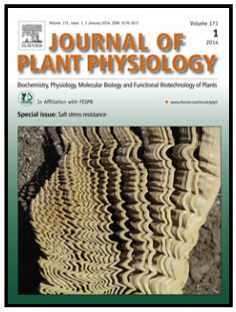

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Identification of genes coding for functional zeaxanthin epoxidases in the diatom *Phaeodactylum tricornutum*

Running title: Zeaxanthin epoxidase genes in *Phaeodactylum tricornutum*

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Abstract

Phaeodactylum tricornutum like other diatoms synthesizes fucoxanthin and diadinoxanthin as major carotenoid end products. The genes involved have recently been assigned for early pathway steps. Beyond β-carotene, only gene candidates for β-carotene hydroxylase, zeaxanthin epoxidase and zeaxanthin de-epoxidase have been proposed from the available genome sequence. The two latter enzymes may be involved in the two different xanthophyll cycles which operate in *Phaeodactylum tricornutum*. The function of three putative zeaxanthin epoxidase genes (*zep*) was addressed by pathway complementation in the *Arabidopsis thaliana* Zep mutant *npq2*. Genes *zep*2 and *zep*3 were able to restore zeaxanthin epoxidation and a functional xanthophyll cycle but the corresponding enzymes exhibited different catalytic activities. Zep3 functioned as a zeaxanthin epoxidase whereas Zep2 exhibited a broader substrate specificity additionally converting lutein to lutein-5,6-epoxide. Although *zep*1 was transcribed and the protein could be identified after import into the chloroplast in *A. thaliana*, Zep1 was found not to be functional in zeaxanthin epoxidation. The non-photochemical quenching kinetics of wild type *A. thaliana* was only restored in transformant npq2-zep3.

Keywords: *Arabidopsis thaliana npq2* mutant, diatoms; fucoxanthin synthesis; *Phaeodactylum tricornutum;* xanthophyll cycle; zeaxanthin epoxidase

1. Introduction

Diatoms (Bacillariophyceae) are widely spread in the oceans and are non-active swimmers. As not motile, they are exposed to water current and thus to strong changes in light conditions (MacIntyre et al., 2000, Wagner et al., 2006).Their ecological success is probably due to their ability to drive photosynthesis also with green light as well as their fast acclimation to the fluctuating light conditions. For both functions, carotenoids play an important role. Fucoxanthin bound to the light-harvesting complexes is the major carotenoid that absorbs light in the range around 500 – 565 nm in vivo (Owens and Wold, 1986; Premvardhan et al., 2010). Its biosynthesis via zeaxanthin, violaxanthin and neoxanthin has recently been established and some genes involved have been identified (Dambek et al., 2012).
Diatoms possess two xanthophyll cycles (Lohr and Wilhelm, 1999). The major xanthophyll cycle is the reversible diadinoxanthin-diatoxanthin cycle, in which diadinoxanthin is de-epoxidzed to diatoxanthin under high-light conditions (Fig. S1). The other violaxanthin-zeaxanthin cycle also found in higher plants undergoes a similar de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin (Fig. S1) under light stress and vice versa. At least the diadinoxanthin-diatoxanthin cycle is involved in the dissipation of excess light energy by non-photochemical-quenching (Müller et al., 2001; Lavaud et al., 2003). The light-dependent epoxidation and the de-epoxidation reactions are catalysed by different enzymes. However, little is known about the interaction of these xanthophyll cycle enzymes. It is an open question whether the same epoxidases and de-epoxidases are active in both xanthophyll cycles since the section of the molecules of diadinoxanthin and violaxanthin on one hand and diatoxanthin and zeaxanthin on the other which are modified in both reactions are identical. For violaxanthin de-epoxidase (Vde) from spinach and a green alga which possess only the violaxanthin-zeaxanthin cycle, their potential de-epoxidize diadinoxanthin in substantial rates compared to antheraxanthin has been demonstrated (Goss, 2003).

To date three putative violaxanthin de-epoxidase genes, vde, and three putative zeaxanthin epoxidase genes, zep, all with two typical lipocalin motifs were assigned in the genome of *P. tricornutum* (Coesel et al., 2008). However, their function has not been fully assigned yet. Only one of the vde genes (Pt44635) has been silenced in *P. tricorntum* (Lavaud et al., 2012). This led to a decreased synthesis of diatoxanthin upon transfer of transformants to high light in combination with a different NPQ phenotype. For the *zep* genes, pathway complementation in *Escherichia coli* has been attempted but this approach was not successful (Dambek et al., 2012). From *Arabidopsis thaliana* several mutants with defective carotenoid biosynthesis exist. Among them is mutant npq2 (= aba1), a non-photochemical quenching mutant with inactivated Zep (Niyogi et al., 1998). By complementing the *npq2* mutant with the *zep* gene candidates from *P. tricornutum* and analysis of the transformants, we could demonstrate zeaxanthin epoxidase activity for some of the expressed *zep* gene. A possible role of individual zeaxanthin and diatoxanthin epoxidases in the different xanthophyll cycles of diatoms is discussed.

2. Materials and Methods

2.1. Strains, cultivation, and transformation

Cells of *Phaeodactylum tricornutum* UTEX 646 were grown under continuous shaking at 18±1°C in a 16 h day 8 h night regime (40 µmol photons m^{-2} s^{-1} in ASP-media modified after Provasoli et al., (1957) as previously described (Eilers et al., 2015).

Wild-type plants of *Arabidopsis thaliana* Col-0 and the zeaxanthin accumulating *npq2*-mutant with a point mutation in the *zep* gene (Niyogi et al., 1998) were grown under 80-100 µmol photons m^{-2} s^{-1} in a 9 h day and 15 h night regime at 19 to 21 °C. Flowers were inoculated overnight (modified after Clough and Bent, 1998) with *A. tumefaciens* strains carrying one of the three gene copies of the *zep*-genes of *P. tricornutum* in the p-Green vector with a buffer containing sucrose 5%, benzylaminopurin 0.04 µM, Tween20 0,01% and Murashige-Skoog salts (Serva Heidelberg, Germany) 2.2 mg ml^{-1}. Inoculation was repeated after one week. Second generation plants were selected spraying a 300 µM aqueous solution of glufosinate. All experiments were performed with four to six week old second generation plants. *Escherichia coli* XL1 blue (Bullock et al., 1987) used for plasmid amplification of the genes in LB-Medium with appropriate antibiotics according to Sambrook et al., (1989) at 37 °C.

Agrobacterium tumefaciens (GV3101) containing plasmids pSoup und pMP90 (Hellens *et al.*, 2000) was transformed according to Berberich et al., (2008). Cells were grown under continuous shaking at 28 °C in YEP-Medium (yeast extract 10g l^{-1}; pepton, 10 g l^{-1}; NaCl 5g l^{-1}).

2.2. DNA and RNA isolation

A. thaliana leaves frozen in liquid nitrogen were homogenized by grinding and DNA extracted with DEX- buffer and chloroform/isoamylalkohol modified after Murray and Thomson (1980). After precipitation with isopropanol DNA was used either to amplify the transit peptide of Zep *A. thaliana* or for molecular screening of *A. thaliana* transformants. Leaf powder obtained the same way was used for RNA isolation using the innuPREP Plant RNA Kit (AnalytikJena). DNA was removed by phenol/chloroform treatment and RNAse free DNAse. For expression studies of the *zep*-genes complementary DNA (cDNA) was synthesized using „Reverse Transcriptase RevertAid™ Premium" (#EP0723, MBI Fermentas, St. Leon-Rot, Germany).

2.3. Cloning and construct design of zep genes

According to Coesel et al., (2008) and after confirmation in the *P. tricornutum* genome database DOE Joint Genome Institute website (http://genome.jgi-psf.org/Phatr2) primers were designed to amplify the gene sequences of the *zep1* (Pt45845 / EEC48429.1), *zep2* (Pt56488 / EEC51398.1) and *zep3* (Pt56492 / EEC50032.1) coding regions shortened of the putative signal and transit peptides from their cDNAs obtained by PCR according to Juhas et al., (2014). For sequencing amplified fragments were cloned into pJet1.2.BLUNT (MBI Fermentas, St. Leon-Rot, Germany) and for *A. thaliana* transformation into the vector pGreen (Hellens et al., 2000) together with the transit peptide of Zep from *A. thaliana*. The structures of the plasmids are shown in Supplementary Fig. S2. Details on primers and size of inserted

reading frames can be found in Supplementary Table S1A. For the fusion with *zep1*, the GFP sequence was amplified plasmid from pRTdSGFP (Weber *et al.,* 2008).

2.4. Carotenoid analysis of A. thaliana lines

Freeze-dried *A. thaliana* leaves were pulverized and pigments were extracted in methanol containing 6% KOH at 60°C. Partitioning into 10% ether in petrol and HPLC analysis was as previously described (Dambek et al., 2012) on a 25 cm Nucleosil C18 column at 32°C with a mobile phase of acetonitrile/methanol/2-propanol (85:10:5). Separation and quantification of antheraxanthin and lutein-5,6-epoxide started by enrichment of both carotenoids on TLC (silica plates, toluene/ethyl acetate/methanol 76:20:4). The band at R_f 0.35 was scraped off and eluted with methanol followed by HPLC in a second system. This procedure was previously described for other carotenoids (Breitenbach et al., 2013). This second HPLC system with a C18 Vydac 218TP54 column and 2% water in methanol as mobile phase allows baseline separation of lutein and zeaxanthin (Zhu et al., 2008) and separated lutein-5,6-epoxide from antheraxanthin with retention times of 29.4 (absorbance maxima 414, 439, 470 nm) and 31.2 min (absorbance maxima 422, 443 and 472 nm), respectively. Absorbance at 450 nm and spectra of individual peaks were recorded with a photodiode array detector 440 (Kontron, Straubenhard, Germany). Individual carotenoids were quantified and identified with authentic standards by their retention times and absorbance spectra.

2.5. Chlorophyll fluorescence measurements

In vivo chlorophyll a fluorescence parameters (van Kooten and Snell, 1990) of three week old *A. thaliana* lines were analyzed using the FluorCam 800 MF device (Photon Systems Instruments, Brno, Czech Republic) in combination with the FluorCam 7 software with actinic red light (500 µmol photons m^{-2} sec^{-1}) for 70 sec and saturating light pulses (>5000 µmol photons m^{-2} sec^{-1}) followed by 90 sec relaxation in the dark.

2.6. Expression of zep genes

Quantitative real time PCR was performed to determine transcript levels of transgenic *zep* from *P. tricornutum* in *A. thaliana* lines using the Master Mix SensiFAST™ SYBR Lo-ROX (BIOLINE, Luckenwalde, Germany) according to the manufacturer's instructions. The primers used and the sizes of the amplified fragments are listed in Supplementary Table S1B. The PCR program used is shown in Supplementary Table S1C. Primer efficiencies were higher than 80% in all cases. Relative expression of *zep1, zep2 and zep3* versus endogenous *atzep* was calculated.

2.7. Protoplast isolation for cellular localization of zep1

For demonstrating chloroplast import of *zep1,* a GFP-construct was designed and transient expression was tested in protoplasts of wild type *A. thaliana*. Protoplast isolation and transformation were

as described in Machettira et al. (2011) and Sommer et al. (2011). Analysis was performed with an Axiophot fluorescence-microscope (Zeiss, Germany). The small subunit of Rubisco of *Nicotiana plumbifolium* was used as a positive control.

2.8. Homology modelling

We modeled the tertiary and quaternary structure of predicted Zep proteins using the SWISS-MODEL workspace (Arnold et al., 2006; Biasini et al., 2014). First, we searched for the closest known structure which was urate oxidase (Hicks et al., 2013) for atZep and Zep1. For Zep2 the structure of 4-hydroxybenzoate hydroxylase (Wang et al., 2002) and for Zep3 the structure of 3-hydroxybenzoate 6-hydroxylase (Montersino et al., 2013) was used for homology modeling.

3. Results

3.1. A. thaliana zep transformants

In order to assign the function of the three gene-copies of zeaxanthin epoxidase 1, 2 and 3 of *P. tricornutum* in a gene complementation approach, the zeaxanthin accumulating *A. thaliana npq2* mutant was transformed individually with the three *zep*-containing plasmids. Analysis with the stable transformants was performed on different levels and compared with *npq2* mutants and the wild type plants. Each of the constructs was equipped with the appropriate sequence of *P. tricornutum zep 1, 2* or *3*, shortened in their putative signal and transit peptide region. To ensure chloroplast import of the constructs, the transit peptide of the Zep of *A. thaliana* was cloned N-terminal of each sequence.

After 6 weeks of growth, all plants exhibited an F_v/F_m ration of around 0.8 (data not shown) which indicates an absence of photo stress. Nevertheless, reduced growth of the npq2 mutant compared to wild type plant was evident as already described (Kalituho et al., 2007). In contrast, the npq2 transformants *zep2* and *3* recovered in growth and reached the same size as wild type. However, in the npq2 transformant with *zep1* the WT phenotype was not restored.

Integration of *zep* genes from *P. tricornutum* into the genome of *npq2* was demonstrated by genetic analysis of the transformants (Fig 1). The presence of the expected genomic DNA fragments demonstrates genome integration. In Fig 1A three positive lines of npq2-zep1 (a, b, c) show the expected DNA band at 1547 bp (Supplementary Table S1A). Also in npq2-zep2 (three lines) and npq2-zep3 transformants the transgene was present indicated by the DNA sizes of 1733 bp and 1609 bp, respectively. The data presented from the following investigations were of the npq2-zep1 line from lane a and from the npq2-zep2 lane c.

3.2. Carotenoid analysis

The changes in the carotenoid composition of the *npq2* transformants were determined by HPLC analysis (Fig. 2). In wild type plants, lutein (peak 6) is the dominating carotenoid. Among the xanthophyll

cycle carotenoids, zeaxanthin is hardly detectable therein and antheraxanthin is present in very small amounts. The quantities of the individual carotenoids are shown in Table 1. Mutant *npq2* exhibits a different carotenoid pattern (Fig. 2). Lutein is still the most abundant carotenoid but substantial amounts of zeaxanthin accumulate additionally. From the epoxy carotenoids, only very small amounts of antheraxanthin but no violaxanthin or neoxanthin were detectable. Both transformants npq2-zep2 and npq2-zep3 were completely different in carotenoid composition compared to *npq2*. With respect for violaxanthin formation, these two transformants resembled the wild type (Fig. 2, Table 1). The biggest differences compared to the wild type were obtained with npq2-zep2 which showed a comparably low lutein content and a high content of antheraxnthin and lutein-5,6-epoxide. The latter epoxy carotenoid was below detection in the other lines. In contrast to the other transformants, npq2-zep1 exhibited no significant changes to the *npq2* mutant in the concentrations of zeaxanthin or the epoxy carotenoid antheraxanthin derived directly from it. Violaxanthin is absent from npq2-zep1 as well as from the *npq2* mutant and the very low antheraxanthin concentration in the latter resembles that of *npq2*.

3.3. Non-photochemical quenching (NPQ)

The transformants with complemented violaxanthin synthesis and npq2-zep1 were further investigated to demonstrate an operative xanthophyll cycle by NPQ kinetics of dark adapted *A. thaliana* plants with wild type plants and mutant npq2 as controls (Fig. 3). Under our light conditions, the npq2 mutant exhibited a faster NPQ kinetics in the light phase and a slower relaxation in the dark phase compared to wild type plants. The maximum NPQ values were reached within 70 sec with values around 1.0 for WT and 1.2 for mutant npq2. Transformant npq2-zep1 exhibited exactly the same kinetics as *npq2* (Fig. 3A). All values were identical. The NPQ value for npq2-zep2 with about 0.3 (Fig. 3B) was even below the value of the *A. thaliana* wild type. Transformant npq2-zep3 showed maximum NPQ values close to npq2 but with a slower initial rise and a much faster relaxation (Fig. 3C). These results indicate that by reconstitution of epoxy carotenoid synthesis also the xanthophyll cycle is at least partially restored as well albeit to a different extent in the *zep2* and *zep3* transformants.

3.4. Expression of zep genes in A. thaliana and localization of Zep1

In contrast to npq2-zep2 and npq2-zep3, npq2-zep1 did not show an altered phenotype with respect to epoxy carotenoid formation and NPQ kinetics although *zep1* integration was confirmed. Therefore, expression of *zep* genes was determined by quantitative real-time PCR (qRT-PCR) using equal amounts of mRNA as template for cDNA synthesis. Due to a similar expression pattern of the *zep* gene of *A. thaliana* in all lines, it was used as reference gene. Primer efficiencies for all *zep* genes including the one from *A. thaliana* were higher than 83%. Fig 4 shows the amounts of *zep* mRNA in the transformants related to the *A. thaliana zep* RNA which differed strongly. The highest transcript values were obtained for *zep3* which was about 160-fold higher than for *zep2* with 1718±538. The relative mRNA amounts for *zep1* were in between. These results indicate that expression of *zep1* is not the limiting factor for Zep

functionality. Another reason for lack of Zep1 function could be its import into the chloroplast. Therefore, the GFP sequence was fused to the C-terminus of *zep1* to determine chloroplast import and protein accumulation in this organelle. Protoplasts of *A. thaliana* WT were transformed transiently with this construct. Chloroplasts can be identified by their red auto fluorescence and GFP proteins by their green fluorescence (Fig. 5). As positive control, a GFP fusion of the small subunit of ribulose-bisphosphate carboxylase (Rubisco) of *N. plumbifolium* with a bright green GFP signal is shown together with *A. thaliana* WT protoplasts as negative control. In WT protoplasts only a faint greenish background is visible. For zep1-GFP, a green fluorescence substantially above the WT background was obtained. Details are better visible with higher magnification (Fig. 5, bottom). The results from this localization experiment give definite evidence that the expressed protein of *zep1* reaches the chloroplast in *A. thaliana* in a processed form.

4. Discussion

Although the carotenoid biosynthesis pathway to diadinoxanthin and fucoxanthin has been established, none of the genes and enzymes beyond β-carotene have been assigned (Dambek et al., 2012). The step from zeaxanthin to violaxanthin is catalyzed by zeaxanthin epoxidase. Zeaxanthin epoxidases are present in plants in only one gene copy whereas in several algal groups multiple copies of putative ZEP genes exist (Frommolt et al., 2008; Goss and Jakob, 2010). Diatoms possess higher copy numbers. For example, the centric *Thalasiosira pseudonana* has two copies (*zep1* and *zep2*), whereas in the pennate *P. tricornutum* three copies (*zep1, 2 and 3)* are present (Coesel et al., 2008). However in contrast to plants and green algae, an additional epoxidase for the conversion of diatoxanthin to diadinoxanthin as part of the major xanthophyll cycle may exists in *P. tricornutum*. The functional assignment of the three *zep* genes was not successful to date (Dambek et al., 2012). Therefore, the three epoxidase candidates, *zep1*, *zep2* and *zep3* from *P. tricornutum* were used to complement an epoxidase mutant of *A. thaliana*. Several positive transformants with integrated *zep* cDNAs into the genome of *npq2* mutant were obtained (Fig. 1). However, only npq2-zep2 and npq2-zep3 showed a restoration of the wild type phenotype. This correlated with the newly acquired epoxidase activities. In case of npq2-zep3 antheraxanthin and violaxanthin were synthesized which resembles the formation of epoxy carotenoids in the wild type (Table 1). In the npq2-zep2 transformant, the concentration of antheraxanthin is much higher than for violaxanthin. Futhermore, lutein-5,6-epoxide is formed at the expense of lutein. This indicates that Zep2 is highly efficient in mono-epoxidation but poorly catalyzes the second epoxidation step from antheraxanthin to violaxanthin. In addition, Zep2 possesses affinity for lutein which is not the case for Zep3. The ability for light-dependent epoxidation of lutein to lutein-5,6-epoxide is common for shade leaves in canopies of woody plants (Förster et al., 2009). Due to its broad substrate specificity epoxidizing zeaxanthin and lutein at similar activity, Zep2 is the most likely candidate for the epoxidase which converts diathoxanthin to diadinoxanthin in the xanthophyll cycle of *P. tricornutum* (Fig. S1). In contrast to Zep2 and Zep3, Zep1 expressed under the CAMV35S promoter is inactive as a zeaxanthin epoxidase.

The amount of zeaxanthin determines non-photochemical quenching activity as already shown for *A. thaliana* mutants exhibiting higher values for npq2 than the wild-type (Niyogi et al., 1998). The decrease of the NPQ values of the npq2-zep2 and npq2-zep3 transformants compared to the non-transformed mutant (Fig. 3) resembles their formation of epoxy carotenoids (Table 1). The NPQ value in transformant npq2-zep3, ranging between the wild type and *npq2*, and the lowest value for npq2-zep2 are negatively related to the sum of antheraxanthin and violaxanthin. Either the high antheraxanthin and lutei-5,6-epoxide contents or the low lutein content of npq2-zep2 may be responsible for its non-photochemical quenching activity which is lower than for the wild type (Fig. 3). This feature of npq2-zep2 resembles two *A. thaliana* mutants, *lut1* and *lut2*, both with increased antheraxanthin and decreased or zero lutein (DellaPenna, 1999). Most likely, the low lutein content causes this negative influence since it has been shown that one of the lutein molecules in LHCII is directly involved in energy dissipation (Ruban et al., 2007).

Attempts were made to elucidate the reason for lack of zeaxanthin epoxidase activity in the npq2-zep1 transformant. In all three *A. thaliana* transformants, *zep* transcripts including *zep1* were detected (Fig. 4). All three *zep* genes are also expressed upon light stimulation in *P. tricornutum* cells (Coesel et al., 2008). In this diatom, the transcript level of *zep1* was down-regulated immediately during the first 6 h after shifting algae from low-light to high-light and increased only later, whereas levels of *zep2* were not influenced at all and levels of *zep3* were directly up-regulated after high-light exposure (Nymark et al., 2009). In comparison to npq2-zep2 and npq2-zep3 (Fig. 4), it could be shown that the *zep1* transcript level in the *A. thaliana* transformant was high enough for substantial protein synthesis. Finally, Zep1 protein could be identified in the chloroplast. This indicates that *zep1* mRNA is translated and that the resulting protein is imported into the chloroplast. However, this Zep1 enzyme is unable to utilize zeaxanthin as a substrate in our transformant. One possibility for this missing activity is that Zep1 encodes an enzyme which lost its activity. Looking at the models of the three Zep proteins from *P. tricornutum* and the enzyme from *A. thaliana* we found a strong structural correlation to flavin containing aromatic hydroxylases. Zep1 lacks the C-terminal part which might be involved in either dimerization as known from structurally related enzymes (Montersino et al., 2013) or in substrate specificity. In Zep2 and 3 this C-terminal helix is close to the putative lipocalin domain (Fig 6) probably giving rise to its substrate selectivity. Overall, Zep2 and Zep3 are structurally more related to the *A. thaliana* enzyme which might explain that the heterologous enzymes from *P. tricornutum* are functional in *A. thaliana* and can revert the *npq2* phenotype to wild type levels.

To date, we do not know which epoxidases are part of the violaxanthin-zeaxanthin cycle, the diadionxanthin-diatoxanthin cycle or both cycles, simultaneously (Fig. S1). We cannot rule out that Zep1 which is also present in *Thalasiosira pseudonana* (Coesel et al., 2008) may be a highly specific epoxidase unable to accept either zeaxanthin or antheraxanthin as substrates. However, a strict substrate specificity seems unlikely since the diatoxanthin half of the molecule to be epoxidized is identical to the zeaxanthin part where the same reaction occurs. In general, a broad substrate specificity is known for carotenogenic

enzymes (Steiger et al., 2000). This has also been shown for violaxanthin de-epoxidases from spinach and the green alga *Mantoniella squamata*, other lipocalin enzymes of the violaxanthin-zeaxanthin cycle, which convert the mono-epoxides antheraxanthin and also diadinoxanthin equally well (Goss, 2003). A better candidate for an epoxidase converting diatoxanthin to mono-epoxy diadinoxanthin may be Zep2 due to its high mono-epoxidase and poor di-epoxidase activity.

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Figure Captions

Fig. 1. PCR amplification and separation on agarose gel from genomic DNA of *A. thaliana* plants wild type (WT), *npq2* mutant and transformants with integrated *zep1* (**A,** 1547 bp), *zep2* (**B,** 1733 bp) and *zep3* (**C,** 1609 bp) of *Phaeodactylum tricornutum* genes. No template control (NTC).

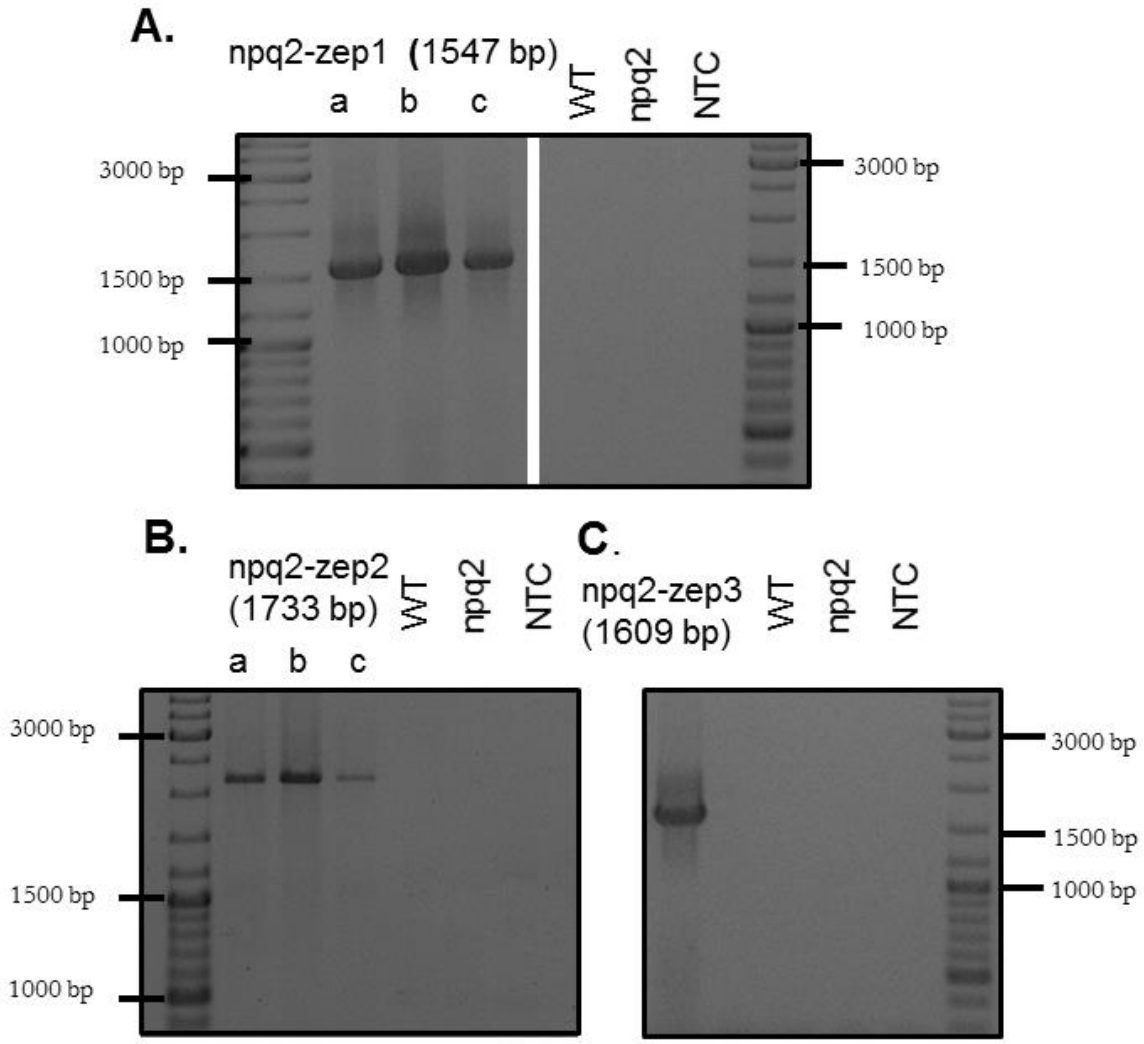


Fig 2. HPLC separation of carotenoids from *Arabidopsis thaliana* wild type, *npq2* mutant and *npq2* transformants in comparison with xanthophyll cycle carotenoid standards. Carotenoid peaks 1 neoxanthin, 2 violaxanthin; 3 antheraxanthin, 4 zeaxanthin, 5 β-carotene, 6 lutein. Peak 3 of npq2-zep2 consists of antheraxanthin plus lutein-5,6-epoxide. It could by separated on a polymeric Vydac C18 218TP54 column.

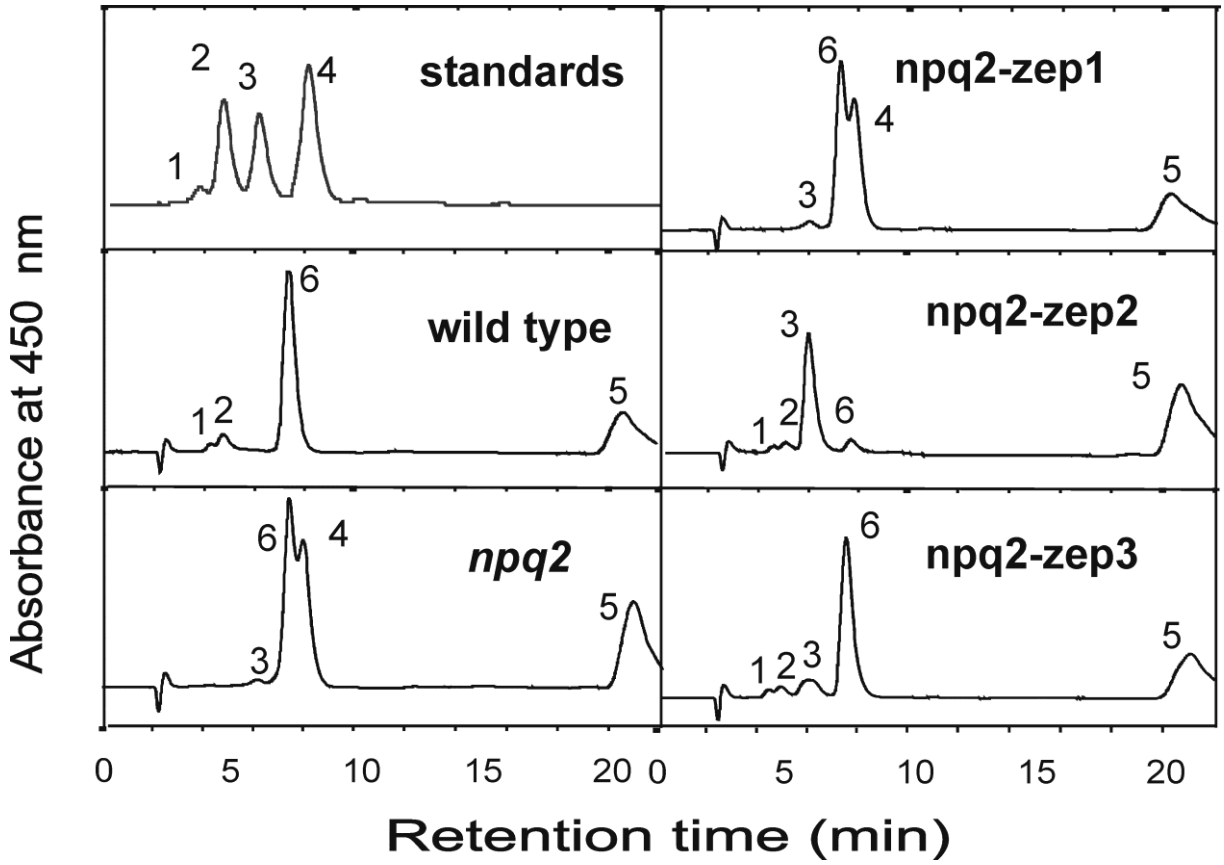


Fig. 3. Non photochemical quenching (NPQ) kinetics of different *Arabidopsis thaliana* plants of wild type (•), *npq2* mutant (>) and transformants(■), npq2-zep1 with integrated *zep1* (A), npq2-zep2 with integrated *zep2* (B) or npq2-zep3 with integrated *zep3* (C) genes from *Phaeodactylum tricornutum*. Means from determinations ± SD of determinations from six representative plants.

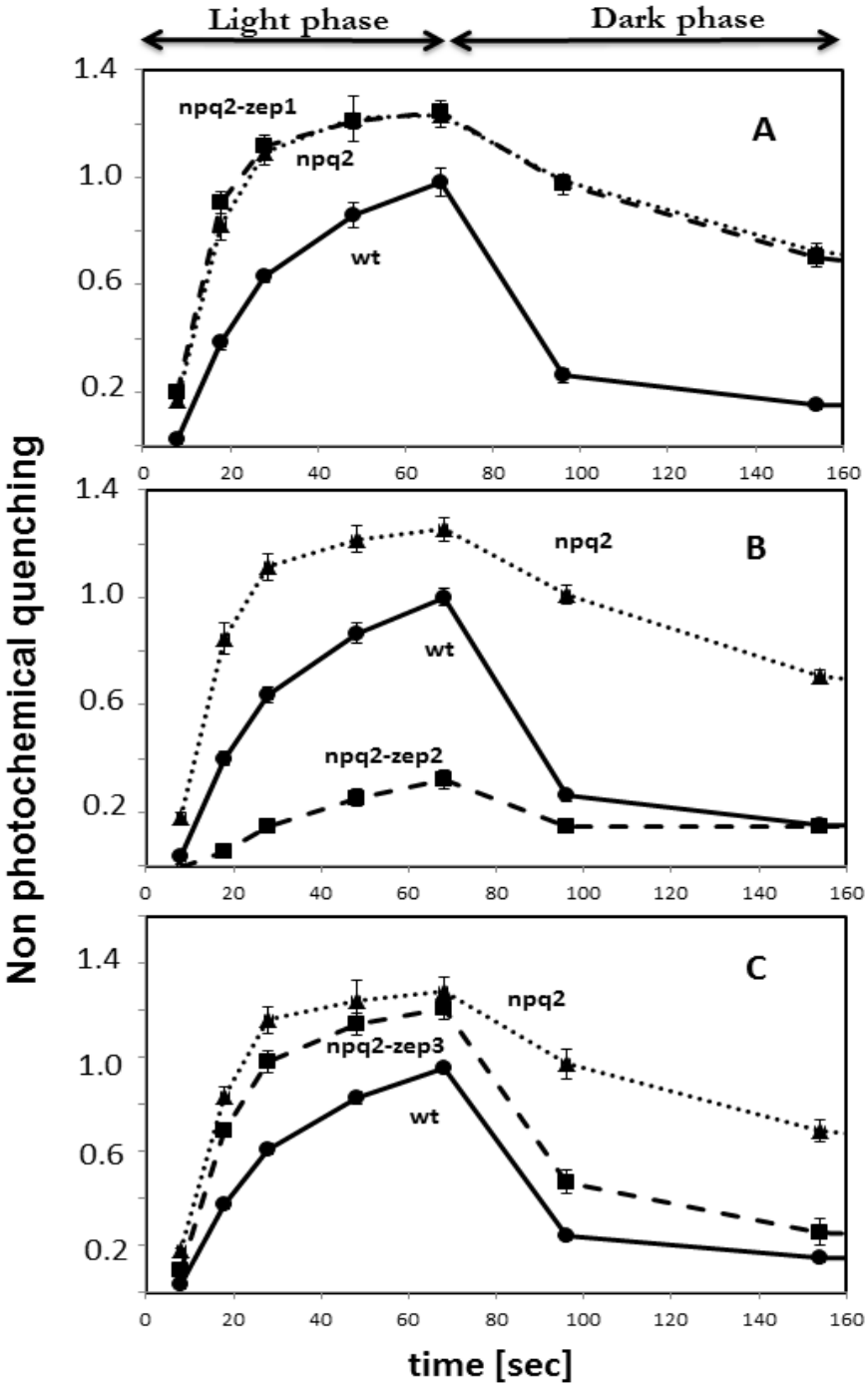


Fig 4. Relative amounts of transcript level of *A. thaliana npq2* transformants with integrated *zep1, zep2* and *zep3* of *Phaeodactylum tricornutum* genes determined by real time PCR. Mean values of 3 independent replicates, normalized to *zep* expression of *Arabidopsis thaliana* wild type.

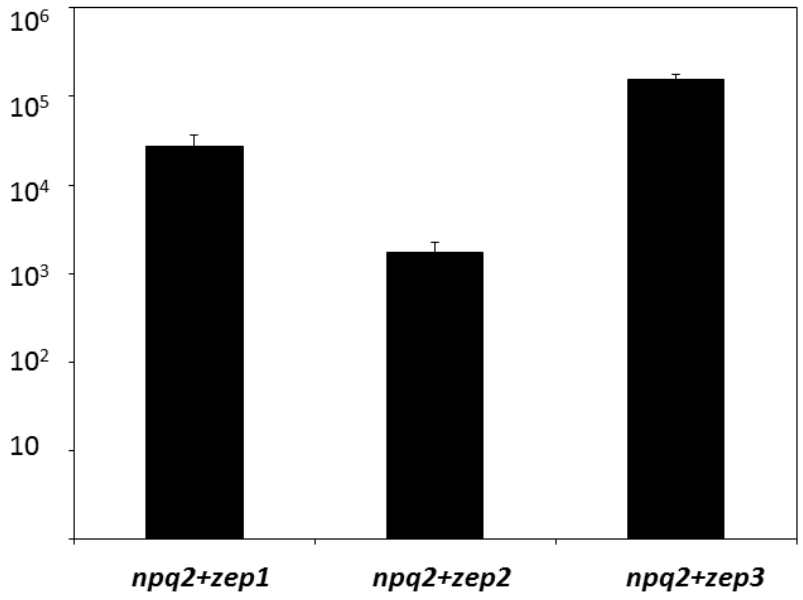


Fig 5. Fluorescence images of expression and cellular localization of *zep1* construct in wild type protoplasts of *Arabidopsis thaliana*. Images showing the red auto fluorescence of chloroplast (left) and the green GFP signal (right). A. small subunit of ribulose-bisphosphate carboxylase of *Nicotiana plumbifolium* served as positive control; B. non-transformed wild type plant as negative control; C. *zep1* construct; bottom images of zep1- GFP fluorescence at 63x magnification.

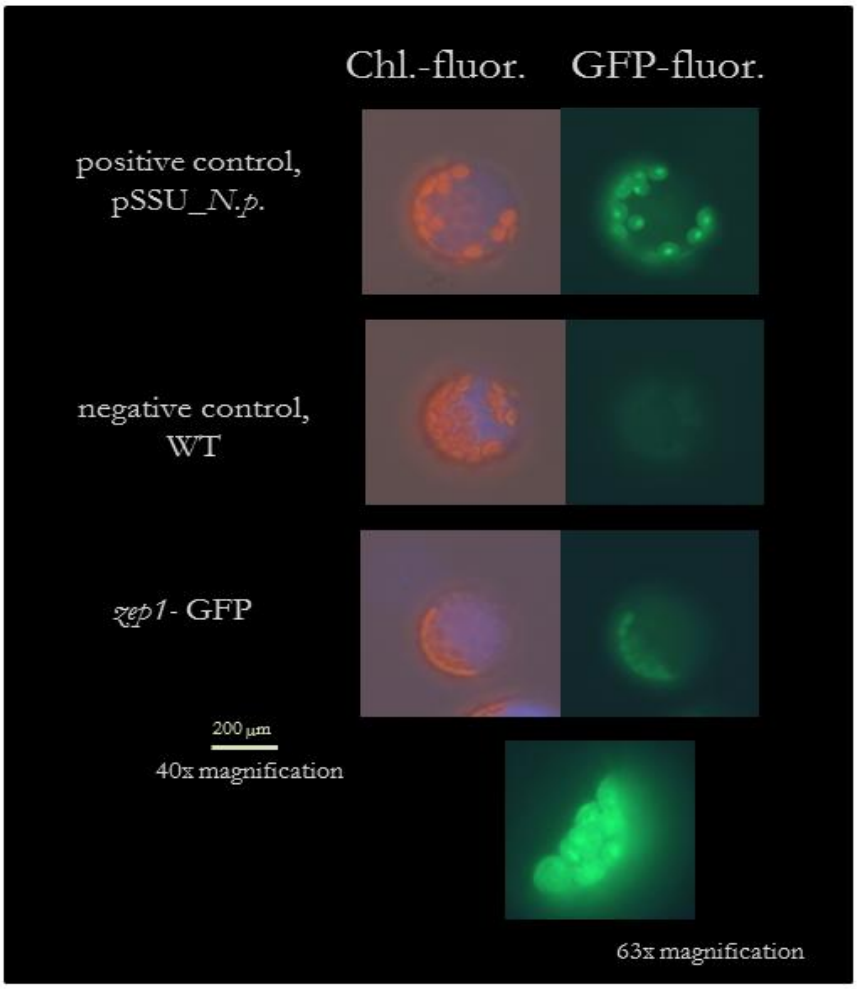
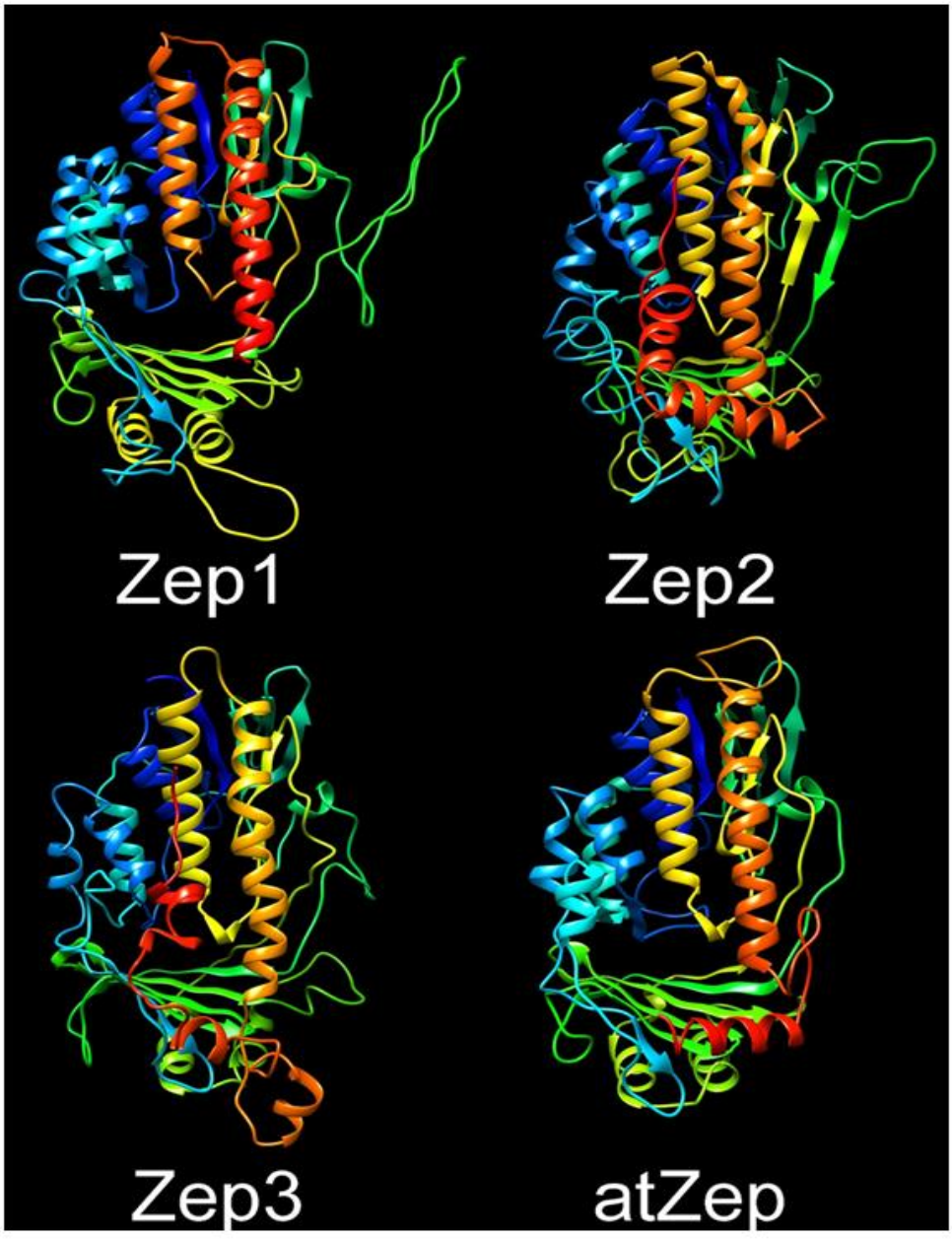


Fig. 6. Homology models of Zep1, Zep2 and Zep3 of *Phaeodactylum tricornutum* and Zep of *Arabidopsis thaliana*. The blue N-terminal part depicts the putative flavin-binding domain. The green beta-sheets represent the putative substrate binding lipocalin domain. The C-terminal helices with unknown function are given in red. Note that only conserved regions of have been modeled.



Table

Table 1 Carotenoid content of *Arabidopsis thaliana* wild type, npq2 mutant and zep transformants. Tzep1 is npq2-zep1, Tzep2 is npq2-zep2 and Tzep3 is npq2-zep3; Nx neoxanthin, Vx violaxanthin, Lut lutein, Zx zeaxanthin bC β-carotene

Line	Carotenoids (μg g^{-1} dw)					
	Nx	Vx	Ax	Lut	Zx	bC
WT	20.8±2.7	71.3±9.2	12.1±1.6	612.0±78.5	0	378.4±48.5
npq2	0	0	26.0±1.1	474.0±17.6	431.6±16.1	388.5±34.0
Tzep1	0	0	33.1±5.7	630.6±91.9	559.4±96.3	432.0±74.4
Tzep2	16.1±1.4	36.2±3.3	503.5±45.7*	52.4±4.7	0	418.1±18.3
Tzep3	29.8±4.5	50.5±8.5	53.5±6.3	603.2±100.9	0	453.8±65.9

*Antheraxanthin plus lutein-5,6-epoxide in a ratio of 41.2 versus 58.8%