We cloned the promoter of the 9-cis-epoxycarotenoid dioxygenase gene from *Arachis hypogaea* L. β-Glucuronidase (GUS) histochemical staining and GUS activity assay indicated that the activity of the promoter was exhibited predominantly in the leaves and enhanced by water and NaCl stresses, and by application of abscisic acid (ABA) and salicylic acid (SA) in transgenic *Arabidopsis*. Moreover, two novel ABRE-like (abscisic acid response element) elements were identified in the promoter region.

Drought is a major abiotic stress that limits the growth and production of plants. The mechanisms of drought responses have been investigated most extensively in *Arabidopsis*; but the responses of agricultural crops to drought stress have not been extensively studied. Abscisic acid (ABA) is a key regulator of seed de-germination, root growth, stomatal aperture, and plant responses to dehydration stress. Moreover, ABA regulates the expression of many genes, the products of which can function in the dehydration tolerance in both vegetative tissues and seeds. ABA is an apocarotenoid compound derived from oxidative cleavage of the 11, 12 double bond of 9-cis-epoxycarotenoids (neoxanthin and/or violaxanthin). The 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme was identified by analysis of the maize *viviparous 14* (vp14) mutant. Induction of *NCED* genes by drought stress in leaves of *Arabidopsis* is closely correlated with stress-induced ABA biosynthesis.

Investigation of tissue-specific expression of *AtNCEDs*:GUS has been performed in transgenic *Arabidopsis* only, and it indicated that *AtNCED3* is the major stress-induced gene in leaves, with minor contributions from other *AtNCEDs*. The relationship between drought tolerance and the levels of *NCED* gene expression and endogenous ABA in agricultural crops must be examined. We have isolated and characterized a dehydration-inducible *NCED* gene, *AhNCED1* (GenBank accession no. AJ574819), from dehydrated peanut (*Arachis hypogaea* L.) leaves.

To gain further understanding of the regulatory mechanism of *AhNCED1* gene expression, we isolated the *AhNCED1* promoter and analyzed its specific expression by GUS reporter-aided histochemical staining and fluorometric assay in transgenic *Arabidopsis*.

The *AhNCED1* promoter was isolated by the PCR-based genomic walking procedure as described by Siebert et al. Based on the cDNA sequence of the *AhNCED1* gene (GenBank accession no. AJ574819), two non-overlapping gene-specific primers were designed to amplify the region upstream of the coding sequence. Primary PCR was carried out using an adaptor primer, 5′-GTAATACGACTCATATAAGGC-3′, and a gene-specific primer, 5′-CAATACAAAACTTCCATCATCGCTCCTC-3′, followed by a second PCR with a nested adaptor primer, 5′-ACTATAGGCGACCGGTGGTTG-3′, and a nested gene-specific primer, 5′-GCCAACATGGGACACATTTCAAACATGGG-3′.

Ex Taq DNA Polymerase (Takara, China) with proof reading activity was used for amplification. The 2,446-bp PCR walking product from the *SacI* adaptor-ligated DNA library was cloned and sequenced (GenBank accession no. EU497940) (Fig. 1). Nucleotide BLAST searches of sequences upstream of the start codon did not reveal any significant homology to any known nucleotide sequences in the GenBank database. The promoter sequence was analyzed for potential cis-elements using the programs PLACE and PlantCare. A number of motifs were found in the *AhNCED1* promoter upstream of two putative basic cis-elements, TATA box (−318 to −308) and CAAT box (−323 to −319). These included two copies of the ABRE motif (−1,380 to −1,375 and −1,323 to −1,319) involved in drought and ABA responsiveness, one TCA-element (−382 to −373) involved in SA responsiveness, and one WUN-motif (−1,780 to −1,772) involved in wound responsiveness.

One TC-rich motif (−1,213 to −1,256) involved in defense and stress responsiveness, one skin-1 motif (−1,260 to −1,256) involved in endosperm expression, and several cis-elements in response to light (data not shown) were also found in the promoter region. The fact that many cis-elements related to various stresses are present in the promoter region suggests that the *AhNCED1* gene is controlled by a complicated regulatory mechanism and that it can respond to various stresses.

To characterize the *AhNCED1* promoter, we made and analyzed transgenic *Arabidopsis* expressing the...
AhNCED1::GUS fusion gene and its base-substituted mutants, mut-1 and mut-2, with vector pCAMBIA1301. The promoter size of AhNCED1 is 1,418-bp, including two putative ABRE, motif A (−1,380 to −1,375) and motif B (−1,332 to −1,319). Site-directed mutagenesis was carried out as described by Seyfang and Jin. The following synthetic oligonucleotides were used to introduce mutations into the putative ABRE-like elements: mut-1 (5′-AGGCACATCGAGAGGAGAATG-3′) and mut-2 (5′-GGCGACACGGGGGTTCGTCGGTTATG-3′). The underlined sequences show the nucleotides mutated. Arabidopsis ecotype Col-0 was transformed with Agrobacterium strain GV3101 harboring the constructs described above by the floral dip method. Regenerated plants were collected from three independent lines for each construct. The transgenic AhNCED1::GUS construct generated in our previous work and GUS histochemical staining observed in a majority of the transgenic lines respectively in GUS histochemical negative controls respectively in GUS histochemical staining. Figure 2A shows the representative GUS staining in the hypocotyls and radicles (Fig. 2A a). In the hypocotyls, GUS activity was barely detected in the initial growth stage, GUS expression was undetectable in the initial reproductive stage and the inflorescence development stage (Fig. 2A a and j). During the reproductive growth stage, GUS activity was observed in the floral buds, stigmas, stamens, sepal, and pollen, but not in the petals, pistils, or pedicels (Fig. 2A f–h). In the mature silique, intense GUS expression was restricted to the abscission zone and the stigmatic papillae (Fig. 2A i). The above results indicated that the AhNCED1 promoter possesses a specific developmentally regulated expression in vegetative and reproductive tissues, consistently with previous results that ABA is involved in various growth and developmental stages of plants.

For abiotic stresses, 20 6-d-old transgenic seedlings were transferred onto filter papers soaked with a solution containing 300 mmol/l sorbitol for 2.5 h, 250 mmol/l NaCl for 8 h, or 100 μmol/l ABA for 6 h. Salicylic acid (SA) treatment was done by wetting the filter papers with 1 mmol/l SA solution and 20 10-d-old transgenic seedlings were allowed to grow on it for 5 h. Seedlings grown in 1/2 MS media under normal conditions were used as controls. All treatments were done independently, and three independent lines were used in each comparison. The transformant with the AhNCED1::GUS fusion gene without mutation exhibited dehydration-induced expression of GUS activity in the leaves, but not in the roots (Fig. 2B c and h). GUS activity was increased 1.87-fold (Fig. 3A). However, the transformants of mut-1 and mut-2 barely exhibited dehydration-induced expression of GUS activity (Fig. 2B d, e, i and j). The dehydration-induced GUS activities were 43.7% and 34.9% in mut-1 and mut-2 respectively, as compared to the transformants without mutation (Fig. 3A). Previous reports have indicated that many dehydration- and ABA-inducible genes contain a conserved cis-element named ABRE (abscisic acid response element; ACGTGG/TC) in their promoter regions. Various types of ABRE-like elements have been reported, including the G-box sequence (CACCGTG) and coupling element (CGCGTG), CE3, hex3, and motif III in their promoter regions. Most of the known ABRE-like elements contain an A/GCCT core motif.
Fig. 2. Histochemical Localization of GUS Activity in Transgenic Arabidopsis.

A, GUS expression of the AhNCED1::GUS recombinant transgene in the developing transgenic plants under normal conditions: a, 2-d-old seedling; b, 5-d-old seedling; c, 10-d-old seedling; d, 2-week-old plant; e, floral buds; g, mature flower; h, stamen; i, mature siliques; j, plant in the inflorescence development stage. B, Histochemical analysis of GUS expression driven by the AhNCED1 promoter and its mutations in response to dehydration. a and f, wild-type Arabidopsis seedlings (negative control); b and g, transgenic Arabidopsis seedlings harboring the 35S::GUS fusion construct (positive control); c and h, transgenic Arabidopsis seedlings harboring the AhNCED1::GUS fusion construct without mutation; d and i, transgenic Arabidopsis harboring the AhNCED1::GUS fusion construct with substitution mutation mut-1 for motif A (−1,380 to −1,375); e and j, transgenic Arabidopsis harboring the AhNCED1::GUS fusion construct with substitution mutation mut-2 for motif B (−1,323 to −1,319). Seedlings were grown in 1/2 MS media and then exposed to dehydration for 2.5 h (f, g, h, i, and j) or were grown under normal conditions (a, b, c, d, and e).

Fig. 3. Fluorometric GUS Assay of AhNCED1::GUS Fusion Constructs in Response to Abiotic Stresses and Hormone Treatment in Transgenic Arabidopsis Seedlings.

A, Transgenic Arabidopsis seedlings harboring the AhNCED1::GUS fusion construct and its base-substituted mutants (mut-1 and mut-2) were treated with 300 mmol/l sorbitol for 2.5 h. B, Transgenic Arabidopsis seedlings harboring the AhNCED1::GUS fusion construct were treated with 250 mmol/l NaCl for 8 h, 100 μmol/l ABA for 6 h and 1 mmol/l SA for 5 h. Error bars represent the standard deviations of three independent transgenic lines.
copy of these cis-elements is not sufficient for the full drought response. On the other hand, GUS activities were enhanced by NaCl stress, ABA application, and SA treatment in the transgenic seedlings transformed with the \textit{AhNCED1::GUS} fusion gene. Activities were increased 85.2%, 1.49-fold and 44.6% respectively as compared to the untreated controls (Fig. 3B). These data above indicate that the \textit{AhNCED1} promoter exhibits a significant response to NaCl stress, ABA application, and SA treatment.

In conclusion, we isolated the \textit{AhNCED1} promoter from peanut genomic DNA and characterized its spatial and temporal expression patterns in transgenic \textit{Arabidopsis}. We found that the activity of the promoter was predominantly exhibited in the leaves, and that promoter activity was enhanced by water stress, high salinity stress, ABA application, and SA treatment. Two novel ABRE-like elements were identified in the promoter region by site-directed mutagenesis. It was found that the \textit{AhNCED1} gene plays a crucial role in regulating plant growth and development. The present work perhaps provides useful information for further investigation of the initial perception of drought stress and the signal transduction pathway leading to elevated \textit{AhNCED1} expression and ABA levels in peanut. Furthermore, our work perhaps opens up the possibility of isolating the abscisic acid-responsive element binding protein (AREB), and might lead to practical approaches to the genetic modification of drought tolerance in crops.

Acknowledgments

We are grateful to Professor N. N. Wang of the Department of Plant Biology and Ecology, Nankai University, China, for technical support and critical reading of the manuscript. This work was supported by the National Natural Science Fund of China (30771297), the Natural Science Program of Guangdong Province (06025049), and the Higher Education Natural Science Research Project of Guangdong Province (06Z0099), China.

References