Seed Science Research (2017) **27**, 251–261
doi:10.1017/S0960258517000216

Transcriptomics of *nine-cis-epoxycarotenoid dioxygenase 6* induction in imbibed seeds reveals feedback mechanisms and long non-coding RNAs

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(Received 3 April 2017; accepted after revision 30 May 2017; first published online 11 September 2017)

Abstract

Induction *of nine-cis-epoxycarotenoid dioxygenase 6* (*NCED6*), an abscisic acid (ABA) biosynthesis gene, alone is sufficient to suspend germination in testa-ruptured seeds, which are at the final step of germination. Molecular consequences of *NCED6* induction in imbibed seeds were investigated by RNA sequencing. The analysis identified many unknown and uncharacterized genes that were up-regulated by *NCED6* induction, in addition to the major regulators of ABA signalling. Interestingly, other *NCED*s were up-regulated by *NCED6* induction, suggesting that the major rate-limiting enzymes in the ABA biosynthesis pathway are subject to positive-feedback regulation. *ZEAXANTHIN EPOXIDASE* and *ABSCISIC ALDEHYDE OXIDASE3*, which function upstream and downstream of *NCED*, were also up-regulated in seeds by *NCED6* induction, which suggests that the distinct layers of positive feedback loops are coordinately operating in the *NCED6*-induced seeds. *SOMNUS* (*SOM*), which was also up-regulated by *NCED6* induction, was the major mediator of enhanced ABA signalling in *NCED6*-induced seeds. *SOM* exerted negative effects on GA biosynthesis, which also contributes to a high ABA–GA ratio and reinforces the suppressive state of germination. Besides these coding genes, long intergenic non-coding RNAs (lincRNAs) were also up-regulated upon *NCED6* induction (termed *N6LINCRs*). Conditional expression of *N6LINCR1* altered gene expression profiles in seeds. Twenty-six genes were up-regulated and 66 genes were down-regulated by the induction of *N6LINCR1*. These results suggest that some of *N6LINCRs* have a regulatory role in gene expression in seeds, which potentially contributes to the regulation of germination by ABA.

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Keywords: ABA, *Arabidopsis*, long non-coding RNA, NCED, positive feedback

Introduction

Seed dormancy is an important strategy of plants, which has evolved to ensure successful establishment of their offspring in natural environments (Bewley *et al*., 2013). Seed dormancy is also an important agricultural trait because the absence of dormancy in crop species could result in precocious germination on the maternal plants, such as pre-harvest sprouting (PHS) (Gubler *et al*., 2005). Many genes associated with seed dormancy have been identified. Rice *Seed dormancy 4* (*Sdr4*), a quantitative trait locus (QTL) associated with PHS resistance, was identified by the comparison of *japonica* and *indica* cultivars (Sugimoto *et al*., 2010). The major seed dormancy QTL *PM19*s in wheat were identified by the novel pipeline that analyses multiple near isogenic lines with next generation sequencing (Barrero *et al*., 2015). In *Arabidopsis*, *DELAY OF GERMINATION*s (*DOG*s) have been identified as the major QTLs for seed dormancy (Alonso-Blanco *et al*., 2003; Bentsink *et al*., 2006, 2010).

Strong genetic evidence indicates that the genes mentioned above are the major determinants of seed dormancy. For example, the single mutation *dog1-1* causes a no-dormancy phenotype (Alonso-Blanco *et al*., 2003; Bentsink *et al*., 2006, 2010). However, conditional expression of *DOG1* is not sufficient to reconstitute dormancy in after-ripened seeds (Nakabayashi *et al*., 2012). This suggests that integrated function of multiple factors (Nonogaki, 2014) and/or a suitable chemical environment in seeds that modifies a single determinant (Nakabayashi *et al*., 2012, 2015; Nee *et al*., 2016) is critical for seed dormancy.

An exception that has been demonstrated to be capable of suspending germination of imbibed

non-dormant seeds is the induction of *nine-cis-epoxycarotenoid dioxygenase 6* (*NCED6*), a rate-limiting abscisic acid (ABA) biosynthesis gene. Induction of *NCED6* alone by a chemically inducible system causes ABA increase and suppression of germination in *Arabidopsis* seeds at the testa-rupture stage, the final step of germination (Martinez-Andujar *et al.*, 2011). *NCED6* induction provides a good experimental system for the analysis of the ABA-dependent seed dormancy pathway.

An understanding of the mechanisms of ABA perception and signal transduction has been advanced greatly in the last decade (Cutler *et al.*, 2010). However, molecular mechanisms downstream of enhanced ABA biosynthesis and signalling in seeds are still elusive. It is necessary to expand the understanding of the known pathways and also decode unrecognized mechanisms in the ABA-dependent seed dormancy pathway.

In this study, the *NCED6*-inducible system was employed to investigate the molecular consequences of *NCED6* induction by RNA sequencing (RNA-seq). The analysis identified unknown and uncharacterized coding genes, in addition to known factors, which mediate seed response to *NCED6* induction. The expression analysis was also extended to long non-coding RNAs (lncRNAs). The significance of positive feedback regulation in hormone metabolism and possible regulatory roles of lncRNAs in gene expression in seeds are evaluated in this paper.

Materials and methods

Inducible gene expression

Chemically inducible gene expression was performed following the previously published method and using the previously described lines (*AGE:NCED* 5-125, 8-181, 15-132) (Martinez-Andujar *et al.*, 2011). Briefly, Intrepid2F (IP, Dow AgroSciences) was diluted 10,000 times with water, which contained methoxyfenozide at approximately 62 μM, and was used to moisten filter papers for seed imbibition. Seeds were incubated until the testa rupture stage and then used for RNA extraction for gene expression analysis by RNA-seq or RT-PCR.

RNA sequencing and data analysis

RNA was extracted from 10 mg of IP-treated (+) or -untreated (–) seeds of three independent inducible transgenic lines and three wild-type (WT) plants, using a standard phenol/chloroform extraction method. RNA was purified by oligo-dT beads. Samples for RNA-seq were prepared using a TrueSeq™ RNA Sample Preparation v2 Guide Low-Throughput (LT) Protocol or Illumina TruSeq™ Stranded RNA LT (Illumina). All libraries were sequenced by the Center for Genome Research and Biocomputing at Oregon State University with HiSeq 2000. The RNA-seq data were analysed using Bowtie2 (Langmead and Salzberg, 2012), Tophat2 (Kim *et al.*, 2013), Cufflinks, Cuffmerge, Cuffdiff (Trapnell *et al.*, 2013), HISAT2 (Kim *et al.*, 2015) and StringTie (Pertea *et al.*, 2015). The numbers of transcript reads were counted and compared for each sample by Cuffdiff, which determined differentially expressed genes with statistical significance ($q < 0.05$).

RT-PCR

RT-PCR was performed using RNA extracted from seeds as described above. Two micrograms of total RNA was reverse transcribed with MMLV-RT (Promega) and oligo dT primer. For strand-specific PCR, either of a gene-specific forward or reverse primer was used for RT. The resulting RT product (1 μl) was used for PCR. PCR was performed using ExTaq DNA polymerase (Takara, Mountain View, CA, USA). The conditions for PCR were: one cycle at 94°C (1 min), touchdown cycles (94°C for 15 s, 66°C → 59°C for 15 s, and 72°C for 30 s) (one cycle for each temperature), and 30 cycles at 94°C (15 s), 59°C (15 s), and 72°C (30 s), followed by extension at 72°C (7 min). For semi-quantitative PCR, the numbers of the major cycles at 59°C were limited to 15, 20 or 25 cycles, depending on expression level of each gene. Primers used in this study are listed in Table S1.

Vector construction

To generate inducible lines for a non-coding RNA, a 1185-bp fragment was amplified from the *Arabidopsis* genomic DNA (gDNA) with the InFusion forward and reverse primers containing the *BstB*I sites. The PCR product was inserted into the inducible AGE vector (Martinez-Andujar *et al.*, 2011) at the *BstB*I site. The transformation vectors were introduced into *Agrobacterium tumefaciens*, which were used to transform *Arabidopsis thaliana* Columbia-0 by the floral dip method (Clough and Bent, 1998).

Results and Discussion

Molecular consequences of NCED6 induction in imbibed seeds

The induction of *NCED6* with the Gene Switch (GS) system, a chemically inducible gene expression system

https://doi.org/10.1017/S0960258517000216 Published online by Cambridge University Press

(Martinez-Andujar *et al*., 2011), is sufficient to cause germinating seeds to suspend or reverse the physiological processes proceeding towards radicle protrusion (Fig. 1A). While NCED is a rate-limiting enzyme for ABA biosynthesis, induction of an enzyme should not be effective if the substrates are not present. Therefore, the suppression of germination by *NCED6* induction alone suggests that the pathways upstream of the rate-limiting reaction are operational and a substantial amount of the NCED substrates 9′-*cis*-neoxanthin and/or 9-*cis*-violaxanthin is continuously supplied even in non-dormant seeds. The steady supply of the substrates for the rate-limiting ABA biosynthesis enzyme even during germination is probably a critical strategy for seeds to respond to environmental changes promptly and cease the germination process immediately when they sense marginal conditions. The same mechanism may function in intact seeds before testa rupture to cause secondary dormancy, which could be induced by seed imbibition at high temperatures, in osmotica, or under darkness (Bewley *et al*., 2013).

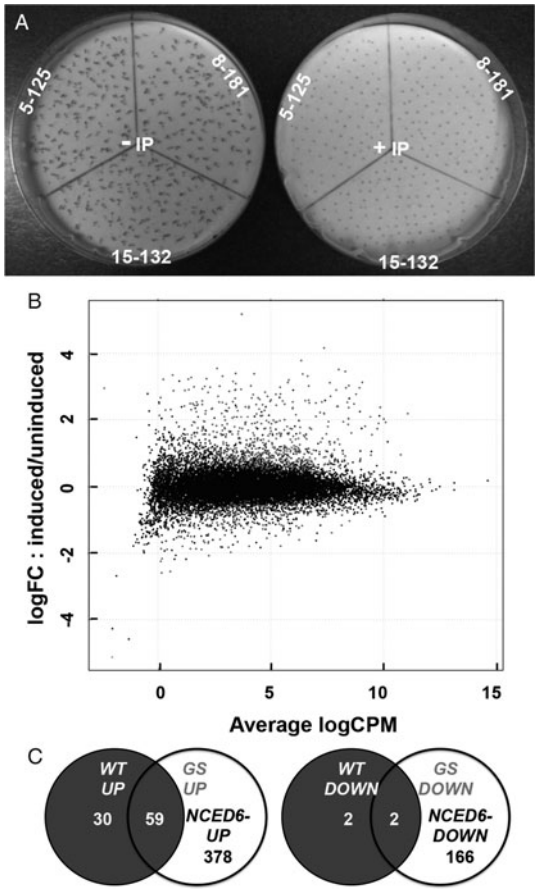

Figure 1. The effects of *nine-cis-epoxycarotenoid dioxygenase 6* (*NCED6*) induction on germination and gene expression. (A) Suppression of germination in the *NCED6*-induced *Arabidopsis* seeds. Germination of *NCED6*-inducible seeds (three independent lines 5-125, 8-181 and 15-132) was suppressed in the presence of the chemical ligand Intrepid2F (+IP) while those seeds germinated normally in the absence of the chemical ligand (–IP). (B) Differential gene expression between *NCED6*-induced and -uninduced seeds. Smear plot shows gene switch up-regulated (*GS-UP*) and down-regulated (*GS-DOWN*) genes (grey dots) upon *NCED6* induction. CPM, count per million; FC, fold change. (C) Venn diagrams showing genes specifically up- (left, *NCED6-UP*) or down-regulated (right, *NCED6-DOWN*) in the *NCED6*-inducible seeds, excluding the *GS-UP* (59) and *GS-DOWN* (2) genes that were also differentially expressed in wild-type (WT) upon ligand application (*WT-UP*, *WT-DOWN*).

The ecological significance of seed responses to the environment is clear, however the molecular and biochemical mechanisms underlying the suppression of germination by ABA is not fully understood. To obtain more insights into ABA-regulated molecular events in seeds, RNA-seq was performed using the *NCED6*-induced and -uninduced *Arabidopsis* seeds. Induction of *NCED6* using GS caused differential expression of a number of genes (Fig. 1B). The RNA-seq analysis identified 437 genes up-regulated (*GS-UP*) and 168 genes down-regulated (*GS-DOWN*) in the *NCED6*-induced seeds with statistical significance ($q < 0.05$). The genes that were also differentially expressed between ligand-treated or -untreated WT seeds were subtracted from the original *GS-UP* and *GS-DOWN* groups and the remainders were termed *NCED6-UP* (378) and *NCED6-DOWN* (166) (Fig. 1C, Table S2). *NCED6* was one of the highly differentially expressed genes, which verified successful induction of gene expression during sample preparation for the RNA-seq experiments and the propriety of the computational and statistical analyses (Table S2). *NCED6-UP* genes included known ABA signalling components (e.g. *ABA INSENSITIVE 5*), annotated yet uncharacterized genes (e.g. *DELAY OF GERMINATION1-LIKE4*) and unknown proteins (e.g. *At3g48510*). Differential expression of the representative genes was confirmed by RT-PCR (Fig. 2).

As anticipated, many of the differentially expressed genes were associated with abiotic stresses, including cold, high temperature and drought (Table S2). The ABA-induced protein phosphatases 2C (PP2C) *HIGHLY ABA-INDUCED1* (*HAI1*), *HAI2* and *HAI3* (Bhaskara *et al*., 2012) were detected as *NCED-UP* genes. In addition to *ABI5*, a major regulator of seed maturation, typical seed maturation genes, such as *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) and storage proteins were also detected as *NCED6-UP* genes (Table S2, Fig. 3). These results suggest that the seed maturation programmes can, at least in part, be re-introduced to the testa-ruptured seeds if *NCED* expression reaches a certain threshold, even though they are at the very final step of germination.

The misguided expression of *NCED6* at the last stage of germination also triggered expression of the major regulators of seed dormancy, such as

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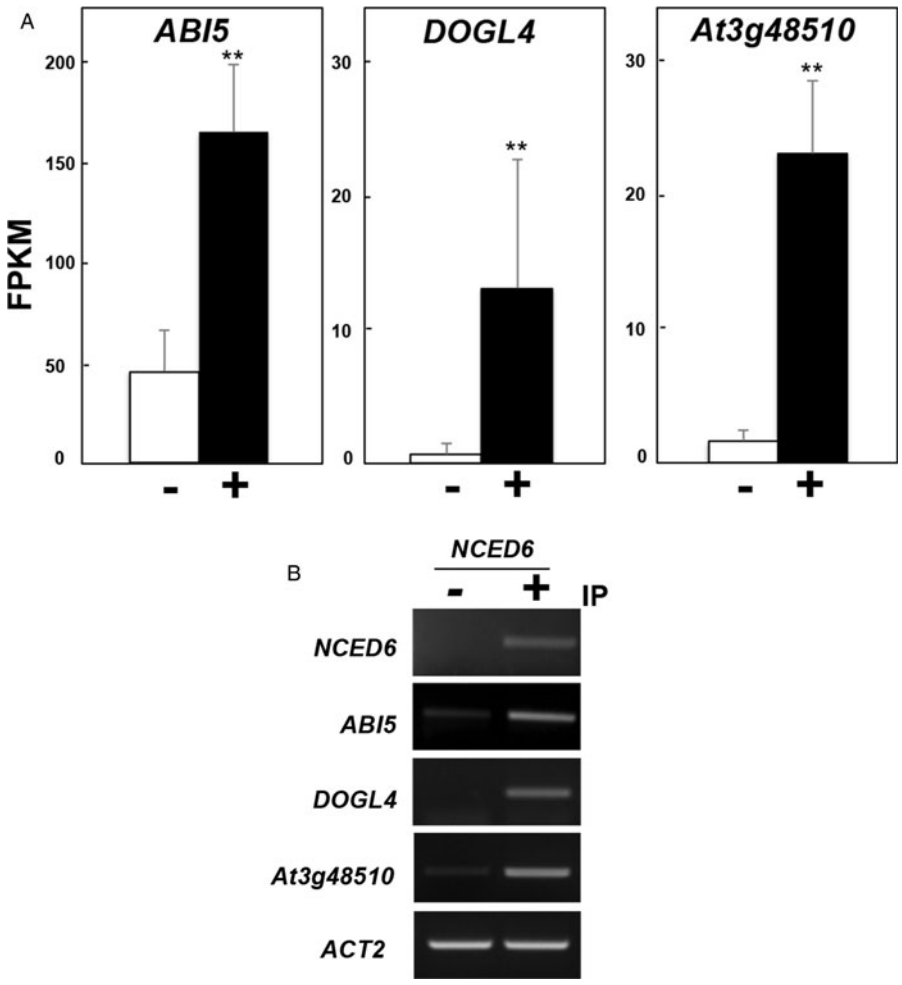

Figure 2. Differential expression of representative *NCED6-UP* genes. (A) Differential expression detected by RNA-seq analysis between induced (+, filled bar) and uninduced (–, open bar) seeds. FPKM, fragments per kilobase of exon per million fragments mapped. *ABI5*, *ABA INSENSITIVE5*; *ACT2*, *ACTIN 2*, *DOGL4*, *DELAY OF GERMINATION1-LIKE 4*. $^{**}P < 0.01$ (Student's *t*-test compared with control). (B) RT-PCR of the same group of genes. Representative gel images are shown.

SOMNUS (*SOM*), a seed-specific nucleus-localized CCCH-type zinc finger protein (Kim *et al*., 2008; Park *et al*., 2011) and *MOTHER OF FT AND TFL1* (*MFT*), a phosphatidylethanolamine-binding protein (Yoo *et al*., 2004; Xi *et al*., 2010). *SOM* is activated by PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5) and ABI3 (Park *et al*., 2011), which directly bind to the *SOM* promoter while ABI3, ABI5 and DELLA proteins can also interact with each other to bind the *SOM* promoter at high temperature (Lim *et al*., 2013). It has been demonstrated that expression of the GA biosynthesis genes *GA3ox1* and *GA3ox2* are reduced by *SOM* expression, suggesting that SOM suppresses seed germination by reducing GA levels in seeds (Kim *et al*., 2008). In fact, *GA3ox1* and *GA3ox2* were detected as *NCED-DOWN* genes in our analysis, together with *SOM* as an *NCED-UP* gene (Fig. 3).

Down-regulation of *GA3ox1* and *GA3ox2* can be mediated by suppression of the Jumonji domain-containing proteins 20 and 22 (JMJ20/22), which are histone arginine demethylases. JMJ20/22 activate *GA3ox1* and *GA3ox2* by removing the inhibitory methyl marks from these genes (Cho *et al*., 2012). It is known that the transcript levels of *JMJ20/22* are increased in the *pil5* and *som* mutants, suggesting that SOM suppresses *JMJ20/22* (Cho *et al*., 2012). Therefore, it is conceivable that the down-regulation of *GA3ox1* and *GA3ox2* in the *NCED6*-induced seeds was the consequence of the up-regulation of *SOM* (Fig. 3). However, *JMJ20* and *JMJ22* were not detected as *NCED-DOWN* genes in our analysis. Thus the mechanism of *GA3ox* regulation by SOM in the *NCED6*-induced seeds is not clear.

MFT is a gene that is specifically induced in the radicle–hypocotyl transition zone of the embryo in response to ABA (Xi *et al*., 2010). Up-regulation of *MFT* in the induced seeds also verifies the strong ABA response occurring in the *NCED6*-induced seeds, which must be the consequence of increased expression of *ABI5*, because *MFT* is directly regulated

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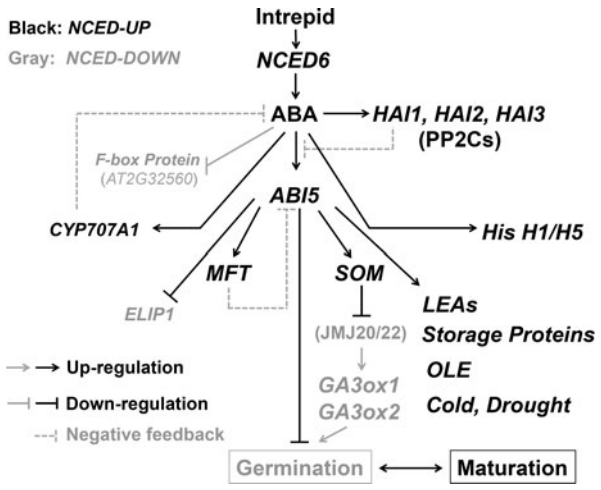

Figure 3. Schematic representation of the major *NCED-UP* (black) and *NCED-DOWN* (grey) genes found in the *NCED6*-induced seeds and their mechanistic association. See text for details. *ELIP, EARLY LIGHT-INDUCIBLE PROTEIN*; *HAI, HIGHLY ABA-INDUCED*; *JMJ, JUMONJI*; *LEA, LATE EMBRYOGENESIS ABUNDANT*; *MFT, MOTHER OF FT AND TFL1*; *OLE, OLEOSIN*; PP2C, PROTEIN PHOSPHATASE 2C; *SOM, SOMNUS*.

by ABI5 (Xi *et al*., 2010) (Fig. 3). MFT exerts a negative feedback effect to *ABI5* (Xi *et al*., 2010) (Fig. 3). The extremely high levels of ABA production in the *NCED6*-induced GS seeds (Martinez-Andujar *et al*., 2011) probably triggered the negative feedback regulation to counteract the excessive ABA levels and maintain a certain level of ABA–GA balance in seeds. However, as *ABI5* was still detected as an *NCED-UP* gene, the robustness of the GS system most likely overrode and masked the effects of the negative feedback function by MFT.

The *NCED* GS system was originally created to gain a proof of concept for technology development for prevention of pre-harvest sprouting (PHS) in cereals (Martinez-Andujar *et al*., 2011; Nonogaki *et al*., 2014). In wheat, potential of altered expression of a *MFT* orthologue for dormancy regulation and PHS control has been demonstrated (Nakamura *et al*., 2011). Therefore, the GS system (Martinez-Andujar *et al*., 2011), which can increase both ABA biosynthesis (*NCED*) and response (*MFT*), is expected to be effective for PHS prevention in cereals. A more advanced system of PHS prevention, which is devoid of potential pleiotropic effects with the GS system, has also been developed based on the knowledge obtained for seed responses to ABA (Nonogaki *et al*., 2014). Therefore, the basic mechanisms analysed here in *NCED6*-induced seeds will also be important for translational biology for the future (Nonogaki and Nonogaki, 2017).

Other negative feedback responses, besides *MFT-ABI5*, were also observed in the *NCED6*-induced seeds. *HAI PP2Cs* mentioned above are negative regulators of ABA signal transduction (Fig. 3). *CYP707A1*, an ABA deactivation gene, was also an *NCED-UP* gene, presumably as another counteracting response to ABA increase in seeds (Fig. 3). *NCED6* induction seems to cause a number of changes in hormone metabolism genes themselves (discussed below).

Positive feedback mechanisms in ABA biosynthesis

The initial analysis using the Tophat2 program had identified *NCED2* and *NCED9* as *NCED6-UP* genes, which suggests the presence of positive-feedback regulation in the ABA biosynthesis pathway. The positive-feedback regulation has been suggested for *NCED5* (Okamoto *et al*., 2010; Nonogaki *et al*., 2014) and *NCED9* (Okamoto *et al*., 2010) in the previous studies. Although the list of *NCED6-UP* by the HISAT2 analysis did not include these *NCED*s (Table S2), *NCED2*, *NCED5* and *NCED9* seemed to be enhanced by *NCED6* induction in the same dataset (Fig. 4, histograms). Experiments by RT-PCR confirmed that *NCED2*, *NCED5* and *NCED9* are also subject to positive feedback (Fig. 4, top right inset). In a separate project, we prepared *DOGL4*-inducible lines (authors' unpublished observations) and found that induction of *DOGL4*, which was one of the *NCED6-UP* genes (Fig. 2), increased *NCED9* expression (Fig. 4, bottom right inset). This observation also confirms that *NCED9* is subject to positive feedback regulation in the *NCED6*-inducible system (*NCED6*→ABA→*DOGL4*→*NCED9*). The positive feedback mechanism seems to play a central role in the regulation of the rate-limiting step of (and hence the entire) ABA biosynthesis pathway (Fig. 4, pathway scheme).

The high magnitude of *NCED* expression, which leads to over-production of the rate-limiting enzymes, could deplete its substrate 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin from the *NCED6*-induced seeds (Fig. 4). Interestingly, *ZEAXANTHIN EPOXIDASE* (*ZEP*), which catalyses the upstream reaction and subsequently provides the substrates for NCED, was an *NCED-UP* gene (Table S2, Fig. 4), indicating that there is another positive-feedback loop upstream of ABA biosynthesis pathway (Fig. 4).

While the steady supply of the substrates could meet the demands of enhanced NCED activities, the products of enzyme reaction could over-accumulate if the downstream pathways are not operating accordingly. Therefore, another positive feedback loop may be present downstream as well. Indeed, *Arabidopsis ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*), which catalyses the last step of abscisic aldehyde conversion to ABA, was also detected an *NCED-UP* gene (Table S2) (Fig. 4). At least the major steps of the ABA biosynthesis pathway in seeds seem to function coordinately to enhance ABA biosynthesis (Fig. 4). These results

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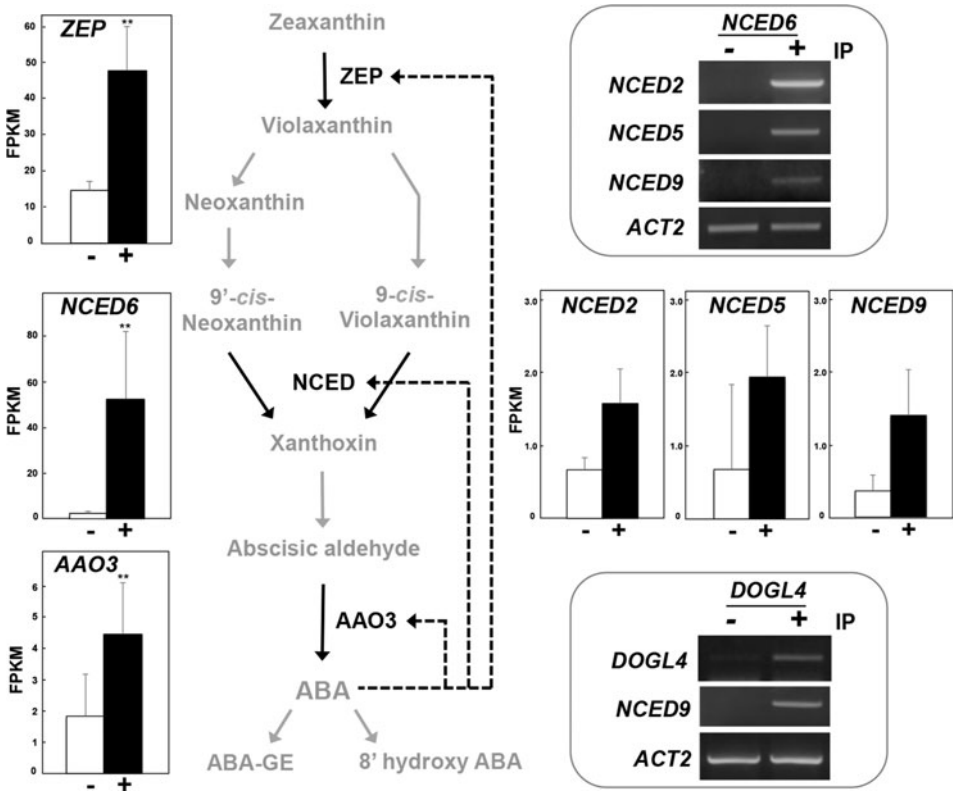

Figure 4. Positive feedback mechanisms in the ABA biosynthesis pathway found in the *NCED6*-induced seeds. Histograms show differential expression of the ABA biosynthesis genes in the *NCED6*-induced (+, filled bar) and -uninduced (–, open bar) seeds. $^{**}P < 0.01$ (Student's *t*-test compared with control). Schematic representation of the major ABA biosynthesis pathway is shown in the center. *Top right inset*, RT-PCR for expression of other NCEDs in *NCED6*-induced (+) or uninduced (–) seeds. *Bottom right inset*, RT-PCR for *NCED9* expression in *DOGL4* (*DELAY OF GERMINAITON1-LIKE4*)-induced (+) or -uninduced (–) seeds (see text for *DOGL4*). Induction of *NCED6* caused up-regulation of *NCED2*, *NCED5* and *NCED9*, suggesting positive feedback regulation of *NCED*s in the native system (dashed black arrow in the centre). Representative gel images are shown. *ZEAXANTHIN EPOXIDASE* (*ZEP*) and *ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*), which function up- and downstream of *NCED*s, were also subject to positive feedback (top and bottom dashed arrows), which suggests coordinated enhancement of ABA biosynthesis in seeds. FPKM, fragments per kilobase of exon per million fragments mapped.

explain the robustness of the induction of *NCED6*, a single gene, for the suspension of seed germination.

Positive feedback mechanisms were suggested for *ZEP* and *AAO3* in non-seed tissues (Xiong *et al.*, 2002) and for *NCEDs* in seeds (Okamoto *et al.*, 2010; Nonogaki *et al.*, 2014) previously. Our data provided strong evidence of multiple layers of positive-feedback loops present in the *NCED6*-induced seeds. The mechanistic details of the positive feedback regulation in ABA metabolism in seeds are not clear, except for the *DOGL4–NCED9* interaction. However, some of the *NCED-UP* genes described above, such as *SOM* and *MFT* (Fig. 3), probably contribute to the positive feedback mechanism in ABA metabolism in the *NCED6*-induced seeds. *ZEP* expression is reduced in the *som* mutant, suggesting that SOM up-regulates *ZEP* (Kim *et al.*, 2008). Therefore, it is possible that the positive feedback to *ZEP*, which was observed in the *NCED6*-induced seeds (Fig. 4), was mediated through the enhancement of *SOM* expression by *NCED6* induction (Fig. 3). SOM also up-regulates *NCED6* (Kim *et al.*, 2008). Therefore, it is possible that expression from the native *NCED6* gene in the genome was stimulated by SOM, in addition to the ligand-dependent expression of the switchable *NCED6* transgene. Probably, the expression of the native *NCED6*, which was enhanced by the increased SOM activity, also contributed to the large fold changes of *NCED6* expression between induced and -uninduced seeds (Table S2). The major ABA deactivation gene *CYP707A2* in *Arabidopsis* seeds (Kushiro *et al.*, 2004) is negatively regulated by SOM (Kim *et al.*, 2008), which could also contribute to maintaining high levels of ABA in seeds, although this gene was not detected as an *NCED-DOWN* (i.e. *SOM-DOWN*) gene in our data set. All of these events by SOM that enhance ABA accumulation in seeds, together with the down-regulation of the GA biosynthesis genes by SOM (possibly through JMJ20/22 described above), are thought to have increased the ABA–GA ratio in seeds and prevented seed germination. Thus SOM is also a potential target of modification for future applications.

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Long non-coding RNAs

Many unknown proteins that are induced by ABA have been detected by our RNA-seq analysis (Table S2). Some known but uncharacterized genes, such as *DOGL4*, were also found as *NCED-UP* or ABA-responsive genes. In addition, RNA-seq reads were assembled to the intergenic regions where protein coding genes were not annotated by the Salk T-DNA express *Arabidopsis* Gene Mapping Tools (http://signal.salk.edu/cgi-bin/tdnaexpress) (examples shown in Fig. 5A). These RNAs, which were expressed from intergenic regions, were considered as long intergenic non-coding RNAs (lincRNAs). We found five lincRNAs responding to *NCED6* induction in seeds (termed *N6LINCRs*) (Fig. 5B). *N6LINCR1-5*, which are about 400–800 bases in size spread over different chromosomes and show distinct sequences while all of them were found between coding genes without overlapping them (Fig. 6) (except for some known non-coding RNAs discussed later).

To examine a potential role of lincRNAs in seeds, we focused on and characterized *N6LINCR1*, which exhibited the most obvious response to *NCED6* induction (Fig. 5B). RT-PCR analysis of *N6LINCR1* using the *NCED6*-inducible seeds showed that it was expressed in seeds without induction but its expression was enhanced by *NCED6* induction (Fig. 7A). *N6LINCR1* expression was enhanced by exogenous ABA (10 μM) in WT seeds and also in leaves (Fig. 7B). Strand-specific RT-PCR showed that *N6LINCR1* was expressed preferentially from one strand (Fig. 7C). In this case, enhancement of its expression by *NCED6* induction was also observed, confirming the *N6LINCR1* response to the

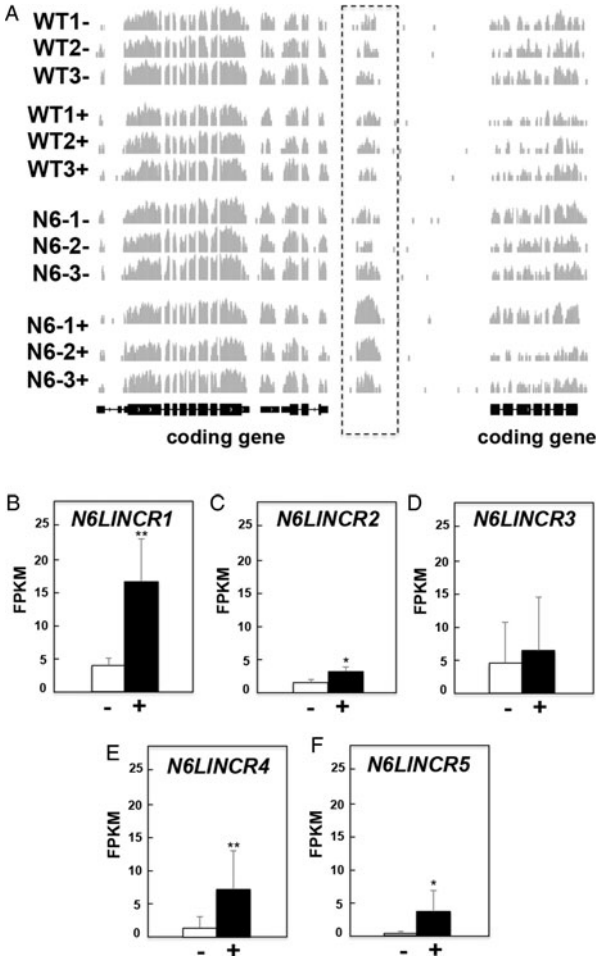

Figure 5. Expression of lncRNAs from intergenic regions (lincRNA) of the *Arabidopsis* genome upon *NCED6* induction. (A) Example of assembly of RNA-seq reads at the coding genes (black boxes) and an intergenic region (boxed by dashed line). RNA-seq reads from three independent lines of the wild-type (WT1, WT2, WT3) and *NCED6*-inducible (N6-1, N6-2, N6-3) seeds with (+) or without (–) ligand treatment are assembled. (B–F) Expression of lincRNAs (*N6LINCRs*) in the *NCED6*-inducible seeds with (+, filled bar) or without (–, open bar) ligand application. $^{**}P < 0.01$, $^{*}P < 0.05$ (Student's *t*-test compared with control). FPKM, fragments per kilobase of exon per million fragments mapped.

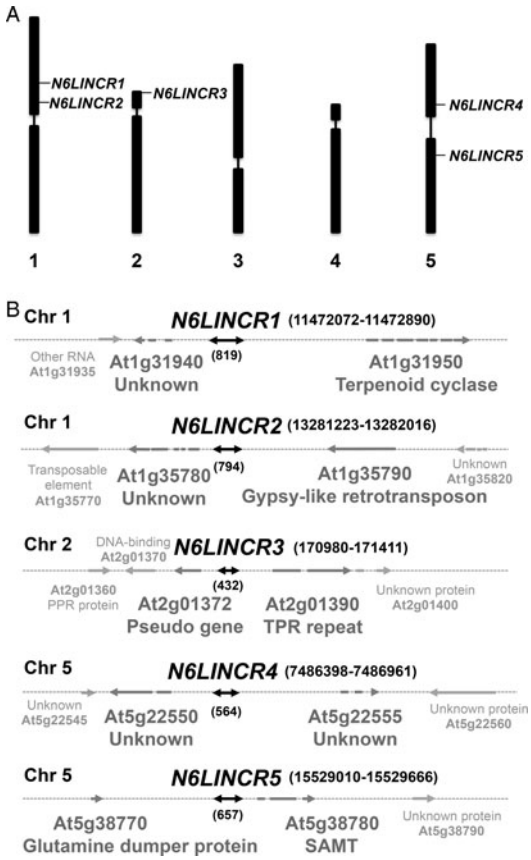

Figure 6. The *N6LINCR* loci in the *Arabidopsis* genome. (A) Schematic representation of the approximate positions of *N6LINCR1-5* in the *Arabidopsis* chromosomes. (B) Schematic representation of the genome positions of *N6LINCR1-5* relative to the neighbouring genes.

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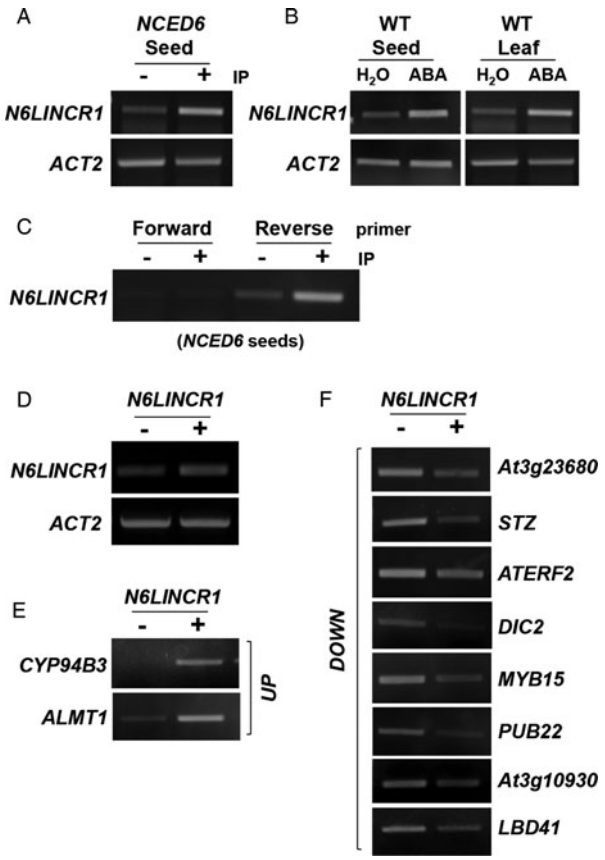

Figure 7. Expression of *N6LINCR1* and *N6LINCR1*-regulated genes. (A) Expression of *N6LINCR1* in the *NCED6*-inducible seeds with (+) or without (–) ligand application. (B) Expression of *N6LINCR1* in wild-type (WT) seeds (left) or leaf (right) in water (H_2O) or ABA. (C) Strand-specific RT-PCR using the forward (N6LINCR1-F0) or reverse (N6LINCR1-R0) primer (see Table S1). RNA was extracted from the *NCED6*-inducible seeds incubated with (+) or without (–) the ligand. (D) RT-PCR showing enhanced expression of *N6LINCR1* in the *N6LINCR1*-inducible seeds upon ligand application (+) compared with control (–). (E) and (F), RT-PCR showing up-regulation (E, *UP*) and down-regulation (F, *DOWN*) of the representative genes that were identified by RNA-seq analysis of the *N6LINCR1*-inducible seeds. *ACT2*, *ACTIN2*; *ALMT1*, *ALUMINUM-ACTIVATED MALATE TRANSPORTER 1*; *ATERF2*, *ETHYLENE RESPONSE FACTOR2*; *CYP94B3*, *CYTOCHROME P450, FAMILY 94*; *DIC2*, *DICARBOXYLATE CARRIER 2*; LBD41, *LOB DOMAIN-CONTAINING PROTEIN 41*; *MYB15*, *MYB DOMAIN PROTEIN 15*; *PUB22*, *PLANT U-BOX 22; STZ*, *SALT TOLERANCE ZINC FINGER*. Representative gel images are shown.

increase in endogenous ABA. The other strand was also expressed at a detectable level (discussed below).

Transcription from part or the vicinity of the *N6LINCR1* genomic region was also detected by previous EST (expressed sequence tag), tiling array or RNA-seq analysis (Matsui *et al.*, 2008; Nakashima *et al.*, 2009; Okamoto *et al.*, 2010; Richter *et al.*, 2010; Visscher *et al.*, 2010; Qin *et al.*, 2011; Jin *et al.*, 2013) (Fig. S1). Two non-coding RNAs (At1g06473 and At1g06483) are now annotated at this genomic region in the Salk T-DNA express *Arabidopsis* Gene Mapping Tools, although they are in opposite orientation to *N6LINCR1*. In any case, this genomic region, which does not code a protein, appears to be activated for transcription, and in the case of *N6LINCR1* it is hormone-dependent transcription.

While *N6LINCR1* was originally identified as an 819-bp lncRNA, the results of tiling RT-PCR using fine-mapped primers suggested that the broader genomic region of *N6LINCR1* (1185 bp) can be transcribed (Fig. S2). This possible longest *N6LINCR1* region (1185 bp) was used for further analyses. LincRNAs could regulate gene expression through sequence specificity to their targets (Turner *et al.*, 2014). The 1185-bp *N6LINCR1* sequence contained the oligonucleotides that completely or nearly completely matched part of the *Arabidopsis* genome (Fig. S3A and B). The duplicated sequence AAGAAATATATTAGTAATT of unknown function was found in *N6LINCR1*, which matched the vicinity of potential microRNA (miRNA) target genes in the *Arabidopsis* genome (Fig. S3C). The potential triplex forming oligonucleotides (TFO) (Buske *et al.*, 2012) sequence TTTCCTCTTTCTC TTATCTCTC, through which a lncRNA could form a triplex with double-stranded DNA, was also found in *N6LINCR1* (Fig. S3A). LncRNA could repress genes through TFO formation (Buske *et al.*, 2012) or de-repress genes by serving as a miRNA sponge (Hansen *et al.*, 2013; Turner *et al.*, 2014) or target mimic (Liu *et al.*, 2015). However, any of the genes in the vicinity of the *N6LINCR1*-matched regions in the *Arabidopsis* genome, including the potential miRNA targets and TFO targets, did not show differential expression in the original RNA-seq data. The possibility that *N6LINCR1* acts as a transregulator of genes located on other chromosomes (Galupa and Heard, 2015), through the TFO, miRNA or other mechanisms, should not be excluded yet because our examination was not comprehensive. However, it turned out to be difficult to speculate on possible targets of *N6LINCR1* only through sequence specificity.

The importance of the secondary structure of lncRNAs, rather than their sequence homology to the target genes, has been suggested (Glazko *et al.*, 2012; Turner *et al.*, 2014). It is possible that *N6LINCR1* regulates gene expression through its secondary structure (Fig. S4), which hinders identification of its targets only by computational sequence analysis. Therefore, we decided to create *N6LINCR1*-inducible lines to see whether its induction could affect gene expression profiles. We used the GS system to generate *N6LINCR1*-inducible lines. Resulting transgenic plants increased *N6LINCR1* expression upon ligand application (Fig. 7D). Unlike *NCED6* induction, apparent

https://doi.org/10.1017/S0960258517000216 Published online by Cambridge University Press

phenotypic changes were not observed in ruptured seeds by *N6LINCR1* induction. However, when we ran RNA-seq for ligand-treated and -untreated seeds of the three independent *N6LINCR1* lines, we observed obvious changes in gene expression profiles by *N6LINCR1* induction (Table S3), some of which have been confirmed by RT-PCR (Fig. 7E and F). These results suggest that *N6LINCR1* might function as a regulatory RNA, which potentially mediates ABA responses in seeds through transcriptional control. *N6LINCR1* induction caused more down-regulation (66 *N6LINCR1-DOWN*) than up-regulation (26 *N6LINCR1-UP*) (Table S3).

Regulatory roles of lncRNAs in gene expression, particularly their suppressive roles, have been well documented. LncRNAs could interact with Polycomb Repressive Complex 2 (PRC2) through a secondary structure (Zhao *et al*., 2010), which triggers histone H3 lysine 27 (H3K27) trimethylation to cause gene silencing (Simon and Kingston, 2009). Possible regulation of the dormancy gene *DOG1* through the PRC2 pathway has been suggested (Bouyer *et al*., 2011). Antisense *DOG1* lncRNA (*asDOG1*), which suppresses *DOG1* expression and negatively affects seed dormancy, has also been found (Fedak *et al*., 2016). Seed dormancy mechanisms appear to be regulated by gene repression through chromatin remodelling, part of which could be mediated by lncRNA (Liu *et al*., 2007; Wang *et al*., 2013; Nonogaki, 2014). Therefore, it is possible that *N6LINCR1* plays a suppressive role in gene expression through epigenetic mechanisms.

While the major role of *N6LINCR1* could be suppression of gene expression, 26 genes were detected as *N6LINCR1-UP* (Table S3). LncRNAs could promote gene expression by binding to transcription factors through a secondary structure, which in turn binds to the promoter regions of target genes (Turner *et al*., 2014). We do not know which genes are directly or indirectly regulated by *N6LINCR1*. However, it is interesting that some genes are up-regulated by *N6LINCR1* induction.

In terms of individual genes, *EARLY LIGHT-INDUCED PROTEIN*2 (*ELIP2*), which has been characterized for seed germination mechanisms (Rizza *et al*., 2011), was found as a *N6LINCR1-DOWN* gene (Table S3). *ETHYLENE RESPONSE FACTOR105* (*ERF105*), another gene identified as *N6LINCR1-DOWN* (Table S3), is known to be repressed through chromatin remodelling in seeds (Wang *et al*., 2013), which can be regulated by a lncRNA. While these are interesting targets and possibilities, it probably does not make much sense to speculate on a possible role of *N6LINCR1* in germination mechanisms just by extracting individual genes out of the lists and discussing available information. Understanding the mechanisms underlying how *N6LINCR1* regulates those genes and seed germination will require a number of experiments in the future. Nonetheless, as demonstrated by this study, *N6LINCR1* response to endogenous and exogenous ABA is unequivocal and its induction obviously alters gene expression profiles in seeds. Therefore, it is possible that *N6LINCR1* mediates, at least in part, the suppressive effects of *NCED6* induction on germination, through regulation of gene expression.

Thus, the transcriptome analysis in this study identified multiple layers of positive feedback regulation for coding genes and possible involvement of lncRNA in seed transcriptome.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S0960258517000216.

Acknowledgements

We are grateful to Roger Beachy, Washington University in St Louis, for collaboration to create the *NCED6*-inducible system; Kelly Vining, Oregon State University, for suggestions for sequence analysis, and Mariko Nonogaki, Oregon State University, for critical reading and helpful suggestions for the manuscript.

Funding

This project was supported by American Seed Research Foundation in part.

Conflicts of interest

T.S., Y.K. and R.K. were trainees from Snow Brand Seed.

Supplementary Materials

Supplementary Figures S1–S4 and Table S1 (Word)

Figure S1. Transcripts detected in the previous and present studies that were originated from the *N6LINCR1* genomic region.

Figure S2. Identification of 1185-bp genomic region of *N6LINCR1* by tiling PCR.

Figure S3. Analysis of the 1,185-base *N6LINCR1* sequence.

Figure S4. The structure of *N6LINCR1* predicted by RNAfold.

Table S1. List of primers used in this study.

Supplementary Table S2 (Excel)

NCED6 UP and *DOWN* genes

Supplementary Table S3 (Excel)

N6LINCR1 UP and *DOWN* genes.

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