EXPRESSION AND LOCALIZATION OF ARACHIS HYPOGAEA 9-CIS EPOXYCAROTENOID DIOXYGENASE 1 (AHNCED1) OF PEANUT UNDER WATER STRESS

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ABSTRACT
The oxidative cleavage of cis-epoxycarotenoids catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) is considered to be the rate-limiting step in abscisic acid (ABA) biosynthesis. The optimal induction conditions for expressing the AhNCED1 (Arachis hypogaea 9-cis epoxycarotenoid dioxygenase 1) protein in E. coli BL21 (DE3) were established. The purified recombination AhNCED1 protein was used to generate a polyclonal antibody in rabbit. This purified antibody was used for western blotting analysis of protein samples extracted from different organs of peanut. Under normal growth conditions, water contents of leaves and stems in peanut were higher than that of roots, and AhNCED1 mRNA and protein were low in stems and leaves, but was not detectable in roots. After treatment with 20% PEG6000 for 7 h, endogenous ABA predominantly accumulated in leaves and stems, and water contents of leaves and roots reduced more than that of stems. At the transcriptional level, AhNCED1 mRNA was strongly induced in roots and leaves. At expression of protein level, AhNCED1 were enhanced in both leaves and roots, but no change in stems. In transgenic Arabidopsis, AhNCED1-GFP fluorescence was detected in the root tips and cotyledons. Intense GFP-fluorescence signals were localized in chloroplasts of cotyledons in transgenic Arabidopsis treated with 20% PEG6000 for 3h, and AhNCED1 targeted into chloroplasts was also indicated by western blotting in peanut. The results suggest that different spatio-temporal expression of AhNCED1 in leaves and roots related to water stress and these areas may be the main places that synthesize ABA in response to water stress.

Keywords: expression, AhNCED1, different organs, peanut, localization


Introduction
Abscisic acid (ABA) is an important plant hormone that mediates plant responses to abiotic stresses, including drought, salinity, and low temperature, and acts with other phytohormones to regulate plant growth and dormancy (21). In addition, ABA may enable plants to drought and cold stresses (18). It is now well established that ABA in plants is synthesized from carotenoids (C40) and is an apo-carotenoid compound derived from oxidative cleavage of the 11, 12 double-bonds of 9-cis-epoxycarotenoids (27). Biochemical and genetic evidence has demonstrated that the cleavage of 9-cis-epoxycarotenoids is the rate-limiting step in the ABA biosynthetic pathway, which is catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) (22).

The NCED enzyme was first identified by analysis of the maize viviparous 14 (vp14) mutant (17). NCED genes have subsequently been identified in several species, including tomato (1), bean (13), avocado (3), Arabidopsis (10), grape (20), peanut (23), and orange (15). Induction of NCED genes by water stress in leaves was correlated with stress-induced ABA biosynthesis (3, 10, 13, 22). Moreover, ectopic expression of a tomato NCED gene caused overproduction of ABA in tomato and tobacco (22). Over-expression of AtNCED3 in transgenic Arabidopsis and PvNCED1 in tobacco plants resulted in increased ABA accumulation and resistance to water stress (10, 13). All of these results strongly suggest a key regulatory role of NCED in stress-induced ABA biosynthesis, at least in photosynthetic tissues.

AhNCED1 (Arachis hypogaea 9-cis epoxycarotenoid dioxygenase1, GenBank Accession No. AJ574819) was previously cloned from water-stressed peanut plants (23). Multiple sequence alignment of the deduced amino acids of AhNCED1 shows that the AhNCED1 protein shares 75%, 74%, 71%, 68%, and 60% identity with the NCED from bean, cowpea, tomato, Arabidopsis, and maize, respectively. The protein sequences are highly homologous at the C terminus. Blocks of similarity are clustered around four conserved histidines. The amino terminus of the AhNCED1 protein is basic, with a high content of Ser and Thr residues characteristic of chloroplast transit peptides (23), which is similar to that found at the amino terminus of ZmVP14 (21). The constitutive expression of the AhNCED1 gene in wild-type Arabidopsis resulted in an increase of ABA accumulation in transgenic plants subjected to water stress. Ectopic expression of the AhNCED1 gene in the 129B08/nced3 Arabidopsis mutant driven by the 35S promoter restores its ability to accumulate ABA during water stress and reverses hypersensitivity to
nonionic osmotic stress and soil drought. These results indicate that the expression of the *AhNCED1* gene plays an important role in the regulation of ABA levels in peanut during water stress, and that water-stress tolerance of Arabidopsis plants can be improved by ectopic expression of the *AhNCED1* gene, causing accumulation of endogenous ABA (23). Immunohistochemical analysis revealed that in water-stressed Arabidopsis plants, AtNCED3 was detected exclusively in the vascular parenchyma cells together with AtABA2 and AAO3. In situ hybridization, using the antisense probe for AtNCED3, showed that the drought-induced expression of AtNCED3 was also restricted to the vascular tissues (8). The spatial constraint of AtNCED3 expression in vascular tissues provides a novel insight into plant systemic response to water stress. Little is known, however, about the expression and location of the NCED proteins of peanut under water stress.

In order to better understand the regulatory mechanism of ABA biosynthesis, an investigation was carried out with an emphasis on characterizing the expression and location of the AhNCED1 protein. This study reports the induction and expression of the AhNCED1 recombination protein in *E. coli*. The protein expression in the organs of the peanut and the localization of the AhNCED1 in transgenic Arabidopsis under normal and water-stressed conditions were investigated. Transgenic Arabidopsis model was used because it is unsuitable to detected GFP-fluorescence signal under a confocal laser scanning microscopy for peanut. The study provides new insight for understanding the function of AhNCED1 and the regulatory mechanism of ABA biosynthesis.

**Materials and Methods**

**Plant materials**

Seeds of peanut (*Arachis hypogaea* L. cv. YueYou 7) were sown in pots with a potting mixture of vermiculite, perlite, and soil (1:1:1) and grown in a growth chamber with 16 h light from fluorescent and incandescent lamps (200 u mol m^{-2} s^{-1}) at 26°C, followed by 8 h darkness at 22°C. Plants were watered daily with half-strength Murashige and Skoog (MS) nutrient solution (12). For western blotting analysis, RNA gel blot analysis, endogenous ABA level and organ water content determination experiments, plants were treated with 20% PEG6000 (polyethylene glycol) for 7h.

Seeds from wild-type *Arabidopsis thaliana* ecotype Columbia and transgenic plants were sterilized, plated on solid medium (1×MS medium, 1% sucrose, and 0.7% agar, pH 5.7), and cold treated for three days to break dormancy and germination. After two weeks, the seedlings were transferred to soil and grown in growth chambers with a 16 h-light/8 h-dark cycle at 23°C in 70% humidity. For the detection of GFP-fluorescence, transgenic plants were treated with 20% PEG6000 for 3h.

**Expression and purification of the recombinant AhNCED1**

*AhNCED1* cDNA were obtained by RT-PCR from total RNA of leaves in peanut using QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA). PCR products was ligated into pProEx HTa expression vector at the ECOR I/PST I cloning site, in frame with a coding sequence for the six His epitope tag to obtain pPr-AhNCED1. pPr-AhNCED1 was transformed into *E. coli*, BL21 (DE3). For the expression of AhNCED1 in *E. coli*, the BL21 (DE3) strain harboring the pPr-AhNCED1 plasmid was grown at 28°C in LB (Luria-Bertani) medium broth supplemented with 100 mg ml^{-1} ampicillin for 15 h. IPTG (isopropyl-b-D-thiogalactopyranoside) was then added to the cell culture with the final concentration of 0.2 mmol l^{-1} to 1.0 mmol l^{-1}. Samples were taken at 1, 4, 8, and 12 h after addition of the 0.2 mmol l^{-1} IPTG and cells were harvested by centrifugation at 5000 rpm for 5 min. The cells were resuspended in 0.1×culture volume of ice-cold His-Bind buffer (0.5 M NaCl, 5 mM imidazole, and 20 mM Tris-HCl, pH 7.9). The cells were lysed by sonication with an ultrasonic processor (Vibrocell VC600 sonicator, 600 W max; Sonics & Materials inc., USA) for 10 s at 4°C and centrifuged at 25 000g for 15 min. Supernatants (soluble fraction) and pellets harvested by centrifugation at 5000 rpm for 5 min. The cells were resuspended in 0.1×culture volume of extraction buffer (0.5 M NaCl, 2 M urea, 1 mM DTT, 2% Triton X-100, and 20 Mm Tris-HCl, pH 7.5), sonicated for 10 s at 4°C and centrifuged at 25 000g for 15 min. The pellets were washed twice with washing buffer (0.5 M NaCl, 2 M urea, 20 mM Tris-HCl, pH 7.5), resuspended in regeneration buffer (0.5 M NaCl, 6 M urea, 20 mM Tris-HCl, pH 7.5), and incubated at room temperature (25°C) for 30 min. The incubated mixture was centrifuged at 25 000g for 10 min and the supernatant was used for further purification. The AhNCED1 from the insoluble fraction was also purified with a His-Bind purification kit (QIAGEN, USA). In addition, the insoluble fraction was resuspended in 0.1×culture volume of extraction buffer (0.5 M NaCl, 2 M urea, 1 mM DTT), resuspended in regeneration buffer (0.5 M NaCl, 6 M urea, 20 mM Tris-HCl, pH 7.5), and incubated at room temperature (25°C) for 30 min. The incubated mixture was centrifuged at 25 000g for 10 min and the supernatant was used for further purification. The AhNCED1 from the insoluble fraction was also purified with a His-Bind purification kit (QIAGEN, USA). To prevent protein aggregation during the purification steps, all of the buffers used for the insoluble fraction (binding buffer, washing buffer, and elution buffer) contained 6 M urea. The purified AhNCED1 from both the soluble and insoluble fractions was desalted with an Amicon Ultra-15 filter (Millipore, USA). Desalting buffer (100 mM Tricine, pH 7.5, 10% glycerol, and 1 mM DTT) was added to the Amicon filter, and the purified AhNCED1 was further added drop by drop, followed by centrifugation at 2800g for 30 min. This desalting step was repeated three times. Thereafter, a refolding buffer (100 mM Tricine, pH 7.5, 10% glycerol) including 10 mol l^{-1} FeSO_{4} was added to the AhNCED1 to remove DTT, followed by centrifugation at 2800g for 30 min. The refolding step was also repeated three times. The His-tag was removed with a Thrombin Cleavage Capture Kit (QIAGEN, USA). The concentration of purified AhNCED1 after His-tag cleavage was measured with the Bradford assay.
Antibody preparation, protein extraction, and western blotting

The AhNCED1 protein purified from BL21 (DE3) cells was used to raise polyclonal antibodies in rabbit against NCED. The crude antiserum was purified by affinity purification. Each 100 mg of AhNCED1 antigen was run on a 12% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel and electroblotted to a PVDF (polyvinylidene fluoride) membrane. The blot membranes were first blocked with a 5% solution of skim milk in TBS and then incubated with crude antiserum for 1 h. Subsequently, the blots were washed in TBS containing 0.05% Tween 20 to remove the unbound and nonspecific antisera. The specific anti-AhNCED1 antibody bound to the membrane was eluted with 100 mmol l⁻¹ glycine solution of skim milk in TBS and then incubated with crude antiserum for 1 h. Subsequently, the blots were washed in TBS containing 0.05% Tween 20 to remove the unbound and nonspecific antisera. The specific anti-AhNCED1 antibody bound to the membrane was eluted with 100 mmol l⁻¹ glycine buffer (pH 8.0) and stored at 4°C.

Leaf, stem and root samples (0.5 g) were ground in a mortar with pestle in liquid nitrogen, and extracted in a sample buffer (62.5 mmol l⁻¹ Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% l-1 Tris buffer, pH 8.0) and stored at 4°C.

After centrifugation, the supernatant was boiled for 5 min. The boiled protein sample (40-60 mg per lane) was loaded on a 12% SDS-PAGE gel, and separated by electrophoresis. Following protein separation by SDS-PAGE, proteins were transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked in 10% BSA/PBS for 1 h and incubated for 1 h with anti-AhNCED1 antibody (1:2000 in 5% BSA/PBS; Invitrogen). After washing in 0.1% PBS-Tween (PBS-T) three times, the blots were incubated for 1 h with an anti-rabbit secondary antibody conjugated to alkaline-phosphatase (Sigma) and developed with BCIP/NBT.

RNA gel blot analysis

Total RNA was extracted from the frozen samples using the modified phenol-chloroform method as described in Wan and Li (23). To investigate the expression pattern of AhNCED1 gene in imbibed peanut seeds in response to ABA or naproxen treatment, RNA gel blot analysis was carried out according to the instructions of the Digoxigenin Nucleic Acid Detection Kit (Roche, USA). Each total RNA (20 μg) was separated by 1.5% agarose gel electrophoresis and then blotted onto the Hybond N membrane (Amersham, USA). The PCR Digoxigenin Probe Synthesis Kit (Roche) was used to generate the AhNCED1 gene specific probe with peanut cDNA (GenBank accession number AJ574819) as a template according to the manufacturer's instructions. The gene-specific primers used were GSP1 (GTT CAG GCC GTG AAA TTC CAC) and GSP2 (GCG CTT CAA TCC ACC GGA TAC CA). Hybridization and detection were performed according to the standard procedures as specified by the manufacturer (Roche).

Measurement of endogenous ABA level

Endogenous ABA was extracted, as described by Xiong et al. (25), from drought-stressed or control peanut plants and Arabidopsis plants. Extraction in non-oxidative methanol:water (80:20, v/v), pre-purification through SepPak C18 cartridges (Waters, Milford, MA, USA) and HPLC fractionation in a Nucleosil C18 column (Macherey-Nagel, Germany) have been previously described (4). Recovery of ABA on purification was determined by means of [³H]ABA added to the extracts and scintillation counting of aliquots of the purified fractions. The ELISA procedure was based upon the competition, for a limited amount of monoclonal anti-ABA antibody (Xia, China), between standard ABA–BSA conjugate adsorbed on the wells of a microtitration plate and free ABA extracted from the samples. Bound antibodies were labelled with a peroxidase-conjugated goat antibody to mouse immunoglobulins (Sigma), and peroxidase activity was then measured. A standard curve was established on each microtitration plate. ABA content was determined five times for each sample.

Plant organ water content

Following the 7 h water stress treatment, leaves, stems, and roots were cut and collected at midday to determine the fresh weight (FW). Organ water content (OWC) was determined from organ fresh weight (OFW) and organ dry weight (ODW) using the equation OWC = (OFW - ODW)/OFW × 100. ODW was determined after drying the organ for 2 h at 95°C. OWC was expressed as g H₂O·g FW⁻¹.

Plant transformation and Determination of AhNCED1-GFP protein localization

To produce transgenic plants in which GFP-tagged AhNCED1 protein was expressed under the control of the AhNCED1 promoter. Transformation to the wild-type Arabidopsis (Col) plants was performed according to the procedure described by Bechthold et al. (2). Transformants were selected on Murashige and Skoog agar with 50 mg ml⁻¹ kanamycin. Plants with a single copy of the transgene insertion were selected based on segregation of the selection marker gene in T2 progeny, and homozygous transgenic lines were generated. For the detection of GFP-fluorescence, Arabidopsis foliage leaves and roots detached from 2-week-old seedlings and etiolated hypocotyls were mounted on slides. For the control, 35S-pPR(pPRoEX HTa -GFP transgenic Arabidopsis were used. GFP-fluorescence was observed under a confocal laser scanning microscopy (LSM 510 Meta, Zeiss) using a blue laser light at 488 nm and emission through a 505-530 bandpass filter.

Chloroplast Isolation from peanut Leaves

Chloroplast isolation and fractionation were performed according to the method of Robinson and Mant (16). Briefly, chloroplasts were isolated from 3-week-old peanut plants grown on a potting mixture of vermiculite, perlite, and soil (1:1:1). Two grams of leaves from 3-h drought-stressed plants was homogenized (Physectron) in 30 ml of ice-cold grinding...
buffer, and intact chloroplasts were fractionated on a Percoll gradient as described.

Results and Discussion
The NCED enzyme is thought to be the rate-limiting enzyme in ABA biosynthetic pathway. The full-length NCED cDNA was previously cloned from dehydrated peanut (Arachis hypogaea L.) leaves (23), and subsequently characterized (24). There was, however, little data about the expression and localization of the NCED proteins of peanut.

Expression of fusion AhNCED1 and purification

Fig. 1. The optimal induction conditions and expression of AhNCED1 in E. coli

(A) SDS-PAGE analysis of the expression level of AhNCED1 induced with different concentrations of IPTG. Final concentrations of IPTG were 0.2 mmol l⁻¹ to 1.0 mmol l⁻¹ for 8 h;

(B) SDS-PAGE analysis of the expression level of AhNCED1 induced with IPTG for different hours. AhNCED1 was induced for 0, 2, 4, 8, and 12 h after the addition of 0.2 mmol l⁻¹ IPTG;

(C) SDS-PAGE analysis of AhNCED1 expressed in E. coli harboring pPR-AhNCED1 induced by 0.2 mmol l⁻¹ IPTG for 12 h. M: molecular markers; Lane I: total protein transformed with pPR vector as a control; Lane II: total protein of the pPR-AhNCED1 transformant; Lane III: insoluble protein of the pPR-AhNCED1 transformant; Lane IV: soluble protein of the pPR-AhNCED1 transformant;

(D) Purification of recombinant AhNCED1 and western blotting analysis with antibody AhNCED1. M: molecular markers; 1: total cell protein of the pPR-AhNCED1 transformant after 0.2 mmol l⁻¹ IPTG induction for 12 h; 2 and 3: purified pPR-AhNCED1 protein with Ni-NTA superflow column; 4: pPR negative control for hybridization; 5: hybridization of pPR-AhNCED1 protein with antibody

To express AhNCED1 in E. coli, the coding region was cloned into the expression vector pProEX HTa. The expression construct was checked by restriction enzyme digestion and DNA sequencing. pPR-AhNCED1 was introduced into an E. coli host (BL21- DE3) and induced with IPTG. SDS-PAGE showed that the expression level of AhNCED1 with different concentrations of IPTG for the same induction time was nearly identical (Fig. 1A). However, under conditions with the same concentration of IPTG, the expression level of AhNCED1 was enhanced as the induction time was increased (Fig. 1B).

The suitable induction condition for protein expression was set to 0.2 mmol l⁻¹ IPTG for 12 h. These conditions enabled a shorter induction time and resulted in the expression of AhNCED1 recombination protein. Under this condition, SDS-PAGE of the crude cell extracts identified a 66-kDa protein band from the IPTG induced pPR-AhNCED1 cell extracts, but not from the pPR control cell extracts, and the recombination protein was soluble (Fig. 1C). It is known that AhNCED1 encodes a polypeptide of 601 amino acids, with a calculated molecular weight of 66.86 KD (23). The resulting recombinant AhNCED1 protein was soluble and had a molecular weight of about 66 kDa, which was consistent with that of the previously reported AhNCED1 protein (23). The expressed AhNCED1 protein in E. coli was purified and the purity of the resulting protein sample was verified by SDS-PAGE, exhibiting specific AhNCED1 protein band run at the position equivalent to 66 kDa (Fig. 1D). The purified recombination AhNCED1 protein was used to generate a polyclonal antibody in rabbit. This antibody was able to identify a single band of AhNCED1 homologous protein run also at the 66 kDa position in the total protein extract from E. coli (Fig. 1D). So, the AhNCED1 antibody was used for western blotting analysis of protein samples extracted from different tissues in order to examine the responsive organ of peanut. It could help us better understand the function of AhNCED1.

Expression of AhNCED1 mRNA and AhNCED1 protein in different organs of peanut under water stress

To determine the organ specificity of AhNCED1 protein expression and endogenous ABA accumulation under water stress, total mRNA and protein was prepared separately from leaves, stems, and roots of 7-h 20% PEG6000 treatment or control peanut plants. The expression of AhNCED1 mRNA and protein, as well as ABA accumulation and organ water contents, in different organs under water stress is shown at Fig. 2. In the control plants (fully hydrated), leaves, stems and roots contained low ABA (Fig. 2A). The water contents of leaves and stems was higher than that of roots (Fig. 2B), and AhNCED1 mRNA and protein were low-levels expression in stems and leaves, but not detectable in roots (Fig. 2C). After water stress, ABA was rapidly synthesized and accumulated after 7h (Fig. 2A), and the reduced water contents of leaves and roots were more than that of stems (Fig. 2B). At the transcriptional level, the AhNCED1 mRNA was strongly induced in roots and leaves (Fig. 2C). At the protein level, AhNCED1, were enhanced in both leaves and roots, but there was no change in stems (Fig. 2C). The endogenous ABA also predominantly accumulated in leaves and stems under the dehydration condition (Fig. 2A).
Fig. 2. Organ specificity of the accumulation of endogenous ABA (A); organ water contents (B); AhNCED1 mRNA and protein levels (C) in peanut plants in response to water stress. Actin was detected on the same blot as a control for protein loading. Total mRNA and protein was prepared separately for roots (R), stems (S), and leaves (L) of 7-h water stress or control peanut plants. The ABA levels and organ water contents (OWC) in the root, stem, and leaf of peanut plants at the presence or absence of 7-h water stress treatment were measured in triplicate for each sample as described in “Materials and Methods”.

In dehydrated cowpea plants, the VuNCED1 gene (GenBank Accession No. AB030293) was strongly expressed in leaves and stems, but not in roots. The endogenous ABA also mainly accumulated in leaves and stems under conditions of water stress (9). In the present study, expression of AhNCED1 mRNA and protein in peanut under normal growth conditions was predominantly in the leaves and stems, with no expression in roots. In response to water stress, however, the AhNCED1 mRNA and protein, and endogenous ABA all predominantly accumulated in leaves and roots, with only a slight change in the stems (Fig. 2). The organ-specific pattern of ABA accumulation under water stress was consistent with that of AhNCED1 mRNA and protein expression, as shown in Fig. 2. Liang et al (11) isolated the AhNCED1 promoter from peanut genomic DNA and found that the activity of the promoter was predominantly exhibited in leaves and relatively weaker in terms of staining in the hypocotyls and radicles. The promoter activity was enhanced by water stress. Thompson et al (22) indicated that in leaves of tomato 9-cis-epoxycarotenoids is present in abundance, and the enzyme activities involved in the reaction from xanthoxin to ABA are constitutive (14). Therefore, these results provide evidence that leaves and roots may be responsive to water stress resulting in increased ABA accumulation. Accumulation of the AhNCED1 protein in these organs would therefore permit a prompt response to water stress.

Location of AhNCED1 in Arabidopsis and peanut plants under water stress

Fig. 3. Intracellular localization of AhNCED1 protein. GFP-fluorescence images of transgenic plant that did not introduce AhNCED1 (A, C, E, F, G, H) and transgenic plant introduced GFP-tagged AhNCED1 protein (B, D, I, J, K, L,) are shown. Bars = 100 µm for A to D and 10 µm for E and L. (A) and (C) GFP-fluorescence images of a transgenic plant with introduced...
of a protein (26). In previous studies, most nceD proteins transformation and detection of GFP-fluorescence signal Arabidopsis plants were usually used for the purpose of probed with antiserum raised against recombinant AhnceD1 sample. each lane was loaded with 30 µg of protein. the membranes were cytosolic (cyt) and chloroplast (chlp) fractions were separated for each leaf either were kept turgid (0 h), or were treated with 20% PeG6000 for 3h. (Fig. 4). immunoblot of protein extracted from detached peanut leaves that treating plants with 20% PeG6000 for 3 h

K

L

Merged immunofluorescence and phase contrast images of (K) and (L) GFP-fluorescence images of GFP-tagged AhNCED1 protein in plant cells of the main root under normal growth conditions and treating plants with 20% PEG6000 for 3h;

(K) and (L) Merged immunofluorescence and phase contrast images of transgenic plant cell with introduced GFP-tagged AhncD1 protein after treating plants with 20% PEG6000 for 3 h

Fig. 4. Immunoblot of protein extracted from detached peanut leaves that either were kept turgid (0 h), or were treated with 20% PEG6000 for 3h. Cytosolic (Cyt) and chloroplast (Chlp) 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 AhNCED1

Arabidopsis plants were usually used for the purpose of transformation and detection of GFP-fluorescence signal of a protein (26). In previous studies, most NCED proteins in plant cells were targeted to chloroplasts in the cytoplasm (19, 21). AhNCED1 was reported to be located in plastids because the first 30 amino acids of the protein function as a chloroplast transit peptide (23). It has been suggested that the ABA biosynthesis pathway could occur inside chloroplasts (9). However, biochemical analyses of ABA biosynthetic enzymes have indicated that the last steps of ABA biosynthesis may occur in the cytosol (5). The possibility of dynamic mobilization of the ABA precursor and/or ABA in Arabidopsis has been proposed since reporter expression has been shown to be highly restricted to specific vascular tissues that are not thought to be target sites of ABA action (6). In this study, two constructs for expression of the promotor-AhNCED1-GFP and 35S-pPR-GFP fusion proteins for Arabidopsis transformation were created and confirmed by DNA sequencing before they were introduced into the plant. The intracellular localization of AhNCED1 protein is documented on Fig. 3A to L. At the level of tissues, the GFP-fluorescence signal from a 35S-pPR-GFP transgenic plant was detected in hypocotyls and the edge of cotyledons (Fig. 3A, C). However, the fluorescence signal of GFP-tagged AhNCED1 protein was detected in tissues with active cell division, such as the main root tips (Fig. 3B) and cotyledons (Fig. 3D) of Arabidopsis. For a long time it has generally been regarded that root tips are the main site for ABA biosynthesis and that ABA is transported from there to the target tissues (7). Under conditions of water stress, the fluorescence signal of AhNCED1-GFP fusion protein in the chloroplast of these tissues was enhanced (Fig. 3J). The use of a blue laser light at 488 nm and emission through a 505-530 bandpass filter, allowed for verification that this observation was not due to autofluorescence from the chloroplasts in the transgenic plants. Thus, both in vitro studies and western blotting indicate that AhNCED1 is targeted into chloroplasts. This assures that the protein has access to the carotenoid substrates.

Conclusions

AhNCED1 was effectively expressed in E. coli. The AhNCED1 mRNA and protein in both the leaves and roots of peanut were responsive to water stress. Then in these place ABA accumulation were increased, thereby improving the drought tolerance of peanut plants. AhNCED1 is targeted into chloroplasts. It will be interesting to determine the initial perception of water stress and the signal transduction pathway that up-regulates the AhNCED1 protein expression and the ABA level in plants during drought-stress response.

Acknowledgements

We are grateful to Dr Marnie Light at Research Centre for Plant Growth and Development, University of KwaZulu-Natal in South Africa and Prof. Hai Hang Li at college of life science, South China Normal University in China, for technical support and critical reading of the manuscript. This study was supported by grants from the National Natural Science Foundation of China (30771297), Natural Science Program of Guangdong Province (06025049), Science and Technology Programs of Guangdong Province (2006B20101002).
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