BRIEF COMMUNICATION

High irradiance effects on the xanthophyll cycle pigments and the activity of violaxanthin de-epoxidase in soybean callus

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Abstract

High irradiance (HI) effects on xanthophyll cycle pigments (XCP) and activity of violaxanthin de-epoxidase (VDE) in terms of de-epoxidation index (DEI) were studied in soybean calli. The calli from the hypocotyl segments of 5-d seedlings were induced on a solid (1.1 % agar) MS medium (pH 5.8) supplemented with 4.52 μM 2,4-dichloro-phenoxyacetic acid, 2.32 μM kinetin, and 3 % sucrose. After a 30 d cultivation, the green calli were irradiated for 24 h with "white light" (HI, 1 300 μmol m^{-2} s^{-1}) and VDE was isolated from the photosystem 2 (PS2) particles. In the control (0 h irradiation) callus, the reaction of PS2 particles with VDE in the presence or absence of *Tween 20* resulted in the decrease of VIO content and the increase of ZEA content. In the 24 h HI-callus, the reaction of PS2 particles in the absence of VDE led to the decrease of VIO and ANT contents and increase of ZEA content. In the control, DEIs in the presence of VDE with or without 0.1 % *Tween 20* (1.04 and 1.06, respectively) were significantly higher than the DEI (0.76) in the absence of VDE. In the HI-callus, DEIs in the presence of VDE with or without 0.1 % *Tween 20* (0.98 and 0.96, respectively) were similar to that (1.03) in the absence of VDE.

Additional key words: antheraxanthin; *Glycine max;* HPLC; neoxanthin; violaxanthin; zeaxanthin.

Xanthophyll cycle (XC) plays an essential role in the protection of plants and algae against excess photons (Demmig-Adams and Adams 1992, Schubert *et al*. 1994) with the involvement of two enzymes: (*1*) violaxanthin de-epoxidase (VDE) which is located in the thylakoid lumen and catalyzes conversion of violaxanthin (VIO) to zeaxanthin (ZEA) *via* the intermediate antheraxanthin (ANT); (*2*) zeaxanthin epoxidase (ZE) which is located on the stromal surface of thylakoid membranes and catalyzes the reverse reaction (Yamamoto 1979). ANT and ZEA are effective in the dissipation of excess photons and protect the plants from photoinhibition (Gilmore 1997, Goss *et al*. 1998). The accumulation of ZEA and ANT, along with the trans-thylakoid pH gradient, mediates non-radiative dissipation of photon energy in the antennae of chlorophylls (Björkman and Demmig-Adams 1993). This non-radiative dissipation of photon energy is an alternative energy path that diverts energy from photosystem 2 (PS2) (Rockholm and Yamamoto 1996).

Exposure of leaves to high irradiance (HI) causes the reduction of photosynthetic efficiency, a phenomenon referred to as photoinhibition (Powles 1984, Demmig-Adams *et al*. 1996). Irradiation induces the forward de-epoxidase reaction by establishing the necessary acidic lumen through the proton pump. The required acidity for de-epoxidase activity can also be generated by ATP hydrolysis or supplied by buffer (Yamamoto *et al*. 1972).

The PS2 particles are derived from granal thylakoids treated with *Triton X-100* and exposed to both the lumenal and stromal surfaces of thylakoid membranes (Murata

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Abbreviations: ANT – antheraxanthin; DEI – de-epoxidation index; HPLC – high-performance liquid chromatography; NEO – neoxanthin; PS2 – photosystem 2; VDE – violaxanthin de-epoxidase; VIO – violaxanthin; XC(P) – xanthophyll cycle (pigments); ZE – zeaxanthin epoxidase; ZEA – zeaxanthin.
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et al. 1984). In the isolated chloroplasts, addition of ascorbate, which is presumably lost during isolation, is also required as an essential and specific reductant for de-epoxidase activity (Neubauer and Yamamoto 1992).

Biochemical study on VDE is more or less restricted, due to localization of the enzyme in the thylakoid lumen. The PS2 particles may be useful for the study of VDE. Simple mixing the enzyme with the particles did not manifest VDE activity, and the enzyme required a neutral detergent such as *Tween 20, 40, 60, 80, etc*. In the reaction with intact thylakoids, the exogenously added VDE did not require *Tween 20* for its functioning. It remained unknown why the detergent was necessary for the VDE reaction with the substrate in PS2 particles (Kuwabara *et al*. 1998).

VDE is stereospecific for xanthophylls that have the 3-hydroxy-5,6-epoxide group in a 3S,5R,6S configuration. Also, the polyene chain of the carotenoid must be all-*trans*. Thus NEO, which is 9-*cis*, is an inactive substrate and becomes active when isomerised to all-*trans* form (Yamamoto and Higashi 1978). VDE is a nuclear-encoded protein synthesized in the cytoplasm as a precursor with a transit peptide that directs transport of the protein into the thylakoid lumen (Bugos and Yamamoto 1996). VDE is soluble at near neutral pH, but when the lumen is acidified along with the photosynthetic electron transport, it becomes hydrophobic through protonation and binds to the membranes (Yamamoto *et al*. 1972, Büch *et al*. 1994, Hager and Holocher 1994). According to Yamamoto and Higashi (1978) and Hager and Holocher (1994), VDE is a protein of about 60 kDa, but according to Rockholm and Yamamoto (1996) and Bugos and Yamamoto (1996) of only about 40 kDa. However, depending upon the two-dimensional IEF/SDS-PAGE results, Rockholm and Yamamoto (1996) described that VDE is a 43 kDa protein. Kinetics of VDE has been investigated in the leaf of Romaine lettuce (*Lactuca sativa*) (Rockholm and Yamamoto 1996), spinach (Kuwabara *et al.* 1998), and *Arabidopsis thaliana* and *Nicotiana tabacum* L. (Hieber *et al*. 2002). Also, dynamic changes of NEO, VIO, ANT, ZEA, α-carotene, and β-carotene under HI were investigated in soybean callus (Pandey *et al*. 2003). However, the activity of VDE in XC with the PS2 particles of the callus, which contains less chlorophylls and incomplete granal and stromal thylakoids, could exhibit different responses under HI. Therefore, we investigated the xanthophyll cycle pigments (XCP) and activity of VDE with HI in green callus of soybean.

Soybean (*Glycine max* cv. Pungsannamulkong) callus culture was made according to Pandey *et al*. (2003). After 30 d of cultivation, the green calli 0.4–0.8 cm thick were irradiated with "white light" from a 150 W *HQI* lamp (irradiance of 1 300 μmol m^{-2} s^{-1}) for 0 and 24 h.

The PS2 particles from the 0 and 24 h-HI-calli were prepared as described by Kuwabara and Hashimoto (1990) and Kuwabara *et al*. (1998) with some modifications. Typically, each 100 g of the calli was macerated with 10 cm^3 of 50 M Na-K phosphate buffer (pH 6.4)/0.3 M sucrose/100 mM NaCl (1 : 1 : 1, v/v). Next, 30 cm^3 of 20 % (m/v) *Triton X-100* was added with gentle stirring. The mixture was centrifuged with a *Beckman J2-MC* centrifuge at 180×*g* for 2 min. The supernatant was centrifuged at 17 420×*g* for 40 min; the chloroplast pellet was suspended in 4 cm^3 of 40 mM Na-K phosphate buffer (pH 6.4) and centrifuged at 180×*g* for 3 min. The supernatant was again centrifuged at 23 700×*g* for 40 min; the pellet was suspended in 3 cm^3 of 25 mM 4-morpholineethanesulfonic acid/NaOH buffer (pH 6.5)/0.3 M sucrose/10 mM NaCl (medium A, 1 : 1 : 1, v/v) and centrifuged at 23 700×*g* for 15 min. The thylakoid pellet (PS2 particles) was suspended in 4 cm^3 of medium A, and 3 cm^3 of the thylakoid suspension (PS2 particles) was used for the extraction of VDE.

Extraction of VDE was made with some modifications in the method of Kuwabara *et al*. (1998). The thylakoid suspension (PS2 particles, 3 cm^3) was centrifuged at 17 420×*g* for 30 min; the thylakoid pellet was re-suspended in 3 cm^3 of 50 mM Tris-HCl buffer (pH 7.5)/1 mM $MgCl_2$ (1 : 1, v/v) and then sonicated with *1210R-DTH Branson Ultrasonic Cleaner* (Danbury, USA) for 20×12 s at 10 s intervals. The VDE suspension was centrifuged at 27 220×*g* for 90 min and the supernatant was designated the VDE extract.

Assay of VDE activity was carried out as described by Kuwabara *et al*. (1998) with some modifications. The VDE extract was brought with 0.025 cm^3 of 0.1 % (m/v) *Tween 20* or without. Volume of 1.2 cm^3 of 50 mM sodium acetate–HCl buffer (pH 4.9) were added to 0.1 cm^3 of the suspension of PS2 particles and 0.2 cm^3 of the VDE extract supplemented with 0.1 % *Tween 20* or without. The reaction was initiated by addition of 0.06 cm^3 of 0.8 M sodium ascorbate. Then, the mixture was incubated at 30 °C for 1 h. The reaction was stopped by addition of 0.06 cm^3 of 0.2 M dithiothreitol (Yamamoto and Kamite 1972). The particles were collected by centrifugation at 27 220×*g* for 30 min and analysed for the content of XCP by HPLC (Kuwabara *et al*. 1998, de las Rivas *et al*. 1989). The de-epoxidation state of the membranes was described in terms of the de-epoxidation index (DEI) = (ANT+2 ZEA)/(VIO+ANT+ZEA), which indicates the average number of the de-epoxidized residue in the molecules of the XCP, and hence $0 \leq DEI \leq 2$ (Kuwabara *et al*. 1998).

The XCP from the PS2 particles was extracted with 0.5 cm^3 of acetone-methanol (7 : 2, v/v) by vortexing for 5 min and centrifuged at 17 420×*g* for 30 min. To the supernatant, 0.025 cm^3 of dichloromethane was added. Dichloromethane had been mixed with 1/100 vol. of 1 M Tris-HCl buffer (pH 8.0) to prevent acidification that causes the rearrangement of 5,6-epoxide of VIO to the furanoid-5,8-epoxide. The colourless insoluble materials were precipitated by centrifugation at 27 220×*g* for 30 min (Kuwabara *et al*. 1998). The supernatant was used for analyses of XCP with a high-performance liquid chro-

matography (HPLC) according to Pandey *et al*. (2003). According to de las Rivas *et al*. (1989), the mobile phase consisting of acetonitrile : methanol : water : ethyl acetate (7.00 : 0.96 : 0.04 : 2.00, v/v) was used for the separation. The injection volume was 0.02 cm^3 with the flow rate of 0.8 cm^3 min^{-1} for 15 min to separate various XCP. Peaks were identified according to Val *et al*. (1986), de las Rivas *et al*. (1989), and Pandey *et al*. (2003). Data of three replications were analysed using *t*-test and LSD test after ANOVA.

In the control (0 h) callus, the reaction of VDE with the PS2 particles in the absence of *Tween 20* at 30 °C for 1 h resulted in the decreases in VIO and ANT contents (25.9 and 5.2 %, respectively) and increase in ZEA content (107.3 %). Further, the reaction of VDE with PS2 particles in the presence of *Tween 20* induced the reduction of NEO and VIO contents (7.9 and 17.8 %, respectively), but an enhancement of ANT and ZEA contents (34.1 and 145.6 %, respectively) (Fig. 1, *top*). In the 24 h-HI-callus, the reaction with PS2 particles in the absence of VDE led to the decreases of VIO and ANT contents (17.9 and 30.3 %, respectively) and increase of ZEA content (117.0 %), but the content of NEO remained similar to that in the control callus. Also, the reaction of VDE with PS2 particles in the absence of *Tween 20* showed that ANT content increased (31.5 %), but VIO and ZEA contents decreased (10.0 and 29.3 %, respectively) as compared to the reaction in the absence of VDE. However, the reaction of VDE with PS2 particles in the presence of *Tween 20* increased NEO and ANT contents (5.7 and 26.2 %, respectively), but decreased VIO and ZEA contents (41.9 and 50.7 %, respectively) as compared to the reaction in the absence of VDE (Fig. 1, *top*).

In the control callus, DEI in the reaction with PS2 particles in the absence of VDE was the lowest (0.76±0.02). However, DEIs in the presence of VDE supplemented with or without 0.1 % *Tween 20* (1.04±0.01 and 1.06±0.05, respectively) were significantly higher than that (0.76±0.02) in the absence of VDE (p<0.01). In the 24 h HI-callus, DEI (1.03±0.05) in the absence of VDE was similar to those in the presence of VDE supplemented with or without 0.1 % *Tween 20* in the control callus. And also, DEIs in the presence of VDE supplemented with or without 0.1 % *Tween 20* (0.98±0.03 and 0.96±0.04, respectively) were similar to that (1.03±0.05) in the absence of VDE (Fig. 1, *bottom*).

We found that in the control callus, VDE enhanced the conversion of VIO into ZEA and the presence of *Tween 20* stimulated the reaction. Our findings support that of Kuwabara *et al.* (1998) who showed that *Tween 20*

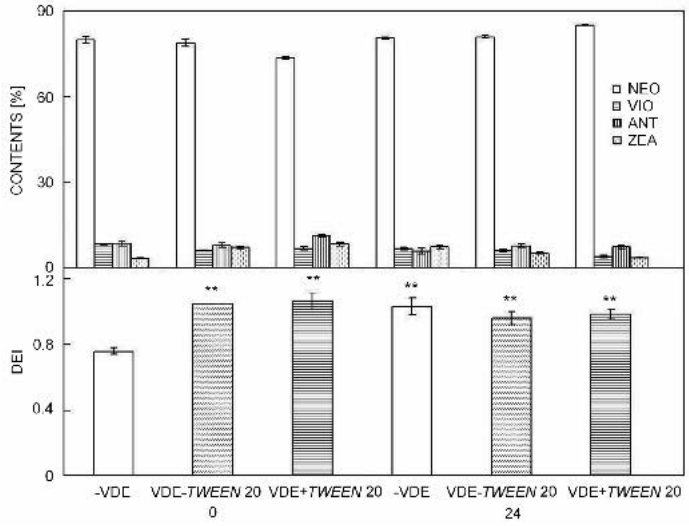

Fig. 1. The contents [%] of the xanthophyll cycle pigments, neoxanthin (NEO), violaxanthin (VIO), antheraxanthin (ANT), and zeaxanthin (ZEA) (*top*), and de-epoxidation indexes (DEI) (*bottom*) in the presence or absence of violaxanthin de-epoxidase (VDE) and *Tween 20* from the reactions with photosystem 2 particles at 30 °C for 1 h in 0 and 24 h calli of soybean irradiated with 1 300 µmol m^{-2} s^{-1}. Means ± SE, n = 3, **values significantly different at p<0.01 in the LSD test.

is essential for VDE activity when PS2 particles are used as a substrate, but its presence is not essential when VIO is used as a substrate. In the 24 h HI-callus, the PS2 particles reaction in the absence of VDE resulted in the decreases of VIO and ANT contents and in increase of ZEA content. Similarly, HI-treated soybean callus contained less VIO (by 22.8 %) and more ZEA (by 72.0 %) (Pandey *et al.* 2003). According to Woitsch and Römer (2003) the "white light"-irradiation of de-etiolating seedlings of tobacco (*Nicotiana tabacum* L. cv. Samsun) resulted in increases in the amounts of mRNAs of carotene hydroxylase, ZE, and VDE.

In the 24 h HI-callus, the reaction of PS2 particles in the presence of VDE and 0.1 % *Tween 20* induced an increase in NEO and ANT contents and a decrease in VIO and ZEA contents. These results could be explained by (*1*) the findings of Woitsch and Römer (2003) in which HI has activated the mRNAs of ZE that stimulated the

conversion of ZEA into ANT and VIO. HI-stimulation of endogenous ZE has overcome the cumulative effect of endogenous and exogenous of VDE, and/or (*2*) there was a reversible inter-conversion of VIO into ZEA, operating when photons are in excess for photochemistry (Demmig-Adams 1990, Pfündel and Bilger 1994). The cycle comprises de-epoxidation and epoxidation sequences catalyzed by two different enzymes. The de-epoxidase that converts di-epoxide VIO *via* ANT (mono-epoxide) into the epoxide free ZEA is located on the luminal surfaces of thylakoid membranes. The enzyme activity requires ascorbic acid and optimal luminal pH of 7.5 (Sierfermann and Yamamoto 1975). The presence of *Tween 20* decreased the VDE activity by affecting the luminal pH or the substrate structure (Kuwabara *et al.* 1998) that resulted in conversion of VIO into ANT, but not into ZEA. In the present study we found that in the control callus the DEIs for the reaction of PS2 particles in the absence or presence of VDE without or with *Tween 20* were 0.76, 1.04, and 1.06, respectively. In agreement with our findings, Kuwabara *et al.* (1998) reported that under the normal irradiance, the DEIs for spinach leaf under the above conditions were 0.5, 1.1, and 1.0, respectively.

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