The 9-*cis*-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean

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Abscisic acid (ABA), a cleavage product of carotenoids, is involved in stress responses in plants. A well known response of plants to water stress is accumulation of ABA, which is caused by *de novo* synthesis. The limiting step of ABA biosynthesis in plants is presumably the cleavage of 9-*cis*-epoxycarotenoids, the first committed step of ABA biosynthesis. This step generates the C_{15} intermediate xanthoxin and C_{25}-apocarotenoids. A cDNA, *PvNCED1*, was cloned from wilted bean (*Phaseolus vulgaris* L.) leaves. The 2,398-bp full-length *PvNCED1* has an ORF of 615 aa and encodes a 68-kDa protein. The PvNCED1 protein is imported into chloroplasts, where it is associated with the thylakoids. The recombinant protein PvNCED1 catalyzes the cleavage of 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin, so that the enzyme is referred to as 9-*cis*-epoxycarotenoid dioxygenase. When detached bean leaves were water stressed, ABA accumulation was preceded by large increases in *PvNCED1* mRNA and protein levels. Conversely, rehydration of stressed leaves caused a rapid decrease in *PvNCED1* mRNA, protein, and ABA levels. In bean roots, a similar correlation among *PvNCED1* mRNA, protein, and ABA levels was observed. However, the ABA content was much less than in leaves, presumably because of the much smaller carotenoid precursor pool in roots than in leaves. At 7°C, *PvNCED1* mRNA and ABA were slowly induced by water stress, but, at 2°C, neither accumulated. The results provide evidence that drought-induced ABA biosynthesis is regulated by the 9-*cis*-epoxycarotenoid cleavage reaction and that this reaction takes place in the thylakoids, where the carotenoid substrate is located.

Abscisic acid (ABA) is one of the five "classical" plant hormones (1). It is ubiquitous in higher plants and algae and is also produced by several fungi (2). In higher plants, ABA plays important roles in embryo development and seed dormancy. ABA is also involved in adaptation of plants to various stresses (drought, salinity, cold). When ABA is applied to plants, it causes rapid stomatal closure and thus reduces water loss via transpiration. Furthermore, water stress causes a rapid increase in the ABA content of plants. Evidence has accumulated that this increase in ABA is attributable to *de novo* synthesis (3). The increase in ABA biosynthesis in water-stressed plants can be prevented by application of actinomycin D, cordycepin, or cycloheximide (4, 5), indicating that nuclear gene transcription and protein synthesis in the cytosol are required before an increase in ABA synthesis can occur.

To determine which step(s) in ABA biosynthesis is (are) stimulated by water stress, it was essential to first establish the ABA biosynthetic pathway. ABA is a sesquiterpenoid (C_{15}), and two pathways for its biosynthesis have been proposed: (*i*) The direct pathway from isopentenyl pyrophosphate (C_5) via farnesyl pyrophosphate (C_{15}) to ABA. Current evidence indicates that this pathway operates in fungi (2). (*ii*) The indirect pathway in which ABA is a cleavage product of carotenoids (C_{40}). Evidence that this pathway functions in green plants has been obtained with mutants deficient in carotenoids, from $^{18}O_2$ labeling experiments, and from the finding that, in water-stressed roots and etiolated leaves, there is a 1:1 stoichiometry between the disappearance of violaxanthin and neoxanthin and the formation of ABA and its catabolites, phaseic acid, and dihydrophaseic acid (2, 6).

Direct evidence for the indirect pathway of ABA biosynthesis has come from the ABA-deficient mutant *vp14* of maize and the cloning of the corresponding gene *Vp14* (7). In enzyme assays, the recombinant protein VP14 cleaves 9-*cis*-epoxycarotenoids (8), thus giving rise to 2-*cis*,4-*trans*-xanthoxin and C_{25}-apocarotenoids (Fig. 1). Because there appears to be no isomerization at the C_{15} precursor level (11), the carotenoid precursors must be in the 9-*cis* configuration to give ABA, which is by definition 2-*cis*,4-*trans*. Available evidence indicates that the conversion of xanthoxin to ABA is not rate limiting and that these later steps in the pathway are not up-regulated by water stress (12). Because the carotenoid substrate is abundantly available in photosynthetic tissues (13), it follows by the process of elimination that the cleavage of 9-*cis*-epoxycarotenoids is the rate-limiting step in the ABA biosynthetic pathway. With the cloning of the *Vp14* gene from maize, which encodes nine-*cis*-epoxycarotenoid dioxygenase (NCED), it is possible to investigate the regulation of this and homologous genes in other species at the molecular level. In maize, it was found that expression of *Vp14* was up-regulated by water stress (7). The homolog *LeNCED1* of tomato (*Lycopersicon esculentum* Mill.), which is the wild-type allele of the ABA-deficient mutant *notabilis*, was also induced by drought (14, 15). We have cloned a gene from bean, *PvNCED1*, that encodes the cleavage enzyme and have analyzed its enzymatic activity and localization. Its response to water stress has been characterized at the mRNA and protein levels in both leaves and roots. The results demonstrate that the cleavage reaction is the key regulatory step in water stress-induced ABA biosynthesis.

Materials and Methods

Plant Material. Bean seedlings (*Phaseolus vulgaris* L., cv. Top Crop) were grown in trays with vermiculite in a growth chamber and were watered daily with half-strength Hoagland nutrient solution. The conditions in the growth chamber consisted of 18 h of light from fluorescent and incandescent lamps (200 μmol·m^{-2}·s^{-1} photon) at 25°C, followed by 6 h of darkness at 22°C. When the primary leaves had fully expanded (12–14 days after planting), they were detached, and the fresh weight was reduced rapidly (<10 min) by 12–15%, using a hair drier. The stressed leaves were stored in a polyethylene bag in darkness at room temperature (22–23°C) until harvest. Wilted leaves were rehydrated by submergence in distilled water for 5 min.

Abbreviations: ABA, abscisic acid; NCED, 9-*cis*-epoxycarotenoid dioxygenase; RACE, rapid amplification of cDNA ends.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF190462).

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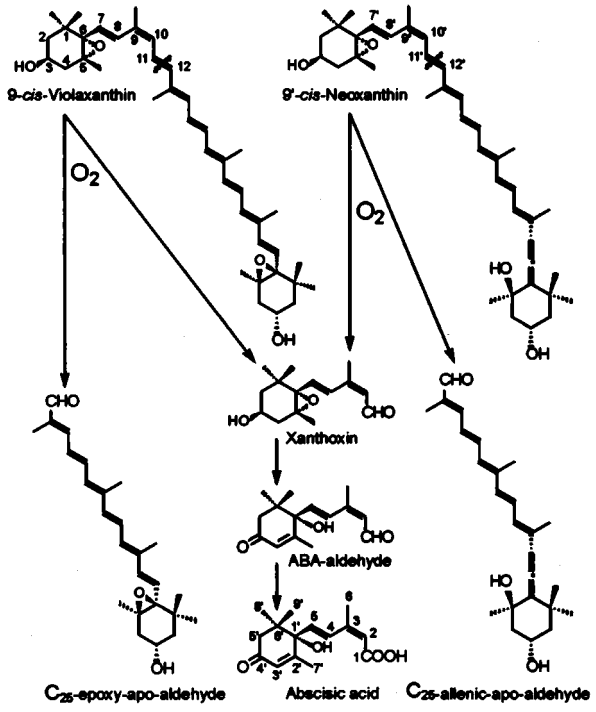

Fig. 1. Pathway of ABA biosynthesis from 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin. Oxidative cleavage of the C (11, 12) double bonds of 9-*cis*-epoxycarotenoids is the first committed step of ABA biosynthesis. C_{25}-apocarotenoids are byproducts of the cleavage reaction. The numbering system for ABA (9) is different from that in use for carotenoids (10).

For experiments with roots, the seeds were planted in vermiculite; after germination, the seedlings were suspended from perforated boards over trays containing aerated half-strength Hoagland nutrient solution (16). At the start of an experiment, the root systems were detached from the seedlings and were blotted dry. Next, the detached roots were submerged in an aerated solution of half-strength Hoagland containing 180 g/liter of polyethylene glycol 6000 (Fluka), which had an osmotic potential of ≈−0.5 MPa (17). For recovery from osmotic stress, the roots were rinsed in distilled water and further kept in aerated half-strength Hoagland solution. Upon harvest, all samples were frozen immediately in liquid N_2 and were stored at −80°C until use. The same plant material was used for RNA, protein, and ABA measurements. Material to be used for ABA determinations was lyophilized, and the dry weight was determined.

Cloning of *PvNCED1* by Reverse Transcription–PCR and Rapid Amplification of cDNA Ends (RACE). Degenerate primers for NCED (JZ101, 5′-TTT/C GAT/C GGN GAT/C GGN ATG G-3′; JZ117, 5′-GCA/G TTC CAN AGA/G TGA/G AAA/G CAA/G AAA/G CAA/G TC-3′) were synthesized based on the sequences of the corresponding genes from maize (7) and tomato (14) (Fig. 2). RNA was obtained from bean leaves that had been water-stressed for 2 h, and the first-strand cDNA was synthesized by M-MLV Reverse Transcriptase (GIBCO/BRL) with oligodT_{16}N as primer. Subsequent PCR fragments were sequenced and compared with the above-mentioned NCED sequences. After the product had been confirmed as an NCED homolog, its missing 5′ and 3′ ends were obtained by a 5′- and 3′-RACE System (GIBCO/BRL), respectively, according to the manufacturer's protocol. The gene-specific primer for 5′-RACE was JZ156 (5′-GGA ACA CCG GGC GGC CTA AAG ATT-3′), and the gene-specific primer for 3′-RACE was JZ157 (5′-TCC GAA GAC GAT TTA CCA TAC CAC-3′). To obtain the full-length *PvNCED1* clone, primers were designed corresponding to the untranslated region of the 5′ end (JZ166, −34 to −11, 5′-CCT CAA AAA TAC AAC ACC AAC AAA-3′) and the 3′ end (JZ163, +2,233 to +2,257, 5′-TGG GGA AAC AAA ACA AGA AGA ATA-3′). The end-to-end PCR product

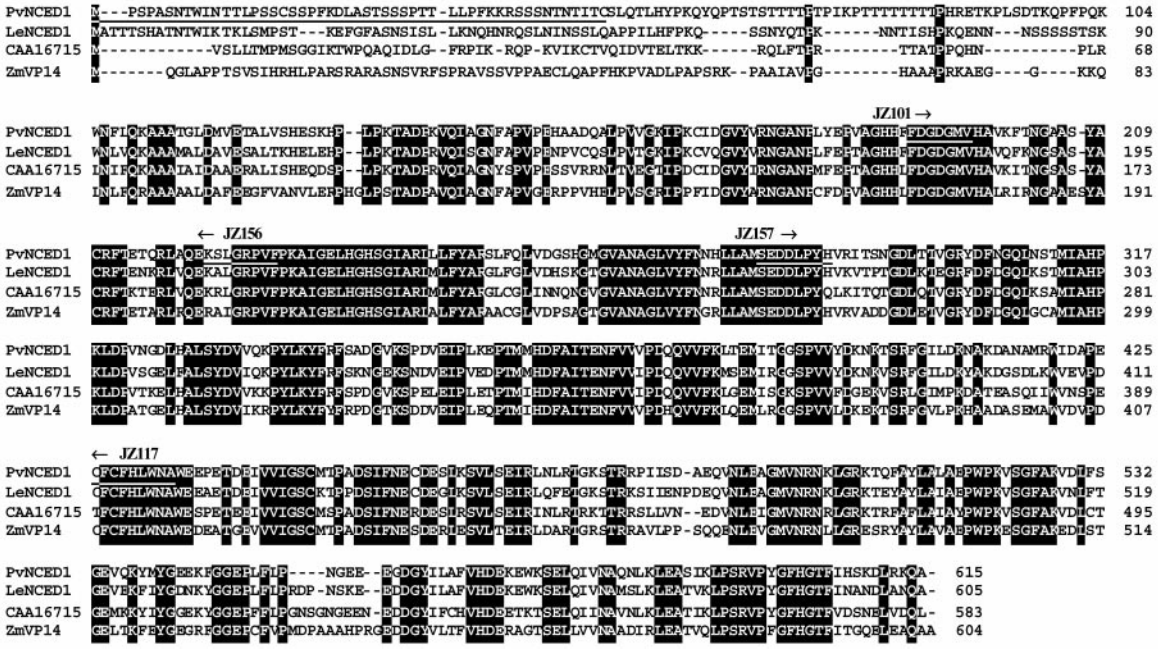

Fig. 2. Alignment of the deduced amino acids of PvNCED1 (AF190462) with tomato LeNCED1 (Z97215), an *Arabidopsis* NCED (CAA16715), and maize VP14 (U95953). The dark shading with white letters indicates amino acid identity for the aligned residue for all four proteins. The locations of the degenerate primers JZ101 and JZ117, and gene-specific primers JZ156 and JZ157, are indicated by arrows and underlining. A putative chloroplast-targeting peptide of PvNCED1 is underlined.

PvNCED1–2291 was obtained by eLONGase Enzyme Mix (GIBCO/BRL) instead of *Taq* DNA Polymerase (GIBCO/BRL), used in all other PCRs.

In all cloning experiments, PCR fragments were gel-purified with a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) and were ligated into the pGEM-T Easy Vector (Promega). Plasmids were isolated by Wizard miniprep SV (Promega) and were sequenced from both strands. Sequence analysis was performed by using LASERGENE software (DNAstar, Madison, WI).

***In Vitro* Import of PvNCED1 into Pea Chloroplasts.** The pGEMPvNCED1–2291 plasmid was linearized with *Nde*I, which has a single cleavage site downstream of the *PvNCED1–2291* coding region. Δ*495-PvNCED1–2291*, a 495-bp deletion at the 5′-end of *PvNCED1–2291* lacking the chloroplast targeting peptide, was used as a negative control. *In vitro* transcription was performed with the RiboMAX Large Scale RNA Production System (Promega) with T7 RNA polymerase according to the manufacturer's protocol. The *in vitro* translation product, nascent PvNCED1, was obtained by the rabbit reticulocyte translation system (Promega). Intact chloroplasts were isolated from 2-week-old pea seedlings (*Pisum sativum* L.) by density gradient centrifugation on a Percoll gradient (18). The import reaction consisted of 50 μl of 1 mg/ml chloroplasts, 2×10^5 dpm ^{35}S-labeled PvNCED1, and 2 mM Mg-ATP, in a total volume of 150 μl. The Rubisco small subunit was used as a control for the import assay. The mixture was incubated at room temperature for various time periods, after which intact chloroplasts were pelleted through 40% Percoll in import buffer at 3,000 × *g* for 6 min, were washed with import buffer, and then were treated with thermolysin. The intact chloroplasts were reisolated, and a portion was subsequently fractionated into a soluble and a membrane fraction. The proteins were analyzed by a 9% SDS/PAGE gel.

Expression and Purification of Recombinant PvNCED1 and PvNCED1-ΔN Protein. A primer corresponding to amino acids S(52) to P(59) of *PvNCED1–2291* and JZ163 was used to amplify the truncated *PvNCED1* (*PvNCED1-ΔN*) clone from the pGEMPvNCED1–2291 plasmid. The *PvNCED1–2291* and *PvNCED1-ΔN* fragments were separated from the pGEM-T Easy Vector by *Eco*RI digestion and were inserted into the *Eco*RI site of the pGEX-5X-2 vector (Pharmacia). The resulting plasmids were named pGEXPvNCED1 and pGEXPvNCED1-ΔN, respectively, and were maintained in the Dh5α strain of *Escherichia coli*. For better expression, both plasmids were introduced into the BL21 strain of *E. coli*. An overnight culture of 5 ml was diluted to 100 ml with 2 × YTA medium (16 g/liter tryptone/10 g/liter yeast extract/5 g/liter NaCl/100 μg/ml ampicillin) and was grown at 30°C until mid-log phase. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM. Growth continued for 2 h at 30°C. The cells were harvested, were resuspended in 10 ml PBS containing 10 mg lysozyme, and were sonicated. Triton X-100 was added to the mixture to give a final concentration of 0.1%. The mixture was centrifuged at 18,000 × *g* for 10 min. The supernatant was applied to 1 ml of a 50% slurry of glutathione Sepharose 4B (Pharmacia) in PBS buffer. After binding, the beads were washed with PBS buffer, were equilibrated with factor Xa cleavage buffer, and were treated with 25 units of Factor Xa Protease (Pharmacia) for 4 h at 22°C. Finally, the purified PvNCED1 and PvNCED1-ΔN proteins were eluted in the presence of 0.1% Triton X-100. Protein was quantified by the modified Lowry method (19).

Preparation of C_{40}-Epoxycarotenoids. The C_{40}-epoxycarotenoids all-*trans*-violaxanthin and 9′-*cis*-neoxanthin were isolated from spinach leaves as described (20). After isomerization with iodine, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, all-*trans*-neoxanthin, and 9′-*cis*-neoxanthin were separated by normal phase HPLC with a μPorasil semipreparative column (0.78 × 30 cm, Waters), using a linear gradient from 10–100% ethyl acetate in hexane for 65 min at a flow rate of 2.5 ml/min. The isomers of violaxanthin and neoxanthin were verified by their absorption spectra in ethanol and were quantified by spectrophotometry (10).

Cleavage Enzyme Assays. The cleavage enzyme assay was performed as described (8). Appropriate amounts of protein and substrates were added in a total volume of 100 μl. Assays were incubated for 15 min at 22°C. Me-ABA was added as an internal standard before partitioning with ethyl acetate. The reaction products were analyzed by HPLC with a 0.4- × 30-cm μPorasil column (Waters). The putative products, xanthoxin and C_{25}-apocarotenoids, were monitored at 285 and 436 nm, respectively, were collected, and were identified by mass spectrometry (8).

DNA and RNA Gel Blotting. Bean genomic DNA (10 μg) was digested with the enzymes indicated and then was subjected to electrophoresis in a 0.8% agarose gel. Total RNA from leaves and roots was extracted in TRIzol reagent (GIBCO/BRL). Leaf (10 μg) or root (20 μg) RNA was run in denaturing agarose gels. Both DNA and RNA were transferred to Hybond-N+ nylon membranes (Amersham Pharmacia) and were probed with the *PvNCED1–2291* fragment, which was labeled with ^{32}P-dCTP by the Random Primers DNA Labeling System (GIBCO/BRL). Hybridization was performed in a 50% formamide system (21). For low stringency, hybridization was performed at 37°C, followed by washing in 2 × standard saline citrate (SSC)/0.5% SDS at 53°C for 30 min; high stringency hybridization was performed at 42°C, followed by washing at 65°C.

Antibody Preparation, Protein Extraction, and Immunoblotting. Purified PvNCED1-ΔN protein from BL21 cells was used for raising polyclonal antibodies in rabbit against NCED (Cocalico Biologicals, Reamstown, PA). Crude antiserum was purified by affinity purification (22). PvNCED1-ΔN antigen (100 μg) was run on a 10% SDS/PAGE gel and was electroblotted to a nitrocellulose membrane. Membranes were blocked with a 5% solution of nonfat milk in TBS and were incubated with crude antiserum for 1 h. The nonspecific antisera were washed away by TBS containing 0.05% Tween 20. The specific anti-PvNCED1 antibody bound to the membrane was eluted with 100 mM glycine at pH 2.5. The eluate was neutralized with 0.1 volume of 1 M Tris buffer (pH 8.0) and was stored at −20°C.

For plant protein extraction, 0.3 g of frozen tissue was pulverized in liquid N_2 and extracted in sample buffer (62.5 mM Tris·HCl, pH 6.8/10% glycerol/2% SDS/5% mercaptoethanol) containing a proteinase inhibitor mixture (Sigma) and 1 mM phenylmethanesulfonyl fluoride (PMSF). After centrifugation, the supernatant was boiled for 5 min. Protein (40–60 μg per lane) was loaded on a 10% SDS/PAGE gel. After electrophoresis, protein was electroblotted to a Protran BA nitrocellulose membrane. Filters were blocked with a 5% solution of nonfat dry milk in TBS buffer (10 mM Tris·HCl/150 mM NaCl, pH 7.4) for 1 h at 22°C. An appropriate amount of purified anti-PvNCED1 antibody was added to the same blocking buffer, and incubation was continued for another 2 h. The filters were then washed and treated with the second antibody according to the standard method (23). The second antibody goat anti-rabbit HRP conjugate (Pierce) was applied at a dilution of 1:10,000. After washing, antigenic bands were visualized by using SuperSignal Substrate (Pierce).

Results

Cloning of *PvNCED1*. cDNA was prepared by reverse transcription of poly(A)$^+$ RNA isolated from bean leaves that had been

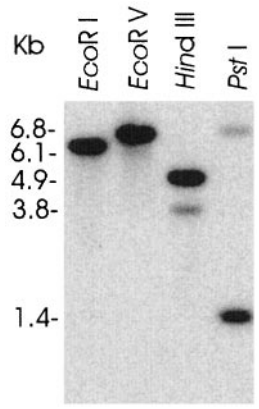

Fig. 3. Southern blot analysis of DNA from bean. Each lane contained 10 μg of genomic DNA that had been digested with *Eco*RI, *Eco*RV, *Hin*dIII, or *Pst*I. The filter was probed with the full-length *PvNCED1–2291* clone at low stringency. The size of the fragments is indicated on the left.

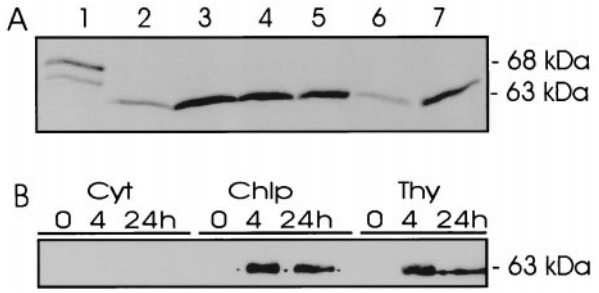

Fig. 4. Localization of PvNCED1. (*A*) Import of PvNCED1 into pea chloroplasts. Lanes: 1, *in vitro* translated nascent PvNCED1; 2–5, pea chloroplasts incubated with PvNCED1 for 15 s, 5, 10, and 20 min, respectively, then treated with thermolysin. The chloroplasts were reisolated and analyzed in 9% SDS/PAGE; 6 and 7, soluble and membrane fractions, respectively, of chloroplasts that had been incubated with PvNCED1 for 20 min. (*B*) Immunoblot of protein extracted from detached bean leaves that were either kept turgid (0 h), or were dehydrated for 4 or 24 h. Cytosolic (Cyt), chloroplast (Chlp), and thylakoid (Thy) fractions were separated for each leaf sample. Each lane was loaded with 30 μg of protein. The membranes were probed with antiserum raised against recombinant PvNCED1.

dehydrated for 2 h. A 738-bp fragment was amplified by degenerate primers JZ101 and JZ117 (Fig. 2). The sequence of this fragment showed high similarity with the maize and tomato *NCED* sequences. The missing 5′ and 3′ ends were isolated by 5′-RACE and 3′-RACE with gene-specific primers JZ156 and JZ157, respectively (Fig. 2). The 2,398-bp full-length cDNA sequence has an ORF of 615 aa with a 123-bp 5′ untranslated region and a 427-bp 3′ untranslated region. Clone *PvNCED1–2291* was generated with primers JZ166 and JZ163 and sequenced from both strands. The sequence of the *PvNCED1–2291* clone was found to be identical to the corresponding region of the *PvNCED1* cDNA sequence. The *PvNCED1–2291* clone was used for all studies described in this paper. The deduced amino acid sequence of PvNCED1 is a polypeptide of 68.07 kDa with an isoelectric point of 6.77. It shares 68.3, 64.3, and 58.6% identity at the amino acid level with LeNCED1 of tomato (14), *Arabidopsis* (CAA16715), and maize VP14 (7), respectively. A putative 51-aa chloroplast-targeting peptide is located at the N terminus of the PvNCED1 sequence (Fig. 2).

A Southern blot probed with the *PvNCED1–2291* fragment gave the same bands, both at high and low stringency (Fig. 3). Considering that there are two *Hin*dIII restriction sites (+1,763, +1,841) and two *Pst*I sites (+326, +1,733) within the gene, these results indicate that *PvNCED1* is present as a single copy in the bean genome without intron. In maize (7), tomato (15), and *Arabidopsis* (ref. 24; *Arabidopsis* homologs of *PvNCED1* from the *Arabidopsis* genome sequencing project can be found at the National Center of Biotechnology Information Entrez Web site, http://www.ncbi.nlm.nih.gov/Entrez/), families of *Vp14*-related genes have been reported. By using other degenerate primers, a 520-bp partial sequence, *PvNCED2*, was amplified from bean. It showed more similarity with *AtNCED1* from *Arabidopsis* (24) than with *PvNCED1*. Southern blots probed with *PvNCED1–2291* or the partial *PvNCED2* sequence showed no cross-hybridization (data not shown). This indicates that there is more than one *NCED*-related sequence in bean, but the homology among them is apparently quite low.

PvNCED1 Is Targeted into Chloroplasts. Because the carotenoid substrates for NCEDs are associated with the chloroplast membranes, it has been proposed that the cleavage reaction is localized in chloroplasts (2). The N-terminal of PvNCED1 has a putative 51-aa chloroplast-targeting peptide (Fig. 2), which indicates that the mature form of PvNCED1 is ≈63 kDa. Serine and threonine constitute 25 and 17%, respectively, of the total amino acid content of this region. It has been suggested that the high content of serine and threonine is involved in targeting nuclear-encoded proteins into the chloroplast (25). To test whether PvNCED1 is localized in the chloroplast, the *PvNCED1–2291* sequence was transcribed *in vitro* and was translated. When the 68-kDa ^{35}S-labeled PvNCED1 protein was incubated with intact pea chloroplasts, it was imported into chloroplasts and processed to a 63-kDa mature protein, as predicted. The import reaction was completed within 5 min (Fig. 4*A*). No signal was detected in the chloroplasts incubated with the protein Δ495-PvNCED1, whose transit peptide had been deleted (data not shown). To further localize PvNCED1 inside the chloroplast, the chloroplasts were fractionated into soluble and insoluble fractions after completion of import. Autoradiography showed (Fig. 4*A*, lanes 6 and 7) that the majority of the imported PvNCED1 protein was associated with the insoluble fraction, which contained both thylakoid membranes and chloroplast envelopes. The location of PvNCED1 in chloroplasts was confirmed by immunoblotting. Proteins from various fractions of turgid and stressed bean leaves were separated by 10% SDS/PAGE. The blot was reacted with specific anti-PvNCED1 antibody. A single 63-kDa band was found only in chloroplasts and thylakoids from stressed leaves (Fig. 4*B*). Thus, both *in vitro* import studies and Western blotting indicate that PvNCED1 is targeted into chloroplasts. This assures that the protein has access to the carotenoid substrates.

Characterization of PvNCED1 Enzyme Activity. Purified PvNCED1 and PvNCED1-ΔN from the glutathione *S*-transferase fusion vector expressed in strain BL21 of *E. coli* are ≈68 and 63 kDa, respectively, as predicted from the nucleic acid sequences. Both proteins were used to perform enzyme assays. Of the four substrates tested, the all-*trans* isomers of violaxanthin and neoxanthin were not cleaved by either protein. By contrast, both 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin served as substrates for the proteins. Thus, the substrate specificity of PvNCED1 is the same as that reported for the VP14 protein of maize (8). As detected by HPLC, a decrease in the substrate peak was accompanied by the concomitant appearance of a C_{25}-apocarotenoid peak (436 nm) and a xanthoxin peak (285 nm). The cleavage products of the enzyme assays were identified by mass spectrometry as before (8). With a given amount of substrate, the cleavage reaction was protein-dependent. As the amount of enzyme increased, more xanthoxin was produced (Fig. 5*A*). When the effectiveness of PvNCED1 and PvNCED1-ΔN was

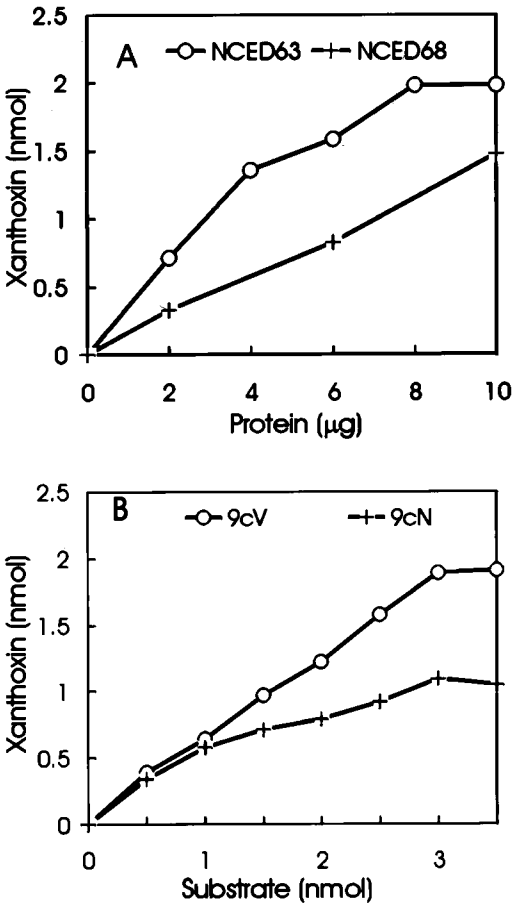

Fig. 5. Enzyme assays with recombinant PvNCED1. (*A*) Protein concentration-dependent cleavage enzyme activity of nascent PvNCED1 (NCED68) or mature protein (NCED63). The reaction mixture in 100 μl contained 4 nmol of 9-*cis*-violaxanthin as substrate. (*B*) Substrate concentration-dependent cleavage enzyme activity with 9-*cis*-violaxanthin (9cV) or 9′-*cis*-neoxanthin (9cN) as substrates. The reaction mixture contained 10 μg of NCED63 protein.

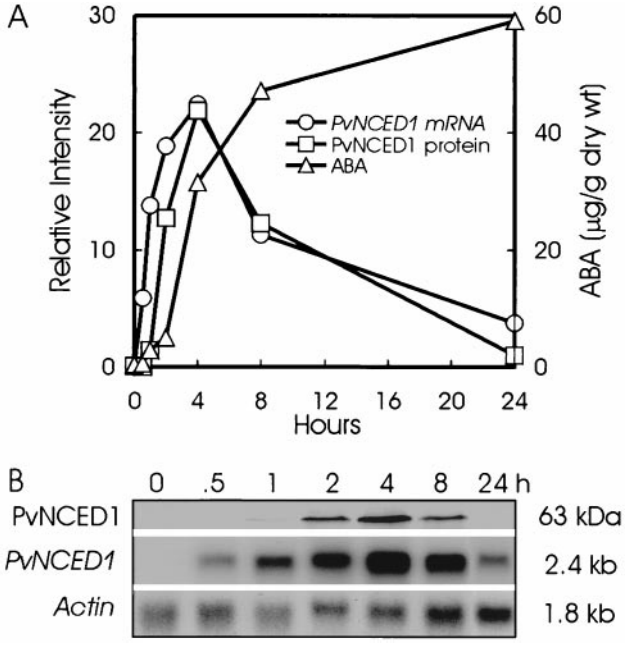

Fig. 6. Effect of dehydration on *PvNCED1* transcript and protein levels, and ABA accumulation in leaves. (*A*) Time course of changes in *PvNCED1* mRNA, PvNCED1 protein, and ABA levels in detached bean leaves in response to dehydration. (*B*) Protein gel blot cross-reacted with antiserum raised against recombinant PvNCED1 (*Top*), and RNA gel blot analysis of the expression of *PvNCED1* (*Middle*), or *Actin* (*Bottom*) as loading control. The ratio of *PvNCED1*/*Actin* is plotted in *A* as relative intensity.

compared, the mature form ΔN was approximately twice as efficient as the precursor PvNCED1 (Fig. 5*A*). Considering that PvNCED1 functions in chloroplasts in the mature form, the PvNCED1-ΔN used in the *in vitro* enzyme assay should more closely reflect the *in vivo* conditions. With 10 μg of PvNCED1-ΔN protein in the assay, the cleavage reaction also showed a substrate dependency, and xanthoxin production increased with increasing amounts of substrate (Fig. 5*B*). Consistent with the maize VP14 protein, PvNCED1-ΔN converted more 9-*cis*-violaxanthin than 9′-*cis*-neoxanthin in the assay, although the latter substrate is generally more abundant in plants than the former (13).

***PvNCED1* mRNA, PvNCED1 Protein, and ABA Accumulation in Response to Leaf Water Status.** Fig. 6 shows the expression of *PvNCED1* mRNA and protein, as well as ABA accumulation, in detached bean leaves during the course of wilting. Turgid leaves contained little ABA (Fig. 6*A*), and *PvNCED1* mRNA and protein were not detectable (Fig. 6*B*). After dehydration, ABA was rapidly synthesized and continued to accumulate for more than 8 h. The highest rate of accumulation took place between 2 and 4 h after the beginning of stress (Fig. 6*A*). At the transcriptional level, the 2.4-kb *PvNCED1* mRNA was strongly induced within 0.5 h of stress treatment (Fig. 6*B*). At the protein level, a single 63-kDa band, representing the mature form of PvNCED1, was detected in total leaf protein after 1 h of stress (Fig. 6*B*). The induction of *PvNCED1* mRNA and protein was transient; the levels of both started to decline after 4 h of dehydration. This coincided with a much decreased rate of ABA accumulation (Fig. 6*A*). Clearly, the time course shows a close correlation between mRNA, protein, and accumulation of the end product, ABA, thus indicating that biosynthesis of ABA under stress conditions is regulated by *PvNCED1* at the transcriptional level.

PvNCED1 was not visible in Fig. 6*B* after 24 h of stress, but it was detectable after longer exposure of the immunoblot. However, in chloroplasts and thylakoids, PvNCED1 was detected after 24 h (Fig. 4*B*) because they are enriched in PvNCED1 compared with total leaf protein analyzed in Fig. 6*B*.

When stressed leaves were rehydrated, they became turgid again. As a result, ABA levels started to decrease after 1 h of rehydration and further decreased to the prestress level within 4 h (Fig. 7*A*). During this period, the first detectable decreases in *PvNCED1* mRNA and protein were observed after 0.5 and 1 h, respectively (Fig. 7*B*). The rapid disappearance of ABA indicates that ABA 8′-hydroxylase, which converts ABA to phaseic acid, must be strongly induced upon rehydration. Indeed, it has been shown in bean leaves that accumulation of ABA catabolites is concomitant with the disappearance of ABA during recovery from stress (26).

***PvNCED1* mRNA, PvNCED1 Protein, and ABA Accumulation in Roots upon Dehydration and Rehydration.** When detached bean roots were subjected to osmotic stress in an aerated PEG6000 solution, a rapid increase in the ABA level was observed (Fig. 8*A*). After 4 h of submergence in the PEG6000 solution and subsequent recovery in half-strength Hoagland solution, ABA was secreted into the medium by the roots. In response to rehydration, the ABA level in the roots decreased close to that present before dehydration over a 4-h period (Fig. 8*A*). Preceding the increase

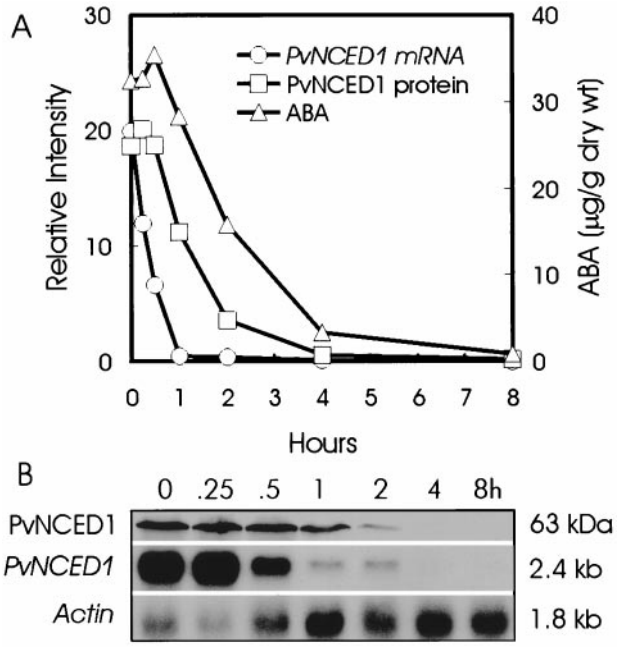

Fig. 7. Effect of rehydration on *PvNCED1* transcript and protein levels, and ABA loss in wilted leaves. (*A*) Time course of *PvNCED1* mRNA, protein, and decrease in ABA level in detached bean leaves in response to rehydration after 4 h water stress. (*B*) Protein gel blot cross-reacted with antiserum raised against recombinant PvNCED1 (*Top*), and RNA gel blot analysis of the expression of *PvNCED1* (*Middle*), or *Actin* (*Bottom*) as loading control. The ratio of *PvNCED1*/*Actin* mRNA is plotted in *A* as relative intensity.

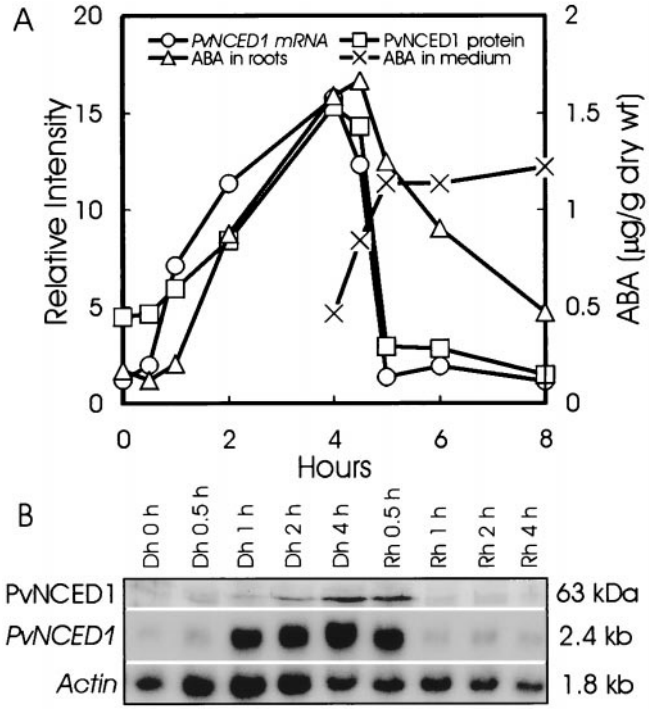

Fig. 8. Effect of dehydration for 4 h and rehydration during the subsequent 4 h on *PvNCED1* transcript and protein levels, and ABA content in roots. (*A*) Time course of changes in *PvNCED1* mRNA, PvNCED1 protein, and ABA levels in detached bean roots in response to dehydration and rehydration. (*B*) Protein gel blot cross-reacted with antiserum raised against recombinant PvNCED1 (*Top*), and RNA gel blot analysis of the expression of *PvNCED1* (*Middle*), or *Actin* (*Bottom*) as loading control. The ratio of *PvNCED1*/*Actin* mRNA is plotted in *A* as relative intensity. Dh, dehydrated; Rh, rehydrated.

in ABA level, both *PvNCED1* mRNA and protein increased, and both decreased rapidly after rehydration (Fig. 8*B*). As in dehydrated leaves, the accumulation of ABA in stressed roots is preceded by induction of *PvNCED1* mRNA and protein. This indicates that in both leaves and roots ABA biosynthesis is regulated by the abundance of *PvNCED1* mRNA.

***PvNCED1* mRNA and ABA Accumulation at Low Temperatures.** Application of ABA to plants at warm temperatures enhances their freezing tolerance. Moreover, ABA increases transiently in plants during cold acclimation (27). In contrast, water-stressed leaves maintained at 2°C did not accumulate ABA (28). This observation led Wright (28) to suggest that the increase in ABA must involve enzymatic conversion from a precursor pool. We have investigated the effects of low temperature on *PvNCED1* mRNA and ABA accumulation in both turgid and dehydrated leaves. Immediately after detached leaves were dehydrated, they were placed in polyethylene bags and were kept at 22°C or were transferred to a cold room (7°C) or a refrigerator (2°C). Detached turgid leaves were kept under the same conditions. Leaf samples were harvested after 5 and 24 h and were analyzed for ABA and *PvNCED1* transcript levels. Fig. 9 shows that there was neither ABA accumulation nor *PvNCED1* expression in any of the turgid leaf samples. Likewise, in dehydrated leaves stored at 2°C, there was no increase in ABA or *PvNCED1* transcript levels. However, *PvNCED1* mRNA did accumulate at a low rate in dehydrated leaves kept at 7°C, and the ABA content increased from 0.3 to 4.4 μg/g dry weight over a 24-h period in these leaves (Fig. 9). These results demonstrate that turgid bean leaves do not respond to low temperature by accumulation of ABA. In agreement with Wright's results (28), dehydrated leaves kept at 2°C did not synthesize ABA, but, at a slightly higher temperature (7°C), ABA accumulated at a slow rate and, as at 22°C, expression of *PvNCED1* was correlated with ABA biosynthesis.

Discussion

We have isolated a full-length *PvNCED1* cDNA from water-stressed bean leaves. *PvNCED1* encodes a protein that is targeted to chloroplasts, where it is associated with thylakoids (Fig. 4). Heterologous expression as a glutathione *S*-transferase fusion protein in *E. coli* and enzyme assays with the purified recombinant protein established that PvNCED1 catalyzes the cleavage of 9-*cis*-epoxycarotenoids (Fig. 5). In response to water stress, a close correlation was found between the abundance of *PvNCED1* mRNA and protein, and increase in ABA levels in leaves and roots (Figs. 6 and 8). In leaves, there is an abundance of 9-*cis*-epoxycarotenoids (13), and the enzyme activities converting xanthoxin to ABA are constitutive (12, 29). Therefore, these results provide evidence in support of the long-standing hypothesis that drought-induced ABA biosynthesis is regulated by the 9-*cis*-epoxycarotenoid cleavage step at the transcriptional level, assuming that the abundance of *PvNCED1* mRNA after dehydration is attributable to increased transcription. However, a decrease in mRNA degradation cannot be ruled out, although unlikely on the basis of results with inhibitors of transcription (4, 5).

Although dehydration strongly induced *PvNCED1* expression in leaves and roots (Figs. 6 and 8), the former accumulated ≈30× more ABA than the latter. Similar differences in ABA levels between stressed leaves and roots have been reported for tomato and *Xanthium* (16). Whereas in leaves there is an abundance of substrate for the cleavage reaction, availability of substrate is probably the limiting factor for ABA biosynthesis in roots (30). Developing bean embryos also accumulate large amounts of ABA (31). Although some of this ABA may be of

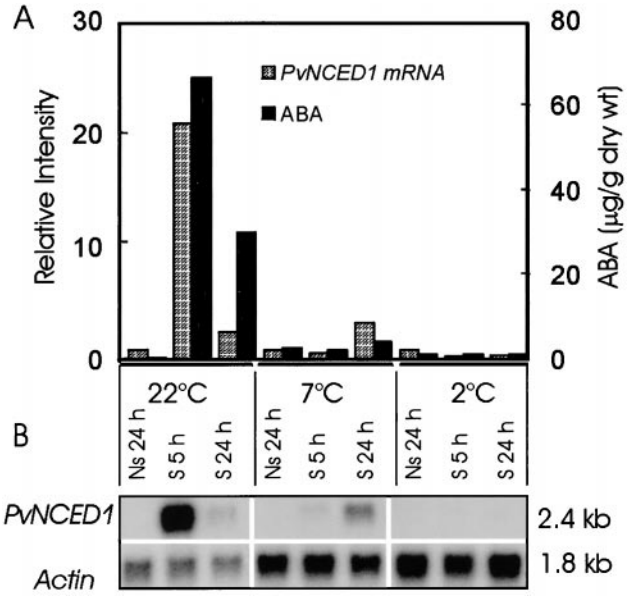

Fig. 9. The effect of low temperature on stress-induced ABA and expression of *PvNCED1*. (*A*) ABA levels and relative intensity of *PvNCED1* mRNA of nonstressed (Ns) and dehydrated leaves (S) kept at different temperatures. (*B*) RNA gel blot analysis of *PvNCED1* mRNA (*Upper*) or *Actin* as control. The ratio of *PvNCED1*/*Actin* mRNA is plotted in *A* as relative intensity. Dehydrated leaves (S) were kept at 22, 7, or 2°C for 5 or 24 h. Nonstressed (Ns) leaves were kept at the same temperatures for 24 h.

maternal origin, at least part of it is synthesized *in situ*, and, again, it is likely that the substrate is limiting (32).

Recombinant PvNCED1 protein from bean and VP14 from maize both show substrate specificity for 9-*cis*-epoxycarotenoids. The cleavage of an epoxycarotenoid in the 9-*cis* configuration leads to the formation of 2-*cis*,4-*trans*-xanthoxin and 2-*cis*,4-*trans*-ABA, which is the naturally occurring isomer (Fig. 1). Both VP14 and PvNCED1 show higher affinity for 9-*cis*-violaxanthin than for 9′-*cis*-neoxanthin. However, in leaves, the latter is much more abundant than the former (13). So far, no native cleavage enzyme has been purified and assayed. In the *in vitro* cleavage enzyme assay, the substrate has to be emulsified with a detergent, which is undoubtedly very different from the *in vivo* conditions. At present, the native substrate for the *in vivo* cleavage reaction remains unknown.

The localization of PvNCED1 in chloroplasts follows from the sequence analysis (Fig. 2), *in vitro* import assays (Fig. 4*A*), and Western blot analysis (Fig. 4*B*). Transport of the nascent protein into the organelle must proceed very rapidly because only the mature form of PvNCED1 was detected in leaves and roots (Figs. 6–8). The size of the PvNCED1 protein in roots was the same as of that detected in leaves. Considering that roots contain non-green plastids, presence of the 63-kDa PvNCED1 protein in roots implies that nascent PvNCED1 protein is imported into these plastids via a mechanism similar to that operating in chloroplasts. Therefore, PvNCED1 provides a tool with which to study protein import into non-green plastids.

The gene for the cleavage enzyme may be a member of a small gene family. As indicated by Southern blotting, there are several *Vp14*-like genes in maize (7). For *Arabidopsis*, the sequences of several NCED homologs are present in the database (ref. 24; http://www.ncbi.nlm.nih.gov/Entrez/). *AtNCED1* lacks the N-terminal chloroplast-targeting peptide (24). We also found a *PvNCED1*-like gene, *PvNCED2*, in bean. The partial sequence shows higher similarity to *AtNCED1* than to *PvNCED1*, which indicates that it may lack the chloroplast-targeting sequence. Northern blot analysis showed that *PvNCED2* is constitutively expressed in bean embryos, as well as in turgid and stressed leaves (data not shown). It is unlikely that *NCED* homologs without a chloroplast-targeting peptide play a role in ABA biosynthesis. More *NCED*-like genes need to be isolated and characterized to give a better understanding of the regulation of ABA biosynthesis in different organs, at different stages of development, and under different environmental conditions.

We found that *PvNCED1* mRNA and ABA accumulated at a low rate at 7°C, but not at all at 2°C (Fig. 9). Moreover, no ABA accumulated in turgid leaves kept at low temperatures. This contrasts with reports that ABA accumulates during cold acclimation (27). However, transfer of plants (or of the roots only) to low temperature causes dehydration because of a reduction in root hydraulic conductance (33, 34). We interpret ABA accumulation during cold acclimation, therefore, to be caused by dehydration rather than the low temperature *per se*. When plants are cold-acclimated in light, it is likely that their leaf temperature will be several degrees Celsius above freezing. Our results obtained at 7°C (Fig. 9) show that these conditions would be conducive for ABA synthesis at a low rate in dehydrated plants.

After dehydration, the ABA content of leaves increases at a rapid rate during the first 4–5 h and then levels off (26, 35). This decline in the rate of ABA accumulation is associated with a decrease in *PvNCED1* mRNA and protein (Fig. 6). Gibberellin levels are subject to feedback regulation of gibberellin 20-oxidase (36, 37) and 3β-hydroxylase (38) at the mRNA level. However, a similar mechanism may not be involved in the regulation of ABA levels because we established in preliminary experiments that dehydration of leaves that had been fed large amounts of ABA still showed induction of *PvNCED1* (data not shown). Some other type of feedback, e.g., at the enzyme level, remains a possibility. Besides a decline in *PvNCED1* mRNA and protein after 4 h of stress (Fig. 6), the rate of conversion of ABA to phaseic acid increases steadily during water stress until it equals that of ABA synthesis (26, 35). This increased rate is caused by induction of ABA 8′-hydroxylase activity by its own substrate (39). In detached leaves, the level of ABA is determined by the rates of biosynthesis and catabolism. The two pivotal enzymes in these processes are 9-*cis*-epoxycarotenoid dioxygenase and ABA 8′-hydroxylase, respectively. Because ABA induces its own catabolism, it is more likely that ABA levels in plants can be genetically manipulated by repression of 8′-hydroxylase than by over-expression of *NCED*.

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