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Short-term down-regulation of zeaxanthin epoxidation in *Arabidopsis thaliana* in response to photo-oxidative stress conditions

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ABSTRACT

The epoxidation of zeaxanthin (Zx) to violaxanthin after exposure to different light stress conditions has been studied in *Arabidopsis* (*Arabidopsis thaliana*). Formation of Zx was induced by illumination of intact leaves for up to 8 h at different light intensities and temperatures. The kinetics of epoxidation was found to be gradually retarded with increasing light stress during pre-illumination, indicating a gradual down-regulation of the Zx epoxidase activity. Retardation of the epoxidation rates by a factor of up to 10 was inducible either by increasing the light intensity or by extending the illumination time or by decreasing the temperature during pre-illumination. The retardation of the epoxidation kinetics was correlated with a decrease of the PSII quantum efficiency after the pre-illumination treatment. Experiments with the *stn7/stn8* mutant of *Arabidopsis* indicated that the thylakoid protein kinases STN7 and STN8, which are required for the phosphorylation of PSII proteins, are not involved in the short-term down-regulation of Zx epoxidation. However, the retardation of Zx epoxidation was maintained in thylakoids isolated from pre-illuminated leaves, indicating that a direct modification of the Zx epoxidase is most likely involved in the light-induced down-regulation.

1. Introduction

The light-induced formation of reactive oxygen species is an extremely harmful stress factor for photosynthetic organisms. Higher plants have developed a number of strategies to avoid or minimize damage related to such photo-oxidative stress. The carotenoid zeaxanthin (Zx), which is formed from violaxanthin (Vx) via the intermediate antheraxanthin (Ax) in the de-epoxidation reactions of the so-called xanthophyll cycle [1–3], plays a central role in different photoprotective mechanisms in chloroplasts: It is essentially required for thermal dissipation of excess light energy (= non-photochemical quenching, NPQ) in the antenna of PSII (so-called qE-quenching) [4–6] and additionally acts as an antioxidant in the lipid phase of the thylakoid membrane [5] or at the protein/lipid interface [7,8]. Furthermore, a role of Zx has been proposed for photoinhibitory processes (qI-quenching) related to events in the reaction centre of PSII [9,10]. In over-wintering evergreen plants, additional involvement of Zx (and Ax) in sustained down-regulation of PSII has been reported [11–14].

The Zx content in the thylakoid membrane has to be regulated very carefully to avoid undesirable annihilation of absorbed light energy at non-saturating light intensities on the one hand and to allow rapid activation of protective mechanisms under stress conditions on the other hand [15]. The activity of the VxDE – and by that the rate of Zx formation – is strictly regulated by the lumen pH [16] and the enzyme is activated at pH values below 6.2 [17,18]. At low light intensities which are not sufficient to generate a lumen pH below 6.2, or in the dark, Zx is reconverted back to Vx in the epoxidation reactions. The epoxidation is catalyzed by the Zx epoxidase (ZxE) [19,20] which is localized at the stroma side of the thylakoid membrane and requires molecular oxygen as second substrate [21,22] and NADPH as cofactor [23].

The regulation of the rate of Zx epoxidation is thus central to control the level of Zx in chloroplasts of higher plants. After short (up to 15 min) illumination at moderate light intensities up to 600 µmol photons m^{-2} s^{-1} Zx is completely reconverted to Vx within 1 h under *in vitro* and *in vivo* conditions [23,24]. Prolonged illumination (180 min) of pea leaves at very high intensities (2500 µmol photons m^{-2} s^{-1}) at 2 °C induced a retardation of Zx epoxidation by a factor of about 10 [24]. In intact leaves, a kinetic correlation of Zx epoxidation and the relaxation of the qI-component of NPQ (= photoinhibition) has been described [9,25]. Such a correlation can be understood by a direct or indirect involvement of Zx in energy dissipation processes located in the reaction centre of PSII. In contrast to qE, this Zx-dependent qI-quenching was found to be independent of a transthylakoid pH gradient [10,26]. In extreme cases of severe stress, like in over-wintering evergreen plants high levels of Zx can be permanently retained during winter along with sustained down-regulation of PSII

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Abbreviations: Ax, antheraxanthin; DEPS, de-epoxidation state; NPQ, non-photochemical quenching; PSII, photosystem II; Vx, violaxanthin; VxDE, violaxanthin de-epoxidase; Zx, zeaxanthin; ZxE, zeaxanthin epoxidase

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activity [12,27]. Obviously, persistent Zx accumulation provides efficient photoprotection of the photosynthetic apparatus under unfavourable environmental conditions [28].

The molecular basis of the retainment of Zx under extreme stress conditions is unclear. In general, either a restriction of the substrate availability or an inactivation of the ZxE might control the rate of Zx epoxidation. From studies with antenna-depleted pea plants it was concluded that the ZxE prefers protein-bound xanthophylls rather than non protein-bound ones as a substrate [24,29], so that light-induced changes in the substrate availability might be involved in the down-regulation of Zx epoxidation. Alternatively, a down-regulation of the ZxE itself can be proposed. There is some experimental evidence that phosphorylation reactions may be involved in the sustained down-regulation of the activities of ZxE [30] and PSII [14,31]. Ebbert et al. [31] observed that sustained D1 phosphorylation during winter acclimation is accompanied by the up-regulation of the protein phosphatase inhibitor TLP40 in an evergreen conifer. TLP40 is a thylakoid lumen protein being involved in the regulation of a thylakoid membrane phosphatase which is responsible for the dephosphorylation of PSII core proteins [32]. It has further been shown in more recent studies that also the levels of PsbS and particularly PsbS-related proteins like early-light-inducible proteins (ELIPs), high-light-inducible proteins (HLIPs) and one helix proteins (OHPs) are up-regulated during winter acclimation of evergreen plants [13,33,34]. However, the possible functions of these proteins in the regulation of persistent energy dissipation are unknown. A role of the NADPH concentration as a critical parameter for the ZxE activity was recently proposed in a study with the *nadk2* mutant of *Arabidopsis* [35]. This mutant, which is defective in a chloroplast NAD kinase and thus contains only about 50% of the amount of NADPH found in wild-type plants, accumulates high levels of Zx even in low light and in the dark [35]. In a recent study with duckweed (*Lemna trisulca*) it was shown that amino sugars are efficient inhibitors of Zx epoxidation and it was speculated that this class of substances may be involved in the sustained down-regulation of Zx epoxidation under *in vivo* conditions [36].

While most of the previous work on the regulation of the xanthophyll cycle has been concerned with the regulation of the VxDE, this work presents a thorough study of the characteristics of Zx epoxidation and the regulation of ZxE activity in response to light stress conditions in the short term. We could show that Zx epoxidation is gradually retarded upon increasing high-light stress and nearly completely inhibited within 2 h of high-light treatment at low temperature. The retardation of Zx epoxidation was correlated with a decrease of the PSII quantum efficiency. The down-regulation of Zx epoxidation was further found to be maintained in thylakoids isolated from pre-illuminated leaves, but to be independent on the phosphorylation of PSII proteins. Therefore our data provide evidence that a light-stress induced modification of the ZxE is responsible for the down-regulation of Zx epoxidation.

2. Material and methods

2.1. Plant material and growth conditions

Wild-type and *stn7/stn8* mutant plants *Arabidopsis thaliana* (ecotype Columbia 0) were grown in soil at a light intensity of 150 µmol photons m^{-2} s^{-1} and a constant temperature of 20 °C under long-day conditions (14 h light/10 h dark). 4–5 week-old plants were used for all experiments.

2.2. Fluorescence measurements

The PSII quantum efficiency (F_V/F_M) in the dark-adapted and light-adapted state was determined at 20 °C using a pulse-amplitude-modulated fluorometer (PAM 2000, Walz). Prior to each measurement leaves were dark-adapted for 10 min to allow the relaxation of the transthylakoid pH gradient. Control measurements of the NPQ relaxation kinetics in presence and absence of the uncoupler nigericin confirmed, that the pH-dependent NPQ completely relaxed within this time, even after extreme pre-illumination conditions (e.g. 4 or 8 h at 2000 µmol photons m^{-2} s^{-1} and 5 °C).

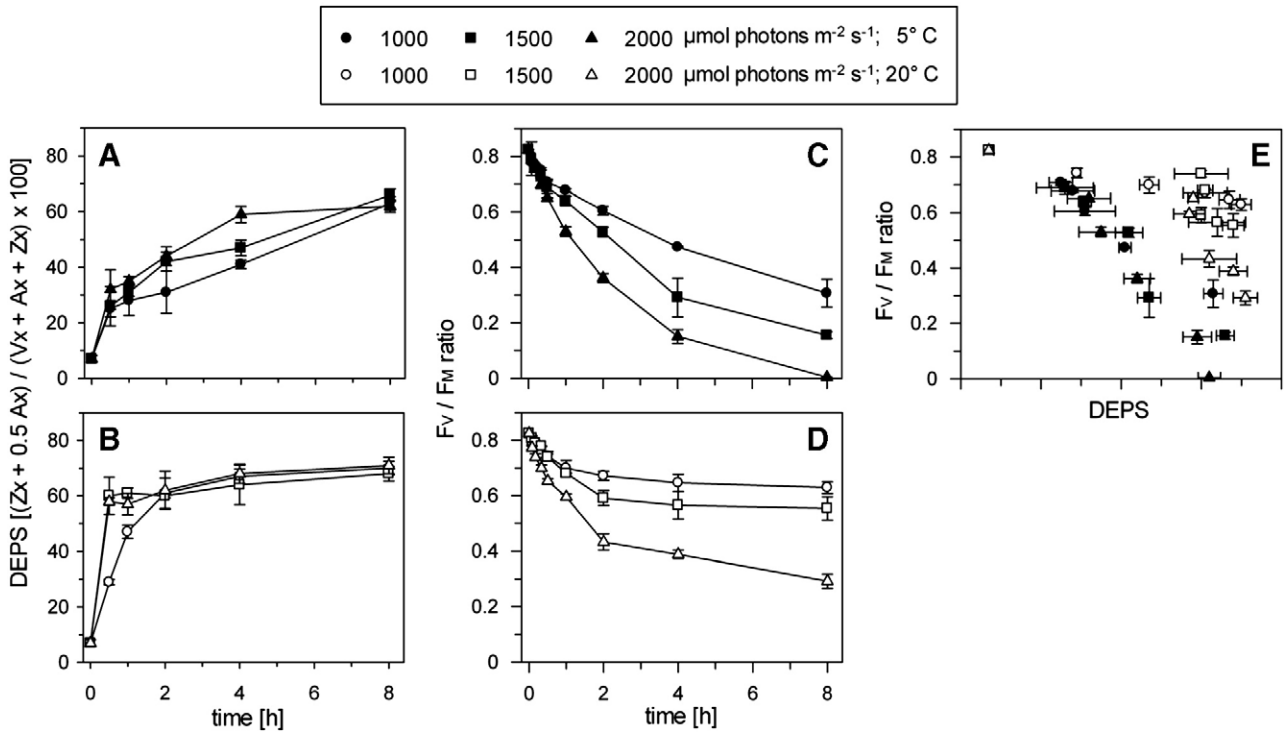

Fig. 1. Time course of Vx de-epoxidation and PSII quantum efficiency at different illumination intensities. A and B, Time course of Vx de-epoxidation at 5 °C and 20 °C, respectively. C and D, Time course of PSII quantum efficiency at 5 °C and 20 °C, respectively. E, Relationship between DEPS and PSII quantum efficiency. 5 week-old wild-type plants were dark-adapted overnight. Leaf discs were placed on water in a temperature-controlled cuvette and illuminated for up to 8 h at indicated light intensities. For pigment analysis, leaf discs were frozen in liquid nitrogen at indicated times prior to pigment extraction. For fluorescence measurements, leaf discs were dark-adapted for 10 min to allow for the relaxation of the transthylakoid pH gradient. Mean values (±SD) of 3 independent experiments are shown.

2.3. Pigment analysis

For pigment analysis, leaves were frozen in liquid N_2 and stored at −80 °C. Pigments were extracted with acetone and quantified by reverse phase HPLC [37].

2.4. Immunoblot analysis

Thylakoids were isolated from dark-adapted and pre-illuminated leaves from 5 week-old plants. Proteins were separated by SDS-PAGE and transferred to poly (vinylidene difluoride) membranes as described [38]. Samples were probed with phosphothreonine antibodies (New England Biolabs) and signals were visualized by enhanced chemiluminescence (Amersham Biosciences).

2.5. In vitro epoxidation

Thylakoids were isolated from pre-illuminated leaves from 5 week-old plants. Epoxidation was performed in the dark in a medium containing 0.4 M sucrose, 50 mM HEPES/NaOH pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 0.3 mg/ml bovine serum albumine, 0.5 mM NADH and 1 µM FAD. Epoxidation was started by the addition of thylakoids (equivalent to 20 µg/ml Chl) to the reaction medium. At indicated times an aliquot of 100 µl of the suspension was mixed with 900 µl acetone to stop the reaction.

3. Results

3.1. Violaxanthin de-epoxidation and inactivation of PSII

The time course of Vx de-epoxidation (Fig. 1A, B) and of PSII inactivation (Fig. 1C, D) was determined during 8 h of illumination at three different light intensities (1, 1.5 and 2 mmol photons m^{-2} s^{-1}) and at two different temperatures (5° and 20 °C). At 20 °C the maximum de-epoxidation states (DEPS) was reached after about 0.5 h of illumination at the two highest light intensities and after about 2 h of illumination at a light intensity of 1 mmol photons m^{-2} s^{-1} (Fig. 1B). By contrast, the maximum DEPS was reached not until 4 to 8 h when illumination was done at 5 °C (Fig. 1A). It should be noted, however, that the same maximum DEPS developed under all conditions. The activity of PSII, as derived from the quantum efficiency (F_V/F_M) of PSII, was gradually decreased with increasing light intensities at both temperatures (Fig. 1C, D). At 20 °C a biphasic decay of F_V/F_M was detected: The major decrease during the first 2 h of illumination was followed by a further slow decay (Fig. 1D) reaching final F_V/F_M values of about 0.65, 0.55 and 0.3 after illumination at 1, 1.5 and 2 mmol photons m^{-2} s^{-1}, respectively. At 5 °C the decrease of the F_V/F_M ratio was much more pronounced (Fig. 1C). While the initial decay during the first 2 h was similar as at 20 °C, no retardation of the decrease was observable at longer illumination time, leading to final F_V/F_M values of about 0.3 and 0.15 after illumination at 1 and 1.5 mmol photons m^{-2} s^{-1}, respectively and to complete inactivation of PSII after 8 h of illumination at 2 mmol photons m^{-2} s^{-1}. Notably, no linear correlation of the DEPS and the quantum efficiency of PSII was found at both temperatures (Fig. 1E).

We determined the dynamics of Zx epoxidation after pre-illumination of leaves under the same conditions as shown in Fig. 1. Zx epoxidation was induced by transfer of pre-treated leaves to low light (10 µmol photons m^{-2} s^{-1}) and the xanthophyll conversion was followed for up to 4 h at 20 °C.

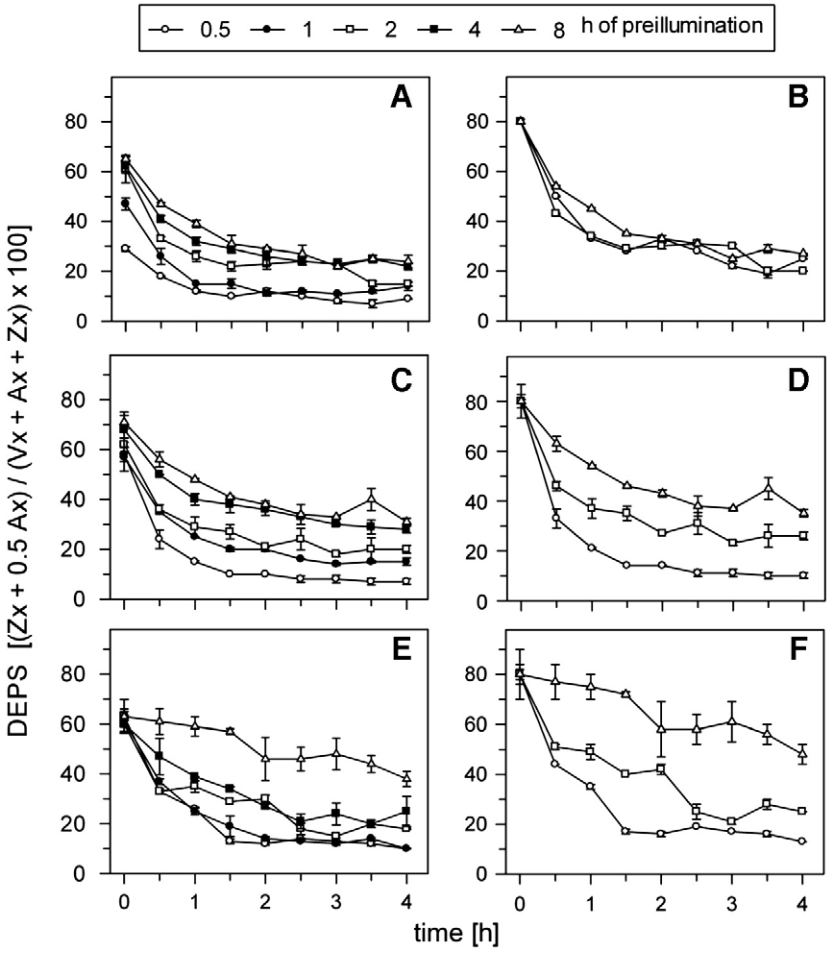

Fig. 2. Time dependence of epoxidation after pre-illumination at 20 °C. A, C and E, Epoxidation after pre-illumination at 1, 1.5 and 2 mmol photons m^{-2} s^{-1}, respectively. B, D and F, Normalized data corresponding to the data shown in the respective part on the left side. Epoxidation was induced by illumination of leaf discs at 10 µmol photons m^{-2} s^{-1}. At indicated times, leaf discs were frozen in liquid nitrogen and then used for pigment extraction. Mean values (±SD) of 3 independent experiments are shown.

3.2. Dynamics of zeaxanthin epoxidation

Fig. 2 summarizes the dynamics of Zx epoxidation in leaves which have been pre-illuminated at 20 °C at 1 mmol photons m^{-2} s^{-1} (Fig. 2A, B), 1.5 mmol photons m^{-2} s^{-1} (Fig. 2C, D) or 2 mmol photons m^{-2} s^{-1} (Fig. 2E, F). For each light intensity, five different traces are shown which represent the time course of epoxidation after five different times of pre-illumination (from 0.5 to 8 h). The left part of Fig. 2 shows the DEPS in each sample as determined from pigment analysis (Fig. 2A, C, E). For a better comparison of the epoxidation kinetics, selected data were normalized to the same initial DEPS (Fig. 2B, D, F). After pre-illumination at 1 mmol photons m^{-2} s^{-1}, the epoxidation kinetics were very similar in each case, independent of the time of pre-illumination and the initial DEPS (Fig. 2A, B). Pre-illumination at 1.5 mmol photons m^{-2} s^{-1}, however, induced a gradual retardation of expoxidation with increasing pre-illumination time. The same trend was observable at the highest light intensity, 2 mmol photons m^{-2} s^{-1}, of pre-illumination, but the retardation of Zx epoxidation was much more pronounced after 8 h of pre-illumination. The kinetics of expoxidation was thus independent of the light intensity during pre-illumination when only short illumination times of up to about 1 h were applied but clear differences became visible at longer illumination times.

The dynamics of Zx epoxidation after pre-illumination at 5 °C was analyzed under the same conditions as before (Fig. 3). To minimize the temperature-dependent but light-independent reduction of the ZxE activity at this lower temperature, all samples were placed immediately after pre-illumination on water with a temperature of 20 °C. After the shortest pre-illumination time of 0.5 h, similar epoxidation kinetics was observed for all light intensities (Fig. 3B, D, F). Interestingly, the kinetics was nearly identical to that observed after short pre-illumination at 20 °C (cf. Fig. 2B, D, F). The retardation of epoxidation at longer pre-illumination time, however, was much more pronounced after low temperature treatment. After pre-illumination for 8 h at the two lower light intensities (1 and 1.5 mmol photons m^{-2} s^{-1}), the DEPS at the end of the low light treatment reached not less than 50% of the initial value obtained directly after pre-illumination (Fig. 3A–D). After pre-illumination for 2, 4 and 8 h at the highest light intensity of 2 mmol photons m^{-2} s^{-1}, Zx epoxidation was nearly completely abolished (Fig. 3E, F). Obviously the increased photo-oxidative stress at lower temperature induced a pronounced retardation of Zx epoxidation.

3.3. Down-regulation of zeaxanthin epoxidation and reduction of PSII quantum efficiency

The above data imply that the Zx epoxidation is down-regulated under *in vivo* conditions in dependence of the extent of photo-oxidative stress. A very similar down-regulation could be induced either by increasing the light intensity or by prolongation of the illumination time or by lowering the temperature, as shown for two representative examples in Fig. 4. To quantify the retardation in terms

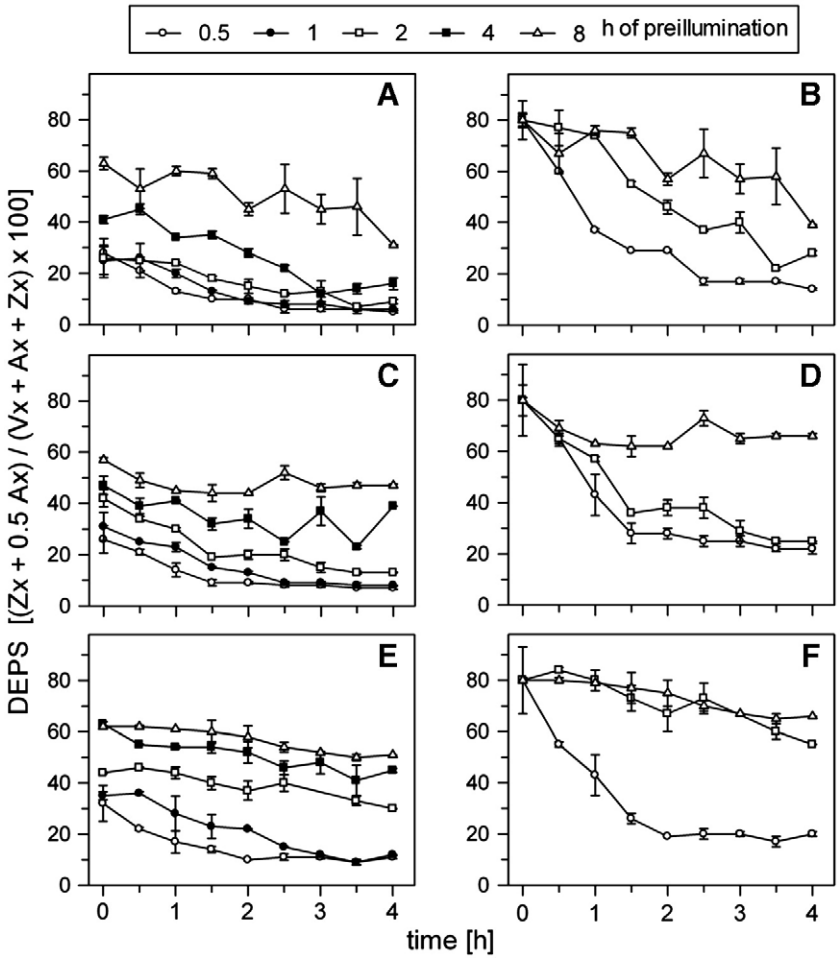

Fig. 3. Time dependence of epoxidation after pre-illumination at 5 °C. A, C and E, Epoxidation after pre-illumination at 1, 1.5 and 2 mmol photons m^{-2} s^{-1}, respectively. B, D and F, Normalized data corresponding to the data shown in the respective part on the left side. After pre-illumination leaf discs were immediately placed on water with a temperature of 20 °C and illuminated at 10 µmol photons m^{-2} s^{-1}. At indicated times, leaf discs were frozen in liquid nitrogen and then used for pigment extraction. Mean values (±SD) of 3 independent experiments are shown.

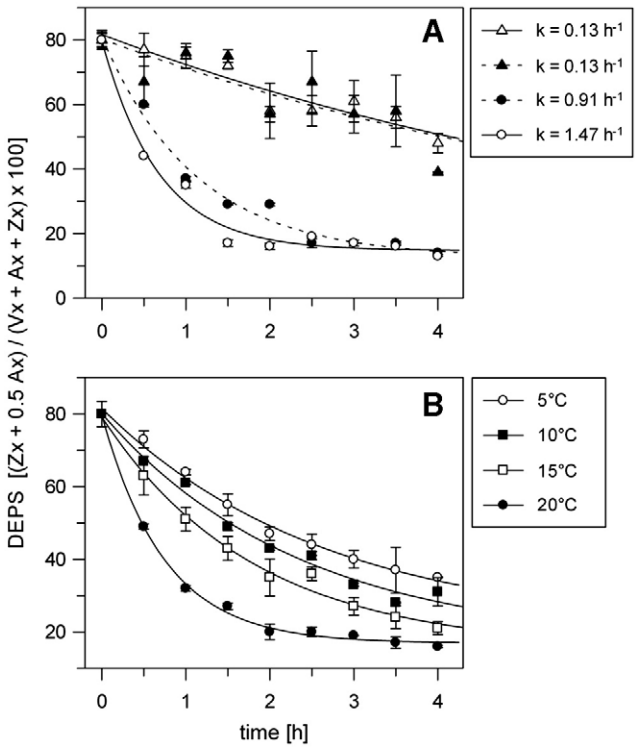

Fig. 4. Analysis of the epoxidation kinetics. A, Selected normalized data from Figs. 2 and 3 were fitted with a single exponential decay according to the equation $y=A*\exp(-k*t)+B$. The constant B represents the residual minimal DEPS which yielded values between 12 and 17 in all cases. Data obtained after pre-illumination under the following conditions have been used: 2 mmol photons m^{-2} s^{-1}, 0.5 h, 20 °C (open circles); 1 mmol photons m^{-2} s^{-1}, 0.5 h, 5 °C (filled circles); 2 mmol photons m^{-2} s^{-1}, 8 h, 20 °C (open triangles) and 1 mmol photons m^{-2} s^{-1}, 8 h, 5 °C (filled triangles). The determined rate constants (h^{-1}) for the respective fits (solid or dotted line) are given in the figure. B, Temperature dependence of Zx epoxidation. Leaf discs were illuminated for 1 h at a light intensity of 2 mmol photons m^{-2} s^{-1} and at 20 °C and then placed immediately on water with temperatures of 5, 10, 15 and 20 °C, as indicated in the figure. Solid lines represent fitted curves according to the equation given above. The resulting rate constants were 0.38, 0.42, 0.55 and 1.35 h^{-1} for the data obtained at 5, 10, 15 and 20 °C, respectively. All data points represent mean values (±SD) of 3 independent experiments.

of apparent rate constants of epoxidation, the data were fitted as a single exponential decay (for details see legend to Fig. 4). The fitted data are shown as solid and dotted lines in Fig. 4. These analyses of the epoxidation kinetics revealed that Zx epoxidation after pre-illumination at 20 °C and a light intensity of 2 mmol photons m^{-2} s^{-1} was about 10 fold faster after 0.5 h of illumination than after 8 h of illumination (Fig. 4A). A similar difference was obtained for the pre-illumination at 5 °C and a light intensity of 1 mmol photons m^{-2} s^{-1} (Fig. 4A). The slightly slower epoxidation found after short illumination at 5 °C in comparison with the respective data at 20 °C might be related to the fact that the leaf temperature was still below 20 °C in the beginning of the epoxidation experiment. To evaluate the possible effect of the leaf temperature on the ZxE activity we determined the temperature dependence of epoxidation. Leaves were illuminated for 1 h at a light intensity of 2 mmol photons m^{-2} s^{-1} and at 20 °C and then placed immediately on water with temperatures of 5, 10, 15 and 20 °C. As illustrated in Fig. 4B, the rate of Zx epoxidation was gradually reduced upon lowering of the temperature, but the most pronounced effect was observed between 20° and 15 °C. Thus the slight reduction of Zx epoxidation after short pre-illumination times at low temperature (cf. Fig. 4A) could indeed be related to a slightly lower leaf temperature in the beginning of the epoxidation experiment.

As shown in Fig. 4A for selected conditions we performed the same kinetics analysis for all epoxidation experiments shown in Figs. 2 and 3. In all cases it was possible to fit the data with a single exponential decay. Plotting the resulting rate constants for this exponential decay against the quantum efficiency of PSII determined at the end of the pre-illumination treatment exhibited a clear dependence of the epoxidation rate on the activity state of PSII (Fig. 5). The deviation of some few data from the fitted function (represented by the solid line in Fig. 5) is likely to be related to errors in fitting the original data (Figs. 2 and 3) with a single exponential decay kinetics. These errors arise from the fact that (1) only a limited number of nine data points was available for fitting the time course of each trace (Figs. 2 and 3) and (2) that these few data additionally showed rather large standard deviations at least in some cases. Irrespective of these uncertainties, however, the dependence of the epoxidation rate on the activity state of PSII exhibited two different phases. At higher F_V/F_M values (above 0.4) the epoxidation rate was gradually down-regulated with decreasing PSII quantum efficiency. Within this range, the apparent rate constant of epoxidation was reduced by a factor of about 5 (from about 2 h^{-1} at a F_V/F_M of 0.74 to about 0.4 h^{-1} at a F_V/F_M of 0.5). Further reduction of F_V/F_M below a threshold F_V/F_M of about 0.4 induced a further reduction of the apparent rate constant of epoxidation by a factor of 4 (from 0.4 h^{-1} to about 0.1 h^{-1}), leading to nearly complete inhibition of epoxidation within the time frame of the experiment.

3.4. Regulation of zeaxanthin epoxidation and phosphorylation of PSII proteins

To evaluate the possible involvement of light-dependent phosphorylation of thylakoid proteins in the down-regulation of ZxE activity, we studied the regulation of Zx epoxidation in the *stn7/stn8* mutant of *Arabidopsis* (Fig. 6). In this mutant, which is deficient in the two thylakoid protein kinases STN7 and STN8 [39], the light-dependent phosphorylation of PSII proteins is blocked under both low-light and high-light conditions ([39]; Fig. 6A). Leaves from wild-type and mutant plants were pre-illuminated at 20 °C for 30 min at 1.5 mmol photons m^{-2} s^{-1} and at 5 °C for 2 h at 2 mmol photons m^{-2} s^{-1}. As before, the high-light treatment at low temperature led to a strong reduction of Zx epoxidation in wild-type

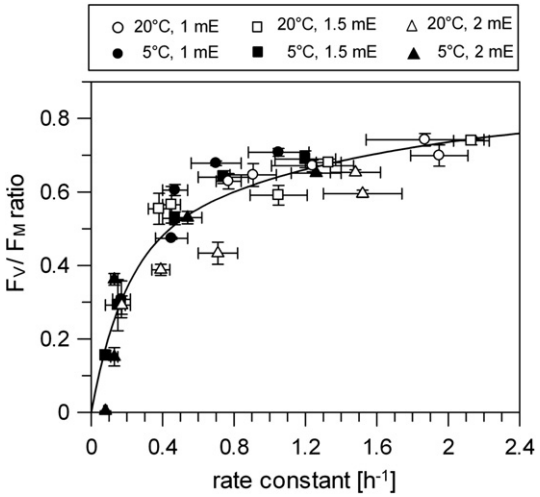

Fig. 5. Relationship between the epoxidation rate and the PSII quantum efficiency. The F_V/F_M ratios correspond to the respective values determined after pre-illumination and prior to the onset of epoxidation as given in Fig. 1 C and D at $t=0$. The rate constants have been determined by fitting all curves with a single exponential decay according to the equation given in the legend to Fig. 4. The pre-illumination conditions of the respective data sets are indicated in the legend of the figure. For simplicity, the light intensity is expressed as mE instead of mmol photons m^{-2} s^{-1}. The solid line represents a fit of the data as a first order reaction with two exponentials according to the equation $y=A1*(1-\exp(-k1*t))+A2*(1-\exp(-k2*t))$. The amplitudes of the two phases, A1 (slow phase) and A2 (fast phase) were determined with 0.45 and 0.4, respectively.

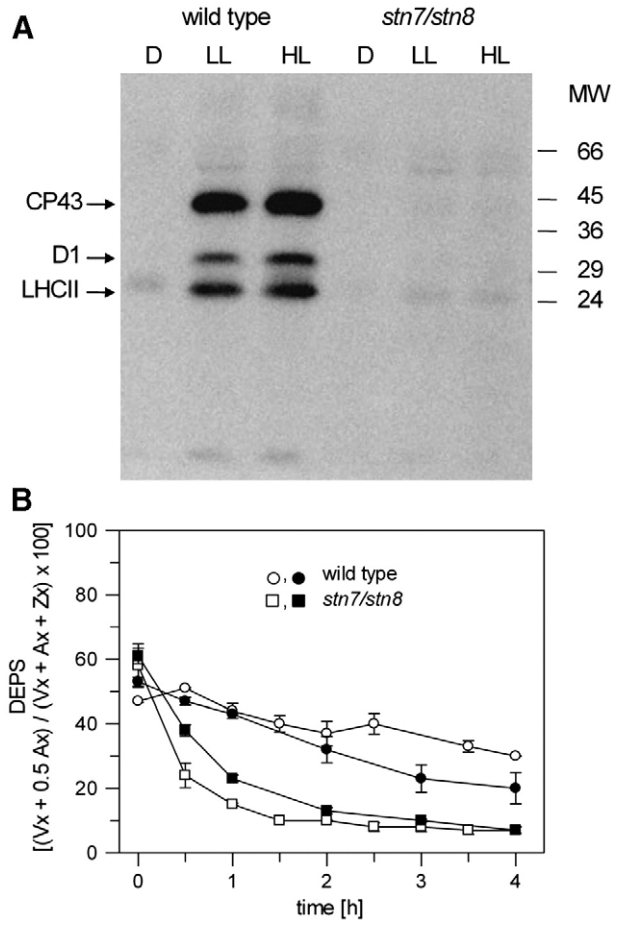

Fig. 6. Phosphorylation of thylakoid proteins and Zx epoxidation in wild-type and *stn7/stn8* plants. A, Phosphorylation of LHCII and PSII core proteins as detected by immunoblot analysis. D = dark-adapted plants; LL = plants illuminated for 1 h at 80 µmol photons m^{-2} s^{-1}; HL = plants illuminated for 1 h at 1 mmol photons m^{-2} s^{-1}. B, Time course of epoxidation after pre-illumination of plants for either 30 min at 1.5 mmol photons m^{-2} s^{-1} and 20 °C (squares) or 2 h at 2 mmol photons m^{-2} s^{-1} and 5 °C (circles). Each data point represents the mean value (±SD) of 3 independent experiments.

plants in comparison with the pre-illumination treatment at 20 °C (cf. Figs. 2 and 3). Notably, a very similar down-regulation of ZxE activity was also found for *stn7/stn8* mutant plants (Fig. 6B), indicating that phosphorylation of PSII proteins is not involved in the regulation of ZxE activity.

3.5. Zeaxanthin epoxidation in isolated thylakoid membranes

To clarify whether the down-regulation of Zx epoxidation is possibly related to the action of soluble stromal factors (e.g. limiting NADPH or suboptimal pH) or to continuing VxDE activity, epoxidation was studied in thylakoids that were isolated after pre-illumination. For pre-illumination, two different conditions were chosen which induce strongly different epoxidation kinetics (Fig. 7). After pre-illumination at 2 mmol photons m^{-2} s^{-1} for 30 min and at 20 °C, similar rates of Zx epoxidation were found under *in vivo* and *in vitro* conditions. After pre-illumination at 2 mmol photons m^{-2} s^{-1} for 2 h and at 5 °C, epoxidation rates were strongly retarded to a similar extent in both leaves and isolated thylakoids (Fig. 7). Since de-epoxidation cannot contribute to xanthophyll conversion under these *in vitro* conditions (high pH, no ascorbate present) this experiment clearly shows that the retardation of Zx epoxidation under *in vivo* conditions is not influenced by a continuing VxDE activity. It can further be excluded from this result that the down-regulation of ZxE activity is related to a soluble stromal factor (e.g. NADPH, pH). Thus, most likely a direct modification of the enzyme is responsible for its down-regulation.

4. Discussion

Retention of Zx under severe stress has been observed in several studies [9–14]. In this work we showed for the first time, that (1) inhibition of ZxE activity is responsible for Zx retention and that (2) ZxE activity can be down-regulated very rapidly, i.e. within a few hours. The kinetics of Zx epoxidation showed a pronounced dependence on the light stress experienced by a leaf prior to the onset of epoxidation. Zx epoxidation was generally completed within about 1 h after mildest treatments (i.e. 30 min pre-illumination at 20 °C and up to 1.5 mmol photons m^{-2} s^{-1}) and nearly completely inhibited after severe light stress (i.e. more than 2 h of pre-illumination at 5 °C and 2 mmol photons m^{-2} s^{-1}). This reduction of the epoxidation rate by a factor of more than 10 was closely related to the activation state of PSII (Fig. 5), but was independent of the DEPS (Fig. 1). Down-regulation of ZxE activity during long-term winter acclimation has been reported in particular for a variety of evergreen species [11,12,27,40]. Retainment of Zx under these conditions is accompanied by sustained energy dissipation [40] but in contrast to the well-known qE-component of NPQ [41] this process is independent of a high transthylakoid pH gradient [10]. Instead, a direct role of Zx in lasting forms of energy dissipation has been proposed [10,42]. Short-term down-regulation of PSII activity, also termed as qI-component of NPQ or photoinhibition, has also be related to a pH-independent but Zx-dependent mechanism of energy dissipation in pea [9] and several evergreen species [25]. The observed light-induced inactivation of PSII concomitant with the down-regulation of ZxE activity in the short-term reported here is thus likely to reflect a lasting form of energy dissipation similar to that developing during winter acclimation of evergreen species. In physiological terms, the down-regulation of ZxE activity in the short-term could therefore simply be understood as to retain Zx concomitant with the generation of lasting (pH-dependent or pH-independent) forms of energy dissipation. The gradual down-regulation of ZxE activity thus allows a flexible adjustment of the retention time of Zx in response to daily light-stress periods experienced by the plant. By that, the Zx activity could be assigned as an indicator of the physiological state of a plant with respect to its photoprotective capacity. Notably,

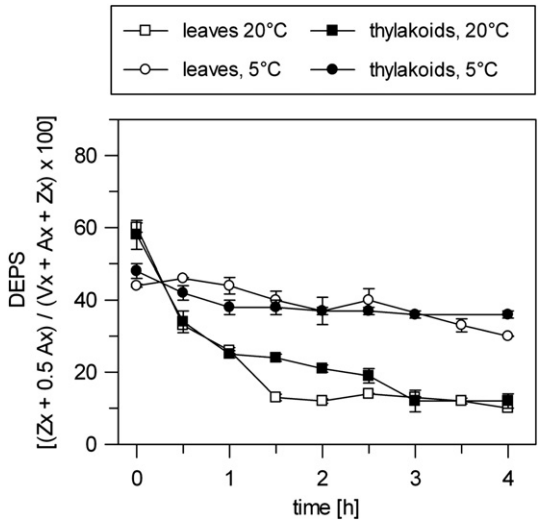

Fig. 7. Time course of epoxidation in leaves and isolated thylakoids from pea plants. Leaves were pre-illuminated at 2 mmol photons m^{-2} s^{-1} for either 30 min at 20 °C (squares) or 2 h at 5 °C (circles). Epoxidation was determined in leaves (open symbols) or thylakoids isolated after pre-illumination treatment (closed symbols). Each data point represents the mean value (±SD) of 3 independent experiments.

Zx epoxidation is nearly completely inhibited when the PSII quantum efficiency drops below 50% (F_V/F_M of 0.4) of unstressed controls (F_V/F_M of 0.8). Since the decrease in F_V/F_M in leaves was found in numerous studies to be linearly correlated with a decline in the optimal quantum yield of photosynthesis (e.g. [43,44]) or the reduction of PSII activity of isolated thylakoids [45], it might be speculated whether this threshold reflects the inactivation of one PSII centre per PSII dimer. Possibly, the retention of Zx in inactive PSII centres is required to maintain a photoprotective dissipative state of these centres.

The molecular mechanism of the regulation of ZxE activity is still unclear. Recent work suggested that sustained D1 phosphorylation may be involved in the generation of lasting dissipative states of PSII [14]. However, our data obtained with the *stn7/stn8* mutant of *Arabidopsis*, which lacks phosphorylation of PSII core and antenna proteins [39], but still shows the light-dependent down-regulation of ZxE activity (Fig. 6) contradict this hypothesis. Thus either another unknown kinase is involved in the regulation of Zx epoxidation or the ZxE is regulated by another factor. The reported up-regulation of TLP40, an inhibitor of PSII core protein phosphorylation, along with the generation of sustained energy dissipation in PSII [14] and the observed retardation of Zx epoxidation by the phosphatase inhibitors sodium fluoride (NaF) and sodium molybdate (Na_2MoO_4) in rice leaves [30] indicate that rather dephosphorylation reactions than phosphorylation reactions might be responsible for the down-regulation of ZxE activity. However, experiments with different phosphatase inhibitors applied to *Arabidopsis* leaves did not result in significant changes of Zx activity (data not documented). We also isolated homozygous TLP40 knockout plants obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) to study the role of this protein in the regulation of ZxE, but these mutants were unable to grow on soil under our growth conditions. Possibly, TLP40 serves additional essential functions for the development of plants.

It has further been shown recently for the *Arabidopsis nadk* mutant that the reduction of NADPH to about 50% of wild-type levels results in accumulation of high levels of Zx [35]. This indicates that the NADPH concentration is a critical factor for ZxE activity. However, limitation of the epoxidation reaction by reduced levels of NADPH can be excluded to be involved in the light-dependent down-regulation of ZxE activity reported here, since the light-induced down-regulation of Zx epoxidation was maintained under *in vitro* conditions in presence of high concentrations of NAD(P)H (Fig. 7). This experimental finding further implies that a direct modification of the ZxE is most likely responsible for its regulation. To clarify this point it would be necessary to study the purified ZxE, but so far only in one study the heterologous expression and purification of an active enzyme has been reported some ten years ago [19]. Thus more work, particularly with the native enzyme is urgently needed. Finally, also altered substrate properties might be responsible for the light-dependent reduction of the epoxidation rate. It has been shown earlier, that the ZxE – in contrast to the VxDE – prefers protein-bound xanthophylls rather than non-protein-bound xanthophylls [24,29]. Although binding of Zx to specific binding sites may indeed be induced in distinct antenna proteins upon increasing light stress, possibly mediated by rearrangement of PSII antenna proteins, it seems unlikely that such changes will apply to all Zx binding sites in all xanthophyll binding proteins of both photosystems.

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