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New transgenic line of *Arabidopsis thaliana* with partly disabled zeaxanthin epoxidase activity displays changed carotenoid composition, xanthophyll cycle activity and non-photochemical quenching kinetics

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Summary

Zeaxanthin epoxidase (ZE, E.C. 1.14.13.90), an enzyme belonging to the lipocalin superfamily, catalyses the conversion of zeaxanthin to antheraxanthin and violaxanthin. These reactions are part of the xanthophyll biosynthetic pathway and the xanthophyll cycle. The role of carotenoids in the dissipation of excessive light energy has been widely studied using mutants with a disabled carotenoid biosynthetic pathway. In this paper, the transgenic line MaZEP7 with partially disabled ZE activity is described and compared with wild-type plants and *npq2* mutant lacking active ZE. We examined the presence and the abundance of *aba1* transcripts, measured pigment composition, xanthophyll cycle functioning and chlorophyll fluorescence in all three lines. The MaZEP7 line contains additional copies of the *aba1* gene introduced by agroinfiltration, but no enhanced *aba1* transcript level was observed. In addition, ZE activity in MaZEP7 was impaired, resulting in an altered xanthophyll profile. In dark-adapted plants, violaxanthin and

Abbreviations: ABA, abscisic acid; Ax, antheraxanthin; β-Car, β-carotene; ETR, electron transport rate; F_0, minimal fluorescence; F_m, maximal fluorescence; $F_{m'}$, maximal fluorescence in illuminated sample; F_s, steady-state fluorescence; LHC, light harvesting complex; Lt, lutein; LtE, lutein epoxide; NPQ, non-photochemical quenching of chlorophyll fluorescence; Nx, neoxanthin; qN, parameter representing non-photochemical quenching; qP, parameter representing proportion of open PS II centers; VDE, violaxanthin de-epoxidase; VAZ, xanthophyll cycle pigments total pool; Vx, violaxanthin; Y, quantum yield of photosystem II; ZE, zeaxanthin epoxidase; Zx, zeaxanthin.

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neoxanthin levels were lower than in wild-type plants, whereas antheraxanthin and zeaxanthin levels were considerably higher. The presence of lutein epoxide was also observed. Violaxanthin levels changed only minimally during light exposition, whereas antheraxanthin was converted to zeaxanthin and there was no epoxidation during the course of the experiment indicating disturbed xanthophyll cycle functioning. The amounts of carotenoids and chlorophylls on a dry weight basis and chl *a*/chl *b* ratio were similar in all lines. The presence of epoxidated pigments in MaZEP7 plants indicates that epoxidation occurs, but it is likely very slow. Chlorophyll fluorescence measurements showed that the dependence of electron transport rates on light intensity for the MaZEP7 line resembled the *npq2* mutant. Kinetic measurements showed that the MaZEP7 line exhibited very rapid induction and a high steady-state value of non-photochemical quenching.

Introduction

Photosynthetic organisms have developed several mechanisms for protection against an excess of light, as any overexcitation of the photosynthetic apparatus leads to photoinhibition (Powles, 1984). Carotenoids, which are antenna pigments necessary for the proper assembly and functioning of photosynthetic complexes (Pogson et al., 1998), also play an important photoprotective role. One of the main photoprotective mechanisms is the light-dependent interconversion of violaxanthin (Vx) to antheraxanthin (Ax) and zeaxanthin (Zx), which is called the xanthophyll cycle (Latowski et al., 2004) and occurs in green algae and higher plants. Two opposite reactions of the xanthophyll cycle are catalyzed by two enzymes belonging to the lipocalin superfamily: violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE) (Grzyb et al., 2006). Both are encoded by nuclear genes: *vde1* (coding VDE) and *aba1* (coding ZE). Primary products of these genes contain N-terminal transit peptides targeting these proteins to chloroplasts. After reaching the chloroplast, these peptides are cut off, yielding the final product. Localized in thylakoid lumen and activated by acidic pH, VDE catalyses the de-epoxidation of Vx to Ax (faster reaction) and Ax to Zx. The extent of the conversion of Vx to Zx is limited by Vx accessibility and is usually between 50% and 80% (Eskling and Åkerlund, 1998). A reverse reaction, the epoxidation of Zx, is catalyzed by ZE, an enzyme thought to be constantly associated with the thylakoid membrane and light harvesting complex (LHC) (Gruszecki and Krupa, 1993). The optimal pH for its activity is 7.5, which suggests that epoxidation takes place on the stromal side of the membrane (Siefermann and Yamamoto, 1975). Epoxidation occurs in darkness, but is faster in the light (Goss et al., 2006), although in the light it competes with the much faster de-epoxidation reaction. Therefore, the effect of ZE activity in light-exposed leaves is visible only when low light intensities or VDE inhibitors such as dithiothreitol are used (Eskling and Åkerlund, 1998; Goss et al., 2006). The first step of epoxidation is faster than the second (Goss et al., 2006).

The role of Zx in the ΔpH-dependent, fast-inducible non-photochemical quenching of chlorophyll fluorescence (NPQ) mechanism connected with nonradiative energy dissipation called qE has been widely studied (Demmig et al., 1987; Gilmore and Yamamoto, 1991). In *Arabidopsis thaliana*, Zx is responsible for about 85% of qE (Niyogi et al., 1997, 1998), but Zx-independent qE occurring in barley has also been reported (Finazzi et al., 2004). There are two models for participation of xanthophylls in qE. In one, Zx acts indirectly as an allosteric effector enabling (in cooperation with proton binding) conformational changes of LHCs leading to dissipative state of the antenna (Horton et al., 2005; Perez-Bueno et al., 2008). The other model assumes direct involvement of xanthophylls in quenching of excited chlorophyll (Holt et al., 2004; Ruban et al., 2007). Recent experiments based on femtosecond transient absorption measurements in intact thylakoid membranes confirmed participation of xanthophylls in chlorophyll quenching. Formation of xanthophyll–Chl heterodimer, in which energy is transferred from excited Chl to xanthophyll, and formation of Zx cation radical in the PS II antenna as an effect of energy transfer have been reported (Ma et al., 2003; Holt et al., 2005). Most likely, both proposed mechanisms are responsible for NPQ. The participation of Zx in the qI component of NPQ connected with photoinhibition has also been postulated (Farber et al., 1997). Other roles of Zx include free radical quenching, preventing lipid peroxidation (Havaux et al., 2000) and membrane physical property

modulation (Gruszecki and Strzalka, 1991, 2005; Kostecka-Gugała et al., 2003). Zx is also an intermediate in the biosynthetic pathway of ABA, an important plant hormone, connected with stress response and dormancy regulation. The reversible, light-induced conversion of Vx to Zx enables rapid adaptation to changing light conditions and protection when exposed to light stress.

Several mutants of *Arabidopsis* affected in xanthophyll pigment composition or NPQ have been examined (Pogson et al., 1998; Niyogi et al., 1998, 2001; Havaux et al., 2000, 2004). Mutants without ZE activity have been characterized as well (Tardy and Havaux, 1996; Hurry et al., 1997; Niyogi et al., 1998; Pogson et al., 1998). In this paper, we describe the transgenic line MaZEP7 with partially disabled ZE activity. The MaZEP7 line has additional copies of the *aba1* gene introduced by agroinfiltration and was predicted to over-express ZE. Unexpectedly, epoxidation of Zx in the transgenic MaZEP7 line was disturbed and no enhanced levels of *aba1* transcript were observed. The transgenic line was compared to wild-type plants and an *npq2* mutant (*aba1-6*) defective in the gene encoding ZE (Niyogi et al., 1998). This mutant accumulates Zx, whereas levels of Ax, Vx and neoxanthin (Nx) are very small, displays very rapid induction of NPQ and is not susceptible to wilting. We examined the xanthophyll profiles, the xanthophyll cycle functioning and measured chlorophyll fluorescence parameters in all three lines. The results obtained for the transgenic line differed from WT and also from the *npq2* mutant.

Materials and methods

Plant material and growth conditions

Three lines of *Arabidopsis thaliana* were used: Columbia as WT, *npq2* mutant (CS3772, *aba 1-6*) ordered from TAIR (The *Arabidopsis* Information Resource) and the transgenic MaZEP7 line.

Arabidopsis seeds were surface-sterilized (10 min in 50% sodium hypochlorite), washed with sterile water (5 × 1 mL) and sown on Petri plates containing a B5 medium with 3% sucrose and 0.7% agar. The medium for MaZep7 additionally contained kanamycin (25 μg/mL) as a selection factor.

The plates were incubated in darkness, in 4 °C for 4 d and then placed in a growth chamber (temperature 20 °C, photoperiod of 16 h light, 8 h dark, humidity 70%). After 10 d the seedlings were transferred into a soil culture and grown for 6 weeks (temperature 25 °C, 16/8 h photoperiod, humidity 70%, illumination 80–100 $\mu E/m^2 s$).

Transgenic line construction

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions from 15-d-old leaves of *A. thaliana*. Reverse transcription was performed using ImProm-II Reverse Transcription System (Promega) with 1 μg of total RNA. Obtained ZEP cDNA was amplified using primers ZEPcDNAF (5′-GGCATCACAACAGATAAAGTC-3′) and ZEPcDNAR5′PcPCNA1R (5′-GACCTATATATACATTGTCATGC-3′). The PCR reaction mixture (50 μL) contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 U of Expand High Fidelity System (Roche), 1 μM of each primer and 1 μL of cDNA. The samples were heated at 95 °C for 5 min and then subjected to 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min and then incubated at 72 °C for 7 min in thermocycler (MJ Research). The purified PCR product was reamplified using previous PCR conditions with new set of primers MaZEPFORF (5′-AACGATAGCCATGGGTTCAACTCCGTTTTGC-3′) and MaZEPRORF (5′- GAAGATCTTCAGTGATGGTGATGGTGATGAGCTGTCTGAAGTAATTTATCG-3′) and 10 ng of template. The final PCR product was purified, digested with *Bgl*II and *Nco*I and cloned into pAVA319 expression vector containing 35S promoter (von Arnim et al., 1998, full sequence Genebank accession AF078810), digested previously with *Bgl*II and *Nco*I, followed by sequencing. The constructed pAVA319 ZEP vector, together with the destination vector pGreen0029 (http://www.pgreen.ac.uk; Hellens et al., 2000), was digested with *Bam*HI and *Kpn*I and the expression cassette containing ZEP ORF with sequence coding C-terminal 6-His-Tag added was cloned into the pGreen0029 carrying a kanamycin resistance gene for transgenic plant selection. The resulting recombinant pGreen0029ZEP was introduced into *Agrobacterium tumefaciens* LBA4404 strain together with pSoup helper plasmid (Hellens et al., 2000). Transgenic *Arabidopsis* seeds were obtained using the floral dip method. There was only one transgenic line obtained and tested.

RT-PCR analysis

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions from 4-week-old leaves of *Arabidopsis* collected in the middle of the light period, because the transcription level of *aba1* gene is highest at that time (Thompson et al., 2000). Reverse transcription was performed using Revert Aid™, a First Strand cDNA Synthesis Kit (Fermentas) with 1 μg of total RNA. Next, PCR reactions mixtures (10 μL) containing 1 × PCR buffer, 1.5 mM $MgCl_2$,

200 μM dNTPs, 0.5 U of *Taq* polymerase (A&A Biotechnology), 200 nM of each primer were prepared. Amplification of violaxanthin de-epoxidase transcript was performed using VDEF 5′-AACAAGGTCATCTCAGTCCTACC-3′ and VDER 5′-AACTTGTGCATGCTTATTGAAGG-3′, and for zeaxanthin epoxidase ZDEF 5′-TCGAAGATCCATCAAGAAGAAAGC-3′ and ZDER 5′-TCCTGGTTTTACTTGGGGTAAAGG-3′ primers were used. The primers were complementary to sequences located in different exons of *vde1* or *aba1* genes. For semi-quantitative expression analysis of the *aba1* gene, 1 μL of cDNA, 40 nM 18S rRNA primer and 360 nM 18S rRNA competimer (Quantum RNA™Universal 18S, Ambion Inc.) were used to amplify 18S RNA transcript. For *vde1* expression level analysis, 2 μL of cDNA, 21 nM 18S rRNA primer and 379 nM 18S rRNA competimer were used. The samples were heated at 94 °C for 5 min and then subjected to 30 cycles of 94 °C for 30 s, 57 °C for 35 s, 72 °C for 45 s and then incubated at 72 °C for 5 min in thermocycler (MJ Research). The obtained products were separated in ethidium bromide-containing 1% agarose and relative expression levels of the tested genes were analyzed using densitometric analysis of gel images obtained with advanced tools of Adobe Photoshop 6.0.

DNA gel blot analysis of PCR amplified fragment DNA (PCR-Southern)

Genomic DNA was extracted from leaves of 1-month-old *Arabidopsis* plants using a Genomic Mini Kit (A&A Biotechnology). The purified DNA (10 ng) was used for PCR reaction. For *aba1* amplification, the gene-specific primers ZDEF and ZDER were used. The reaction was carried out in 15 μL volume containing 1 × PCR buffer, 200 μM dNTPs, 1 U of ExTaq DNA polymerase (TaKaRa) and 2 μM of each primer. The samples were heated at 94 °C for 5 min, and then subjected to 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and then incubated at 72 °C for 7 min in master cycler gradient thermocycler (Eppendorf). The resulting amplified PCR products (5 μL) were separated in 0.8% (w/v) agarose gel, blotted on a positively charged nylon membrane (Roche), following standard hybridization protocol (Sambrook and Russel, 2001). Hybridization was performed for 16 h at 68 °C with 730 bp long DIG-labeled probe generated by PCR using cDNA as a template and ZDEF and ZDER primers. The filter was incubated with anti-digoxigenin-AP Fab fragments, following CSPD (Roche) chemiluminescent substrate and finally was exposed to the X-ray film.

Quantitative pigment analysis

Pigments were extracted from lyophilized *Arabidopsis* leaves with 80% acetone and determined by absorbance measurements at 663.2, 646.8 and 470 nm according to Lichtenthaler and Wellburn (1983). The amounts of pigments on a dry weight basis and chl *a*/chl *b* molar ratio were calculated.

Carotenoid analysis and xanthophyll cycle activity measurement

A. thaliana plants of all three lines were kept in darkness for 12 h, then exposed to white light (intensity 1000 μE/m^2 s) and placed in darkness again. Sets of samples (leaves) were taken as follows: before light exposure, after 0.5 and 1 h of light exposure and after 1, 2, 3, 5, 10, 24 h of subsequent darkness. Samples were frozen in liquid nitrogen. Pigments were extracted from leaf samples by grinding in a mortar with solvent A (acetonitrile:methanol:water in proportion 72:8:1) containing a small amount of $CaCO_3$. The suspension was centrifuged (5 min, 8000*g*). The supernatant was passed through a 0.2 μm syringe filter and separated using RP-HPLC. The column used was Nucleosil 100 C-18 (5 μm particle size, 25 × 0.4 cm, Teknokroma, Spain) with octadecylsilan as a stationary phase. Solvent A was used as a mobile phase, flow rate 2 mL/min, sample volume 200 μL, detection by measuring absorbance at 440 nm. The obtained chromatograms were analyzed using a Borwin program calculating areas corresponding with manually selected peaks. In further calculations, different molar extinction coefficients of xanthophyll cycle pigments were taken into account. The coefficients were calculated on the basis of data from the literature (Britton et al., 1995) and normalized spectra of xanthophylls. They were Ax – 113,440, lutein (Lt) – 120,250, lutein epoxide (LtE) – 151,850, Nx – 147,900, Vx – 149,200, Zx – 108,000.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence from intact leaves was measured at 25 °C using modulated fluorometer PAM 210 (Walz). Leaves of dark-acclimated plants (40 min in darkness) were put on a measuring head with the upper side facing the detector, and a piece of wet paper towel on the lower side. Fluorescence parameters obtained were electron transport rate (ETR), maximum quantum yield of PS II photochemistry (F_v/F_m), quantum yield of PS II photochemistry (Y), proportion of open PS II (qP)

calculated as described in Maxwell and Johnson (2000) and non-photochemical quenching represented as qN parameter (Van Kooten and Snell, 1990). Quantum flux density of photosynthetic active radiation for ETR calculation was estimated as 0.84. Two types of measurements were carried out: light curves and NPQ induction kinetics. At the beginning of every measurement, minimal fluorescence (F_0) and maximal fluorescence (F_m) were determined. The measuring light parameters were 650 nm emission peak, intensity less than 0.5 $\mu E/m^2 s$, frequency of modulation 32 Hz when only measuring light was applied and 8 kHz when actinic and/or saturating light were also applied. The saturating light parameters were emission peak 660 nm, intensity 3500 $\mu E/m^2 s$, time of pulse 1 s. In the light curve measurements, red actinic light (emission peak 650 nm) with intensity increasing in steps was applied. The intensities used were 59 $\mu E/m^2 s$ (for 5 min), 89, 119, 149, 209, 309, 439, 599, 849, 1249, 1859 $\mu E/m^2 s$ (applied for 3 min each). In NPQ induction kinetic measurements, leaves were illuminated with actinic light (309 $\mu E/m^2 s$) for 30 min. The actinic light was then switched off. Measurements of F and $F_{m'}$ were carried out after 20 s, 2 min 20 s, 4 min 20 s, 6 min 20 s, 10 min 20 s, 15 min 20 s, 20 min 20 s, 25 min 20 s and 30 min 20 s of illumination, and after 0.5, 1.5, 2.5, 5, 7.5, 10, 15, 25 min of darkness.

Results

Differences in the growth rate of the examined *Arabidopsis* plants were observed from the seedling stage. In *in vitro* culture, *npq2* mutant seedlings were smaller than WT plants at the same age, although no delay in greening was observed. MaZEP7 seedlings seemed to be a bit bigger than WT and their leaves expanded faster – i.e. when WT seedlings had mostly four leaves, many MaZEP7 plants had six. The difference in size was clearly visible for the first 4 weeks of soil culture; for older plants it was less marked, but MaZEP7 rosettes were thicker than WT. The phenotype of *npq2* mutants was completely different – plants grew slowly, were small, their leaves were often elongated and had rolled up edges. No susceptibility to wilting was observed. Entrance into the generative phase occurred at the same time in all lines. *Npq2* mutants had many infertile floral shoots and only a few seeds were obtained, whereas the MaZEP7 line did not differ from WT. The DNA gel blot analysis of the transgenic line and WT showed the presence of three different specific PCR products when primers designed for *aba1* gene amplification were used (Figure 1). PCR products characteristic for wild copy of *aba1* gene containing introns (about 1400 bp long) were observed in both lines. A shorter product (about 730 bp) corresponding to the size of the introduced extra copy of transgene (cDNA), which did not contain introns, was observed only in MaZEP7 line. Moreover, in the transgenic line, an additional product (about 1000 bp) was also observed. Transcripts of *vde1* and *aba1* genes were present in all three lines (Figure 2). Results of semi-quantitative analysis of gene transcript levels are shown in Table 1. It is clearly visible that *vde1* transcript levels were similar in all lines, whereas *aba1* gene transcript levels differed. The *aba1* transcript level was the highest in WT, and in the transgenic line it was little lower. The *npq2* mutant exhibited the lowest transcript levels.

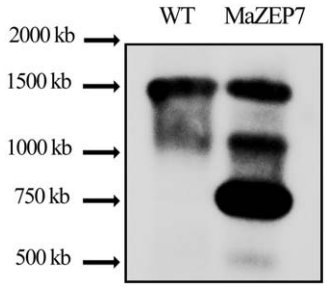

Figure 1. DNA gel blot analysis of PCR products amplified with primers designed for *aba1* gene. WT – wild type, MaZEP7 – transgenic line. Aliquot of 5 μL were taken to analysis.

Amounts of photosynthetic pigments chl *a*, chl *b* and total carotenoids on a dry weight basis, and chl *a*/chl *b* molar ratios were similar in all lines (Table 2). HPLC separation of leaf extracts revealed differences in carotenoid profiles (Figure 3). Control plants had a typical carotenoid profile: Nx, Lt, β-carotene (β-car) and xanthophyll cycle pigments: mostly Vx and a little of Ax and Zx, which is typical of dark-adapted leaves. In MaZEP7 plants, increased levels of Ax and Zx and decreased levels of Nx and Vx were observed. Moreover, an additional peak corresponding to LtE was present in transgenic plants. The *npq2* mutant had Zx as a nearly sole xanthophyll cycle pigment (Vx and Ax were present, but in small amounts). Levels of Lt and β-car were similar to WT, whereas Nx levels were very low. The total amount of β-car pathway pigments was similar in all examined lines (Table 2).

To examine how the xanthophyll cycle operates in the examined lines, we exposed dark-acclimated plants to high light (1000 $\mu E/m^2 s$) and placed them in darkness again. HPLC separation of extracts from

leaf samples taken in certain periods of time allowed us to observe changes in xanthophyll cycle pigments levels. Differences in xanthophyll cycle functioning were clearly visible (Figure 4). In dark-acclimated leaves of WT plants, Vx made up about 95% of the xanthophyll cycle pigments total pool (VAZ); there were about 5% Ax and less than 0.5% Zx (Figure 4A). Light exposure led to a rapid decrease in Vx content associated with a significant Zx increase and a smaller Ax increase – we observed levels of 60% Vx, 13% Ax and 27% Zx after 30 min of illumination. During the subsequent 30 min of light treatment, Zx levels decreased to 17.5%, Vx levels increased to 65.5%, while Ax levels were still increasing and reached a value of 17%. Prolonged illumination did not cause further changes. In a sample taken in an additional experiment after 2 h of light treatment, pigment levels were nearly the same as after 1 h (data not shown). After placing plants in darkness, Vx levels increased slowly, Zx levels decreased, and Ax levels increased during the first 2 h of darkness (reaching a maximal level of 19%), and decreased after that

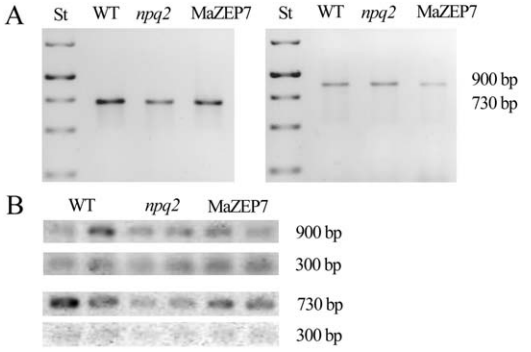

Figure 2. RT-PCR analysis of *aba1* (730 bp product) and *vde1* (900 bp product) gene transcripts in *Arabidopsis* leaves. Products of PCR reaction for transcript presence checking (A) and semi-quantitative analysis where 18S rRNA (300 bp product) was used as an internal standard (B). St – MW standards, WT – wild type, MaZEP7 – transgenic line, *npq2* – *npq2* mutant.

Table 1. Semi-quantitative RT-PCR analysis of *aba1* and *vde* genes expression levels.

	VDE/18S	ZE/18S
Wild type		
1	0.96	1.71
2	1.04	1.43
Npq2 mutant		
1	0.98	1.23
2	0.97	1.25
MaZep7		
1	0.96	1.40
2	0.97	1.38

Relative expression was calculated as ratio of VDE or ZE mRNA band intensity and 18S RNA used as internal standard.

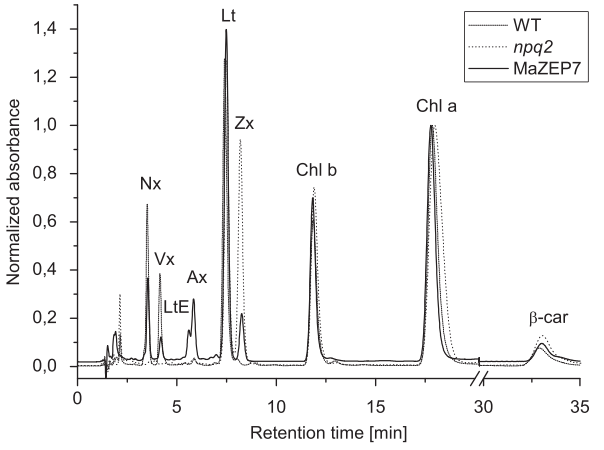

Figure 3. Chromatograms of extracts from dark-adapted leaves of *Arabidopsis*. WT – wild type, MaZEP7 – transgenic line, *npq2* – *npq2* mutant. Because fresh weight of samples differed, absorbance was normalized in respect to chl *a* content.

Table 2. Amounts of pigments in dark-adapted leaves of Arabidopsis.

	WT	*npq2* mutant	MaZEP7 line
Amounts of pigments (mg of pigment/g of dry weight)			
Chl *a*	7.78 ± 1.68	7.77 ± 1.20	7.61 ± 0.60
Chl *b*	3.81 ± 0.57	4.09 ± 0.89	3.83 ± 0.39
Total carotenoids	1.56 ± 0.46	1.40 ± 0.18	1.60 ± 0.16
Chl *a*/Chl *b*	2.07	1.93	2.02
Xanthophylls ratios			
(Ax+Nx+Vx+Zx)/Lt	0.59 ± 0.07	0.62 ± 0.03	0.58 ± 0.06
Nx/Lt	0.30 ± 0.01	0.002 ± 0.001	0.18 ± 0.05

Values are mean ± SE ($n = 3$). Relative amounts of chosen xanthophylls in dark-adapted leaves obtained by chromatogram analysis and expressed as ratios of peak areas. Values are mean ± SE ($n = 9$).

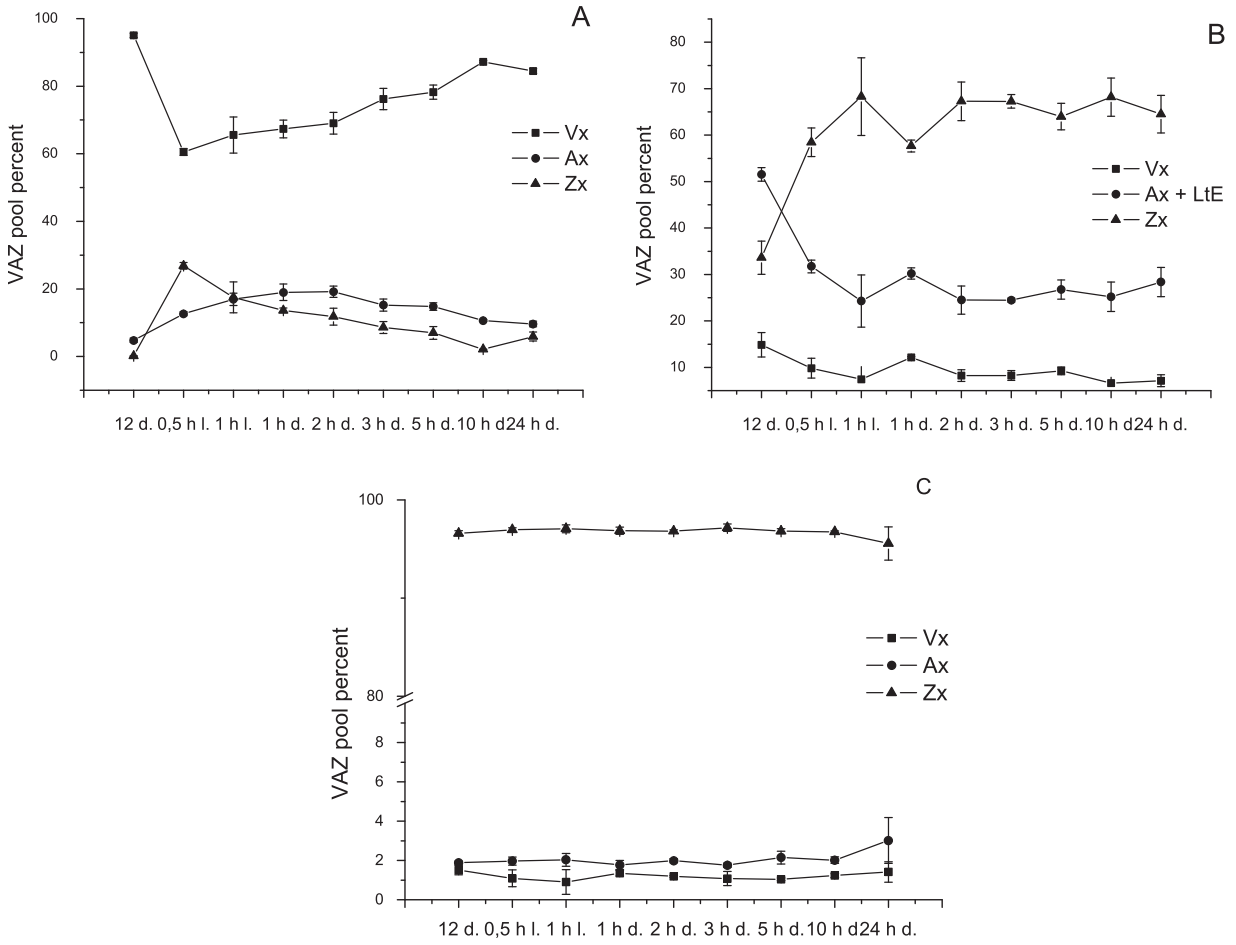

Figure 4. Relative percentage of xanthophyll cycle pigments during 1 h of light exposure (1000 μE/m^2 s) and subsequent 24 h of darkness. Differences between extinction coefficients of the xanthophyll cycle pigments at wavelength 440 nm were taken into account: (A) wild type, (B) MaZEP7, (C) *npq2* mutant, d. – darkness, l. – light. Values are mean ± SE ($n = 3$). SE is indicated by bars when larger than symbol.

time. After 24 h of darkness, Vx was about 84.5%, Ax – 9.5% and Zx – 6% (Figure 4A). In MaZEP7 plants, the initial Ax+LtE level was about 51%, Zx constituted about 34% and Vx had only about 15% share of the VAZ pool (Figure 4B). After 30 min of light treatment, Ax+LtE levels decreased to 32%, Vx levels decreased to 10% and Zx levels increased to 58%. Thirty minutes of additional illumination caused further decreases of Ax+LtE (to 24%) and Vx (to 7%), as well as an increase of Zx (to 68%). Of the three epoxidated xanthophylls, Ax was the preferentially used substrate for VDE (see Figure 5). After placing plants in darkness, no significant changes in the xanthophyll cycle pigments levels were observed. In the *npq2* mutant, the Zx level was about 97%, Ax – 2%, Vx – 1% and no changes in pigment amounts were observed during light and dark periods (Figure 4C).

Light response curves revealed differences between MaZEP7, WT and *npq2* plants (Figure 6).

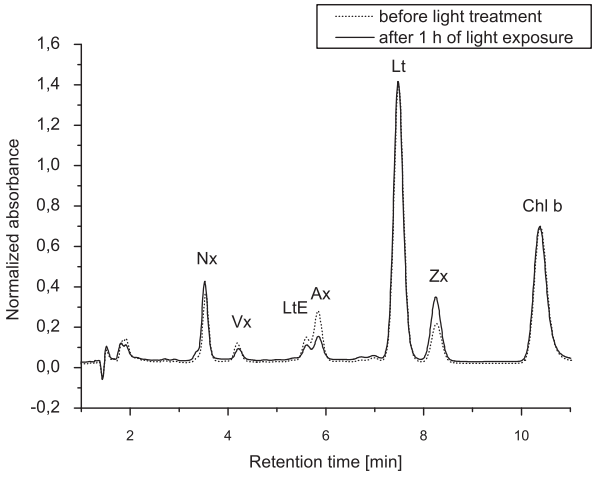

Figure 5. Light-induced changes in xanthophyll profile in MaZEP7 plants – examples of chromatograms obtained from leaf extracts taken before and after 1 h of light treatment. Absorbance was normalized in respect to chl *a* content.

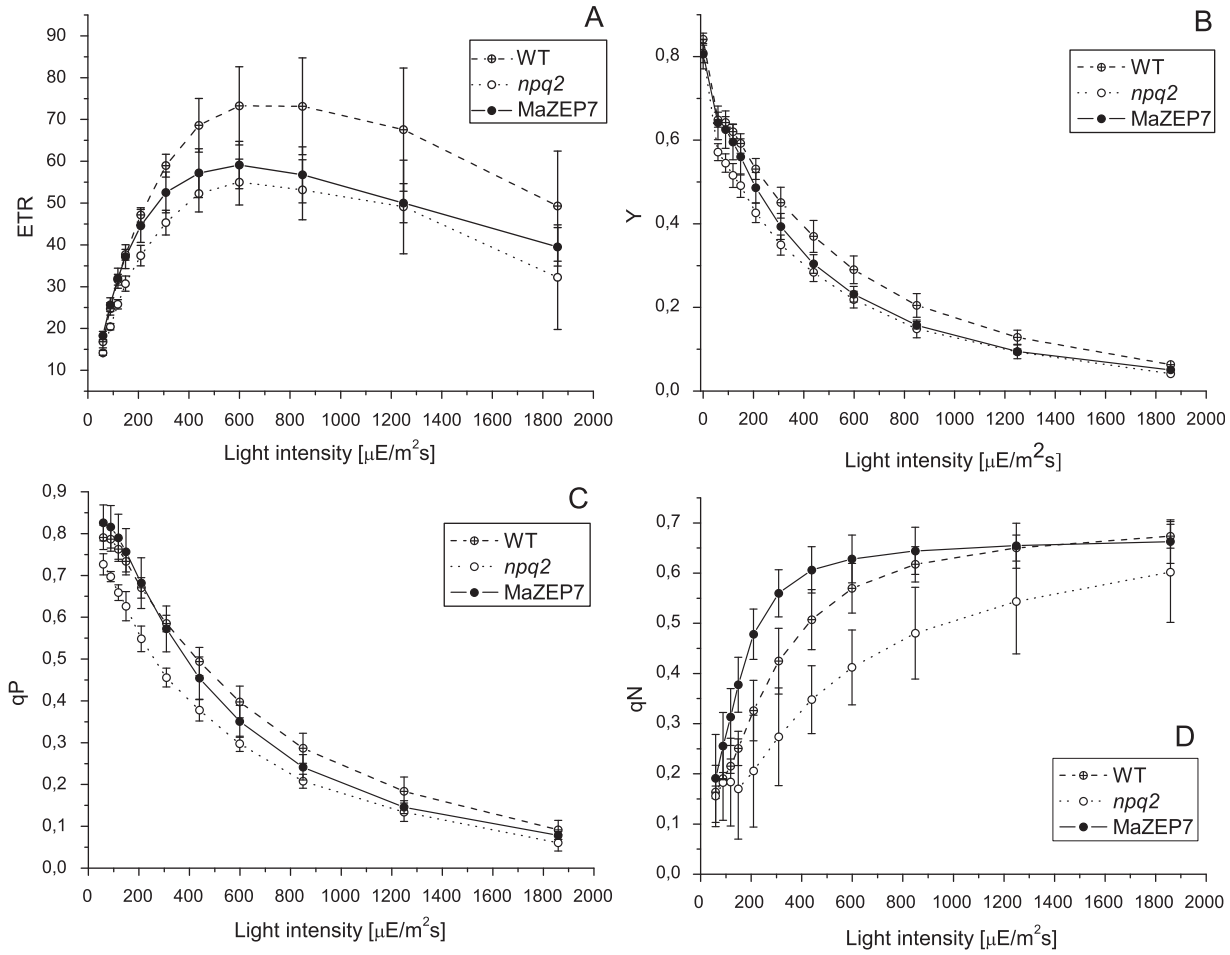

Figure 6. Light response curves. WT – wild type, MaZEP7 – transgenic line, *npq2* – *npq2* mutant. (A) ETR calculated as $Y \times PAR \times 0.5 \times 0.84$; (**B**) Y – quantum yield of PS II: maximal quantum yield calculated as $(F_m - F_0)/F_m$ in dark acclimated leaf is shown as value for light intensity = 0 and quantum yield for light exposed leaf calculated as $(F_{m'} - F_s)/F_{m'}$ for all other points; (C) qP calculated as $(F_{m'} - F_s)/(F_{m'} - F_0)$; (D) qN calculated as $(F_m - F_{m'})/(F_m - F_0)$. Before experiments, plants were dark-adapted for 40 min. Values are mean ± SE ($n = 5$). SE is indicated by bars when larger than symbol.

In low light intensities (below 200 $\mu E/m^2 s$), ETR values for WT and MaZEP7 plants were nearly the same, whereas for the *npq2* line they were slightly lower (Figure 6A). In stronger light, the ETR of WT reached values significantly higher than corresponding ETR values of the other lines. In the case of the MaZEP7 line, as light intensity increased it behaved more like the *npq2* mutant. Maximal ETR values were observed for a light intensity of 600 $\mu E/m^2 s$ in all lines, although in the case of WT, nearly the same ETR was measured for 850 $\mu E/m^2 s$, whereas in other lines the ETR parameter started to decrease. For intensities above 600 $\mu E/m^2 s$ ETR values for *npq2* mutant and transgenic plants were similar. The maximal quantum yield of PS II photochemistry was similar in all lines. It was a little higher for WT (0.84) than for transgenic plants and the *npq2* mutant (both about 0.8) (Figure 6B). Regularities observed for ETR changes in the case of MaZEP7 were true for the real efficiency of the PS II photochemistry, Y (Figure 6B) and the qP parameter (Figure 6C), representing the proportion of open PS II centers. In low light the transgenic line behaved like WT and in high light, it behaved like the *npq2* mutant, which had the lowest values of Y and qP. The values of qN parameter were higher for transgenic plants than for other lines excluding the lowest (where qN values of all lines were similar) and two highest (when qN values of MaZEP7 and WT were similar) intensities of light applied, whereas the *npq2* mutant had the lowest values. We noted that qN values initially increased rapidly, but seemed to reach plateau for high light intensities. The fastest growth and earliest plateau were observed in the MaZEP7 line, and next for WT. The *npq2* mutant did not reach a plateau for light intensities used in our experiment.

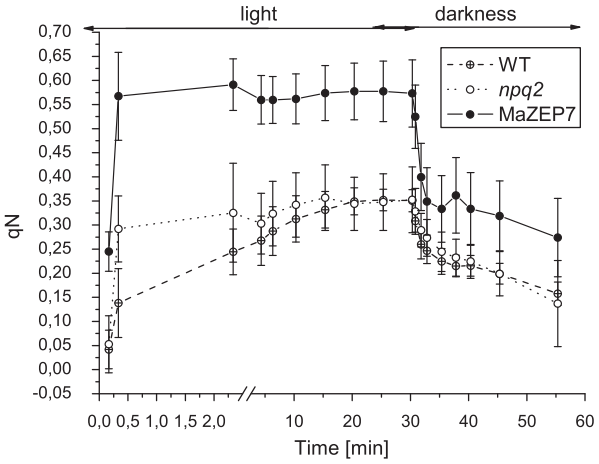

Figure 7. Time courses for induction and relaxation of qN calculated as $(F_m-F_{m'})/(F_m-F_0)$. WT – wild type, MaZEP7 – transgenic line, *npq2* – mutant *npq2*. Before experiments plants were dark-adapted for 40 min. After first measurement, actinic light with intensity 309 $\mu E/m^2 s$ was applied for 30 min, then 30 min of darkness was applied. Values are mean $\pm$ SE ($n = 5$). SE is indicated by bars when larger than symbol.

Time courses of NPQ induction (Figure 7) showed that, in the MaZEP7 and *npq2* mutant, the qN parameter rose very fast and reached a steady-state value in 20 s, while WT plants needed about 20 min to reach steady state. Steady-state values for WT and *npq2* plants were similar, while for MaZEP7, they were much higher. After switching off actinic light qN of transgenic plants decreased the fastest, but stayed on rather high level. *Npq2* mutants' qN decreased a little slower than WT, but values reached at the end of experiment were similar.

Discussion

By creating a transgenic line, we hoped to obtain plants with enhanced epoxidation due to the over-expression of ZE. Surprisingly, epoxidation in MaZEP7 plants was disturbed, but the transgenic line also differed from the *npq2* mutant without active ZE. The DNA gel blot analysis (Figure 1) showed that the MaZEP7 line contained an introduced transgene, although the number of copies of the gene could not be determined (more than one transgene was probably inserted). Detection of an additional PCR product observed in the transgenic line (around 1000 bp) suggests that T-DNA might have been tandemly integrated and also underwent rearrangements that led to truncation of one of the inserted transgene copy. In all three lines, *aba1* transcripts were present, which means that transcription is not the main factor responsible for the observed differences. The occurrence of ZE mRNA in the *npq2* mutant is also interesting. The genetic characteristics of this line are not known, but it was obtained by ethylmethane sulphonate-induced mutagenesis, which causes mainly point mutations. It is likely that there were only minor changes in the DNA sequence, which did not make transcription impossible, but the obtained protein product was inactive. The presence of *aba1* transcript in the MaZEP7 line on a level little lower than in WT means that, in this case, no over-expression occurred (in such case transcript level should be higher). The possibility exists that the localization of the transgenic protein was improper, which resulted in disturbed activity. On the other hand, *A. thaliana* has its own endogenous ZE, in which activity was also affected by an unknown mechanism. Partial silencing of the *aba1* gene could not be excluded.

Similar levels of *vde1* gene transcript in all lines led us to draw the conclusion that variations in xanthophyll cycle pigments composition and also ZE activity are not directly connected with *vde1* gene transcription, at least under the growth conditions utilized here (low light).

The differences in growth rate are also an unexplained issue. Even in the case of relatively well-studied mutants with a changed *aba1* gene (Tardy and Havaux, 1996; Hurry et al., 1997; Niyogi et al., 1998; Pogson et al., 1998; Dall'Osto et al., 2005), the resulting data are contradictory. The growth retardation was related to disabled ABA synthesis, sustained energy dissipation due to the accumulation of high levels of Zx or disturbed antenna assembly due to improper xanthophyll composition. In some of the experiments, exogenously added ABA and growing mutants in high humidity caused growth rates to revert to those of WT (Tardy and Havaux, 1996). In other experiments (Pogson et al., 1998), exogenous ABA did not cause such effects. The transgenic plants we obtained also had elevated levels of Ax and Zx, but grew even better than WT. Possibly, only portions of the Vx, Nx and Ax present in leaves are needed for proper antennae assembly, or Ax can effectively substitute for other xanthophylls. The high NPQ observed in the MaZEP7 line also did not retard plant growth, although at low light intensity (as used in growth chamber), qN values of all lines were similar. The difference in amounts of fertile seeds may indicate a role of ABA in causing growth rate differences. Similar amounts of Chl and total carotenoids as well as chl *a*/chl *b* ratios in all three lines (Table 1) showed that changes in the carotenoid profile could be compensated to some

extent. In the case of blocked epoxidation, epoxy-xanthophylls are substituted by Zx in an equimolar manner (Pogson et al., 1998), so the total amount of β-car pathway xanthophylls remains unchanged. Our experiment confirmed these results.

The xanthophyll profile in the MaZEP7 line (Figure 3) shows that epoxidation occurs but is less effective than in WT. Large amounts of Ax suggest that the second step of epoxidation is more disturbed than the first step. The presence of LtE suggests a changed specificity of ZE. Kruk and Szymańska (2008) hypothesized that the substrate specificity of ZE is broad enough to allow LtE synthesis, but this reaction depends on Lt availability. The level of Vx in the MaZEP7 mutant is low but rather constant, which indicates that the efficiency of subsequent reactions leading to Nx is lowered. This suggests their down-regulation in the case of Vx shortage or limited Vx accessibility. The occurrence of a Vx fraction unavailable for converting enzymes has been postulated (Jahns, 1995; Farber et al., 1997). It is now known that there are different Vx pools in thylakoids. There is a little free Vx in membranes, but most of it is present in different antenna complexes and binding to proteins causes limited convertibility. There are at least 11 antenna proteins belonging to chl *a*/chl b binding proteins family, 6 associated to PS II and 5 to PS I. They share a highly conserved secondary structure and there are known carotenoid-binding sites occurring in these proteins: L1, L2, N1 and V1 (N1 and V1 occur not in all LHC proteins). In all LHC proteins, Lt is bound to L1 site, but occupancy of other sites differs. In V1 and N1 sites, pigments are bound relatively loosely, and if Vx is bound to them (Lhca3-V1, Lhca1-N1) it is easily convertible, while Vx bound to L2 site (Lhcb4, Lhcb5, Lhcb6, Lhca1, Lhca2, Lhca4) remains non- or very slowly convertible (Jahns et al., 2001; Wehner et al., 2004, 2006). The de-epoxidation reaction observed in MaZEP7 plants resulted in only a very small decrease in Vx. The main substrate for VDE was Ax (Figure 5), which could be an effect of its abundance, enabling effective competition with Vx. Light treatment caused de-epoxidation of about 35% of total epoxy-xanthophylls in WT (Vx was mainly de-epoxidated) as well as in MaZEP7 (de-epoxidation in this case concerned mainly Ax and ELt).

The epoxidation reaction in WT plants started to predominate during light exposure and occurred in darkness, leading to a regeneration of the initial xanthophyll level. In the MaZEP7 line, no epoxidation was observed during the experiment examining the xanthophyll cycle, although epoxy-xanthophylls were present (but in lesser amounts). In *A. thaliana* and other examined plants, only one enzyme responsible for epoxidation of Zx has been discovered to date – ZE encoded by the *aba1* gene, showing distinct homology with other plant lipocalins (Charron et al., 2005). This led us to exclude two epoxidating enzymes – one active in xanthophyll cycle and another responsible for Vx and Nx biosynthesis and situation in which one of this protein is somehow disabled, whereas the another remains active. In the transgenic line, ZE most likely catalyzes epoxidation, but at a very slow rate.

An altered xanthophyll profile caused different behavior of MaZEP7 plants during chlorophyll fluorescence measurements (Figure 6). In low light conditions, transgenic plants were more like WT, which suggests that an initial extremely high Zx level and/or extremely low Vx level are/is needed to evoke the lowering of ETR, Y and qP. It should be noted that, at low light intensities, when capacity of photosynthesis is far from saturation, mechanisms of acclimation to high light are simply not acting, i.e. pH-dependent nonradiative quenching does not operate because of lack of sufficient pH gradient needed for conformational changes of antenna proteins. The exposure of transgenic plants to stronger light makes them behave like the mutant *npq2*, suggesting that after activation of high light acclimating processes, enhanced levels of epoxy-xanthophylls start to play a role. Moreover, in high light, Ax, abundant in MaZEP7, is converted to Zx. The *npq2* mutant is considered to dissipate excitation energy continuously (Kalituho et al., 2007) so the $F_{m'}$ value is similar to F_m; thus, the qN parameter calculated as $(F_m-F_{m'})/(F_m-F_0)$ could be low in comparison to WT. In the case of the transgenic line, qN values are high, suggesting that energy dissipation is activated in high light.

The rapid induction of NPQ in the case of the *npq2* mutant has been described previously (Niyogi et al., 1998) and was explained by a high initial Zx level, enabling Zx to participate in quenching from the beginning of the light treatment. The rapid NPQ induction in transgenic lines seems to be similar to the effect observed for the *npq2* mutant and can be explained by high levels of Zx and Ax in dark-acclimated leaves. The reason for increased NPQ in transgenic plants in comparison to *npq2* mutant and wild type remains unclear.

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