Lhcx proteins provide photoprotection via thermal dissipation of absorbed light in the diatom *Phaeodactylum tricornutum*

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Diatoms possess an impressive capacity for rapidly inducible thermal dissipation of excess absorbed energy (qE), provided by the xanthophyll diatoxanthin and Lhcx proteins. By knocking out the *Lhcx1* and *Lhcx2* genes individually in *Phaeodactylum tricornutum* strain 4 and complementing the knockout lines with different Lhcx proteins, multiple mutants with varying qE capacities are obtained, ranging from zero to high values. We demonstrate that qE is entirely dependent on the concerted action of diatoxanthin and Lhcx proteins, with *Lhcx1*, *Lhcx2* and *Lhcx3* having similar functions. Moreover, we establish a clear link between *Lhcx1/2/3* mediated inducible thermal energy dissipation and a reduction in the functional absorption cross-section of photosystem II. This regulation of the functional absorption cross-section can be tuned by altered Lhcx protein expression in response to environmental conditions. Our results provide a holistic understanding of the rapidly inducible thermal energy dissipation process and its mechanistic implications in diatoms.
Upon absorption of a photon, the singlet excited state of a chlorophyll molecule has three major fates: photochemistry, heat dissipation, and fluorescence emission. The optimization of photochemistry is achieved when the rate of photon absorption is equal to the rate of electron transfer. Although this requires a large absorption cross-section of photosystem II under low light (LL) conditions, the same absorption cross-section would lead to an overexpansion of ΦE capacity, which indicates the involvement of various Lhcx proteins in this knockout background. In addition, we knocked out the Lhcx2 gene and express the Lhcx2 and Lhcx3 protein in this knockout background. This experimental design allows us to quantify the influence of each Lhcx protein on ΦE and ΦPSII.

**Results**

**The effect of different Lhcx proteins on ΦE.** By using the TALEN method, we independently targeted two different sites of the Lhcx1 gene in *P. tricornutum* strain Pt4 (UTEX 646). Three Lhcx1 knockout (x1KO) lines were obtained (cf. Supplementary Fig. I, Supplementary Fig. 2 for genetic characterization). Three Lhcx1 knockout (x1KO) lines were obtained (cf. Supplementary Fig. I, Supplementary Fig. 2 for genetic characterization). First, we predicted the Lhcx1 knockout in *P. tricornutum* x1KO lines, which lacked the Lhcx1 protein under LL growth conditions (Fig. 1a). Two of these are based on TALEN pair 1 (x1KO_1a/1b), and the third on TALEN pair 2 (x1KO_2). Although the wild-type cells activated ΦE rapidly during 3 mins of supra-optimal light exposure, the three x1KO clones lacked this ΦE induction (Fig. 1b). Moreover, the very low ΦPSII values obtained after 3 min of supra-optimal light exposure in the x1KO lines even increased during the following dark phase, indicating it is not of ΦE origin. To prove that the observed phenotype is indeed related to the knockout of Lhcx1, we complemented the independent x1KO_1a and x1KO_2 lines with an Lhcx1 gene that was modified at the TALEN-binding sites by synonymous codon usage, in order to prevent a re-cutting by the TALEN system. To express the gene, we used either the native Lhcx1 promoter/terminator or the FcpA (Lhcf1) promoter/terminator. Out of several lines created (Supplementary Fig. 1a and b), some were further characterized regarding their NPQ characteristics. These lines showed a rescue of the ΦE phenotype to different extents (Fig. 1b).

For closer examination, we used the x1KO_1a strain, as it showed no statistically significant difference in growth compared with the wild-type (Fig. 1c), which is expected for cells cultivated under LL conditions where ΦE is not induced. We transformed this strain with each of the three other Lhcx genes to generate x1KO + x2/x3/x4-supplemented strains. To ensure similar regulation and expression of the other Lhcx genes as the original Lhcx1, we used the Lhcx1 promoter and terminator in all transformed strains. Our aim was to see the possible effects of each Lhcx protein on triggering ΦE. Normally, these effects are hidden or dampened by the presence of Lhcx1, which is the most expressed Lhcx protein under LL cultivation (Fig. 1a). From the obtained clones, we chose two for the Lhcx2 and Lhcx3, respectively, and three for the Lhcx4 gene, all of which showed strong expression of the respective gene (Supplementary Fig. 3) as well as protein under LL growth conditions (Fig. 2). We then investigated the ΦE pattern upon exposure to 10 min of supraoptimal actinic light and subsequent recovery under LL conditions known to relax ΦE in *P. tricornutum* better than darkness. Although the x1KO line showed only a slight linear increase in NPQ, which did not relax under LL conditions and may rather be related to photoinhibition processes (qI), the x1KO + x2 and x1KO + x3-supplemented lines recovered ΦE capacity (Fig. 3a; Supplementary Fig. 4). The extent of ΦE varied in the two chosen strains supplemented with the same gene (i.e., x2a vs. x2b, and x3a vs. x3b; Fig. 3a; Supplementary Fig. 4), most likely caused by differential expression of the respective genes.
owing to positional effects on the inserted vector and the amount of inserted copies. Interestingly, Lhcx4-supplemented lines were unable to restore qE capacity (Fig. 3a; Supplementary Fig. 4). Diatoms show a strong correlation between qE and the concentration of Dt, which is formed via de-epoxidation of Dd. However, Dd de-epoxidation was similar in all our strains regardless of qE capacity (Fig. 3b; Supplementary Fig. 5). When the Dd to Dt conversion was inhibited by dithiothreitol (DTT) in the qE containing strains (wild-type, x1KO + x1a, x1KO + x1b), the qE capacity was lost (Fig. 3; Supplementary Fig. 4). Instead, those strains exhibited NPQ characteristics similar to the x1KO mutants without DTT treatment. Hence, Dt can only confer qE in the presence of Lhcx1/2/3 proteins, and vice versa, and the slower NPQ phase, observed under prolonged high light intensities, is independent of both compounds.

Under LL conditions, Lhcx1 is the highest expressed Lhcx gene, followed by Lhcx2, whereas Lhcx3 and Lhcx4 are hardly expressed. We created an Lhcx2-KO line (x2KO, verified by allele specific PCR and sequencing, qPCR and western blot, Supplementary Fig. 6, 7, and 8), which did not exhibit a reduction in qE capacity under LL cultivation (Inset Supplementary Fig. 9). However, Lhcx2 had been proposed to provide additional qE capacity under prolonged exposure to supra-optimal light. Indeed, the x2KO strain, which lacks the high light induction of Lhcx2 (Supplementary Fig. 7 and 8), showed a lower increase of qE capacity compared with the wild-type when exposed to

Fig. 1 qE capacity in P. tricornutum Lhcx1 mutants. a Western blot of low light grown strains, from left to right: wild type, three x1KO lines (x1KO_1a,1b and 2), two complemented lines of the x1KO_1a with the Lhcx1 gene cloned between the Lhcx1 promoter and terminator (x1KO + x1a,b) and between the FcpA promoter (x1KO + Fcp_x1a,b), respectively, and one complemented line of the x1KO_2 line (x1KO_2 + x1). After blotting, the blot was cut and the upper part was incubated with a Rubisco antibody, whereas the lower part was incubated with the Lhcx antibody. Protein mass marker bands are indicated on the left. b NPQ development during 3 min of actinic high light (white bar) followed by 6 min of recovery in darkness (black bar). Representative traces are depicted. c Mean doubling time in the exponential growth phase in the wild type and three x1KO lines during low light growth. SE is given. Statistically significant differences between the x1KO lines and the wild-type are indicated by a * (unpaired t test, eight degrees of freedom, p < 0.05, n = 5 biological replicates). Source data are provided as a Source Data file.
The acceptor QA, from which electron transport rates and the transfer of one electron from the PSII reaction center to exposed to a short background, termed x2KO. For these strains we measured variable capacity as the wild-type and the x2KO line under LL cultivation. The relationship between qE and OPsII. The different Lhcx-mutated lines generated offered us a unique opportunity to investigate the extent to which Lhcx-mediated qE can influence OPsII in diatoms. OPsII, the functional absorption cross-section of PSII, represents the probability of an absorbed photon of a given wavelength to drive a successful charge separation. To examine this phenomenon, we selected those strains that exhibited varying degrees of qE when grown under LL conditions. For reference, we ordered these lines from high to no qE capacity: x2KO + x2 > wild-type = x2KO > x1KO + x3a > x1KO + x2a > x1KO = x1KO + x4a. In addition, we included another strain, in which Lhcx3 has a similar qE capacity as the wild-type and the x2KO line under LL cultivation. For these strains we measured variable fluorescence as a function of light intensity at 15 light steps. After each light step, cells were exposed to a short flash leading to a single turnover of PSII, hence the transfer of one electron from the PSII reaction center to the acceptor QA, from which electron transport rates and OPsII can be calculated, the latter by fitting the rise in fluorescence to a cumulative one-hit Poisson function. Electron transport rates started to saturate at ~130 µmol photons m^{-2} s^{-1} for all cultures (Supplementary Fig. 10). qE became apparent at similar light intensities in wild-type, x1KO + x2a, x1KO + x3a, x2KO + x2 and x2KO + x3. Following the initiation, qE rapidly increased until light intensities of ~350 µmol photons m^{-2} s^{-1}, after which NPQ rose slowly until reaching its maximum values of 0.6–0.7 at 800 µmol photons m^{-2} s^{-1}; in the x2KO + x2 line NPQ reached even 0.8 (Supplementary Fig. 11; Fig. 4a). In contrast, in the x1KO and x1KO + x4a strains NPQ decreased to negative values at light intensities up to 250 µmol photons m^{-2} s^{-1} and reached maximum values of ~0.2 at 800 µmol photons m^{-2} s^{-1}. In the x1KO + x2a strain, qE started at a higher light intensity in comparison to the wild-type, and maximum NPQ was in between the x1KO/x1KO + x4a and the other strains. When inhibiting Dt synthesis with DTT, the rapid phase of NPQ development in the qE-possessing strains was completely abolished, resulting in NPQ traces resembling those of the x1KO and x1KO + x4a strains without DTT (Supplementary Fig. 11). In the two latter strains, DTT application did not change NPQ characteristics at all. We do not consider the slight linear increase of NPQ at higher light intensities as qE. We observed this increase in all strains and it was independent of the presence of Lhcx proteins or of a functional Dd de-epoxidation.

OPsII values of low light grown strains, measured in the dark, were between 500 and 550 Å² PSII upon blue light (450 nm) exposure, typical for P. tricornutum. We observed a slight decline in OPsII as light intensities increased to 70–100 µmol photons m^{-2} s^{-1} (Fig. 4a; Supplementary Fig. 12). At higher light intensities up to 350–400 µmol photons m^{-2} s^{-1}, OPsII increased in the x1KO and the x1KO + x4a lines and remained at the same level in x1KO + x2a line. In contrast, OPsII decreased to a greater extent in the strains with high qE capacity (wild-type, x1KO + x3a, x2KO, x2KO + x2/x3), down to values of 400–450 Å² PSII (a reduction of 15–20%). Further increased light intensities, which induced a slight linear increase of NPQ capacity not related to qE (see above), did not further downregulate OPsII. When inhibiting Dt synthesis by addition of DTT, qE-possessing strains showed a OPsII development similar to the x1KO and the x1KO + x4a strains where OPsII was unaffected by the addition of DTT (Supplementary Fig. 12). Besides calculating the changes in OPsII based on single turnover saturating flashes, leading to one Q_{A}\rightleftharpoons Q_{B} per PSII, OPsII is also often determined by applying prolonged weak flashes in the presence of DCMU, which yields to a full reduction of Q_{A}, but has some side effects due to the application of DCMU (reviewed in ref. 10). In order to corroborate our results, we compared the effect of three minutes supra-optimal light exposure on NPQ establishment and OPsII behavior in wild-type and x1KO strains using the DCMU method and applying the calculation and correction procedure as described by Tian et al. Using this method, we also observed a significant reduction in OPsII in the wild type after supra-optimal light exposure, which was absent in the x1KO strain (Supplementary Fig. 13 and 14). Based on these results, we conclude that there is a pronounced influence of qE on changes in OPsII. Furthermore, we investigated the relationship between OPsII and NPQ after 1 day of high light growth. qE capacity increased in strains, which already possessed qE capacity under LL growth and was now present even in the x1KO and x1KO + x4a line (Fig. 4b). This is owing to the high amounts of Lhcx2 and Lhcx3 (cf. protein levels under LL and high light cultivation, Fig. 2, Supplementary Fig. 8), which can partially rescue qE capacity in the absence of Lhcx1, as already demonstrated by the analysis of the x1KO supplemented lines (cf. Fig. 3). As opposed to LL grown cells, qE initiated below 100 µmol photons m^{-2} s^{-1} and leveled off at 300–400 µmol photons m^{-2} s^{-1}, thereafter only slowly rising with higher light intensities. The highest NPQ values were obtained in the x2KO + x2/x3 and the x1KO + x3a lines, with NPQ values of 1.6–1.7 at 400 µmol photons m^{-2} s^{-1}, i.e., when the rapid phase of qE induction was complete. Simultaneously, OPsII decreased in all lines substantially, by up to 40–45% in wild-type, x1KO + x3a, x2KO, and x2KO + x2/x3 and by up to 30% in x1KO and x1KO + x4a (Fig. 4b; Supplementary Fig. 12). Strain x1KO + x2a displayed a decrease in between these two groups. As for LL grown cells, the rapid decline of OPsII up to light intensities of 250–400 µmol photons m^{-2} s^{-1} well correlated with the rapid development of qE, with the exception of the decline of OPsII under very weak light intensities in the beginning (Fig. 4b). The weaker and linear increase of NPQ under further increased light intensities did not have any effect on OPsII.

We also investigated the excitation pressure on PSII (monitored as 1-qL, a proxy for the reduction state of the plastoquinone pool) required to reduce OPsII. Strains possessing a higher qE capacity exhibited a substantial decrease of OPsII...
at much lower PSII excitation pressure. While for LL grown cultures $1-q_L$ values of 0.6–0.8 were necessary to reduce $\sigma_{PSII}$ in $q_E$ possessing strains, high light-acclimated cultures started to decrease $\sigma_{PSII}$ at $1-q_L$ values of ~0.2–0.3 (Supplementary Fig. 15). This correlates with the onset of $q_E$ at lower light intensities in high light vs. LL grown cultures (cf. Fig. 4).

NPQ is the most widespread parameter used to characterize thermal dissipation. However, owing to its derivation method, it may exaggerate the effect of thermal dissipation at higher values. To bound NPQ between 0 and 1, the parameter $Y(NPQ)$ has been proposed to better visualize the effect of thermal dissipation. By plotting NPQ versus $Y(NPQ)$ we observed an almost linear correlation up to NPQ values of ~0.6 (Supplementary Fig. 16). Above this threshold, the relative increase of NPQ vs $Y(NPQ)$ was enhanced. The maximum $Y(NPQ)$ values were obtained for high light grown cultures and reached ~0.6, i.e., an induced thermal dissipation of 60% of absorbed photons. We plotted all $\sigma_{PSII}$ values against $Y(NPQ)$ for both LL and high light grown strains together, excluding those points where $Y(NPQ)$ was absent or did not further downregulate $\sigma_{PSII}$ (see details legend Fig. 5). The latter points represent $\sigma_{PSII}$ values measured when NPQ shifted from the fast $q_E$ component to a slower component and had no

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**Fig. 3** NPQ kinetics and xanthophyll cycle activity in wild type and mutant strains. Strains were concentrated to a chlorophyll $a$ amount of 10 mg L$^{-1}$. a NPQ capacity of wild type (four biological replicates (BR)), x1KO_1a (four BR), and x1KO_1a supplemented lines x1KO + x2a (three BR), x1KO + x3a (four BR) and x1KO + x4a (three BR) during 10 min exposure to 1700 µmol photons m$^{-2}$ s$^{-1}$ (white bar), followed by 18 min of low light (gray bar). Red points indicate samples that had been incubated with DTT prior to high light exposure in order to prevent diatoxanthin formation; b pool size of diadinoxanthin + diatoxanthin (Dd + Dt) per chlorophyll $a$ and de-epoxidation state (DES) following 10 min illumination with 1700 µmol photons m$^{-2}$ s$^{-1}$ without and with prior application of DTT. Three BR were measured. Source data are provided as a Source Data file.
Fig. 4 NPQ development and changes in σPSII during increasing light intensities. Wild-type and mutant strains cultivated under low light (a) and 24 h of high light (~ 400 µmol photons m$^{-2}$ s$^{-1}$) (b) were exposed to 15 steps of increasing light intensity (1 min duration each), and NPQ (black trace) and σPSII (red trace) were recorded. Values are the mean of six (low light cultures) or five (high light cultures) biological replicates. SD is given. Dashed black line denotes zero NPQ. Source data are provided as a Source Data file
before returning to the ground state through emitting fluorescence. With the induction of qE and the resultant quenching, fluorescence lifetimes shorten. In line with the rapid onset of qE (cf. Fig. 3), we saw a pronounced decrease of fluorescence lifetimes when qE was induced by 10 min of continuous supra-optimal illumination. Fluorescence lifetime is a measure of the time span a molecule remains in the excited state before returning to the ground state through emitting fluorescence. With the induction of qE and the resultant fluorescence quenching, fluorescence lifetimes shorten. In line with the rapid onset of qE (cf. Fig. 3), we saw a pronounced decrease of fluorescence lifetimes in the LL grown qE possessing strains (wild-type, x1KO + x2a, x1KO + x3a, x2KO, x2KO + x2, x2KO + x3) during the first minute of illumination, which was absent in the x1KO and the x1KO + x4a strain (Supplementary Fig. 18). Thereafter, there was a slight linear decline in all strains. In contrast, in high light grown cells a rapid decrease of fluorescence lifetimes was visible in all strains already after 1 min of illumination. Overall, the decreases in lifetime well reflected the capacity for qE in the different strains.

**Discussion**

By knocking out the Lhcx1 gene, we obtained the *P. tricornutum* strain (x1KO) that had no capacity for qE, i.e., the rapid component of NPQ (Fig. 3; Fig. 4). Our results support previous findings by Bailleul et al. regarding the involvement of Lhcx1 in qE. We suggest that Lhcx1 provides essentially all qE capacity in *P. tricornutum* grown under LL conditions. This is different to *C. reinhardtii*, where no qE is developed under LL growth conditions, but both existing Lhcx proteins provide qE after prolonged exposure to high light. Our results also revealed that Lhcx2 and Lhcx3 provide qE capacity in *P. tricornutum*, alongside Lhcx1, but cannot specify a role for Lhcx4 in this process, at least not for high light inducible, Dt-dependent qE and independent of Lhcx1. This is in line with the strong upregulation of Lhcx4 under prolonged dark conditions, which requires future research to elucidate its function.

As a consequence of the lack of Lhcx1, the x1KO strain, as well as the x1KO + x4a strain, exhibit no decrease in qE under high light exposure. Instead, they show a slight increase in qE, as expected when PSI reaction centers become progressively closed, causing excitons to jump from one PSI unit to another, in search for an open PSI center. On the other hand, the onset of qE in wild-type and the five other strains leads to a substantial decrease in both fluorescence lifetimes and qE. This decrease in qE clearly indicates a reduction in the flux of energy to the PSI core, which provides photoprotection under supra-optimal light. While previously it has been shown that the decrease in qE correlates to a certain degree with the NPQ capacity in different algal species, here, we prove that in the diatom *P. tricornutum* this decrease is mediated in a concerted manner via Lhcx proteins (Lhcx1, Lhcx2, and Lhcx3) and the conversion of Dd to Dt. Lhcx proteins or the xanthophyll cycle alone—at least under LL growth conditions - do not activate qE or cause a reduction in qE under supra-optimal irradiances.

As Dt binds to LHC antenna proteins and Lhcx proteins are not part of the PSI core, the decrease in qE is indicative of thermal dissipation of absorbed energy in the LHC antenna complexes, as originally proposed by Genty et al. Accordingly, analysis of picosecond lifetime kinetics revealed that the exposure to supra-optimal irradiances leads to functional modifications in both antennae and PSI reaction centers, but the thermal dissipation occurs only in the antennae. Our results also showed that the slow phase of NPQ, which is activated under prolonged exposure to higher light levels, has no effect on qE. A similar slow NPQ phase with no reduction in qE is observed in the mutants lacking qE (x1KO and x1KO + x4) as well as in all strains treated with DTT (which inhibits the formation of Dt). These results suggest that the slow phase of NPQ is related to photoinhibitory damage to the PSI reaction center (qI).
by increasing thermal dissipation, but without further affecting oPSII. These processes, together with photoinhibited PSII reaction centers, may explain the observation of very high NPQ values, which do not affect oPSII.

There had been several reports on two potential qE quenching loci in diatoms based on fluorescence lifetime kinetics. The first is associated with an uncoupling of antenna complexes from the PSII core, which is independent of Dt formation. The second is taking place close to the PSII reaction center and is directly dependent on the xanthophyll cycle supposedly.

These reports are corroborated by classical PAM analyses in centric diatoms, where one component of qE is independent of the conversion of Dd to Dt and clearly different antenna organizations exist. However, the two qE-quenching sites concept is difficult to reconcile with the prime dependency of qE on the amount of Dt in the pennate *P. tricornutum* and clearly different antenna organizations exist.

Because the PSI reaction center is energetically a shallow trap, excitons that reach a PSI core may transfer back to the peripheral antennae where they can be thermally dissipated once all PSI reaction centers are closed. Such a mechanism has been identified in plants, termed "economic photoprotection", and later was also postulated to exist in diatoms. This economic photoprotection, however, does not involve a decrease in qE upon induction of qE, which is in sharp contrast to our results. As we obtained a linear correlation between the extent of Y(NPQ) and the decrease in qE driven directly by Dt (Fig. 5, Supplementary Fig. 17), we can use the regression equation to calculate oPSII for a theoretical maximal Y(NPQ) of 1, i.e., where all absorbed energy is dissipated as heat. This leads to a value of oPSII = 111 Å² at Y(NPQ) = 1 (and 115 Å² if both parameters are measured after 1 s darkness). Using a chlorophyll a specific absorption coefficient of 9.8 m² (g chlorophyll a)⁻¹ for blue light as used here for the oPSII measurements) and assuming a dimeric PSI core for diatoms in vivo, containing 70 chlorophyll a molecules, we calculated a functional PSI core cross-section of 112 Å². This value is virtually identical to the one calculated at Y(NPQ) = 1 based on our oPSII vs. Y(NPQ) regression. In a similar approach for the centric diatom *T. pseudonana*, Campbell, and co-workers calculated with a monomeric PSI and concluded that at Y(NPQ) = 1 the remaining oPSII is provided by the PSI core and some LHCs that are not thermally downregulated. If one assumes that the existing dimeric PSI is excitonically coupled and shares a common peripheral LHC antenna, then the regeneration to a Y(NPQ) of 1 provides a residual oPSII, which also in *T. pseudonana* corresponds to the functional absorption cross-section of the dimeric PSI core. In either cases, the results of Xu et al. and this study indicate that regulation of oPSII upon induction of qE does not involve the PSI core, otherwise the regression at Y(NPQ) of 1 would lead to oPSII values close to 0. The most parsimonious explanation is that Lhcx-mediated qE mechanistically leads to a disconnection of the peripheral LHC antennae—very much in line with recent results obtained by a different experimental approach—and these functionally disconnected LHC antennae dissipate the excess absorbed light as heat. Such a mechanism has been identified as one compound of NPQ in *Arabidopsis thaliana* using electron microscopy.

It is not clear yet how this disconnection of the LHC antennae is achieved, but recent results indicate a strong role of Dt molecules in influencing thylakoid membrane rigidity. Moreover, traces of Lhcx proteins have been detected in photosystem II preparations, indicating that Lhcx proteins might be more strongly connected to the PSI core than the peripheral LHC proteins. Both aspects could influence peripheral LHC antenna connectivity with the PSI cores in such a way that, upon induction of qE, the peripheral antennae are moved away slightly, exceeding the maximal distance for functional Förster resonance energy transfer to the PSI core. In such functionally disconnected LHC antennae, internal heat dissipation and chlorophyll fluorescence emission—the remaining energy dissipation pathways—would compete and a further synthesis of Dt under prolonged supra-optimal light would decrease fluorescence yield even more. Although some individual Lhcx proteins, such as Lhcx4, could possess other functions than qE, our results suggest distinguishable. Accordingly, the most rapid NPQ process (qE) can be easily identified by a pronounced reduction in oPSII upon exposure to high light. Whether this scenario holds true also for other algal taxa remains to be tested, but recent results in the green alga *Ostreococcus taurii* suggest a similar correlation between qE and oPSII. Our measurements of a significantly decreased oPSII under qE conditions are fully consistent with the proposal that qE shortens the time an exciton can travel before being thermally dissipated, and thus decreases the effective excitation diffusion length for an exciton to reach an open PSI reaction center.

Finally, it had been speculated that different Lhcx proteins have different capacities to provide NPQ, with Lhcx1 being the most effective. We indeed observed no qE in the LL grown cells if Lhcx1 is knocked out, but this is rather owing to a much lower expression of Lhcx2 and Lhcx3 under these conditions. Although we observe different extents of qE in the x1KO supplemented lines as compared with the wild-type, it remains unclear whether this is owing to an altered efficiency of Lhcx2 and Lhcx3 in providing qE or owing to differential protein expression. Also, whether qE provided by the different Lhcx proteins is activated at different light exposure conditions (either light intensity or exposure duration) remains to be elucidated. However, our results clearly demonstrate that the extent of qE always correlates with a reduction in oPSII with the same linear relation in all lines (Fig. 5, Supplementary Fig. 17). This implies that Lhcx1/2/3 have the same capacity for decreasing the remaining energy dissipation pathways—would compete and a further synthesis of Dt under prolonged supra-optimal light would decrease fluorescence yield even more...
that the primary role of Lhcx proteins is providing qE by decreasing the functional absorption cross-section.

Then, why do diatoms have several different Lhcx genes, in the case of the polar strain *Fragilariaopsis cylindrus* even 11? As has been shown in 32,33,36,67, all four Lhcx proteins of *P. tricornutum* are modulated in their expression by different environmental triggers, such as, e.g., low light (Lhcx1), high light (Lhcx2, Lhcx3), blue light (Lhcx3-3), iron limitation (Lhcx2), or nitrogen starvation (Lhcx3, Lhcx4). We propose that one gene alone cannot contain all required regulatory cis-elements in order to respond to the multitude of environmental triggers. Instead, through gene duplications during evolution, several regulatory cis-elements could be integrated into several Lhcx gene promoters to modulate the expression of proteins with very similar functions and thus to tune the environmental conditions.

Overall, our study reveals the molecular mechanism of how diatoms fine tune qE, allowing them to thrive in continuously changing light environments, where NPQ is one of the most important physiological processes.68

**Methods**

**Cell culturing.** Experiments were performed in *Phaeodactylum tricornutum* strain 4 (Pt4, JGI ID: 44733). Cells were cultured in a shaker at 20 °C in a 16 h day/8 h night cycle exposed to white light with an intensity of 40 µmol photons m−2 s−1 (onset at 8:00 a.m.) defined as low light (LL). Cells were cultured in sterile Provasoli’s enriched F/2 seawater using Tropic Marin Classic arti.

**Generation of TALEN and complementation constructs.** TALEN-knockout plasmids for Lhcx1 and Lhcx2 were generated following the procedure for creating TALEN-KO lines in *P. tricornutum* by Serif et al.27. This method relies on Golden Gate reactions, where digestion with TfiIIs restriction enzymes and ligation are carried out in one step27. For constructing the specific TALENs, we assembled the respective TALEN monomers into the respective backbone vectors available at Addgene. For Lhcx1 we constructed two TALEN pairs, targeting two different sites in the Lhcx1 gene. For Lhcx2 we constructed one TALEN pair. All genomic target sites contained a thymine before the actual TALE recognition site. For each TALEN we assembled the corresponding promoter, each with a half monomer, the FokI nuclease-coding region as well as the antibiotics resistance cassette, and do not occur in wild type cells. For long-term light stress experiments, cells were exposed to white light with an intensity of ~ 400 µmol photons m−2 s−1 for 24 h. Light intensities were measured with a spherical quantum sensor (US-SQ/W, Walz, Germany).

**Determination of mutated Lhcx promoter activity.** Wild-type Pt4 cells were biologically transformed with the TALEN bearing vectors according to71. Positive clones were selected on Zeocin (75 µg mL−1; Invitrogen, USA) and Nourseothricin (150 µg mL−1; ClonNAT, Werner Bioagents, Germany) containing solid medium plates in low-salt F/2 medium (8.3 g L−1 sea salt). Pre-screening of the colonies was performed using an Imaging PAM system (Walz, Germany), searching for clones with altered NPQ characteristic. Further characterization included western blot, PCR, and DNA sequencing. Confirmed KO clones were spread on individual agar plates in a suitable dilution to obtain clones from single cells. Three out of these were reanalyzed for each knockout line and one of each KO line was used for all follow-up experiments. In addition, whole-genome sequencing (WGS) of one of each allele was performed to verify germ line integration.

**DNA isolation and allele specific PCR.** Genomic DNA was isolated using the Nexttec 1step DNA isolation kit (Biozyn, Germany) according to the manufacturer’s instructions. A cell pellet corresponding to 10 mL of culture was used as starting material. The concentration of genomic DNA was measured with a Nanodrop 2000 (Thermo Fisher Scientific, Germany). In order to prove that both alleles were mutated, allele specific PCR was applied for the x2KO mutants only, as the Lhcx1 gene of Pt4 does not contain allele specific differences in the TALEN-targeted region. The sequences of the two primer pairs for both alleles as well as of allelic pairs are specified in Supplementary Table 3. The genotypic sequence for Lhcx1 of Pt4 is depicted in Supplementary Fig. 20. Allele specific PCR, provided no PCR product in the x2KO line (Supplementary Fig. 6a). We additionally used the primer combination Lhcx2_prom-fw and Lhcx2_term-rev, which amplifies the Lhcx2 promoter, Lhcx2 gene and Lhcx2 terminator in a length of 347 bp in wild-type. In the x2KO mutant, one of the alleles a deletion of 518 bp occurred (Supplementary Fig. 6). The sequence of this mutated allele is indicated in Supplementary Fig. 21.

**Whole-genome sequencing.** In order to identify the correct DNA sequence for both alleles of Lhcx1 and Lhcx2 in Pt4 and in order to verify the biallelic knockout of the Lhcx1 gene in the x1KO lines, the genomic DNA of Pt4 and the three x1KO lines were sequenced using the MasterPure DNA Purification Kit (Epicentre, USA) according to the manufacturer’s instructions and sequenced by Illumina 125 bp paired end sequencing by GATC Eurofins (Germany). Quality control for raw reads was done using FastQC. Low quality reads (quality score ≤ 20) were trimmed by FastX/AT Trimmer (http://hannonlab.cshl.edu/fastx_toolkit/index.html). As no reference genome existed so far, we first produced a new Pt4 reference genome, by assembling and aligning the trimmed reads of Pt4 with Bowtie2 against the available genome of Pt1 CCAP 1055/1 deposited in Ensembl, which is
an update of the previously deposited genome in JGI36. Then, the SAM files created by Bowtie2 were converted into BAM files using SAM tools37 and a new consensus sequence was created by the aid of the BCF tools pipeline34. Finally, the quality controlled and trimmed reads of the xIKO mutants were mapped against the reads of the new Pt4 reference genome by aid of Bowtie2.

Fluorescence analyses. Pre-screening of mutants was performed with an Aqua-Pen (PSI Instruments, Czech Republics) or an Imaging PAM (Walz, Germany). Fine fluorescence kinetics were recorded with a Dual-PAM or a Fluorescence Induction and Relaxation instrument (a mini-FIRE). For the Dual-PAM measurements, cells were stained with 10 µg mL−1 of chlorophyll a mL−1 and 4.6 µM NaHCO3 was added. Before starting experiments, cells were acclimated to dim light for 30–45 min. Kinetic measurements were performed by exposing the cells for 10 min of actinic light with an intensity of 1700 µmol photons m−2 s−1, consisting of similar proportions of red and blue light photons, followed by 18 min of recovery conditions with 40 µmol photons m−2 s−1. Saturation flashes (8000 µmol photons m−2 s−1 red light, 800 ms) were applied every 30 s. For some experiment, DTT (500 mM final concentration) was added 5 min prior to fluorescence recording. To monitor acclimation under prolonged high light, cells were exposed for 130 min to 1700 µmol photons m−2 s−1, followed by 30 min recovery conditions with 40 µmol photons m−2 s−1. NPQ was calculated from Fm/Fm′. Here, we set Fm to the first light step Fm′, as this value is usually higher than the Fm obtained for dark acclimated *P. tricornutum* cells.

The variable fluorescence signatures of minimal (Fo) and maximal (Fm) fluorescence, corresponding to states where all PSI reaction centers are open or closed, respectively, as well as the functional absorption cross-sections were measured using a mini-FIRE75. Fluorescence induction was achieved with blue light emitting diodes (450 nm, 30-nm half bandwidth) and measured in the red light region (680 nm, 20 nm bandwidth). An 80 µs pulse, with peak optical power of 1 W/cm2, ensured that all PSI reaction centers were reduced with a single turnover of PSI. From this single turnover protocol, parameters such as F0, electron transfer rates, NPQ and Y(NPQ) can be calculated46. For all strains, these parameters were measured in response to increasing actinic light in 15 steps with 1 min acclimation at each step (i.e., light curves, 0–800 µmol photons m−2 s−1 of blue light), where variable fluorescence (resulting in F′ and Fm′) was recorded at the end of each light step. In addition, after each light step, the actinic light was turned off for 1 s to allow for re-opening of reaction centers. This 1 s dark period was short enough to prevent NPQ relaxation14, but did allow us to directly measure F0, which is needed for the calculation of 1–Q. Electron transfer rates were calculated from E x (F′/Fm′−F′/Fm′)−1. NPQ and 1–Q were calculated following46. F ′ was derived from F′−Fm′ transition induced by an 80 µs single turnover flash76. Using the F′−Fm′ transition allowed us to make more acquisitions, improving the signal-to-noise ratio, and reducing the error in the fitting procedure. A comparison of oPSII and oPSIIa (i.e., obtained after 1 s darkness) showed little difference in the values or the interpretation of our results (cf. Fig. 5, Supplementary Fig. 17).

Fluorescence lifetimes were measured using a custom-built picoquant lifetime fluorometer as described in ref. 46. We measured lifetimes for Fo and Fm levels in the dark, followed by measurements of Fo′ and Fm′ lifetimes during exposure to 850 µmol photons m−2 s−1 (blue light) for 10 min to induce NPQ. The fluorescence acquisition interval was 20 s, short enough to avoid changes in the state of the photosynthetic units. Average fluorescence lifetimes were calculated by fitting the collected data to a sum of three exponential decays, which were deconvoluted from the instrument response function46.

**Pigment analysis.** Pigment extraction and HPLC separation followed the protocol established by37. In short, pigments of cells filtered on a 1.2 µm isopore membrane (Millipore) were extracted with a mixture of 81% methanol/9% 0.2 M ammonium acetate at pH 7.0. Extracts were obtained for dark acclimated *P. tricornutum* cultures33. Primer sequences for *Lhcx1* or *Lhcx2* were designed to recognize both alleles, in contrast to that used by33. qPCR was run on a 7500 Fast RT-PCR system (Applied Biosystems, USA). Each strain was measured in biological triplicates, and on top each gene per sample was measured three times. Cycle threshold values and gene amplification efficiencies were obtained by utilizing PCR Miner 4.078. Relative transcript levels were calculated according to79.

**Western blot.** Protein extraction and separation followed the protocol described by ref. 80, but using 14% lithium dodecyl sulfate-polyacrylamide gel electrophoresis for protein separation. Samples corresponding to an amount of 1 µg chlorophyll a were loaded on the gel. Proteins were blotted on a BioTrace PVDF ( Pall Corporation, USA) or an Amersham Protran nitrocellulose membrane (GE Healthcare, GBR) using the semidy blotting technique by means of a BioRad Trans-Blot Turbo System (Hercules, USA). Previously, either an anti-Lhcx, raised against a recombinant Chlamydomonas Lhcx protein, had been used to quantify Lhcx protein expression in *P. tricornutum*82–84 or it had been the anti-FCP6 raised against a specific C-terminal peptide of the FCP6 protein in *Cyclotella cryptica*35,36. We designed a new polyclonal anti-*P. tricornutum*-Lhcx (manufactured by Agrisera, Sweden) raised against the peptide MAQELVNGKGILEHL, because 14 (Lhcx1, Lhcx3), 13 (Lhcx2), and 12 (Lhcx4) out of these 15 amino acids are contained specifically in the C-terminus of the respective Lhcx proteins in *P. tricornutum*, thus making this antibody superior in detecting the different Lhcx proteins with a similar affinity. Anti-Lhcx was applied in 1:10,000 dilution overnight. Anti-Rubisco (AS03 037, Agrisera, Sweden, 1:10,000 dilution) served as a loading control. After binding of the secondary antibody for 1 h (goat anti-rabbit IgG, (H&L) HRP) conjugated, AS09 602, Agrisera, Sweden; 1:10,000 dilution for Lhcx, 1:20,000 for Rubisco), signals were detected using Roti-Lumin Plus (Carl Roth, Germany) in an Odyssey FC Imaging System (LI-COR, USA).

**Statistics.** Significance with a P value ≤0.05 was determined with a two-tailed unpaired Student’s t test calculated with SigmaPlot 12 on biological replicates as indicated in the respective legends. For gene expression we used the Pairwise Fixed Reallocation Randomization Test performed by REST2006 according to80 with 2000 randomizations.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Source data for the figures and supplementary figures are provided with the article. Any further data related to the article is available from the corresponding author upon reasonable request. Whole-genome sequencing reads for Pt4, xIKO_1a, xIKO_1b, and xIKO_2 are deposited at the European Nucleotide archive (ENA) under the following accession code: "PRJEB38325".

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**Author contributions**

J.M.B., J.S., J.L., A.F., P.G.K., M.Y.G., P.G.F., and B.L. designed experiments. J.M.B., C.R.B., M.S., and B.L. screened the mutants via PCR, western blot, growth, and NPQ experiments. J.H., C.R.B., and B.L. analyzed the whole-genome sequencing data. J.M.B. and B.L. performed in-depth NPQ, HPLC, and gene expression analyses. J.S., J.M.B., and B.L. recorded FIRE and fluorescence lifetime data. J.S., M.Y.G., P.G.F., and B.L. analyzed the FIRE and fluorescence lifetime data. J.M.B., J.S., A.F., J.L., M.Y.G., P.G.K., P.G.F., and B.L. wrote the article.

**Additional information**

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