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Molecular cloning of violaxanthin de-epoxidase from romaine lettuce and expression in *Escherichia coli*

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ABSTRACT **Plants need to avoid or dissipate excess light energy to protect photosystem II (PSII) from photoinhibitory damage. Higher plants have a conserved system that dissipates excess energy as heat in the light-harvesting complexes of PSII that depends on the transthylakoid ΔpH and violaxanthin de-epoxidase (VDE) activity. To our knowledge, we report the first cloning of a cDNA encoding VDE and expression of functional enzyme in *Escherichia coli*. VDE is nuclear encoded and has a transit peptide with characteristic features of other lumen-localized proteins. The cDNA encodes a putative polypeptide of 473 aa with a calculated molecular mass of 54,447 Da. Cleavage of the transit peptide results in a mature putative polypeptide of 348 aa with a calculated molecular mass of 39,929 Da, close to the apparent mass of the purified enzyme (43 kDa). The protein has three interesting domains including (*i*) a cysteine-rich region, (*ii*) a lipocalin signature, and (*iii*) a highly charged region. The *E. coli* expressed enzyme de-epoxidizes violaxanthin sequentially to antheraxanthin and zeaxanthin, and is inhibited by dithiothreitol, similar to VDE purified from chloroplasts. This confirms that the cDNA encodes an authentic VDE of a higher plant and is unequivocal evidence that the same enzyme catalyzes the two-step mono de-epoxidation reaction. The cloning of VDE opens new opportunities for examining the function and evolution of the xanthophyll cycle, and possibly enhancing light-stress tolerance of plants.**

Light is essential for photosynthesis but potentially can damage the photosynthetic apparatus when the intensity exceeds photosynthetic capacity. Plants have evolved various mechanisms to cope with the excess-light conditions that, in fact, are experienced by most plants in natural environments. In higher plants, a highly conserved feedback mechanism, the zeaxanthin and antheraxanthin-dependent dissipation of energy in the light-harvesting complexes of photosystem II (PSII), is now generally accepted as a system that protects PSII by dynamically modulating energy transfer from the antennae to the reaction center, thus regulating the quantum efficiency of PSII (1–6). There is clear evidence from the effects of dark ATP-hydrolysis-induced acidification of thylakoids that this system is "energy dependent" and obviously independent of electron transport or associated redox changes induced by light (7). Under such conditions, a short-lifetime fluorescence component or putative quenching complex is formed (8).

Zeaxanthin and antheraxanthin are formed from violaxanthin by violaxanthin de-epoxidase (VDE) activity in the so-called xanthophyll or violaxanthin cycle (9). De-epoxidation-dependent energy dissipation is capable of responding to light environments over a wide dynamic range from sun-flecks to long-term growth conditions mainly due to the properties of the xanthophyll cycle (10, 11). The response range apparently gives higher plants the ability to adapt photosynthetically to diverse environments and, for this reason, may have been an important factor in higher-plant evolution.

VDE is specific for xanthophylls that have the 3-hydroxy-5,6-epoxy group in a *3S, 5R, 6S* configuration and are all-trans in the polyene chain (12). It is a soluble protein, localized in the lumen of thylakoids (13), and *in vitro* requires ascorbate and monogalactosyldiacylglycerol for activity (14). The enzyme was recently purified to one major polypeptide fraction using conventional techniques and a novel lipid-affinity precipitation step with monogalactosyldiacylglycerol (15). Here we describe the cloning and characterization of a cDNA for VDE from romaine lettuce and the expression of an active protein in *Escherichia coli*.

MATERIALS AND METHODS

Primers and Conditions for PCR. Peptides from a tryptic digest, along with the N terminus of VDE purified from romaine lettuce chloroplasts, were previously sequenced (15). Two peptides (N terminus and fragment no. 15; see Fig. 1) were used to develop the oligonucleotides 5′-GAYGCHYT-BAAGACHTGYGC-3′ (216-fold degeneracy) and 5′-TTGVARRTTDGGRATRAT-3′ (144-fold degeneracy). The PCR mixture contained template cDNA synthesized from poly(A)$^+$ RNA from romaine lettuce leaves (*Lactuca sativa* L. cv Romaine), 0.2 mM of each dNTP, 25 pmol of both primers, and 1.25 units of *Taq* DNA polymerase (Promega). Amplification conditions consisted of 35 cycles of 94°C for 45 sec, 50°C for 1 min, and 72°C for 1 min. The amplified DNA fragment was subcloned into the *Sma*I site of pGEM-7Zf (Promega) and sequenced.

cDNA Cloning of VDE. A cDNA library was constructed from poly(A)$^+$ RNA isolated from a pooled sample of various age romaine lettuce leaves using the Timesaver cDNA Synthesis Kit (Pharmacia) and ligated into λZAPII (Stratagene). An initial screening of 2.5×10^5 recombinant plaques using the random primed ^{32}P-labeled VDE PCR product as a probe resulted in identification of 11 positive clones, three of which were plaque purified followed by *in vivo* excision of the plasmid. The cDNAs (vde1, vde2, and vde3) were subcloned into the *Not*I site of pGEM-5Zf and both strands of cDNA were sequenced completely using an Applied Biosystems model 373A automated sequencer.

***In Vitro* Transcription/Translation.** The templates for *in vitro* transcription/translation were VDE cDNAs subcloned into the *Not*I site of pGEM-5Zf. Full-length sense RNA was transcribed by T7 RNA polymerase from uncleaved plasmid and translation was carried out in the presence of [^{35}S]methionine using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturers instructions. After incubation, aliquots from the coupled transcription/translation mixture were mixed with SDS sample buffer and

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Abbreviations: VDE, violaxanthin de-epoxidase; PSII, photosystem II.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U31462).
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electrophoresed by SDS/PAGE. Fluorography of dried polyacrylamide gels was carried out at −70°C.

Expression of Recombinant VDE, Enzyme Assay, and Quantification of Xanthophyll Pigments. Vde2 cDNA was subcloned into the *Not*I site of pGEM-5Zf and the constructs transformed into *E. coli* DH5α. The cells were grown in Luria–Bertani broth at 37°C and induced with 10 mM isopropyl β-D-thiogalactopyranoside as described (16). Following the 2-hr induction, the cells were centrifuged at 4000 × *g* for 10 min at 4°C. The cells were resuspended in 3 ml of 50 mM Tris (pH 7.4) and 1 mM EDTA, and lysed using an ultrasonic cell disruptor equipped with a microprobe for 10 cycles (30 sec on/30 sec off) in an ice bath. The resulting extract was centrifuged at 10,000 × *g* for 10 min at 4°C and the supernatant was collected. The pellet was washed with 3 ml of 50 mM Tris (pH 7.4) and 1 mM EDTA, and resuspended in 3 ml of the same buffer. VDE activity in 100 μl of extract or pellet resuspension was tested using the *in vitro* assay at pH 5.1 and absorbance change at 502 nm − 540 nm as described (14). For quantification of xanthophyll pigments, the reactions were stopped at various times with addition of solid Tris base and the xanthophylls were extracted three times with diethyl ether. The ether was evaporated under a stream of N_2 and the xanthophylls were solubilized in 100 μl 90% acetone followed by HPLC analysis (17).

Western Blot Analysis. Intact chloroplasts were isolated as described (18) and lysed with five freeze-thaw cycles using liquid N_2 (19). Expression of vde2 in *E. coli* DH5α was as described above and the cells were lysed by freeze-thaw. Proteins were resolved on a 12% SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane. The blot was developed by immunodetection using alkaline phosphatase-conjugated secondary antibodies. Protein was estimated using a prepared reagent (Bio-Rad) and bovine gamma globulin as the standard.

Northern Blot Analysis. Total RNA was isolated from lettuce leaves using guanidine thiocyanate as described (20). Poly(A)$^+$ RNA (2 μg per lane) was fractionated by electrophoresis in a 0.66 M formaldehyde/1% agarose gel and transferred by alkaline downward blotting to a nylon membrane. The RNA was hybridized at 65°C with a random-primed ^{32}P-labeled vde1 cDNA probe and washed at the same temperature as described (21). To demonstrate equal loading of RNA samples, the same blot was probed with a random-primed ^{32}P-labeled soybean ubiquitin genomic clone at 55°C and washed at the same temperature as described (21). The signals were visualized by autoradiography using preflashed film.

RESULTS AND DISCUSSION

VDE was purified from romaine lettuce (*L. sativa* L. cv Romaine) by conventional chromatography and a novel lipid-affinity precipitation step with monogalactosyldiacylglycerol (15). Tryptic peptides from the purified protein along with the N terminus were sequenced and a set of degenerate oligonucleotide primers were synthesized based on these sequences. Using the PCR, a 681-bp cDNA fragment was amplified from cDNA prepared from romaine lettuce leaves. The fragment was used as a probe to screen a romaine lettuce cDNA library and three near full-length cDNAs (vde1, vde2, and vde3) encoding VDE were isolated (Fig. 1). The vde cDNAs encompassed an open reading frame encoding a 473-aa protein with a calculated M_r of 54,447. Four peptides (including the N-terminal peptide) determined by amino acid sequencing of purified VDE were identified in the deduced peptide sequence (Fig. 1). The molecular mass of the VDE pre-protein was verified by *in vitro* transcription/translation of vde1 and vde2 cDNAs, which produced a 55-kDa product when analyzed by SDS/PAGE (Fig. 2). The deduced pre-protein contained an 125 aa putative transit peptide for transport into the chloroplast and thylakoid lumen. It is a typical bipartite transit peptide similar to those of the thylakoid lumen proteins plastocyanin (22) and the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with PSII (23). In higher plants, the bipartite transit peptide is composed of an N-terminal part for targeting to the chloroplast stroma (24) followed by a short, hydrophobic, signal peptide-like domain that functions as a thylakoid-targeting and -translocation signal (25, 26). This short hydrophobic segment is readily observed in the hydropathy plot for lettuce VDE (Fig. 3*A*) and the hydropathy analysis of the transit peptide is very similar to analyses of the bipartite transit peptides of the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with PSII (23). The N terminus of the mature VDE protein corresponds to amino acid 126 as indicated by N-terminal sequencing of purified VDE from romaine lettuce (15). Therefore, mature VDE consists of a 348-aa protein with a calculated M_r of 39,929 and a calculated pI of 4.57.

The primary structure of the deduced mature VDE exhibits some characteristic features. The protein is hydrophilic overall as observed from the hydropathy plot (Fig. 3*A*) with 57.2% of the total amino acid residues having polar side chains. The only regions of the protein exhibiting hydrophobicity are the area around the initiation methionine and the area surrounding the putative transit peptide cleavage site. Three interesting domains were identified in the deduced mature VDE including (I) a cysteine rich domain, (II) a lipocalin signature, and (III) a highly charged domain (Fig. 3*B*). In the first domain, 11 of the 13 total cysteines in the mature VDE are present suggesting that this is most likely the site where dithiothreitol (DTT), a known inhibitor of VDE (28), has its effect. The cysteines probably form more than one disulfide linkage because partial inhibition of VDE activity with DTT results in an accumulation of antheraxanthin (29). The lipocalin signature is a domain identified in a number of diverse proteins that bind small hydrophobic molecules (30). For example, crustacyanin, a protein from lobster carapace that contains a lipocalin signature, binds the carotenoid astaxanthin (31). Similarly, this domain may play a role in binding the substrate violaxanthin. In the third domain, ≈47% of the residues have charged side chains. The most striking feature of this domain is the high concentration of glutamic acid residues; 27.6% of the residues in this domain (69.2% of the total in the mature VDE) are glutamic acids whereas only 2% are aspartic acids. The functional significance of this domain is not apparent. The partial protonation of glutamic acid residues in this domain at pH 5.2, the optimum for de-epoxidase activity, possibly increases binding to the thylakoid membrane.

Authenticity of the vde cDNA was confirmed by expression of vde2 in *E. coli*. The vde2 cDNA was subcloned into the *Not*I site of pGEM-5Zf in both sense and antisense orientations with respect to *lac*Z and the constructs transformed into *E. coli* DH5α. Extracts from *E. coli* expressing vde2 orientated with *lac*Z (sense) had strong VDE activity, whereas no detectable activity was observed from extracts of *E. coli* transformed with vde2 in antisense orientation or pGEM-5Zf alone (Fig. 4*A*). Furthermore, addition of DTT, a strong inhibitor of de-epoxidase activity (28), abolished all VDE activity. Trace activity was detected in the membrane fraction of vde2 sense suggesting either that some of the enzyme was not washed away following lysis or that lysis was not complete. The vde2 cDNA (sense construct) is in frame with respect to the *lac*Z fusion protein as determined from sequence analysis. However, a VDE-*lac*Z fusion protein would not be translated because there are two termination codons in the same reading frame as the VDE pre-protein upstream of the VDE initiation methionine. These termination codons are near the beginning of the vde2 sequence so a rather short lacZ fusion protein would be synthesized. Therefore, translation of the VDE

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    gtgggttcgaattttacccaccacaagttttgtcctaccataattgggataaggagtctaatttcccttgtacaa  -159
ttttccaatttcttcctccgccacaccatatatatactgtacgccacttcgaacgctacaatgtttgaaaaagacgca  -80
gattttacaaagacggagaagataataagcttcaagtactccgatcgtcaggtggcctttggaagccaacaaactggct  -1

atg gct ctt tct ctt cac act gta ttt ctc tgc aaa gag gaa gcc ctc aat tta tat gca   60
met ala leu ser leu his thr val phe leu cys lys glu glu ala leu asn leu tyr ala   20

aga tca cca tgt aat gaa agg ttt cac agg agt gga caa cct cct acc aac ata atc atg  120
arg ser pro cys asn glu arg phe his arg ser gly gln pro pro thr asn ile ile met   40

atg aaa att cga tcc aac aat gga tat ttt aat tct ttc cgg ttg ttt aca tct tat aag  180
met lys ile arg ser asn asn gly tyr phe asn ser phe arg leu phe thr ser tyr lys   60

aca agt tct ttc tca gat tct agc cat tgc aag gat aaa tct cag ata tgc agc atc gat  240
thr ser ser phe ser asp ser ser his cys lys asp lys ser gln ile cys ser ile asp   80

aca agt ttt gag gaa ata caa aga ttt gat ctc aaa agg ggc atg act ttg att ctt gaa  300
thr ser phe glu glu ile gln arg phe asp leu lys arg gly met thr leu ile leu glu  100

aag caa tgg aga caa ttc ata caa ttg gct atc gta ttg gtt tgc aca ttt gtt atc gtt  360
lys gln trp arg gln phe ile gln leu ala ile val leu val cys thr phe val ile val  120

ccc aga gtt gat gcc gtt gat gct ctt aaa act tgt gct tgt tta ctc aaa gaa tgc agg  420
pro arg val asp ala val asp ala leu lys thr cys ala cys leu leu lys glu cys arg  140
                   ↑             n-terminal peptide
att gag ctt gca aaa tgt ata gca aac cca tct tgt gcg gca aac gtt gcc tgt cta cag  480
ile glu leu ala lys cys ile ala asn pro ser cys ala ala asn val ala cys leu gln  160

act tgc aac aat cgt cct gac gag acc gaa tgt cag ata aaa tgt ggt gac ttg ttc gaa  540
thr cys asn asn arg pro asp glu thr glu cys gln ile lys cys gly asp leu phe glu  180

aac agt gtg gtg gac caa ttc aac gag tgt gcg gtt tcc cga aag aaa tgt gtg ccc cgg  600
asn ser val val asp gln phe asn glu cys ala val ser arg lys lys cys val pro arg  200

aaa tcg gat gtg ggt gaa ttc ccg gtt ccg gat cgt aat gca gtg gtt caa aat ttt aac  660
lys ser asp val gly glu phe pro val pro asp arg asn ala val val gln asn phe asn  220

atg aaa gac ttt agt ggg aag tgg tat ata aca agt ggt tta aat cct aca ttt gat gca  720
met lys asp phe ser gly lys trp tyr ile thr ser gly leu asn pro thr phe asp ala  240

ttt gat tgt caa ctt cat gag ttt cat atg gaa aat gat aaa ctt gtt ggg aac tta aca  780
phe asp cys gln leu his glu phe his met glu asn asp lys leu val gly asn leu thr  260

tgg cgc ata aaa act ttg gat ggt ggt ttc ttt act cga tct gct gtg caa aca ttt gtt  840
trp arg ile lys thr leu asp gly gly phe phe thr arg ser ala val gln thr phe val  280
                     tryptic fragment #11
caa gat cca gat ctt cct gga gca ctt tat aat cat gac aat gag ttt ctt cac tac caa  900
gln asp pro asp leu pro gly ala leu tyr asn his asp asn glu phe leu his tyr gln  300
     tryptic fragment #21
gat gac tgg tac ata tta tct tcc caa atc gaa aac aaa ccc gat gat tac ata ttc gta  960
asp asp trp tyr ile leu ser ser gln ile glu asn lys pro asp asp tyr ile phe val  320

tac tac cga ggt cga aac gac gca tgg gat gga tac ggt ggg tcc gtg atc tac acc cga 1020
tyr tyr arg gly arg asn asp ala trp asp gly tyr gly gly ser val ile tyr thr arg  340

agc ccg aca ctc ccc gaa tcg atc atc cca aac cta caa aaa gca gcc aaa tcc gtg ggt 1080
ser pro thr leu pro glu ser ile ile pro asn leu gln lys ala ala lys ser val gly  360
                 tryptic fragment #15
cga gac ttt aac aat ttc ata aca acc gac aat agt tgt ggg cct gag cct cca ttg gtg 1140
arg asp phe asn asn phe ile thr thr asp asn ser cys gly pro glu pro pro leu val  380

gaa agg ctt gag aaa aca gcg gaa gag ggc gag aag ttg ttg ata aaa gaa gct gta gag 1200
glu arg leu glu lys thr ala glu glu gly glu lys leu leu ile lys glu ala val glu  400

ata gaa gaa gag gtt gaa aaa gag gtg gag aag gtt aga gat act gag atg act ttg ttt 1260
ile glu glu glu val glu lys glu val glu lys val arg asp thr glu met thr leu phe  420

cag agg ttg ctt gaa ggg ttt aag gag ttg caa caa gat gaa gag aat ttt gtg agg gag 1320
gln arg leu leu glu gly phe lys glu leu gln gln asp glu glu asn phe val arg glu  440

ttg agt aaa gaa gag aag gaa att ctg aat gaa ctt caa atg gaa gcg act gaa gtt gaa 1380
leu ser lys glu glu lys glu ile leu asn glu leu gln met glu ala thr glu val glu  460

aag ctt ttt ggg cgc gcg tta ccg att agg aaa ctt aga taa atttcgatgattgattcagacaa 1445
lys leu phe gly arg ala leu pro ile arg lys leu arg ***                          473

tatatatagtcatatggattatgtagatactagagaaaacccaaaaaaactttgtatacgtgataaacgtgtttgtga 1524
tttgtttattggcttaaaattgtagaatagctttttaattctttacaaaaaaattgattgtctattggtagccaagag 1603
gttcacgaaaagactgaaagggtcttgccggtttgcgggttaggccaaatttttggggcgggatcggtcttgatcggg 1682
tttttctttaaaacatgtatttttataaatgatgagttattttcaattttggctaaaaaaaaa                1747
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FIG. 1. Nucleotide and deduced amino acid sequence for romaine lettuce VDE. Three cDNAs were sequenced and their regions are as follows: vde1 (−233–1526 bp), vde2 (−65–1578 bp), and vde3 (−83–1747 bp). The polypeptide sequence begins at the first methionine of the open reading frame and is preceded by three termination codons in the same reading frame. Amino acid sequences confirmed by amino acid sequencing of tryptic fragments of the purified enzyme or determination of the N terminus of VDE are underlined. The arrow indicates the putative site of cleavage of the transit peptide.

protein is occurring from a translation initiation site within the vde2 cDNA.

To verify the products of de-epoxidation, the reaction with vde2 sense extract was analyzed by HPLC (Fig. 4*B*). Antheraxanthin and zeaxanthin appeared consistent with sequential de-epoxidation and concomitant with the rapid decrease in violaxanthin, similar to observations reported over three decades earlier for de-epoxidation in lima bean (*Phaseolus leunatus*) leaves exposed to high light (9). This is the first unequivocal evidence that the same enzyme catalyzes the two-step mono de-epoxidation reaction. In future studies, expression of the VDE cDNA in *E. coli* will allow analysis of structure/function relationships in VDE by mutagenesis (site-directed and deletion of domains noted in Fig. 3*B*).

An attempt to express vde1 was unsuccessful. *E. coli* transformed with vde1 subcloned in pGEM-5Zf and orientated with

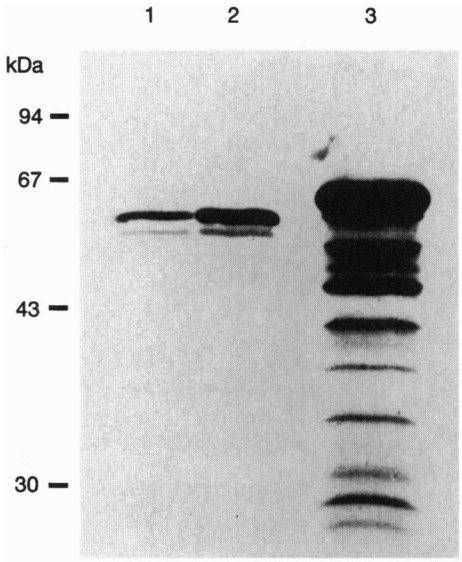

FIG. 2. Analysis of the *in vitro* translation products by SDS/PAGE and fluorography. Lanes: 1, translation product from vde1; 2, translation product from vde2; 3, translation product from firefly luciferase control (62 kDa). Molecular mass markers are indicated in kilodaltons.

*lac*Z (vde1 cDNA in frame with *lac*Z fusion protein) did not grow, whereas *E. coli* transformed with vde1 cDNA in antisense orientation with respect to *lac*Z grew well. The main difference between vde1 and vde2 is that vde1 has a longer 5′ untranslated region and, because there are no termination codons in frame with the fusion protein in the first 141 nucleotides of vde1, a much larger *lac*Z fusion protein would be produced. It is possible that this *lac*Z fusion protein may be lethal to *E. coli*.

Various molecular masses for the VDE protein have been reported. For example, in early studies, M_rs of 54,000 (32) and 60,000 (12) were determined for VDE using size-exclusion chromatography. Recently, the M_r of spinach and romaine lettuce VDE were reported as 58,000 (19) and 43,000 (15) by

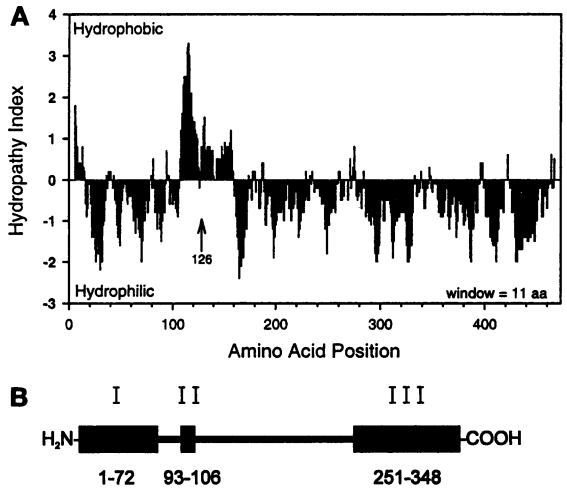

FIG. 3. Analysis of the deduced amino acid sequence of VDE. (*A*) Hydropathy plot of the deduced amino acid sequence of VDE calculated as described (27) with a window of 11 amino acids. The N terminus of the mature VDE polypeptide (amino acid 126) is indicated with an arrow. (*B*) Map of the deduced amino acid sequence of the mature protein for VDE illustrating the three domains identified: (I) a cysteine rich domain, (II) a lipocalin signature, and (III) a highly charged domain. The amino acid spanning regions are indicated below the domains with numbering beginning at the N terminus of the mature protein.

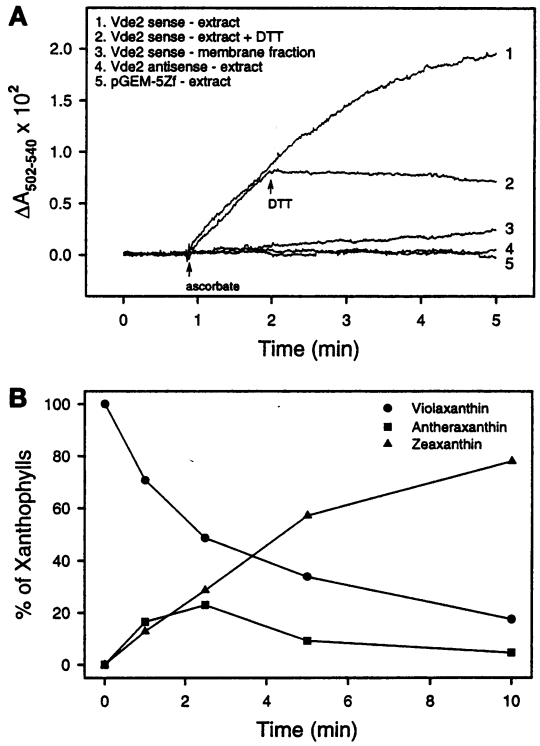

FIG. 4. Expression of vde2 in *E. coli*. (*A*) Kinetics of absorbance change demonstrating expression of active VDE by vde2 in *E. coli* DH5α. Expression was assayed from vde2 constructs in both sense and antisense orientations with respect to *lac*Z along with *E. coli* containing the vector only (pGEM-5Zf). DTT (3 mM, final concentration) was added directly to the assay 70 sec after ascorbate (30 mM, final concentration) addition. Specific activity of the enzyme was 64.9 ± 5.4 nmol violaxanthin de-epoxidized min^{-1} mg $protein^{-1}$. (*B*) Timecourse of xanthophyll conversions by expressed vde2 (sense construct) in *E. coli*. Specific activity of the enzyme was 19.4 ± 0.9 nmol of violaxanthin de-epoxidized min^{-1} mg $protein^{-1}$.

SDS/PAGE, respectively. Except for the last report, these values are considerably greater than the M_r of the deduced mature VDE (39,900), which may be due to the method in which the molecular weight was estimated. Size-exclusion chromatography does not always give an accurate assessment of the true M_r of a protein. Estimation of the M_r of spinach VDE was based on an observation that increasing amounts of VDE activity were released from the lumen of thylakoid vesicles by increasing numbers of freeze-thaw cycles using liquid nitrogen (19). When this total protein was analyzed by SDS/PAGE, a 58-kDa protein was identified that also increased with increasing numbers of freeze-thaw cycles. However, this 58-kDa protein was not purified so its identity remains unknown.

Lipid-affinity purification resolved VDE to a single active component of 43 kDa by two-dimensional SDS/PAGE (15). Using an antibody prepared against a synthetic peptide derived from the N terminus of lettuce VDE (15), Western blot analysis was performed on VDE isolated from chloroplasts of market romaine lettuce, tobacco (*Nicotiana tabacum* L. cv Xanthi), and market spinach (*Spinacia oleracea* L.), and also in bacterially-expressed VDE protein. The antibody recognized VDE in these three plant species demonstrating that the N terminus is conserved and all migrated with approximately the same M_r of 43,000 (Fig. 5). Expressed vde2 in *E. coli* also migrated at the same M_r as the romaine lettuce VDE, whereas extracts from *E. coli* containing only pGEM-5Zf produced some minor cross-reacting proteins, none of which having a M_r of 43,000.

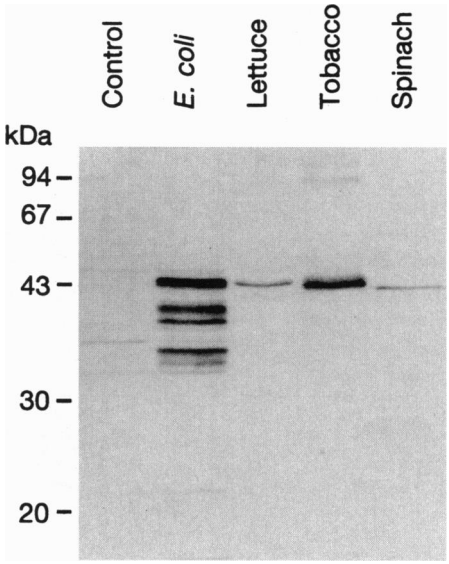

FIG. 5. Western blot analysis of VDE from chloroplasts of various C_3 plants and expressed vde2 in *E. coli*. The blot was probed with a polyclonal antibody prepared against a synthetic peptide derived from the N terminus of lettuce VDE (VDALKTCACLLK) (15). The following samples were loaded at 10 μg per lane: chloroplast soluble protein from market romaine lettuce, tobacco and market spinach; total soluble protein from *E. coli* expressing vde2 subcloned in pGEM-5Zf (lane *E. coli*); and total soluble protein from *E. coli* containing only pGEM-5Zf (lane Control). VDE migrates with an approximate size of 43 kDa. Molecular mass markers are indicated in kilodaltons.

Two interesting observations are evident from VDE expressed in *E. coli* (Fig. 5). The first is that the *E. coli*-expressed VDE produced many immunoreactive bands of lower molecular weight. This may be due to some processing occurring at the C terminus of the protein by *E. coli* DH5α (since the antibody recognizes the N terminus) or due to translational pausing. The second is that the bacterial-expressed VDE protein migrates at the same molecular mass as mature VDE from romaine lettuce and not as the expected size of the deduced VDE pre-protein with the transit peptide (54.4 kDa). This suggests that *E. coli* may recognize the chloroplast transit peptide and cleave it. The N terminus of the bacterially-expressed VDE will need to be sequenced to determine the actual site where cleavage is occurring. A similar observation was also reported for the nuclear-encoded chloroplast enzyme acetolactate synthase from *Arabidopsis* when expressed in *E. coli* (33).

Expression of the VDE transcript was analyzed in market romaine lettuce leaves that were dark adapted for an undetermined period of time. The same level of transcript was detected in both yellow leaves and rapidly expanding green leaves (Fig. 6). Because there is some expression in immature yellow leaves of lettuce, this may suggest that there is some constitutive level of expression of VDE. Other experimental evidence that supports this observation is that VDE activity was detected in etiolated bean (*Phaseolus vulgaris* L. var. Commodore) leaves, a tissue lacking an active photosynthetic apparatus (34). However, a greater transcript level was detected in mature green leaves, indicating that VDE may also be developmentally expressed. Two hybridizing transcripts were observed for each sample indicating the possibility that the upper larger transcript may be processed to the slightly smaller transcript (1.7 kb) having the greater level of hybridization. Both transcripts are most likely from the same gene because Southern blot hybridization demonstrated the presence of only one gene for VDE (data not shown). The increased level of transcript in mature green leaves of lettuce may be due to two

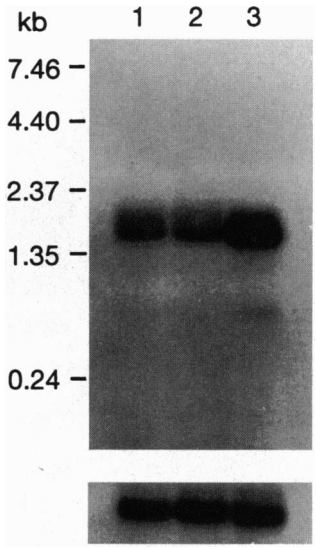

FIG. 6. Expression of the VDE transcript in leaves of romaine lettuce. Lanes: 1, 2 μg poly(A)$^+$ RNA from immature yellow leaves; 2, 2 μg poly(A)$^+$ RNA from rapidly expanding green leaves; 3, 2 μg poly(A)$^+$ RNA from mature green leaves (upper panel, 28 hr film exposure). The same blot was hybridized with a soybean ubiquitin genomic clone probe to demonstrate equal loading of RNA samples (lower panel, 15 min film exposure). The position of migration of RNA standards (GIBCO/BRL) is shown.

possible reasons: (*i*) higher expression occurs in tissues with a higher density of fully developed chloroplasts or (*ii*) expression may be regulated by light intensity because the mature green leaves receive a higher intensity of light than the immature leaves that are partially shielded in the center of the head of lettuce.

Further research will analyze whether VDE is developmentally regulated and what effect light has on expression. This first molecular cloning of a xanthophyll-cycle enzyme opens new opportunities for testing the mechanism of de-epoxidation-dependent nonphotochemical fluorescence quenching, examining the evolution of the xanthophyll cycle, and possibly enhancing the light-stress tolerance of plants.

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