A Distal ABA Responsive Element in AtNCED3 Promoter Is Required for Positive Feedback Regulation of ABA Biosynthesis in Arabidopsis

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Abstract
The plant hormone abscisic acid (ABA) plays a crucial role in plant development and responses to abiotic stresses. Recent studies indicate that a positive feedback regulation of ABA exists in ABA biosynthesis in plants under dehydration stress. To understand the molecular basis of this regulation, we analyzed the cis-elements of the AtNCED3 promoter in Arabidopsis. AtNCED3 encodes the first committed and highly regulated dioxygenase in the ABA biosynthetic pathway. Through delineated and mutagenesis analyses in stable-transformed Arabidopsis, we revealed that a distal ABA responsive element (ABRE: GGCACGTG, -2372 to -2364 bp) is required for ABA-induced AtNCED3 expression. By analyzing the AtNCED3 expression in ABRE binding protein ABF3 over-expression transgenic plants and knock-out mutants, we provide evidence that the ABA feedback regulation of AtNCED3 expression is not mediated by ABF3.

Introduction
Abscisic acid (ABA) plays a critical role in several plant developmental processes including inhibition of seed germination and promotion of seed dormancy [1], and mediates plant responses to various environmental stresses, particularly drought and salinity [2,3]. Under drought or salinity stress, plants quickly synthesize and accumulate up to 40-folds of ABA [4], which facilitates stomatal closure and expression of ABA responsive genes to protect plants from further water loss and damage. For this reason, understanding the mechanism by which stress induces ABA biosynthesis will provide insights to engineering stress tolerant crops.

ABA is synthesized by oxidative cleavage of epoxy-carotenoids in plants [4]. Key steps in the pathway have been identified in various plant species [5]. The epoxidation of zeaxanthin and antheraxanthin to form violaxanthin and neoxanthin is catalyzed by zeaxanthin epoxidase (ZEP/AtABA1) [6,7]. The products are isomerized to produce 9-cis isomers which are cleaved by nine-cis-epoxy-carotenoid dioxygenase (NCED) to form xanthoxin [8–10]. The later is subsequently converted to ABA by two oxidases, a short-chain dehydrogenase/reductase (SDR) and aldehyde oxidase 3 (AAO3) [11–13]. The activity of the AAO3 enzyme requires a sulfurred molybdenum cofactor (MoCo), which is converted from the desulfo- to the sulfo-form by the MoCo sulfurrease ABA3 [14,15]. Although the pathway is clear, the regulation of ABA synthesis remains poorly understood. Evidence indicates that the oxidative cleavage of epoxy-carotenoids by NCED is the first committed, highly regulated, step in ABA biosynthesis [9,10,16,17], and that the accumulation of ABA is the result of de novo expression of NCEDs in response to various stresses [9,10,16]. In Arabidopsis, loss of AtNCED3 compromises ABA accumulation under drought stress, causing wilty plants [17]. Conversely, over-expression of AtNCED3 in Arabidopsis and LeNCED1 in tomato (Lycopersicon esculentum) enhances endogenous ABA accumulation and increases dehydration resistance [17,18].

Synthetic pathways are frequently feedback-regulated by end-products. Plant hormones show both negative and positive regulations. Bioactive gibberellins (GA) negatively regulate GA biosynthesis by curtaining the transcription of GA20ox and GA3ox [19,20]. Ethylene can both positively and negatively regulate ethylene biosynthesis by up regulating LeACS2 and LeACS4 and down regulating LeACS6 transcription during fruit ripening in tomato [21]. Recent evidence indicates that a positive feedback regulation exists also in ABA biosynthesis. Indeed, exogenous ABA significantly enhanced the expression of ABA1, AAO3 and ABA3 and AtNCED3 was induced by ABA in different Arabidopsis accessions [7,12,14,22]. Furthermore, in the ABA deficient mutants aba1 and aba3, the transcript of all the inducible ABA biosynthetic genes under stress were significantly lower than those in the wild-type [7,14]. In addition, AtNCED3 transcript were dramatically reduced in ABA insensitive mutants snk2.2/2.3/2.6 and psy1gyp1gyp2gyp4 in comparison to the wild-type under ABA treatment [23,24]. Taken together, these results strongly demonstrate that a positive feedback regulation exists in ABA biosynthesis, which contributes to ABA accumulation under drought stress.
Many ABA responsive genes contain conserved ABA responsive elements (ABRE, PyACGGTG/TG) in their promoter regions [25]. ABREs can be recognized by ABRE binding proteins/factors (AREBs/ABFs), which are a family of basic leucine zipper (bZIP) transcription factors [26,27]. In general, a single copy of ABRE is not sufficient for ABA responsive gene expression; it requires two ABREs or one ABRE coupled with a coupling element (CE) [28–30]. Two ABREs are necessary for ABA-responsive expression of RD29B in Arabidopsis [27]. One ABRE and one CE are necessary for ABA induction of VP1 expression in maize (Zea mays) [31]. Similar case is also found in HVA22 from barley (Hordeum vulgare) [22].

To understand the regulation of ABA biosynthesis, it is necessary to identify the cis-elements in the AtNCED3 promoter that mediate the positive feedback regulation. In this study, we mapped the ABA responsive elements by deletion and mutation analysis in transgenic Arabidopsis. The results indicate that a distal ABRE (GGCACGTG) plays a crucial role in conferring ABA responsive expression of AtNCED3. This element shares conserved core sequence with drought induced NCED promoters from other species. We further show the evidence that ABF3, a known ABRE binding factor, does not regulate the expression of AtNCED3.

Materials and Methods

Plant materials, growth conditions and ABA treatment

Arabidopsis ecotype Columbia (Col-0) was used and ab3 mutant (SALK_096965) was kindly provided by Prof. Qi Xie (Institute of Genetics and Developmental Biology, Chinese Academy of Science, China). Seeds were sterilized, sown on 1/2 MS [32] plates, and subjected to cold treatment for 3 days. The plates were transferred to a growth chamber with 16-h-light (PPFD = 100 µmol m$^{-2}$s$^{-1}$) /8-h-dark cycle at 22°C for two weeks. Two-week-old seedlings were used for treatment or grown on soil at 22°C with 16 h of light daily.

For ABA treatment, 100 µM ABA in water was sprayed on leaves of two-week-old seedlings, or with water in the case of the controls, and the plants were incubated at 22°C for 5 h.

Histochemical GUS staining and quantitative measurement of GUS activity

Histochemical GUS staining was performed as described previously [33]. 10 independent lines and at least 10 individual plants from each line were analyzed. Briefly, tissues were incubated in staining solution (0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 50 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, and 1 mM X-Gluc) at 37°C for 16h. Stained tissues were bleached in 70% ethanol for 16h.

Measurement of GUS activity in whole seedling was carried out by the method of Jefferson et al [34]. 10 independent lines and 10 individual plants from each line were analyzed. The fluorescence was measured by an Infinite M1000 microplate reader (TECAN Group Ltd). Protein concentration was determined by Protein assay reagent (Bio-Rad).

Sequence analysis

The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database was used to analyze the promoter sequence of AtNCED3. Sequence logos were created online using the Weblogo resource (http://weblogo.berkeley.edu/).

Quantitative RT-PCR analysis of gene expression

Total RNA was purified using Qiagen Plant RNeasy kit according to the manufacturer’s instructions and then treated with DNase I (New England Biolabs) to eliminate genomic DNA contamination. Reverse transcription reactions were performed using 1 µg of total RNA by SuperScriptII reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed in the iQ5 real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad). Each experiment was replicated three times. To quantify the copy number of each RNA, the Ct (threshold cycle) value was compared with a standard curve. The amplified RNA fragment was purified, quantified spectrophotometrically to generate a standard curve for each gene. All the transcripts were normalized as copy number per ng of total RNA. Sequences of primers are listed in Table S1.

Plasmid construction and plant transformation

We made five constructs to identify ABA responsive region in –2664 bp AtNCED3 promoter. Five DNA fragments containing 5’-deletion AtNCED3 promoter regions (-2664, -2018, -1470, -774 or -349 to -4 bp) were amplified by PCR with forward primers (N3-PDF1, N3-PDF2, N3-PDF3, N3-PDF4 or N3-PDF5) and reverse primer (N3-PDR1) using Phusion DNA polymerase (New England Biolabs). PCR fragments were ligated into pENTR D/Topo (Invitrogen), and then recombined into pGWB3 vector using Gateway LR Clonase II enzyme mix kit (Invitrogen).

Four constructs were designed to further confirm that the region between -2664 and -2018 bp contains ABA responsive cis-element. Two 3’-deletion fragments (-2664 to -2018 bp and -2664 to -1470 bp) were amplified by forward primer (N3-PDF1) and reverse primers (N3-FR1 or N3-FR2). The CaMV 35S minimal promoter (−46 to +88 bp) and −349 bp AtNCED3 promoter (−349 to −4 bp) were amplified with 35SM-F1, 35SM-R1 and N3-PDF3, N3-PDR1, respectively. −2664 to −2018 bp and −2664 to −1470 bp regions were ligated with CaMV 35S minimal promoter or −349 bp AtNCED3 promoter by overlap extension PCR (OE-PCR) as described by Wurch et al [35]. Briefly, −2664 to −2018 bp region was ligated with CaMV 35S minimal promoter or −349 bp AtNCED3 promoter by OE-PCR with complementary primers (S35S-F, S35S-R and SN3-F, SN3-R). −2664 to −1470 bp region was ligated with CaMV 35S minimal promoter or −349 bp AtNCED3 promoter by OE-PCR with complementary primers (L35S-F, L35S-R and LN3-F, LN3-R). The resultant fragments were ligated into pENTR D/Topo, and then recombined into pGWB3 vector using Gateway LR Clonase II enzyme mix kit (Invitrogen).

We designed constructs with mutation of ABAE base substitution to investigate the effect of ABRE in −2664 bp AtNCED3 promoter. The mutated promoter was generated with complementary primers (mABRE-F and mABRE-R) by OE-PCR. The resultant fragment was ligated into pENTR D/Topo (Invitrogen), and then recombined into pGWB3 vector using Gateway LR Clonase II enzyme mix kit (Invitrogen).

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To narrow down ABA responsive cis-element region between the −2664 to −2018 bp region of AtNCED3 promoter, additional 5’-deletion constructs (np6-np9) were generated. AtNCED3 promoter regions (−2414, −2327, −2214 or −2092 to −4 bp) were amplified with forward primers (N3-PDF6, N3-PDF7, N3-PDF8 or N3-PDF9) and reverse primer (N3-PDR1) using Phusion DNA polymerase (NEB). PCR fragments were ligated into pENTR D/Topo (Invitrogen), and then recombined into pGWB3 vector using Gateway LR Clonase II enzyme mix kit (Invitrogen).

We designed constructs with mutation of ABRE base substitution to investigate the effect of ABRE in −2664 bp AtNCED3 promoter. The mutated promoter was generated with complementary primers (mABRE-F and mABRE-R) by OE-PCR. The resultant fragment was ligated into pENTR D/Topo, and then recombined into pGWB3.

To create three repeats of 87 bp region or mutated 87 bp region followed by the −349 bp AtNCED3 promoter or CaMV 35S minimal promoter, firstly, 87 bp region or mutated 87 bp region were amplified by complementary primers (R87R-F, R87R-R), and then generated three tandem repeats of each fragment by OE-PCR. Three tandem repeats of 87 bp region and mutated 87 bp region was ligated with CaMV 35S minimal promoter or −349 bp AtNCED3 promoter by OE-PCR with complementary primers (L35S-F, L35S-R and LN3-F, LN3-R). The resultant fragments with the cis-element Required for AtNCED3 Expression
promoter with complementary primers (R87S-F, R87S-R and R87N3-F, R87N3-R). The fused fragments were ligated into pENTR D/Topo, and then recombinated into pGWB2.

The open reading frame of ABF3 was amplified using Phusion DNA polymerase and primers (ABF3-35S and ABF3-ER) to give overexpression vector. The PCR product was introduced into pENTR D/Topo, and then recombinated into pGWB2. The information of primers is listed in Table S1.

Arabidopsis transformation was carried out using floral-dip method as described previously [36]. T1 generation transgenic lines were selected on 1/2 MS plates containing hygromycin (final concentration of 25 mg/l). For each construct, more than 10 independent lines with a single insertion locus were selected based on the segregation of seedlings (3:1) that were resistant to hygromycin (final concentration of 25 mg/l) in T2 generation. T2 plants for a single insertion locus were used in histochemical GUS staining and quantitative measurement of GUS activity.

Results

The 2664 bp region of AtNCED3 promoter from the translation start site possesses various regulatory elements, including ABA responsive cis-element

We previously described that transgenic Arabidopsis expressing the β-glucuronidase (GUS) gene under the control of a 1.5 kb-long region of the AtNCED3 promoter lacked GUS expression in response to dehydration stress [33]. In opposite, a 1.0 kb AtNCED3 promoter driven firefly luciferase (LUC) in transgenic Arabidopsis has been reported to respond to osmotic stress [37,38]. To analyze AtNCED3 promoter, we developed in Arabidopsis a reporter assay taking into consideration various regions of the AtNCED3 promoter ranging from 2.7 kb and 0.3 kb upstream of translation start site. The A of the ATG codon at the translational site was defined as +1. We generated five constructs that contained the AtNCED3 promoter region between –2664 to –4 bp, –2018 to –4 bp, –1470 to –4 bp, –774 to –4 bp or –349 to –4 bp fused with GUS gene (Figure 1A, np1-np5). Transgenic Arabidopsis plants were generated, as named after the constructs. At least 10 independent plants from each individual transgenic line were analyzed by histochemical GUS staining. Three developmental stages were observed: 36 h after germination, 5-day-old seedlings and the reproductive stage.

In early seedling developmental stage, GUS activity was found in cotyledons of transgenic plants containing any length promoter (Figure 1B, A-E), whereas GUS expression was only observed in radicle tips of np1 transgenic plants (Figure 1B, A). In 5-day-old seedlings, GUS expression was observed in vascular tissue of cotyledons and stems of transgenic plants containing any promoter fragment (Figure 1B, F-O), however, the expression in maturation zone of root was only found in np1 transgenic plants (Figure 1B, P). During the reproductive stage, only np1 transgenic plants showed GUS activity in styles and vascular tissue of sepals (Figure 1B, U). These results indicate that the 2664 bp of AtNCED3 promoter from translation start site possesses various regulatory elements involving in tissue specific expression of AtNCED3.

The in silico analysis of the promoter sequence of AtNCED3 revealed the presence of five hypothetical ABREs [39]. In the five predicted ABREs, two were conserved in core sequence (CAGGTG), one proximal (CAGTTGGCC, –206 to –198 bp) and one distal (GGACAGTG, –2372 to –2364 bp). A putative TATA box (TATATA) was located between –155 and –149 bp (Figure 2A).

In order to identify regions containing ABA responsive cis-element, at least 10 independent transgenic lines from each construct were sprayed with 100 μM ABA and analyzed after 5 h. The np1 transgenic plants with the 2664 bp AtNCED3 promoter exhibited a 2.0-fold increase in GUS activity when treated with ABA, whereas no induction was detected in transgenic plants harboring shorter promoter regions (Figure 2B). GUS staining showed elevated expression in vascular tissue of true leaves in np1 transgenic plants after exogenous ABA application (Figure 2C). These data indicate that the 2664 bp region of AtNCED3 promoter from the translation start site confers the ABA responsive expression of AtNCED3 and the ABA responsive cis-element is located between –2664 and –2018 bp from the translation start site.

A 87 bp region of AtNCED3 promoter contains ABA responsive cis-element

To further confirm that the ABA responsive cis-element is located in the region between –2664 and –2018 bp, we employed a deletion-fusion approach. The 3’-deletion fragment (–2664 to –2018 bp) was fused to either a CaMV 35S minimal promoter (–46 to +68 bp from transcription start site) [40] or a –349 bp AtNCED3 promoter (Figure 3). To rule out the possibility that one cis-element pairs with distal ones to establish a functional unit, the longer fragment (–2664 to –1470 bp) was ligated to a CaMV 35S minimal promoter and a –349 bp AtNCED3 promoter, respectively (Figure 3). The –349 bp AtNCED3 promoter has been demonstrated not in response to ABA (Figure 2B, np5). GUS activity driven by the –349 bp AtNCED3 promoter was stronger than the CaMV 35S minimal promoter either with or without ABA treatment (Figure 3). GUS activity assay showed that all the transgenic plants harboring four different constructs displayed higher GUS activity after sprayed with 100 μM ABA (Figure 3). These results further confirm that the 546 bp region between –2664 and –2018 bp contains the cis-element responsive to ABA.

To further delineate the region between –2664 and –2018 bp of the AtNCED3 promoter, additional 5’ deletion constructs containing the regions –2414 to –4 bp, –2327 to –4 bp, –2214 to –4 bp or –2092 to –4 bp were made, and the corresponding transgenic plants were generated (Figure 4, np6-np9). When treated with ABA, the plants containing the 2414 bp promoter-GUS (np6) transgene showed a 1.5-fold increase in GUS expression; whereas, those containing promoters shorter than 2414 bp (np7, np8 and np9) did not show any ABA induction (Figure 4). These results indicate that the putative ABA responsive cis-element is located in the 87 bp region between –2414 and –2327 bp from the translation start site of the AtNCED3 promoter.

An ABRE in 87 bp region is required for ABA induced expression of AtNCED3

The analysis of this 87 bp region identified a putative ABRE (Figure 5A). Thus, we tested whether this putative ABRE mediates the ABA responsive expression of AtNCED3. To do so, the region containing the putative ABA responsive cis-element, between –2414 and –2327 bp, was duplicated 3 times and then fused with either a CaMV 35S minimal promoter or –349 bp AtNCED3 promoter, followed by GUS gene and named R87x3-35S and R87x3-N3 (Figure 5B). A mutant version (mR87x3-35S and mR87x3-N3), in which the putative ABRE (GGCACGGT) was mutated to TTTCGCGGG, was constructed in the same way to identify the function of this cis-element in response to ABA (Figure 5B). At the same time, we introduced base substitutions into the putative ABRE in –2664 bp AtNCED3 promoter and named mABRE (Figure 5B). All constructs were transformed into Arabidopsis and transgenic plants were sprayed with 100 μM
ABA GUS activity assay showed that GUS activities were induced in R87x3-35S and R87x3-N3 lines (2.7- and 8.4-folds, respectively), but not in mR87x3-35S, mR87x3-N3 and mABRE lines (Figure 5B). The results indicate that the putative ABRE (GGCACGTG) is a functional ABA responsive cis-element in AtNCED3 promoter, and GUS expression driven by -349 bp AtNCED3 promoter is stronger than that by the CaMV 35S minimal promoter with or without ABA treatment.

AtNCED3 expression is not transactivated by ABF3

Previous studies showed that ABF3 can recognize ABRE, and that its expression was induced by ABA and dehydration stress,
providing a possibility that AtNCED3 expression is directly regulated by ABF3 [26, 27]. To test this possibility, we examined the expression of AtNCED3 in both ABF3 over-expression (OE) transgenic plants and ABF3 mutant (abf3). The abf3 mutant (SALK_096965) was proven to be a null mutant with a T-DNA insertion in intron 2 [41]. The ABF3-OE lines produced several times expression of ABF3 (Acc. 001036708) (Figure 6A). Quantitative RT-PCR analysis showed that application of 100 μM ABA induced AtNCED3 expression in control and ABF3-OE plants (Figure 6B). However, its expression in the ABF3-OE plants was similar to that observed in the control plants, suggesting that over-expression of ABF3 did not enhance the ABA-induced AtNCED3 expression (Figure 6B). Consistent with this result, loss of ABF3 function in the abf3 mutant did not affect AtNCED3 expression either with or without ABA treatment (Figure 6B). An ABF3 regulated gene, RD29B (Acc. D13044), was used as positive control. These results together indicate that AtNCED3 expression is not transactivated by ABF3.

Discussion

Significant progress has been made in understanding ABA perception and downstream gene activation mechanisms when plants encounter stresses [42, 43]. The mechanism by which plants perceive stress and initiate de novo synthesis of ABA is obscure, although stress-induced ABA synthesis has been known since 1960’s [44]. Stress-induced ABA accumulations can be ABA-dependent or independent [22, 45]. But the molecular basis for such accumulation is poorly understood. In Arabidopsis, AtNCED3 is the key step regulating ABA biosynthesis. AtNCED3 expression is highly induced by various stresses leading to enhanced ABA biosynthesis [45]. By analyzing a series of delineated promoters of AtNCED3, coupled with mutagenesis, we revealed that a distal ABRE (-2372 to -2364 bp) is critical for the ABA induced AtNCED3 expression. This element escaped detection in earlier reports where promoters shorter than 1.5 kb were used in promoter-reporter analysis [33]. Indeed, these

Figure 2. The 2664 bp region of AtNCED3 promoter contains ABA responsive cis-element. (A) The analysis of putative cis-elements in 2664 bp AtNCED3 promoter by Plant Cis-Acting Regulatory Element (PlantCARE) database. The A of the ATG codon at the translational site is defined as +1. Octothorpes indicate the ABREs with conserved core sequence. (B) GUS activities of transgenic plants for the 5'-deletion constructs. Values represent means of the activities of ten independent lines. Error bars represent the SD of three independent measurements. Data were analyzed by Student’s t-test (*P<0.05, **P<0.01). The ratios indicate the fold change increases of the GUS activities after ABA treatment compared with values obtained from control treatment. (C) Histochemical analysis of GUS expression in the 2664 bp AtNCED3 promoter-GUS transgenic plants with or without 100 μM ABA treatment for 5 h (scale bars represent 2.5 mm). 10 independent lines and 15 individual plants from each line were analyzed.

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constructs failed to show an expression pattern consistent with the endogenous AtNCED3, particularly the expression in vascular tissues of leaves and stress induction [46]. The identification of this distal element in AtNCED3 promoter provides the molecular basis for the ABA-dependent ABA accumulation.

Recently Behnam et al [47] reported that the same distal ABRE is required for dehydration induction of AtNCED3 expression. Thus, this element is critical to ABA and dehydration induced AtNCED3 expression, hence ABA accumulation in plants under stresses. This raises the question whether the activation of AtNCED3 expression by ABA and dehydration involved identical or different mechanisms. In one scenario, dehydration may increase AtNCED3 expression by an ABA-independent manner; then the increased ABA triggers further increase in AtNCED3 expression in an ABA-dependent manner. As a result, an increase of ABA content is achieved quickly after the plant encounters stresses. In another scenario, dehydration may increase locally ABA content independently from AtNCED3 expression, but via hydrolysis of ABA-GE to produce active ABA [48]. The accumulation of ABA induces/stimulates AtNCED3 expression which causes further increase of ABA. It can also be argued that the application of ABA is perceived as a stress by the plant; hence it initiates stress response, which leads to increased ABA biosynthesis. However, this interpretation is not in agreement with the reduction of AtNCED3 expression in ABA-deficient mutants under stresses [7,14]. In any cases, this distal ABRE

Figure 3. The region between -2664 and -2018 bp of AtNCED3 promoter contains ABA responsive cis-element. Left, the different 3'-deletion constructs of the AtNCED3 promoter-GUS. In constructs np-35L and np-35S, –2664 to –1470 bp or –2664 to –2018 bp fragments are fused to a CaMV 35S minimal promoter (–46 to +8 bp from transcription start site) respectively, followed by a GUS reporter gene. In constructs np-N3L and np-N3S, –2664 to –1470 bp and –2664 to –2018 bp fragments are fused to –349 bp AtNCED3 promoter (–349 to –4 bp) respectively, followed by a GUS reporter gene. Right, GUS activities of transgenic plants for the 3'-deletion constructs are shown. Values represent means of the activities of ten independent lines. Error bars represent the SD of three independent measurements. Data were analyzed by Student’s t-test (*P<0.05; **P<0.01). The ratios indicate the fold change increases of the GUS activities after ABA treatment compared with values obtained from control treatment. doi:10.1371/journal.pone.0087283.g003

Figure 4. The region between –2414 and –2092 bp of AtNCED3 promoter contains ABA responsive cis-element. Left, the different 5'-deletion constructs of the AtNCED3 promoter between –2414 and –2092 bp region. The AtNCED3 promoter regions between –2414 to –4 bp, –2327 to –4 bp, –2214 to –4 bp or –2092 to –4 bp are cloned upstream of GUS coding region. Right, GUS activities of transgenic plants for the 5'-deletion constructs are shown. Values represent means of the activities of ten independent lines. Error bars represent the SD of three independent measurements. Data were analyzed by Student’s t-test (*P<0.05; **P<0.01). The ratios indicate the fold change increases of the GUS activities after ABA treatment compared with values obtained from control treatment. doi:10.1371/journal.pone.0087283.g004
element provides the molecular basis for ABA/stress regulation of AtNCED3 expression. This information can be used to identify the transcription factors that directly regulate AtNCED3 transcription, which may be an entry point to dissect the signaling pathway from stress perception to ABA synthesis.

Many drought-induced NCEDs have been studied. By comparing AtNCED3 promoter with the drought-induced NCED promoters from grape (Vitis vinifera) [49], tomato [18], peanut (Arachis hypogaea L.) [50], rice (Oryza sativa) [51] and maize [9], a conserved 6 nucleotide core (C/GACGTG) emerged as a distal ABA responsive cis-element (Figure 7A, B). Two of them, the distal ABRE in AhNCED1 promoter (–1386 to –1379 bp) and AtNCED3 promoter (–2372 to –2364 bp) have been identified to respond to drought [47,50], suggesting the ABRE from different species may have the similar function. However, there is no evidence whether the distal ABA responsive cis-elements from other species are involved in drought responsive transcription. Further studies will be needed to identify whether these ABREs contribute to ABA response.

Functional ABREs are frequently found close to transcription start site. Two ABREs for ABA induction of HVA22 expression in barley are located between –240 and –84 bp of the HVA22 promoter from transcription start site [28]. An ABRE required for ABA responsive expression of the n294 in Arabidopsis is located between –63 to –55 bp transcription start site [30]. Similar cases were found in the MIR168a in Arabidopsis (–126 to –122 bp from transcription start site) and Vp1 in maize (–84 to –75 bp from transcription start site) [31,52]. In the promoter of Arabidopsis and other five species, we found a putative ABRE (CACGTG) near the transcription start site (Figure 7A, B), but this proximal putative ABRE in Arabidopsis was not necessary for ABA induced AtNCED3 expression. Firstly, progressive deletion of the promoter sequences up to 2327 bp position did not detect ABA responsive expression of the reporter gene (Figure 4A, np6). Secondly, the –2664 to –2018 bp or –2664 to –1470 bp region fused to minimal CaMV 35S promoter is sufficient for ABA induced expression of AtNCED3 (Figure 3A, np-35L and np-35S). These data indicate that the proximal putative ABRE in Arabidopsis was not necessary for ABA induced AtNCED3 expression. Firstly, progressive deletion of the promoter sequences up to 2327 bp position did not detect ABA responsive expression of the reporter gene (Figure 4A, np6). Secondly, the –2664 to –2018 bp or –2664 to –1470 bp region fused to minimal CaMV 35S promoter is sufficient for ABA induced expression of AtNCED3 (Figure 3A, np-35L and np-35S). These data indicate that the proximal putative ABRE is not necessary for ABA response. However, we noted that any promoter fragments fused to –349 bp AtNCED3 promoter containing the proximal putative ABRE can enhance the response to ABA (Figure 3A, np-N3L and np-N3S). Figure 5A, R87x3-N3 and mR87x3-N3, and the –349 bp AtNCED3 promoter was enough for AtNCED3 expression in early development stage (Figure 1A, E, G and O), suggesting that this proximal ABRE may possess enhancer function or confer specific AtNCED3 expression in response to developmental cues.

The 1.5 kb promoter-GUS failed to confer stress induced GUS expression although it is known that AtNCED3 is strongly induced in stressed leaves [33]. The 1 kb promoter, on the other hand, delivered stress induced expression of LUC [37,38]. However, this

![Figure 5. The distal ABRE of the AtNCED3 promoter is involved in ABA-responsive expression.](image-url)
study and others [47] convincingly demonstrated that the cis-elements responsible for ABA and stress induction are located on the distal end of the \( \text{AtNCED3} \) promoter. Besides the possibility of repressors in the \( \approx 500 \) bp (1 to 1.5 kb) region, we tend to believe that detection methods are responsible for the discrepancy. Detection of LUC is much more sensitive than GUS staining. Based on existing data, we think that the distal cis-elements defined by this study play a predominant role in stress induced \( \text{AtNCED3} \)

**Figure 6. The expression of \( \text{AtNCED3} \) in WT, ABF3 overexpression transgenic plants (OE) and \( \text{abf3} \).** (A) Expression of the ABF3 assayed by quantitative RT-PCR in Col-0 (C), ABF3 OE1 (OE1) and ABF3 OE2 (OE2) two-week-old whole seedlings under control conditions or after 5 h of exposure to 100 \( \mu \)M ABA. Data are means \pm SE (\( n = 3 \)). Data were analyzed by Student’s t-test (*) \( P < 0.05 \); ** (*) \( P < 0.01 \). (B) Expression of the RD29B and \( \text{AtNCED3} \) assayed by quantitative RT-PCR in Col-0 (C), ABF3 OE1 (OE1), ABF3 OE2 (OE2) and ABF3 mutant (\( \text{abf3} \)) two-week-old whole seedlings under control conditions or after 5 h of exposure to 100 \( \mu \)M ABA. Data are means \pm SE (\( n = 3 \)). Data were analyzed by Student’s t-test (*) \( P < 0.05 \); ** (*) \( P < 0.01 \).

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**Figure 7. The distal and proximal ABREs are conserved in drought induced NCED promoters.** (A) Comparison of the conserved ABRE in the distal region and proximal region of drought induced NCED promoters from Arabidopsis (\( \text{AtNCED3} \); Acc. NM_112304), grape (\( \text{VvNCED1} \); Acc. AY337613), tomato (\( \text{LeNCED1} \); Acc. Z97215), peanut (\( \text{AhNCED1} \); Acc. EU497940), rice (\( \text{OsNCED3} \); Acc. AY838899) and maize (\( \text{Vp14} \); Acc. U95953). Red letter represents non-conserved nucleotide. (B) Sequence logos for distal ABRE (left) and proximal ABRE (right). Sequence logos were created online using the Weblogo resource [59].

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expression, whereas the proximal ones (such as the ones included in the 1 kb promoter) may play a minor role. Conceivably even with a 1 kb promoter, there will be many genes that can affect the promoter activity, either directly or indirectly. In fact, the cuticle synthetic gene reflects an indirect regulation on promoter activity, either directly or indirectly. In fact, the cuticle reduction of ABA content. ABA inactivation pathway whereas a line with stopper bar represents Arrow lines indicate stimulation of gene expression, ABA production or ABA inactivation pathway whenever a line with stopper bar represents reduction of ABA content.

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It is unexpected to find that ABF3 can not activate the expression of AtNCED3, in view of that ABF3 is known to recognize ABRE and activates ABA responsive genes. Indeed, ABRE is very similar to the G box (CACGTG) which is involved in the red light signaling [53], jasmonic acid response [54], and auxin response [55], suggesting that different transcription activators can recognize the core sequence (CACGTG). In addition, transcriptome analysis of ABF3 over-expression transgenic Arabidopsis demonstrated that AtNCED3 was regulated similarly in over-expression and control plant lines under drought condition as well as in the wild type [37].

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Supporting Information

Table S1 Primers used in this study.

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Author Contributions

Conceived and designed the experiments: BCT YZY. Performed the experiments: YZY. Analyzed the data: BCT YZY. Contributed reagents/materials/analysis tools: YZY. Wrote the paper: BCT YZY.

Figure 8. Schematic representation of the balance between ABA biosynthesis and inactivation under different conditions. When plants encounter drought stress, ABA is accumulated by de novo synthesis or activation of inactive ABA pool (ABA-GE hydrolysis); then further increased by positive feedback biosynthetic pathway. ABA inactivation pathway is also activated by drought stress to maintain endogenous ABA levels within the permissible range. Once stress is relieved, ABA inactivation pathway allows for rapid degradation of ABA inactivation pathway is also activated by drought stress to maintain endogenous ABA levels within the permissible range. This notion is supported by the quick reduction of ABA when stresses are relieved.

The cis-Element Required for \textit{AtNCED3} Expression
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