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Zeaxanthin epoxidation — an *in vitro* approach*

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Zeaxanthin epoxidase (ZE) is an enzyme operating in the violaxanthin cycle, which is involved in photoprotective mechanisms. In this work model systems to study zeaxanthin (Zx) epoxidation were developed. Two assay systems are presented in which epoxidation of Zx was observed. In these assays two mutants of *Arabidopsis thaliana* which have active only one of the two xanthophyll cycle enzymes were used. The *npq1* mutant possesses an active ZE and is thus able to convert Zx to violaxanthin (Vx) but the violaxanthin de-epoxidase (VDE) is inactive, so that Vx cannot be converted to Zx. The other mutant, *npq2*, possesses an active VDE and can convert exogenous Vx to Zx under strong light conditions but reverse reaction is not possible. The first assay containing thylakoids from *npq1* and *npq2* mutants of *A. thaliana* gave positive results and high efficiency of epoxidation reaction was observed. The amount of Zx was reduced by 25%. To optimize high efficiency of epoxidation reaction additional factors facilitating both fusion of the two types of thylakoids and incorporation of Zx to their membranes were also studied. The second kind of assay contained *npq1* mutant thylakoids of *A. thaliana* supplemented with exogenous Zx and monogalactosyldiacylglycerol (MGDG). Experiments with different proportions of Zx and MGDG showed that their optimal ratio is 1:60. In such system, due to epoxidation, the amount of Zx was reduced by 38% of its initial level. The *in vitro* systems of Zx epoxidation described in this paper enable analysis some properties of the ZE without necessity of its isolation.

Key words: zeaxanthin epoxidation, xanthophyll cycle, model system, *Arabidopsis thaliana* mutants *npq1*, *npq2*

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INTRODUCTION

Violaxanthin cycle is the most common of the six xanthophyll cycle types described so far in the literature. It occurs in higher plants, mosses, lichens and some groups of algae protecting them against overexcitation (Garcia-Plazaola *et al.*, 2007; Goss *et al.*,2006). Two reversible reactions occur in the violaxanthin cycle. One of them is de-epoxidation of a di-epoxide xanthophyll, violaxanthin (Vx), by violaxanthin de-epoxidase (VDE). This enzyme is located in the thylakoid lumen and for activation requires ascorbate as a donor of protons, monogalactosyldiacylglycerol (MGDG) or another non-bilayer lipids such as, phosphatidylethanolamine (PE) and an acidic pH which is necessary for binding VDE to thylakoid membrane (Hager & Holocher, 1994; Latowski *et al.*, 2004, Jahns *et al.*, 2009). In the reverse reaction catalysed by zeaxanthin epoxidase (ZE), zeaxanthin (Zx), an epoxy-free pigment is converted to Vx. ZE, which is located on the stromal side of thylakoids, is a monooxygenase epoxidizing 3-hydroxy β-ionone rings of xanthophylls in 5, 6 position. NADPH, FAD, molecular oxygen and pH 7.5 are required for Zx epoxidation (Siefermann & Yamamoto, 1975; Büch *et al.*, 1995). There exist model systems to study Vx de-epoxidation with the use of isolated enzyme (Yamamoto & Higashi, 1978; Latowski *et al.*, 2002). Such approach in the case of Zx epoxidation is difficult because the respective enzyme has not been isolated and purified so far. In this paper two kinds of model systems for studies on Zx epoxidation *in vitro* are presented for the first time. In both systems reduction in the Zx amount was observed. In these assays two mutants of *Arabidopsis thaliana* which have active only one of the two xanthophyll cycle enzymes were used. The first one, *npq1*, produces active ZE and it converts Zx to Vx, while VDE is inactive and photoprotection by xanthophyll cycle mechanism is not observed (Havaux *et al.*, 2000). The other mutant, *npq2*, possesses active VDE and it is able to convert exogenous Vx to Zx under strong light but reverse reaction is not possible. This mutant can accumulate Zx and it was used as a source of this pigment in our experiments.

MATERIALS AND METHODS

Two model systems of Zx epoxidation *in vitro* were tested using thylakoids isolated from *Arabidopsis thaliana*. Six week old plants grown in a 12 h/12 h photoperiod at 20°C, after 0.5 h dark incubation were illuminated for 30 min with 1500 $\mu E \cdot m^{-2}s^{-1}$ and homogenized 2 times by 10 s in isolation medium (330 mM Sorbitol, 20 mM Tricine, 5 mM EGTA, 5 mM EDTA, 10 mM Na_2CO_3, pH 7.6). After centrifugation for 5 min at 4000 rpm, the supernatant was discarded and residue was resuspended in resuspension medium (300 mM Sorbitol, 20 mM Hepes, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.6). At the end chlorophyll concentration in 80% acetone was measured by the method of Arnon (Arnon, 1949). The capability of Zx epoxidation in thylakoids isolated by above described method, was tested first for wild type of *A. thaliana*.

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Abbreviations: Ax, antheraxanthin; MGDG, monogalactosyldiacylglycerol; PE, phosphatidylethanolamine; VDE, violaxanthin de-epoxidase; Vx, violaxanthin; ZE, zeaxanthin epoxidase; Zx, zeaxanthin.

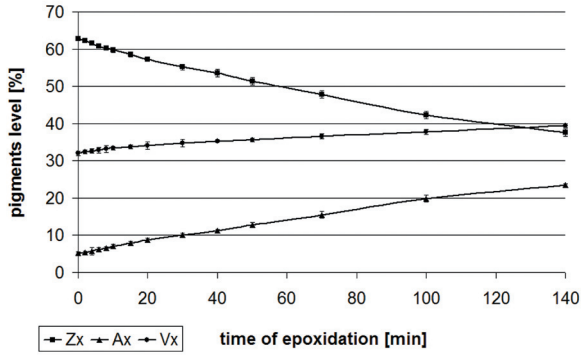

Figure 1. Kinetics of Zx epoxidation in thylakoids isolated from *Arabidopsis thaliana* wild type.
Thylakoids were sonicated for 7 seconds with 3 repeats and the amount of chlorophyll was 100 µg per ml of reaction mixture. Zx epoxidation assay was performed for 140 min. Ax, antheraxanthin; Vx, violaxanthin; Zx, zeaxanthin. The average concentrations of pigments at the beginning of epoxidation was: Vx, 14.8±0.09; Ax, 2.4±0.007; Zx, 29.0±0.029 nmol/ml.

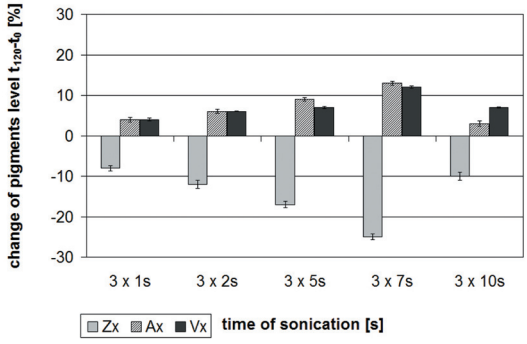

Figure 2. Change in xanthophylls level in dependence on time of sonication.
Assay system contains thylakoids isolated from *npq1* and *npq2* mutants and mixed in the amounts 1:1 and 50:50 µg of chlorophyll per ml of reaction mixture. Reaction was performed for 120 min. Ax, antheraxanthin; Vx, violaxanthin; Zx, zeaxanthin. The average concentrations of pigments at the beginning of epoxidation was: Vx, 6.97±0.04; Ax, 1.16±0.01; Zx, 20.9±0.44 nmol/ml.

In the case of assay using the mutants for Zx epoxidation *in vitro*, thylakoids from two *A. thaliana* mutants (*npq1* and *npq2*) were mixed together and then sonicated. The first tested parameter to develop the optimal model system for Zx epoxidation was the time of sonication of the *npq1*/*npq2* thylakoids mixture. Five different times of sonication were tested (1, 2, 5, 7, 10 seconds with 125 kHz and 20% amplitude) always with three repetitions. The second parameter studied in this assay system was the ratio of thylakoids from *npq1* and *npq2* mutants. The amount of thylakoids was determined by chlorophyll concentration. There were tested following *npq1* to *npq2* ratios: 1:1 (50:50 µg/ml), 1:1 (100:100 µg/ml), 3:1 (75:25 µg/ml), 3:1 (150:50 µg/ml).

In another kind of model system tested, only thylakoids from *npq1* mutant were used because they retain a capability of Zx epoxidation *in vivo*. In this system, instead of *npq2* thylakoids as the only source of Zx, the effect of different ratios of exogenous Zx and MGDG on epoxidation efficiency were studied. 20 nmol of the pigment was mixed with MGDG in different proportion (1:15, 1:30, 1:60, 1:100), using methanolic stock solutions of both compounds. After mixing, the solution was evaporated by nitrogen and then the dry residue was dissolved in ethanol. This solution was added into reaction mixture. The concentration of ethanol in the reaction medium was less than 2%. Epoxidation reaction was performed for 120 minutes at room temperature and the level of three pigments (Zx, Vx, antheraxanthin (Ax)) were measured by HPLC as described earlier (Latowski *et al.*, 2002). The reaction mixture contained: thylakoids, epoxidation medium (400 mM Sorbitol, 50 mM Hepes, 5 mM $MgCl_2$, pH 7,6), albumin, sodium ascorbate, FAD, NADPH.

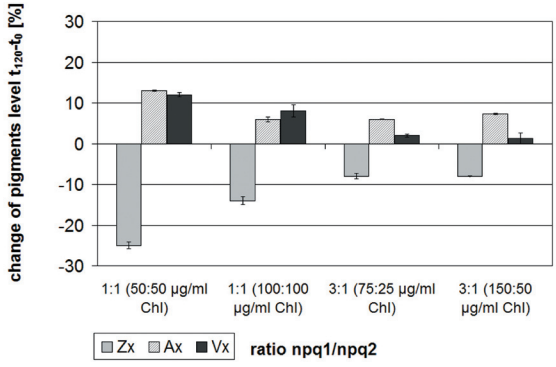

Figure 3. Change in xanthophylls level dependent on the ratio of thylakoids from both mutants.
Thylakoids were sonicated for 7 seconds with three repeats. Reaction was performed for 120 min. Ax, antheraxanthin; Chl, chlorophyll; Vx,– violaxanthin; Zx, zeaxanthin. The average concentrations of pigments at the beginning of epoxidation was: for 1:1 (50:50 µg/ml) Vx — 6.97±0.04, Ax — 1.16±0.01, Zx — 20.9±0.44 nmol/ml; for 1:1 (100:100 µg/ml) Vx — 13.4±0.08, Ax — 2.23±0.002, Zx — 40.2±0.84 nmol/ml; for 3:1 (75:25 µg/ml) Vx — 4.63±0.05, Ax — 0.56±0.003, Zx — 6.1±0.02 nmol/ml; for 3:1 (150:50 µg/ml) Vx — 7.33±0.15, Ax — 0.9±0.003, Zx — 10.5±0.05 nmol/ml.

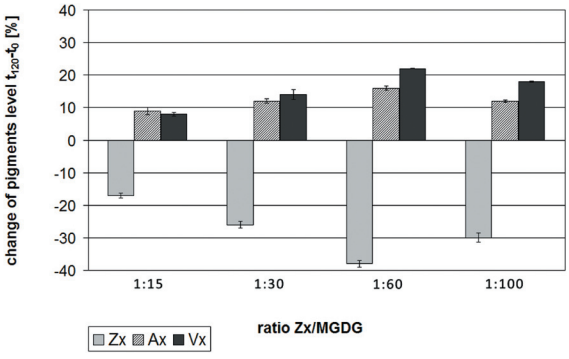

Figure 4. Change in xanthophylls level dependent on the ratio of zeaxanthin and MGDG.
Ethanolic solution of both compounds was added into reaction mixture. Reaction was performed for 120 min. Ax, antheraxanthin; MGDG, monogalactosyldiacylglycerol; Vx, violaxanthin; Zx, zeaxanthin. The concentration of exogenous Zx at the beginning of the epoxidation was 20 nmol/ml.

RESULTS AND DISCUSSION

The studies on Zx epoxidation in *in vitro* systems were not possible because up today the enzyme involved in this reaction, zeaxanthin epoxidase, has not been isolated and no model system for analysis of kinetics or molecular mechanism of this reaction has been developed. It was also difficult to study of Zx epoxidation in isolated thylakoids although two methods for thylakoids isolation

where ZE was working were described for plants. One of them was for lettuce (Siefermann & Yamamoto, 1974) and the other for spinach (Büch *et al.*, 1995). The obtained results show that our method of thylakoids isolation from model plant, *A. thaliana,* which is presented in this work, also permits to study Zx epoxidation in isolated thylakoids (Fig. 1). In fact the presented method uses a fraction containing fragmented chloroplasts with thylakoids and stroma and therefore ZE, which is located at the stromal side of thylakoids, is also present in the final suspension. In assay system using thylakoids from *npq1* and *npq2* mutants the optimal time of their sonication was defined as 7 seconds with 125 kHz and 20% amplitude with 3 repeats. It was observed that after that time of sonication, epoxidation is the most efficient (Fig. 2). Probably such time of sonication allow better fusion of the two types of thylakoids and, on the other hand, it does not cause significant destruction of isolated structures. The second parameter studied in this model system was the ratio of thylakoids from *npq1* and *npq2*. The optimal ratio was estimated as 1:1 (Fig. 3). The amount of thylakoids in reaction mixture was also important for Zx epoxidation efficiency. The optimum was defined as 100 μg of chlorophylls per 1 ml of epoxidation mixture. Higher concentration is not advantageous. In this model system with optimal proportion of the thylakoids from both mutants and optimal sonication conditions the amount of Zx was reduced by 25% (Fig. 3).

The most simplified system we studied consists of *npq1* mutant thylakoids supplemented with exogenous Zx with MGDG in varying proportions. The optimal ratio of Zx to MGDG was defined as 1:60. The low amount of ethanol added to the assay mixture with Zx and MGDG did not reduce the efficiency of the reaction. In optimal conditions the amount of Zx was reduced by 38% (Fig. 4). The *in vitro* assay with exogenous Zx, is a very promising model system permitting studies on the Zx epoxidation. The use of exogenous substrate allows better controlling the reaction conditions and provides new possibilities to investigate molecular mechanism of epoxidation in the xanthophylls cycle.

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