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TECHNICAL ADVANCE

Amplification of ABA biosynthesis and signaling through a positive feedback mechanism in seeds

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SUMMARY

Abscisic acid is an essential hormone for seed dormancy. Our previous study using the plant gene switch system, a chemically induced gene expression system, demonstrated that induction of *9-cis-epoxycarotenoid dioxygenase* (*NCED*), a rate-limiting ABA biosynthesis gene, was sufficient to suppress germination in imbibed Arabidopsis seeds. Here, we report development of an efficient experimental system that causes amplification of *NCED* expression during seed maturation. The system was created with a *Triticum aestivum* promoter containing ABA responsive elements (ABREs) and a *Sorghum bicolor NCED* to cause ABA-stimulated ABA biosynthesis and signaling, through a positive feedback mechanism. The chimeric gene *pABRE:NCED* enhanced *NCED* and *ABF* (ABRE-binding factor) expression in Arabidopsis Columbia-0 seeds, which caused 9- to 73-fold increases in ABA levels. The *pABRE:NCED* seeds exhibited unusually deep dormancy which lasted for more than 3 months. Interestingly, the amplified ABA pathways also caused enhanced expression of Arabidopsis *NCED5*, revealing the presence of positive feedback in the native system. These results demonstrated the robustness of positive feedback mechanisms and the significance of *NCED* expression, or single metabolic change, during seed maturation. The *pABRE:NCED* system provides an excellent experimental system producing dormant and non-dormant seeds of the same maternal origin, which differ only in zygotic ABA. The *pABRE:NCED* seeds contain a GFP marker which enables seed sorting between transgenic and null segregants and are ideal for comparative analysis. In addition to its utility in basic research, the system can also be applied to prevention of pre-harvest sprouting during crop production, and therefore contributes to translational biology.

Keywords: ABA, seed, dormancy, *Arabidopsis thaliana*, *Triticum aestivum*, *Sorghum bicolor*, technical advance.

INTRODUCTION

Seed dormancy is defined as a failure of germination under conditions that are otherwise favorable for the emergence of an embryo from a seed (Bewley *et al.*, 2013). The key genes that play a central role in induction of seed dormancy have been identified through genetic and molecular approaches (Bentsink *et al.*, 2006; Liu *et al.*, 2007; Graeber *et al.*, 2013). Some of these are not directly associated with hormonal regulation (Nakabayashi *et al.*, 2012), while others function directly through hormone balance in seeds (Kushiro *et al.*, 2004; Lefebvre *et al.*, 2006).

The major plant hormone involved in the induction and maintenance of seed dormancy is ABA (Seo *et al.*, 2009; Bewley *et al.*, 2013), which induces transcription factors and other regulatory proteins involved in suppression of germination (Piskurewicz *et al.*, 2008; Lee *et al.*, 2010). Abscisic acid is also associated with the seed maturation program including the induction of storage proteins and other proteins associated with desiccation tolerance (Galau *et al.*, 1987). The levels of ABA in seeds are determined by its biosynthesis, deactivation and transport (Seo and Koshiba, 2011). Biosynthesis of ABA in seeds is mainly regulated by the rate-limiting enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Lefebvre *et al.*, 2006; Martinez-Andujar *et al.*, 2011), while 8′-hydroxylase plays an important role in ABA deactivation (Kushiro *et al.*, 2004; Millar *et al.*, 2006; Barrero *et al.*, 2009).

It has been demonstrated that the induction of *NCED6,* using the plant gene switch system (PGSS), a chemically

induced gene expression system, was sufficient to suppress the germination of imbibed seeds of wild-type (WT) *Arabidopsis thaliana*, ecotype Columbia-0 (Col), after 3-days' pre-chilling (dormancy release) treatment (Martinez-Andujar *et al.*, 2011). The suppression of germination by *NCED6* induction was cancelled by fluridone, an inhibitor of carotenoid (hence ABA) biosynthesis, suggesting that the dormancy phenotypes in the PGSS *NCED6* seeds were dependent on *de novo* ABA biosynthesis. In fact, ABA levels in the *NCED6*-induced seeds were more than 20-fold higher than the ABA levels in uninduced seeds (Martinez-Andujar *et al.*, 2011). These results showed that *NCED* expression could serve as a sole determinant of seed dormancy under certain conditions.

While *NCED* induction in imbibed seeds suppresses germination, seed dormancy in natural systems is induced in developing seeds during maturation and results obtained from imbibed seeds do not necessarily confirm the role of *NCED* as a single determinant of seed dormancy induction. Conditional expression of *DOG1*, one of the best-characterized seed dormancy-specific genes, which is expressed during the seed maturation stage (Bentsink *et al.*, 2006), does not suppress seed germination when it is induced in imbibed seeds (Nakabayashi *et al.*, 2012). Thus, induction of dormancy-associated genes only in imbibed seeds may not represent their roles in the natural system. It is necessary to create an efficient experimental system that enables enhanced *NCED* expression in developing seeds for analysis of the biochemical and molecular mechanisms of seed development and dormancy regulated by ABA.

Chemical induction (Piskurewicz *et al.*, 2008; Martinez-Andujar *et al.*, 2011) of *NCED* in developing seeds can be tested by painting siliques with a ligand. However, genes might not be induced efficiently in seeds due to impermeability of the testa to a ligand. In addition, application of a ligand to siliques affects the maternal tissues, such as the replum, valves and funicular tissues, which could alter seed development and dormancy. Drenching the soil or media surrounding the roots of the maternal plants could deliver a chemical ligand to developing seeds via the vascular system. However, in this case also, the maternal plants can be affected by ectopic gene induction. The major objective of this study was to develop an efficient experimental system to enhance *NCED* expression in developing seeds in a specific manner that does not depend on chemical induction but still allows us to experimentally examine whether *NCED* expression alone or a single metabolic change during seed maturation can serve as a determinant of seed dormancy in mature seeds.

To this end, we expressed *NCED* with an ABA-regulated promoter which is activated during the seed maturation stage. This system is expected to cause positive feedback through ABA biosynthesis and signaling (details in Results). Although we planned all experiments in Arabidopsis, we were interested in creating the experimental system using genes from cereal crops, because if the dormancy controlling system is established successfully it would not only provide an efficient experimental system for basic research but also establish a basic technology for preventing pre-harvest sprouting (PHS), a serious problem in crop production, which could contribute to food security in our society. A *NCED* was isolated from *Sorghum bicolor* and used with an ABA-regulated promoter from wheat (*Triticum aestivum*), which is activated in developing seeds during the maturation stage. The role of *NCED* expression during seed maturation as a determinant of seed dormancy, its relation to ABA biosynthesis in mature seeds during imbibition and positive feedback mechanisms in the natural seed dormancy system will be discussed in this paper. The utility of the experimental system for seed dormancy research and its potential for PHS prevention in crop species will also be discussed.

RESULTS

Isolation of sorghum *NCED*

Sorghum *NCED* sequences were searched through the Sorghum GDB database (http://www.plantgdb.org/SbGDB/) using the cDNA sequence of *A. thaliana NCED6* (*AtNCED6*, NM_113327.2), which identified *Sb01g013520.1* (and *Sb02g003230.1* later). The *Sb01g013520.1* gene was used for further analyses and experiments. The deduced amino acid sequence of *Sb01g013520.1* contained the domains conserved throughout the known NCEDs in both monocots and dicots (Figure S1 in Supporting Information). *Sb01g013520.1* was termed *SbNCED*. Since the *SbNCED* gene does not contain introns, the coding sequence was isolated from sorghum genomic DNA and used for functional analyses.

The SbNCED sequence was also aligned with the sequences of AtNCED5, AtNCED6 and AtNCED9 that are expressed in Arabidopsis seeds (Figure S2). For AtNCED9, the protein sequence starting with the second methionine (position 51, MASTT…) was used for alignment because it has been suggested that the first 150 bp of the annotated coding sequence (NM_106486.2) might include the 3′ region of the *NCED9* promoter sequence, which adds 50 extra amino acids (position 1, MTIIT…) to the N-terminus (Frey *et al.*, 2012). The AtNCED3 sequence was also included in the alignment because *SbNCED* was annotated as a putative ortholog of *AtNCED3* in the Sorghum GDB database. SbNCED exhibited high similarities to both AtNCED3 and the seed-expressed NCEDs (Figure S2). *SbNCED* was used to create amplification of the ABA biosynthesis and signaling pathways (see next section).

A positive feedback mechanism through ABA biosynthesis and signaling

In Arabidopsis, *NCED* induction increases ABA biosynthesis in seeds and suppresses germination (Martinez-Andujar

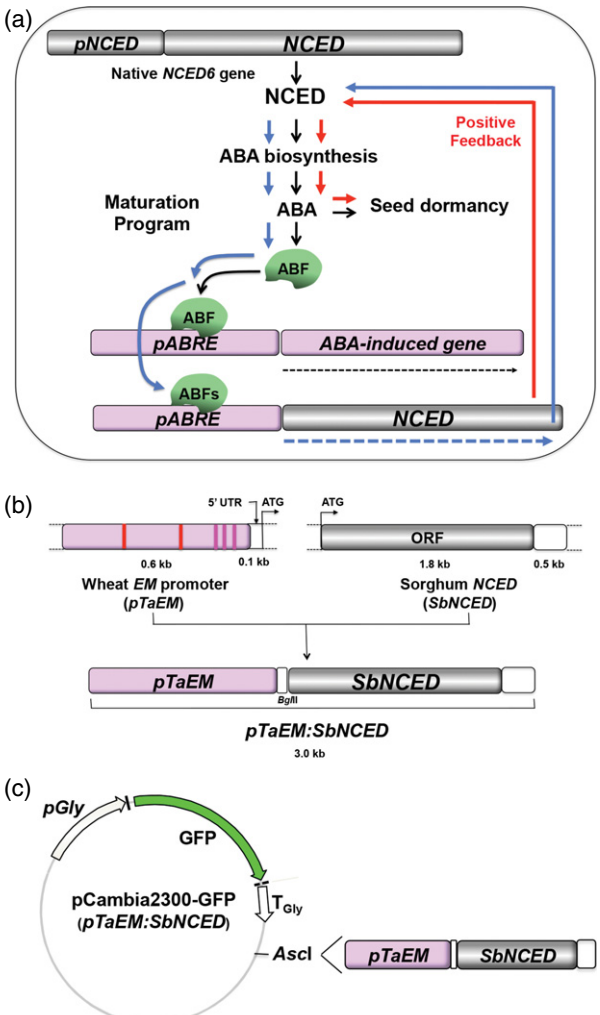

Figure 1. Hypothesis and strategies for a positive feedback mechanism to amplify ABA biosynthesis and signaling.
(a) Schematic representation of amplification of the ABA biosynthesis and signaling pathways. Black arrows indicate the native pathway. Expected positive feedback (red arrows) and amplified feedback (blue arrows) are indicated (see text for details). ABF, ABRE-binding factor; ABRE, ABA responsive element; NCED, 9-*cis*-epoxycarotenoid dioxygenase.
(b) Schematic representation of *pABRE:NCED* used in this study. *Sorghum bicolor NCED* (*SbNCED*) was driven by the wheat (*Triticum aestivum*) *Early Methionine-labeled* promoter (*pTaEM*). Pink and red bars, ABRE and RY motifs.
(c) Schematic representation of the pCambia 2300-GFP vector. The GFP construct in this vector contains the *glycinin* promoter (*pGly*) and terminator (T_{Gly}).

et al., 2011), most likely through induction of ABA responsive element (ABRE)-binding factors (ABFs) (Figure 1a). The ABFs upregulate genes containing ABRE or the ABRE-like motifs in their promoters (*pABRE*) (Yamaguchi-Shinozaki and Shinozaki, 2005). The targets of ABFs include genes encoding storage proteins, such as 2S albumin (Devic *et al.*, 1996) and LATE EMBRYOGENESIS ABUNDANT (LEAs), which are associated with the desiccation tolerance of seeds (Devic *et al.*, 1996; Carles *et al.*, 2002). We were interested in placing *NCED* under the control of *pABRE*. The chimeric gene *pABRE:NCED* should create positive feedback regulation through NCED production, which was expected to enhance ABA biosynthesis and seed dormancy at least to some extent (Figure 1a, red arrows). At the same time, the other side of the cascades producing ABFs (Figure 1a, blue vertical arrows) should also be enhanced by this system, resulting in the activation of the target genes through their binding to ABRE (Figure 1a). The enhanced ABA signaling pathway should eventually come back through expression of *NCED* that is placed under *pABRE*. This system, triggered by the native NCED and ABA, was expected to cause amplification of the magnitude of the ABA biosynthesis and signaling pathways (Figure 1a).

To drive the expression of *SbNCED* using the positive feedback mechanism, the *SbNCED* sequence was fused to the 5′ upstream sequence of wheat (*T. aestivum*) *Early Methionine-labeled* (*EM*) that contains an ABRE (Guiltinan *et al.*, 1990) (Figure S3). The cereal chimeric gene was termed *pTaEM:SbNCED* (Figure 1b). We reasoned that, if the regulatory mechanisms of ABA-induced seed dormancy are conserved between monocot and dicot species, the monocot *pABRE:NCED* chimeric gene *pTaEM:SbNCED* should be functional in Arabidopsis and cause amplification of the ABA biosynthesis and signaling pathways, leading to enhanced seed dormancy in this heterologous system. A potential problem associated with this approach was that suppression of germination, an expected phenotype when the hypothesis was correct, would hinder identification of the *pTaEM:SbNCED* plants, because screening of transgenic plants in antibiotic media requires germination. To address this issue, we used a green fluorescent protein (GFP), which was driven by the seed-specific *glycinin* promoter (Schmidt and Herman, 2008), as a visible transgenic marker (*pGly:GFP*, Figure 1c) (see Experimental Procedures for details).

Recovery of the *pTaEM:SbNCED* seeds using a GFP marker

Arabidopsis WT Col plants were transformed at the flower-bud stages with *Agrobacterium tumefaciens* containing the pCambia 2300-GFP vector with or without *pTaEM:SbNCED*. When siliques reached the maturation stage, they were examined for GFP signals under a dissection microscope equipped with a UV lamp. Spotted fluorescent signals, indicating GFP signals from seeds, were visible through silique valves (Figure 2b,c). Elimination of the valves confirmed that the signals were indeed derived from individual seeds (Figure 2d,e), although GFP signals in different seed tissues (e.g. testa, endosperm, embryo) were not inspected. The GFP signals were still intense in harvested dry seeds, which were used for propagation.

Initial investigation suggested that putative transgenic seeds with strong GFP signals (Figure S4a, 'S') did not

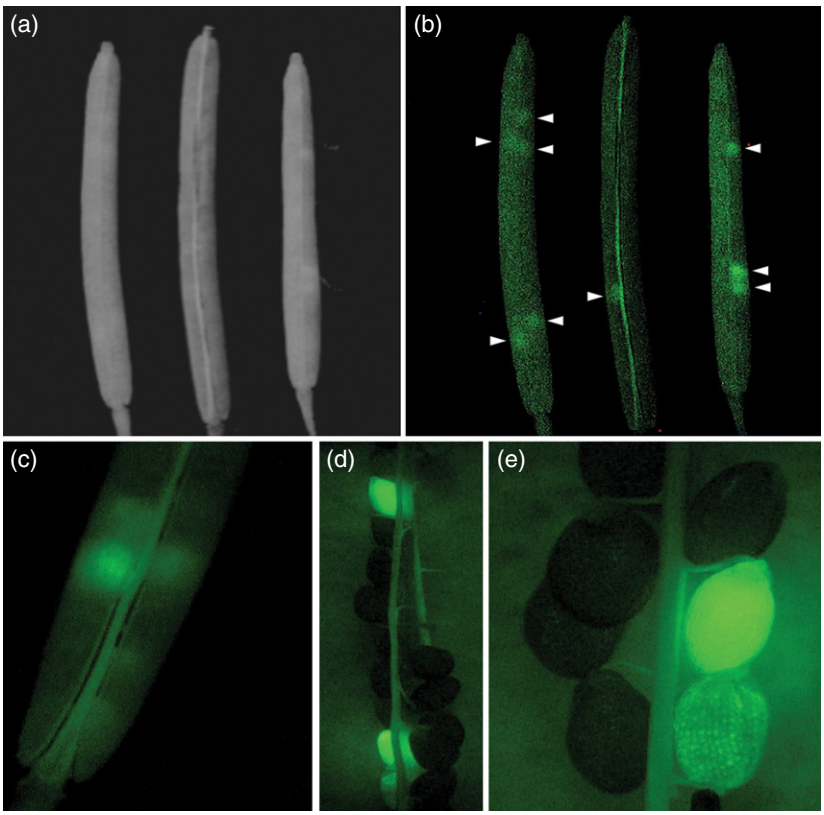

Figure 2. The GFP signals from putative T_1 transgenic seeds of *pTaEM:SbNCED* lines.
(a), (b) T_0 siliques under light and UV, respectively. The positions of the GFP signals are highlighted by white arrowheads.
(c) The GFP signals from seeds inside a T_0 silique.
(d, e) The GFP signals from T_1 seeds retained on the replum (the valves were removed).

germinate after 3-days' pre-chilling, which usually enhances seed germination in Col, while seeds with weak GFP signals (Figure S4a, 'W') germinated. T_1 plants were grown from germinated GFP seeds regardless of signal intensity [termed SW (strong + weak)]. The embryos were dissected out from ungerminated 'S' seeds (Figure S4b). From these, a total of 24 T_1 plants with stable transformation were obtained.

Hyperdormancy phenotypes in *pTaEM:SbNCED* seeds

The GFP-positive T_1 *pTaEM:SbNCED* plants produced siliques containing T_2 GFP seeds and null-segregant WT seeds (Figure 3a). Eight Independent lines exhibiting a 3:1 ratio for GFP:WT were obtained. Seeds of *pTaEM:SbNCED* and null-segregant WT were sorted for each line based on GFP fluorescence (Figure 3b–e) and subjected to initial examination for germination immediately after harvest. In all independent lines, *pTaEM:SbNCED* seeds remained highly dormant even with 3 days' pre-chilling, while the majority of null-segregant WT seeds germinated (Figure 3f). These seeds were stored for further analysis.

Col seeds do not have very deep dormancy compared with highly dormant ecotypes, such as Cape Verde Islands (Cvi), and start to germinate without pre-chilling a few weeks after harvest. However, *pTaEM:SbNCED* seeds still exhibited dormancy after 3-months' storage, while null-segregant WT seeds, which had experienced exactly the same environmental conditions on the maternal plant and storage conditions over the 3-month period as did *pTaEM:SbNCED* seeds, germinated well without pre-chilling (Figure 4a). The hyperdormancy phenotypes were observed consistently in the eight independent transgenic lines (Figure 4b). Three days of pre-chilling enhanced germination of the 3-month-old *pTaEM:SbNCED* seeds but did not result in full alleviation of dormancy (Figure 4c, green). Application of 10 μM fluridone, an inhibitor of carotenoid (and hence ABA) biosynthesis, allowed full recovery of germination in some *pTaEM:SbNCED* lines (Figure 4c, light green), suggesting that the hyperdormancy phenotypes were due to enhanced ABA biosynthesis, although there were still dormant seeds in other *pTaEM:SbNCED* lines. Dormancy levels varied among the different transgenic lines, as is often also observed in different batches of WT seeds. All germination data presented here were obtained from heterozygous *pTaEM:SbNCED* lines alone, because comparison between transgenic and null-segregant seeds, which was devoid of the maternal effects, was ideal for this study. It should be noted that homozygous lines also exhibited variation with a tendency for more enhanced dormancy, which requires further analysis.

Possible effects of *GFP* expression on developing seeds

The *pTaEM:SbNCED* construct was introduced to the Arabidopsis genome using the pCambia 2300-GFP vector,

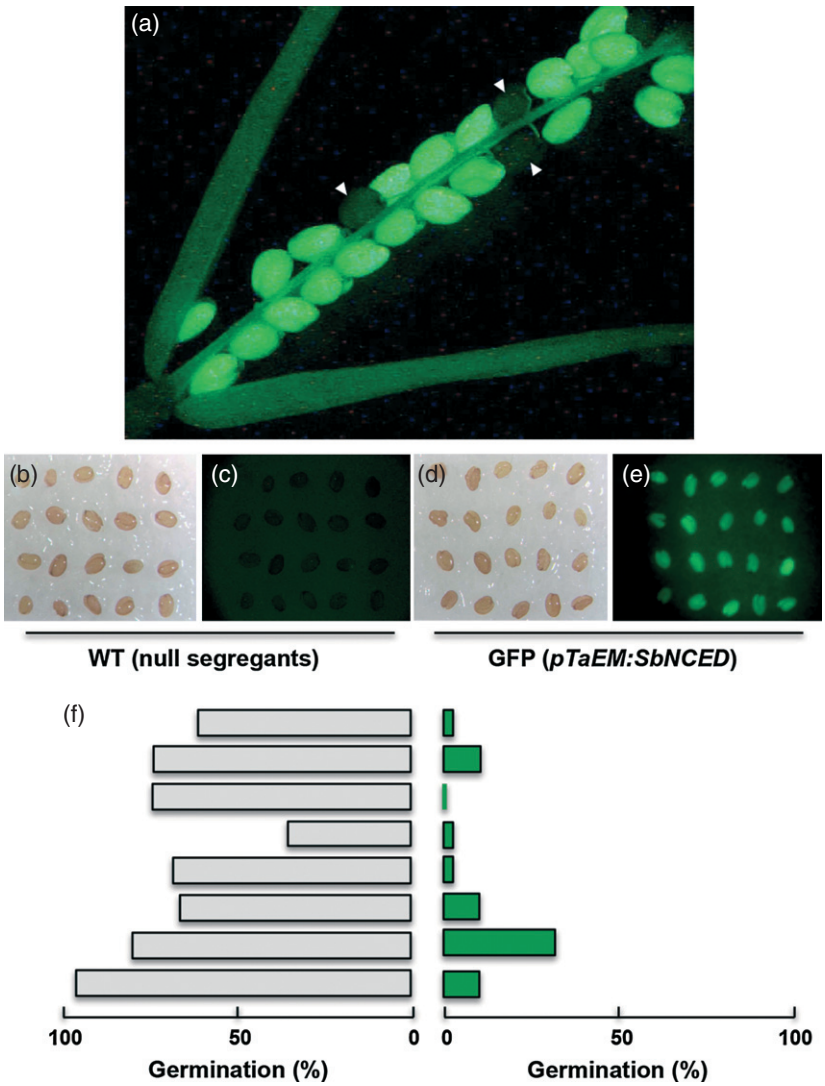

Figure 3. Germination of *pTaEM:SbNCED* T_2 and null-segregant wild-type (WT) seeds at harvest.
(a) T_1 silique showing segregation of the T_2 *pTaEM:SbNCED* (GFP) and null-segregant WT (white arrowheads) seeds.
(b, c) Light and UV images of sorted WT (null-segregant) seeds, respectively.
(d, e) Light and UV images of sorted GFP (*pTaEM:SbNCED*) seeds, respectively.
(f) Initial examination of germination of sorted wild-type (WT, gray bar) and *pTaEM:SbNCED* (GFP, green bar) seeds in eight independent lines after 3-days' pre-chilling at 4°C and 5-days' incubation at 22°C. Initial examination was done without replicates.

which contained a *GFP* driven with the *glycinin* promoter, a seed-specific promoter of a storage protein (Figure 1c). Therefore, it is possible that the hyperdormancy phenotypes observed in *pTaEM:SbNCED* seeds were a consequence of GFP accumulation in seeds, which could reduce the native storage proteins and affect dormancy levels. In fact, we observed reduction in some peptides in GFP seeds, compared with null-segregant WT seeds, which could be storage proteins similar to glycinin (Figure 5a). To see whether GFP expression itself affects seed dormancy levels, we also analyzed GFP and null-segregant seeds from Arabidopsis lines transformed with an empty pCambia 2300-GFP vector. The GFP seeds from these lines normally germinated after 3 days' pre-chilling (Figure 5b, c), which was consistently observed in multiple independent lines (Figure 5d). These results suggested that GFP expression in seeds exerted little effect on seed dormancy and that the hyperdormancy phenotypes observed in *pTaEM:SbNCED* seeds were caused specifically by *pTaEM:SbNCED* construct or *SbNCED* expression.

SbNCED expression, *ABF* enhancement and ABA overproduction in *pTaEM:SbNCED* seeds

To see whether *SbNCED* was actually expressed in *pTaEM:SbNCED* lines, reverse transcription (RT)-PCR was performed using *SbNCED* primers. The *SbNCED* primers did not amplify products from *AtNCED5*, *AtNCED6* and *AtNCED9* in the Arabidopsis genome (Figure 6a) or from pure templates (Figure 6b), confirming their specificity. The *SbNCED*-specific primers were then used to examine its expression during seed development. While the *glycinin* promoter driving GFP was activated around the middle stages of seed development (Figure 6d), *pTaEM* was expected to be active at the late stage. Therefore, RNA was extracted from maturing siliques that developed a yellow color (Figure 6d, stages 24–27) and used for expression

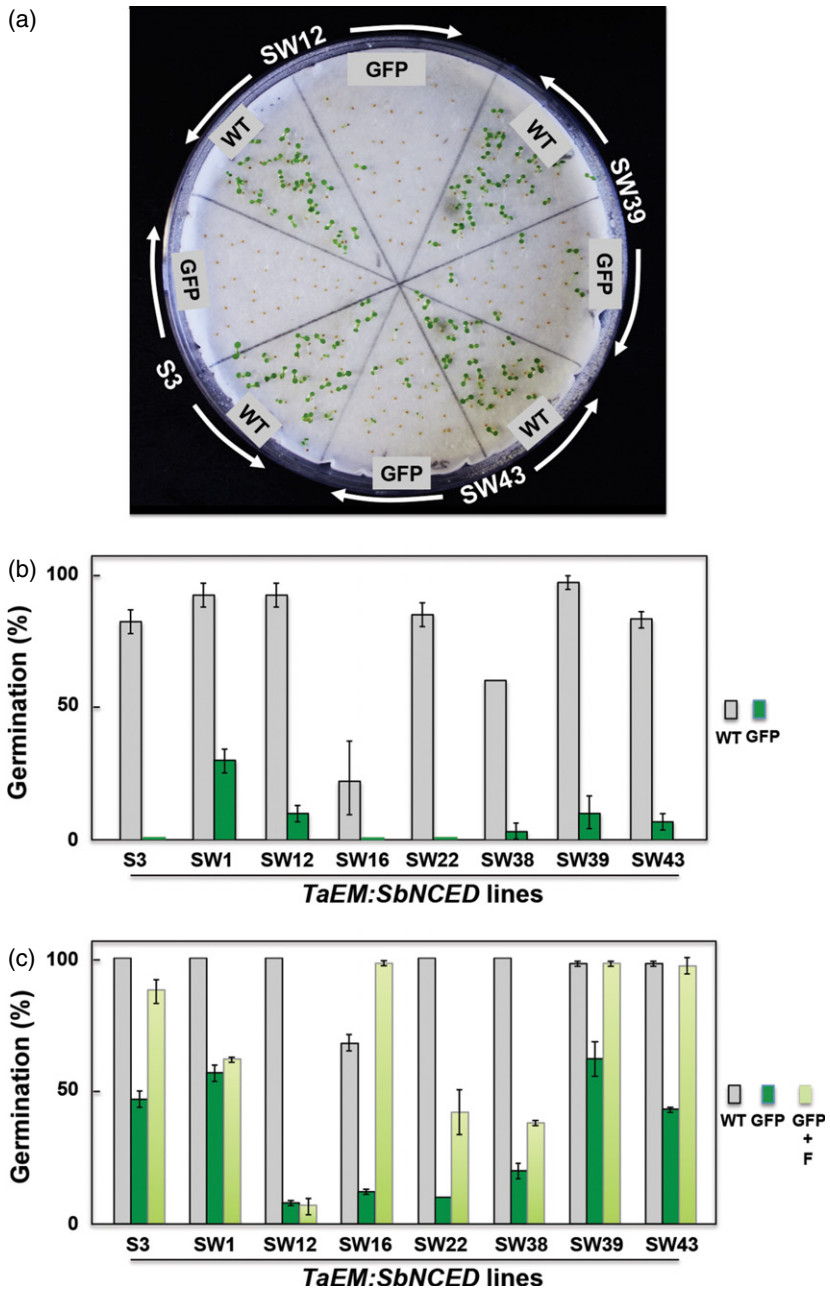

Figure 4. Germination of *pTaEM:SbNCED* T_2 and null-segregant wild-type (WT) seeds after 3-months' storage.
(a) Germination of *pTaEM:SbNCED* (GFP) and WT seeds segregating from the same maternal plant (S3, SW12, SW39 or SW43) after 3-months' storage without pre-chilling.
(b) Germination of WT (gray bar) and *pTaEM:SbNCED* (GFP, green bar) seeds segregating from the same maternal plant (S3, SW1, SW 12, SW16, SW22, SW38, SW39 or SW43) after 3-months' storage, without pre-chilling.
(c) Germination of WT (gray bar) and *pTaEM:SbNCED* (GFP, green bar) seeds segregating from the same maternal plant (S3, SW1, SW 12, SW16, SW22, SW38, SW39 or SW43) after 3-months' storage, with 3-days' pre-chilling. Germination of *pTaEM:SbNCED* seeds with 10 μM fluridone (GFP+F, light green bar) is also shown. Values in (b) and (c) are averages of two data points for eight independent transgenic lines.

analysis. An expected size (1.25 kb) of product was detected in RT-PCR (Figure 6c). Since the cDNA sequences of *SbNCED* and *AtNCEDs* are very similar, the RT-PCR product was sequenced and then analyzed focusing on the 5′ region, which varies among *NCEDs* (Figure 6e). Comparison of the sequence of the RT-PCR product with the authentic sequences indicated that RT-PCR product was indeed the product of *SbNCED* mRNA (Figure 6f).

SbNCED accumulated in dry *pTaEM:SbNCED* seeds, which was not observed in the vector control (VC) lines (Figure 7a). Accumulation of *SbNCED* mRNA in dry seeds confirmed its expression during the maturation stage. Interestingly, *NCED5* expression was enhanced in *pTaEM:SbNCED* seeds in all of the three independent lines examined, compared with its expression in the VC seeds (Figure 7a). These results suggest that amplified ABA biosynthesis, which was caused by *SbNCED* expression, positively affected expression of the native *NCED5*. These results suggest that positive feedback regulation is present in the native system (see Discussion). In contrast, *NCED6* and *NCED9* expression was not enhanced but rather decreased by the enhanced ABA biosynthesis (Figure 7a).

The RT-PCR results indicated that *SbNCED* was actually expressed in Arabidopsis seeds during the maturation

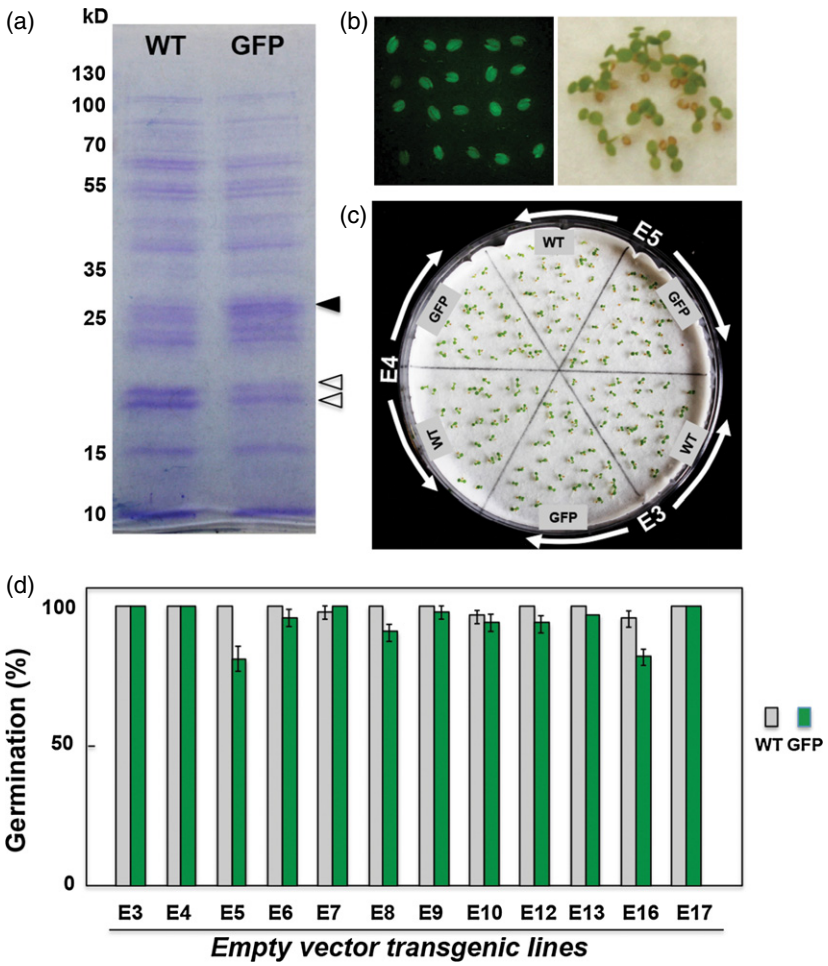

Figure 5. Germination of GFP and null-segregant wild-type (WT) seeds from transgenic control carrying an empty pCambia 2300-GFP vector.
(a) Coomassie staining of proteins extracted from null-segregant WT and *pTaEM:SbNCED* (GFP) seeds. Closed and open arrowheads indicate approximate positions of GFP peptide and putative storage proteins, respectively. Molecular masses are indicated to the left.
(b) Photograph of GFP seeds from an empty vector control (left) and their germination (right).
(c) Comparison of GFP and null-segregant WT seeds from transgenic control lines with an empty vector (E3, E4, E5).
(d) Germination of WT (gray bar) and GFP (green bar) seeds segregating from the same individual plant (E3–E10, E12, E13, E16, E17) immediately after harvest, with 3-days' prechilling. Values are averages of two data points for 12 independent transgenic lines.

stage, which suggested that the wheat *EM* promoter was recognized by the Arabidopsis system, including ABFs, and functioned during seed maturation (Figure 1). *SbNCED* expression was also detected in imbibed *pTaEM:SbNCED* seeds. *pTaEM:SbNCED* seeds imbibed for 40 h expressed *SbNCED* while WT Col seeds did not (Figure 7b). *ABA INSENSITIVE5* (*ABI5*), an *ABF,* was also expressed in *pTaEM:SbNCED* seeds but was not observed in WT Col seeds (Figure 7b). These results suggested that hyperdormancy in *pTaEM:SbNCED* seeds was caused by enhanced ABA biosynthesis and signaling during the maturation stage, which was carried over from the developmental stage through the imbibitional stage of mature seeds, as is natural dormancy.

The levels of ABA in dry *pTaEM:SbNCED* seeds were increased compared with those in null-segregant WT seeds (Figure 8a), suggesting that *SbNCED* expression in developing seeds indeed enhanced ABA biosynthesis during the maturation stage. The dramatic increases in ABA levels in *pTaEM:SbNCED* seeds were consistent with the hyperdormancy phenotypes in these lines (Figure 8b). The ABA levels in *pTaEM:SbNCED* seeds at 40-h imbibition (immediately before radicle emergence in WT) was 9- to 73-fold higher than those in WT seeds (Figure 8c). These results suggested that the *pABRE:NCED* system caused amplification of ABA biosynthesis and signaling and imposed deep dormancy in Arabidopsis seeds over generations.

DISCUSSION

Are there positive feedback mechanisms in the native dormancy pathways as well?

High levels of *NCED* expression are maintained in imbibed seeds during primary dormancy (Cadman *et al.*, 2006). *NCED* is also upregulated when thermoinhibition occurs in imbibed non-dormant seeds at high temperatures (Argyris *et al.*, 2008; Toh *et al.*, 2008). Mature seeds of Cvi, a highly dormant Arabidopsis ecotype (Alonso-Blanco *et al.*, 2003), from which the *DOG1* Cvi allele was identified (Bentsink *et al.*, 2006), also seem to require *NCED* expression or ABA biosynthesis for maintenance of seed dormancy in imbibed seeds (Ali-Rachedi *et al.*, 2004; Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007). There are factors other

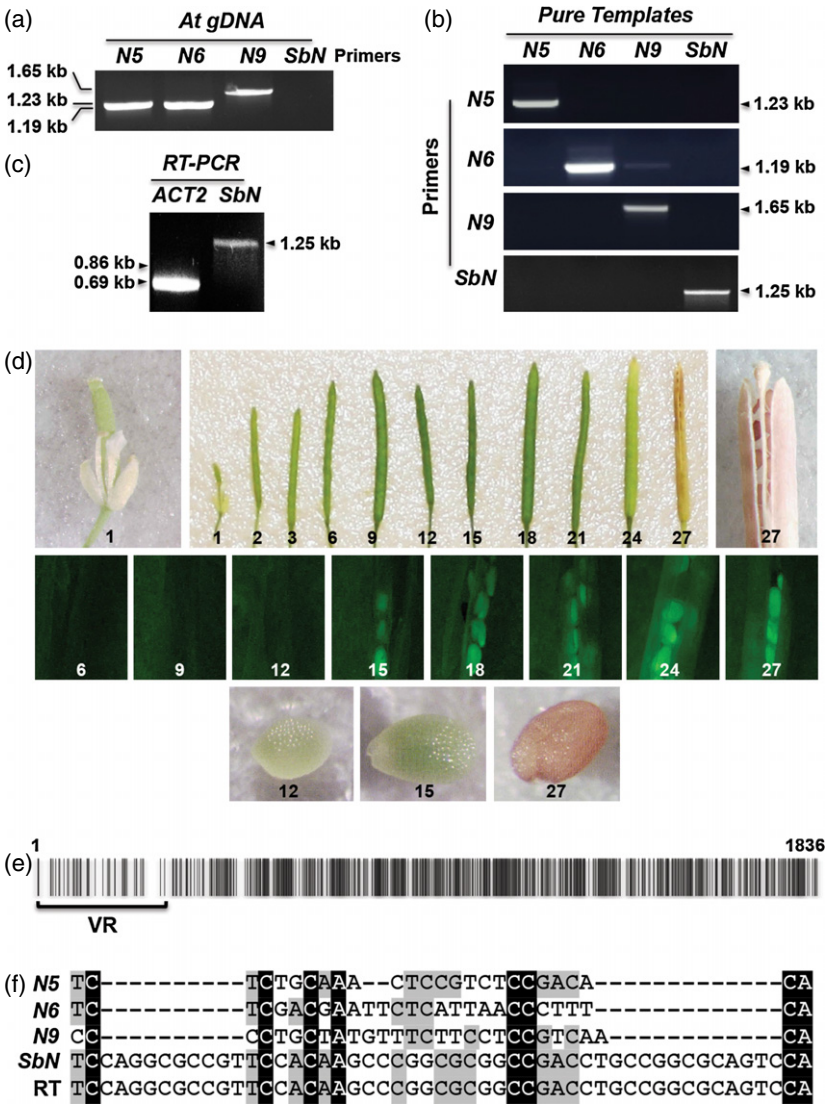

Figure 6. Expression of *SbNCED* in *pTaEM:SbNCED* seeds during maturation.
(a, b) Specificity of *AtNCED5* (*N5*), *AtNCED6* (*N6*), *AtNCED9* (*N9*) and *SbNCED* (*SbN*) primers used for RT-PCR. Each set of primers was tested with Arabidopsis genomic DNA (a) or pure templates (b) (PCR products shown in panel a for *N5, N6* and *N9* or cDNA in a vector for *SbNCED*).
(c) Reverse transcriptase-PCR of *SbNCED* and *ACTIN2* (*ACT2*) using RNA extracted from yellow siliques containing maturing *pTaEM:SbNCED* (line SW43) seeds. The sizes of *ACT2* (0.69 kb) and *SbNCED* (1.25 kb) cDNAs are indicated, together with the predicted size of the *ACT2* gDNA fragment including an intron (0.86 kb), which was not amplified, verifying no contamination by genomic DNA in RT products.
(d) Top: developing siliques from a single inflorescence, including the youngest stage before petal abscission (stage 1) through to the oldest stage with mature seeds in a dehiscent silique (stage 27). Middle: GFP signals from developing *pTaEM:SbNCED* seeds. Bottom: appearance of immature seeds before (stage 12) and after (stage 15) GFP accumulation and a mature seed (stage 27).
(e) Schematic representation of the conserved sequences among *AtNCED5*, *AtNCED6*, *AtNCED9* and *SbNCED*. VR, variable region.
(f) Part of the VR sequences of *AtNCED5* (*N5*), *AtNCED6* (*N6*), *AtNCED9* (*N9*) and *SbNCED* (*SbN*), aligned with the sequence of the RT-PCR product (RT).

than *NCED* expression that could affect ABA levels in seeds, such as ABA hydroxylases (Kushiro *et al.*, 2004; Millar *et al.*, 2006; Barrero *et al.*, 2009) and conjugating enzymes (Chiwocha *et al.*, 2003; Feurtado *et al.*, 2004). However, continuous expression of *NCED* in imbibed seeds appears to be an indispensable mechanism for maintenance of the dormant status of imbibed seeds.

Previous study using PGSS demonstrated that induction of *NCED6* was sufficient to suppress germination during seed imbibition, suggesting that *NCED* can be a single determinant of seed dormancy (Martinez-Andujar *et al.*, 2011). However, in nature, dormancy is induced spontaneously in developing seeds on the maternal plant (Bewley *et al.*, 2013) and it was critical to experimentally examine alteration of *NCED* expression during seed maturation and its effects on dormancy imposition in mature seeds. The present study using the wheat *LEA* promoter, which is under the control of ABA and activated during seed maturation, provided direct evidence that *NCED* expression during seed maturation indeed functions as a determinant of dormancy in mature seeds (Figure 4).

In the experimental system developed in this study (Figure 1), *pABRE* enabled *NCED* expression during seed maturation as designed (Figure 6). However, *NCED* was also expressed during imbibition of *pABRE:NCED* seeds (Figure 7b). This is very similar to what happens in naturally dormant seeds. In WT Arabidopsis seeds, dormancy is induced during seed development, concomitant with an increase in ABA levels, which is most likely through *NCEDs* expressed in seeds (Lefebvre *et al.*, 2006; Frey *et al.*, 2012), and dormancy is maintained in mature seeds, which continue to express *NCED* during imbibition (Cadman *et al.*, 2006).

NCED expression during both seed maturation and imbibition appears to be essential for seed dormancy. As fluridone releases seed dormancy in *pABRE:NCED*, at least in

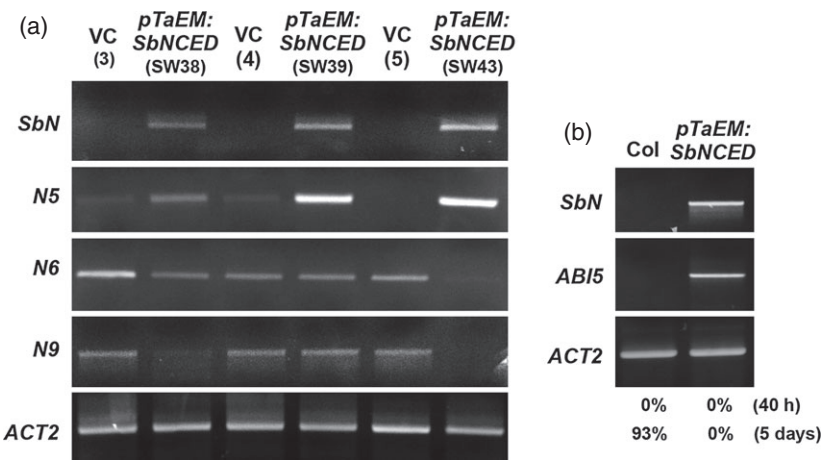

Figure 7. Accumulation of *SbNCED* in dry and imbibed *pTaEM:SbNCED* seeds.
(a) Reverse transcriptase-PCR of *SbNCED* (*SbN*), *AtNCED5* (*N5*), *AtNCED6* (*N6*), *AtNCED9* (*N9*) and *ACTIN2* (*ACT2*) using RNA extracted from dry seeds of *pTaEM:SbNCED* (SW38, SW39 and SW43) lines and empty vector control (VC: 3, 4, 5) lines.
(b) Reverse transcriptase-PCR of *SbNCED* (*SbN*), *ABA INSENSITIVE5* (*ABI5*), and *ACTIN2* (*ACT2*) using RNA extracted from wild-type Columbia (Col) and *pTaEM:SbNCED* (homozygous SW39 line) seeds imbibed for 40 h without pre-chilling. The germination percentage of each line after 40 h or 5 days is indicated below the panel.

part (Figure 4), and in WT seeds (Yoshioka *et al.*, 1998; Grappin *et al.*, 2000), the maintenance of dormancy in imbibed seeds depends on *de novo* ABA biosynthesis. It is possible that increase in *NCED* during seed maturation affects *NCED* levels and ABA biosynthesis in mature WT seeds during imbibition. This could be mediated by ABA itself or ABA-induced factors (including epigenetic signatures), which accumulate during the maturation stage and stimulate *NCED* expression in imbibed seeds.

If ABA biosynthesis during seed maturation affects its synthesis in mature seeds during imbibition (which in turn determines the final output of dormancy or germination), what are the mechanisms that cause this feedforward ABA biosynthesis over the two discontinuous developmental stages of seed maturation and imbibition? In the case of *pABRE:NCED* seeds, *pABRE* is stimulated by ABA and ABF (Figure 1). Therefore, enhanced accumulation of ABA itself in *pABRE:NCED* seeds could trigger the positive feedback mechanism as soon as the seed water content reaches a sufficient level for transcriptional and translational activities and initiate ABA biosynthesis. An important biological question is whether there is also a similar positive feedback mechanism in the natural system of seed dormancy, which could be triggered by ABA or other factors accumulated during seed maturation.

The effects of environmental factors, such as light (Seo *et al.*, 2006; Oh *et al.*, 2007; Gubler *et al.*, 2008) and temperature (Argyris *et al.*, 2008) or other hormones (Sawada *et al.*, 2008; Jacobsen *et al.*, 2013), on *NCED* expression in imbibed seeds are well understood. However, direct (transcription) factors controlling *NCED* expression during seed maturation and imbibition are not known. FUSCA3 (FUS3) (Gazzarrini *et al.*, 2004; Suzuki and McCarty, 2008) and XERICO (Zentella *et al.*, 2007; Piskurewicz *et al.*, 2008) promote ABA biosynthesis in seeds. *XERICO* expression is stimulated by DELLA proteins (Zentella *et al.*, 2007; Piskurewicz *et al.*, 2008), which are destabilized or inactivated by gibberellin (GA) and GID1 (GIBBERELLIN INSENSITIVE DWARF1) through the 26S proteasome pathway (McGinnis *et al.*, 2003; Ariizumi and Steber, 2007). Since ABA negatively regulates GA levels through up- and downregulation of GA deactivation and biosynthesis genes, respectively (Seo *et al.*, 2006, 2009), promotion of ABA biosynthesis by XERICO could form a positive feedback loop through reduction of GA levels and stabilization of RGL2. However, XERICO does not induce *NCED* (Zentella *et al.*, 2007). Likewise, there is no evidence to indicate direct induction of *NCED* by FUS3. Thus, the precise regulatory mechanisms of *NCED* expression, which is involved in the induction and maintenance of dormancy, remain elusive.

A recent study provided evidence for a positive feedback mechanism in ABA deactivation. ABI4, an ABA-inducible gene, is associated with primary dormancy (Shu *et al.*, 2013) and sensitivity of seedlings to sugar, which is mediated by DOG1 (Teng *et al.*, 2008). Chromatin immunoprecipitation quantitative PCR and genetic analyses showed that ABI4 directly binds to the promoter regions of *CYP707A1* and *CYP707A2*, ABA hydroxylase genes, and represses their expression. The ABI4-binding elements were essential for this repression (Shu *et al.*, 2013). Reduction in ABA hydroxylases leads to the accumulation of ABA. Therefore, the originally produced ABA in seeds could increase its own level through *ABI4* activation and *CYP707A* repression. These results suggest that a positive feedback mechanism could contribute to ABA levels in seeds and dormancy, at least through ABA deactivation.

It has been suggested that positive feedback mechanisms are also operational for ABA biosynthesis. In Arabidopsis plants, ABA induces *ZEAXANTHIN EPOXIDASE* (*ZEP*/*ABA1*/*LOS6*) and *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*/*ABA3*/*LOS5*) (Xiong *et al.*, 2002). Zeaxanthin epoxidase catalyzes conversion of zeaxanthin to violaxanthin, which is upstream of the site of *NCED* action, while AAO3 catalyzes the last step of active ABA production from abscisic aldehyde (Nambara and Marion-Poll, 2005). These results indicate that the initially produced, possibly small, amount of

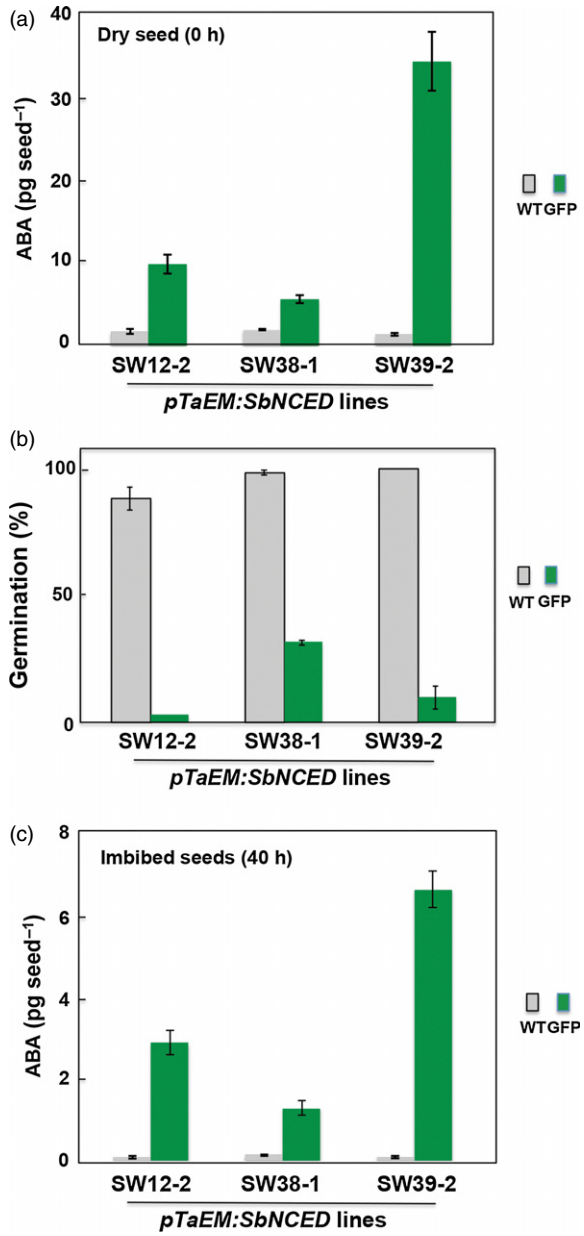

Figure 8. Enhanced ABA biosynthesis and dormancy in *pTaEM:SbNCED* seeds.
(a) The ABA levels in dry seeds of *pTaEM:SbNCED* (GFP, green bar) and null-segregant wild-type (WT, gray bar) sorted from *pTaEM:SbNCED* siliques (SW12-2, SW38-1 and SW39-2). Values are averages ± SD (n = 3 replicates).
(b) Germination of *pTaEM:SbNCED* seeds of the SW12-2, SW38-1 and SW39-2 lines used for ABA quantification in (a) and (c) after 5 days. The first radicle started to emerge after 40 h. Values are averages of two data points for three independent transgenic lines.
(c) The ABA levels in 40-h imbibed seeds of *pTaEM:SbNCED* (GFP, green bar) and null-segregant WT (gray bar) sorted from *pTaEM:SbNCED* siliques (SW12-2, SW38-1 and SW39-2). Values are averages ± SD (n = 3 replicates). Note that the scales in panels (a) and (c) are different.

ABA in certain tissues and organs can be amplified through positive feedback mechanisms during plant development in Arabidopsis.

An important question is whether the rate-limiting enzyme NCED is subjected to positive feedback regulation. Our data suggest that *NCED5* expression in seeds is regulated by a positive feedback mechanism while *NCED6* and *NCED9* may not be regulated by the same pathway and could be subjected to a negative feedback regulation (Figure 7a). Examination of *NCED* expression in the published tiling array data using the *cyp707a1a2a3* triple mutant (Okamoto *et al.*, 2010), in which ABA levels were increased 70-fold compared with wild-type seeds, suggests that *NCED5* expression (and also *NCED9* in this case) was enhanced by ABA. These results also support that *NCED5* expression in seeds is subject to positive feedback regulation.

Drought and sugar response studies have clearly demonstrated that ABA produced in Arabidopsis plants exerts a positive feedback effect on *NCED3* expression (Cheng *et al.*, 2002; Barrero *et al.*, 2006). Interestingly, the inducibility of *NCED3* by ABA varies (3- to 18-fold) among different accessions of Arabidopsis (Barrero *et al.*, 2006), suggesting that there is natural variation in the strength of the positive feedback loops in the ABA biosynthesis and signaling pathway. For future research it will be important to identify the factors and detailed mechanisms associated with the *NCED5* positive feedback loop and to see whether the strength of the feedback mechanism varies between highly dormant and less dormant accessions. These approaches will provide more insights into the regulatory mechanisms of the seed dormancy pathway controlled by ABA.

pABRE:NCED as an efficient experimental system for seed research

It is well known that the maternal plants, and the environment surrounding them, could affect seed dormancy (Munir *et al.*, 2001; Chiang *et al.*, 2013). The maternal effects on dormancy levels of the next generation of seeds are important for plant adaptation and interesting aspects of biology in general that contribute to our understanding of ecology and evolution. However, the maternal effects sometimes hinder the interpretation of data associated with seed dormancy. For example, ABA is produced in both the maternal plant and developing seeds while seed dormancy is determined mainly by the latter, zygotic ABA (Karssen *et al.*, 1983; Groot and Karssen, 1992; Kanno *et al.*, 2010). Over-expression or knockdown mutants associated with ABA metabolism provide useful information for seed dormancy, but when ABA levels are altered in both the maternal plant and developing seeds it could complicate the interpretation of data obtained from these mutants. Increased or reduced ABA levels in the maternal plant affect its own development and could indirectly affect the physiology of seeds and dormancy, as artifacts. The possible artifacts resulting from altered plant growth can

be addressed by reciprocal crosses between the mutant and the WT. However, it is still difficult to distinguish and sort the mutant and null-segregant seeds to make pure seed populations for phenotypic comparison.

The heterozygous *pABRE:NCED* lines, which produce segregating dormant and non-dormant seeds with and without a visible GFP marker, enable seed sorting (Figure 3) and direct comparison for germination tests (Figures 4 and 5) or biochemical experiments (Figure 8). The *pABRE:NCED* and null-segregant WT seeds share the same testa properties, which are also known to affect seed dormancy (Debeaujon *et al.*, 2000, 2007). Thus, *pABRE:NCED* allows precise comparison of dormant and non-dormant seeds of the same maternal origin and under the same developmental conditions, and which differ only in embryonic and endospermic ABA, for seed dormancy research.

pTaEM:SbNCED for translational biology

Our study demonstrated the importance of *NCED* amplification for the control of seed dormancy and also established an efficient experimental system for basic research on the mechanisms of seed dormancy. In addition, it also provided a proof of the robustness of a positive feedback mechanism which can be applied to the development of technology. The *pABRE:NCED* system created by the cereal genes (*pTaEM:SbNCED*) offers a direct tool for the prevention of PHS in food crops. Sorghum is an important staple crop for people in developing countries. Therefore, PHS in sorghum could be a serious issue for food security. Wheat is another major world crop for which PHS often causes problems during grain production (Gubler *et al.*, 2005).

The *NCED* PGSS system (Martinez-Andujar *et al.*, 2011) can be used for prevention of PHS. However, it may not be practical to spray a chemical ligand in large-scale production such as with cereals. Besides, unnecessary induction of *NCED* in plant organs other than seeds could cause pleiotropic effects such as slow growth and increased susceptibility to diseases (Fan *et al.*, 2009). In the *pABRE:NCED* system, the positive feedback mechanism is initiated only in the specific tissues in seeds and timings where/when the native ABA is produced. Therefore, this system could be considered as an enhancement, or recovery, of the original seed dormancy system that has been reduced or lost over the course of crop domestication. Deep dormancy can be released by inducing 'germination rescue' genes, such as ABA hydroxylases, and/or *NCED* RNA interference via PGSS by chemical application, which is a highly feasible wet treatment to seeds after harvest. Thus, the outcomes of this study can also contribute to translational biology via the control of seed dormancy and germination for crop production and food security.

EXPERIMENTAL PROCEDURES

Sequence alignment and homology analysis

The DNA and protein sequences were analyzed using the Bioinformatics Resource Portal ExPASy (http://www.expasy.org/genomics/sequence_alignment). Protein sequences were aligned using the CLUSTALW program (http://embnet.vital-it.ch/software/ClustalW.html) with the conserved sequences highlighted by the BOXSHADE 3.21 program (http://embnet.vital-it.ch/software/BOX_form.html).

Vector construction

A 0.75-kb DNA fragment, including the promoter and 5′ untranslated region of *TaEM* (Figure S3), was amplified from wheat genomic DNA using the forward (TaEM-P724-F) and reverse (TaEM-P724-R) primers (primer sequences are listed in Table S1), which was reamplified with the second set of primers containing restriction sites (TaEM-P724-F-*Asc*I and TaEM-P724-R-*Bgl*II). The *pTaEM* fragment with restriction sites was cloned to the Blunt-end TOPO PCR4.0 (Invitrogen, http://www.invitrogen.com/). The TOPO vector containing *pTaEM* was digested with *Bgl*II, PCR-purified and used for the construction of *pTaEM:SbNCED.*

A 2.3-kb DNA fragment, including the *SbNCED* coding region and the 3′ downstream sequence (0.5 kb, Figure S5), was amplified from sorghum genomic DNA using the forward (SbNCED-F) and reverse (SbNCED-R) primers, which were re-amplified with the second set of primers (Wheat-Sorghum Infusion F2 and -R2). The *SbNCED* fragment was inserted to the *Bgl*II site of the Blant TOPO vector containing *pTaEM* using an InFusion kit (Clontech, http://www.clontech.com/) (Figure 1b). The chimeric gene *pTaEM:SbNCED* was excised by *Asc*I, gel-purified and then ligated to the *Asc*I site of the pCambia2300-GFP vector (Figure 1c). The resulting transformation vector was introduced into *A. tumefaciens*, which was used to transform *A. thaliana* Col-0 by the floral dip method (Clough and Bent, 1998).

All PCR reactions for vector construction were done using a high-fidelity DNA polymerase PrimeSTAR (Takara, http://www.takara-bio.com/). The conditions for PCR were: one cycle at 94°C (1 min), touchdown cycles (94°C for 15 sec, 66°C → 59°C for 15 sec, and 72°C for 30 sec) (one cycle for each temperature) and 30 cycles at 94°C (15 sec), 59°C (15 sec) and 72°C (30 sec), followed by extension at 72°C (7 min).

Germination tests

The GFP and null-segregant GFP seeds from heterozygous *pTaEM:SbNCED* plants were sorted by examination under a dissection microscope (Leica MZ6, http://www.leica.com/) equipped with a UV lamp. Thirty GFP and 30 WT seeds were plated on the same plate containing two layers of filter papers (Whatman No. 2, 9 cm in diameter) moistened with 3.5 ml of deionized water or 10 μM fluridone solution and incubated at 22°C under light for 5 days, with or without 3-days' pre-chilling treatment at 4°C. Averages of duplicates and their data points are shown as germination data, except for the initial examination which was briefly done before seed storage.

Expression analysis

For RT-PCR to analyze gene expression, RNA was extracted from yellow siliques at the seed maturation stage (Figure 6d) using standard phenol-SDS extraction. Two micrograms of total RNA was reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Promega, http://www.promega.com/). The

resulting RT product (1 μl) was used for PCR. The conditions of PCR were as described above. Specific primers were designed for *NCED5* (NCED5-specific-F and -R), *NCED6* (NCED6-specific-F: and -R), *NCED9* (NCED9-specific-F and -R) and *SbNCED* (SbNCED-RT-F2: and SbNCED-RT-R1:) (Table S1) and used for PCR.

Quantification of ABA

Procedures for ABA extraction, purification and measurement are described elsewhere (Preston *et al.*, 2009). Briefly, 40-h-imbibed *pTaEM:SbNCED* seeds or null segregants were homogenized by using TissueLyserII (Qiagen, http://www.qiagen.com/) in 80% (v/v) methanol containing 1% (v/v) acetic acid, d_6-ABA (Icon Isotopes, http://www.iconisotopes.com/) was added as an internal standard. After extraction, three cartridge columns, Oasis HLB, MCX, WAX (Waters, http://www.waters.com/) were used to purify ABA according to the manufacturer's instructions. The ABA measurement was performed by a triple Quad LC-ESI-MS/MS (G6400 Series, Agilent Technologies, http://www.agilent.com/). The MS/MS transitions for quantification are as follows: ABA (m/z = 263/153), d6-ABA (m/z = 269/159).

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CONFLICT OF INTEREST

A patent application has been filed for an indirectly related technology.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Alignment of the amino acid sequences of monocot and dicot 9-*cis*-epoxycarotenoid dioxygenases (NCEDs).

Figure S2. Alignment of the amino acid sequences of sorghum and Arabidopsis 9-*cis*-epoxycarotenoid dioxygenases (NCEDs).

Figure S3. The 5′ upstream and coding sequences of the wheat (*Triticum aestivum*) *Early Methionine-labeled* (*TaEM*).

Figure S4. Diagram showing the procedures used for T_1 plant and T_2 seed production.

Figure S5. The 3′ downstream sequence of sorghum 9-*cis*-epoxycarotenoid dioxygenase (NCED) used in this study.

Table S1. List of primers used in this study.

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