plant in their antioxidant defence, the second redox system—the reaction of tocopherol with singlet oxygen (Fig. 1) produced in PSII—is.

Singlet oxygen ( $^1\text{O}_2$ ) is typically produced in the reaction of oxygen ( $^3\text{O}_2$ ), which is a triplet state in its ground state, with the triplet state of photosensitized pigments. Chlorophyll, as the main light-absorbing pigment in the light-harvesting complex, the inner antenna, and also in the reaction centres, is very efficient in absorbing light and has the additional advantage that the excited states are long-lived enough (up to a few nanoseconds) to allow conversion of the excitation energy into an electrochemical potential via charge separation. If the energy is not efficiently used, the spins of the electrons in the excited state can rephase and give rise to a lower energy excited state: the chlorophyll triplet state. The chlorophyll triplet state has an even longer lifetime (about a few  $\mu\text{s}$ ) and can react with  $^3\text{O}_2$  to produce the very reactive  $^1\text{O}_2$  if no efficient quenchers are around. Chl triplet states may be populated directly by intersystem crossing (changing of the spin) from a singlet excited chlorophyll, or by charge recombination reactions (reversal of the charge separation and electron transfer reactions) in the reaction centres.

$^1\text{O}_2$  formation by charge recombination is favoured under certain physiological conditions like exposure to high light intensities or drought, leading to closure of the stomata and low  $\text{CO}_2$  concentrations in the chloroplasts (Hideg, 2004). Under such conditions the plastoquinone pool can be in a very reduced state and recombination reactions in PSII are favoured (for review see Adir *et al.*, 2003).

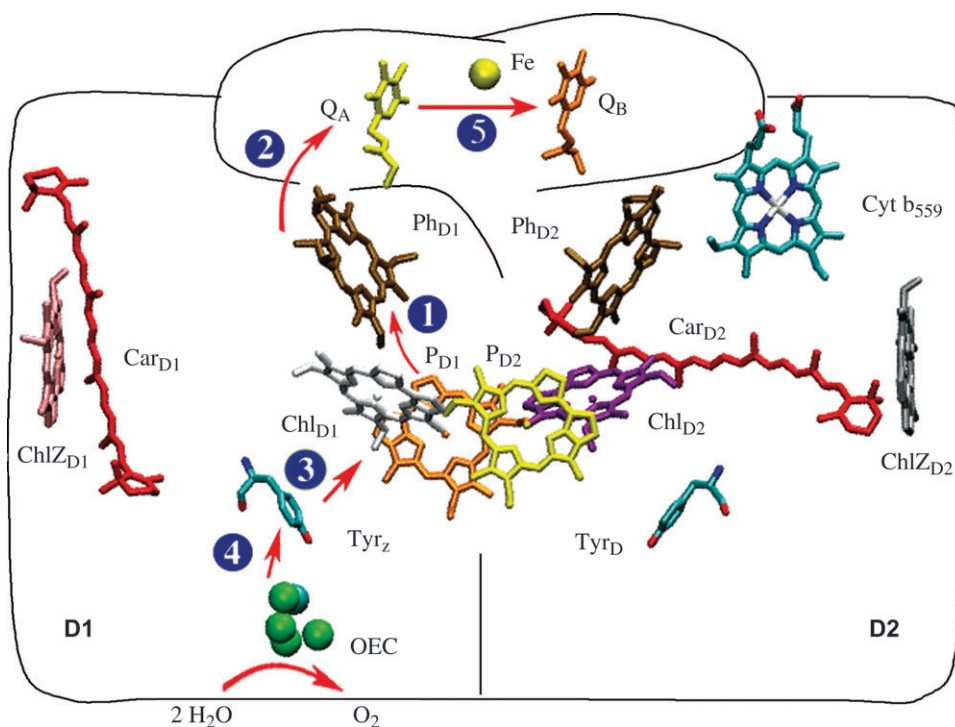
$^1\text{O}_2$  can react with proteins, pigments, and lipids and is thought to be the most important species responsible for light-induced loss of PSII activity, the degradation of the D1 protein (protein of the reaction centre of PSII), and for pigment bleaching (for reviews on photoinhibition see Prasil *et al.*, 1992; Aro *et al.*, 1993; Nixon *et al.*, 2005). It was assumed that  $^1\text{O}_2$  has a short life-time of about 200 ns in cells (Gorman and Rodgers, 1992), and that it reacts with target molecules in the immediate neighbourhood. The possible diffusion distance of  $^1\text{O}_2$  has been calculated to be up to 100 nm in a physiologically relevant situation

(Sies and Menck, 1992). However, recent measurements (Skovsen *et al.*, 2005) show that its lifetime is much longer (6  $\mu\text{s}$ ) than stated earlier. Skovsen and coworkers showed that  $^1\text{O}_2$  is able to diffuse over larger distances (about 270 nm) and could even diffuse out of the chloroplast into the cytosol.

Trebst and coworkers (Trebst *et al.*, 2002, 2004) provided evidence that  $^1\text{O}_2$  is the important damaging species during photoinhibition (i.e. the light-induced loss of PSII activity and of the D1 protein) of *Chlamydomonas reinhardtii* cells. This is supported by the fact that  $^1\text{O}_2$ -generating substances cause the same fragmentation pattern of the D1 protein as does photoinhibition (Mishra *et al.*, 1994; Okada *et al.*, 1996). The conformation of the D1 protein seems to be crucial for the high susceptibility of this protein to damage by  $^1\text{O}_2$  (Okada *et al.*, 1996).  $^1\text{O}_2$  formation *in vivo* was measured in leaves of *Arabidopsis thaliana* by the use of a fluorescent dye (Hideg *et al.*, 2001; op den Camp *et al.*, 2003). It seems reasonable to assume that not all  $^1\text{O}_2$  formed within PSII reacts with the D1 protein but that it may also damage chlorophyll, or cause lipid peroxidation. A certain quantity may even diffuse into the cytosol and act as a signalling molecule (see below).

The dangerous triplet state of chlorophylls, which is the origin of the observed  $^1\text{O}_2$ , can be quenched directly by carotenoids in close vicinity. The edge-to-edge distance between the two molecules must be less than the van der Waals distance (3.6 Å), i.e. the electron orbitals must have some overlap (Edge and Truscott, 1999). In this case, a spin exchange reaction occurs, the triplet state of the carotenoid is formed which either can dissipate the excess energy directly as heat or by physical quenching via enhanced intersystem crossing with  $^3\text{O}_2$  (Edge and Truscott, 1999). A quenching of triplet chlorophyll occurs in the antenna system but not in the reaction centre, although two  $\beta$ -carotene molecules are present in the PSII reaction centre (for the location of the carotenes in the reaction centre, see Loll *et al.*, 2005 and Fig. 2). In the reaction centre, the distance between the carotenes and the triplet chlorophyll is too large to allow a direct triplet quenching. The primary function of these  $\beta$ -carotenes is probably the quenching of  $^1\text{O}_2$  produced via the triplet state of  $\text{P}_{680}$  (Telfer *et al.*, 1994; Telfer, 2002, 2005). The latter was generated by charge recombination in PSII of the primary pair,  $\text{P}_{680}\text{Ph}$  (Telfer, 2002).  $^1\text{O}_2$  can react with carotenoids in an energy transfer mechanism, deactivating  $^1\text{O}_2$  (Gorman and Rodgers, 1992). In addition, tocopherol acts as a scavenger of  $^1\text{O}_2$  in the reaction centre.

In the reaction centre of PSII (Fig. 2), the first radical pair formed after excitation by light is  $\text{P}_{680}^+\text{Ph}^-$  (reaction 1), with  $\text{P}_{680}$  being the primary electron donor and pheophytin the primary electron acceptor (for a recent review on PSII see Goussias *et al.*, 2002; for the X-ray structure of PSII see Zouni *et al.*, 2001; Kamiya and Shen, 2003; Ferreira *et al.*, 2004; Loll *et al.*, 2005). The next step of electron



**Fig. 2.** A simplified model of the reaction centre of photosystem II showing only the main subunits D1 and D2 which carry the redox active cofactors. The forward electron transfer reactions are indicated by numbers. 1, Primary charge separation (formation of  $P^+$  and  $Ph^-$ ); 2, reduction of the primary quinone acceptor  $Q_A$ ; 3, reduction of  $P^+$  by tyrosine Z; 4, reduction of tyrosine Z by the oxygen evolving complex; 5, reduction of the secondary quinone acceptor  $Q_B$ . Abbreviations: Chl<sub>Z</sub>, peripheral chlorophylls; Car, carotenes; Fe, non-haem iron. OEC, oxygen evolving complex; P, the chlorophylls that become photo-oxidized; Chl, accessory chlorophylls; Ph, peophytins. The subscripts D1 and D2 indicate on which protein the cofactors are located.  $Q_A$  and  $Q_B$ , the first and second plastoquinone acceptors. Tyr<sub>Z</sub> and Tyr<sub>D</sub>, redox active tyrosine residues. The model is based on the X-ray structure by Loll *et al.* (2005).

transfer after the formation of the primary radical pair ( $P_{680}^+Ph^-$ ) leads to the reduction of the primary quinone acceptor  $Q_A$  (2). Subsequently,  $P_{680}^+$  is reduced by electron donation from the redox active tyrosine Tyr<sub>Z</sub> (3), which itself obtains an electron from the water oxidizing complex (4). The last step (5) is the reduction of the secondary quinone  $Q_B$ , which is a two electron acceptor. After double reduction,  $Q_B$  leaves the binding site and diffuses into the plastoquinone pool. The  $Q_B$  binding site is occupied by an oxidized plastoquinone molecule.

In photosynthetic electron transport, forward electron transfer reactions are much faster than charge recombination reactions. However, charge recombination reactions can occur when the forward electron transport cannot proceed. If the primary quinone acceptor stays reduced because of a block in forward electron transport due to a reduced plastoquinone pool, charge recombination takes place which leads to the formation of  $P_{680}^+Ph^-$  and the repopulation of the excited state of  $P_{680}$ . The excited state formed can be either a singlet or a triplet state. The triplet state is mainly localized on one of the accessory chlorophylls, Chl<sub>D1</sub> or Chl<sub>D2</sub> (van Mieghem *et al.*, 1991). It is generally accepted that the triplet is mainly on Chl<sub>D1</sub> because this is the branch of the reaction centre active in

forward electron transport and back reactions (recombinations) yielding the triplet state (Kamlowski *et al.*, 1996; Diner *et al.*, 2001; Lubitz, 2002). According to Noguchi *et al.* (2001) up to 30% of the triplet is located at  $P_{D1}$  at room temperature and it is highly probable that the triplet state is delocalised over all four pigments (Chl<sub>D1</sub>,  $P_{D1}$ ,  $P_{D2}$ , and Chl<sub>D2</sub>) at room temperature. The  $^3Chl$  reacts with  $^3O_2$  leading to the toxic and very reactive  $^1O_2$ .

The yield of charge recombination reactions and thereby the production of  $^3Chl$  and  $^1O_2$ , respectively, can be modified by the midpoint potential of  $Q_A$ . Prior to photoactivation newly assembled photosystem II, which still does not contain a functional manganese cluster, has about a 150 mV more positive midpoint potential of  $Q_A$  (Johnson *et al.*, 1995). In the presence of  $Q_A$  with a more positive midpoint potential charge recombination and  $^3P_{680}$  formation is less probable because a higher activation energy is needed for recombination via the primary radical pair ( $P_{680}^+Ph^-$ ). Furthermore, the midpoint potential of  $Q_A$  and the yield of  $^1O_2$  formation can be modified by PSII herbicides (Krieger-Liszka and Rutherford, 1998; Rutherford and Krieger-Liszka, 2001; Fufezan *et al.*, 2002; for a more detailed discussion see Krieger-Liszka, 2005). Rappaport and co-workers (Rappaport *et al.*, 2002)



investigated the influence of the midpoint potential of the Ph/Ph<sup>-</sup> redox couple on charge recombination between S<sub>2</sub>Q<sub>A</sub><sup>-</sup> and showed that the recombination rate is also sensitive to the free energy gap between Pheo and Q<sub>A</sub>.

As discussed above, chlorophyll triplet states of the reaction centre of PSII are formed in the recombination reaction of the primary radical pair (P<sub>680</sub><sup>+</sup>Ph<sup>-</sup>). The two β-carotenes bound to the reaction centre proteins are not able to quench P<sub>680</sub> triplet states because they are too far away. Oxygen, however, does react with the triplet state and singlet oxygen is formed (Telfer, 2002, 2005; Trebst, 2003). The yield of the triplet state of P<sub>680</sub> and of singlet oxygen therefore depends on the light intensity for P<sub>680</sub> excitation and of the redox state of the electron flow system that slows oxidation of Q<sub>A</sub><sup>-</sup>. The singlet oxygen formed in the P<sub>680</sub> triplet state quench induces the degradation of the D1 protein (Keren *et al.*, 1997; Szilard *et al.*, 2005). After degradation, the protein is newly synthesized and reassembled into a new functional PSII, the phenomenon called the rapid turnover of the D1 protein in photo-inhibition (Mattoo *et al.*, 1989; Aro *et al.*, 1993).

An experimental approach to show the participation of tocopherol in PSII stability under high light conditions has recently been described. For that the biosynthesis of tocopherol was inhibited in the alga *Chlamydomonas reinhardtii* (Trebst *et al.*, 2002, 2004; Kruk *et al.*, 2005) with inhibitors (herbicides) of the HPP dioxxygenase (Pallett *et al.*, 1998; Matsumoto *et al.*, 2002). This enzyme is a key enzyme in the biosynthetic pathway from tyrosine to plastoquinone and to tocopherol. It converts hydroxyphe-nylpyruvate to homogentisate. As the inhibitors prevent both plastoquinone and tocopherol biosynthesis, the experimental conditions were set such that plastoquinone was not limiting the system. It is possible to maintain a sufficient amount of plastoquinone by adding the inhibitors after a time which had allowed the algae and their photosynthetic system to get saturated with plastoquinone, that is the inhibitors were added to a green autotrophic culture growing in the logarithmic growth phase with optimal photosynthetic activity. Such cells with the inhibitor now added showed no change in photosynthetic activity and growth rate in low light. Therefore, tocopherol biosynthesis did not limit growth and photosynthesis at low light intensities (70 μE). If, however, the cells were exposed to strong light (1200 μE equal to 20 times growth light) after an hour the photosynthesis rate had already dropped to almost zero, due to an inactive PSII, as there was only a small change in PSI activity. Immunoblots showed the disappearance of the D1 protein (Trebst *et al.*, 2002). Important for the interpretation is the tocopherol content. The α-tocopherol pool was not appreciably lowered when cells were grown in low light in the presence of the inhibitor. After 1 h high light, however, the α-tocopherol pool approached very low levels, to be almost nil after 2 h strong light. Loss of α-tocopherol correlated with the loss of

photosynthesis and of the D1 protein. Clearly α-tocopherol has a turnover that is small in low light, but 20 times increased in high light (Trebst *et al.*, 2002). The loss of α-tocopherol in this turnover is compensated in untreated algae by resynthesis. In the presence of the biosynthesis inhibitor, tocopherol can no longer be synthesized and then the pool gets exhausted. With little tocopherol left, PSII function is no longer sustained. The effect of the inhibitor may be reversed by homogentisate. This is easily understood in that, by adding the product of the inhibited reaction, tocopherol synthesis can continue. In addition, this experiment shows that the inhibitor reduced specifically the tocopherol content and did not damage photosynthesis by reducing the amount of plastoquinone. The inhibition can be reversed, furthermore, by supplying a chemical singlet oxygen scavenger, like diphenylamine or derivatives of it (Trebst *et al.*, 2002). This is taken as a strong indication that it is indeed the singlet oxygen produced that is responsible for the α-tocopherol loss and the loss of tocopherol scavenging singlet oxygen is responsible for inactivation of photosynthesis. It is also a proof that it is singlet oxygen that induces D1 protein degradation, as concluded earlier from other experimentation, in particular by Ohad and coworkers (Prasil *et al.*, 1992; Keren *et al.*, 1997), echoed in the many reviews (Aro *et al.*, 1993), but sometimes still in doubt.

An attempt was made to reverse the inhibitor effect on α-tocopherol biosynthesis in *Chlamydomonas* by the end product, i.e. tocopherol. But tocopherols cannot permeate into the algal cell. Therefore short chain tocopherols were synthesized that were taken up by the algae. In bromoethyl-tocopherol, such a short chain tocopherol derivative was found that reverses the inhibitor effect. The inhibition could also be reversed by adding γ-tocopherol derivatives (Trebst *et al.*, 2004; Kruk *et al.*, 2005). It indicates that non-functional mechanistic reasons induce plants to accumulate α-tocopherol in the leaves and γ-tocopherol in the seed.

Summarizing the results with the inhibitors of tocopherol biosynthesis, it was concluded that tocopherol has an obligatory function in plants in protecting photosystem II structure and function and its D1 protein by scavenging the singlet oxygen produced in the PSII reaction centre. This obligatory function becomes apparent in high light conditions, when other singlet oxygen quenching (like carotene bound to the reaction centre) or scavenging (in the degradation mechanism of the D1 protein) cannot cope with the amount of <sup>1</sup>O<sub>2</sub> produced. It is also shown that it is not the change of concentration of tocopherol that is indicative of functional significance but rather its turnover rate. It might be noted that, in these inhibition experiments, not only were changes in tocopherol turnover and PSII activity in high light in *Chlamydomonas* observed, but there was also a change in carotenoid concentration and composition, not yet understood (P Jahns, unpublished results). The authors' conclusion on the role of tocopherol in PSII protection in

high light stress has also recently been supported by the data and interpretation in studies with the mutants deficient in tocopherol. These data with deletions in a double mutation showed that the protective system (here by tocopherol and the xanthophyll cycle) for overexcitation in high light is due to damage to PSII (Havaux *et al.*, 2005).

### Tocopherol and ROS signalling

As discussed, it is the general role of tocopherol in the antioxidant network of the chloroplast to reduce the highly damaging potential of ROS and of singlet oxygen in particular. To reduce but not to eliminate either species, as it appears established that ROS are important signals in communicating the (redox) state of the electron transport system to gene expression systems both in the chloroplast and in the nucleus in high light acclimation (Baier and Dietz, 1999; Foyer and Noctor, 2003, 2005; Apel and Hirt, 2004). Recently, singlet oxygen is also shown to be involved in such signalling pathways. Different strategies were used to produce  $^1\text{O}_2$  in a gene expression system: (i) chemical generation by Rose Bengal (Fischer *et al.*, 2005); (ii) by the photosensitizing activity of accumulated chlorophyll precursors in *flu* mutants of *Arabidopsis* (op den Camp *et al.*, 2003; Apel and Hirt, 2004); and (iii) by charge recombination reactions in PSII (Fischer *et al.*, 2006). Mechanistic details of the signalling pathways by which the plastid controls nuclear gene expression are not yet understood. It is difficult to imagine that  $^1\text{O}_2$  functions directly as a signalling molecule, although in the light of the new data by Skovsen *et al.* (2005) a direct action of  $^1\text{O}_2$  may even be possible. Alternatively, chlorophyll breakdown products or lipid peroxides may act as signalling molecules (for a review see Beck, 2005). Tocopherol is part of the modulation of ROS concentration in adjusting their concentration sufficient for second messenger activity but below damaging effects.

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