S2-028

Suppression of the light sensitivity of a zeaxanthin- and lutein-deficient mutant of *Chlamydomonas reinhardtii*: Zeaxanthin is sufficient for protection from high light stress

I Baroli, AD Do, KK Niyogi

Department of Plant and Microbial Biology, University of California, 111 Koshland Hall, Berkeley, CA 94720-3102, USA. ibaroli@nature.berkeley.edu

Keywords: xanthophylls, light stress, photoprotection, Chlamydomonas

Introduction

Xanthophylls are oxygenated carotenoids that are integral components of the photosynthetic apparatus. They participate in light harvesting, energy dissipation under excessive light and quenching of excited intermediates, such as singlet and triplet chlorophylls and singlet oxygen (Frank and Cogdell, 1996).

To understand the contribution of individual xanthophylls in protection from light stress and to identify other components in photoprotection, we are studying several mutant strains of *Chlamydomonas reinhardtii* that are unable to synthesize specific xanthophylls. The *npq1* mutant of *Chlamydomonas* was isolated in a screen for mutants that are defective in quenching the singlet excited state of chlorophyll when exposed to high light (HL). This defect in nonphotochemical quenching (NPQ) is due to the lack of de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin in the mutant, but it does not prevent survival in HL (Niyogi et al., 1997a). The *lor1* mutation affects the accumulation of lutein and its derivative loroxanthin in *Chlamydomonas*. Even though the *lor1* strain has a normal xanthophyll cycle, it is partially defective in NPQ, but can survive in HL. The double mutant *npq1 lor1*, which accumulates violaxanthin and neoxanthin as the only oxygenated carotenoids irrespective of light conditions, shows a severe lack of NPQ and light-mediated xanthophyll interconversion and is unable to grow in HL conditions (Niyogi et al., 1997b), where it rapidly bleaches. This bleaching phenotype is not solely due to the lack of NPQ in *npq1 lor1*, because a similar NPQ-defective mutant that has normal xanthophyll content is not particularly prone to chlorophyll bleaching in HL (Niyogi et al., 1997b). Thus, in *Chlamydomonas*, both lutein and zeaxanthin contribute to NPQ-mediated photoprotection, but they may also play a role in the inactivation of other intermediate excited states produced during photosynthesis.

In this report we describe the isolation and characterization of stable genetic suppressors of the HL-sensitivity phenotype of the *npq1 lor1* strain. Two classes of suppressors are described, those with an abnormal xanthophyll composition and those with an abnormal response of the light-harvesting complex to growth in HL.

Materials and methods

The *npq2* mutant and the *npq1 lor1* double mutant have been described earlier (Niyogi et al., 1997a; Niyogi et al., 1997b). The wild-type strain CC125 was obtained from the *Chlamydomonas* Genetics Center (Duke University). For physiological studies, cells were grown photoautotrophically on a shaker in 100 mL minimal (HS) medium at 25 ^{o}C. Continuous

illumination was provided from above by cool-white fluorescent lights, at a photon flux density of 50 µmol photons m^{-2} s^{-1} (low light, LL) or 500 µmol photons m^{-2} s^{-1} (high light, HL). For HPLC determination of pigment content and for suppressor screening, cells were exposed to continuous high or low light on minimal medium agar plates. Photosynthetic pigments were analyzed by HPLC after extraction in acetone (García-Plazaola and Becerril, 1999). For the isolation of suppressor mutations, the double mutant *npq1 lor1* was grown in LL to the exponential phase. A volume containing 10^6 cells was plated on agar in minimal medium and placed in HL. Suppressor colonies were detected 7 d after plating, from a background of bleached cells. The frequency of suppression was approximately 10^{-4}. Oxygen evolution of exponentially growing intact cells was measured with a polarographic, Clark-type oxygen electrode (Hansatech DW1), in the presence of 4 mM $NaHCO_3$, with a saturating illumination of 600 µmol photons m^{-2} s^{-1}. The rate of photosynthetic oxygen evolution was calculated on a per-cell basis. The extent of lipid peroxidation was determined from 10-mL culture aliquots as described (Vavilin et al., 1998). Western blots were performed as described (Baroli and Melis, 1996), with the anti-LHC1/CP1 antibody kindly provided by R. Bassi (Univ. of Verona, Italy).

Results

The zeaxanthin- and lutein-deficient *npq1 lor1* double mutant of *Chlamydomonas* undergoes extensive bleaching if transferred from normal growing conditions (LL) to an irradiance of 500 µmol photons m^{-2} s^{-1} (HL), which is not excessive for the wild type (Niyogi et al., 1997b). Fig. 1 shows the growth phenotypes of the wild type and *npq1 lor1* under these two light conditions. The double mutant showed no growth at HL, whereas the wild type grew normally. The HL sensitivity of *npq1 lor1* was partially rescued by growth under low oxygen tension, indicating that oxygen is involved in cell death in HL in the double mutant (data not shown).

Fig. 1. Growth of *Chlamydomonas* strains under continuous LL or HL photoautotrophic conditions. The wild type, the HL-sensitive double mutant *npq1 lor1* and three zeaxanthin-accumulating suppressor strains are shown**.**

Fig. 2 shows the time course of chlorophyll bleaching in the double mutant after transfer from LL to HL conditions. While the wild type was unaffected by the treatment, except for a slight decrease in chlorophyll content and an increase in photosynthetic activity, the *npq1 lor1* strain gradually lost its chloroplast pigment content and the thylakoid membranes became disorganized and disappeared (not shown). The bleaching was

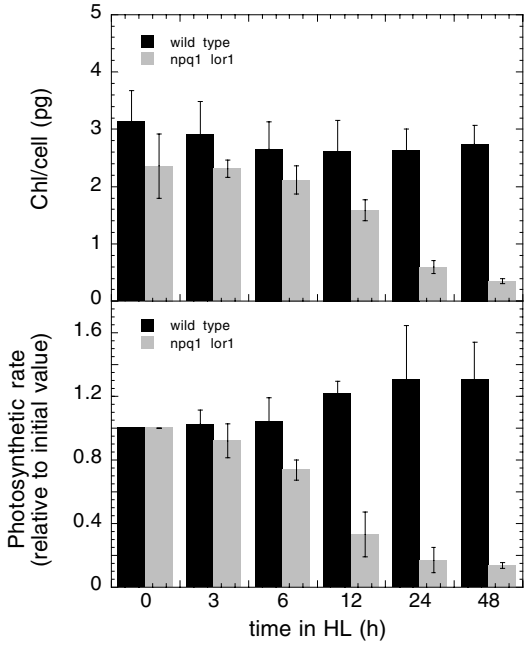

Fig. 2. Effect of a shift from LL to HL growth conditions on total chlorophyll content and photosynthesis in wild type and the *npq1 lor1* double mutant. n = 3 different cultures; error bars = SD.

associated with loss of photosynthetic function, measured as evolution of oxygen (bottom panel, Fig 2), and with the accumulation of lipid peroxides, an indication of oxidative stress, in the cell membranes (see Fig. 4).

To understand the contribution of the different xanthophylls to the process of photoprotection, we isolated genetic suppressors of the HL sensitivity of the *npq1 lor1* mutant. When the double mutant was exposed to HL, stable suppressors arose spontaneously as green colored colonies in a bleached background, with a frequency of approximately 10^{-4}. Of a population of approximately 200 suppressors, 40 % exhibited a change in the pigment composition relative to the parental strain, and within this class the most abundant suppressors showed accumulation of zeaxanthin in both LL and HL. Three such suppressor strains, which showed varying degrees of constitutive accumulation of zeaxanthin, were selected for further analysis. The *npq1 lor1 npq2-2* suppressor strain showed negligible epoxidation of zeaxanthin in both light conditions, whereas *npq1 lor1 npq2-3* and *npq1 lor1 npq2-4* showed intermediate epoxidation levels (Fig. 3), with slight changes depending on light intensity. All three strains grew normally in both LL and HL in photoautotrophic conditions (Fig. 1).

The bleaching of *npq1 lor1* after transfer to HL conditions was accompanied by an accumulation of lipid peroxides. Suppression of the HL sensitivity phenotype slowed the formation of lipid peroxides in all three zeaxanthin-accumulating suppressors, as shown in Fig. 4. The extent of lipid peroxidation during the treatment appears to be inversely proportional to the relative amount of xanthophyll accumulated as zeaxanthin in the suppressors.

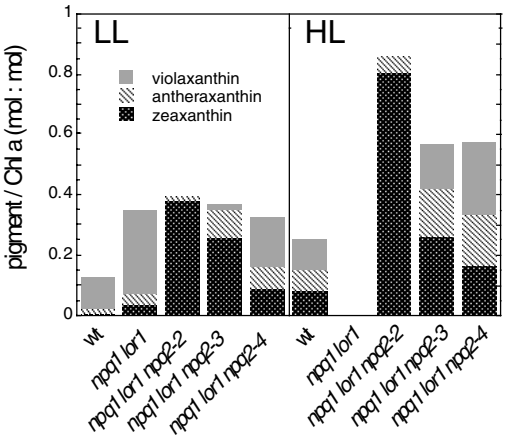

Fig. 3. Xanthophyll pigment content of wild type, *npq1 lor1* and the three selected *npq1 lor1 npq2* suppressor strains under continuous LL or HL conditions. Cells were grown on agar plates under either continuous LL or HL. n = 3-5; SD was <10% of the means.

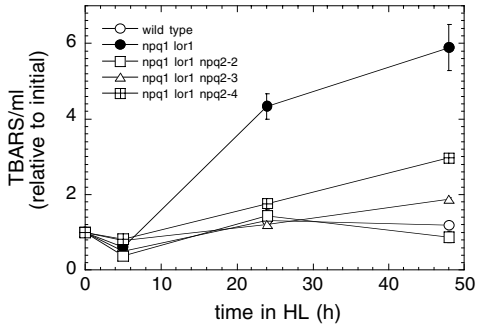

Fig. 4 . Effect of a shift from LL to HL growth conditions on the formation of lipid peroxides in the wild type, the *npq1 lor1* mutant and the zeaxanthin-accumulating suppressors. Lipid peroxides were measured as thiobarbituric acid-reactive substances (TBARS). n= 3 different cultures; error bars = SD.

Genetic analysis showed that, in addition to the original *npq1* and *lor1* mutations, the suppressors carried a single mutation in the *npq2* locus described previously in *Chlamydomonas* (Niyogi et al., 1997a). The suppressor strain *npq1 lor1 npq2-2* was crossed with the reference *npq2-1* mutant strain, and no recombinants with wild-type zeaxanthin epoxidation were observed among the progeny, demonstrating allelism to *npq2*. Stable diploid strains were constructed from

crosses of *npq1 lor1 npq2-2* and *npq1 lor1 npq2-3* or *npq1 lor1nq2-3*. The pigment content of these diploids, as analyzed by HPLC, revealed no complementation of the zeaxanthin epoxidation defect. To investigate whether the suppression was caused by a single gene, the three suppressor strains were backcrossed with the parental *npq1 lor1* strain. In all three cases, vegetative diploids obtained from these crosses showed that the suppressors were recessive in terms of the HL-sensitivity phenotype. The phenotypic analysis of the meiotic progeny, with regard to light sensitivity and pigment composition, is shown in Table 1. The suppressor mutation segregated as a single Mendelian gene in all three strains. From this genetic analysis we conclude that the three suppressor strains carry different alleles of the *npq2* gene, conferring different degrees of zeaxanthin epoxidation, and that the *npq2* mutations are responsible for the suppression of the HL-sensitivity phenotype.

Table 1. Genetic analysis of three suppressor strains that showed a constitutive accumulation of zeaxanthin. In all cases the strain *npq1 lor1 NPQ2* was used as the (+) mating type and the suppressed strains as mating type (-).

cross	number of progeny analyzed	ratio of HL resistant/ HL sensitive	ratio of parental/mutant pigment composition	recombinants
npq1 lor1 NPQ2 x *npq1 lor1 npq2-2*	41	21 : 20	21 : 20	0
npq1 lor1 NPQ2 x *npq1 lor1 npq2-3*	18	10 : 8	10 : 8	0
npq1 lor1 NPQ2 x *npq1 lor1 npq2-4*	37	18 : 19	18 : 19	0

Another kind of suppressor strain recovered in our screen presented no changes in the parental pigment content but had an abnormal light-harvesting complex (LHC) when grown under HL conditions. Fig. 5 shows a Western blot after a denaturing SDS-PAGE, with a polyclonal antibody that recognizes most of *Chlamydomonas* light-harvesting complex proteins (Bassi and Wollman, 1991). The cells had been grown in liquid media for several generations under continuous LL or HL conditions. The wild type showed the same LHC composition irrespective of the growth light intensity. However, the suppressor strain, temporarily named 10-22, shows a much decreased content of the LHCII b proteins under continuous HL. The LHC composition of the double mutant *npq1 lor1* in LL was similar to that of the wild type at this level of resolution (not shown). Genetic analysis of the strain 10-22 suppressor phenotype showed it to be caused by a single nuclear mutation. Further characterization of this suppressor strain is underway.

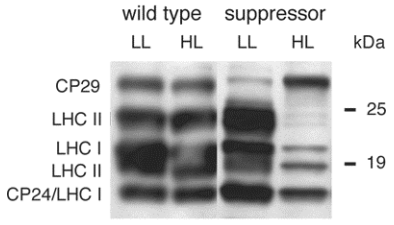

Fig. 5. Western blot analysis of the light-harvesting complex of wild-type *Chlamydomonas* and a suppressor of the HL sensitivity of the *npq1 lor1* double mutant. Cells were grown under continuous LL or HL conditions in minimal medium. The same number of intact cells was loaded in each lane.

Conclusions

The genetic dissection of the carotenoid biosynthetic pathway in green organisms is allowing us to assign specific roles for each xanthophyll in the chloroplast. Lutein and zeaxanthin have both been shown to participate in the quenching of the singlet excited state of chlorophyll

(Niyogi et al., 1997b). Lack of these two pigments leads to the accumulation of lipid peroxides in cell membranes, disappearance of thylakoids, loss of chlorophyll and cell death. All these are symptoms of oxidative stress. Other plant and algal mutants that have normal xanthophyll content but are incapable of performing NPQ do not show this type of bleaching. This suggests that the protection afforded by lutein and zeaxanthin against photo-oxidative stress does not come about only by NPQ, but that these xanthophylls may also be necessary for the efficient quenching of singlet oxygen, a major initiator of oxidative stress in the chloroplast. We isolated several suppressors of the HL-sensitivity phenotype of a lutein- and zeaxanthin-deficient *Chlamydomonas* strain. Our data show that zeaxanthin as the only xanthophyll is sufficient for preventing photo-oxidative stress in *Chlamydomonas*. Intermediate epoxidation states of the xanthophyll pool also lead to protection from bleaching in HL. Another, different form of photoprotection is shown by a suppressor strain that retained the lutein and zeaxanthin deficiency but was able to reduce the size of its light-harvesting antenna when grown at HL, perhaps thereby diminishing the excitation pressure on photosystem II.

References

Baroli I, Melis A (1996) *Planta* **198**: 640-646.
Bassi R, Wollman F-A (1991) *Planta* **183**: 423-433
Frank HA, Cogdell RJ (1996). *Photochem. Photobiol*. **63**: 257-264.
García-Plazaola JI, Becerril JM (1999) *Phytochem. Anal*. **10**: 307-313
Niyogi KK, Björkman O, Grossman AR (1997a) *Plant Cell* **9**: 1369-1380
Niyogi KK, Björkman O, Grossman AR (1997b) *Proc. Nat. Acad. Sci*. USA **94**: 14162-14167.
Vavilin DV, Ducruet JM, Matorin DN, Venediktov PS, Rubin AB (1998) *J. Photochem. Photobiol*. B Biol. **42**: 233-239

Acknowledgements

This research was supported by a grant from the National Institutes of Health (GM5879901) to K. K. N.