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# Expression of *vde* gene integrated into tobacco genome in antisense and overexpressed ways

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## Abstract

Violaxanthin de-epoxidase (VDE) is localised in the thylakoid lumen of chloroplasts and catalyses de-epoxidation of violaxanthin into antheraxanthin and zeaxanthin. Tobacco *vde* gene was inserted into a binary vector pCAMBIA1301 with the hygromycin resistant gene for selection in antisense and overexpressed ways. Two constructs with antisense and overexpressed *vde* gene were introduced in tobacco (*Nicotiana tabacum* L.) using *Agrobacterium tumefaciens* strain LBA4404. PCR and Southern blot analyses demonstrated that the exogenous gene was integrated into genome of tobacco plants. VDE activity assay and HPLC analysis of pigments showed that the *vde* gene was expressed in the overexpressed transformants, whereas suppressed in the antisense ones. The chlorophyll fluorescence measurements proved that the contents of VDE in transgenic plants have a significant function in non-photochemical quenching.

*Additional key words*: *Nicotiana*; non-photochemical quenching; transgenic plants; violaxanthin de-epoxidase; xanthophyll cycle.

## Introduction

When plants absorb more photons through photosynthesis, the excess energy can result in photoinhibition, or photo-oxidative damage to photosynthetic apparatus by the formation of toxic substances. In nature, higher plants have evolved various mechanisms to cope with the excess-photon conditions (Niyogi 1999, Ort 2001). Much interest has been focused on the possible role of zeaxanthin (Z), a component of the xanthophyll cycle, in photoprotection (Demmig-Adams *et al.* 1996). The xanthophyll cycle consists of the enzymatic interconversions of three carotenoids, which are violaxanthin (V), antheraxanthin (A), and Z. Violaxanthin de-epoxidase (VDE) is a key enzyme in this cycle. The de-epoxidation of V to Z is catalysed by VDE localised in the thylakoid lumen (Hager 1969, Yamamoto *et al.* 1972), and requires ascorbate and optimal pH near 5.0 (Bratt *et al.* 1995). Later, the experiments demonstrated that both Z and A mediate the non-radioactive dissipation of energy as heat (Gilmore *et al.* 1992, 1993, 1995) to protect photosynthetic apparatus from photoinhibition.

Recently, a number of studies have been made on biochemistry and molecular biology of VDE. The *vde* gene was isolated from plants and identified (Bouvier *et al.* 1996, Bugos *et al.* 1996), and amino acid sequence of the VDE was determined, showing that it belongs to the member of lipocalin family (Bugos *et al.* 1998). The relationship between Z+A and energy dissipation measured as NPQ was conclusively demonstrated using an *Arabidopsis* mutant lacking VDE activity (Niyogi *et al.* 1998). The *vde* gene is developmentally expressed, but the reason for it has not been understood yet (Bugos *et al.* 1999). In tobacco plants with inserted antisense *vde* gene the VDE activity was reduced by more than 95 % (Chang *et al.* 2000, Sun *et al.* 2001, Verhoeven *et al.* 2001). These results demonstrate that the protective function of the violaxanthin cycle is apparent only under stress. To determine the function of different domains in the VDE protein, also Hieber *et al.* (2002) constructed tobacco plants that overexpress the VDE protein, and confirmed the protective function of the VDE in the xanthophyll cycle and provided the evidence that photoprotection is a complex process.

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*Abbreviations*: A – antheraxanthin; Chl – chlorophyll; NPQ – non-photochemical quenching; PFD – photon flux density; PS2 – photosystem 2; V – violaxanthin; VDE – violaxanthin de-epoxidase; Z – zeaxanthin.
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In this report, we tried to further evaluate the relationship between Z formation and NPQ, and to con-firm the function of the xanthophyll cycle in heat dis-sipation. We put the *vde* gene into tobacco plants using both overexpressed and antisense approaches and de-termined the VDE protein activity in two types of transgenic tobaccos.

## Materials and methods

**Materials**: Tobacco (*Nicotiana tabacum* L.) Wisconsin 38 was provided by Prof. Mi Ma. *Escherichia coli* strain DH5α and *Agrobacterium tumefaciens* strain LBA4404 were kept in our laboratory. Plasmid pCAMBIA1301 (pCB1301) with CaMV 35s promoter, reporter gene *gusA*, and plant-selected gene *hpt* encoded for hygromycin phosphate transferase was used as a binary vector to do transformation.

**Tobacco *vde* cDNA gene cloning and expression vectors constructing**: Total RNA was extracted from wild tobacco leaves by *Trizol* according to the manufacturer's instruction, and tobacco cDNA was done by RT-PCR. The polymerase chain reaction (PCR) was performed using cDNA as a template and synthetic oligo-nucleotides as primers A (5' primer, ACG CTA GCA TGG CTC TTG CCC CTC; 3' primer, GCG GTC ACC TAC TTA CC TTA ATT TCC), and primers B (5' primer, CTG AGC TCG GTT GAT GCT CTC AAG; 3' primer, ACG GAT CCT TAT TGG TAA AGC ACG). The PCR product A was inserted in forward way into *Nhe* I-*BstE* II site of pCAM-1301 by partial enzyme digestion and the sequence was analysed to confirm whether it encodes the sequence of VDE region. The PCR product B was inserted in reverse way into pCB-GUS (R.-C. Lin, unpublished) containing a cauliflower mosaic virus (CaMV) 35s promoter using *BamH* I and *Sac* I site (data not shown).

**Transformation**: The overexpressed and antisense constructs were, respectively, transformed into *Agrobacterium tumefaciens* strain LBA4404 by means of triparental mating (Hoekema *et al.* 1983). Tobacco transformation was carried out as described by Horsch *et al.* (1985) and Fisher *et al.* (1995) with modification. Transformants were selected on MS medium containing 3 % sucrose, 100 g $m^{-3}$ hygromycin, and 500 g $m^{-3}$ carbenicillin.

**PCR and Southern blot analyses**: Total genomic DNAs were extracted from young leaves of transformed and wild-type plants according to SDS method (Sambrook *et al.* 1989). PCR was carried out in a system of 20 $mm^3$ containing 50 pmol of each oligonucleolide primer, 250 mmol of dNTP, 1 U of Taq polyerase, and PCR reaction buffer, 30 ng template DNA to detect the *hpt* gene, and the plasmid used for positive control. Thermal cycles included a beginning of 94 °C for 3 min and 35 repeats of 94 °C for 40 s, 55 °C for 50 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

DNAs isolated from some of the transformed plants were analysed in detail using Southern hybridisation method (Sambrook *et al.* 1989). Twenty μg of total genomic DNAs from transgenic and non-transformed plants were digested with *Hind* III restriction enzyme and separated by electrophoresis in 0.8 % agarose gels. Moreover, DNA from the antisense plant was digested with *BamH I/Sac* I and that from the overexpressed plant was digested with *Nhe I/BstE* II. The digested DNAs in gels were blotted onto *Hybond-N* (*Amersham*) nylon membranes and hybridised with probe $^{32}$P-dCTP labelled Tvde fragment. After washing the membrane, it was exposed to X-ray film for 7 d.

**VDE extraction and assay**: Extraction and assay of VDE were performed as previously described by Bugos *et al.* (1999). A unit of VDE activity is defined as 1 nmol of V de-epoxidised per min. Activity was calculated from the initial rate of absorbance change using the difference extinction coefficient of 63 $mM^{-1}$ $cm^{-1}$ determined at 502 nm against 540 nm reference wavelength (Yamamoto *et al.* 1978, Yamamoto 1985). V was isolated from a saponified methanol extract of spinach leaves by gradient chromatography on silica gel column using the acetone: *n*-hexane (4 : 6) system.

**Analysis of pigments** extracted from tobacco leaf discs (1 $cm^2$) near the tip was conducted by reverse phase HPLC according to Thayer and Björkman (1990) and Haldimann *et al.* (1995). Isocratic separation was carried through by methanol : acetonitrile : ethylacetate (50 : 40 : 10) from 0 to 17 min and ethylacetate from 17 to 20 min on a *Zorbax ODS C18* (4.6×250 nm, 20 % C, 5 μm particle size) reverse phase column protected by a guard column [*Agilent Zorbax Original, RP18* (non-endcapped), 10 μm particle size]. The column was re-equilibrated with methanol : acetonitrile : ethylacetate (50 : 40 : 10) for 10 min. The flow rate was 16.7 $mm^3$ $s^{-1}$.

**Chlorophyll (Chl) fluorescence induction** was measured with a *PAM-2000* portable fluorometer (*Walz*, Effeltrich, Germany) equipped with a leaf holder (Schreiber *et al.* 1986). Leaves were dark-adapted for approximately 2 h prior to analysis and were exposed to actinic irradiation (2 000 μmol $m^{-2}$ $s^{-1}$) for 5 min and dark-adapted for additional 15 min. Saturating pulses (1.2 s) of "white light" (~7 000 μmol $m^{-2}$ $s^{-1}$) were applied to determine $F_m$ or $F_m'$ values. Conventional fluorescence nomenclature was that of Van Kooten and Snel

(1990) and NPQ was calculated as $(F_m - F_m')/F_m'$. Fluorescence parameters used are the maximal efficiency of photosystem 2 (PS2) photochemistry for dark-acclimated leaves, $F_v/F_m$. Following high irradiance treatment, the leaves were relaxed for 30 min under dark-adaptation, and $F_v/F_m$ was determined.

## Results and discussion

**Transformed plants**: Leaf discs of tobacco were transformed by *Agrobacterium tumefaciens* LBA4404 containing the binary vector. The regenerated hygromycin resistant plants were transferred to soil in plastic pots. Both 80 transformed $T_0$ plants with antisense and overexpressed *vde* gene were grown in greenhouse.

**PCR and Southern blot identification of $T_0$ transformed plants**: PCR amplification of the *htp* gene showed a 1.0 kb band as expected in each transformed plant, whereas no such product appeared in wild-type (WT) tobacco (data not shown).

Integration of *Tved* gene into genome of tobacco plants in two expressed constructs was verified by Southern hybridisation (Fig. 1). The total genomic DNA extracted from WT and transformed plants and restricted only with *Hind* III resulted in a single signal band after hybridisation with a random-primed labelled probe prepared from the tobacco VDE cDNA (data not shown). The total genomic DNA extracted from antisense transgenic plant TA69 and cut with *BamH* I/*Sac* I, and that

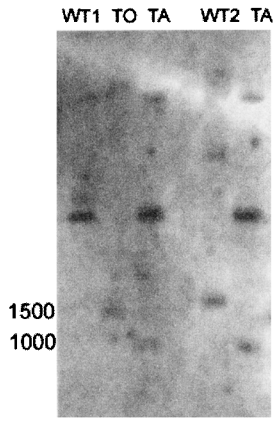


Fig. 1. Southern blot analysis of $T_0$ transformed tobacco plants. WT1, WT2, wild-type plants; TO, the overexpressed plant; TA, the antisense plant. DNA from WT1, TA cut with *BamH* I/*Sac* I, and from WT2, TO cut with *Nhe* I/*BstE* II.

from overexpressed transgenic plant TO13 with *Nhe* I/*BstE* II cutting resulted in different hybridising signals (Fig. 1). This could suggest that the *Tvde* gene in the two expressed constructs was integrated into the tobacco genomes. Hybridisation of the genomic DNAs from the wild-type plants with the probes showed two bands (Fig. 1).

**VDE activity**: Specific activities of VDE extracted from two types $T_0$ transgenic and WT plants were analysed in relation to total Chl *a*+*b* content (Fig. 2). The VDE activity in the plants TA7 and TA69 was decreased by more than 60 % and in the plants TO120 and TO13 was increased approximately by 75 %. It means that the *Tvde* gene inserted in two ways was expressed.

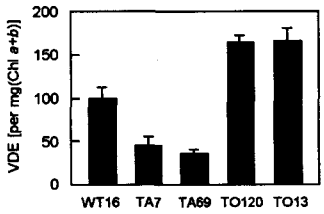


Fig. 2. Violaxanthin de-epoxidase (VDE) activity based on total chlorophyll content in leaves of wild-type (WT) and TA and TO plants in $T_0$ generation. *Error bars* represent SD values ($n = 3$).

**Changes in contents of photosynthetic pigments**: The pigments in the leaves of TA7, TA69, TO13, TO120, and WT plants were quantified by HPLC. The Z content and the extent of de-epoxidation in leaves from dark-adapted plants and those treated with high PFD (2 000 μmol $m^{-2}$ $s^{-1}$ for 30 min) were analysed. The TA plants either in dark or under high PFD had a similar content of Z, but the WT and TO plants showed very high content of Z under high PFD. However, TO13 and TO120 plants had more Z than WT plants (Fig. 3*A*). The de-epoxidation state (DES) [(Z+A/2)/(V+A+Z)] of the TO plants was obviously in-creased, whereas that of the TA plants was changed only slightly (Fig. 3*B*). The xanthophyll-cycle pool (V+A+Z) did not significantly differ between WT and transgenic plants (data not shown).

**Chl fluorescence during high PFD**: Chl fluorescence indicated as NPQ of WT, TA69, and TO120 plants was examined in the laboratory using detached leaves under PFD of 2 000 μmol $m^{-2}$ $s^{-1}$ (Fig. 4). Under irradiation, all plants showed a different level of NPQ increase. The response rate of NPQ induction to high PFD in TO120 was the highest within 5 min. However, in our experiments there was almost no difference in the final extent of NPQ in contrast to that reported for WT plants by Hieber *et al.* (2002). Certainly, it is necessary to explain this different result by further work. The de-epoxidation of xanthophyll cycle was significantly lower in TA69 than WT, which corresponds to its lower NPQ.

The kinetics of $F_v/F_m$ recovery in detached leaves of TA69, TO120, and WT after exposure to high PFD (2 000 μmol $m^{-2}$ $s^{-1}$) for 1 h was reduced. The $F_v/F_m$ of TA69 was lower than that of WT, while that of TO120 decreased only slowly. At sun acclimation, the maximal yield of photochemistry ($F_v/F_m$) in the TA69 leaves treated by high PFD was thus significantly reduced as compared to the WT plant (Fig. 5).

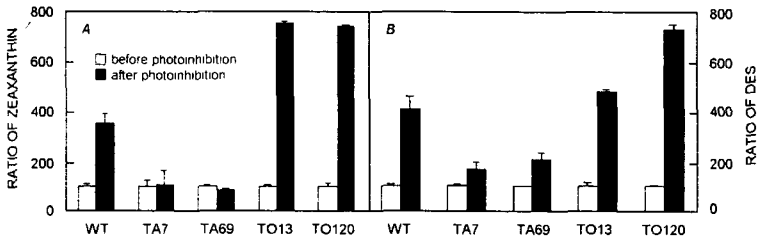


Fig. 3. Zeaxanthin content and de-epoxidation state (DES) [(Z+A/2)/(A+Z+V)] from the dark-adapted and photoinhibited leaves of WT, TA, and TO plants determined by HPLC. *Error bars* represent SD values ($n = 5$).

Our results demonstrate that the *Tvde* gene in the forms of either overexpressed or antisense constructs was successfully transferred into tobacco genome and could be expressed (Fig. 2). This report for the first time shows that the VDE activities were simultaneously confirmed in TO plants with increased level and in TA plants with decreased level. Similar reports in the past were presented only in overexpressed pattern or only in antisense pattern.

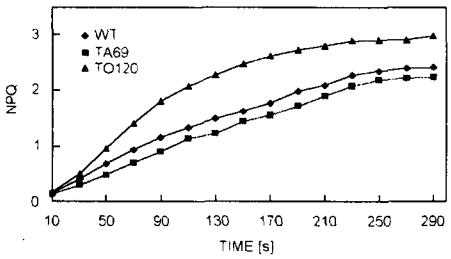


Fig. 4. Time course of NPQ induction in the detached leaves of tobacco plants WT, TA69, and TO120. Dark-adapted leaves were exposed to 2 000 μmol(photon) $m^{-2}$ $s^{-1}$ and the NPQ of each sample was measured in 20 s intervals.

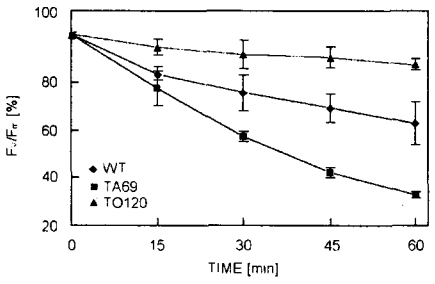


Fig. 5. Effect of irradiation at 2 000 μmol(photon) $m^{-2}$ $s^{-1}$ on the photochemical efficiency ($F_v/F_m$) in detached leaves of WT, TA69, and TO120 plants. $F_v/F_m$ value before high PFD irradiation was used as 100 %. *Error bars* represent SD values ($n = 3$).

This study shows that two types of transgenic plants specifically respond to photoinhibitory treatments and that there is a strong quantitative correlation between the formation of Z in the xanthophyll cycle and a type of fluorescence quenching which is indicative of increased non-radioactive energy dissipation. The transgenic and WT plants in the high PFD treatment have a different content of Z (Fig. 3*A*) and corresponding decreased trends in the maximal yield of PS2 photochemistry ($F_v/F_m$) (Fig. 5). This supports our current understanding of the functional significance of Z-dependent energy dissipation. The dissipation of excitation energy is measured as the non-photochemical quenching of Chl fluorescence (NPQ). NPQ consists of three components: $q_E$, which is dependent on thylakoid pH gradient, $q_T$, state-transition-dependent quenching, and $q_I$, photoinhibition-dependent quenching (Horton *et al.* 1988). The possible role of xanthophyll cycle as a determinant of quenching capacity was examined by the $q_I$ component. However, in the TO plant the response to high PFD showed a substantially higher initial rate of NPQ induction (Fig. 4), similar to that observed by Hieber *et al.* (2002). The TA, TO, and WT plants had differently increased NPQ trends with the 5-min high PFD treatment. The possible reasons for this would be that the biochemical control of the xanthophyll cycle is related to the initial effect of the non-photochemical quenching mechanism.

Our results provide the evidence that the interconversions of V to Z, or accumulation of Z may be correlated to variable fluorescence ($F_v/F_m$) decline in the experimental conditions when the *vde* gene from tobacco is inserted into tobacco itself and in forward and reverse ways simultaneously. The results probably reflect the verity of the *Tvde* gene expression and its role in catalysis of V to Z as compared with the results of Sun *et al.* (2001) and Hieber *et al.* (2002).

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