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 C15 intermediate xanthoxin and C15-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 (C15), and two pathways for its biosynthesis have been proposed: (i) the direct pathway from isopentenyl pyrophosphate (C5) via farnesyl pyrophosphate (C15) 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 (C40). Evidence that this pathway functions in green plants has been obtained with mutants deficient in carotenoids, from 18O2 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 C25-apocarotenoids (Fig. 1). Because there appears to be no isomerization at the C15 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 submersion 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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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 $\sim -0.5$ 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 $-80^\circ C$ 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 CGC GAT CGN GAT CGN 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 oligodT16N 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, 234 to 211, 5'-CCT CAA AAA TAC AAC ACC AAC AAA-3') and the 3' end (JZ163, 12,233 to 12,257, 5'-TGG GGA AAC AAA ACA AGA AGA ATA-3'). The end-to-end PCR product

![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. C25-apocarotenoids are byproducts of the cleavage reaction. The numbering system for ABA (9) is different from that in use for carotenoids (10).](image1)

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![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.](image2)
\(Pv\text{NCED1\textendash}2291\) 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 pGEM\(\text{Pv}\text{NCED1\textendash}2291\) plasmid was linearized with \(\text{NdeI}\), which has a single cleavage site downstream of the \(Pv\text{NCED1\textendash}2291\) coding region. \(\Delta\text{955-Pv}\text{NCED1\textendash}2291\), a 495-bp deletion at the 5′-end of \(Pv\text{NCED1\textendash}2291\) 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 \(Pv\text{NCED1}\), was obtained by the rabbit reticulocyte translation system (Promega). Intact chloroplasts were isolated from 2-week-old pea seedlings (\(P\)isum \(s\)ativum \(L\)) by density gradient centrifugation on a Percoll gradient (18). The import reaction consisted of 50 \(\mu\)l of 1 mg/ml chloroplasts, 2 × \(10^7\) dpm \(^3\)S-labeled \(Pv\text{NCED1}\), and 2 mM Mg-ATP, in a total volume of 150 \(\mu\)l. 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 \(\times g\) 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\textendash}\Delta\text{N} Protein.** A primer corresponding to amino acids S(52) to P(59) of \(Pv\text{NCED1\textendash}2291\) and JZ163 was used to amplify the truncated \(Pv\text{NCED1} (\text{PvNCED1\textendash}\Delta\text{N})\) clone from the pGEM\(\text{Pv}\text{NCED1\textendash}2291\) plasmid. The \(Pv\text{NCED1\textendash}2291\) and \(Pv\text{NCED1\textendash}\Delta\text{N}\) fragments were separated from the pGEM-T Easy Vector by EcoRI digestion and were inserted into the EcoRI site of the pGEX-5X-2 vector (Pharmacia). The resulting plasmids were named pGEX\(\text{Pv}\text{NCED1}\) and pGEXP\(\text{v}\text{NCED1\textendash}\Delta\text{N}\), respectively, and were maintained in the \(\text{Dh5}\alpha\) 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 × YT medium (16 g/liter tryptone/10 g/liter yeast extract/5 g/liter NaCl/100 \(\mu\)g/ml ampicillin) and was grown at 30°C until mid-log phase. Isopropyl-\(\beta\)-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 \(\times g\) 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 \(Pv\text{NCED1}\) and \(Pv\text{NCED1\textendash}\Delta\text{N}\) proteins were eluted in the presence of 0.1% Triton X-100. Protein was quantified by the modified Lowry method (19).

**Preparation of C40-Epoxycarotenoids.** The C40-epoxycarotenoids all-\(\text{trans}\)-violaxanthin and 9′-cis-neoxanthin were isolated from spinach leaves as described (20). After isomerization with iodine, all-\(\text{trans}\)-violaxanthin, 9-cis-violaxanthin, all-\(\text{trans}\)-neoxanthin, and 9′-cis-neoxanthin were separated by normal phase HPLC with a \(\mu\)Porasil 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 \(\mu\)l. 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 \(\mu\)Porasil column (Waters). The putative products, xanthoxin and C25-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 \(\mu\)g) 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 TR Izol reagent (GIBCO/BRL). Leaf (10 \(\mu\)g) or root (20 \(\mu\)g) 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 \(Pv\text{NCED1\textendash}2291\) 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 \(Pv\text{NCED1\textendash}\Delta\text{N}\) 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). \(Pv\text{NCED1\textendash}\Delta\text{N} antigen (100 \(\mu\)g) was run on a 10% SDS/PAGE gel and was electrophoretically transferred 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-\(Pv\text{NCED1}\) 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 \(N\) and extracted in sample buffer (62.5 mM Tris\(\text{HCl}\), pH 6.8/10% glycerol/2% SDS/5% mercaptoethanol) containing a protease inhibitor mixture (Sigma) and 1 mM phenylmethanesulfonyl fluoride (PMSF). After centrifugation, the supernatant was boiled for 5 min. Protein (40–60 \(\mu\)g 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\(\text{HCl}\)/150 mM NaCl, pH 7.4) for 1 h at 22°C. An appropriate amount of purified anti-\(Pv\text{NCED1}\) 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...
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 PnNCED1–2291 was generated with primers JZ166 and JZ163 and sequenced from both strands. The sequence of the PnNCED1–2291 clone was found to be identical to the corresponding region of the PnNCED1 cDNA sequence. The PnNCED1–2291 clone was used for all studies described in this paper. The deduced amino acid sequence of PnNCED1 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 PnNCED1 sequence (Fig. 2).

A Southern blot probed with the PnNCED1–2291 fragment gave the same bands, both at high and low stringency (Fig. 3). Considering that there are two HindIII restriction sites (+1,763, +1,841) and two PstI sites (+326, +1,733) within the gene, these results indicate that PnNCED1 is present as a single copy in the bean genome without intron. In maize (7), tomato (15), and Arabidopsis (ref. 24; Arabidopsis homologs of PnNCED1 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, PnNCED2, was amplified from bean. It showed more similarity with AtNCED1 from Arabidopsis (24) than with PnNCED1. Southern blots probed with PnNCED1–2291 or the partial PnNCED2 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.

PnNCED1 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 PnNCED1 has a putative 51-aa chloroplast-targeting peptide (Fig. 2), which indicates that the mature form of PnNCED1 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 PnNCED1 is localized in the chloroplast, the PnNCED1–2291 sequence was transcribed in vitro and was translated. When the 68-kDa 35S-labeled PnNCED1 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. 4A). No signal was detected in the chloroplasts incubated with the protein Δ495-PnNCED1, whose transit peptide had been deleted (data not shown). To further localize PnNCED1 inside the chloroplast, the chloroplasts were fractionated into soluble and insoluble fractions after completion of import. Autoradiography showed (Fig. 4A, lanes 6 and 7) that the majority of the imported PnNCED1 protein was associated with the insoluble fraction, which contained both thylakoid membranes and chloroplast envelopes. The location of PnNCED1 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-PnNCED1 antibody. A single 63-kDa band was found only in chloroplasts and thylakoids from stressed leaves (Fig. 4B). Thus, both in vitro import studies and Western blotting indicate that PnNCED1 is targeted into chloroplasts. This assures that the protein has access to the carotenoid substrates.

Characterization of PnNCED1 Enzyme Activity. Purified PnNCED1 and PnNCED1-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 PnNCED1 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 C25-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. 5A). When the effectiveness of PnNCED1 and PnNCED1-ΔN was
band, representing the mature form of PvNCED1, was detected
stress treatment (Fig. 6B). The reaction mixture contained 4 nmol of
9-cis-violaxanthin as substrate. (B) Substrate concentration-dependent cleavage
zyme activity with 9-cis-violaxanthin (9cV) or 9'-cis-neoxanthin (9cN) as
substrates. The reaction mixture contained 10 μg of NCED63 protein.

compared, the mature form ΔN was approximately twice as
efficient as the precursor PvNCED1 (Fig. 5A). 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. 5B). Consis-
tent 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. 6A), and PvNCED1 mRNA and protein were
not detectable (Fig. 6B). 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. 6A). At the transcriptional level, the
2.4-kb PvNCED1 mRNA was strongly induced within 0.5 h of
stress treatment (Fig. 6B). 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. 6B). 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. 6A). 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. 6B after 24 h of stress, but
it was detectable after longer exposure of the immunoblot.
However, in chloroplasts and thylakoids, PvNCED1 was de-
dected after 24 h (Fig. 4B) because they are enriched in
PvNCED1 compared with total leaf protein analyzed in Fig. 6B.

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. 7A). During this period, the first detectable decreases
in PvNCED1 mRNA and protein were observed after 0.5 and 1 h,
respectively (Fig. 7B). The rapid disappearance of ABA indi-
cates that ABA 8'-hydroxylase, which converts ABA to phasic
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. 8A). 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. 8A). Preceding the increase
in ABA level, both PvNCED1 mRNA and protein increased, and both decreased rapidly after rehydration (Fig. 8B). 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 2°C or were transferred to a cold room (7°C), or a refrigerator (2°C). 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, 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...
leaves (S) were kept at 22, 7, or 2°C for 5 or 24 h. Nonstressed (Ns) leaves were of PvNCED1 of maize both show substrate specificity for 9-

It is likely that the substrate is limiting (32).

The localization of PvNCED1 in chloroplasts follows from the import assays (Fig. 4B). 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 homologues 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 homologues 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 phasic 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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