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

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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 stresses.[6–12]

ABA is synthesized during dehydration and degraded during rehydration. Key genes involved in ABA biosynthesis during dehydration have recently been identified

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based on genetic and molecular approaches, particularly in *Arabidopsis*.[13] Many of these are induced by dehydration stress, including the *AtNCED3* gene for 9-*cis*-epoxycarotenoid dioxygenase (NCED),[14] the *AAO3* gene for abscisic aldehyde oxidase,[15] the *AtABA3* gene for molybdenum cofactor sulphurase,[16] and the *AtZEP* gene for zeaxanthin epoxidase.[17] The carotenoid cleavage reaction is a key irreversible step in the ABA biosynthesis pathway.[18] 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*.[14] 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.[14] This indicates that the induction of *AtNCED3* regulates the accumulation of ABA during dehydration.[14]

The induction of *AtNCED3* occurs in the early stages of the response to dehydration stress.[19] 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.[20] 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 stress and contains *cis*-acting elements involved in dehydration-induced 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.[21] 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.[22,23]

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 *Eco*RI and *Hin*dIII sites of the pBE2113 vector.[24] 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 *Not*I restriction enzyme and then ligated. The ligated fragment and pBE-CD vector were digested using the *Sfi*I 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 *Sal*I restriction enzyme and ligated into the *Sal*I site of the pBI101*GFP* 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 *Sfi*I restriction enzyme and ligated into the *Sfi*I 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 *Sfi*I restriction enzyme. In addition, each promoter fragment was ligated into the *Sfi*I-digested 0.1-kb promoter region fused with *GFP* fragments and then inserted into the *Sfi*I-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 *Bam*HI restriction enzyme. Each promoter fragment was inserted into the *Bam*HI site of the pBluescript SK+ vector, amplified by PCR, ligated into the *Sfi*I-digested 0.1-kb promoter region fused with

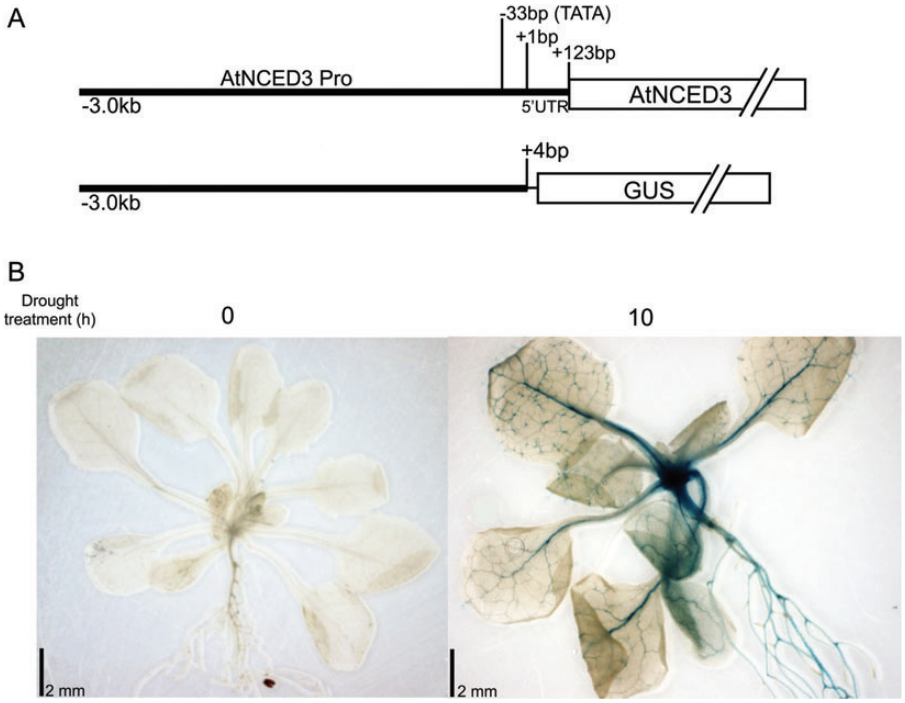

Figure 1. Tissue-specific expression of the 3.0-kb *AtNCED3* promoter fused to the *GUS* gene in transgenic *Arabidopsis* plants under dehydration stress conditions. (A) Diagram of the construction of the 3.0-kb *AtNCED3 promoter::GUS* gene. (B) Histochemical analysis of GUS expression in the 3.0-kb *AtNCED3 promoter::GUS* transgenic *Arabidopsis* lines with or without dehydration treatment for 10 h.

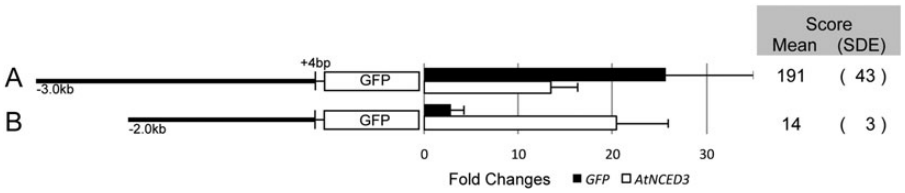

Figure 2. Deletion fragment of the *AtNCED3* promoter fused to *GFP*. Schematic of 1.0-kb 5′ deletion analysis of 3.0-kb *AtNCED*3 promoters fused to the *GFP* reporter gene in related *Arabidopsis* transgenic T2 lines (left, constructs A and B). Expression of *GFP* and endogenous *AtNCED3* in response to dehydration stress treatment (see the Materials and methods section for details) measured by qRT-PCR (right, transgenic lines containing the A and B constructs). The ratios of *GFP* mRNA to endogenous *AtNCED3* mRNA are expressed as fold changes in expression and are denoted by filled and open boxes, respectively. Error bars indicate the standard deviation of the error (SDE) ($n = 3$). The score index to the right indicates the mean and related standard error value ($n = 3$) for each construct (A or B) among related transgenic lines (the details of the score index are described in the Materials and methods section).

GFP fragments, and then inserted into the *Sfi*I-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).[25,26] 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 *Bam*HI 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 *Sfi*I restriction enzyme and inserted

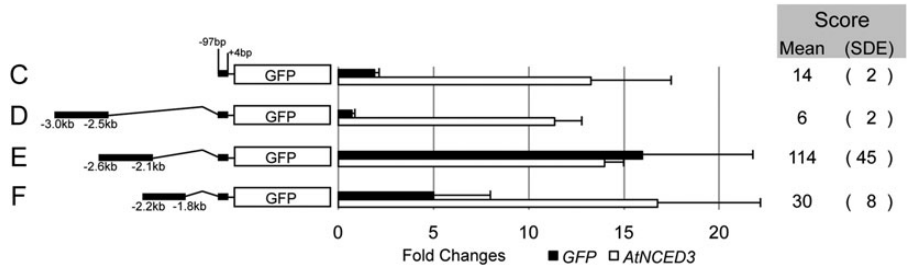

Figure 3. The 5′ deletion analysis of the −3.0- to −1.8-kb *AtNCED*3 promoter region involved in dehydration-responsive expression, based on gain-of-function experiments in related transgenic *Arabidopsis* T2 lines. Schematic diagram of construct C with −97 to +4 bp DNA fragments as a minimal TATA sequence 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.

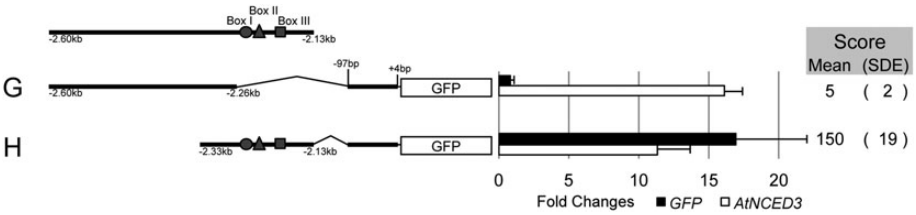

Figure 4. Analysis of the 0.5-kb region within the −2.60- and −2.10-kb region of the *AtNCED*3 promoter region involved in dehydration-responsive expression, based on gain-of-function experiments on related transgenic *Arabidopsis* T2 lines. (Left) Schematic diagram of construct G with the −2.60- to −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.

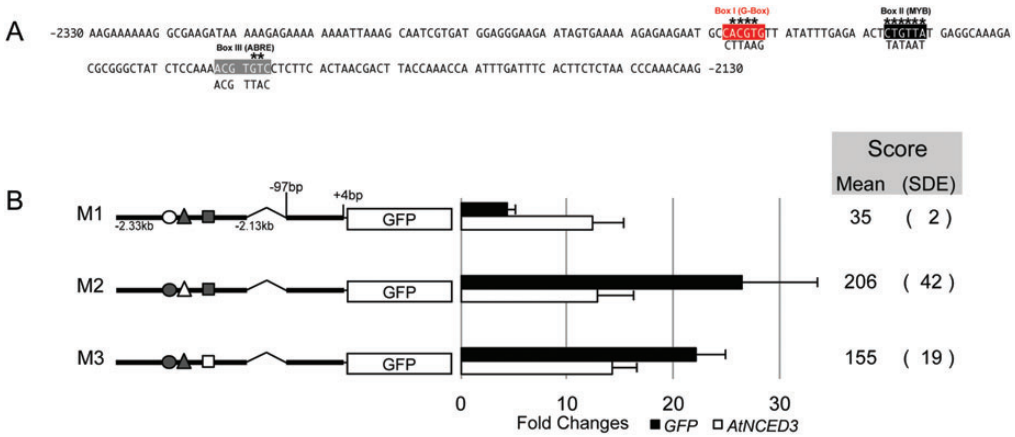

Figure 5. Base substitution analysis of the 0.2-kb region between −2.33- and −2.13-kb of the *AtNCED*3 promoter involved in dehydration-responsive expression, based on gain-of-function experiments on related transgenic *Arabidopsis* T2 lines. (A) A 200 bp promoter sequence of *AtNCED3*, the area between −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 (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.

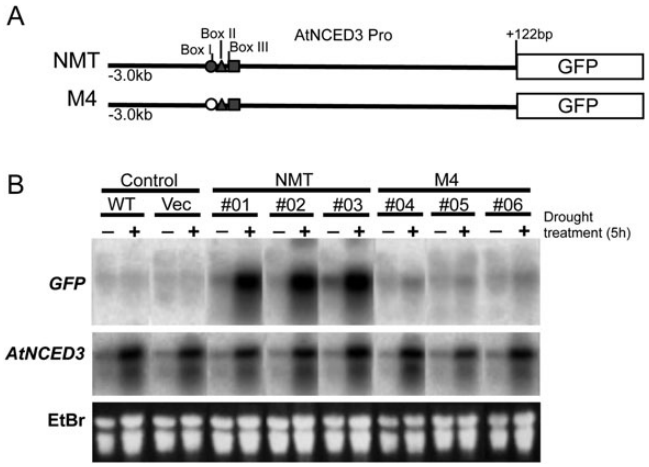

Figure 6. Analysis of the 3.0-kb *AtNCED3* promoter region (including the transcription initiation site) involved in dehydration-responsive expression, based on the RNA gel blot analysis of non-mutated and Box I base substitution-related transgenic *Arabidopsis* T2 lines. (A) Schematic diagram of construct NMT with the 3.0-kb *AtNCED3* promoter region without a base substitution at Box I (filled circles), and construct M4 with a base substitution at Box I (CACGTG changed to CTTAAG between −2248 and −2243 bp of the *AtNCED3* promoter; opened circles) fused to the *GFP* reporter gene. (B) *GFP* and endogenous *AtNCED3* expression patterns under dehydration conditions among NMT and M4 transgenic lines. RNA gel blot analysis of Columbia wild-type (WT), three independent *Arabidopsis* transgenic lines for each construct (NMT and M4), and a control transgenic line consisting of an empty pBE-CD vector with a kanamycin-resistance marker gene (Vec). Two-week-old plants were subjected to dehydration for 5 h (see Material and methods for details). Ethidium bromide-stained total RNA is shown as a loading control. Expression patterns of *GFP* (upper blot membrane) and endogenous *AtNCED3* (lower blot membrane) among control and transgenic plants were analysed by hybridization with ^{32}P-labelled cDNA probes and detected by autoradiography.

into the *Sfi*I site of the pBE-CD vector. The method for introducing the mutation was basically the same as that previously described. Two types of promoter fragments were amplified by PCR using primer set 1 or set 2 from the NMT construct as a template. 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 PCR products were inserted into the *Sfi*I site of the pBE-CD vector.

The insert regions of all constructs were sequenced to confirm cloning errors. *Arabidopsis* plants (Col ecotype) were transformed by these constructs using *Agrobacterium* infiltration methods. Generated T2 plants were used.

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:

$$\text{Score} = \frac{\text{Fold changes in } \textit{GFP} \text{ reporter gene}}{\text{Fold changes in } \textit{AtNCED3} \text{ gene}} \times 100 \quad (1)$$

where

$$\text{Fold changes of } \textit{GFP} \text{ reporter gene} = \frac{\textit{GFP} \text{ expression after treatment}}{\textit{GFP} \text{ expression before treatment}} \quad (2)$$

$$\text{Fold changes of } \textit{AtNCED3} \text{ reporter gene} = \frac{\textit{AtNCED3} \text{ expression after treatment}}{\textit{AtNCED3} \text{ expression before treatment}} \quad (3)$$

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

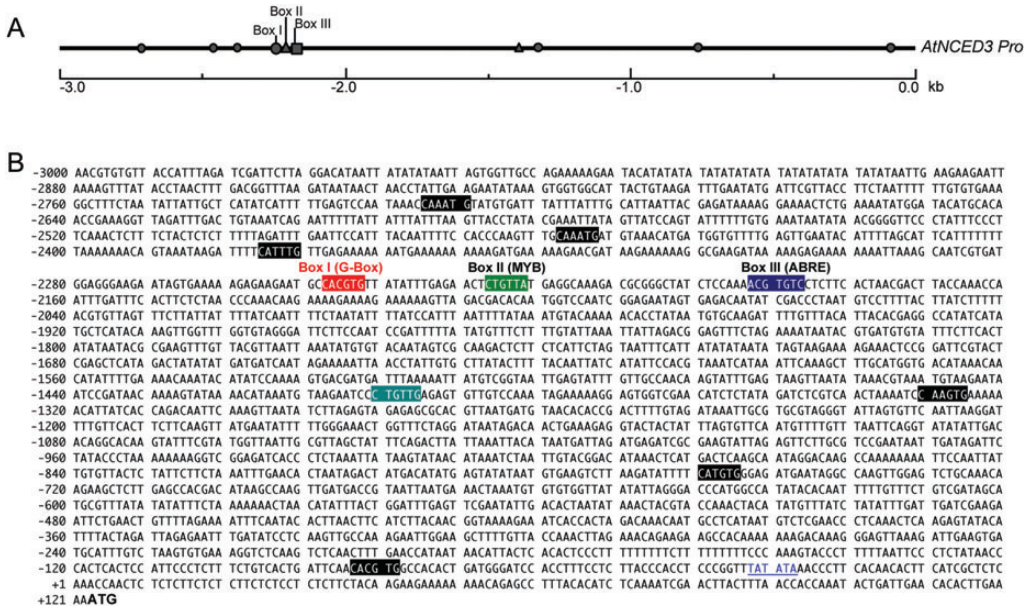

Figure 7. The sequence of the 3.0-kb *AtNCED3* promoter. (A) Schematic diagram of the 3.0-kb *AtNCED3* promoter region, showing the relative *cis*-acting elements. G-Box and Myc recognition consensus sequences (Myc RS) are denoted by filled circles; Myb recognition sequences (Myb RS) are denoted by filled triangles; and ABREs are denoted by filled square. (B) The DNA sequence of the 3.0-kb *AtNCED3* promoter. The putative transcription initiation site is 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; and Myc RS are in the black box, Box II in green box; Myb RS in dark cyan box, and Box III in the blue box.

fragments were labelled with [α-^{32}P] 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 region of the* 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* plants, 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 of *AtNCED3* was deduced to be located 122 bp upstream of the initiation codon of *AtNCED3*. Using the TAIR database, annotation results showed that there were no neighbouring genes in the 3.0-kb upstream region of *AtNCED3*. *Arabidopsis* transgenic plants were generated by constructing an *AtNCED3 promoter::β-glucuronidase* (*AtNCED3 pro::GUS*) fusion gene (Fig. 1A). Forty-five independent *Arabidopsis* 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 (CTGTTA 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 NMT, 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 ^{32}P-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, Fig. 6B), 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; Supplementary 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.[32,33] Furthermore, Endo *et al*.[34] 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 *OsNCECD3* promoter::*GFP* was induced in response to dehydration and salinity treatments,[35] 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*.[36], 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 *NCED* promoter area (upstream of the start codon) in various species including: *Arabiopsis lyrata*, *Poplus trichocarpa*, *Vitis Vinifera*, *Zea mays*, *Oryza sativa*, *Brachypodium disyachyon*, *Sorgum 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 *NCED*3 (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: ACGTGTC 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 *LfNCED3* gene in ABA-mediated heterophylly induction in *Lilium formosanum*, but not in the *LoNCED3* promoter of *Lilium oriental*.[37] The *Arabidopsis AtNCED3* gene does not contain the E2F-like element in its promoter. Moreover, heterophylly 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.[31,36] 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.[38] 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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References

1. Barnabas, B., Jager, K. and Feher, A. 2008, The effect of drought and heat stress on reproductive processes in cereals, *Plant Cell Environ.*, **31**, 11–38.
2. Zhang, J.Z., Creelman, R.A. and Zhu, J.K. 2004, From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops, *Plant Physiol.*, **135**, 615–21.
3. Reynolds, M. and Tuberosa, R. 2008, Translational research impacting on crop productivity in drought-prone environments, *Curr. Opin. Plant Biol.*, **11**, 171–9.
4. Moore, J.P., Le, N.T., Brandt, W.F., Driouich, A. and Farrant, J.M. 2009, Towards a systems-based understanding of plant desiccation tolerance, *Trends Plant Sci.*, **14**, 110–7.
5. Roy, S.J., Tucker, E.J. and Tester, M. 2011, Genetic analysis of abiotic stress tolerance in crops, *Curr. Opin. Plant Biol.*, **14**, 232–9.
6. Bray, E.A. 1997, Plant responses to water deficit, *Trends Plant Sci.*, **2**, 48–54.
7. Busk, P.K. and Pages, M. 1998, Regulation of abscisic acid-induced transcription, *Plant Mol. Biol.*, **37**, 425–35.
8. Bartels, D. and Sunkar, R. 2005, Drought and salt tolerance in plants, *Crit. Rev. Plant Sci.*, **24**, 23–58.
9. Shinozaki, K. and Yamaguchi-Shinozaki, K. 2000, Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways, *Curr. Opin. Plant Biol.*, **3**, 217–23.
10. Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2006, Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future, *Curr. Opin. Biotechnol.*, **17**, 113–22.
11. McCourt, P. and Creelman, R. 2008, The ABA receptors—we report you decide, *Curr. Opin. Plant Biol.*, **11**, 474–8.
12. Verslues, P.E. and Zhu, J.K. 2005, Before and beyond ABA: upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress, *Biochem. Soc. Trans.*, **33**, 375–9.
13. Nambara, E. and Marion-Poll, A. 2005, Abscisic acid biosynthesis and catabolism, *Annu. Rev. Plant Biol.*, **56**, 165–85.
14. Iuchi, S., Kobayashi, M., Taji, T., et al. 2001, Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*, *Plant J.*, **27**, 325–33.
15. Seo, M., Peeters, A.J., Koiwai, H., et al. 2000, The *Arabidopsis* aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves, *Proc. Natl Acad. Sci. USA*, **97**, 12908–13.

16. Xiong, L., Ishitani, M., Lee, H. and Zhu, J.K. 2001, The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression, *Plant Cell*, **13**, 2063–83.
17. Vogel, J.T., Zarka, D.G., Van Buskirk, H.A., Fowler, S.G. and Thomashow, M.F. 2005, Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*, *Plant J.*, **41**, 195–211.
18. Qin, X. and Zeevaart, J.A. 1999, The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean, *Proc. Natl Acad. Sci. USA*, **96**, 15354–61.
19. Shinozaki, K. and Yamaguchi-Shinozaki, K. 2007, Gene networks involved in drought stress response and tolerance, *J. Exp. Bot.*, **58**, 221–7.
20. Tan, B.C., Joseph, L.M., Deng, W.T., et al. 2003, Molecular characterization of the *Arabidopsis* 9-cis epoxycarotenoid dioxygenase gene family, *Plant J.*, **35**, 44–56.
21. Valvekens, D., Montagu, M.V. and Van Lijsebettens, M. 1988, *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection, *Proc. Natl Acad. Sci. USA*, **85**, 5536–40.
22. Seki, M., Ishida, J., Narusaka, M., et al. 2002, Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray, *Funct. Integr. Genomics*, **2**, 282–91.
23. Oono, Y., Seki, M., Nanjo, T., et al. 2003, Monitoring expression profiles of *Arabidopsis* gene expression during rehydration process after dehydration using ca 7000 full-length cDNA microarray, *Plant J.*, **34**, 868–87.
24. Mitsuhara, I., Ugaki, M., Hirochika, H., et al. 1996, Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants, *Plant Cell Physiol.*, **37**, 49–59.
25. Higuchi, R., Krummel, B. and Saiki, R.K. 1988, A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions, *Nucleic Acids Res.*, **16**, 7351–67.
26. Wurch, T., Lestienne, F. and Pauwels, P.J. 1998, A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes, *Biotechnol. Tech.*, **12**, 653–7.
27. Urano, K., Yoshiba, Y., Nanjo, T., Ito, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2004, *Arabidopsis* stress-inducible gene for arginine decarboxylase AtADC2 is required for accumulation of putrescine in salt tolerance, *Biochem. Biophys. Res. Commun.*, **313**, 369–75.
28. Seki, M., Narusaka, M., Ishida, J., et al. 2002, Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray, *Plant J.*, **31**, 279–92.
29. Urano, K., Hobo, T. and Shinozaki, K. 2005, *Arabidopsis* ADC genes involved in polyamine biosynthesis are essential for seed development, *FEBS Lett.*, **579**, 1557–64.
30. Umezawa, T., Okamoto, M., Kushiro, T., et al. 2006, CYP707A3, a major ABA 8′-hydroxylase involved in dehydration and rehydration response in *Arabidopsis thaliana*, *Plant J.*, **46**, 171–82.
31. Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. 1999, Plant cis-acting regulatory DNA elements (PLACE) database, 1999, *Nucleic Acids Res.*, **27**, 297–300.
32. Cornish, K. and Zeevaart, J.A. 1985, Movement of abscisic acid into the apoplast in response to water stress in Xanthium strumarium L, *Plant Physiol.*, **78**, 623–6.
33. Thompson, A.J., Mulholland, B.J., Jackson, A.C., et al. 2007, Regulation and manipulation of ABA biosynthesis in roots, *Plant Cell Environ.*, **30**, 67–78.
34. Endo, A., Sawada, Y., Takahashi, H., et al. 2008, Drought induction of *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells, *Plant Physiol.*, **147**, 1984–93.
35. Bang, S.W., Park, S.H., Jeong, J.S., et al. 2013, Characterization of the stress-inducible *OsNCED3* promoter in different transgenic rice organs and over three homozygous generations, *Planta*, **237**, 211–24.
36. Maruyama-Nakashita, A., Nakamura, Y., Watanabe-Takahashi, A., Inoue, E., Yamaya, T. and Takahashi, H. 2005, Identification of a novel cis-acting element conferring sulfur deficiency response in *Arabidopsis* roots, *Plant J.*, **42**, 305–14.
37. Chen, H.C., Hwang, S.G., Chen, S.M., Shii, C.T. and Cheng, W.H. 2011, ABA-mediated heterophylly is regulated by differential expression of 9-cis-epoxycarotenoid dioxygenase 3 in lilies, *Plant Cell Physiol.*, **52**, 1806–21.
38. Yoshida, T., Fujita, Y., Sayama, H., et al. 2010, AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation, *Plant J.*, **61**, 672–85.