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Identification of a slowly inducible zeaxanthin-dependent component of non-photochemical quenching of chlorophyll fluorescence generated under steady-state conditions in *Arabidopsis*

Manuela Nilkens [a], Eugen Kress [a], Petar Lambrev [b], Yuliya Miloslavina [b], Marc Müller [b], Alfred R. Holzwarth [b], Peter Jahns [a,*]

[a] Biochemie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany
[b] Max-Planck-Institut für Bioanorganische Chemie, D-45470 Mülheim a.d. Ruhr, Germany

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ABSTRACT

The induction and relaxation of non-photochemical quenching (NPQ) under steady-state conditions, i.e. during up to 90 min of illumination at saturating light intensities, was studied in *Arabidopsis thaliana*. Besides the well-characterized fast qE and the very slow qI component of NPQ, the analysis of the NPQ dynamics identified a zeaxanthin (Zx) dependent component which we term qZ. The formation (rise time 10–15 min) and relaxation (lifetime 10–15 min) of qZ correlated with the synthesis and epoxidation of Zx, respectively. Comparative analysis of different NPQ mutants from *Arabidopsis* showed that qZ was clearly not related to qE, qT or qI and thus represents a separate, Zx-dependent NPQ component.

1. Introduction

The non-photochemical quenching (NPQ) of excitation energy represents an important photoprotective mechanism in plants. At least three different processes contribute to NPQ [1]: (i) the pH-regulated energy dissipation in the antenna of photosystem II (PSII) [2], (ii) state transitions [3] and (iii) photoinhibition [4]. These processes affect the fluorescence and have been frequently termed as the qE, qT and qI components of NPQ, respectively. They were originally derived from the relaxation kinetics of chlorophyll fluorescence quenching after preillumination of barley leaves at saturating light intensities [5]: A fast phase ($\tau_{½}$ = 1–2 min) was assigned to qE, a middle phase ($\tau_{½}$ = 5–10 min) to qT and a slow phase ($\tau_{½}$ > 60 min) to qI. Later studies of the light-dependence of the extent and relaxation kinetics of the different components indicated, however, that the middle phase observed under those conditions is unlikely to be related to state transitions, since state transitions occur under low light conditions only, while the middle phase of NPQ relaxation was found to be rather large at high light intensities [6]. It was thus speculated that this phase of NPQ relaxation might be related to a modified relaxation of qE processes [6] and may thus represent a second component of qE. Alternatively, the middle phase has been discussed to correspond to qI and thus may reflect a photoinhibitory state of photosystem II (PS II) [7]. Since the fast qE represents the dominating NPQ component under moderate light conditions, only rapidly (within 10 to 15 min) inducible and relaxing NPQ processes have been determined in most studies on NPQ, and more slowly developing processes contributing to NPQ are usually neglected.

The molecular bases of NPQ in general and of the qE part in particular are still under debate. The rapidly forming qE component of NPQ has been assigned to a synergistic action of the lumen pH, PsbS and the xanthophyll zeaxanthin (Zx) [8]. The formation of the maximum qE requires not only a high ΔpH but also the conversion of violaxanthin (Vx) to Zx [9,10]. During the past years, various models have been proposed for both the site and mechanism of qE. It is in particular unclear whether the qE-quenching site is located in the minor antenna proteins [11] or the major antenna complexes of PSII, LHC II, [12] and whether qE quenching is caused by electron transfer from Zx to Chl [13], by energy transfer from Chl to lutein [12] or by Chl–Chl charge transfer state formation [14]. It is common to all models

Abbreviations: Ax, antheraxanthin; Chl, chlorophyll; NPQ, non-photochemical quenching; PS II, photosystem II; qE, energy-dependent quenching; qI, photoinhibitory quecnhing; qT, state transition quenching; qZ, zeaxanthin-dependent quenching; Vx, violaxanthin; Zx, zeaxanthin

* Corresponding author. Heinrich-Heine-Universität Düsseldorf, Biochemie der Pflanzen, Universitätsstrasse 1, D-40225 Düsseldorf, Germany. Tel.: +49 211 8113862; fax: +49 211 8113706.
E-mail address: pjahns@uni-duesseldorf.de (P. Jahns).

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that pH- and PsbS-dependent rearrangements in the antenna structure and/or conformation of antenna proteins essentially control qE and that Zx is either directly or indirectly involved in the generation of total NPQ quenching. Recent analysis of ultrafast Chl fluorescence kinetics in diatoms [15] and in intact leaves of *Arabidopsis* [16] provided evidence that at least two different quenching sites contribute to NPQ under steady state NPQ conditions *in vivo*: (1) The PsbS-dependent site is located in PSII antenna complexes (preferentially LHCII) that become functionally detached from PSII upon illumination and (2) a second Zx-dependent site is located in antenna complexes (preferentially minor antennae) that remain attached to PSII [16]. The existence of two different quenching sites acting in parallel could not only consistently explain all available data on NPQ but could provide also an explanation for the proposed Zx-dependent and Zx-independent components of NPQ, including more slowly developing processes under steady-state conditions.

The molecular basis of qI is even less understood than qE. In general, the term qI (= photoinhibitory quenching) is rather ill-defined, but frequently used for all NPQ processes relaxing with slower kinetics than the transthylakoid pH gradient and thus comprises all processes contributing to the light-induced down-regulation, inactivation and damaging of PS II in the middle- and long-term. It has to be noted, however, that under moderate light intensities, only a small fraction of qI will be related to photoinhibition in the classical sense which is defined as the light-induced reduction of the quantum yield of photosynthetic carbon fixation [17] and which is most likely caused in its initial phase by Q_A double reduction followed by photo-oxidative damage to the D1 protein [18–21].

Nearly all former studies on NPQ have been focused either only on the rapidly (within few minutes) inducible and relaxing qE component of NPQ (which represents the dominating NPQ component under moderate illumination conditions) or only on photoinhibitory processes (qI) developed under long lasting (several hours) high light stress, while intermediate NPQ processes have been usually neglected. Such processes, which can be supposed to be developed independent of the generation of photoinhibitory states under moderate illumination conditions at longer time scale than qE (>10 min) may be related to NPQ processes that represent an additional NPQ state different from qI. Since the xanthophyll Zx is known (i) to be synthesized in this time range [22,23] and (ii) to be involved not only in qE [24,25] but also in qI [26,27], Zx may play a key role in such processes. In fact, recent analysis of ultrafast Chl fluorescence kinetics under NPQ conditions in intact leaves of *Arabidopsis* identified a PsbS-independent but Zx-dependent quenching site in the PSII antenna [16]. Furthermore, a NPQ mechanism with similar characteristics (PsbS-independent, but Zx-dependent) has recently been related to Zx-dependent conformational changes in PSII antenna proteins [28].

It would thus be of particular interest to characterize the dynamics of different NPQ processes in one and the same experimental approach with sufficient time resolution in the range from minutes up to more than an hour. For this purpose we studied the induction and relaxation of NPQ and its correlation with the xanthophyll cycle activity in different *Arabidopsis* mutants affected in PsbS (*npq4* and L17), xanthophyll conversion (*npq1* and *npq2*) and state transitions (*stn7*) during illumination for up to 90 min under moderate, but saturating light intensities. The aim of this approach was to obtain more information about the characteristics and correlation of more slowly developing NPQ states and the role of PsbS, Zx and state transitions in these processes. Our data provide evidence that aside from qE an additional slow NPQ process is induced under steady-state conditions, which is characterized by different dependencies on PsbS, Zx and the transthylakoid pH gradient than qE, but which is neither related to state transitions nor to photoinhibition. Based on these data we derived a more refined model of the full NPQ quenching developing under steady-state conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana wild-type and mutant plants (ecotype Columbia 0) were grown on soil at a light intensity of 150 µmol photons $m^{-2} s^{-1}$ and a constant temperature of 20 °C under short-day conditions (10 h light/14 h dark). 6–8 week-old plants were used for all experiments. Following mutants were used: *npq1* and *npq2* [10], *npq4* [29], L17 [30] and *stn7* [31]. The replicates shown in the different experiments represent data obtained with leaves from different plants grown at different time.

2.2. Fluorescence measurements

Room temperature Chl a fluorescence was measured under *in vivo* conditions at 20 °C using a pulse-amplitude-modulated fluorometer (PAM 101, Walz, Effeltrich, Germany). For all experiments leaves were dark-adapted at least 2 h prior to the onset of illumination. Leaves were positioned on wet filter paper in a self-built cuvette and continuously supplied with moistened air throughout the experiment. For the induction of NPQ, leaves were illuminated with white light for up to 90 min at a light intensity of 900 µmol photons $m^{-2} s^{-1}$. Relaxation of NPQ was determined during a subsequent dark phase for up to 100 min. For the determination of the induction of NPQ, saturating white light pulses (2500 µmol photons $m^{-2} s^{-1}$, duration 800 ms) were applied every 20 s during the first 100 s of illumination, followed by 9 flashes given every 100 s and up to 22 flashes given every 200 s. For the determination of the relaxation of NPQ in the dark, saturating light pulses were spaced 20 s for the first 100 s, followed by 9 flashes spaced 100 s and up to 10 flashes spaced 500 s. Control experiments in absence of actinic light indicated that under these experimental conditions (i) nearly no NPQ (<0.02) is induced by the saturating light pulses during the induction of NPQ and (ii) the NPQ relaxation kinetics are not affected by the saturating light pulses. Stern–Volmer type of NPQ was calculated as described [1].

2.3. Pigment analysis

For the induction of Zx formation, detached leaves from dark-adapted plants were floated on water and illuminated with white light for up to 90 min at a light intensity of 900 µmol photons $m^{-2} s^{-1}$. Reconversion of Zx to Vx was induced by transfer of pre-illuminated leaves to darkness. At the indicated times, leaves were frozen in liquid N_2 and stored at −80 °C. Pigments were extracted with acetone and quantified by reverse phase HPLC [32].

3. Results

3.1. NPQ induction

The induction of NPQ during 90 min of illumination at a light intensity of 900 µmol photons $m^{-2} s^{-1}$ has been measured in *Arabidopsis* wild-type (WT) and different NPQ mutant plants: *npq1* (Zx-deficient) and *npq2* (Zx-enriched), *npq4* (PsbS-deficient), L17 (PsbS over-expressing) and *stn7* (deficient in LHCII phosphorylation and thus in state transitions) (Fig. 1). At this light intensity, photosynthesis is saturated in plants grown at a light intensity of 150 µmol photons $m^{-2} s^{-1}$ [29] so that maximum values for qE can be induced without inducing pronounced photo-oxidative stress and thus photoinhibition of PSII [23]. Indeed, when calculating the electron transport rate through PSII from the fluorescence data, saturation of electron transport was established after about 20 min of illumination and no reduction of electron transport was detectable at longer illumination time (Fig. 2). Moreover, recent time-resolved fluorescence kinetics analyses under similar experimental conditions did not reveal

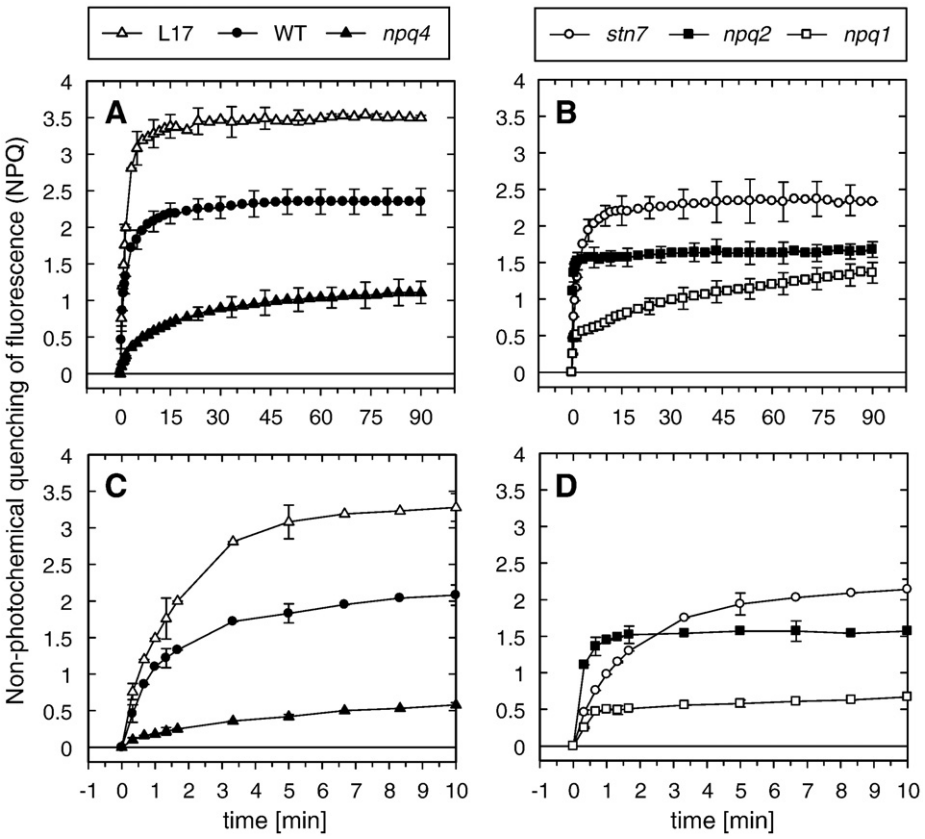

Fig. 1. The induction of NPQ in detached leaves from dark-adapted plants. A and B, NPQ induction during 90 min of illumination at a white light intensity of 900 µmol photons m^{-2} s^{-1}. C and D, NPQ induction during the first 10 min of illumination. Plants were dark-adapted for at least 2 h. Detached leaves were placed on wet filter paper in a temperature-controlled cuvette (20 °C). For the determination of NPQ, saturating white light pulses (2500 µmol photons m^{-2} s^{-1}, duration 0.8 s) were applied every 20 s during the first 100 s of illumination, followed by 9 flashes given every 100 s and 22 flashes given every 200 s. Stern–Volmer type of NPQ ($F_m/F_m'-1$) was calculated as described by [1]. Mean values ± SD of 4 to 15 independent measurements are shown.

any indications for the formation of photoinhibited PSII [16]. Hence, these illumination conditions are optimal for studying NPQ under steady-state conditions. To minimize the contribution of the saturating light flashes (which are required to determine the NPQ parameter) to NPQ formation, flashes were separated 20 s for the first 100 s of induction, 100 s for the time range between 100 s and 1000 s, and 200 s in the time range from 1000 s to 90 min. In WT plants, a stable maximum NPQ value of about 2.3 was reached after about 30 min. Analysis of the kinetics of NPQ induction revealed the existence of at least two different phases (Table 1). The kinetics of the dominating rapid phase (amplitude about 1.5, overall rise time about 1 min) reflects the generation of the transthylakoid pH-gradient and can thus be assigned to qE. This rapid phase showed the well-known characteristics with respect to PsbS (abolished in *npq4* and increased in L17; Fig. 1A, C) and Zx (reduced amplitude in *npq1* and accelerated kinetics in *npq2*). The kinetics of the slower phase (amplitude about 0.8, rise time about

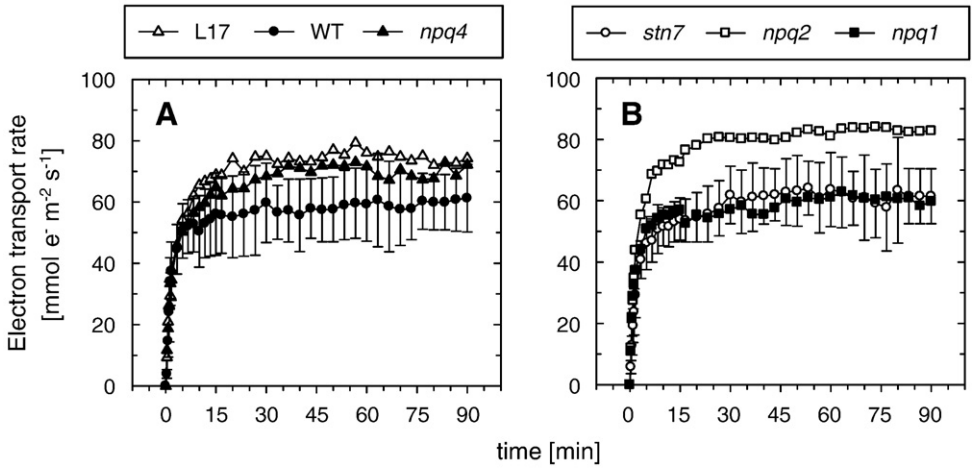

Fig. 2. Electron transport rates. *In vivo* electron transport rates during illumination of intact leaves, J_F, were derived from the fluorescence parameter Φ_{PSII} [47] as described in [7]. All experimental conditions were as described in the legend to Fig. 1. Mean values ± SD of 4 to 15 independent measurements are shown. For clarity, SD is shown for WT and *stn7* mutants.

Table 1
Kinetic parameters of NPQ induction.

	A_1	τ_1 [s]	A_2	τ_2 [s]
WT	1.52 ± 0.05	59 ± 3	0.81 ± 0.04	570 ± 37
stn7	1.91 ± 0.03	96 ± 3	0.41 ± 0.04	980 ± 120
npq4	0.53 ± 0.05	460 ± 50	0.57 ± 0.07	2600 ± 700
L17	3.00 ± 0.07	96 ± 4	0.41 ± 0.06	770 ± 100
npq1	0.60 ± 0.03	9 ± 2	0.63 ± 0.02	1600 ± 120
npq2	1.55 ± 0.02	16 ± 1	0.09 ± 0.03	1020 ± 90

The average data of NPQ induction shown in Fig. 1 were fitted with two exponentials according to the equation $y = y_0 + A_1*(1-\exp(-t/\tau_1)) + A_2*(1-\exp(-t/\tau_2))$. SD represents the fitting error of the respective parameter, r^2 was >0.99 in all cases.

10 min) was found to be in the same range as the conversion of Vx to Zx [22]. Analysis of this slow phase of NPQ induction in different NPQ mutants (Figs. 1 and 3, Table 1) revealed the following differences in comparison with wild-type plants:

(1) State transitions do not influence the slow phase of NPQ induction formation under these experimental conditions. This phase is also present in *stn7* and the overall NPQ amplitude and the kinetics of induction are only marginally altered in this mutant (Fig. 3).
(2) The slow phase of NPQ induction was found to be independent of PsbS and was detectable in *npq4* and L17 to a similar extent and kinetics (Fig. 1A, C). However, a pronounced very slow phase of NPQ induction was additionally generated in *npq4*. Such a slow phase of NPQ induction was not found in all other genotypes except for *npq1* (see below).
(3) The formation of the slow phase of NPQ induction is strongly modified by Zx (Figs. 1B, D and 2). In the presence of high levels of Zx (*npq2*), NPQ induction was accelerated and no pronounced slow phase of NPQ induction was detectable. By contrast, the slow phase of NPQ induction was strongly slowed down in absence of Zx (*npq1*) (see Fig. 1B, D).

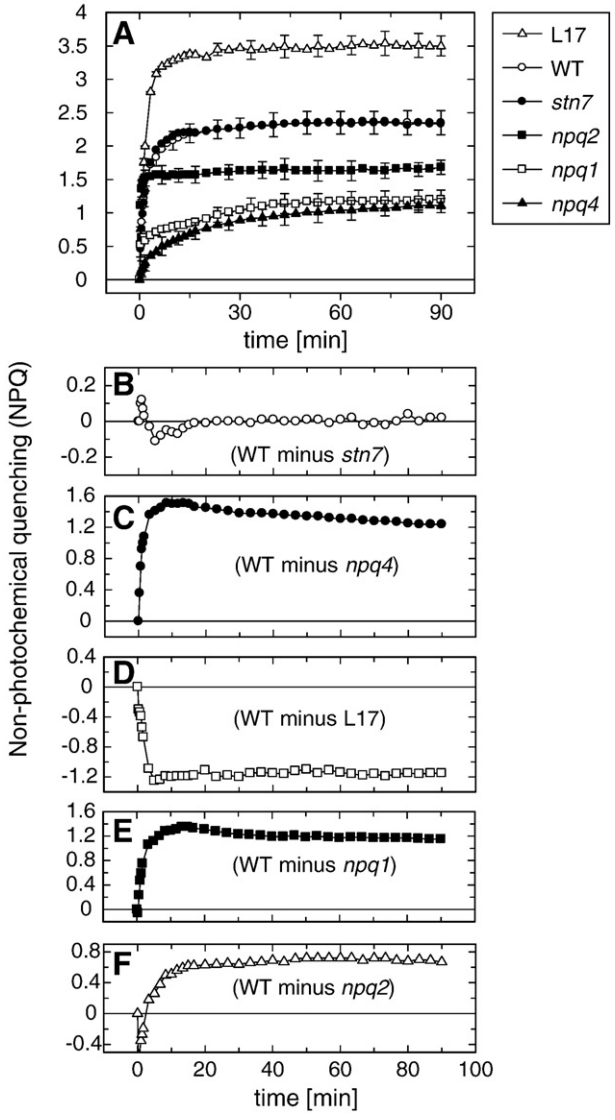

Fig. 3. Differences between the induction of NPQ in WT and in different mutant plants. A, NPQ induction in WT and mutant plants (taken from Fig.1). B–F, Differences of NPQ induction in the WT and different mutant plants, as derived from the traces shown in A.

3.2. NPQ relaxation

The NPQ relaxation kinetics is frequently used as a tool for the characterization of NPQ processes. We studied the relaxation of NPQ in darkness after different times (5, 15, 30, 60 and 90 min) of preillumination (Fig. 4, Table 2). In WT plants (Fig. 4A), three different phases contributed to NPQ relaxation in *Arabidopsis* under the given illumination conditions: a fast phase (lifetime of 35 to 50 s), a middle phase (lifetime 800 to 1000 s) and a slow phase, which was nearly irreversible within the time range of the experiments (lifetime >2 h, referred to as 'y_0' in Table 2).

The rapidly relaxing phase represented the dominating NPQ component under all conditions (70–80% of the total NPQ). It reached its maximum amplitude after 15 min with a steady state value of about 1.6 and can clearly be assigned to the relaxation of the qE component of NPQ. Note that a similar amplitude has been determined for the induction of qE (Table 1, Fig. 1). Accordingly, this rapid phase represents pH-regulated and PsbS-dependent processes which relax independent of Zx reconversion to Vx, since Zx epoxidation occurs on a longer time range (see below).

The middle phase developed more slowly (fully developed after about 30 min) and contributed to about 20–25% of the overall NPQ (maximum amplitude of about 0.6). The kinetics (and amplitude) of this middle phase (lifetime of about 10–15 min) of NPQ relaxation was in the similar range as that of the slow phase of NPQ induction (Table 1, Fig. 1). Strikingly, this kinetics resembled the kinetics of Zx formation and relaxation (see below), so that the slow phase of NPQ induction and the middle phase of NPQ relaxation are likely to be related to Zx-dependent processes, which do not relax on the same time scale as the transthylakoid pH gradient.

The slow phase of NPQ relaxation was found to increase slowly with prolonged illumination. After the shortest illumination time (5 min), no contribution of the slow phase was detectable and the maximum amplitude of about 0.2 (corresponding to a relative portion of about 10% of the maximal NPQ) was reached after 90 min of preillumination (Fig. 5). This slowly developing NPQ component might thus be assigned to photoinhibitory processes.

In the *stn7* mutant (Fig. 4B) the relaxation of NPQ was very similar to that observed in wild-type plants (cf. also Table 2 and Fig. 5). Obviously, state transitions do not contribute significantly to both the formation and relaxation of NPQ in *Arabidopsis* under the given illumination conditions. The only difference in comparison with WT plants was found for the kinetics of the middle phase, which was faster after the shortest preillumination time of 5 min (lifetime of about 300 s in *stn7* vs 800 s in the WT), but reached similar kinetics after 90 min of preillumination (lifetime of about 900 s in both genotypes).

In absence of PsbS (*npq4*), the kinetics of NPQ relaxation was strongly slowed down in comparison with WT plants. No fast relaxation with a lifetime in the range of 1 min was observable in

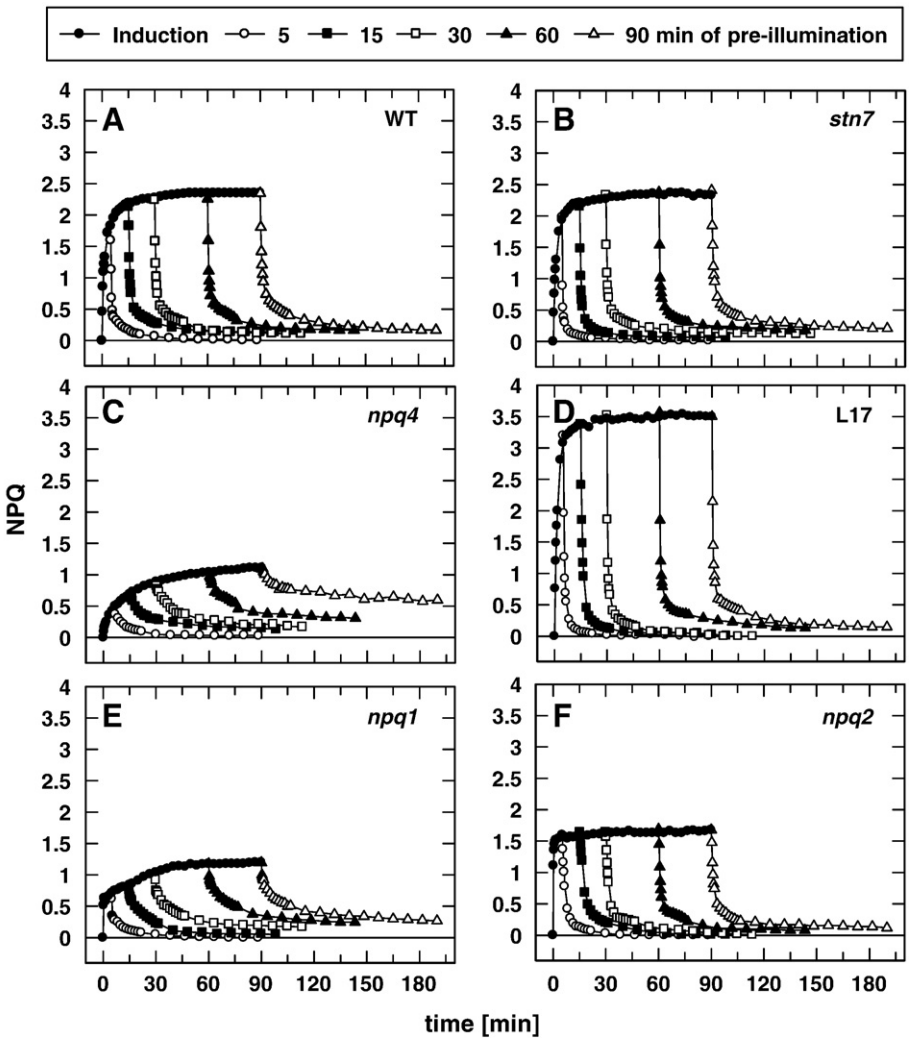

Fig. 4. NPQ relaxation after different times of preillumination. Dark-adapted plants were illuminated under essentially the same condition as described in the legend to Fig. 1. The relaxation of NPQ was measured after 5, 15, 30, 60 and 90 min of preillumination. At indicated time, the actinic light was switched off and NPQ was determined for up to 120 min. Saturating light pulses were spaced 20 s for the first 100 s, followed by 9 flashes spaced 100 s and up to 10 flashes spaced 500 s. Under these conditions nearly no NPQ (<0.02) was induced by the saturating light pulses. Mean values of 3 to 5 independent measurements are shown. For clarity, SD is not shown, but was in the range <0.2 (see also Figs. 1 and 5).

npq4, confirming the absence of the pH- and PsbS-dependent qE component in this mutant. The lifetime of the most rapidly relaxing phase detectable in *npq4* was in the same range as that of the middle phase in WT and *stn7* plants (Figs. 4 and 5, Table 2), indicating that Zx-dependent quenching processes are active also in absence of PsbS. Moreover, an additional slower phase (lifetimes of about 20–60 min) of NPQ relaxation was found after preillumination for at least 15 min. Notably, the amplitude of the slowest, irreversible phase reached much higher values in comparison to all other genotypes. After 90 min of preillumination, about 50% of the total NPQ was found to relax with this slowest kinetics (Table 2, Fig. 5). This indicates that the induction of photoinhibitory processes is more pronounced in *npq4* plants underlining the photoprotective function of PsbS-dependent NPQ processes.

In the presence of higher PsbS levels (Fig. 4D), the amplitude of the rapidly relaxing NPQ phase was strongly increased. As found for WT plants, the amplitude of this phase was similar to that of the rapid phase of NPQ induction (cf. Table 1) confirming the essential role of PsbS for qE. In contrast to the WT, the maximal amplitude of the middle phase of NPQ relaxation was already fully developed after 5 min of illumination and the kinetics of its relaxation was faster, particularly after shorter preillumination time (Table 2, Fig. 5). On the other hand, the slowest phase of NPQ relaxation (and thus photoinhibitory processes) was not induced during preillumination times shorter than 60 min. Consistent with the opposite observations in *npq4* these data underline again the photoprotective role of PsbS-dependent NPQ processes.

Also in absence of Zx (*npq1*), three different phases of NPQ relaxation were found. The kinetics of the rapid phase was clearly faster than in the WT, particularly after the shortest preillumination time (Table 2), in agreement with other studies [33,34]. However, the amplitude of the rapidly relaxing phase was rather small (0.1–0.4) and significantly smaller than that of the rapid phase of NPQ induction in *npq1* (0.6). Obviously, the reversibility of these pH controlled NPQ processes is altered in absence of Zx. On the other hand, the middle phase of NPQ relaxation in *npq1* showed similar characteristics in comparison with the middle phase of NPQ relaxation in the WT: it followed not only similar kinetics but also reached similar maximal amplitudes after about 15 min of preillumination. As for all other genotypes, the amplitude of this phase was similar to that of the slow phase of NPQ induction. It should be noted, however, that both the rapid and middle phase of NPQ relaxation were strongly retarded in *npq1* after the longest preillumination time of 90 min (Table 2, Fig. 5). Like in *npq4*, the amplitude of the slow, irreversible phase of NPQ relaxation was increased in *npq1* in comparison with all other genotypes.

Table 2
Kinetic parameters of NPQ relaxation.

	Time	A_1	τ_1 [s]	A_2	τ_2 [s]	y_0
WT	5	1.28 ± 0.05	33 ± 3	0.34 ± 0.03	770 ± 150	0.01 ± 0.01
	15	1.70 ± 0.05	53 ± 3	0.43 ± 0.04	910 ± 165	0.13 ± 0.02
	30	1.54 ± 0.03	42 ± 2	0.56 ± 0.02	800 ± 65	0.11 ± 0.01
	60	1.56 ± 0.03	34 ± 2	0.58 ± 0.02	910 ± 80	0.17 ± 0.02
	90	1.54 ± 0.03	48 ± 1	0.61 ± 0.02	980 ± 60	0.19 ± 0.01
stn7	5	1.59 ± 0.02	19 ± 1	0.35 ± 0.02	310 ± 25	0.03 ± 0.01
	15	1.63 ± 0.03	39 ± 2	0.44 ± 0.02	390 ± 30	0.08 ± 0.01
	30	1.67 ± 0.03	34 ± 2	0.51 ± 0.02	620 ± 40	0.14 ± 0.01
	60	1.67 ± 0.04	28 ± 2	0.51 ± 0.03	530 ± 60	0.21 ± 0.01
	90	1.60 ± 0.04	53 ± 2	0.54 ± 0.03	880 ± 90	0.25 ± 0.01
npq4	5	0.39 ± 0.01	490 ± 15			0.04 ± 0.01
	15	0.36 ± 0.03	250 ± 35	0.24 ± 0.02	2400 ± 800	0.11 ± 0.03
	30	0.46 ± 0.07	430 ± 70	0.27 ± 0.08	4100 ± 2000	0.09 ± 0.05
	60	0.22 ± 0.04	150 ± 30	0.52 ± 0.04	1000 ± 150	0.32 ± 0.02
	90	0.33 ± 0.03	210 ± 40	0.34 ± 0.05	3600 ± 1000	0.58 ± 0.07
L17	5	2.52 ± 0.05	32 ± 1	0.65 ± 0.02	220 ± 20	0.01 ± 0.01
	15	2.74 ± 0.07	55 ± 2	0.56 ± 0.07	420 ± 60	0.01 ± 0.01
	30	2.78 ± 0.05	24 ± 2	0.69 ± 0.04	440 ± 50	0.01 ± 0.01
	60	2.73 ± 0.06	22 ± 1	0.64 ± 0.04	570 ± 75	0.13 ± 0.02
	90	2.73 ± 0.03	31 ± 1	0.60 ± 0.02	1020 ± 120	0.15 ± 0.01
npq1	5	0.28 ± 0.01	9 ± 2	0.33 ± 0.01	590 ± 40	0.01 ± 0.01
	15	0.10 ± 0.01	29 ± 8	0.59 ± 0.02	770 ± 30	0.05 ± 0.01
	30	0.17 ± 0.02	12 ± 3	0.55 ± 0.01	730 ± 30	0.19 ± 0.01
	60	0.28 ± 0.02	26 ± 4	0.63 ± 0.01	930 ± 50	0.26 ± 0.02
	90	0.40 ± 0.04	86 ± 15	0.49 ± 0.02	1600 ± 200	0.26 ± 0.01
npq2	5	1.44 ± 0.05	97 ± 5	0.29 ± 0.05	710 ± 130	0.01 ± 0.01
	15	1.41 ± 0.07	160 ± 10	0.32 ± 0.08	1600 ± 700	0.01 ± 0.01
	30	1.35 ± 0.05	110 ± 9	0.36 ± 0.06	2500 ± 900	0.02 ± 0.02
	60	1.27 ± 0.04	55 ± 4	0.41 ± 0.03	1100 ± 240	0.07 ± 0.02
	90	1.16 ± 0.05	69 ± 4	0.42 ± 0.05	650 ± 110	0.15 ± 0.01

The average data of NPQ relaxation shown in Fig. 3 were fitted with two exponentials according to the equation $y = A_1 * \exp(-t/\tau_1) + A_2 * \exp(-t/\tau_2) + y_0$. SD represents the fitting error of the respective parameter, r^2 was >0.99 in all cases.

In the presence of high levels of Zx (*npq2*) the fast phase of NPQ relaxation was clearly retarded when compared to the WT, particularly after shorter times of preillumination (Table 2) in agreement with the literature [10,33,34]. Notably, the kinetics of NPQ relaxation became faster at longer illumination time, while the amplitude of the fast phase was reduced and the amplitude of the middle phase increased. By contrast, the kinetics of the middle phase of NPQ relaxation showed a very untypical behaviour in comparison with the other genotypes: it became slower up to 30 min of preillumination but increased again at longer preillumination time. As observed for the PsbS over-expressing line L17, the amplitude of the slow, irreversible phase was very low in *npq2*, underlining the general photoprotective effect of Zx [35,36].

In conclusion, the analyses of the NPQ relaxation kinetics support the view that at least three different processes contribute to NPQ under steady-state conditions: A pH- and PsbS-dependent and rapidly relaxing component (which can be assigned to qE) and a nearly irreversible component which can be assigned to qI. The middle phase, however, cannot be assigned to either qE or qI and is also not related to state transitions (qT). Based on its relaxation kinetics, this component appears to be related to Zx-dependent processes, although a similar phase of NPQ relaxation was found in absence of Zx (*npq1*). We thus determined the formation and reconversion of Zx under the same experimental conditions.

3.3. Xanthophyll conversion

A direct comparison of the dynamics of NPQ and Zx conversion in WT leaves is shown in Fig. 6. The formation of Zx during 90 min of illumination followed clearly slower kinetics than the generation of NPQ (Fig. 6A). In particular, the rapidly formed NPQ phase was not paralleled by the rapid formation of large amounts of Zx. The same held true when the relaxation of NPQ was compared with the reconversion of Zx to Vx (Fig. 6B). Analysis of the extent and kinetics of Zx formation (in those genotypes that are not affected in xanthophyll conversion) revealed a nearly identical amount of the maximal Zx content and monophasic kinetics in all cases with similar

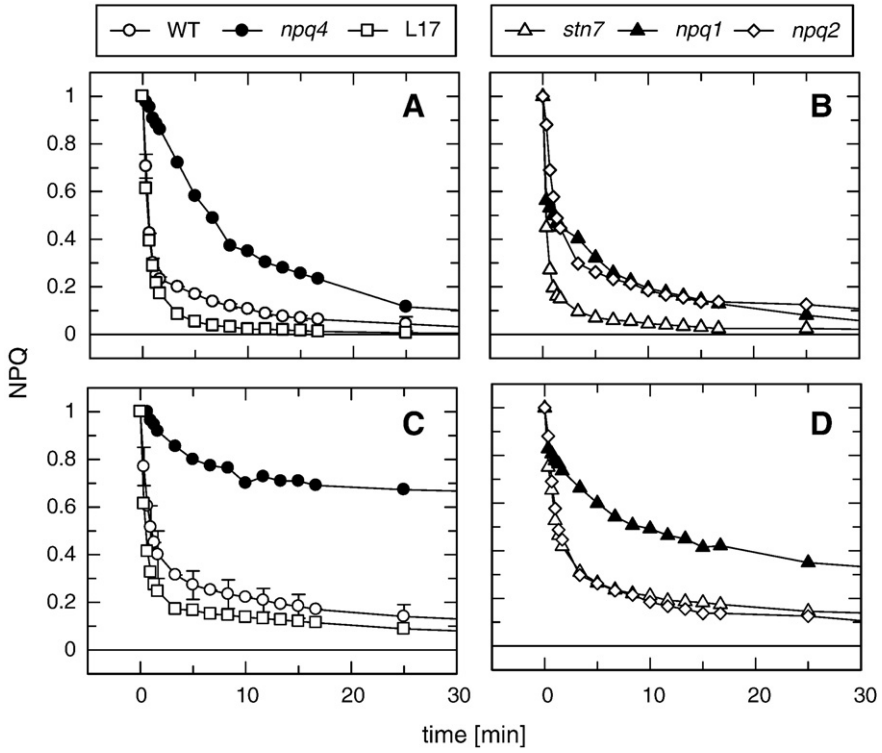

Fig. 5. NPQ relaxation after 5 and 90 min of preillumination. A and B, Initial phase of NPQ relaxation after 5 min of preillumination. C and D, Initial phase of NPQ relaxation after 90 min of preillumination. Original were taken from the experiment shown in Fig. 3. For a better comparison, the NPQ amplitudes determined at the end of the preillumination ($t = 0$) were normalized to 1. Mean values of 3 to 5 independent measurements are shown. For clarity, representative SD values are shown for the WT only.

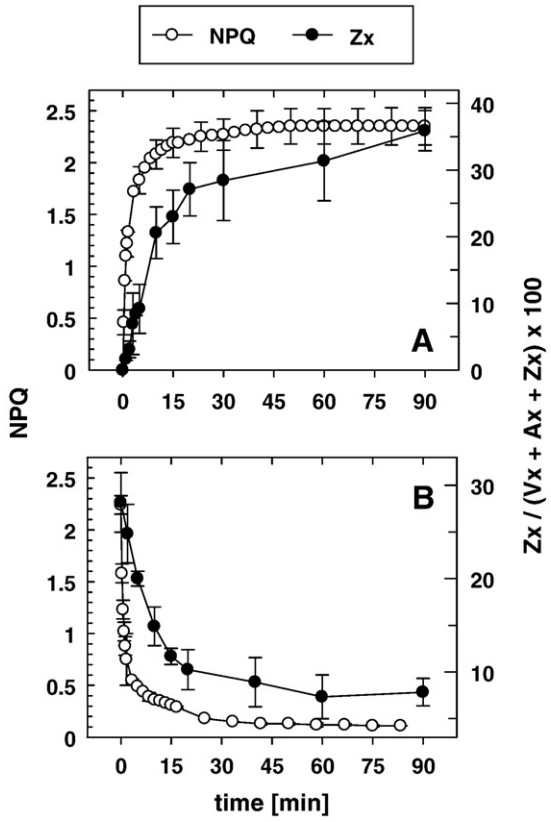

Fig. 6. Comparison of NPQ dynamics and xanthophyll conversion in WT plants. A, NPQ induction and Zx formation during 90 min of illumination. B, Dark relaxation of NPQ and reconversion of Zx to Vx after 30 min of preillumination. Detached leaves from dark-adapted plants were used. NPQ data were taken from Fig. 3. Light-induced Zx formation and reconversion of Zx to Vx in the dark were studied under the same illumination conditions (900 µmol photons $m^{-2} s^{-1}$, white light) as for NPQ measurements. Mean values ± SD from 3 to 5 independent measurements are shown.

rise times of 10–15 min (Fig. 7, Table 3). Only for the *stn7* mutants somewhat slower kinetics were found. Strikingly, the kinetics of Zx formation (Table 3) was very similar to those of the slow phase of NPQ induction (Table 1).

The kinetics of Zx epoxidation after different times of preillumination (under essentially the same conditions as used before for the NPQ parameter) followed a biphasic time course in all cases. The major portion of Zx (70 to 90%) was converted with kinetics of 10–15 min lifetime in most cases, while the remaining portion of Zx (10 to 30%) was not convertible at all within the time range of the experiment (Table 4, Fig. 7). It should be noted that the kinetics of the convertible Zx pool was similar to that of the middle phase of NPQ relaxation (cf. Table 2). Moreover, the portion of the non-convertible Zx pool increased with increasing preillumination time in all genotypes (Table 4, Fig. 7), similar to the irreversible NPQ component (Figs. 4 and 5).

In conclusion, the analysis of the dynamics of xanthophyll conversion underlined a kinetic correlation of (i) the formation and reconversion of Zx and (ii) the slow phase of NPQ induction and the middle phase of NPQ relaxation, respectively.

4. Discussion

We have studied the dynamics of NPQ formation and relaxation, and correlated it with the dynamics of xanthophyll conversion under steady-state conditions. In addition to the well-known qE- and qI components of NPQ, our analyses of the kinetics and amplitudes of different NPQ components under steady-state conditions identified an additional component of NPQ which is formed within the time range of 15 to 30 min and which relaxes in the dark in the same time range. Based on the correlation of the formation and relaxation of this component with the formation and reconversion of Zx, we term this component as the qZ-component of NPQ. The characteristics of NPQ induction/relaxation in the different genotypes are discussed in the following with respect to the regulation, localization and characteristics of different NPQ processes in PSII.

4.1. The rapid phase of NPQ induction (qE)

The rapid phase of NPQ induction is generated with overall rise times from 9 (*npq1*) to about 100 s (*stn7* and L17) and represents the well-known pH-dependent qE component of NPQ. The more rapid and similar kinetics of the fast phase of NPQ induction in *npq1* and *npq2* is known to reflect simply the same pH-dependence of the Zx-independent qE (in *npq1*) and of the sum of the Zx-independent and Zx-dependent qE (in *npq2*) [33]. In dark-adapted WT, *stn7* and L17 plants, the formation of qE is thus rate-limited by the synthesis of qE-active Zx. Based on the very rapid kinetics of generation of the Zx-dependent qE (within about 1–3 min) and the limited amount of Zx that is formed within this short period (Figs. 6 and 7), one has to postulate that a specific pool of Zx is involved in the formation of the Zx-dependent qE. Related to the fact that only Vx bound to the V1 site can be converted in such a short time [37,38], the required Zx is most likely formed from Vx bound to the V1 site of LHCII trimers. Vx conversion at this site might thus occur in parallel with the proposed detachment of LHCII along with qE formation [16].

4.2. The slow phase of NPQ induction (qZ)

The slow phase of NPQ induction, which we term qZ, has been originally attributed to qT [5], but later to either Zx-dependent qE processes [6,39] or photoinhibition [40], and most recently the dissociation of a fraction of PSII antenna complexes was found to occur with similar kinetics [41]. Our data support the view that qZ represents a Zx-dependent NPQ component, which – except for the synthesis of Zx – does not require a low lumen pH nor PsbS and thus does not represent a Zx-dependent qE component. The very similar characteristics of NPQ in WT and *stn7* further exclude any significant contribution of qT to this phase under the given light conditions. We can also exclude that qZ is related to photoinhibitory processes, because qZ was already fully developed after 30 min of preillumination at moderate light intensities which are very unlikely to induce pronounced photoinhibition of PSII. Moreover, the amplitude of qZ did not increase at longer illumination times, which would be expected for a photoinhibitory process (see also [41]). We therefore assign qZ to a PsbS- and pH-independent quenching process which is not related to qI and qT. qZ is generated with rise times of about 10 to 20 min and is thus likely to represent a quenching process that is formed in the antenna of PSII at those xanthophyll binding sites which are more slowly convertible to Zx. Since it is known that Vx bound to LHCII at the V1 site is most rapidly convertible to Zx [38] we speculate that the more slowly formed Zx in the minor LHC proteins of PSII [37] is involved in the generation of qZ. This interpretation would fit with the idea that Zx formation at the L2 site of LHC proteins is involved in the formation of a PsbS- and pH-independent NPQ process [28]. This assignment is further in accordance with the recent finding that a PsbS-independent but Zx-dependent quenching site is formed in the PSII antenna [16].

4.3. NPQ relaxation

Analyses of the NPQ relaxation kinetics identified at least three different NPQ components. Two of these phases are rather well defined: the rapid phase (lifetime of 30 to 60 s) can clearly be assigned to the relaxation of the ΔpH (and thus to qE), while the nearly

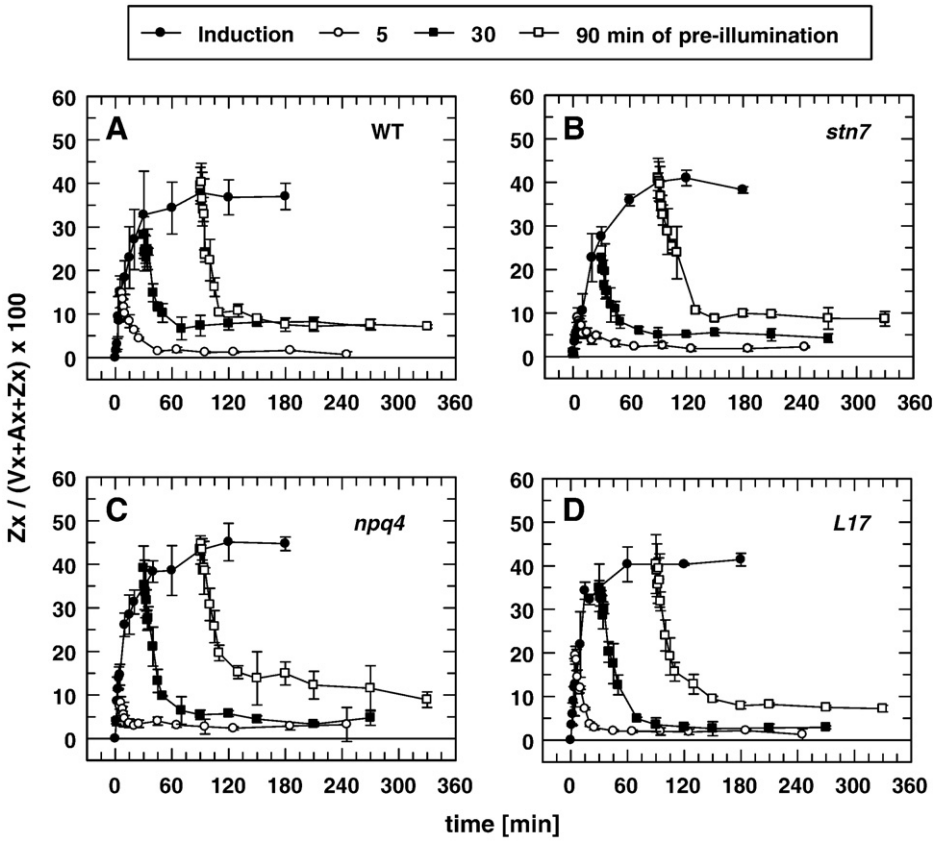

Fig. 7. Dynamics of xanthophyll conversion. Detached leaves from dark-adapted plants were illuminated for up to 180 min with white light at a light intensity of 900 µmol photons m^{-2} s^{-1}. For determination of Zx formation in the light, leaves were frozen in liquid N_2 at indicated time and the pigment content was subsequently analyzed by HPLC. The reconversion of Zx to Vx was measured after 5, 30 and 90 min of preillumination. To induce Zx reconversion, leaves were transferred into darkness. At indicated time, leaves were frozen in liquid N_2 and the pigment content was subsequently analyzed by HPLC. Mean values ± SD of 3 to 5 independent measurements are shown.

irreversible slow phase (lifetime of >120 min) can be assigned to photoinhibitory processes (qI). The basis of the middle phase of NPQ relaxation is under discussion since a long time. The presence of this phase in the *stn7* mutant from *Arabidopsis* (Fig. 4, Table 2) clearly proves that state transitions are not responsible for this NPQ component (as has already been supposed in an earlier study on barley leaves [6]). Instead, we assign this phase of NPQ relaxation to the relaxation of qZ, because (i) Zx reconversion was found to occur in the same time range as qZ relaxation and (ii) the amplitude of this phase was similar to that of the slow phase of NPQ induction (Tables 2 and 4). However in some cases the kinetic correlation of Zx epoxidation and qZ relaxation was not perfect. This is most likely due to limitations in the exact determination of the kinetics of Zx epoxidation: Firstly, the number of data points and the rather large SD in the pigment data are generally limiting the analyses of Zx epoxidation kinetics in comparison with the much better defined NPQ data. Secondly, – and perhaps more important – the pigment conversion kinetics are based on the total pool of xanthophyll cycle pigments, while the NPQ data refer only to those xanthophylls involved at the different specific quenching sites. Thirdly, the pigment analyses represent an average of the xanthophylls of the whole leaf, while the NPQ data reflect fluorescence quenching detected preferentially in chloroplasts at the leaf surface. Demmig-Adams et al. have shown that the extent of NPQ correlates nicely with the de-epoxidation state of xanthophylls and a better correlation was found for the sum of Ax and Zx rather than for Zx [42]. However, this did not apply to the correlation of the kinetics of NPQ relaxation and epoxidation: The kinetics of the decrease of the de-epoxidation state (A + Z)/(V + A + Z) was much slower than the Zx epoxidation kinetics (at least by a factor of 2, data not shown), leading to dramatically

Table 3
Kinetic parameters of Zx formation.

	A_1	τ_1 [s]
WT	36.1 ± 1.8	860 ± 100
stn7	40.9 ± 1.4	1600 ± 150
npq4	39.3 ± 1.8	700 ± 70
L17	40.4 ± 1.6	670 ± 70

The average data of Zx formation shown in Fig. 6 were fitted with a single exponential according to the equation $y = y_0 + A_1 * (1 - \exp(-t/\tau_1))$. SD represents the fitting error of the respective parameter, r^2 was >0.99 in all cases.

Table 4
Kinetic parameters of Zx reconversion to Vx.

	Time	A_1	τ_1 [s]	y_0
WT	5	15.1 ± 0.3	750 ± 40	1.4 ± 0.1
	30	24.9 ± 2.0	580 ± 80	7.5 ± 0.5
	90	33.2 ± 1.6	500 ± 80	8.0 ± 0.4
stn7	5	6.0 ± 0.4	1050 ± 150	2.2 ± 0.1
	30	17.2 ± 0.5	630 ± 90	5.2 ± 0.3
	90	35.8 ± 2.3	900 ± 80	8.2 ± 0.3
npq4	5	5.6 ± 0.5	340 ± 70	2.6 ± 1.2
	30	35.2 ± 1.9	650 ± 30	4.3 ± 0.3
	90	33.8 ± 1.2	990 ± 100	11.9 ± 0.8
L17	5	16.9 ± 1.1	480 ± 50	2.1 ± 0.2
	30	35.8 ± 0.5	880 ± 40	2.8 ± 0.2
	90	33.8 ± 1.7	980 ± 100	7.9 ± 0.2

The average data of Zx reconversion shown in Fig. 6 were fitted with a single exponential according to the equation $y = A_1 * \exp(-t/\tau_1) + y_0$. SD represents the fitting error of the respective parameter, r^2 was >0.99 in all cases.

slower pigment conversion kinetics in comparison with the relaxation of qZ. Thus our data fit much better with the conversion of Zx to Ax only.

Most likely, qZ is identical with the NPQ component, which has been related to a Zx-dependent component of qI in earlier studies on photoinhibition [26,43–45] and in more recent studies of slowly relaxing NPQ processes [28,41]. As pointed out above, our experimental conditions do not lead to rapid (15 to 30 min) formation of photoinhibited PSII, thus qZ can be excluded to represent a rapidly relaxing qI component. In general, qZ revealed remarkable differences and flexibilities among the different genotypes (Table 2, Figs. 3 and 4). In tendency, the relaxation kinetics of qZ was found to be retarded upon increasing illumination times (Table 2, Fig. 4) in all genotypes, most pronounced in *npq2*. This pronounced slowing down of qZ relaxation in the presence of high levels of Zx in *npq2* resembles the features of qE relaxation (which is also retarded in the presence of high levels of Zx) and thus underlines the involvement of Zx in qZ. By contrast, the occurrence of a middle phase of NPQ relaxation in *npq1* plants (which are unable to form Zx) seems to contradict the assignment of this phase to qZ. However, the NPQ dynamics observed in this mutant showed some unusual characteristics. In particular, only 20–50% of the rapidly induced NPQ was found to relax with rapid kinetics. It is thus likely that the middle phase of NPQ relaxation observed in *npq1* reflects a retarded relaxation of a large fraction of qE. This interpretation would imply that a functional xanthophyll cycle is important for the complete rapid relaxation of qE, but more detailed studies on the underlying NPQ mechanism are required to clarify this point.

Similarly, the slowly developing NPQ states in the PsbS-deficient *npq4* mutant remain to be characterized in more detail. The formation of NPQ in *npq4* followed rather slow kinetics only, and NPQ relaxation in *npq4* did not reveal any fast relaxing component. Since PsbS is supposed to be essential for conformational or structural changes in the PSII antenna, it can be speculated that NPQ relaxation in *npq4* is strongly retarded in general, because also the reorganisation of the PSII antenna might be essentially controlled by PsbS. Following this interpretation, the fastest phase of NPQ relaxation in this mutant (lifetime of about 100 to 500 s, Table 2) might represent the relaxation of a retarded Zx-dependent qE. The slower phase of NPQ relaxation (lifetimes 1000 to 4000 s) might then be related to the relaxation of a retarded qZ. Such an interpretation would fit with a recent study on the characteristics of energy dissipation in *npq4* plants [46]. It was concluded from that work, that the slowly inducible and slowly relaxing NPQ generated in *npq4* represents a retarded qE [46]. However, under the experimental conditions used in that study, the NPQ induced in WT plants showed a very untypical behaviour (e.g. very slow induction kinetics and similar maximal amplitudes of NPQ for WT and *npq4* plants after 60 min of illumination) so that different NPQ mechanisms might be active under our experimental conditions.

The qI component of NPQ developed slowly with increasing illumination time and maximal amplitudes are observed in the *npq1* and *npq4* plants, which are known to be more sensitive to light stress than the WT [36]. By contrast, qI was nearly completely absent in L17 and *npq2* plants during the first 30 min of illumination (Table 2, Fig. 4) supporting the photoprotective function of PsbS and Zx. Since the maximum NPQ seems to be fully developed in most genotypes after about 30 min (except for *npq1* and *npq4*, see Fig. 4), the increase of qI (and thus of the amplitude of the slow phase of NPQ relaxation) at longer illumination time should be paralleled by a decrease of the amplitude of either the rapid or middle phase of NPQ relaxation. Due to the rather small amplitude of qI, however, no clear trend could be derived from our data. Only in *npq2* plants, the development of qZ and qI was clearly paralleled by the reduction of the amplitude of the rapidly relaxing qE component. Thus more detailed studies at higher actinic light intensities are required to determine a possible connection among the different NPQ components.

The increase of qI at longer illumination time (Fig. 4) was paralleled by an increase of the irreversible (or very slowly reversible) portion of Zx (Fig. 6). This could point at a role of Zx also in the qI component of NPQ. In fact, the retainment of Zx along with sustained down-regulation of PSII activity [27] and a kinetic correlation of qI and Zx epoxidation [26] has been shown earlier. More recent work further revealed that Zx epoxidation is stepwise down-regulated with increasing photo-oxidative stress [23]. Although no causal or mechanistic connection between Zx and qI can be derived from those correlations, these findings underline at least the important protective role of Zx under photoinhibitory conditions.

5. Conclusion

Our data show that at least three different NPQ processes contribute to the overall NPQ under steady-state conditions at saturating light intensities in *Arabidopsis*: qE, qZ and qI. Considering the data from former characterization of qE and qI, we propose the following model for NPQ development under the given experimental conditions (Fig. 8):

1. qE is formed within 10–200 s as has been reported before. This process is strictly dependent on a high ΔpH and the PsbS protein but requires also Zx synthesis. Fastest rise times can be observed when Zx is already present at the onset of illumination (e.g. in *npq2* plants or in a second illumination cycle applied to pre-illuminated leaves after a short period of re-darkening), while slower rise times (100–200 s) are related to the limitations of qE generation in dark-adapted plants by Zx synthesis. The relaxation of qE depends on the breakdown of the ΔpH.
2. A newly resolved Zx-dependent NPQ component, qZ, is formed within 10–30 min. The formation of qZ is independent of PsbS, but strictly dependent on Zx. The relaxation of qZ depends on Zx epoxidation.

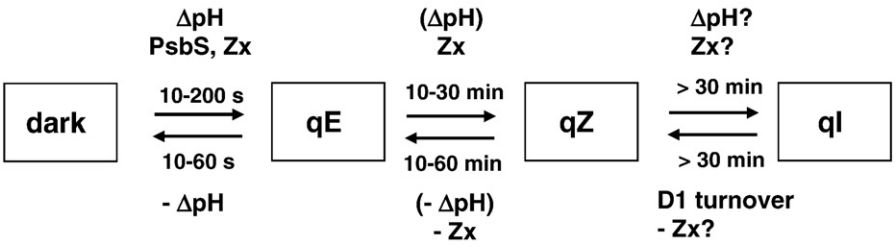

Fig. 8. Model for the different NPQ states generated under saturating light intensities in *Arabidopsis*. Starting from the dark-adapted state, qE is formed within 10–200 s. qE is dependent on the ΔpH, PsbS and Zx. In dark-adapted plants the rate of the formation of qE-active Zx is the time-limiting step for the generation of maximum qE. qE is reversible upon the breakdown of the transthylakoid pH gradient. At longer time scale (10 to 30 min), another Zx-dependent NPQ state is formed (qZ) which depends on the conversion of more slowly convertible Vx and by that indirectly also on the ΔpH. Relaxation of qZ state requires the reconversion of Zx to Vx (and thus also the breakdown of the pH gradient). Finally a photoinhibitory state (qI) is generated after continuing illumination (>30 min) which requires D1 turnover for relaxation. The possible role of Zx in qI is unclear. For further details see text.

3. Photoinhibitory processes (qI) are induced at longer time scale (>30 min). The extent of qI depends on the illumination time, the light intensity, but in particular quite strongly on the genotype. Those genotypes that have a full capacity of NPQ do not develop significant qI under our conditions.
4. State transitions (qT) do not contribute significantly to NPQ at saturating light intensities.

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