Title (82 chars): Improving Photosynthesis and Crop Productivity by Accelerating Recovery from Photoprotection.

Authors: Johannes Kromdijk[1,†], Katarzyna Głowacka[1,5 †], Lauriebeth Leonelli[2], Stéphane T. Gabilly[2], Masakazu Iwai[2,3], Krishna K. Niyogi[2,3*], Stephen P. Long[1,4,*]

Affiliations:
[1]Carl R. Woese Institute for Genomic Biology, University of Illinois, 1206 W Gregory Drive, Urbana, IL 61801, USA

[2]Howard Hughes Medical Institute, Department of Plant and Microbial Biology, 111 Koshland Hall, University of California Berkeley, Berkeley, CA 94720-3102, USA

[3]Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

[4]Lancaster Environment Centre, University of Lancaster, LA1 1YX, UK

[5]Institute of Plant Genetics, Polish Academy of Sciences, ul. Strzeszyńska 34, 60-479 Poznań, Poland

*Correspondence to: niyogi@berkeley.edu and slong@illinois.edu

†Contributed equally to this work.

Abstract (125 words):

Crop leaves in full sunlight dissipate damaging excess absorbed light energy as heat. When sunlit leaves are shaded by clouds or other leaves, this protective dissipation continues for many minutes and reduces photosynthesis. Calculations have shown that this could cost field crops up to 20% of their potential yield. Here we describe the bioengineering of an accelerated response to natural shading events in *Nicotiana* (tobacco), resulting in increased leaf carbon dioxide uptake and plant dry matter productivity by about 15% in fluctuating light. Since the photoprotective mechanism that has been altered is common to all flowering plants and crops, the findings provide proof of concept for a novel route to obtaining a sustainable increase in productivity for food crops and a much needed yield jump.

One Sentence Summary (122 characters):

Altering the regulation of light harvesting increases photosynthetic efficiency and biomass productivity in a crop plant.

Main Text (2411 words):

Based on detailed forecasts of future global food demand, current rates of increase in crop yields per hectare of land are inadequate. Based on prior model predictions of opportunities to improve photosynthetic efficiency and thus improve crop yield (*1*), we here show improvement of photosynthetic efficiency and productivity through genetic manipulation of photoprotection.

Light in plant canopies is very dynamic, and leaves routinely experience sharp fluctuations in levels of absorbed irradiance. When light intensity is too high or increases too fast for photochemistry to utilize the absorbed energy, several photoprotective mechanisms are induced to protect the photosynthetic antenna complexes from over-excitation (*2*). Excess excitation energy in the photosystem II (PSII) antenna complex can be harmlessly dissipated as heat, which is observable as a process named non-photochemical quenching of chlorophyll fluorescence (NPQ, (*3*)). Changes in NPQ can be fast but are not instantaneous, and therefore lag behind fluctuations in absorbed irradiance. In particular, the rate of NPQ relaxation is slower than the rate of induction, and this asymmetry is exacerbated by prolonged or repeated exposure to excessive light conditions (*4*). This slow rate of recovery of PSII antennae from the quenched to the unquenched state implies that the photosynthetic quantum yield of CO_2 fixation is transiently depressed by NPQ upon a transition from high to low light intensity (Fig. 1). When this hypothesis was tested in model simulations and integrated for a crop canopy over a diurnal course, corresponding losses of CO_2 fixation were estimated to range between 7.5% - 30% (*5*-*7*). Based on these computations, increasing the relaxation rate of NPQ has been highlighted as a very promising strategy to improve crop photosynthetic efficiency and in turn yield (*8*).

While the exact NPQ quenching site and nature of the quenching mechanisms involved are still debated (*9*), it is clear that for NPQ to occur, PSII-associated antennae need to undergo a conformational change to the quenched state, which can be induced by a number of different

mechanisms with contrasting time constants (*3*). So-called energy-dependent quenching (qE, (*10*)) requires low thylakoid lumen pH and is greatly aided by the presence of PSII subunit S (PsbS) (*11, 12*) and de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin via the xanthophyll cycle (*13, 14*). Expression of PsbS strongly affects the amplitude of qE formation, and overexpression results in an increased rate of induction and relaxation of qE (*15-17*). As a result, the effects of PsbS overexpression on CO_2 fixation and plant growth depend on the prevailing light environment. Enhancement of qE via PsbS overexpression may offer increased photoprotection under high light or rapidly fluctuating conditions (*18*), but can be at the expense of CO_2 fixation under less stressful conditions (*15*). An alternative route of NPQ manipulation is to modify xanthophyll cycle kinetics. The xanthophyll cycle de-epoxidation state (DES) influences the level of NPQ (*19*), due to the stimulating effect of zeaxanthin on qE and on zeaxanthin-dependent quenching (qZ, (*20*)). qZ has slower relaxation kinetics (10-15 min) than qE (10-90 s), which are linked to the kinetics of the zeaxanthin pool. *Arabidopsis* mutants with increased xanthophyll cycle pigment pool size were shown to have slower rates of NPQ formation and relaxation, due to slower DES kinetics (*21*). Thus, the rate of adjustment of DES appears to be affected by the xanthophyll cycle pool size relative to the rate of turn-over via violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP), which in turn affects the adjustment rate of NPQ.

We hypothesized that by accelerating the xanthophyll cycle and increasing PsbS, NPQ would decline more rapidly on transfer of leaves to shade (Fig. 1), leading to faster restoration of the maximum efficiency of CO_2 assimilation that can be achieved at a given light intensity in the shade, which in turn would allow increased productivity.

Results

Transgene mRNA and protein expression

Nicotiana tabacum was transformed with the coding sequences of *Arabidopsis VDE*, *ZEP* and *PsbS* under the control of different promoters for expression in leaves (Fig. S1). Two transformants with a single T-DNA integration (VPZ-34 and 56) and one transformant with two T-DNA insertions (VPZ-23) were selected based on a seedling NPQ screen (Fig. S2 and S3) and self-pollinated to obtain homozygous T2 progeny for further investigation. All three VPZ-lines showed increases in total (transgenic plus native) transcript levels of *VDE* (10-fold), *PsbS* (3-fold) and *ZEP* (6-fold) relative to wild-type (WT) (Fig. 2A, C and E). For *PsbS* the increase in transcript levels translated into approximately 4-fold higher PsbS protein level (Fig. 2D), as exemplified in bands at 21 kDa (AtPsbS) and 24 kDa (NtPsbS; Fig. 2G). For *VDE* and *ZEP* the increase in transcript levels corresponded to 30-fold for VDE (Fig. 2B and G, 45 kDa) and 74-fold for ZEP (Fig. 2F and G, 73 kDa) increases over WT protein levels. Field-grown plants showed similar increases in protein levels (47-, 3- and 75-fold for VDE, PsbS and ZEP, Fig. S4), although increases in transcript levels were less pronounced (4-, 1.2- and 7-fold for VDE, PsbS and ZEP, Fig. S4).

Faster relaxation of NPQ and recovery of CO_2 fixation rate

To compare the kinetics of dynamic NPQ adjustment, a double exponential model was fitted to dark relaxation of NPQ in young seedlings after exposure to fluctuating light between 2000 and 200 μmol photons m^{-2} s^{-1} (Fig. 3A). The qZ phase of NPQ relaxation (τ_2) was significantly faster in VPZ-lines at an average of 753 s versus 2684 s in WT ($p<0.05$), and qE relaxation (τ_1) was also noticeably faster at an average of 15 s versus 21 s (significant in VPZ-23 and VPZ-56, $p<0.05$). To see if this faster relaxation translated into higher leaf CO_2 uptake, leaves were

exposed to a sharp transition in light from 2000 to 200 μmol photons m^{-2} s^{-1}. CO_2 assimilation declined immediately after the decrease in light intensity in both WT and VPZ lines (Fig. 3B), reaching a minimum at 30 s. During the following 150 s, CO_2 fixation rate increased gradually, but more rapidly in the VPZ lines compared to WT, leading to significantly higher CO_2 fixation rates, averaging an increase of 9% ($p<0.02$).

Effects of fluctuating light on the efficiency of photosynthetic CO_2 assimilation

To evaluate the dynamic effect of VPZ overexpression on the response of leaf CO_2 uptake to light, light intensity was varied in two different ways. First, light intensity was varied from low to high (Fig. S5A), taking care to allow gas exchange and fluorescence to achieve steady state at each light intensity. Second, light intensity was varied in 4 min alternating steps of high to low light (Fig. S5B). The resulting steady-state and fluctuating light response curves of CO_2 fixation and linear electron transport rate were distinctly different between WT and VPZ lines. In steady state, the maximum quantum yield of CO_2 fixation ($\Phi CO_{2\text{-max}}$) was not different between WT and VPZ lines, averaging 0.092 CO_2/absorbed photon (Fig. 4A). Fluctuating light decreased $\Phi CO_{2\text{-max}}$ to 0.058 CO_2/absorbed photon in the WT plants (Fig. 4B), whereas $\Phi CO_{2\text{-max}}$ in the VPZ lines showed a far smaller depression to 0.066 CO_2/absorbed photon ($p<0.05$). Similarly, under fluctuating light, maximum quantum yield of whole chain electron transport ($\Phi PSII_{\text{-max}}$) declined from an average value of 0.73 (Fig. 4C) to 0.54 e^-/absorbed photon in the WT plants (Fig. 4D), compared to 0.60 e^-/absorbed photon in the VPZ lines ($p<0.05$). Thus, under these fluctuating conditions, average $\Phi CO_{2\text{-max}}$ and $\Phi PSII_{\text{-max}}$ of the VPZ lines were 11.3% and 14.0% higher than WT. These differences were also confirmed in plants grown under field conditions (Fig. S6A and B) and were not caused by a difference in photosynthetic capacity, as shown by the lack of differences in $\Phi CO_{2\text{-max}}$ and $\Phi PSII_{\text{-max}}$ between VPZ lines and WT when measured at

steady state (Fig. 4A and C). There were also no differences in the maximum carboxylation capacity (V_{cmax}) or ribulose bis-phosphate regeneration capacity (J_{max}) derived from CO_2 response curves (Table S1) nor were there differences in the levels and stoichiometry of the major photosynthetic complexes (Fig. S7). Instead, the differences under fluctuating conditions corresponded to the faster relaxation of NPQ resulting from VPZ overexpression. Steady-state NPQ below 400 µmol photons m^{-2} s^{-1} was very low (Fig. 4E and S5G) and did not differ between WT and VPZ lines. However, under fluctuating light intensity, NPQ was significantly higher in the WT compared to the VPZ lines at low light (Fig. 4F), whereas NPQ in high light did not differ between WT and VPZ lines (Fig. S5G and H).

Productivity under field conditions

Whether this greater photosynthetic efficiency during shading events would affect productivity was evaluated under field conditions in a randomized block design with 12 blocks (Fig. 5D and S8). Plants from VPZ lines exhibited greater total dry weight per plant by 14 to 20% relative to WT (Fig. 5A), which was evident in increases in leaf, stem and root weights (Fig. S9A-C). Additionally, plants from VPZ lines showed increases in leaf area (Fig. 5B) and plant height (Fig. 5C), relative to WT. Similar productivity increases were found under greenhouse conditions (Fig. S10A-F).

Xanthophyll cycle de-epoxidation as a function of different light treatments

In dark-acclimated leaves from both WT and VPZ lines, the xanthophyll cycle pool was completely epoxidated, i.e., entirely in the form of violaxanthin, (Table 1). Exposure to 400 µmol photons m^{-2} s^{-1} constant light did not lead to substantial de-epoxidation, but 2000 µmol photons m^{-2} s^{-1} constant light led to accumulation of antheraxanthin and especially zeaxanthin. VPZ lines retained more violaxanthin and accumulated less zeaxanthin and antheraxanthin

compared to WT, which led DES in the VPZ lines to be about half that of WT (26% versus 46%). Exposure to fluctuating light led to similar results as high light exposure, but with even less xanthophyll de-epoxidation in the VPZ lines, relative to WT (18% versus 53%), and field-grown plants of VPZ-23 showed significantly lower DES than WT throughout a diurnal period (Fig. S11). Because of the lower DES in the VPZ lines, a concern was that they would be more vulnerable to photoinhibition. However, photoprotection in seedlings after 2 h exposure to excessive light (λ_{max}=470nm, 2000 μmol photons m^{-2} s^{-1}) appeared to be equal (VPZ-56) or even higher (VPZ-23 and VPZ-34; $p<0.05$) than WT (Fig. S12).

Discussion

How does introduction of the VPZ construct accelerate NPQ relaxation on transfer of leaves from high to low light, as would occur in a shading event? NPQ is a compound variable, encompassing several quenching mechanisms with contrasting relaxation kinetics (*22*). Whereas PsbS is exclusively associated with rapidly relaxing energy-dependent quenching (qE), the xanthophyll cycle is involved in multiple components of NPQ, especially qE and qZ. Even though VPZ lines had lower xanthophyll de-epoxidation state (DES) under high and fluctuating light intensity (Table 1), levels of NPQ were similar to WT at high light (Fig. S3B and S5H) implying that the relationship between xanthophyll DES and NPQ has been altered by PsbS overexpression, allowing for higher NPQ at lower DES. The presence of zeaxanthin correlates with faster induction and slower relaxation of NPQ, with respect to qZ and qE (*4, 20, 23*). Consistent with the lower DES in the VPZ-lines, relaxation of both qE (τ_1) and qZ (τ_2) was accelerated by the VPZ overexpression. The faster relaxation of NPQ by VPZ overexpression can thus be explained by two parallel manipulations of NPQ. Combined overexpression of VDE

and ZEP decreased xanthophyll DES, which in turn increased NPQ relaxation rate through qZ, qE and zeaxanthin-associated effects on NPQ kinetics. Second, the overexpression of PsbS led to an increase in qE, which more than offset the decrease due to lower DES (Fig. S3B).

The hypothesis that photosynthetic efficiency could be increased through acceleration of NPQ relaxation (*8, 24*) relies on the inverse correlation between NPQ and photosynthetic efficiency. Under fluctuating light, the VPZ lines showed faster and greater decreases in NPQ following transitions from high to low light, relative to WT (Fig. 4F and S5H), which increased quantum yield of CO_2 assimilation by 14% (Fig. 4B), providing proof that on transition from high to low light, NPQ does indeed limit photosynthetic efficiency. Xanthophyll DES is correlated with NPQ (*19*), which suggests that limiting violaxanthin de-epoxidation may also increase NPQ relaxation rate. However, decreased zeaxanthin formation by antisense *VDE* expression in tobacco in previous studies did not lead to an increase in photosynthetic efficiency and growth (*25, 26*). Reduction in NPQ amplitude (*27*) and anti-oxidant capacity (*28*) leads to greater sensitivity to damage by excessive light in mutants with reduced zeaxanthin (*29*). Here expression of VDE and PsbS was increased to balance the up-regulation of ZEP and avoid such damage (Fig. S12). This conservation of photoprotection in the VPZ lines most likely originates from an increase in qE, reflecting the positive correlation between photoprotection and PsbS content (*18*).

About 50% of canopy carbon gain in crops occurs under light-limitation (*5*). Efficiency of photosynthesis in the shade declines even further with rapid light transitions caused by clouds and wind-driven movement of overshadowing leaves. Higher yields have followed increased planting densities, which also caused denser canopies and increased the proportion of partially shaded leaves, leading to more irregular light conditions for each leaf. Even for upper leaves on a

196 clear day, daily changes in sun angle cause light transitions that are rapid at the chloroplast level
197 (*7*). Thus, light conditions in the field are anything but steady state. Under steady state light, the
198 VPZ lines evaluated here would have shown no yield advantage over WT. Their yield advantage
199 becomes apparent under more realistic, irregular, lighting conditions.

200 Because the xanthophyll cycle and PsbS are common to all vascular plants (*11, 19*), we
201 expect that similar results would pertain to all major crops. Although this work has focused on
202 crop light use efficiency, stomatal conductance also remains high during the first few minutes
203 after transfer to shade. Increasing the rate of relaxation of NPQ will therefore not only increase
204 net carbon gain, but also increase crop water use efficiency. This may be an important attribute
205 given forecast climate change impacts on future crop production (*30*).

206 Transgenic expression of *Arabidopsis* VDE*,* PsbS and ZEP (VPZ) in combination in tobacco
207 led to a marked and statistically significant acceleration of NPQ relaxation on transfer of leaves
208 from high light to shade. As hypothesized, this led to a more rapid recovery of the efficiency of
209 photosynthetic CO_2 assimilation in the shade. Results from field and greenhouse experiments
210 showed that this corresponded to increased productivity in terms of final dry mass. Increases in
211 crop productivity of 15%, as obtained here, demonstrate an important means to achieve the
212 increases in crop yield forecast to be necessary by 2050 (*31, 32*).

References and Notes (891 words)

1. D. R. Ort *et al.*, Redesigning photosynthesis to sustainably meet global food and bioenergy demand. *Proc Natl Acad Sci U S A* **112**, 8529-8536 (2015).
2. Z. Li, S. Wakao, B. B. Fischer, K. K. Niyogi, Sensing and responding to excess light. *Annu Rev Plant Biol* **60**, 239-260 (2009).
3. Müller P, X.-P. Li, K. K. Niyogi, Non-Photochemical Quenching. A response to Excess Light Energy. *Plant Physiology* **125**, 1558-1566 (2001).
4. M. L. Perez-Bueno, M. P. Johnson, A. Zia, A. V. Ruban, P. Horton, The Lhcb protein and xanthophyll composition of the light harvesting antenna controls the DeltapH-dependency of non-photochemical quenching in Arabidopsis thaliana. *FEBS Lett* **582**, 1477-1482 (2008).
5. S. P. Long, S. Humphries, Photoinhibition of Photosynthesis in Nature. *Annu Rev Plant Biol* **45**, 633-662 (1994).
6. C. Werner, R. J. Ryel, O. Correia, W. Beyschlag, Effects of photoinhibition on whole-plant carbon gain assessed with a photosynthesis model. *Plant Cell and Environment* **24**, 27-40 (2001).
7. X.-G. Zhu, D. R. Ort, J. Whitmarsh, S. P. Long, 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. *Journal of Experimental Botany* **55**, 1167-1175 (2004).
8. E. H. Murchie, K. K. Niyogi, Manipulation of Photoprotection to Improve Plant Photosynthesis. *Plant Physiology* **155**, 86-92 (2011).
9. C. D. P. Duffy, A. V. Ruban, Dissipative pathways in the photosystem-II antenna in plants. *Journal of Photochemistry and Photobiology* **152**, 215-226 (2015).
10. C. A. Wraight, A. R. Crofts, Energy-Dependent Quenching of Chlorophyll a Fluorescence in Isolated Chloroplasts. *Eur. J. Biochem* **17**, 319-327 (1970).
11. M. D. Brooks, S. Jansson, K. K. Niyogi, in *Non-photochemical quenching and energy dissipation in plants algae and cyanobacteria*. (Springer, 2014), vol. 40, pp. 297-314.
12. X.-P. Li *et al.*, A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* **403**, 391-395 (2000).
13. B. Demmig-Adams, Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochimica et Biophysica Acta* **1020**, 1-24 (1990).
14. H. Y. Yamamoto, T. O. M. Nakayama, C. O. Chichester, Studies on the Light and Dark Interconversion of Leaf Xanthophylls. *Archives of Biochemistry and Biophysics* **97**, 168-173 (1962).
15. S. Hubbart, O. O. Ajigboye, P. Horton, E. H. Murchie, The photoprotective protein PsbS exerts control over CO_2 assimilation rate in fluctuating light in rice. *The Plant Journal* **71**, 402-412 (2012).
16. X.-P. Li, A. M. Gilmore, K. K. Niyogi, Molecular and global time-resolved analysis of a psbS gene dosage effect on pH and xanthophyll cycle-dependent nonphotochemical quenching in photosystem II. *The Journal of Biological Chemistry* **277**, 33590-33597 (2002).
17. A. Zia, M. P. Johnson, A. V. Ruban, Acclimation- and mutation-induced enhancement of PsbS levels affects the kinetics of non-photochemical quenching in Arabidopsis thaliana. *Planta* **233**, 1253-1264 (2011).
18. X.-P. Li, M.-M. P., A. M. Gilmore, K. K. Niyogi, PsbS-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. *Proc Natl Acad Sci U S A* **99**, 15222-15227 (2002).
19. B. Demmig-Adams, W. W. Adams III, Xanthophyll cycle and light stress in nature: uniform response to excess direct sunlight among higher plant species. *Planta* **198**, 460-470 (1996).
20. M. Nilkens *et al.*, Identification of a slowly inducible zeaxanthin-dependent component of non-photochemical quenching of chlorophyll fluorescence generated under steady-state conditions in *Arabidopsis. Biochimica et Biophysica Acta* **1797**, 466-475 (2010).
21. M. P. Johnson, P. A. Davison, A. V. Ruban, P. Horton, The xanthophyll cycle pool size controls the kinetics of non-photochemical quenching in Arabidopsis thaliana. *FEBS Lett* **582**, 262-266 (2008).
22. A. V. Ruban, Non-photochemical chlorophyll fluorescence quenching: mechanism and effectiveness in protection against photodamage. *Plant Physiol*, (2016).
23. P. Horton, A. V. Ruban, R. G. Walters, Regulation of light harvesting in green plants. *Annu Rev Plant Physiol. Plant Mol. Biol.* **47**, 655-684 (1996).
24. S. P. Long, X.-G. Zhu, S. L. Naidu, D. R. Ort, Can improvement in photosynthesis increase crop yields? *Plant Cell and Environment* **29**, 315-330 (2006).

25. S.-H. Chang, R. C. Bugos, W.-H. Sun, H. Y. Yamamoto, Antisense suppression of violaxanthin de-epoxidase in tobacco does not affect plant performance in controlled growht conditions. *Photosynthesis Research* **64**, 95-103 (2000).
26. W.-H. Sun, A. S. Verhoeven, R. C. Bugos, H. Y. Yamamoto, Suppression of zeaxanthin formation does not reduce photosynthesis and growth of transgenic tobacco under field conditions. *Photosynthesis Research* **67**, 41-50 (2001).
27. K. K. Niyogi, A. R. Grossman, O. Björkman, Arabidopsis Mutants Define a Central Role for the Xanthophyll Cycle in the Regulation of Photosynthetic Energy Conversion. *The Plant Cell* **10**, 1121-1134 (1998).
28. M. Havaux, L. Dall'Osto, R. Bassi, Zeaxanthin Has Enhanced Antioxidant Capacity with Respect to All Other Xanthophylls in Arabidopsis Leaves and Functions Independent of Binding to PSII Antennae. *Plant Physiol* **145**, 1506-1520 (2007).
29. N. Wang *et al.*, Overexpression of zeaxanthin epoxidase gene enhances the sensitivity of tomato PSII photoinhibition to high light and chilling stress. *Physiologia Plantarum* **132**, 384-396 (2008).
30. D. R. Ort, S. P. Long, Limits on Yields in the Corn Belt. . *Science* **344**, 483-484 (2014).
31. J. Kromdijk, S. P. Long, One crop breeding cycle from starvation? How engineering crop photosynthesis for rising CO_2 and temperature could be one important route to alleviation. *Proc R. Soc. B.* **283**, 1-8 (2016).
32. D. Tilman, M. Clark, Food, Agriculture & the Environment: Can We Feed the World & Save the Earth? . *Daedalus* **144**, 8-23 (2015).
33. T. E. Clemente, in *Agrobacterium Protocols,* K. Wang, Ed. (Humana Press Inc, Totowa, 2006), pp. 143-154.
34. L. Leonelli, E. Erickson, D. Lyska, K. K. Niyogi, Transient expression in Nicotiana benthamiana for rapid functional analysis of genes involved in non-photochemical quenching and carotenoid biosynthesis. *The Plant Journal*, (2016).
35. A. C. Thompson, G. F. Nicollier, D. F. Pope, Indole alkylamines of *Desmanthus illinoensis* and their growth inhibition activity. *Journal of Agricultural and Food Chemistry* **35**, 361-365 (1987).
36. K. Głowacka *et al.*, An evaluation of new and established methods to determine T-DNA copy number and homozygosity in transgenic plants. *Plant Cell and Environment* **39**, 908-917 (2016).
37. W. Bilger, O. Björkman, Relationships among violaxanthin deepoxidation, thylakoid membrane conformation, and nonphotochemical chlorophyll fluorescence quenching in leaves of cotton (*Gossypium hirsutum* L.). *Planta* **193**, 238-246 (1994).
38. B. Genty, J.-M. Briantais, N. R. Baker, The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**, 87-92 (1989).
39. K. Oxborough, N. R. Baker, Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - calculation of *qP* and *Fv'/Fm'* without measuring *Fo'*. *Photosynthesis Research* **54**, 135-142 (1997).
40. A. V. Ruban, E. H. Murchie, Assessing the photoprotective effectiveness of non-photochemical fluorescence quencing: A new approach. *Biochimica et Biophysica Acta* **1817**, 977-982 (2012).
41. S. D. Loriaux *et al.*, Closing in on maximum yield of chlorophyll fluorescence using a single multiphase flash of sub-saturating intensity. *Plant Cell and Environment* **36**, (2013).
42. T. D. Sharkey, C. J. Bernacchi, G. D. Farquhar, E. L. Singsaas, Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant Cell and Environment* **30**, 1035-1040 (2007).
43. G. D. Farquhar, S. Von Caemmerer, J. A. Berry, A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. *Planta* **149**, 78-90 (1980).
44. H. Y. Nakatani, J. Barber, An improved method for isolating chloroplasts retaining their outer membranes. *Biochimica et Biophysica Acta* **461**, 500-512 (1977).
45. R. J. Porra, W. A. Thompson, P. E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* **975**, 384-394 (1989).
46. H. Schagger, W. A. Cramer, G. Von Jagow, Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**, 220-230 (1994).
47. L. Van Heukelem, C. S. Thomas, Computer-assisted high-performance liquid chromatography method development with application to the isolation and analysis of phytoplankton pigments. *Journal of Chromatography A* **910**, 31-49 (2001).

48. P. Müller-Moulé, P. L. Conklin, K. K. Niyogi, Ascorbate Deficiency Can Limit Violaxanthin De-Epoxidase Activity in Vivo. *Plant Physiol* **128**, 970-977 (2002).
49. H. Poorter, J. Bühler, D. Van Dusschoten, J. Climent, J. A. Postma, Pot size matters: a meta-analysis of the effects of rooting volume on plant growth. *Functional Plant Biology* **39**, 839-850 (2012).

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Competing financial interests: The University of Illinois has submitted a provisional patent on behalf of J.K., K.G., L.L., K.K.N. and S.P.L on aspects of the findings.

Figure/table captions: 743 words

Fig. 1. **Interaction between photoprotection and CO_2 fixation during sun-shade transitions.** When leaves are exposed to high light, the rate of CO_2 fixation is high and excessive excitation energy is harmlessly dissipated through non-photochemical quenching (NPQ). The level of NPQ is positively correlated with the abundance of Photosystem II subunit S (PsbS) and further stimulated by the de-epoxidation of violaxanthin to zeaxanthin, catalyzed by violaxanthin de-epoxidase (VDE). Upon transition to low light, CO_2 fixation becomes limited by NADPH and ATP derived from photosynthetic electron transport, which in turn is limited by high levels of NPQ. The rate of CO_2 fixation therefore remains depressed until relaxation of NPQ is complete. This can take minutes to hours and is correlated with the rate of zeaxanthin epoxidation, catalyzed by zeaxanthin epoxidase (ZEP). The text underneath the figure describes the strategy employed to accelerate NPQ relaxation compared to wild-type (WT) tobacco.

Fig. 2. **Levels of mRNA and protein of VDE, PsbS and ZEP.**
Native (*Nt*) and transgenic (*At*) violaxanthin de-epoxidase (*VDE*), photosystem II subunit S (*PsbS*) and zeaxanthin epoxidase (*ZEP*) in leaves of wild-type *N. tabacum* (WT) and three lines expressing *AtVDE*, *AtPsbS* and *AtZEP* (VPZ) grown under greenhouse conditions. **(A, C, E)** mRNA levels relative to actin and tubulin. **(B, D, F)** Protein levels relative to WT, determined from densitometry on immunoblots. Error bars indicate SEM (n=5), and asterisk indicates significant differences between VPZ lines and WT ($\alpha = 0.05$). **(G)** Representative immunoblots for VDE, PsbS and ZEP.

Fig. 3. Transient adjustment of NPQ and net CO_2 assimilation
(A) Dark relaxation of NPQ after exposure to alternating high/low light in young seedlings of wild-type *N. tabacum* (WT) and three lines expressing *AtVDE*, *AtPsbS* and *AtZEP* (VPZ). SEM were less than symbol size (n=18). Lines depict best fits of a double exponential model for WT (τ_1 = 21.4 ± 1.2 s and τ_2 = 2641.1 ± 821.2 s), VPZ-23 (τ_1 = 13.3 ± 1.3 s and τ_2 = 792.6 ± 131.7 s), VPZ-34 (τ_1 =19.4 ± 1.4 s and τ_2 = 692.6 ± 77.9 s) and VPZ-56 (τ_1 =13.2 ± 1.0 s and τ_2 = 774.9 ± 94.5 s). **(B)** Time course of net CO_2 fixation rate in fully expanded leaves in response to a decrease in light intensity of 2000 to 200 μmol photons m^{-2} s^{-1} at time zero, indicated by the black arrow. Error bars indicate SEM (n=5). Asterisk indicates significant difference (α = 0.05).

Fig. 4. Photosynthetic efficiency and NPQ under steady-state and fluctuating light. **(A)** Quantum efficiency of leaf net CO_2 assimilation (ΦCO_{2max}) under steady-state light. **(B)** ΦCO_{2max} under fluctuating light. **(C)** Quantum efficiency of linear electron transport ($\Phi PSII_{max}$) under steady-state light. **(D)** Quantum efficiency of linear electron transport ($\Phi PSII_{max}$) under fluctuating light. **(E)** Average NPQ corresponding to (A) and (C). **(F)** Average NPQ corresponding to (B) and (D). Data were derived from light response curves in which light intensity was either increased from low to high PFD, while waiting for steady state at each step (steady-state), or varied from high to low PFD with 4 min of 2000 µmol photons m^{-2} s^{-1} before each light intensity change (fluctuating). Error bars indicate SEM (n=6), and asterisks indicate significant differences (α=0.05) between wild-type *N. tabacum* (WT) and three lines expressing *AtVDE, AtPsbS* and *AtZEP* (VPZ).

Fig. 5. Productivity of field-grown plants *N. tabacum* plants.
Lines expressing *AtVDE, AtPsbS* and *AtZEP* (VPZ) produced 15% larger plants than did the wild-type line (WT). **(A)** Total dry-weight. **(B)** Leaf area. **(C)** Plant height. Data were normalized to WT. Error bars indicate SEM (n=12), asterisk indicates significant differences between VPZ lines and WT (α=0.05). **(D)** Top-view of the field experiment in Urbana, Illinois (40.11 ºN, 88.21 ºW, photo credit: D. Drag) in the summer of 2016.

Table 1. Xanthophyll cycle pigment concentrations and de-epoxidation state (DES). Samples were taken from greenhouse-grown fully expanded leaves of wild-type *N. tabacum* (WT) and three lines overexpressing *AtVDE, AtPsbS* and *AtZEP* (VPZ) in dark-acclimated state or after exposure to constant 400 or 2000 µmol photons m^{-2} s^{-1} (when steady state photosynthesis was reached) or 3 cycles of 3 min 2000 / 3 min 200 µmol photons m^{-2} s^{-1}. Pigment concentrations (mean ± SEM, n = between 3 to 6) were normalized per unit leaf area (g m^{-2}). Asterisks indicate significant differences between VPZ lines and WT ($\alpha = 0.05$). Vio = violaxanthin, Ant = antheraxanthin, Zea = Zeaxanthin. DES (%) = (Zea + 0.5Ant)/(Zea + Ant + Vio), n.d. = not detected.

Light treatment	Pigment	WT	VPZ-23	VPZ-34	VPZ-56
Dark-acclimated	Vio	7.72 ± 0.37	6.64 ± 0.45	6.94 ± 0.64	6.70 ± 0.40
	Ant	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
	Zea	n.d.	n.d.	n.d.	n.d.
	DES	0.0	0.0	0.0	0.0
Constant at 400 µmol photons m^{-2} s^{-1}	Vio	6.68 ± 0.62	7.29 ± 0.47	7.05 ± 0.48	7.07 ± 0.31
	Ant	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
	Zea	0.20 ± 0.10	0.00 ± 0.00	0.05 ± 0.05	0.00 ± 0.00
	DES	2.9 ± 1.4	0.1 ± 0.0	0.7 ± 0.6	0.1 ± 0.0
Constant at 2000 µmol photons m^{-2} s^{-1}	Vio	4.47 ± 0.41	5.09 ± 0.52	3.63 ± 0.59	5.02 ± 0.09
	Ant	0.07 ± 0.00	0.08 ± 0.01	0.06 ± 0.00	0.09 ± 0.01
	Zea	3.81 ± 3.81	*1.48 ± 0.48	*1.23 ± 0.24	*1.94 ± 0.49
	DES	46.2 ± 2.8	*22.9 ± 7.5	*26.2 ± 5.3	*27.4 ± 5.1
Fluctuating between 2000 and 200 µmol photons m^{-2} s^{-1}	Vio	4.20 ± 0.16	*7.11 ± 0.57	*5.72 ± 0.15	*6.14 ± 0.34
	Ant	0.16 ± 0.02	*0.08 ± 0.01	0.13 ± 0.03	*0.08 ± 0.01
	Zea	4.70 ± 0.36	*0.88 ± 0.08	*2.29 ± 0.85	*1.20 ± 0.21
	DES	52.5 ± 5.5	*11.4 ± 0.9	*25.5 ± 17.3	*16.4 ± 4.2

Supplementary Materials:

Materials and Methods

Figures S1-S14

Tables S1-S3

Datasets 1-21

References (*33-49*)