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#### Review

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#### ABSTRACT

Photoprotection of photosystem II (PSII) is essential to avoid the light-induced damage of the photosynthetic apparatus due to the formation of reactive oxygen species (=photo-oxidative stress) under excess light. Carotenoids are known to play a crucial role in these processes based on their property to deactivate triplet chlorophyll ( $^3$ ChI\*) and singlet oxygen ( $^1$ O<sub>2</sub>\*). Xanthophylls are further assumed to be involved either directly or indirectly in the non-photochemical quenching (NPQ) of excess light energy in the antenna of PSII. This review gives an overview on recent progress in the understanding of the photoprotective role of the xanthophylls zeaxanthin (which is formed in the light in the so-called xanthophyll cycle) and lutein with emphasis on the NPQ processes associated with PSII of higher plants. The current knowledge supports the view that the photoprotective role of Lut is predominantly restricted to its function in the deactivation of  $^3$ ChI\*, while zeaxanthin is the major player in the deactivation of excited singlet ChI ( $^1$ ChI\*) and thus in NPQ (non-photochemical quenching). Additionally, zeaxanthin serves important functions as an antioxidant in the lipid phase of the membrane and is likely to act as a key component in the memory of the chloroplast with respect to preceding photo-oxidative stress. This article is part of a Special Issue entitled: Photosystem II.

#### 1. Introduction

Light is the ultimate energy source for photosynthesis and thus represents the most important environmental factor for all photosynthetic organisms. Under natural conditions, nearly all photosynthetic organisms are exposed to changing light intensities which may vary over several orders of magnitude in both the short-term (seconds to minutes) and the long-term (hours to months). To allow optimal photosynthesis at low light intensities, the photosystems possess efficient light harvesting antenna systems. To avoid photo-oxidative damage of the photosynthetic apparatus due to the formation of reactive oxygen species (ROS) under excess light, photosynthetic organisms have developed a number of defense strategies and mechanisms [1], allowing

Abbreviations: ADQ, aggregation dependent quenching; Ax, antheraxanthin; Chl, chlorophyll; CT, Charge transfer; CTQ, charge transfer quenching; DEPS, de-epoxidation state; Lut, Lutein; Lx, lutein epoxide; NPQ, non-photochemical quenching; Nx, neoxanthin; OCP, orange carotenoid protein; PS I, photosystem I; PS II, photosystem II; qE, energy-dependent quenching; qI, photoinhibitory quenching; qT, state transition quenching; qZ, zeaxanthin-dependent quenching; RC, reaction center; ROS, reactive oxygen species; VAZ, sum of violaxanthin, antheraxanthin and zeaxanthin; Vx, violaxanthin; VxDE, violaxanthin de-epoxidase; Zx, zeaxanthin; ZxE, zeaxanthin epoxidase

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either a lowering of ROS formation or the detoxification of already formed ROS. In chloroplasts, two major pathways of ROS formation exist: (i) electron transfer to molecular oxygen at the acceptor side of photosystem I (PSI) [2] or at PSII [3], leading to the formation of the superoxide radical (O<sub>2</sub><sup>-</sup>) and, in subsequent reactions, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or hydroxyl radicals (OH) [4,5]; and (ii) energy transfer from triplet chlorophyll (3Chl\*) to molecular oxygen, leading to the formation of singlet oxygen  $({}^{1}O_{2}^{*})$  [6,7]. In PSII, carotenoids play a central role in the deactivation of <sup>3</sup>Chl\* and <sup>1</sup>O<sub>2</sub>\*, and the reduction of ROS formation due to the thermal dissipation of excess light energy (=nonphotochemical quenching, NPQ) at the level of <sup>1</sup>Chl\*. In the PSII reaction center, the deactivation of  ${}^{1}O_{2}^{*}$  is provided by  $\beta$ -carotene [8], while xanthophylls are involved in the non-photochemical quenching (NPQ) of excitation energy in light harvesting antenna proteins [1,9]. It is worth mentioning here however that low levels of ROS play an important role in signaling for several cellular regulation mechanisms and can thus not simply be considered damaging species [7,10,11]. This review will focus on the photoprotective role of xanthophylls with emphasis on the functions of zeaxanthin (Zx) and lutein (Lut) in NPQ processes associated with PSII of higher plants.

1.1. Antenna proteins, pigment binding sites and xanthophyll cycles in vascular plants

In higher plants, six different peripheral antenna proteins (Lhcb1-6) form the light harvesting antenna of PSII and at least four different

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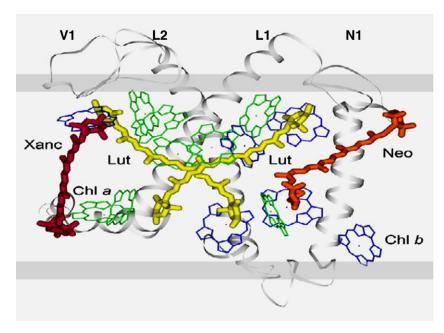
antenna proteins (Lhca1-4) that of PSI [12-15]. The determination of the structure of the trimeric LHCII complex (which is composed of Lhcb1-3 monomers) at a resolution of 2.72 Å [16] and 2.5 Å [17] identified binding of 14 Chl (8 Chl a and 6 Chl b) and 4 xanthophylls (2 luteins (Lut), 1 neoxanthin (Nx) and 1 xanthophyll cycle pigment) (Fig. 1). According to their binding specificity, these latter binding sites have been termed L1, L2, N1 and V1, respectively. Due to the high sequence similarity among the different antenna proteins, a similar protein structure (although with different Chl and xanthophyll binding patterns) has been predicted for the monomeric Lhcb4-6 and Lhca1-4 proteins. However, the different antenna proteins show considerable variations with respect to (i) the oligomeric state (Lhcb1-3 are organized as LHCII trimers, Lhca1/4 and Lhca 2/3 as dimers and Lhcb4-6 as monomers), (ii) Chl binding (14 Chl per monomer for LHCII and LHCI proteins, but considerably less for the monomeric Lhcb proteins) and (iii) xanthophyll binding (4 xanthophyll binding sites in LHCII proteins, and 2-3 xanthophyll binding sites in all other antenna proteins) [14,16–18]. While only Lut binding to the site L1 is common to all antenna proteins, the occupancy of the other xanthophyll binding sites (cf. Fig. 1) differs in each of the other LHC proteins [14].

Two different xanthophyll cycles have been described for land plants: The violaxanthin (Vx) cycle, in which Vx is reversibly converted to Zx via antheraxanthin (Ax) [19-21] and the lutein epoxide (Lx) cycle, in which Lx is reversibly converted into Lut [22,23]. Both cycles are involved in the light-regulated switching of PSII from a light-harvesting state (with epoxidized xanthophylls, Vx and Lx, present in low light or darkness) to an energy dissipating state (with de-epoxidized xanthophylls, Ax, Zx and Lut, present in high light). Therefore, these cycles provide for the short- and longterm acclimation of plants to varying light conditions. While the Vx cycle is present in all land plants, the Lx cycle is restricted to some species only [24]. For the Lx cycle, two different types have been described: (i) a complete Lx cycle, in which Lut is reconverted to Lx overnight and (ii) a truncated Lx cycle, in which Lut reconversion is not observed overnight [23]. Essential photoprotective functions have been assigned to Lut and the xanthophyll cycle pigments Ax and Zx, particularly related to the heat dissipation of excess light energy (NPO).

#### 1.2. General aspects of NPO

The thermal dissipation of excess light energy represents a basic photoprotective principle in photosynthetic eukaryotes. NPQ is generally based on light-regulated and xanthophyll-dependent processes in antenna proteins that are mediated by specific proteins that act as sensors of the lumen pH or the light and thereby activate NPQ through light-induced conformational changes. Several different NPQ principles are known in the plant kingdom:

- (i) In cyanobacteria and red algae, the quenching located in the phycobilisomes is triggered by light-activation of the orange carotenoid protein (OCP) [25–27]. OCP is a water-soluble protein that binds a single carotenoid (3'hydroxyechinenone) as the chromophore [28] and acts as a direct photosensor. Absorption of blue-green light induces structural changes in both the protein and carotenoid, which triggers NPQ induction through interaction with the phycobilisomes in an unknown manner [29]. The relaxation of NPQ under low light or in darkness requires the reconversion of OCP into its inactive state. This process has been shown to be mediated by another protein (termed fluorescence recovery protein, FRP) that interacts with the active form of OCP and accelerates the reconversion of active OCP to the inactive form [30].
- (ii) In brown algae and diatoms, NPQ is mediated by the LHCSR (also termed LI818) protein and additionally requires the conversion of diadinoxanthin (Ddx) to diatoxanthin (Dtx) in the Ddx cycle, or of Vx to Zx in the Vx-cycle [31]. NPQ in the fucoxanthin chlorophyll proteins (FCPs) seems to be strictly dependent on Dtx or Zx and is therefore controlled by the rates of Dtx/Zx formation (during NPQ induction) and Dtx/Zx reconversion (during NPQ relaxation). Although the formation of Dtx or Zx (and thus of NPQ induction) requires a low lumen pH, high NPQ can be maintained even in the absence of a transmembrane proton gradient, once Dtx/Zx has been formed [32,33].
- (iii) In green algae, LHCSR is also essential for NPQ [34]. In contrast to brown algae and diatoms, however, NPQ in green algae is essentially controlled by the  $\Delta pH$  and further depends on the



**Fig. 1.** Structure of LHCII (derived from [16]). The protein backbone with the three transmembrane helices (gray) is shown in the background, the pigments are shown in color. For Chls, only the tetrapyrrole rings are indicated (blue = Chl *a* and green = Chl *b*). Four bound xanthophylls were identified in the structure: Two central luteins (Lut, yellow, binding sites L1 und L2), a xanthophyll cycle pigment (Xanc) on the left (red, V1 site) and neoxanthin (Nx) on the right (orange, N1 site).

action of xanthophylls [35,36] and additionally requires the action of a specific antenna protein, Lhcbm1 [37]. In green algae (as in higher plants) only the Vx cycle is active but not the Ddx cycle found in diatoms and brown algae [21,31,38]. The involvement of Zx - formed in the Vx cycle - in NPQ has been inferred from xanthophyll cycle mutants of Chlamydomonas [36]. However, the maximal NPQ was found to be clearly less dependent on Zx in comparison with higher plants [39] or even independent of Zx [40]. Other xanthophylls than Zx might thus be involved in NPQ in green algae [36]. The relaxation of NPQ in green algae is strictly dependent on the removal of ΔpH. It is important to note that genes encoding the PsbS protein (that acts as a pH sensor and is thus essential for NPQ in land plants, see below) are present in Chlamydomonas, but that the PsbS protein is not expressed [41] even under light stress conditions [42]. However, recent work showed that the transcript levels of both genes encoding PsbS, PSBS1 and PSBS2, were strongly increased following nitrogen deprivation [43], suggesting that PsbS in green algae has either a role unrelated to photoprotection or is required for more efficient photoprotection under these specific stress conditions.

- (iv) Recent studies on the moss *Physcomitrella patens* provided direct evidence that both proteins, PsbS and LHCSR, are active in NPQ in mosses [44]. The knockout of either LHCSR or PsbS led to a reduction of overall NPQ, while simultaneous knockout of both proteins completely abolished the NPQ in *Physcomitrella*. Based on these findings it has been concluded that photoprotective functions of PsbS and LHCSR developed initially in parallel and that LHCSR was lost during the evolution of land plants [44].
- (v) In vascular plants, LHCSR is missing [45] and NPQ is mediated by the PsbS protein [46] that acts as a sensor of lumen pH [47] and may activate NPQ either through inducing conformational changes in the antenna of PSII [9] or through other interactions [1]. So far, a direct interaction of PsbS with LHCs has not been demonstrated, however. NPQ in land plants is furthermore strongly dependent on the xanthophyll Zx [39,48-50] that apparently modulates nearly all components of NPQ [51]. The PsbS dependent qE-component is strictly regulated by the  $\Delta pH$  and some data indicate that all antenna subcomplexes of PSII may be involved in quenching [52-55]. While studies on knock-out mutants affected in the different antenna subcomplexes revealed a high flexibility of NPO, a crucial role has been determined so far for Lhcb6 only [56-58]. Interestingly, Lhcb6 has been shown to be the most recent addition to the PSII antenna during evolution of land plants and is not present in green algae [45,59]. Thus, Lhcb6 can be assumed to have a specific function in NPQ regulation in vascular plants, Studies on the relaxation of the NPQ in land plants demonstrated that only the qE component of NPQ is fully deactivated by collapse of the ΔpH, while Zx-dependent NPQ processes appear to remain active even in the absence of a low lumen pH [51]. The overall quenching mechanisms and locations appear to be more complex, however, than had been assumed until recently. Applying picosecond Chl fluorescence spectroscopy to intact leaves, it has been shown that at least two different quenching sites are active under in vivo conditions in higher plants: A PsbS-dependent quenching site (Q1), located in the major light-harvesting complexes, LHCII, which becomes detached from photosystem II (PSII), and a Zx-dependent quenching site (Q2), located in the remaining antenna of PSII [60] (Fig. 2).

In summary, several NPQ principles have evolved within the plant kingdom, differing with respect to the light stress sensor, the NPQ quenching site, and possibly also the quenching mechanism. While the direct sensing of light by the photoreceptor OCP is a unique

feature of cyanobacteria and red algae, the control of NPQ through a pH sensing protein (LHCSR or PsbS), that transfers the pH signal to (specific) PSII antenna proteins, is common to plants and algae. During evolution, LHCSR-related and PsbS-related NPQ developed in parallel, and the ancestral LHCSR-based mechanism (found in green algae and mosses) was functionally replaced by PsbS only during the evolution of land plants. Furthermore, NPQ in land plants shows unique features with respect to the role of Lhcb6 and the strong modification of energy dissipation by Zx.

#### 2. The role of Lut in photoprotection of PSII

Lutein (Lut) is the most abundant xanthophyll in higher plants and is bound to the L1 (in all antenna subcomplexes) and L2 (in some of the antenna subcomplexes) binding sites of the antenna proteins. Lut serves several important functions: (i) structural stabilization of antenna proteins, (ii) light harvesting (= transfer of excitation energy to Chl) and (iii) quenching of <sup>3</sup>Chl states. Furthermore, Lut has been proposed to also be involved in the quenching of <sup>1</sup>Chl (= NPQ).

#### 2.1. Lut and stabilization of antenna proteins

It is known from reconstitution of antenna proteins that xanthophyll binding is required for the correct folding of antenna proteins [61,62]. Although the replacement of Lut by Vx or Zx during reconstitution of recombinant antenna proteins in the absence of Lut yields functional proteins with respect to Chl binding and light harvesting, the stability of such complexes is often reduced [63,64]. This implies a crucial role of Lut for the stability of antenna proteins, most likely at the binding site L1 that is occupied in all native antenna complexes by Lut. Studying recombinant LHCII, the formation of stable LHC trimers was found to be reduced or completely abolished in absence of Lut [63]. The same conclusion can be drawn from studies with Lut deficient mutants (lut1 or lut2) from Arabidopsis[65]. Analyses of the pigment composition of the different antenna subcomplexes in these mutants showed that Lut is replaced by Vx cycle pigments and that the trimerization of LHCII is strongly reduced [66,67] implying an essential role of Lut for trimerization of LHCII as well [67]. These latter effects were even more pronounced when Lut was replaced by Zx [68]. Since the absence of trimers can be observed even in low-lightgrown Lut-deficient plants as well as in reconstituted recombinant proteins, the destabilization is unlikely to be related to light-induced photo-damage, but rather reflects specific properties of Lut in protein stabilization.

#### 2.2. Lut and light harvesting

Lutein is known to contribute to light harvesting by transferring excitation energy to Chl [69], with high efficiency at both Lut binding sites, L1 and L2 [70–72]. The light-harvesting function of Lut, however, seems to be fully replaced by other xanthophylls (in particular Vx), as can be derived from the unchanged growth of lut2npq1 mutants in comparison with the WT [65,73] and the only slightly reduced energy-transfer properties [67]. A stimulating effect on light harvesting efficiency has been attributed to Lx [74]. Strong accumulation of Lx has been shown to occur particularly in deeply shaded leaves of tropical trees [23,75] in agreement with the proposed function of Lx as an efficient light-harvesting xanthophyll. Lx has been shown to be colocated with Vx, implying that Lx binds to the V1 and L2 sites of antenna proteins [76]. More detailed biochemical and spectroscopic studies on isolated and recombinant antenna proteins demonstrated a higher light-harvesting efficiency of Lx at the V1 and L2 binding site of Lhcb5 [74].

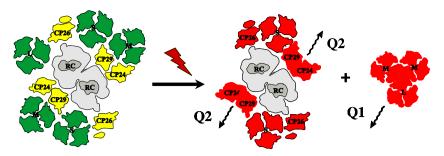


Fig. 2. Schematic view of proposed quenching locations (adapted from [60]). Arrangement and composition of the supramolecular PS II complex in wild-type plants under dark-acclimated (left-hand side) and light-adapted (right-hand side) conditions (adapted from [15]). Binding of Vx to trimeric LHC II and minor LHCs in the dark is indicated by green and yellow color, Zx-containing LHC II and minor LHCs in the light by red color. The formation of quenching site Q1 requires PsbS, while the formation of the quenching site Q2 requires Zx only. We propose that interaction with PsbS causes detachment, migration and aggregation/deaggregation of LHC II (possibly associated with some minor antenna component) depending on the lumen pH. From its dependence on PsbS, the Q1 site is assigned to the fast-forming and fast-reversible qE component of NPQ. L, M and S denote loosely coupled, moderately coupled and strongly coupled trimers of LHC II.

#### 2.3. Lut as quencher of <sup>3</sup>Chl

The most important photoprotective function of Lut is the quenching of <sup>3</sup>Chl, which requires a close proximity of Lut and Chl a [77-79]. Based on the high-resolution crystal structure of LHCII [16,17] suitable Chl a molecules have been identified as possible candidates for triplet energy transfer to Lut at both sites, L1 and L2 [17]. Efficient quenching of <sup>3</sup>Chl by carotenoids has been proven in studies on isolated and recombinant LHCII [67,80-82] and minor PSII antenna complexes [82]. These latter studies confirmed that efficient <sup>3</sup>Chl quenching is related to the action of Lut at binding sites L1 and L2, and that Vx and Nx are much less efficient or not involved in these processes. Replacement of Lut at L1 and/or at L2 by Vx resulted in less efficient <sup>3</sup>Chl quenching [67], strongly increased photoinhibition (i.e. long-lasting depression of the PSII efficiency) and degradation of LHCII under light stress [73] underlining the important specific role of Lut in photoprotection via<sup>3</sup>Chl quenching. Consequently, the high light-induced conversion of Lx to Lut in plants with an active Lx cycle has been interpreted as a photoprotective mechanism that leads to a more efficient <sup>3</sup>Chl quenching at L2 or possibly also at V1 [76].

#### 2.4. Lut as quencher of <sup>1</sup>Chl

Apart from its role in <sup>3</sup>Chl quenching, Lut has also been proposed to act as quencher of excited <sup>1</sup>Chl\* in the pH-regulated and PsbSdependent gE mechanism of NPO. The reduction of NPO and the retardation of NPO induction kinetics in Lut-deficient mutants had originally been interpreted as evidence for a role of Lut in NPQ [83]. Later work on the same mutants showed, however, that the retardation of NPQ induction is due to a slower Zx formation, and it was concluded that Lut plays no direct role in qE [66]. On the other hand, accumulation of Lut in the absence of Zx in the szl1npq1 mutant was shown to restore a large portion of NPQ that is absent in npq1, and the accumulation of Lut in presence of a functional VxDE in szl1 led to a lower Vx (and Nx) content and strongly reduced Zx formation, suggesting that additional Lut may be able to functionally replace Zx with respect to qE [84]. Apparently, xanthophylls are able to replace each other for different functions, so that the use of mutants deficient in single xanthophylls actually may provide information about the role of specific xanthophyll binding sites rather than about the role of specific xanthophylls.

On the basis of resonance Raman and transient absorption spectroscopy applied to isolated LHCII complexes in their oligomerized form, it was proposed that qE is based on energy transfer from  $\operatorname{Chl} a$  to Lut bound in the site L1, and that this mechanism represents the principle mechanism of photoprotection in plants [85]. The proposed mechanism is assumed to be regulated by the lumen pH, which controls the aggregation of LHCII in a PsbS dependent manner.

This quenching may be further modulated by Vx to Zx conversion [9,86-88]. However, the function of Lut in gE and in particular the energy quenching mechanism by the Lut S1 state is still a matter of debate, considering that different quenching mechanisms and quenching sites have been proposed in several studies recently. Recent femtosecond transient absorption studies on isolated LHCII showed, however, that quenching of <sup>1</sup>Chl\* under in vitro conditions in LHCII oligomers is neither dependent on Zx nor on energy transfer to any other carotenoid, such as Lut at L1 [89]. Instead, the latter experimental data provided strong evidence that quenching of <sup>1</sup>Chl\* in LHCII is solely based on Chl-Chl interactions, most likely related to the formation of a fluorescent Chl-Chl CT (charge transfer) state [89]. Strikingly, the spectroscopic signature of the proposed Chl-Chl CT state has also been observed upon induction of NPQ under in vivo conditions [60,90,91] (see also Fig. 2). Thus the involvement of xanthophylls, and in particular of Lut, in aggregation dependent quenching (ADQ) of <sup>1</sup>Chl\* seems very unlikely.

The comparison of ADQ in the different PSII antenna complexes indicated that ADQ is much more efficient in minor complexes (Lhcb4–6) than in LHCII [55]. This may, on one hand, put into question the central role of ADQ in LHCII as the major photoprotective mechanism. On the other hand, this question can only be answered definitively once the detailed mechanism of ADQ quenching has been revealed. In addition to ADQ, a PsbS-dependent charge transfer quenching (CTQ), involving the formation of  $\mathrm{Chl}^-/\mathrm{Zx}^+$  radical pairs, has been suggested to be active in isolated thylakoid membranes [92]. Later work provided support for the view that  $\mathrm{Chl}/\mathrm{Zx}$  CTQ is also active in isolated (recombinant) minor antenna proteins [53,54], but not in LHCII [93]. An additional role of Lut as a partner in CTQ quenching has been proposed for Lhcb5 [54].

In summary, the photoprotective role of Lut is likely to be predominantly related to its function as quencher of <sup>3</sup>Chl, while quenching of <sup>1</sup>Chl\* seems to be achieved more or less independently of Lut.

#### 3. The role of the xanthophyll cycle in the photoprotection of PSII

Although the existence of the light-dependent and reversible formation of Zx in the Vx cycle was established in the early 1960s [20] and biochemically well characterized in the pioneering work by the groups of Yamamoto and Hager [94–102], it took about 25 years before an essential photoprotective role of Zx in energy dissipation was postulated [103]. Demmig and co-workers demonstrated in numerous studies the correlation of NPQ and the de-epoxidation state (DEPS) of the Vx cycle pigments (VAZ) under various experimental conditions (reviewed in [48,49,104,105]). The proposed function of Zx in NPQ was finally proven by the identification and characterization of xanthophyll cycle mutants of *Arabidopsis*, deficient either in

Vx de-epoxidase (VxDE) (npq1 mutant) or Zx epoxidase (Zx) (npq2 mutant) activity [39].

#### 3.1. General aspects of the function and regulation of the Vx cycle

The function of the Vx cycle is based on the photoprotective role of Zx that is formed in the light. At least two different basic functions have been described for Zx: (i) a central role in the deactivation of excited  $^1\mathrm{Chl}^*$  in the antenna of PSII (NPQ, see Section 3.3) [39] and (ii) an antioxidant function as non-protein bound xanthophyll in the lipid phase of the thylakoid membrane (see Section 3.2) [106]. These two different functions imply the presence of different VAZ pools in the thylakoid membrane. In fact, the non-protein-bound Vx fraction in dark-adapted *Arabidopsis* plants grown under moderate light intensities of 120 µmol photons m $^{-2}$  s $^{-1}$  was found to amount to approximately 10% of the total VAZ pool [107], but higher values may be expected for plants acclimated to higher growth light intensities [21].

Strict regulation of the VxDE by the lumen pH [100] ensures that Zx typically accumulates only under conditions where electron transport is saturated, in order to avoid undesirable dissipation of excitation energy under light-limiting conditions [73]. Upon illumination of dark-adapted plants with saturating light intensities, maximal deepoxidation states (DEPS) can be reached within 15 to 30 min [51,95,108,109]. The maximal DEPS in higher plants is rather variable (typically 60-90%) and differs not only among species (e.g. seasonal and evergreen species) but particularly depends also on the growth light conditions (e.g. sun and shade acclimation) [21,104,110,111]. The limitation of Vx convertibility has been attributed to the binding of a fraction of Vx to binding sites in antenna proteins (presumably L2) that are not accessible for de-epoxidation [63,64,108,112]. By contrast, non-protein bound Vx is known to be completely and rapidly convertible to Zx [63,108,109]. A recent study on the convertibility of Vx in isolated spinach LHCII provided further evidence that the thylakoid membrane lipid monogalactosyldiacylglycerol (MGDG) also plays a crucial role for the convertibility of Vx [113].

Reconversion of Zx to Vx is catalyzed by Zx epoxidase (ZxE) and occurs under low light or in the dark. The activity of ZxE, and thus the rate of Zx reconversion, are strongly modified by the light stress experienced by plants [114]. While complete Zx epoxidation can be achieved within about 20–40 min after short pre-illumination with moderate light intensities [51,108,109,115], ZxE activity was found to be gradually retarded after increasing light stress [114]. In extreme cases of severe stress under *in vivo* conditions, such as in overwintering evergreen plants during cold stress, Zx epoxidation might be completely blocked for several weeks [116,117]. The retardation of Zx epoxidation was found to correlate with the down-regulation of PSII activity [114,116,117] implying that Zx has a crucial function in long-lasting quenching states of PSII (see Section 3.3. for further details).

#### 3.2. The role of Zx in the lipid phase of the membrane

The comparison of light stress sensitivity and ROS formation in mutants affected either in qE (npq4), Zx synthesis (npq1), or both (npq1npq4), provided the first evidence that Zx has an important photoprotective function independent from its role in qE [118]. This photoprotective action of Zx has been related to the action of Zx in the lipid phase of the thylakoid membrane [68,119,120] and was furthermore found to be additive to the photoprotective function of tocopherol [106,121]. Since singlet oxygen ( $^{1}O_{2}$ ) has been shown to be the most important ROS involved in photo-oxidative damage in chloroplasts under high light stress [122], any Zx-related enhancement of photoprotection can be expected to be based either on the reduction of  $^{1}O_{2}$  formation or the removal of  $^{1}O_{2}$  or  $^{1}O_{2}$ -related oxidation products. While reduction of  $^{1}O_{2}$  formation is efficiently

provided by NPO processes (see below), the function of non-protein bound Zx is likely due to removal of the <sup>1</sup>O<sub>2</sub> or <sup>1</sup>O<sub>2</sub>-related oxidation products. In fact, the synergistic function of Zx and tocopherol in the protection of membrane lipids against peroxidation has been shown under in vitro [123,124] and in vivo conditions [106,107]. The redundant and synergistic effect of Zx and tocopherol is further supported by the increase in the VAZ pool size in tocopherol-deficient plants and the more pronounced sensitivity to high light stress in the tocopherol- and Zx-deficient *vte1npq1* mutant in comparison with the single mutants vte1 and npq1[121], which leads to a reduction of lipid peroxidation under in vivo conditions independent from the binding of Zx to antenna proteins [106,107]. Finally, the general physicochemical properties of Zx presumably exert a further photoprotective effect related to the stabilization of thylakoid membrane lipids [125], and a unique function of Zx among the carotenoids found in thylakoid membranes has also been supported in several in vitro studies [126-128].

In conclusion, the role of Zx as an antioxidant in the lipid phase of the membrane, particularly under extreme high light stress, is essentially important for plants to minimize photo-oxidative damage of membrane lipids.

#### 3.3. The role of Zx in NPQ

The best-studied photoprotective mechanism against high-light stress is the non-photochemical quenching (NPQ) of excitation energy, which is mostly due to thermal deactivation of excited states of pigments in the antenna of PSII [129]. It is well-established that NPQ is a very complex and finely regulated process, in which the major players are (i) the proton gradient across the thylakoid membrane [130], (ii) the xanthophyll Zx [39,103] and (iii) the PsbS protein [46,47,131]. Based on the induction and relaxation kinetics of NPQ under in vivo and in vitro conditions [51,90,132-134] at least four different NPQ components have been defined which are likely to be based on different molecular mechanisms: (1) qE, energy- or pH-dependent quenching [132], (2) qT, state transition quenching [135], (3) qZ, Zx-dependent quenching [51] and (4) qI, photoinhibitory quenching [136]. In higher plants, state transitions do not significantly contribute to NPQ [51], in contrast to the situation in green algae [137]. For all other components, however, a significant role of Zx, either as direct quencher or modulator, has been proposed [51] (Fig. 3). In general, the relative contribution of the different NPQ processes to the overall light-inducible NPQ in Arabidopsis varies strongly in dependence on light intensity and light-exposure time. The pH-regulated qE dominates the total NPO under short (up to 10 min) illumination times at moderate light intensities, while qZ develops (also at moderate light intensities) in the time range from 10 to 30 min and qI at longer illumination time (>30 min) at high light intensities, as has been derived from the induction and relaxation kinetics of NPQ in dark-adapted Arabidopsis wild type leaves [51,90] (Fig. 3).

A comparative analysis of NPQ dynamics and Zx formation/reconversion in Arabidopsis indicated that the conversion of the major pool of Zx does not match with the dynamics of qE, but rather with the formation and relaxation kinetics of qZ and possibly also qI [51]. As an example, the onset of NPQ and xanthophyll conversion upon illumination of dark-adapted Arabidopsis leaves at two different light intensities is shown in Fig. 4. It is obvious that rapid induction of NPQ (related to the build-up of the  $\Delta pH$ ) is similar under both light conditions, while prolonged illumination at high light intensities induces much more slowly developing NPQ states than at the lower light intensity. As a rough estimate, illumination at 900 µE (for simplicity µE will be used instead of 'µmol photons m<sup>-2</sup> s<sup>-1</sup>) induces mainly qE and qZ, while pronounced induction of qI occurs at 1800 μE (Fig. 4A). The latter can be deduced from the large portion of NPQ that is irreversible within the chosen time. It also follows that Zx synthesis is quite similar at both intensities, but the Zx reconversion in the

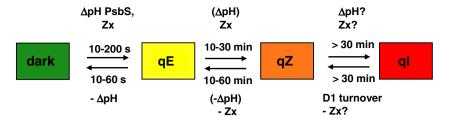


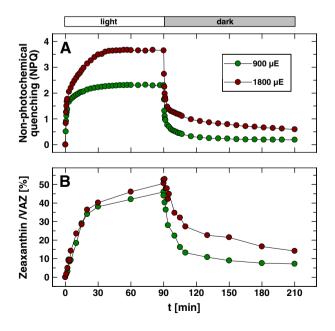
Fig. 3. Model for the different NPQ states in Arabidopsis (adapted from [51]). Starting from the dark-adapted state, illumination of leaves with saturating light intensities leads to the activation of qE within 10–200 s. This process is dependent on  $\Delta pH$  and PsbS, and further modified by Zx. The formation of qE-active Zx is the time-limiting step for the generation of maximum qE upon illumination of dark-adapted leaves. Relaxation of qE is strictly controlled by the breakdown of the transthylakoid  $\Delta pH$ . Prolonged illumination activates another Zx-dependent NPQ state (qZ) within 10 to 30 min and requires the conversion of more slowly convertible Vx. Relaxation of qZ is controlled by the reconversion of Zx to Vx. At longer illumination time (typically > 30 min) a photoinhibitory state (qI) is generated which requires D1 turnover for relaxation.

dark is clearly retarded after illumination at the higher light intensity (Fig. 4B). One should have in mind, however, that the general NPQ capacity and the dynamics of NPQ and xanthophyll conversion shown here for *Arabidopsis* may be highly variable and flexible among different species and under field conditions, particularly when shortlived species are compared with perennial evergreens [138].

In the following, the role of Zx and the proposed molecular mechanisms of the different NPQ processes will be discussed in detail.

#### 3.3.1. The role of Zx in qE

The qE component of NPQ is defined as the NPQ process that is strictly dependent on the pH in the thylakoid lumen [132] and the activation of PsbS that acts as a sensor of the lumen pH [46,47,131]. qE has also been shown to be modulated by Zx [139,140]. Consequently, qE can be activated and deactivated very quickly (1–2 min) in response to rapidly changing light intensities under lab conditions [140–142,145] and in the field [143,144]. The detailed processes giving rise to qE, including the actual quenching site and the underlying molecular mechanism, and the role of Zx in qE are still under intense debate. It was originally proposed that qE is based on pH-induced conformational changes leading to the aggregation of the major LHCII [88,146–148]. Later studies on isolated antenna complexes and on plants with altered composition of the PSII antenna



**Fig. 4.** Dynamics of NPQ and xanthophyll conversion. Dark-adapted *Arabidopsis* WT plants were illuminated for 90 min at light intensities of 900 μE (green) or 1800 μE (maroon) and subsequently transferred to darkness for 120 min. Fluorescence quenching was analyzed using a PAM 101 device (Walz, Effeltrich, Germany), and NPQ was calculated as  $(F_m/F_m'-1)$ . The Zx content was determined from HPLC analyses of the pigment content.

proteins suggested that the minor antenna complexes might also contribute to qE [55,56,149–151]. According to the current view, pH-induced conformational changes or changes in the protein/protein interaction in the PSII antenna proteins are essentially required for qE, and these changes are controlled by PsbS and modulated by Zx. Two different roles of Zx have been proposed: (i) an indirect role as an allosteric modulator of qE, controlling the efficiency, kinetics and the apparent pK of qE [140,152–154] and (ii) a direct role of Zx in the quenching process either due to energy transfer from Chl to Zx [155,156] or due to electron transfer to a neighboring Chl forming a Zx $^+$ /Chl $^-$  CT state [53,54,92,93]. The recent identification of two different NPQ-active quenching sites [60,90] provides a consistent explanation for the two models (see also Fig. 2). However, the latter data put into question whether there always is a dominant combined action of PsbS and Zx.

PsbS-dependent formation of quenching site Q1, i.e. detachment and oligomerization of parts of the PSII antenna complexes — mostly LHCII [60] and likely also some CP24 [57], can explain the proposed LHC II oligomerization model put forward by Horton, Ruban and coworkers [9]. The activation of Q1 in vivo has been shown to be characterized by the formation of a far-red fluorescing component, which can be assigned to an oligomeric state of detached LHCII [60]. This assignment was particularly supported by its spectral characteristics, resembling closely the properties of the far-red fluorescence of isolated aggregated LHCII [157]. Using a multiwavelength spectrometer that allows time-dependent measurement of fluorescence spectra in intact leaves, it was furthermore shown that the far-red fluorescing component has essentially the same characteristics as qE (i.e. it is strictly dependent on PsbS, but not strongly so on Zx, and it is rapidly inducible and rapidly relaxing upon illumination and redarkening, respectively) [90]. Strikingly, a PsbS-dependent dissociation/reassociation of PSII antenna proteins (composed of LHCII, CP29 and CP24) from the PSII-LHCII supercomplex under NPQ conditions has also been demonstrated by biochemical analyses and electron microscopy [57]. Although energy transfer to Lut was proposed to provide the basis of qE in a study of oligomeric LHCII in vitro[85], recent femtosecond transient absorption studies on such samples did not find any evidence for energy transfer to carotenoids, including Zx. Instead, these latter studies supported a model where quenching of <sup>1</sup>Chl\* in LHCII is based on the formation of a fluorescent Chl-Chl CT state [89,91]. Irrespective of the exact molecular mechanism of quenching in LHCII in vitro, Zx seems to play an indirect, albeit important, role in the modulation of PsbS-dependent qE. High levels of Zx accelerate or enhance the formation of qE, and retard its relaxation [39,51,140]. Zx has further been shown to shift the apparent pK of gE to a more alkaline pH [158]. The fluorescence lifetimes associated with the quenched state in chloroplasts have been shown to be significantly shorter in presence of Zx [153] indicating a more efficient energy dissipation, and thus better photoprotection, in the presence of Zx. Interestingly, similar characteristics have been observed for the lifetimes of the far-red fluorescent quenching

component Q1, that was found to be somewhat shortened when Zx was present (*ca.* 400 ps *vs. ca.* 600 ps), although the main quenching effect (from about 4 ns for trimeric LHCII to about 600 ps for the Q1 component) was independent of Zx [60]. Finally, it was concluded from spectroscopic analyses of isolated LHCII that Zx promotes the oligomerization of LHCII [159], which can possibly explain the abovementioned characteristics of Q1.

Upon illumination of dark-adapted leaves, the generation of maximum qE is rate-limited by the formation of qE-active Zx [51]. Since only a small fraction of the overall VAZ pool is converted to Zx in the time range of full activation of qE (within  $\leq$ 3 min), only a rapidly convertible pool of Vx could be responsible for the enhancing effect of Zx on qE under these experimental conditions in *Arabidopsis*. The required rapid conversion kinetics have been determined for non-protein-bound Vx and Vx bound to the V1 site of LHCII [64]. Thus, rapidly convertible VAZ pigments in the close vicinity of LHCII are most likely involved in the modulation of qE by Zx.

In contrast to Q1, the activation of Q2 was found to be independent of PsbS, but strongly dependent on Zx, implying an important role of Zx in the associated quenching process [60]. Although the mechanistic basis of the quenching process in Q2 has not been determined unequivocally, it is possible that the reported formation of Zx-Chl CT states [92] is involved in this type of quenching. Since Zx-Chl CT states have been shown to occur predominantly in the minor antenna proteins (Lhcb4–6), but not in LHCII trimers [93], it can be speculated that the minor LHCs represent the Q2 site of quenching. Such an interpretation would be consistent with the described PsbS- and pHindependent type of quenching found in the minor antenna complex Lhcb5 [160]. Due to missing information on the kinetics of formation and relaxation of quenching at Q2, however, it cannot yet be decided, whether the quenching at Q2 represents strictly pH-controlled processes, and thus a Zx-dependent component of qE quenching, or possibly a more slowly developing and relaxing component of NPQ related to qZ [51] or even qI [57,160]. Irrespective of these remaining questions, however, it is clear that quenching at Q2 is caused by, and identifies, a Zx-dependent NPQ component in the PSII-attached

It is worth to point out here, that the details of the qE characteristics and the role of Zx in qE described above have been derived mainly from investigations performed with *Arabidopsis* (wild-type and mutant) plants. It will be important to determine in future work whether these characteristics may vary or be different in species with generally other properties (*e.g.* long-lived slow growing species, perennial evergreens or sun and shade plants).

#### 3.3.2. The role of Zx in qZ

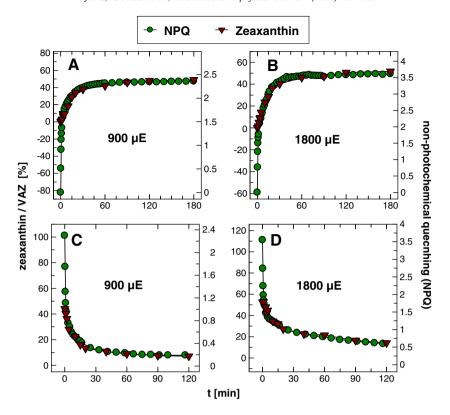
Based on detailed studies on the dynamics of NPQ and xanthophyll conversion, the qZ component of NPQ has been identified as a slowlydeveloping (10-30 min) and slowly-relaxing (10-60 min) component of NPQ [51] (Fig. 3). Due to its dependence on Zx, the formation and relaxation of qZ are also indirectly dependent on the lumen pH, since Zx formation requires a low lumen pH and Zx epoxidation occurs only in the absence of a high  $\Delta pH$ . However, once established, qZ contributes to NPQ even in the absence of a  $\Delta pH$ , thus allowing a sustained dissipation of energy after longer periods of high light illumination, similar to qI (see Section 3.3.3). In physiological terms, such a mechanism can be understood as a kind of 'memory state' of high light stress, allowing a rapid reactivation of highly efficient quenching of plants in case of re-illumination with higher light intensities. It has been speculated that such a 'memory state' may be attributed to the qI component of NPQ [9]. However, the appearance of a more slowly relaxing NPQ component (with similar characteristics as qZ) under prolonged illumination at saturating light intensities was shown not to be associated with a further reduction of the PSII quantum yield and hence not related to the photoinhibitory qI quenching [57]. It is thus very likely that qZ is identical to the NPQ component that was originally related to a Zx-dependent portion of ql [161–165], but which can now better be interpreted as a qE- and ql-independent, but Zx-dependent NPQ component generated in the antenna of PSII [51,57,160]. The Zx-accumulating npq2 mutant can thus be expected to be arrested in the qZ state. In fact, the reduced  $F_v/F_m$  ratio found in unstressed dark-adapted npq2 plants (0.73–0.75) in comparison with wild-type plants (0.82–0.84) is indicative of a permanent NPQ, which is likely to reflect qZ (and not any ql state). Strikingly, the reduction of the  $F_v/F_m$  from about 0.83 to 0.74 corresponds to an NPQ value of about 0.5, which is close to the amplitude of qZ determined in Arabidopsis wild-type plants [51].

These characteristics of qZ hint at a possible connection between qZ and photoinhibitory quenching qI, and might thereby promote the proposed transition from the qE to the qI state [166,167]. Such a connection is further supported by the notion that both NPQ components, qZ and qI, show a retardation of the relaxation kinetics under increasing light stress conditions concomitant with the retardation of Zx epoxidation. As shown in the example in Fig. 5, both Zx formation (during illumination) and reconversion (upon redarkening) correlate kinetically with the formation and relaxation, respectively, of the more slowly developing NPO components qZ and gl, but not with the dynamics of gE. Strikingly, the pronounced slowing of NPO relaxation after illumination at 1800 µE (relative to illumination at 900 µE) was paralleled by a very similar reduction of the Zx epoxidation kinetics. Moreover, the increase of the portion of irreversible (i.e. lifetime > 2 h) NPQ (qI) was paralleled by an increase of the portion of non-convertible (i.e. lifetime >2 h) Zx (Fig. 5). Although this correlation is likely to reflect a pronounced role of Zx in qZ and qI, no conclusions can be drawn from such correlations about the mechanistic basis of such a function. In particular, it remains to be clarified whether Zx has a direct or indirect function in these two photoprotective mechanisms. As already discussed above, qZ may constitute a contribution to quenching at the Zx-dependent site Q2 located at antenna proteins that remain associated with PSII [60] (Fig. 2) and may possibly be attributed to the formation of a Chl-Zea CT state in the minor antenna complexes Lhcb4-6 [53,93], while a part of qI may rather be located in the reaction center (RC) of PSII. Taking all available data together it becomes evident that a substantial part of the slow quenching traditionally assigned to the qI component does not represent genuine photoinhibition (in the sense of impaired photochemistry), but rather a slowly reversible genuine NPQ component that is Zx-dependent.

#### 3.3.3. The role of Zx in qI

It becomes increasingly clear that the term qI is rather ill-defined and comprises all NPQ states that relax more slowly than qE and qZ. Traditionally, qI has been defined rather technically as the very slow or non-relaxing quenching component. Due to this unclear definition, qI reflects all processes that contribute to long-lasting downregulation, inactivation or damaging of PSII. In the classical sense, photoinhibition is defined as the light-induced reduction of the quantum yield of photosynthetic carbon fixation [168], irrespective of the underlying mechanism. Due to the complexity of the high-lightinduced inactivation of PSII in the long-term, however, it is likely that several different processes contribute to this traditional qI term [169], such as the actual irreversible inactivation of PSII due to acceptor side inhibition [170,171], donor side inhibition [172,173] or UV-B related inactivation of PSII [174,175]. While these types of PSII photoinhibition are associated, in a broad sense, with the inactivation of the D1 protein which has to be replaced after degradation by newly synthesized D1, also sustained down-regulation of PSII by maintenance of a ΔpH in the dark due to ATP hydrolysis [176] or by maintenance of sequestered protons in the thylakoid membrane [177] will give rise to qI [178,179].

Independent of the underlying photophysical mechanisms of qI, Zx seems to play a crucial function in all of these processes. This



**Fig. 5.** Comparison of the kinetics of NPQ induction/relaxation with the kinetics of Zx formation/epoxidation. Dark-adapted Arabidopsis WT plants were illuminated for 180 min at light intensities of 900 μE (A) or 1800 μE (B) and subsequently transferred to darkness for 120 min (C, D). For direct comparison of the dynamics of NPQ (circles) and Zx conversion (triangles), the amplitudes of the pigment data were fitted to match the amplitudes of the more slowly developing (>2 min) and relaxing (>2 min) NPQ components, qZ and qI, only.

assumption can be derived from the fact that any sustained downregulation or inactivation of PSII is accompanied by the retention of Zx under in vivo conditions. In Arabidopsis, increasing photo-oxidative stress in the short-term (i.e. during illumination for up to 8 h) induced either by prolonged illumination, increasing light intensities or illumination at low temperature, has been shown to induce a gradual down-regulation of ZxE activity [114], leading to nearly full inactivation of Zx epoxidation after the harshest treatments, as can be found under natural conditions e.g. in evergreen plants during winter acclimation [116,117]. The sustained retention of Zx in evergreen species during winter has been found to correlate not only with the decrease of PSII efficiency but also with the phosphorylation of D1 and an increase of PsbS [180-183]. The differential retention of Zx in response to different photo-oxidative stress conditions thus suggests that the rate of Zx epoxidation represents a long-term memory (i.e. many hours) of the chloroplast with respect to preceding photo-oxidative stress and strongly supports the outstanding importance of Zx as a crucial photoprotective xanthophyll. Although the molecular basis for the repression of Zx epoxidation has not been elucidated so far, it can be speculated that this phenomenon is related to a direct inactivation of the ZxE, since the reduction of the ZxE activity has been shown to be maintained in thylakoids isolated from high-light-treated plants [114]. Such a highlight-induced inactivation of the ZxE might be established e.g. by ROSinduced modifications or by phosphorylation.

The exact role of Zx in qI is still unclear. This uncertainty is related primarily to the lack of sufficient differentiation of the processes summarized traditionally under the qI term and also in part to a contradictory use of this term in the literature. Since at least parts of the processes that contribute to qI are clearly related to the light-induced inactivation of the D1 protein and thus require D1 degradation and resynthesis, any direct effect of Zx might be expected to serve the protection of inactivated PSII RCs. Whether such protective

mechanisms involve a direct interaction of Zx with the RC of PSII or are restricted to the protection of the PSII antenna proteins, remains to be clarified. Alternatively, such a photoprotective function of Zx in qI might also be simply related only to the antioxidative function of Zx in the lipid phase of the membrane (see Section 3.2). In this context, it should be recalled that slowly reversible quenching states, traditionally summarized under the term qI, generally develop more strongly (and relax more slowly) in the absence of Zx (as in the npg1 mutant) and are clearly reduced upon permanent accumulation of high levels of Zx (as e.g. in the npq2 mutant) [51]. Accordingly, Zx deficient plants show increased photo-oxidative stress and Zxaccumulating plants decreased photo-oxidative stress even in the absence of antenna proteins as also judged from the formation of ROS [106]. The nearly complete and sustained depression of  $F_v/F_m$  to values near zero concomitant with the reduction of the photosynthetic capacity in strongly Zx-accumulating over-wintering evergreens demonstrates, however, that a strong reduction of the PSII efficiency is not under all conditions an indicator of undesirable photoinhibition, but may also represent a powerful photoprotective mechanism to reduce ROS formation [144]. These findings underline the important photoprotective role of Zx under a large variety of photooxidative and photoinhibitory conditions.

#### 4. NPQ and photoprotection

Typically, the NPQ response and maximal NPQ capacity of photosynthetic organisms are determined via the PAM pulse fluorometer method that compares the maximal PSII fluorescence intensity of the system under high light stress  $(F_{\rm m}')$  to the maximal fluorescence intensity emitted from the system after dark adaptation  $(F_{\rm m})$ , each measured after a short strong light pulse has been applied to close all PSII centers [184]. This type of measurement yields the so-called "NPQ parameter", defined as  $qN_{\rm SV} = F_{\rm m}/F_{\rm m}' - 1$  based on the assumption of

Stern-Volmer quenching [185]. In general, it is assumed that an increase of gN<sub>SV</sub> results in a corresponding more or less linear increase of the actual photoprotection to the photosystems, in particular of PSII. This means that qN<sub>SV</sub> is actually implicitly taken as a proxy for the degree of photoprotection. Typically, the maximal qN<sub>SV</sub> measured in higher plants at saturating light is up to 5-6 [186,187] but much higher values can sometimes be observed, e.g. in diatom algae [188]. It is worthwhile to point out that the quantitative relationship between qN<sub>SV</sub> and the actual photoprotection of PSII - the actual effect of interest for plant survival - has thus far not been demonstrated nor has a quantitative relationship been derived that would support that notion. In order to judge the physiological importance of the different NPQ processes and their photoprotection effects, it will thus be important to understand the quantitative relationship between a given level of qN<sub>SV</sub> and the actual level of photoprotection on the photosystems under a given set of physiological conditions. Recent estimates from our laboratories (P. Lambrev, Y. Miloslavina, P. Jahns and A.R. Holzwarth, unpublished) indicate that one can by no means assume that the relationship between relative fluorescence quenching - as measured by the qN<sub>SV</sub> parameter - and actual photoprotection should be linear. Detailed future work will have to explore this relationship in detail. Until such detailed insight is available it will be difficult, if not impossible, to judge and differentiate the actual impact and relevance of the various xanthophylls on physiological photoprotection.

#### 5. Conclusions

Two xanthophylls, Lut and Zx, have been shown to serve essential photoprotective functions in PSII. While the role of Lut is mainly restricted to its unique function in the deactivation of excited triplet Chl (3Chl\*) states, Zx is a major player in the deactivation of excited singlet Chl (1Chl\*) state and thus in NPQ. Although the exact role of Zx in NPQ is not completely understood, overwhelming evidence supports that Zx contributes - at least partly - to all NPQ mechanisms except qT (state transitions). It is assumed that Zx is - either directly or indirectly - involved in the activation of NPQ states, first in the peripheral LHCII antenna (qE), and at the longer time scale in the minor antenna complexes (qZ), and finally probably also in the reaction center (qI). In addition to its role in NPQ processes, Zx serves further important functions as antioxidant in the lipid phase of the membrane. The central role of Zx in these photoprotective processes is reflected by the differential down-regulation of Zx epoxidation in response to photo-oxidative stress, which allows the rapid reactivation of the pH-dependent qE state and the differential retention of pH-independent NPO states (qZ and qI) as well as of more general antioxidative properties of Zx. According to these central photoprotective functions, Zx can be suggested to act as a key component in the memory of the chloroplast with respect to preceding photooxidative stress.

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