Characterization of the Promoter Region of an Arabidopsis Gene for 9-cis-Epoxycarotenoid Dioxygenase Involved in Dehydration-Inducible Transcription

BABAK Behnam1,†, SATOSHI Iuchi2,†, MIKI Fujita1, YASUNARI Fujita3, HIROHORI Takasaki1, YURIKO Osakabe1, KAZUKO Yamaguchi-Shinozaki3,4, MASATOMO Kobayashi2, and KAZUO Shinozaki1,*

Gene Discovery Research Group, RIKEN Center for Sustainable Resource Science, 3-1-1 Koyadai, Tsukuba 305-0074, Japan1; Experimental Plant Division, RIKEN Bio-Resource Center, 3-1-1 Koyadai, Tsukuba 305-0074, Japan2; Biological Resources and Post-Harvest Division, Japan International Research Center for Agricultural Sciences, 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan3 and Laboratory of Plant Molecular Physiology, Graduate School of Agricultural and Life Science, The University of Tokyo, 1-1 Yoyoi, Bunkyo-ku, Tokyo 113-8657, Japan4

*To whom correspondence should be addressed. Tel. +81-29-836-4359. Fax. +81-29-836-9060. Email: sinozaki@rtc.riken.jp

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Abstract
Plants respond to dehydration stress and tolerate water-deficit status through complex physiological and cellular processes. Many genes are induced by water deficit. Abscisic acid (ABA) plays important roles in tolerance to dehydration stress by inducing many stress genes. ABA is synthesized de novo in response to dehydration. Most of the genes involved in ABA biosynthesis have been identified, and they are expressed mainly in leaf vascular tissues. Of the products of such genes, 9-cis-epoxycarotenoid dioxygenase (NCED) is a key enzyme in ABA biosynthesis. One of the five NCED genes in Arabidopsis, AtNCED3, is significantly induced by dehydration. To understand the regulatory mechanism of the early stages of the dehydration stress response, it is important to analyse the transcriptional regulatory systems of AtNCED3.

In the present study, we found that an overlapping G-box recognition sequence (5'-CACGTG-3') at −2248 bp from the transcriptional start site of AtNCED3 is an important cis-acting element in the induction of the dehydration response. We discuss the possible transcriptional regulatory system of dehydration-responsive AtNCED3 expression, and how this may control the level of ABA under water-deficit conditions.

Key words: Arabidopsis; abscisic acid (ABA); 9-cis-epoxycarotenoid dioxygenase (NCED); AtNCED3; dehydration response; cis-acting element

1. Introduction

As crop plants are sessile organisms, environmental stresses such as drought and high salinity can compromise economic output and the overall human food supply.1 Drought stress gives rise to biochemical, physiological, and morphological changes that adversely affect the growth, development, and productivity of plants.2–5 The plant hormone abscisic acid (ABA) mediates a variety of physiological and cellular processes, including the response to drought and salt stresses. ABA is synthesized de novo under water-deficit conditions, activates stress-related signal transduction pathways, and modulates the expression of stress-related genes that lead to a variety of physiological and cellular responses, including the accumulation of osmolytes, stomata closure, and tolerance to drought and salt stresses6–12. ABA is synthesized during dehydration and degraded during rehydration. Key genes involved in ABA biosynthesis during dehydration have recently been identified.

† These authors contributed equally to this work.

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based on genetic and molecular approaches, particularly in *Arabidopsis*. Many of these are induced by dehydration stress, including the AtNCED3 gene for 9-cis-epoxy-carotenoid dioxygenase (NCED), the AAO3 gene for asbscisic aldehyde oxidase, the AtABA3 gene for molybdenum cofactor sulphurase, and the AtZEP gene for zeaxanthin epoxidase. The carotenoid cleavage reaction is a key irreversible step in the ABA biosynthesis pathway. This supports the key role of NCED in ABA biosynthesis. In the *Arabidopsis* genome, there are five NCED genes (AtNCEDs). Among them, AtNCED3 is most strongly induced by dehydration and high salinity. Iuchi *et al.* demonstrated that the expression of AtNCED3 is induced by dehydration stress and increases the level of endogenous ABA under dehydration conditions. Overexpression of AtNCED3 improves tolerance to dehydration stress in transgenic plants, whereas its knockout mutant has a dehydration-sensitive phenotype. This indicates that the induction of AtNCED3 regulates the accumulation of ABA during dehydration.

The induction of AtNCED3 occurs in the early stages of the response to dehydration stress. Hence, we believe that it is important to understand the regulation of AtNCED3 transcription to understand the molecular processes involved in early dehydration responses before the production of ABA. Therefore, it is essential to identify and understand the cis-acting elements in the AtNCED3 promoter involved in the responses to dehydration stress. A previous study analysed the effects of a 1.5-kb AtNCED3 promoter on tissue-specific expression in transgenic *Arabidopsis*; however, the GUS reporter gene was not induced in response to water deficit. There has been no comprehensive analysis of the AtNCED3 promoter under dehydration stress.

In the present study, we analysed the longer promoter region of the AtNCED3 promoter to identify cis-acting elements involved in the induction of the gene in response to dehydration. We found that a 3.0-kb region functions in the induced response to dehydration and contains cis-acting elements involved in dehydration-stressed gene expression. Then, we narrowed down the promoter region to a 200 bp region at −2.2-kb and identified cis-acting elements involved in the response of AtNCED3 to dehydration. We found that a G-box-like cis-regulatory element is essential for the dehydration-responsive induction of AtNCED3. We discuss the transcriptional regulation of AtNCED3 in the early dehydration response.

2. Materials and methods

2.1. Plant materials and stress treatment

Transgenic plants of *Arabidopsis thaliana* (Columbia ecotype) were grown on germination medium (GM) agar plates with appropriate antibiotics at 22°C. Plants (transgenic lines or Col ecotype) were grown under continuous light at 22°C for 2 weeks. Plants were exposed to dehydration as described previously.

2.2. Plasmid constructs and transgenic Arabidopsis plants

Linker and primer sequences are described in Supplementary Table S1. To construct the pBE-CD vector, two types of linker were annealed and ligated into the EcoRI and HindIII sites of the pBE2113 vector. The resulting vector was designated the pBE-CD vector.

To make a construct including the 3.0-kb region of the AtNCED3 promoter fused with the GUS gene, the promoter region of *Arabidopsis* genomic DNA and the GUS-NOS terminator region of the pBI101 vector were amplified by PCR. The amplified fragments were digested using the NotI restriction enzyme and then ligated. The ligated fragment and pBE-CD vector were digested using the SfiI restriction enzyme; these were again ligated (Fig. 1A).

Next, we made two constructs: one with the 3.0-kb promoter region of AtNCED3 and another with the 2.0-kb promoter region of AtNCED3 fused to the GFP gene. These promoter regions were amplified by PCR from *Arabidopsis* genomic DNA, and the amplified fragments were digested using the *SalI* restriction enzyme and ligated into the *SalI* site of the pBI101GFP vector (Fig. 2, constructs A and B).

Next, to generate a construct with the 0.1-kb promoter region fused with GFP (Fig. 3, construct C), a fragment containing the 0.1-kb promoter region with GFP was amplified using the 3.0-kb construct as a template with related specific primers (Supplementary Table S1). Then, this was digested with the *SfiI* restriction enzyme and ligated into the *SfiI* site of the pBE-CD vector.

To analyse the −3.0- to −1.8-kb promoter region, three constructs were designed (Fig. 3, constructs D–F). The amplified promoter fragments of the −3.0- to −2.5-kb region, −2.6- to −2.1-kb region, and −2.2- to −1.8-kb region were digested separately using the *SfiI* restriction enzyme. In addition, each promoter fragment was ligated into the *SfiI*-digested 0.1-kb promoter region fused with GFP fragments and then inserted into the *SfiI*-digested pBE-CD vector.

To analyse the −2.6- to −2.1-kb promoter region, two constructs were designed (Fig. 4, constructs G and H). The amplified promoter fragments of the −2.6- to −2.2-kb region and the −2.3- to −2.1-kb region were digested using the *BamHI* restriction enzyme. Each promoter fragment was inserted into the *BamHI* site of the pBluescript SK+ vector, amplified by PCR, ligated into the *SfiI*-digested 0.1-kb promoter region fused with
GFP fragments, and then inserted into the SfiI-digested pBE-CD vector.

To analyse the −2.3- to −2.1-kb promoter region with mutations, three constructs were designed with base substitutions at Box I, Box II, or Box III, designated constructs M1, M2, and M3, respectively (Fig. 5, constructs M1–M3). The base substitutions were introduced via overlap extension PCR (OE-PCR), using two primer sets (Supplementary Table S1). The sequence of primer 2 of set 1 was a complementary sequence of primer 1 of set 2. Two types of promoter fragments were amplified using each primer set. The amplified fragments were mixed and then a second PCR was conducted using primer 1 of set 1 and primer 2 of set 2. The resultant fragments were digested using the BamHI restriction enzyme. The rest of the process for introducing these final fragments into the pBE-CD vector was the same as that described above for the −2.6- to −2.1-kb promoter region.

To investigate the effect of mutations in the 3.0-kb AtNCED3 pro::GFP, constructs with no mutations (NMT) and construct M4 with the Box I base substitution (Fig. 6A) were designed. To make the NMT construct, the 3.0-kb promoter region was amplified by PCR from genomic DNA as a template. The total sequence promoter area is shown in Fig. 7B. The promoter fragments were mixed with GFP::NOS terminator fragments, and re-amplified by PCR using the 5′ primer of the promoter and the 3′ primer of the NOS terminator. The amplified fragments were digested using the SfiI restriction enzyme and inserted...
Figure 3. The 5’ deletion analysis of the −3.0- to −1.8-kb AtNCED3 promoter region involved in dehydration-responsive expression, based on gain-of-function experiments in related transgenic Arabidopsis T2 lines. Schematic diagram of construct C with −97 to +4 bp DNA fragments as a minimal TATA sequence fused to the GFP reporter gene. (Left) In constructs D–F, the GFP reporter gene is fused with the −3.0- to −2.5-kb, −2.6- to −2.1-kb, and −2.3- to −1.8-kb fragments of the minimum promoter, respectively. (Right) Expression of GFP and endogenous AtNCED3 in response to 5 h dehydration treatment among transgenic lines containing constructs C–F, respectively, based on qRT-PCR. The filled and open boxes indicate fold changes in the expression of GFP and endogenous AtNCED3, respectively. Error bars indicate the SDE (n = 3). The score index to the right indicates the mean and related standard error value (n = 3) for each construct (C–F) among related transgenic lines.

Figure 4. Analysis of the 0.5-kb region within the −2.60- and −2.10-kb region of the AtNCED3 promoter region involved in dehydration-responsive expression, based on gain-of-function experiments on related transgenic Arabidopsis T2 lines. (Left) Schematic diagram of construct G with the −2.60- to −2.26-kb fragment and construct H with the −2.33- to −2.13-kb fragment fused to the minimum promoter::GFP. (Right) Expression of GFP and endogenous AtNCED3 in response to dehydration stress treatment (5 h) based on qRT-PCR of transgenic lines containing constructs G and H. The filled and open boxes indicate fold changes in the expression of GFP and endogenous AtNCED3, respectively. Error bars indicate the SDE (n = 3). The score index to the right indicates the mean and related standard error value (n = 3) for each construct (G to H) among related transgenic lines.

Figure 5. Base substitution analysis of the 0.2-kb region between −2.33- and −2.13-kb of the AtNCED3 promoter involved in dehydration-responsive expression, based on gain-of-function experiments on related transgenic Arabidopsis T2 lines. (A) A 200 bp promoter sequence of AtNCED3, the area between −2.33- and −2.13-kb of the complementary strand, shown in FASTA format. The Box I (CACGTG at −2248 bp) as for G-box motif marked in red, Box II (CTGTTA at −2227 bp) as for Myb recognition motif marked in black and Box III (ACGTGTC at −2193 bp) as for an ABRE sequence marked in grey. The letters under the boxes indicate base substitutions at positions marked by asterisks above the boxes. (B) Schematic diagram of constructs M1–M3 with the 200 bp fragment containing base substitutions. (Right) Expression of GFP and endogenous AtNCED3 in response to dehydration stress treatment (5 h), based on qRT-PCR, among transgenic lines containing the constructs M1, M2, or M3. The filled and open boxes indicate fold changes in the expression of GFP and endogenous AtNCED3, respectively. Error bars indicate the SDE (n = 3). The score index to the right indicates the mean and related standard error value (n = 3) for each construct (M1, M2, and M3) among related transgenic lines.
2.3 GUS staining assay

The T2 transgenic plants of AtNCED3 pro::GUS lines were grown on Murashige and Skoog medium containing kanamycin. Transgenic plants at different growth stages (5 and 10 days, and 2 weeks old) were used for GUS staining. Histochemical assays of GUS activity were conducted as described previously.27

2.4 Expression analysis of the GFP reporter gene and AtNCED3

Total RNA was extracted using RNAiso (Takara Co. Ltd., Kyoto, Japan) following methods described previously.28 Quantitative real-time PCR (qPCR) was performed as described previously with modifications.29 The gene-specific primers for qPCR were designed using Primer Express 2.0 (Life Technologies Applied Biosystems, http://www.appliedbiosystems.com/). Specific primers for GFP, AtNCED3, and CBP20 or ubiquitin (UBQ) were generated using SYBR Green qPCR and are listed in Supplementary Table S1. Each measurement was performed in triplicate. In general, three genes were quantified before and after the drought treatments. The expression levels of CBP20 and UBQ were used to determine the amount of cDNA in each sample. The expression level of endogenous AtNCED3 was used to evaluate drought treatments. The expression level of the GFP reporter was used to assess the activity of the promoter fragment upstream of the GFP gene in transgenic plants. For easy evaluation of AtNCED3 promoter activity, we used a score index to compare the promoter activity of the different constructs. The score was calculated using the following formula:

\[
\text{Score} = \frac{\text{Fold changes in GFP reporter gene}}{\text{Fold changes in AtNCED3 gene}} \times 100
\]

where

\[
\text{Fold changes of GFP reporter gene} = \frac{\text{GFP expression after treatment}}{\text{GFP expression before treatment}}
\]

\[
\text{Fold changes of AtNCED3 reporter gene} = \frac{\text{AtNCED3 expression after treatment}}{\text{AtNCED3 expression before treatment}}
\]

RNA gel blot hybridization was performed for Col, Vec, and transgenic plants transformed with the 3.0-kb AtNCED3 pro::GFP or 3.0-kb Box I base-substituted AtNCED3 pro::GFP fused gene. Total RNA was extracted according to a method described previously.22 Total RNA (10 μg) was resolved in a 1% agarose gel containing formaldehyde, and subsequently blotted onto a Hybond-N+ membrane (Amersham Biosciences, Amersham, UK). Two full-length cDNA probes of GFP and AtNCED3 were prepared by reverse-transcription PCR and their nucleotide sequences were verified after cloning into the pGEM-T vector (Promega, Madison, WI, USA). The sequences of primers used to make probes are listed in Supplementary Table S1. The cDNA
fragments were labelled with [α-32P] dCTP (PerkinElmer) using a BcaBEST DNA labeling kit (Takara Co. Ltd.). Subsequent steps of RNA gel blot analysis were performed according to standard protocols with slight modifications.22,30

3. Results

3.1. The 3.0-kb AtNCED3 promoter contains cis-acting regulatory elements involved in dehydration-induced transcription

Previous reports have indicated that the 1.5-kb AtNCED3 promoter region does not contain the cis-acting elements involved in the dehydration-responsive expression of the gene. We also confirmed this in transgenic Arabidopsis. To analyse the AtNCED3 promoter in transgenic Arabidopsis, we used the 3.0-kb upstream region from the transcriptional initiation site of AtNCED3. We defined the AtNCED3 upstream region as the promoter. The transcriptional initiation site was defined as the +1 base. The ATG start codon of AtNCED3 is shown in bold. The sequence between +1 and +122 bp represents the 5’ UTR. The putative TATA sequence from −33 to −28 bp is in blue letters. The cis-acting elements shown in A are marked on the DNA sequence as follows: Box I in red box; Myb RS in dark cyan box, Box II in green box, and Box III in the blue box.

AtNCED3 transgenic lines were generated for the AtNCED3 promoter::GUS construct, and more than 20 lines were analysed by histochemical GUS staining. Transgenic Arabidopsis plants grown 15 or 16 days after germination were used for the dehydration treatment. GUS staining was observed in roots, leaf veins, and vascular tissues when the transgenic plants were subjected to dehydration (Fig. 1B), whereas no or weak GUS staining was observed in non-stressed plants. This indicates that the 3.0-kb AtNCED3 promoter region contains cis-regulatory elements that induce the GUS reporter gene in response to dehydration.

3.2. The AtNCED3 promoter region between 3.0 and 2.0-kb is necessary for the induction of AtNCED3 by dehydration

To determine the regulatory region of AtNCED3 more precisely, transgenic Arabidopsis plants transformed with AtNCED3 pro::GFP fusion genes were analysed. We generated two constructs (A and B) that contained the GFP reporter gene fused with the AtNCED3 promoter region between −3.0 or −2.0-kb and +4 bp from the transcriptional start site of AtNCED3. We used the GFP reporter gene for further analysis of the AtNCED3 promoter, because GFP is sensitive for the detection of promoter induction. The induction score index was highest in the Arabidopsis transgenic plants with construct A (191 ± 43 versus...
14 ± 3 for construct B; Fig. 2). This shows that the 1.0-kb
AtNCED3 promoter region between −3.0- and −2.0-kb contains the dehydration-responsive cis-acting element(s) (Fig. 2).

3.3. A 200 bp region at the 2.13-kb AtNCED3 promoter contains cis-acting elements for dehydration-responsive induction

To narrow down the location of the 1.0-kb AtNCED3 promoter region, we used shorter promoter fragments for further analysis. Four promoter constructs fused to the GFP reporter gene were designed, named constructs C–F. We defined the region between −97 and +4 bp of the AtNCED3 promoter containing the TATA box as the minimum promoter of AtNCED3. Construct C contained the minimum promoter fused to GFP. In constructs D–F, a 0.5-kb region between the −3.0- and −2.5-kb promoter region, a 0.5-kb region between the −2.6- and −2.1-kb promoter region, and a 0.6-kb region between the −2.2- and −1.8-kb promoter region were fused to the minimum promoter and the GFP reporter, respectively (Fig. 3). These constructs were used to generate transgenic Arabidopsis plants, and T2 plants were used for further analysis. After subjecting plants to dehydration stress, the score index of induction of each plant was quantified. Among the four types of transgenic plant, the score index of construct E was significantly higher than that of the other constructs (Fig. 3). We also analysed the dehydration-responsive expression of endogenous AtNCED3, which revealed no significant change in any of the transgenic Arabidopsis plants. This indicates that the 0.5-kb region between the −2.6- and −2.1-kb promoter region contains important cis-acting elements involved in dehydration-responsive transcription.

In the next step, the 0.5-kb region between the −2.6- and −2.1-kb regions of the AtNCED3 promoter was further analysed. We analysed the overlapping 340 bp region between the −2.6- and −2.26-kb promoter region (construct G, Fig. 4) and the 200 bp region between the −2.33- and −2.13-kb promoter region (construct H, Fig. 4), respectively. These promoter fragments were fused to the minimum promoter and GFP for the transgenic Arabidopsis plants. After subjecting the plants to dehydration stress (5 h), the score index was quantified for each transgenic line. The score was higher for construct H (150 ± 19) than for construct G (5 ± 2, Fig. 4). The promoter region between −2.33- and −2.13-kb was analysed using plant cis-acting regulatory DNA elements (PLACE).31 In the 0.2-kb promoter region, we found one sequence (CACGTG at −2248 bp) for the G-box-like motif, one Myb recognition motif (CTGT TA at −2227 bp), and one ABA-responsive element (ABRE)-like sequence (ACGTGTC at −2193 bp); these were named Box I, Box II, and Box III, respectively. In Fig. 4, these Box sequences are denoted by closed black circles, triangles, and squares, respectively. These Box sequences may be important for dehydration-induced AtNCED3 expression.

3.4. Box I is important in the dehydration-responsive induction of AtNCED3

To identify the important cis-acting elements in the 200 bp region between −2.33- and −2.13-kb of the AtNCED3 promoter, we introduced base substitutions into this region. The target regions were the Box I, Box II, and Box III sequences (Fig. 5A). When base substitutions were introduced into Box I (construct M1), the score index of induction was significantly reduced (35 ± 2) under dehydration conditions, whereas the score indices of base substitutions into Box II and Box III were not (constructs M2 and M3, Fig. 5B).

In the next step, to analyse the importance of Box I in the dehydration-responsive induction of AtNCED3, we used the 3.0-kb promoter of AtNCED3 including the 5′ untranslated region (5′ UTR) as the transcription initiation site (Fig. 7). Mutated M4 and non-mutated MNT, constructed as described in the Materials and methods section, are denoted by closed white and black circles, respectively, in Fig. 6A. As described above, RNA gel blot analyses were conducted, and the expression patterns of GFP and AtNCED3 under non-stress and dehydration stress conditions were analysed by hybridization with 32P-labelled cDNA probes. As shown in Fig. 6B, the expression levels of endogenous AtNCED3 were increased by dehydration in all plants (lower blot membrane). GFP expression was not detected in non-transgenic plants or in vector controls (upper blot membrane). GFP in transgenic plants with the 3.0-kb native AtNCED3 pro::GFP construct was highly induced by dehydration (lanes 1–3, upper blot membrane), whereas the induction of GFP in transgenic plants with base-substituted (M4) Box I AtNCED3 pro::GFP was significantly reduced (lanes 4–6, upper blot membrane, Fig. 6B).

Finally, the expression levels of GFP and AtNCED3 were analysed in transgenic Arabidopsis after 5-h dehydration treatment. The score index for the base substitution in Box I was significantly lower (7.6 ± 2; Supplemenary Fig. S1) than that for the 3.0-kb non-mutated AtNCED3 promoter::GFP transgenic plants (191 ± 43, Fig. 2). These results reveal that the Box I element at the −2248 bp position in the 3.0-kb AtNCED3 promoter is important in the dehydration-responsive expression of AtNCED3.
4. Discussion

4.1. The promoter region of AtNCED3 is involved in dehydration-responsive transcription

We found that the 3.0-kb promoter region of AtNCED3 is induced by dehydration stress (Fig. 1), and that the expression is observed mainly in rosette leaf veins and vascular tissues and roots (Fig. 1B). Previous studies have shown that leaves are the main organ in which ABA is produced in response to dehydration or osmotic stress. Furthermore, Endo et al. examined the localization of AtNCED3, AtABA2, and AAO3 proteins in rosette leaves by immunohistochemical analysis and found that, after dehydration stress, these three enzymes were localized mainly in vascular parenchyma cells adjacent to xylem and phloem vessels. The expression profiles of the 3.0-kb AtNCED3 pro::GUS were similar to those of the AtNCED3 protein under water-deficit conditions (Fig. 1), which suggests that the 3.0-kb promoter region contains cis-regulatory elements that are involved in dehydration-responsive expression mainly in vascular tissues of leaves. As detailed above, a series of analyses revealed that a 200 bp region between −2.33- and −2.13-kb of the promoter seems to contain cis-acting elements involved in the dehydration-responsive induction of AtNCED3 (Fig. 5).

In rice, a 2.0-kb OsNCED3 promoter::GFP was induced in response to dehydration and salinity treatments, suggesting that the long 2.0-kb OsNCED3 promoter is also necessary for the dehydration-inducible expression in rice. However, the cis-acting element of the OsNCED3 promoter has not yet been identified. We analysed the 2.0-kb region of the OsNCED3 promoter sequence and found a distal G-box (CACGTG) between −1781 and −1776 bp region. However, there is no evidence whether this distal G-box is involved in dehydration-responsive transcription.

Regulatory cis-acting elements localize within 1.0-kb regions of many stress-inducible promoters. However, the important regulatory region of the AtNCED3 promoter is located in a 200 bp region from −2.33- to −2.13-kb. Another example of far-upstream localization of regulatory cis-acting elements was reported by Maruyama-Nakashita et al., who found that the 16 bp sulphur-responsive element (SURE) is located within the −2777 and −2762 bp promoter region of the SULTR1;1 gene, encoding a high-affinity sulphate transporter. The SURE sequence contains an auxin response factor (ARF)-binding sequence (GAGACA or TGTCTC). Additionally, the presence of the SURE core sequence in S-minus-inducible genes was surveyed using in silico promoter analysis with GeneChip microarrays in Arabidopsis. Of 15 genes, 13 contained ARF-binding sequences in their 3.0-kb regions.

Additionally, genomic alignment analysis of the 3.0-kb AtNCED3 promoter area (upstream of the start codon) in various species including: Arabidopsis lyrata, Populus trichocarpa, Vitis Vinifera, Zea mays, Oryza sativa, Brachypodium distachyon, Sorghum bicolor, and Volvox carteri, resulted in the identification of a G-Box-like cis-acting element at −2.0-kb from the transcription initiation site of NCED3 (http://www.phytozome.net). We found a conserved region around the Box I-like sequence in the AtNCED3 promoter of A. lyrata. This observation also supports the importance of the G-Box in the Box I region (data not shown).

4.2. cis-acting elements that function in the dehydration-responsive induction of the AtNCED3 promoter

The 200 bp region between −2.33- and −2.13-kb of the AtNCED3 promoter contains the G-Box (Box I: CACGTG at −2248 bp), an Myb recognition site (Box II: CTGTTA at −2227 bp), and one ABRE motif (Box III: ACACGTTC at −2193 bp; Fig. 5). A series of analyses, as detailed above, indicated the importance of the G-Box-like element in the Box I region in the dehydration-responsive induction of AtNCED3. More specifically, we found that the G-box at −2248 bp from the initiation codon is an important cis-acting element for induction. This motif may activate the AtNCED3 promoter in response to water-deficit stimuli, because base substitutions of this element had negative effects on dehydration-responsive expression (Figs 6 and 7). We think that the tissue-specific expression in leaf vascular tissues and roots of the 3.0-kb AtNCED3 pro::GUS is mainly regulated by the G-box element (Fig. 1). In Fig. 5, the base substitution in the G-box (M1) in the 3.0-kb promoter caused a significant reduction in the expression of the GFP reporter gene under dehydration conditions, which suggests the important role of the G-box in tissue-specific expression under dehydration conditions, too.

Recently, an E2F-like element (TTTGCCCC) was reported to function in the differential expression of the LnNCED3 gene in ABA-mediated heterophyll induction in Lilium formosanum, but not in the LnNCED3 promoter of Lilium oriental. The Arabidopsis AtNCED3 gene does not contain the E2F-like element in its promoter. Moreover, heterophyll is not induced by ABA in Arabidopsis. Therefore, the E2F-like element seems not to be involved in the ABA-induced expression of AtNCED3.

We found that the observed G-box-like motifs are slightly different from those in the Box I region (Fig. 7). However, they do not seem to be important based on deletion analyses (Figs 2–5). A similar observation was reported in the SULTR1;1 promoter, in which ARF cis-acting elements located at −2777 and −2762 bp
were important for the induction of SULTR1;1 by sulphur deficiency. There are ARF or ARF-like cis-acting elements in promoter regions other than the −2777- and −2762 bp regions that are not important for the induction due to sulphur deficiency.

4.3. Possible transcription factors involved in the dehydration-induced expression of the AtNCED3 promoter

It is important to determine the transcription factors involved in the transactivation of the AtNCED3 promoter. The Box I contains a typical G-box (CACGTG) and its overlapping CANNTG, a typical basic helix-loop-helix (bHLH) recognition sequence. The G-box is recognized and regulated by well-characterized bZIP proteins such as G-binding factors (GBFs) which regulate various cellular processes and environmental responses both positively and negatively. GBF and its family proteins are good candidates of the transcription factor that controls the dehydration-responsive induction of AtNCED3. The bHLH family proteins also function in abiotic stress responses. There is another possibility of the candidate transcription factor as bHLH family protein(s).

We are currently attempting to isolate DNA-binding proteins that specifically interact with the G-box recognition sequence in the Box I, using the yeast one-hybrid screening method. Additionally, we are screening Arabidopsis mutant lines and cDNA overexpressing lines of transcription factor genes for the regulation of ABA levels under dehydration stress conditions to find candidate genes involved in the upstream process of ABA synthesis.

5. Conclusion

We identified a G-box element involved in the induction of AtNCED3 in response to dehydration stress. This cis-acting element is located −2248 bp from the AtNCED3 transcriptional initiation site. There is a high probability that G-box binding factors may recognize cis-elements and induce AtNCED3 gene expression during dehydration stress. Further analysis of these transcription factors will extend our knowledge of the molecular processes involved in plant responses to water deficit.

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