Engineering the lutein epoxide cycle into *Arabidopsis thaliana*

Lauriebeth Leonelli[a,b,1], Matthew D. Brooks[a,b,c,1], and Krishna K. Niyogi[a,b,c,2]

[a]Howard Hughes Medical Institute, University of California, Berkeley, CA 94720; [b]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720; and [c]Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Contributed by Krishna K. Niyogi, June 30, 2017 (sent for review March 16, 2017; reviewed by Jeremy Harbinson and Shizue Matsubara)

Although sunlight provides the energy necessary for plants to survive and grow, light can also damage reaction centers of photosystem II (PSII) and reduce photochemical efficiency. To prevent damage, plants possess photoprotective mechanisms that dissipate excess excitation. A subset of these mechanisms is collectively referred to as NPQ, or nonphotochemical quenching of chlorophyll *a* fluorescence. The regulation of NPQ is intrinsically linked to the cycling of xanthophylls that affects the kinetics and extent of the photoprotective response. The violaxanthin cycle (VAZ cycle) and the lutein epoxide cycle (LxL cycle) are two xanthophyll cycles found in vascular plants. The VAZ cycle has been studied extensively, owing in large part to its presence in model plant species where mutants are available to aid in its characterization. In contrast, the LxL cycle is not found in model plants, and its role in photosynthetic processes has been more difficult to define. To address this challenge, we introduced the LxL cycle into *Arabidopsis thaliana* and functionally isolated it from the VAZ cycle. Using these plant lines, we showed an increase in dark-acclimated PSII efficiency associated with Lx accumulation and demonstrated that violaxanthin deepoxidase is responsible for the light-driven deepoxidation of Lx. Conversion of Lx to L was reversible during periods of low light and occurred considerably faster than rates previously described in nonmodel species. Finally, we present clear evidence of the LxL cycle's role in modulating a rapid component of NPQ that is necessary to prevent photoinhibition in excess light.

lutein | lutein epoxide | nonphotochemical quenching | photoprotection | xanthophyll cycle

The ability to convert solar energy into a chemical form through photosynthesis is essential for maintaining much of life on Earth. For many photosynthetic organisms living in environments with fluctuating light, this dependence on light is a double-edged sword. Light absorption is required to generate chemical energy for metabolic processes; however, too much light can cause damage to photosynthetic complexes and result in generation of toxic reactive oxygen species. To balance these dangers with the need for sufficient light harvesting, plants and other photosynthetic organisms have evolved mechanisms to protect against photodamage caused by the absorption of excess light energy. Several of these photoprotective mechanisms are collectively measured and referred to as nonphotochemical quenching of chlorophyll *a* fluorescence (NPQ), and they function to dissipate excitation nondestructively as heat.

The different mechanisms that constitute NPQ are distinguishable both by the timescales on which they operate and by other individual requirements (1). The most rapidly responding component is qE, or feedback deexcitation, which induces and relaxes within seconds to minutes in response to high light. In plants, qE depends on the presence of the PsbS protein, the generation of a transthylakoid pH gradient, and on the VAZ cycle—a xanthophyll cycle involving the interconversion of violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) (2–4). During exposure to excess light, the enzyme violaxanthin deepoxidase (VDE) is activated and converts V to Z via the intermediate A (5). In limiting light (or darkness), zeaxanthin epoxidase (ZEP) regenerates V through the addition of epoxy groups to the β-ionone rings of Z and A (6). The kinetics of this xanthophyll cycle directly impact the speed of NPQ induction and relaxation, and consequently the efficiency of photochemistry. Recent studies have shown that optimizing the kinetics of photoprotective mechanisms such as NPQ can lead to increases in photosynthetic efficiency and biomass (7) and presents an attractive target for improving crop yields (8–10).

Because the VAZ cycle is the predominant xanthophyll cycle found in most model plants, its role in photoprotection has been extensively characterized (3, 11). However, a second xanthophyll cycle has been identified in large numbers of nonmodel neotropical and woody plant species: the lutein epoxide cycle (LxL cycle) (12–15). The role of the LxL cycle in photosynthetic processes has been more difficult to define. It typically functions in parallel with the VAZ cycle, and because it occurs in nonmodel species, mutants are not easily generated for determining its contribution to photosynthesis and/or photoprotection. In nature, the LxL cycle exists in various permutations. The "truncated" LxL cycle is found in species that accumulate lutein epoxide (Lx) in shade conditions and convert it to lutein (L) when exposed to high light, likely by VDE (16). Additional accumulation of L is thought to be beneficial to plants when exposed to excess light, because it has been shown to increase NPQ in various species (17, 18). In plants with a truncated LxL cycle, newly formed L remains bound to photosynthetic complexes

Significance

Optimizing the balance between light harvesting and photoprotection holds great promise for improving photosynthetic efficiency and ultimately crop yields. The switch between these two states is regulated by xanthophyll cycling, which occurs in response to changing light conditions. Two xanthophyll cycles have been described in vascular plants: the violaxanthin cycle and the lutein epoxide cycle. The contribution of the lutein epoxide cycle to photosynthesis has been difficult to dissect because the violaxanthin cycle often functions in parallel and responds more rapidly. The introduction of the lutein epoxide cycle into *Arabidopsis thaliana* creates a model system in which to study this ecologically significant but less well-characterized xanthophyll cycle and reveals its role in modulating a rapidly reversible component of nonphotochemical quenching of chlorophyll *a* fluorescence in response to light.

Author contributions: L.L. and K.K.N. designed research; L.L. performed research; L.L., M.D.B., and K.K.N. analyzed data; and L.L., M.D.B., and K.K.N. wrote the paper.

Reviewers: J.H., Wageningen University; and S.M., Forschungszentrum Jülich.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. KU980906).

[1]Present address: Center for Genomics and Systems Biology, Department of Biology, New York University, New York, NY 10003.

[2]To whom correspondence should be addressed. Email: niyogi@berkeley.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704373114/-/DCSupplemental.

Table 1. Lutein and lutein epoxide content of *Arabidopsis* lines before and after high light treatments

Plant genotype	Time, min	Lx, mmol/mol Chl	L, mmol/mol Chl	Lx+L	DES L/(L+Lx)
szl1					
	0	*nd*	224.5 ± 12.2	224.5 ± 12.2	1 ± 0
	30	*nd*	215.6 ± 10.7	215.6 ± 10.7	1 ± 0
	60	*nd*	201.1 ± 16.0	201.1 ± 16.0	1 ± 0
	150	*nd*	232.0 ± 17.3	232.0 ± 17.3	1 ± 0
szl1 + *NoZEP1*					
	0	158.5 ± 8.0	45.4 ± 6.0	203.9 ± 7.5	0.22 ± 0.03
	30	71.4 ± 5.8	116.1 ± 16.4	187.6 ± 15.4	0.62 ± 0.04
	60	83.5 ± 22.3	133.7 ± 22.4	217.2 ± 7.62	0.62 ± 0.1
	150	121.2 ± 35.8	73.8 ± 32.8	194.9 ± 17.9	0.38 ± 0.17
szl1npq1					
	0	*nd*	228.8 ± 24.2	228.8 ± 24.2	1 ± 0
	30	*nd*	212.6 ± 30.4	212.6 ± 30.4	1 ± 0
	60	*nd*	189.1 ± 33.3	189.1 ± 33.3	1 ± 0
	150	*nd*	224.5 ± 24.5	224.5 ± 24.5	1 ± 0
szl1npq1 +*NoZEP1*					
	0	175.6 ± 17.7	33.7 ± 9.6	209.4 ± 27.1	0.16 ± 0.02
	30	170.5 ± 22.0	31.6 ± 7.6	202.1 ± 16.8	0.16 ± 0.04
	60	182.8 ± 26.3	29.6 ± 8.1	212.4 ± 25.9	0.14 ± 0.04
	150	181.0 ± 16.9	27.4 ± 3.1	208.3 ± 14.7	0.13 ± 0.02

Plants were exposed to high light (750 μmol photons·m^{-2}·s^{-1}) for 30 min and then returned to low light (125 μmol photons·m^{-2}·s^{-1}) for recovery. Time is measured from the start of the experiment, e.g., 60 min translates to 30 min of recovery (± SE, $n = 4$). *nd* indicates that pigment level was below the detection limit. DES, de-epoxidation state.

long-term, locking in a permanently enhanced capacity for NPQ (18, 19). In contrast, species that contain a complete LxL cycle are able to convert L back to Lx once light levels become favorable, although this conversion often requires many hours to restore initial Lx concentrations (15, 20, 21). Diminished Lx content has been associated with a decrease in efficiency of photosystem II (ΦPSII) (22), suggesting that for plants living in shaded environments that experience intermittent sunflecks, the slow restoration of initial Lx concentrations could have a sizable impact on the net gains of photochemistry.

Because of the higher light-harvesting efficiency of plants containing Lx (22, 23), and the photoprotection conferred by the accumulation of L (17, 24, 25), the LxL cycle is of special interest for optimizing plant growth in environments with variable light. To address the limitations of studying this cycle in nonmodel plants, we took advantage of the *szl1* mutant of *Arabidopsis thaliana* that contains a partial loss-of-function mutation in *lycopene* β-*cyclase* (*LCYB*). This mutation causes an accumulation of xanthophylls derived from α-carotene (such as L) at the expense of the β-xanthophylls that comprise the VAZ cycle (17). By transforming *Arabidopsis* lines containing the *szl1* mutation with *NoZEP1,* a gene encoding a Z epoxidase from *Nannochloropsis oceanica* that generates Lx in planta (26), we introduced a complete LxL cycle into *Arabidopsis* and functionally isolated it from the VAZ cycle. We used these engineered lines to examine the role of the LxL cycle in light harvesting and photoprotection. Through a combination of pigment analysis and chlorophyll fluorescence measurements, we show that Lx increases the dark-acclimated ΦPSII and that NPQ kinetics in the presence of the LxL cycle are similar to those in the presence of the VAZ cycle.

Results

Expression of *NoZEP1* in *Arabidopsis* Results in Accumulation of Lx. A crucial step to building the LxL cycle in *Arabidopsis* was to determine whether the relaxed substrate specificity shown by *NoZEP1* in *Nicotiana benthamiana* (26) was also observed in *Arabidopsis*. We introduced a FLAG-tagged version of *NoZEP1* into different *Arabidopsis* backgrounds (Fig. S1) and examined the pigment composition that resulted from its expression. Irrespective of their genetic background, *Arabidopsis* lines containing the *NoZEP1* transgene consistently accumulated Lx under normal growth conditions (Table 1 and Fig. 1). Because the objective of this study was to engineer a functionally isolated and intact LxL cycle in *Arabidopsis*, we focused on plants containing the *szl1* mutation that causes an enrichment in α-branch carotenoids such as L, while concomitantly reducing accumulation of β-branch carotenoids (17) (Fig. 1*A*).

VDE and NoZEP1 Act Together To Reconstitute the LxL Cycle in *Arabidopsis*. To determine whether the endogenous *Arabidopsis* VDE could produce a complete LxL cycle in *NoZEP1*-expressing plants, we exposed transgenic *szl1* and transgenic *szl1npq1* plants to 30 min of high light followed by a period of low light and monitored changes in pigment concentrations. In addition to the mutation in *LCYB*, *szl1npq1* mutants lack a functional copy of *VDE* and therefore show no deepoxidase activity (17). Upon exposure to high light, the level of Lx in *szl+NoZEP1* lines decreased to 45% of the initial amount, whereas the level of L exhibited a concomitant and stoichiometric increase (Fig. 2*A*). When these plants were returned to low light, the level of Lx began to increase, returning to 77% of the original level after 2 h and to 100% after 8 h of recovery, thus exhibiting a complete LxL cycle. These shifts in ratio of L:Lx are reflected in the deepoxidation states (DES) shown in Table 1 and occur at a rate that is comparable to that of pigment conversion in the VAZ cycle (Table S1 and Fig. S2). Pigment cycling was not observed in *szl1npq1+NoZEP1* lines, because levels of Lx remained constant regardless of high light exposure (Fig. 2*B*), demonstrating that VDE is required for Lx deepoxidation in *Arabidopsis*.

Levels of total α- and β-xanthophylls remained relatively constant throughout the course of the experiment (Fig. S3), indicating that changes in L and Lx ratios were most likely due to the addition and removal of epoxy groups by NoZEP1 and VDE rather than de novo synthesis.

The LxL Cycle Plays an Active Role in Dissipating Excess Light Energy. A surplus of L has been shown to restore quenching of excess light energy in *Arabidopsis* plants that lack Z (17). Because *szl1+NoZEP1* plants showed a light-dependent conversion of Lx to L, we examined

whether an increase in NPQ accompanied this interconversion of pigments. Upon illumination with high light, *szl1+NoZEP1* transgenic lines showed a slower initial increase in NPQ compared with the rapid rise observed in *szl1* and *szl1npq1* lines that already contained high levels of L (Fig. 3 and Table 1). Despite this slower initial rise, NPQ in *szl+NoZEP1* surpassed that of *szl1* after only 5 min and continued to steadily increase over the course of high light exposure. Indeed, the NPQ seen in some transgenic *szl1+NoZEP1* lines approached levels observed in wild-type (Col-0) plants after 20 min of high light exposure (Fig. S4). As in wild type, the majority of this NPQ in *szl+NoZEP1* was rapidly reversible in the dark (Fig. 3 and Fig. S4), with nearly identical kinetics to the qE component of NPQ. In contrast, *szl1npq1+NoZEP1* lines, which were unable to convert accumulated Lx into L, showed markedly reduced NPQ that failed to reach levels comparable to *szl1* or *szl1npq1* lines during the entire 20 min of high light exposure (Fig. 3). Furthermore, none of the NPQ induced in *szl1npq1+NoZEP1* recovered rapidly (Fig. 3), suggesting a specific lack of qE. The NPQ in *szl1npq1+NoZEP1* that was reversible within 10 min was not evident in chlorophyll fluorescence lifetime measurements (27), and it is likely attributable to chloroplast movement (28).

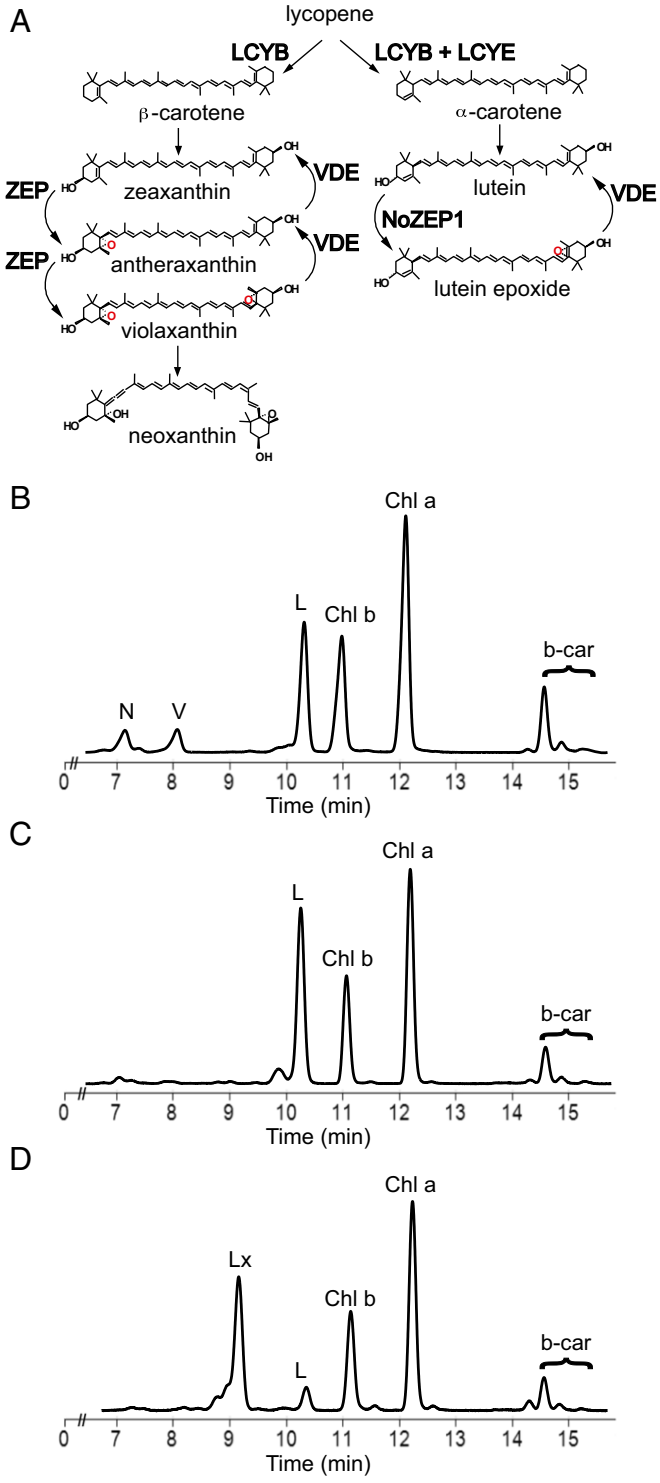

Fig. 1. Pigments in various *Arabidopsis* lines. (*A*) Diagram of xanthophyll biosynthetic pathways in *Arabidopsis*. Enzymes appear in bold, and relevant epoxy groups are shown in red. The *szl1* lines contain mutated LCYB, and *npq1* lines contain mutated VDE. LCYE, lycopene ε-cyclase; NoZEP1, ZEP from *Nannochloropsis oceanica*. (*B*) HPLC analysis of pigments of wild type (Col-0). (*C*) HPLC analysis of pigments of *szl1*. (*D*) HPLC analysis of pigments of *szl1+NoZEP1*. Chl a, chlorophyll *a*; Chl b, chlorophyll *b*; b-car, β-carotene; N, neoxanthin.

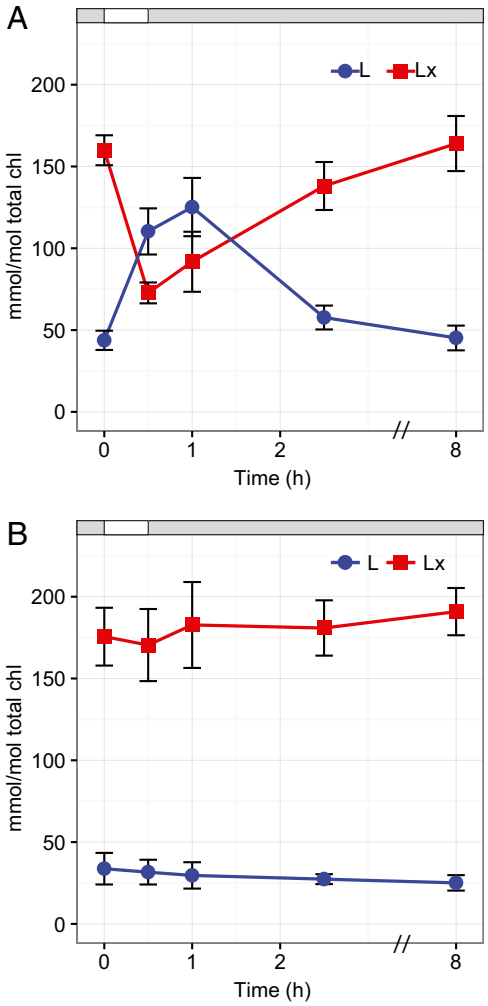

Fig. 2. Light-induced xanthophyll cycling in engineered *Arabidopsis* lines. Changes in pigment composition after 30 min of high light exposure (750 μmol photons·m^{-2}·s^{-1}, white bar) followed by 7.5 h of low light (125 μmol photons·m^{-2}·s^{-1}, gray bar) recovery. (*A*) Lx and L interconversion mediated by VDE and NoZEP1 in an *szl1+NoZEP1* background. (*B*) The absence of Lx and L interconversion in the *szl1npq1+NoZEP1* background that lacks VDE. Error bars denote SD (n = 4).

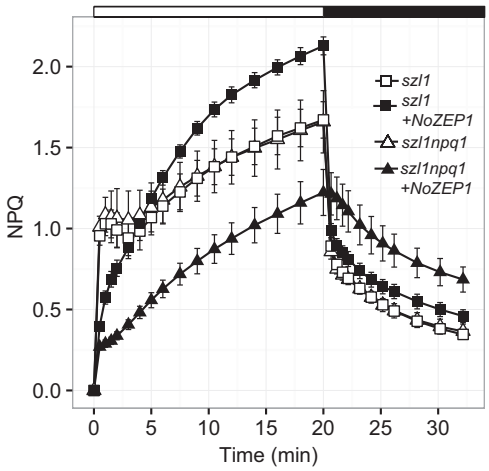

Fig. 3. NPQ in *Arabidopsis* lines containing the LxL cycle. Open symbols indicate *Arabidopsis* mutants that lack the *NoZEP1* transgene, and filled symbols indicate transgenic mutant lines that contain *NoZEP1* and accumulate Lx. White bar represents actinic light intensity of 750 μmol photons·m^{-2}·s^{-1}, and filled bar represents dark recovery. Error bars denote SE (n = 4).

Plants Containing Lx Require a Complete LxL Cycle To Prevent Photoinhibition. Because the substitution of Lx for L appeared to influence NPQ kinetics (Fig. 3), we tested whether photochemistry and/or photoinhibition were altered in *szl1+NoZEP1* plants, which accumulate Lx yet retain the ability to convert it to L in high light. Initial dark-acclimated ΦPSII (F_v/F_m) values in both *szl1+NoZEP1* and *szl1npq1+NoZEP1* were slightly but significantly higher ($P < 0.002$) than in their non-Lx containing counterparts, *szl1* and *szl1npq1* (Table 2 and Fig. 4*A*). This increase in F_v/F_m can be attributed to a decrease in F_o (dark-acclimated minimal fluorescence) rather than an increase in F_m (dark-acclimated maximum fluorescence) (Table S2), possibly indicating that energy transfer between LHCs and PSII reaction centers is more efficient (29). After 3 h of high light exposure (1,300 μmol photons·m^{-2}·s^{-1}), the F_v/F_m values for all genotypes decreased to between 23 and 52% of original values (Fig. 4*B*). The F_v/F_m values in sz*l1* and *szl1+NoZEP1* lines were least affected by prolonged treatment with high light, and after 1 h in low light, were restored to 75% and 71% of their original F_v/F_m values, respectively. After 24 h, F_v/F_m was restored to 98% (0.752) in *szl1* plants and 96% (0.753) in *szl1+NoZEP1* plants, indicating that the presence of a complete LxL cycle had a neutral effect on photoinhibition under the conditions tested (Fig. 4*B*).

The response of *szl1npq1* backgrounds to high light revealed a different pattern. F_v/F_m in both *szl1npq1* and *szl1npq1+NoZEP1* lines was more severely reduced relative to *szl1* and *szl1+NoZEP1* during high light, with *szl1npq1+NoZEP1* experiencing the most dramatic reduction (Fig. 4*B*). The recovery of the *szl1npq1* line matched that of the *szl1* backgrounds after only 5 h in low light, whereas F_v/F_m values in Lx-accumulating *szl1npq1+NoZEP1* plants remained notably depressed until 24 h after high light treatment. Even then, F_v/F_m was restored to only 90% (0.708) in *szl1npq1+NoZEP1* plants, whereas it recovered to 94% (0.714) in the nontransgenic *szl1npq1* lines. The strong photoinhibition observed in *szl1npq1+NoZEP1* compared with *szl1+NoZEP1* demonstrates that the LxL cycle is necessary for effective protection against photoinhibition in plants that have substituted Lx for L.

Discussion

The role of the VAZ cycle in NPQ and photoprotection has been intensively investigated in model plants. Although the accumulation of Lx and the presence of the LxL cycle are widespread in nature (12–15), our understanding of this xanthophyll cycle has been limited by its absence in well-characterized species. Moreover, species that contain a complete LxL cycle also contain a VAZ cycle that functions in parallel, essentially masking the contribution of the LxL cycle to photosynthetic processes. In this study, we introduced *NoZEP1* into the *szl1* mutant of *Arabidopsis*, effectively creating a model plant with a complete LxL cycle that is also functionally isolated from the VAZ cycle (Fig. 5).

The wide distribution of species containing Lx (30), and the correlation of higher photosynthetic efficiencies associated with its presence (22, 23), highlight the LxL cycle as an especially attractive target for further study. Herein we show that despite identical growth conditions, dark-acclimated F_v/F_m values in *Arabidopsis* plants containing Lx are modestly but significantly higher than in plants containing only L (Table 2). It should be noted that the F_v/F_m values in each of these lines are lower than typically observed in unstressed Col-0 plants, consistent with a previous study that showed a decreased F_v/F_m in the *szl1* mutant (29). In vitro studies with reconstituted Lhcb5 antenna proteins have shown that Lx is more efficient at transferring excitation energy to chlorophyll *a* molecules than L (31), which could explain the increase in maximum PSII efficiency measured as F_v/F_m. Alternatively, the observed increase in F_v/F_m might be due to the fact that L, which may function as a constitutive quencher of excitation energy, is sequestered as Lx in these plants, causing less energy to be diverted from photochemistry in light-limited environments. However, it is important to note that the higher F_v/F_m initially observed in Lx-containing plants was not evident after high light treatment, even after a 24-h period of recovery and restoration of initial Lx concentrations (Figs. 2 and 4), suggesting that there may be other slowly relaxing photoprotective components still engaged—a conclusion supported by F_m values that remained lower after 24 h relative to initial F_m values (Table S2). However, with the tools presented in this study, we will now be able to explore the energy transfer dynamics of Lx in vivo and in isolated photosynthetic complexes to learn more about possibilities for improving the efficiency of energy transfer within LHCs and between LHCs and PSII by the addition of Lx to photosynthetic complexes.

Although Lx may increase PSII efficiency, its substitution for L has a negative impact on photoprotection in *Arabidopsis*. Plants containing Lx but lacking a complete LxL cycle are at a disadvantage when it comes to dealing with excess light energy. Photoinhibition in the Lx-accumulating *szl1npq1+NoZEP1* lines was more severe than in the L-accumulating *szl1npq1* plants (Fig. 4*B*). Lutein's role in quenching excess energy has been described in several studies (17, 18, 32), so the effect of its sequestration as Lx on photoinhibition is unsurprising. Lx-accumulating *szl1+NoZEP1* lines, however, were able to convert Lx to L through the action of VDE and, consequently, rates of photoinhibition were indistinguishable from L-accumulating *szl1* plants (Fig. 4*B*). This finding demonstrates that plants containing a complete LxL cycle were able to convert Lx to L rapidly enough to avoid the level of photoinhibition observed in *szl1npq1+*

Table 2. Initial F_v/F_m measurements of plants used in this study

Plant genotype	F_v/F_m
szl1	0.768 ± 0.002
szl1+NoZEP1	0.785 ± 0.003
szl1npq1	0.763 ± 0.002
szl1npq1+NoZEP1	0.784 ± 0.003

F_v/F_m values, indicative of maximum photochemical efficiency (Φ PSII), collected before light treatment from L- and Lx-accumulating *Arabidopsis* lines. Values ± SE (n = 8).

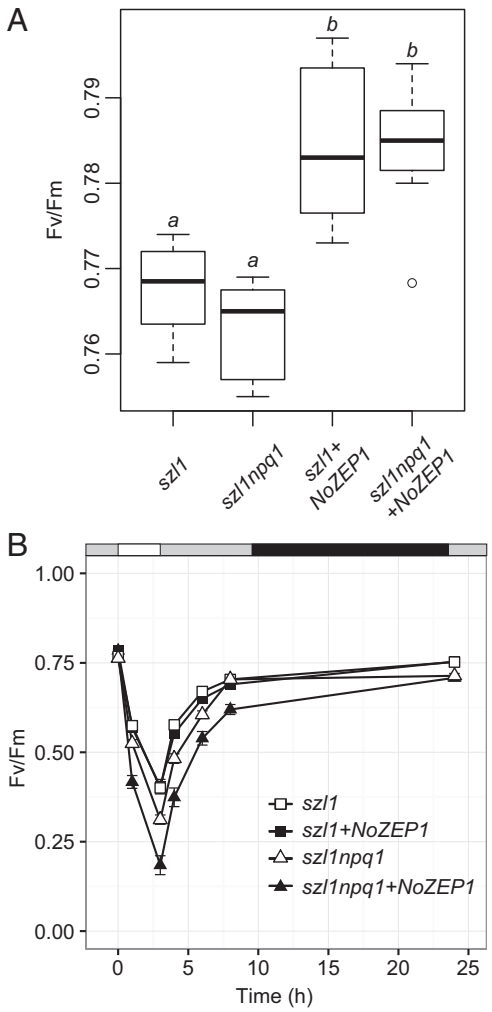

Fig. 4. PSII efficiency (F_v/F_m) of *Arabidopsis* lines containing the LxL cycle. (*A*) Box plots of initial dark-acclimated F_v/F_m measurements in plants containing Lx versus plants containing only L. Bold horizontal lines indicate median per group, boxes represent 50% of the data between the first and third quartiles, and hashes represent the range of the data excluding outliers which are indicated by open circles. Each pair of genotypes was analyzed by using Student's *t* test, and significant differences are indicated by different letters ($P < 0.002$, $n = 8$). (*B*) Changes in F_v/F_m during high light exposure and recovery. Open shapes indicate *Arabidopsis* mutants lacking the *NoZEP1* transgene, and filled shapes indicate transgenic mutant lines that contain *NoZEP1* and accumulate Lx in low light. White bar represents actinic light intensity of 1300 μmol photons·m^{-2}·s^{-1}, gray bar represents light intensity of 125 μmol photons·m^{-2}·s^{-1}, and black bar represents darkness. Error bars denote SE ($n = 8$).

NoZEP1. This observation raised a question about how the rate of Lx to L conversion corresponds to NPQ, a key element of photoprotection.

The role of the VAZ cycle in qE, the rapidly responding component of NPQ, has been studied extensively, yet determining the contribution of the LxL cycle to qE has proven complicated. Many plants that contain the LxL cycle have either truncated versions or exhibit rates of Lx and L interconversion that operate on time frames slower than those typically associated with qE (15, 19–21). Additionally, many species with complete LxL cycles sequester only a small percentage of their L as Lx. In contrast to previous studies (summarized in ref. 33), the *szl1+NoZEP1* lines contained high proportions of Lx (78% of total L+Lx) and showed a light-induced deepoxidation of Lx on a time scale similar to that of V in the VAZ cycle (Fig. 2*A*, Table S1, and Fig. S2). In plants with both the VAZ and LxL cycle, V and A likely compete directly with Lx for the active sites of VDE, which could explain the relatively slow Lx deepoxidation kinetics observed previously. In *szl1* lines, β-xanthophyll accumulation is negligible, allowing Lx greater access to VDE and potentially increasing the rate of Lx deepoxidation. Alternatively (or additionally), it has been proposed that xanthophylls occur in various pools within the chloroplast (33, 34). In species naturally containing Lx, all or a portion of the Lx could be bound in locations

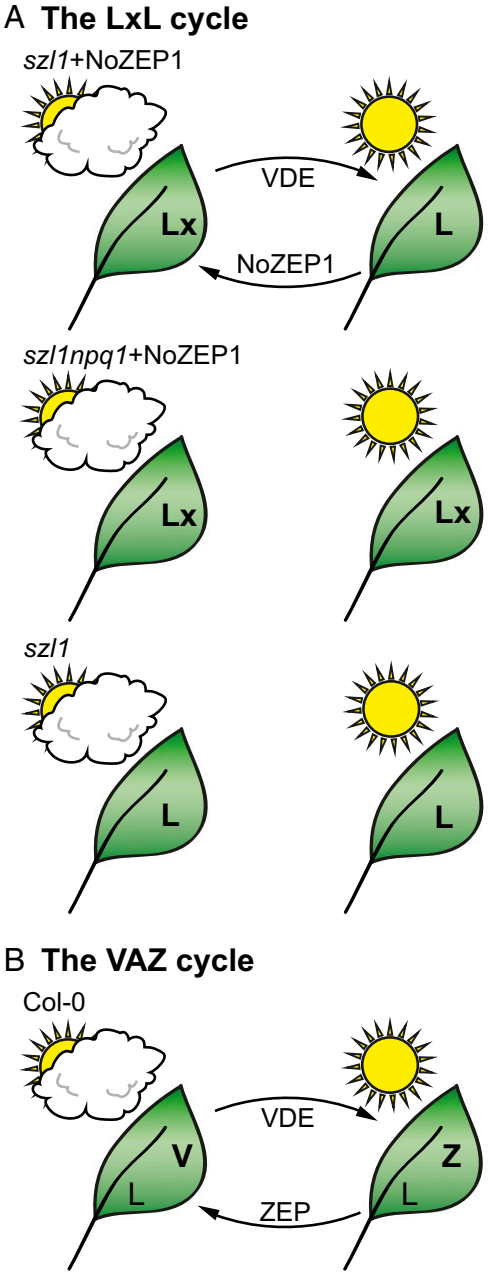

Fig. 5. Diagram illustrating xanthophyll content of leaves from different genotypes used in this study under low and high light conditions. (*A*) Plant lines containing predominantly α-carotenoids that are involved in the LxL cycle. (*B*) Col-0 leaf showing the β-carotenoids involved in the VAZ cycle. Clouds represent periods of low light, and suns represent periods of high light. Arrows indicate enzyme activity, with VDE being activated in high light, and ZEP/NoZEP1 being constitutively active.

where accessibility to VDE is limited. In the *szl1+NoZEP1* lines, the high proportion of Lx suggests a broader occupancy of binding sites in photosynthetic complexes, including peripheral binding sites where Lx would be accessible to VDE, comparable to what is seen when β-xanthophylls are enriched in *Arabidopsis* (35).

Perhaps most importantly, in the context of NPQ, Lx to L conversion directly correlates with the induction of qE-like quenching. Because the majority of α-xanthophylls in *szl1+NoZEP1* lines was sequestered as Lx, NPQ induction was initially slower relative to the *szl1* lines that already contained high levels of L (Fig. 3). However, upon illumination with excess light, VDE began converting the Lx in *szl1+NoZEP1* plants to L (Fig. 2), and the capacity for energy quenching in these plants steadily increased, appearing to surpass that of *szl1* lines after only a few minutes in high light (Fig. 3). This trend is especially intriguing, because the *szl1+NoZEP1* lines have less overall L available after 30 min in HL (Table 1) and yet are able to achieve a higher NPQ than *szl1*. This result suggests that newly converted L has different accessibility to quenching sites and/or that the presence of Lx facilitates quenching, possibly through enhanced energy transfer to quenching sites occupied by L. The quenching in *szl1+NoZEP1* is L-dependent, because it did not occur in *szl1npq1+NoZEP1* plants that are defective in VDE and, thus, unable to convert Lx to L. NPQ in the *szl1+NoZEP1* plants also showed rapid relaxation, resembling that of the qE component observed in wild-type plants (Fig. S4). This kinetic trend was also reflected in the reconversion rate of L to Lx during recovery in *szl1+NoZEP1* plants, which restored Lx concentrations to 77% of original levels after only 2 h of recovery (Table 1 and Fig. 2) rather than the days to weeks required for Lx restoration in native plants containing the LxL cycle (18, 23). This rapid epoxidation of L demonstrates that *NoZEP1* can efficiently complete the LxL cycle in *Arabidopsis*. The observation of L-dependent quenching in *Arabidopsis* plants containing the LxL cycle challenges our perception of what is necessary for rapid photoprotective mechanisms and invites further studies on the role of PsbS and the transthylakoid proton gradient in L-mediated energy dissipation.

Based on the Lx-accumulating species described so far, the LxL cycle appears to be as versatile as the VAZ cycle—existing in either truncated or complete forms, present in diverse tissues, during different seasons, and under various environmental and light conditions (reviewed in refs. 34 and 36). This diversity in form points to multiple functions and also highlights the underlying complexity in deconvoluting contributions of the LxL cycle to different plant processes. In nature, the VAZ cycle and LxL cycle are thought to act synergistically, functioning in parallel to modulate energy harvesting and photoprotection in response to different environmental stimuli. In this study, we introduced a complete LxL cycle into *Arabidopsis* and showed that, in this system, the initial presence of Lx increased ΦPSII, whereas the complete LxL cycle contributed to rapidly inducing and relaxing L-mediated photoprotection. These results present us with an opportunity to explore the LxL cycle as a possible avenue of increasing photosynthetic productivity. Sequestering L as Lx in shaded tissues could potentially increase rates of photochemistry and reduce energy losses due to unnecessary L-mediated quenching, while still maintaining the plant's capacity to deal with influxes of excess light energy through the action of the LxL cycle. Future studies should focus on how the Lx-induced increase in ΦPSII translates into biomass accumulation and whether the energy dissipation observed in plants containing the LxL cycle is mechanistically similar to that mediated by the VAZ cycle. Modeling of the NPQ mediated by the LxL cycle strongly suggests that L-dependent quenching contributes to qE even in wild-type *Arabidopsis* (27).

Materials and Methods

Generation of Transgenic Lines. The following *Arabidopsis* lines were transformed with the pEarleyGate100 plasmid containing the *NoZEP1+FLAG* gene by using the floral dip method described in ref. 37: Col-0, *szl1*, *szl1npq1*, and *npq1*. Transformed seedlings were selected on agar plates supplemented with 1/2 MS salts and 10 $\mu g \cdot mL^{-1}$ ammonium glufosinate. All experiments were performed by using individuals of the T_2 generation segregating for homozygous single insertions with the exception of those involving transgenic *szl1npq1* plants, which used individuals of the T_1 generation that were confirmed for transgene expression through immunoblot and HPLC pigment analysis.

Plant Growth. Transgenic seedlings were transplanted from selection plates after 10 d to 2-inch pots containing Sunshine soil mix no. 4. Plants were maintained in a 10-h day (125 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$)/14-h night photoperiod at 22 °C in a Conviron growth chamber. Materials used in this study were collected from plants that were 5–8 wk old.

Immunoprecipitation and Immunoblot Analysis. One gram of leaf tissue from each transgenic plant line was pooled and flash frozen in liquid nitrogen. Frozen tissue was powdered by using a mortar and pestle and resuspended in 2 mL of extraction buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.2% Nonidet P-40, 6 mM β-mercaptoethanol, with cOmplete protease inhibitor from Roche). Extracts were incubated with 10 μL of anti-FLAG M2 affinity gel (Sigma Aldrich) for 4 h at 4 °C. Purification was carried out as indicated in the manufacturer's protocol. The eluted FLAG-tagged protein was mixed 1:1 with 2× SDS buffer and heated to 85 °C for 3 min. Samples were added to a precast 10–20% Tris-glycine gel (Invitrogen) and run at 125 V for 2 h at 4 °C. Proteins were transferred onto 0.45 μm of PVDF (GE Healthcare) by using the SemiPhor semidry transfer system (Hoefer). Membranes were blocked for 30 min in 5% milk and probed with rabbit anti-FLAG primary antibody (1:5,000) (Sigma Aldrich) at 4 °C overnight. The secondary antibody was donkey anti-rabbit-HRP (1:50,000) (Amersham) incubated for 1 h at room temperature.

Pigment Separation and Analysis. Dark-acclimated leaf discs (0.44 cm^2) from various transgenic lines were placed in Petri dishes on damp Whatman paper in a Conviron under high light (750 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$, 22°C) for 30 min and then returned to a light intensity of 125 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$ for 8 h. Throughout the course of the experiment, leaf discs were flash frozen at various time points in liquid nitrogen and pulverized (6.5 $m \cdot s^{-1}$ for 60 s) by using the cryo rotor of a Fastprep-24 (MP Bio). Pigments were extracted in 200 μL of acetone by gently vortexing for 15 s followed by centrifugation at 20,800 × *g* for 3 min to pellet cell debris. Supernatants were analyzed by using a Spherisorb 5-μm ODS1 column (Waters) as described (38).

Chlorophyll Fluorescence Measurements. Pulse-amplitude-modulation fluorescence measurements were performed by using an FMS2 fluorometer (Hansatech Instruments) on attached leaves of transgenic lines ranging from 5–8 wk in age. Plants were dark-acclimated for 20 min before exposure to 20 min of white actinic light (750 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$) followed by 10 min of dark recovery, unless otherwise stated. Fluorescence measurements and photosynthetic parameters were calculated as described (39).

Damage Assessment. To assess sensitivity to photoinhibition, eight detached leaves from different transgenic lines were floated on distilled water in Petri dishes and placed in high light (1,300 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$, 22 °C) for 3 h, then returned to their original growth conditions (125 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$, 22 °C) for ~6 h until dark (10/14 h day/night cycle) similar to as described (40). F_v/F_m measurements were taken after a 10-min dark acclimation period at various time points by using an FMS2 fluorometer before plants were returned to the appropriate light chamber. Experiments were repeated twice: once using 1,300 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$ and once using 1,800 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$.

ACKNOWLEDGMENTS. We thank Marilyn Kobayashi for assistance with HPLC data analysis and Zhirong Li for providing *szl1* and *szl1npq1 Arabidopsis* seeds. This work was supported by the US Department of Energy, Office of Science, Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division under field work proposal 449B. K.K.N. is an Investigator of the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation (through Grant GBMF3070).

1. Müller P, Li XP, Niyogi KK (2001) Non-photochemical quenching. A response to excess light energy. *Plant Physiol* 125:1558–1566.
2. Demmig-Adams B, Gilmore AM, Adams WW, 3rd (1996) Carotenoids 3: in Vivo function of carotenoids in higher plants. *FASEB J* 10:403–412.
3. Niyogi KK, Grossman AR, Björkman O (1998) Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* 10:1121–1134.
4. Li XP, et al. (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* 403:391–395.
5. Yamamoto HY, Bugos RC, Hieber AD (1999) Biochemistry and molecular biology of the xanthophyll cycle. *The Photochemistry of Carotenoids*, eds Frank HA, Young AJ, Britton G, Cogdell RJ (Springer, Dordrecht, Netherlands), pp 293–303.
6. Hieber AD, Bugos RC, Yamamoto HY (2000) Plant lipocalins: Violaxanthin de-epoxidase and zeaxanthin epoxidase. *Biochimica et Biophysica Acta* 1482:84–91.
7. Kromdijk J, et al. (2016) Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science* 354:857–861.
8. Zhu XG, Ort DR, Whitmarsh J, Long SP (2004) The slow reversibility of photosystem II thermal energy dissipation on transfer from high to low light may cause large losses in carbon gain by crop canopies: A theoretical analysis. *J Exp Bot* 55: 1167–1175.
9. Zhu XG, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. *Annu Rev Plant Biol* 61:235–261.
10. Long SP, Marshall-Colon A, Zhu X-G (2015) Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. *Cell* 161:56–66.
11. Havaux M, Niyogi KK (1999) The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proc Natl Acad Sci USA* 96: 8762–8767.
12. Bungard RA, et al. (1999) Unusual carotenoid composition and a new type of xanthophyll cycle in plants. *Proc Natl Acad Sci USA* 96:1135–1139.
13. García-Plazaola JI, Hormaetxe K, Hernández A, Olano JM, Becerril JM (2004) The lutein epoxide cycle in vegetative buds of woody plants. *Funct Plant Biol* 31: 815–823.
14. Khachik FB, Gary R, Goli MB (1991) Separation, identification, and quantification of carotenoids in fruits, vegetables and human plasma by high performance liquid chromatography. *Pure Appl Chem* 63:71–80.
15. Matsubara S, et al. (2009) Sun-shade patterns of leaf carotenoid composition in 86 species of neotropical forest plants. *Funct Plant Biol* 36:20–36.
16. Goss R (2003) Substrate specificity of the violaxanthin de-epoxidase of the primitive green alga Mantoniella squamata (Prasinophyceae). *Planta* 217:801–812.
17. Li Z, et al. (2009) Lutein accumulation in the absence of zeaxanthin restores nonphotochemical quenching in the *Arabidopsis thaliana npq1* mutant. *Plant Cell* 21: 1798–1812.
18. Förster B, Pogson BJ, Osmond CB (2011) Lutein from deepoxidation of lutein epoxide replaces zeaxanthin to sustain an enhanced capacity for nonphotochemical chlorophyll fluorescence quenching in avocado shade leaves in the dark. *Plant Physiol* 156: 393–403.
19. Matsubara S, et al. (2005) Slowly reversible de-epoxidation of lutein-epoxide in deep shade leaves of a tropical tree legume may 'lock-in' lutein-based photoprotection during acclimation to strong light. *J Exp Bot* 56:461–468.
20. Esteban R, et al. (2010) Operation and regulation of the lutein epoxide cycle in seedlings of *Ocotea foetens*. *Funct Plant Biol* 37:859–869.
21. Matsubara S, Gilmore AM, Osmond CB (2001) Diurnal and acclimatory responses of violaxanthin and lutein epoxide in the Australian mistletoe *Amyema miquelii*. *Funct Plant Biol* 28:793–800.
22. Jia H, Förster B, Chow WS, Pogson BJ, Osmond CB (2013) Decreased photochemical efficiency of photosystem II following sunlight exposure of shade-grown leaves of avocado: Because of, or in spite of, two kinetically distinct xanthophyll cycles? *Plant Physiol* 161:836–852.
23. Matsubara S, et al. (2008) Lutein epoxide cycle, light harvesting and photoprotection in species of the tropical tree genus *Inga*. *Plant Cell Environ* 31:548–561.
24. Niyogi KK, Björkman O, Grossman AR (1997) The roles of specific xanthophylls in photoprotection. *Proc Natl Acad Sci USA* 94:14162–14167.
25. Avenson TJ, et al. (2009) Lutein can act as a switchable charge transfer quencher in the CP26 light-harvesting complex. *J Biol Chem* 284:2830–2835.
26. Leonelli L, Erickson E, Lyska D, Niyogi KK (2016) Transient expression in *Nicotiana benthamiana* for rapid functional analysis of genes involved in non-photochemical quenching and carotenoid biosynthesis. *Plant J* 88:375–386.
27. Leuenberger M, et al. (2017) Dissecting and modeling zeaxanthin- and lutein-dependent nonphotochemical quenching in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA*201704502, in press 10.1073/pnas.1704502114.
28. Dall'Osto L, Cazzaniga S, Wada M, Bassi R (2014) On the origin of a slowly reversible fluorescence decay component in the *Arabidopsis npq4* mutant. *Philos Trans R Soc Lond B Biol Sci* 369:20130221.
29. Cazzaniga S, Li Z, Niyogi KK, Bassi R, Dall'Osto L (2012) The Arabidopsis *szl1* mutant reveals a critical role of β-carotene in photosystem I photoprotection. *Plant Physiol* 159:1745–1758.
30. Esteban R, et al. (2009) Distribution and evolutionary trends of photoprotective isoprenoids (xanthophylls and tocopherols) within the plant kingdom. *Physiol Plant* 135: 379–389.
31. Matsubara S, Morosinotto T, Osmond CB, Bassi R (2007) Short- and long-term operation of the lutein-epoxide cycle in light-harvesting antenna complexes. *Plant Physiol* 144:926–941.
32. Duffy CDP, et al. (2013) Modeling of fluorescence quenching by lutein in the plant light-harvesting complex LHCII. *J Phys Chem B* 117:10974–10986.
33. García-Plazaola JI, Matsubara S, Osmond CB (2007) The lutein epoxide cycle in higher plants: Its relationships to other xanthophyll cycles and possible functions. *Funct Plant Biol* 34:759–773.
34. Esteban R, Garcia-Plazaola JI (2014) Involvement of a second xanthophyll cycle in non-photochemical quenching of chlorophyll fluorescence: The lutein epoxide story. *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria,* Advances in Photosynthesis and Respiration, ed Demmig-Adams B, Garab G, Adams III W, Govindjee (Springer, Dordrecht, Netherlands), pp 277–295.
35. Davison PA, Hunter CN, Horton P (2002) Overexpression of beta-carotene hydroxylase enhances stress tolerance in Arabidopsis. *Nature* 418:203–206.
36. García-Plazaola JI, Esteban R, Fernández-Marín B, Kranner I, Porcar-Castell A (2012) Thermal energy dissipation and xanthophyll cycles beyond the Arabidopsis model. *Photosynth Res* 113:89–103.
37. Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743.
38. Müller-Moulé P, Conklin PL, Niyogi KK (2002) Ascorbate deficiency can limit violaxanthin de-epoxidase activity in vivo. *Plant Physiol* 128:970–977.
39. Brooks MD, Niyogi KK (2011) Use of a pulse-amplitude modulated chlorophyll fluorometer to study the efficiency of photosynthesis in *Arabidopsis* plants. *Methods Mol Biol* 775:299–310.
40. Li X-P, Müller-Moulé P, Gilmore AM, Niyogi KK (2002) PsbS-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. *Proc Natl Acad Sci USA* 99:15222–15227.