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ARTICLE

The NAC Transcription Factor *ANAC089* Modulates Seed Vigor through the ABI5-VTC2 Module in *Arabidopsis thaliana*

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ABSTRACT

Seed viability is an essential feature for genetic resource conservation as well as sustainable crop production. Long-term storage induces seed viability deterioration or seed aging, accompanied by the accumulation of toxic reactive oxygen species (ROS) to suppress seed germination. Controlled deterioration treatment (CDT) is a general approach for mimicking seed aging. The transcription factor *ANAC089* was previously reported to modulate seed primary germination. In this study, we evaluated the ability of *ANAC089* to control seed viability during aging. Compared with that in the wild-type line, the mutation of *ANAC089* significantly increased H_2O_2, thereby reducing seed viability after CDT, while the overexpression of *ANAC089* reduced H_2O_2 and improved seed longevity, indicating a critical role for *ANAC089* in maintaining seed viability through H_2O_2 signaling. A series of studies have shown that *ANAC089* targets and negatively regulates the level of *ABI5*, an important transmitter of abscisic acid (ABA) signals, to affect seed viability after CDT. Furthermore, *ABI5* negatively regulated the expression of *VTC2*, which is involved in the biosynthesis of the antioxidant ascorbic acid and H_2O_2 scavenging. As a result, *ANAC089* attenuates the generation of H_2O_2, thereby enhancing seed viability through the ABI5-VTC2 module during the seed aging process. Taken together, our results reveal a novel mechanism by which ANAC089 enhances seed viability by coordinating *ABI5* and *VTC2* expression, ultimately preventing the overaccumulation of H_2O_2, which would have led to reduced seed viability.

KEYWORDS

Arabidopsis; seed aging; *ANAC089*; *ABI5*; *VTC2*; H_2O_2

1 Introduction

Plant seeds contain plentiful carbohydrates (as in rice seeds) and protein and lipids (as in soybeans), and they are used as staple foods to provide humans with the necessary energy for building human civilization [1,2]. Seeds carry plant genetic information and sexual reproduction to mediate the generational alternation of flowering plants; thus, good germination status after long-term conservation is important for ecological and agronomic value; however, seeds gradually lose their germination viability during storage, a phenomenon called seed aging or deterioration [3–5]. Seed aging is caused by oxidative damage and is influenced by environmental and genetic factors [6,7]. This process is inevitable and irreversible; thus, attenuating seed aging or deterioration can efficiently prolong seed

longevity and sustain germination vigor in a dry state. Identifying genes or regulatory networks involved in seed longevity and understanding the mechanism of seed deterioration is important for germplasm resource preservation and crop safety.

Seed viability gradually increases during seed development, reaches a high seed germination capacity during seed maturation, and then decreases during the postharvest period; therefore, seed maturation status determines seed viability or longevity. Four master genes, namely, ABSCISIC ACID INSENSITIVE 3 (ABI3), LEAFY COTYLEDON 1 (Lec1), LEC2, and FUSCA3 (FUS3), play critical roles in seed maturation, and mutation of any of these four genes causes a deficiency in seed maturation, consequently impairing seed viability [8]. For example, both the *abi3* and *lec1* mutants prevent proper seed storage protein accumulation; thus, they lose their germination viability during the first few weeks of the postharvest period, the *abi3* mutant shows ABA insensitivity during seed germination [9,10], and the *lec1* mutant shows an abnormal embryo phenotype, lacks protein and oil accumulation and prefers to undergo vivipation. The plant hormone abscisic acid (ABA) also significantly contributes to the seed maturation process and seed vigor [9,11]. The bZIP transcription factor ABI5, an ABA transmitter, reportedly controls seed maturation and vigor in Arabidopsis, legumes, and Medicago [12,13]. Other bZIP transcription factors, such as *bZIP23* and *bZIP42*, also regulate ride seed vigor [14]. In addition to plant hormones and genetic factors, environmental stress induces the accumulation of reactive oxygen species, which are often believed to be key factors for regulating seed vigor. An appropriate level of ROS precisely controls the seed germination status and degree of deterioration, and a low concentration of ROS can trigger seed germination, but the overaccumulation of ROS is highly toxic to seed proteins, DNA, and lipids [4,15–17]. Accordingly, plants have also evolved sophisticated strategies for ROS detoxification to avoid oxidative damage and sustain seed vigor; for example, tocopherols (vitamin E) are lipophilic antioxidants that are particularly abundant in mature seeds and are important for seed longevity because they prevent lipid peroxidation during germination [18]. Vitamin C, or ascorbic acid (AsA), is also an efficient nonenzymatic antioxidant that scavenges ROS for seed germination under salt stress [19]. Arabidopsis VITAMIC DEFECTIVE 2 (*VTC2*) encodes the key product of GDP-L-galactose phosphorylation for AsA biosynthesis, and a mutant of *vtc2* induces more H_2O_2 after salt stress [20]. *PER1* encodes the seed-specific antioxidant 1-Cys peroxiredoxin, which scavenges ROS to improve seed viability in rice [14], and even ectopic expression of NnPER1 from lotus also enhances seed vigor and longevity in Arabidopsis [21,22]; however, the detailed mechanism underlying seed vigor or longevity needs further investigation.

Arabidopsis ANAC089 is an NAC transcription factor and is also a redox-responsive protein that regulates seed sensitivity to ABA [23–26]. ANAC089 and its two homologs, *ANAC060* and *ANAC040*, are reported to control seed primary germination and dormancy [27]. ANAC089 is also involved in flowering time regulation by affecting the expression of *FLC*, an important negative factor for initiating flowering [28]. In this study, we investigated the function of ANAC089 in controlling seed vigor and found that ANAC089 attenuated H_2O_2 accumulation, thereby enhancing seed viability, as characterized by a high percentage of seed germination after seed aging. Furthermore, we found that ANAC089 can bind to the promoter of *ABI5*, an important transmitter of ABA signals, to suppress its expression. Consistent with these findings, the mutation of *ABI5* suppressed the generation of H_2O_2 to increase seed vigor, but the overexpression of *ABI5* increased H_2O_2 to decrease seed vigor. Further study revealed that *ABI5* orchestrates endogenous ROS levels by negatively regulating *VTC2*. Thus, our data reveal the novel function of ANAC089 in controlling seed vigor and longevity through the targeting of *ABI5* and downstream *VTC2*, which ultimately scavenges toxic H_2O_2. This study expands our knowledge of the use of ANAC089 for seed conservation and sustainable agricultural production.

2 Materials and Methods

2.1 Plant Materials and Growth

All Arabidopsis (*Arabidopsis thaliana*) materials, including *abi5* and *ABI5-MYC* provided by Chuanyou Li's laboratory (Institute of Genetics, Chinese Academy of Sciences, Beijing, China) [29] and the *vtc2* mutant provided by Kai Shu's laboratory (Northwestern Polytechnical University, Xi'an, China) [19], were surface-sterilized with bleach, plated on half-strength Murashige and Skoog (1/2 MS) media containing 0.8% (w/v) agar and 1% (w/v) sucrose (pH 5.7) and grown at 22°C under long days (16-h light [100 $\mu mol^{-2}s^{-1}$]/8-h darkness) or intermediate days (12-h light [100 $\mu mol^{-2}s^{-1}$]/12-h darkness). Adult plants were grown under long-day conditions at 22°C in a growth chamber with a 16-h white light (50 $\mu mol\ m^{-2}s^{-1}$)/8-h dark cycle. Homozygous seeds in each batch were harvested at the same time and dried in an incubator at 22°C for approximately 2 months before the germination assays.

2.2 Generation of Gene-Editing Lines

The target sequence with the PAM structure (NGG) was predicted and selected by target design online software (http://skl.scau.edu.cn/targetdesign/) according to a previously reported method [30]. The oligonucleotide containing this target sequence was synthesized and digested by the BsaI enzyme and then inserted into the pCAMBIA-CAS9 vector to generate the gene-editing construct. The resulting construct was subsequently transformed into Agrobacterium GV3101 for Arabidopsis transformation. The resulting gene-editing lines were screened on MS media supplemented with 35 mg/L hygromycin, and the nucleotide deletion pattern around the target site within the *ANAC089* genome locus was determined via PCR and Sanger sequencing.

2.3 Transgenic Plant

To obtain the *35S: ANAC089-Flag* or *35S: VTC2-Flag* construct, the full-length fragment of *ANAC089* or *VTC2* was linked with a Flag tag through PCR amplification using the PrimeSTAR enzyme (Takara, Japan), and then recombined into the pRI101-AN vector through the In-Fusion cloning kit (Clontech, Dalian, China). The construct was transformed into *Arabidopsis thaliana* (Col-0) plants through Agrobacterium (*Agrobacterium tumefaciens*)-mediated floral-dipping transformation. The transgenic seeds were collected and sowed on the 1/2 MS medium, and the transgenic line was screened with 50 mg L^{-1} kanamycin. After three generations of screening on MS media supplemented with kanamycin, the homozygous seeds were used for seed viability analysis.

2.4 Seed Viability Measurement by Aged Treatment and Germination Analysis

Seed viability was evaluated by controlled deterioration treatment (CDT) as previously described [24,31]. In brief, the mature seeds were collected and dried in paper bags at room temperature for two months. The dried seeds were loaded in tubes without lids, and the tubes were placed on a copper wire mesh tray in a desiccator. Approximately 1 L of saturated NaCl solution with 0.01% NaClO was kept below the tray, and then the lid of the desiccator was sealed to ensure that the relative humidity inside was approximately 80%. After 2 days of water equilibration at room temperature, the seeds were aged by placing the desiccator in an incubator at 42°C for 4 days (4 d CDT) and then removed from the desiccator and dried at 25°C for 2 days before the germination assay. Seeds were considered to have completed germination when they showed a >2 mm radicle. The percentage of germinated seeds was recorded after 5 days of germination on 1/2 MS solid media.

2.5 Seed Viability Measurement by Tetrazolium Assay

To measure seed viability after aging, a tetrazolium (TTZ, 2,3,5-triphenyl tetrazolium chloride) assay was used as described previously [31]. Briefly, the aged seeds were gently scratched on an emery cloth, incubated in 1% TTZ solution at 25°C for 48 h, and then washed three times with double distilled water. Pictures were taken using a Zeiss stereomicroscope.

2.6 H_2O_2 Content Measurement

The H_2O_2 content was measured according to a previous method [14]. Approximately 10 mg of Arabidopsis seeds were ground in 1 mL of precooled acetone. The homogenate was centrifuged for 10 min at 15,000 × g. A total of 1 mL of supernatant, 0.1 mL of titanium reagent, and 0.2 mL of ammonium solution were mixed to precipitate the titanium–hydroperoxide complex. After centrifugation at 5,000 × g for 10 min, the precipitate was dissolved in 1 mL of 2 M H_2SO_4. H_2O_2 was measured using an ultraviolet (UV)–visible spectrophotometer and the absolute amount was calculated according to a standard curve.

2.7 RT–qPCR Analysis

Total RNA was extracted from hydrated seeds using TRIzol reagent (Invitrogen, Waltham, MA, 02451, USA). RT–qPCR was performed as the used method by Li et al. [32]. Briefly, first-strand cDNA was synthesized from 1.5 μg of DNase-treated RNA in a 20 μL reaction volume using M-MuLV reverse transcriptase (Fermentas, Visalia, CA, 93291, USA) with oligo $(dT)_{18}$ primers. For qPCR, cDNA samples were diluted to 2–10 ng μL^{-1}, and PCR was performed in the presence of SYBR Green I Master Mix on a Roche LightCycler 480 real-time PCR machine according to the manufacturer's instructions. All RT–qPCR experiments were independently performed in triplicate, and representative results are shown. PROTEIN PHOSPHATASE 2A (*PP2A)* was used as the internal control. The prprimerairs used for quantitative RT–qPCR are listed in Table S1.

2.8 Protein Immunoblot Analysis

Total protein was extracted from the seedlings using the method of Li et al. [32,33]. Extracted proteins (15 μg aliquot) were separated with a 12% SDS–polyacrylamide gel, and then the gel was transferred onto polyvinylidene difluoride (PVDF) membranes. After washing with 10 mM PBS buffer (pH = 7.5) three times, the membrane was incubated with the appropriate primary antibody for 30 min at room temperature, and then washed three times with PBS buffer for 15 min each time to remove the unbound antibody, and then the washed membrane was incubated with a secondary horseradish peroxidase-conjugated goat anti-mouse or anti-rat secondary antibody (1:3,000; Promega, Madison, WI, 53711, USA). Signals were detected using the ECL Kit (GeneScript, Nanjing, China). The primary antibody, including anti-ABI5 (1:1,000, Abcam, Shanghai, China), anti-GST (1:3,000, Invitrogen), anti-FLAG (1:3,000; Sigma–Aldrich, St. Louis, MO, 63178, USA), or anti-Actin (1:1,000; Sigma–Aldrich) antibody was used.

2.9 Protoplast Transient Expression Assay

For the transient expression assay, the 2.4-kb *ABI5* fragment upstream of the start codon of ABI5 was used as the promoter, such fragment was inserted into the pGreenII 0800-LUC vector to generate the *pABI5-LUC* reporter construct. The coding sequence of *ANAC089-Flag* was inserted into the pGreenII 62-SK vector under the control of the 35S promoter as the effecter construct. All primer information for generating these constructs is provided in Table S1. The mesophyll protoplasts were produced from *Arabidopsis* leaves as the method of Li et al. [32], the purified protoplast was then subsequently transfections with the constructs as above. A dual-luciferase reporter assay system (Promega) was performed, and the activities of firefly Luc and Renilla luciferase (REN) were measured. Relative LUC/REN ratios were used to compare the transcriptional activity of corresponding promoters.

2.10 Electrophoretic Mobility Shift Assay (EMSA)

The ability of ANAC089 to bind to the *ABI5* promoter was measured by EMSA as described previously [32,33]. Briefly, oligonucleotide probes containing the G-box motif were synthesized, annealed, and labeled using a Biotin-DNA labeling kit (Pierce, Invitrogen, Waltham, MA, 02451, USA). The *E. coli* BL21 strain transformed with *pGST-ANAC089* was induced with 0.1 mM isopropyl β-d-1-thiogalactopyranoside at 28°C

when the OD_{600} was 1.5. The recombination GST-ANAC089 protein was purified from the crude bacterium protein by GST resin affinity chromatography (Invitrogen). The oligonucleotide probes containing the wild-type or mutated CACGTG motif were labeled with biotin and synthesized. These unmutated or mutated oligonucleotides without biotin labeling were used as cold probes or cold mutated probes, respectively. The binding ability of the labeled probe with the purified protein was checked on the nitrocellulose membrane, and the chemiluminescence of the biotin-labeled DNA was detected using a Light Shift Chemiluminescent EMSA Kit (Pierce).

2.11 Data Analysis

The data is presented as the means ± SD of three or five biological replicates for each sample. Using the acquired data, a two-way analysis of variance (ANOVA) following Tukey's test was performed among different groups, or a student's *t*-test was used to compare the difference between only two groups. The statistical analyses were carried out using IBM SPSS Statistics 22 (IBM SPSS, Chicago, IL, USA).

3 Results

3.1 ANAC089 Controls Seed Viability

A previous study showed that *ANAC089* controls the cytosolic redox status during seed germination under ABA stress [26], and we wondered whether *ANAC089* also regulates seed viability. To test this hypothesis, we first applied a gene-editing approach to generate a series of *ANAC089* mutants. As shown in Figs. 1A and 1B, we successfully deleted one nucleotide (deletion A) in the secondary exon of *ANAC089*, causing a codon shift to inactive *ANAC089*; this mutant was named *anac089*-1. We also obtained another gene-editing mutant by deleting a nucleotide (GCAA) in the secondary exon; this line was named *anac089-2*. The *ANAC089* locus in these two lines was sequenced, and successful deletion of this locus was confirmed (Fig. 1B). Moreover, we generated a transgenic line overexpressing the *ANAC089-Flag* fusion gene under the control of the constitutive *35S* promoter. A strong immunoblot signal was also successfully detected in several individual transgenic lines by western blot analysis using an anti-Flag antibody (Fig. 1C).

To determine the effect of *ANAC089* on seed viability, we applied the artificial aging approach (CDT) to simulate the course of seed aging and used the tetrazolium (TDZ) staining method to evaluate seed viability. As shown in Figs. 1D and 1E, after artificial aging treatment, TDZ staining revealed slightly red staining in the wild-type Col seeds and the *anac089-1* and *anac089*-2 gene-edited seeds, suggesting decreased seed viability; in contrast, the seed staining for the *ANAC089-Flag* line was very strong, suggesting high seed viability. We also tested the seed germination potential of these lines after different durations. After 6 months, 1 or 2 years of conservation, both the Col and *ANAC089-Flag* still showed high seed germination, but only approximately 44.5% of the *anac089-1* mutant seeds and 47.8.2% of the *anac089-2* mutant seeds germinated after 1 year of storage, and only 24.3% of the *anac089-1* and 21.4% of the *anac089-2* mutant seeds germinated after 2 years of storage; However, approximately 76.45% of the *ANAC089-Flag* seeds germinated well, which was greater than that of the *anac089-1* and *anac089-2* lines (Fig. S1). Based on these data, we propose that *ANAC089* negatively regulates seed viability.

3.2 ANAC089 Controls ROS Levels

A previous study showed that the ROS level, mainly the H_2O_2 level, affects seed viability [19,20]. Here, we measured the endogenous H_2O_2 level in the seeds after artificial aging treatment. The artificial aging process increased the endogenous H_2O_2 content in wild-type Col seeds on the first day, after which the high level of H_2O_2 was maintained during the following three days of CDT treatment (Fig. 2A), suggesting that a high level of ROS is toxic to seed viability after CDT treatment. We further compared the different H_2O_2 contents among Col, the *anac089* mutant, and its transgenic overexpressing line after

3 days of CDT treatment and found that the H_2O_2 content in the *ANAC089-Flag* line was lower than that in the Col or *anac089* mutant lines (Fig. 2B). It is possible that the relatively lower level of H_2O_2 attenuated the damage caused by CDT to seed viability.

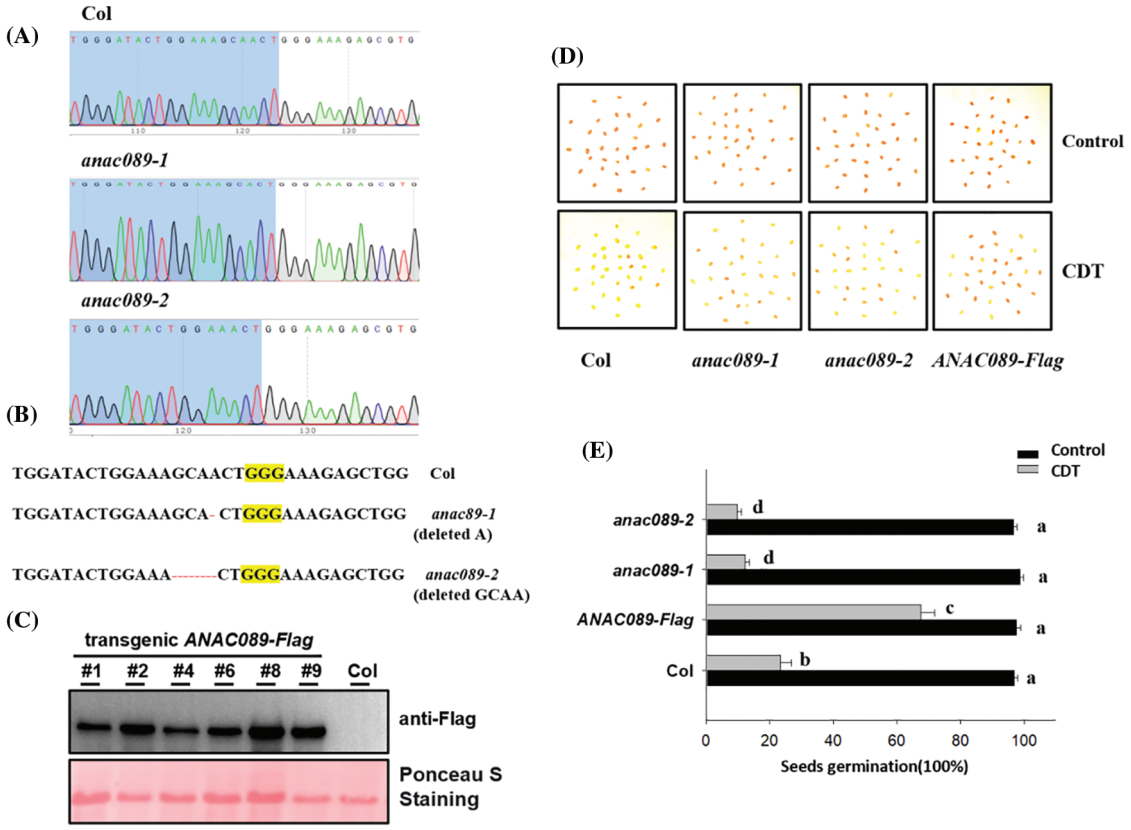

Figure 1: ANAC089 regulates seed viability (A) Identification of the *anac089-1* mutant. A T-DNA fragment is inserted in the first exon to disrupt the functional transcript of *ANAC089*. (B) Sequencing of the gene editing site of *the anac089-2* mutant. One nucleotide was deleted in the *ANAC089* genomic locus by a gene editing approach, and this deletion was confirmed by Sanger sequencing. (C) Verification of the transgenic *ANAC089-Flag* line by western blot analysis. (D and E) Different seed germination percentages among Col, *anac089-1*, *anac089-2*, and *ANAC089-Flag* after 4 days of CDT aging treatment. The seeds were stained with TTC to determine seed viability. Red indicates seeds with high viability, and light yellow indicates that the seeds lost viability. An image was taken after staining (C). After 4 days of CDT treatment, the seeds were placed under normal conditions at 22°C for 5 days, after which the percentage of germinated seeds was recorded. The experiment was performed in triplicate, and the data are presented as the mean ± SD. Bars with different letters are significantly different at $p < 0.05$ (two-way ANOVA with Tukey's test)

VTC2 is an important antioxidant enzyme that scavenges ROS accumulation in Arabidopsis [20]. We also found that the *vtc2* mutant showed low seed viability, accompanied by increased H_2O_2 accumulation after CDT treatment (Fig. 2B), but additional exogenous AsA enhanced the seed germination of *vtc2* after CDT (Fig. S2). These data suggest that *VTC2* may be responsible for H_2O_2 accumulation after CDT treatment. Furthermore, we crossed *vtc2* with *ANAC089-Flag* to obtain the *ANAC089-Flag/vtc2* line.

Unlike the *ANAC089-Flag* line, the *ANAC089-Flag/vtc2* line exhibited lower seed germination after 3 days of CDT treatment, similar to that of *vtc2* (Fig. 2C); the H_2O_2 content in the *ANAC089-Flag/vtc2* seeds was also greater than that in the *ANAC089-Flag* line. However, the addition of AsA increased seed germination and reduced H_2O_2 production in *ANAC089-Flag/vtc2* seeds after CDT treatment (Fig. S3). Meanwhile, we also direct H_2O_2 treatment to suppress the seed germination (Fig. S4). These data further support the hypothesis that high levels of ROS impair seed viability through *ANAC089* and *VTC2*.

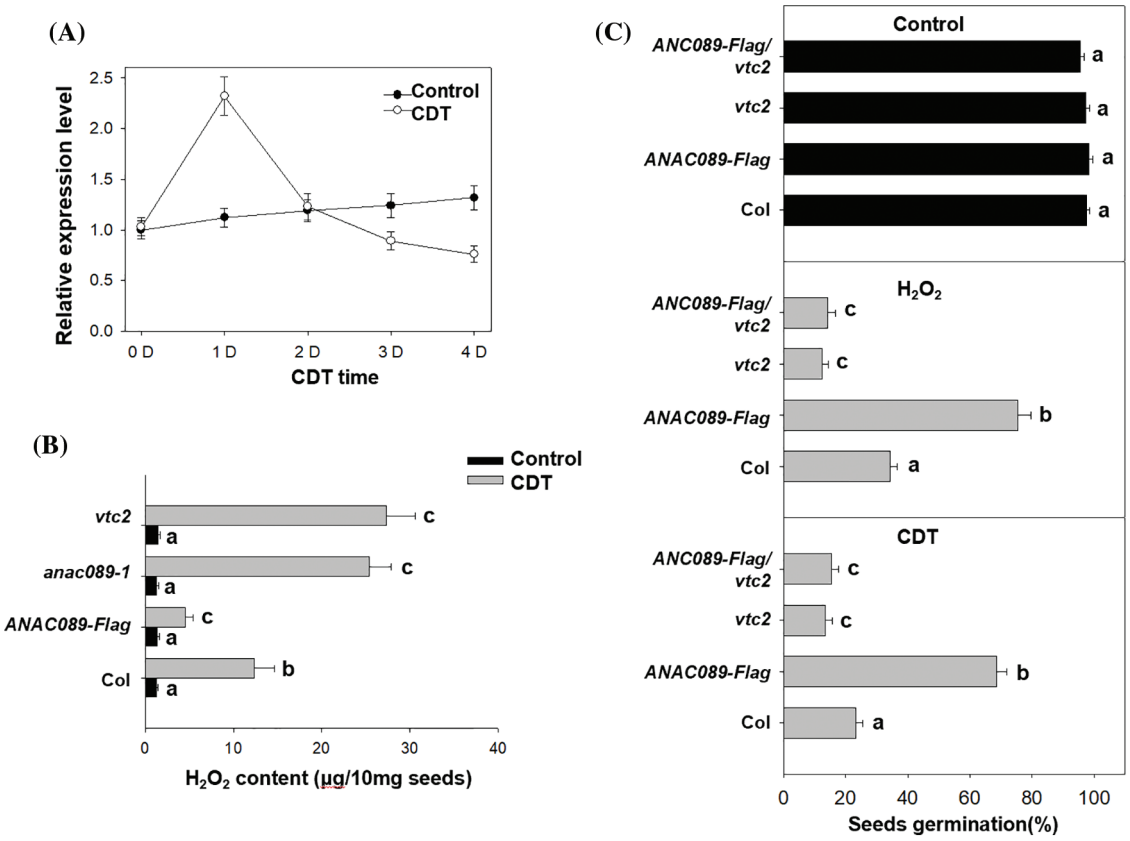

Figure 2: ANAC089 affects the generation of H_2O_2 to control seed viability. (A) The effect of CDT treatment on the transcriptional level of *ANAC089* in wild-type Col seeds. The freshly harvested seeds were dried for two weeks and then treated with CDT for the indicated times, after which the treated seeds were subjected to total RNA extraction. The transcriptional level of *ANAC089* was measured by RT–qPCR analysis, and *PP2A* was used as the internal control. The experiment was performed in triplicate, and the bars indicate ±SD. (B) *ANAC089* affected the accumulation of H_2O_2 after seed aging treatment. Seeds of Col, *anac089*, *vtc2*, and transgenic *ANAC089-Flag* line were treated with CDT for 4 days, after which the H_2O_2 content was measured. Seeds not subjected to CDT treatment were used as controls. The experiment was performed in triplicate, and the bars indicate ±SD. Bars with different letters are significantly different at $p < 0.05$ (two-way ANOVA with Tukey's test). (C) Effects of H_2O_2 and CDT treatment on the percentage of germinated seeds among the Col, *anac89*, and *vtc2* mutant and *ANAC089-Flag* lines. These seeds were treated with 100 nM H_2O_2 for 12 h or CDT for 4 days, germinated under normal conditions at 22°C for 5 days, and the percentage of germinated seeds was recorded. The experiment was performed in triplicate, and the bars indicate ±SD. Bars with different letters are significantly different at $p < 0.05$ (two-way ANOVA with Tukey's test)

3.3 ANAC089 Binds to the Promoter of ABI5

It has been reported that the *ANAC089* homolog *ANAC060* can directly bind to the promoter of *ABI5* to suppress ABI5 expression in Arabidopsis [34]. Here we found that CDT induced the expression of *ABI5* in the wild-type Col seeds, such induction was attenuated in the *ANAC089-Flag* line, but increased in the *anac89* mutant line (Fig. 3A), suggesting that ANAC089 negatively regulated the expression of *ABI5* during CDT. It is possible that *ANAC089* also binds to the promoter of *ABI5* to suppress its expression.

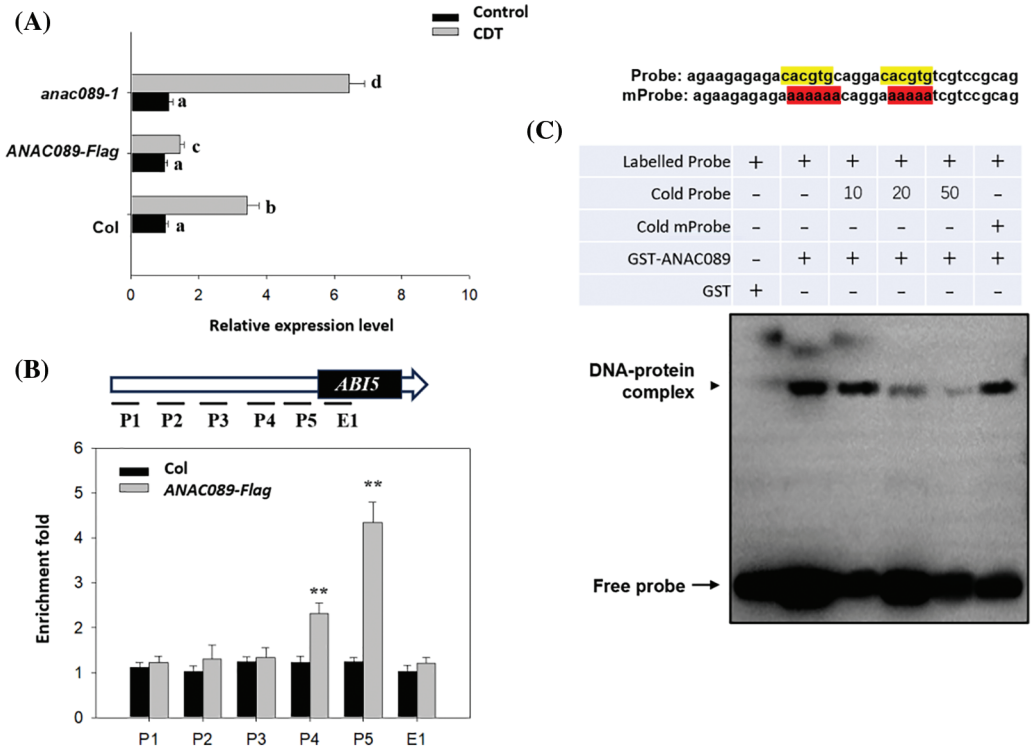

Figure 3: ANAC089 binds to the promoter of *ABI5*. (A) RT–qPCR analysis of the transcriptional level of *ABI5* in the Col, *anac089*-1, and *ANAC089-Flag* lines before or after CDT treatment for 4 days. Seeds treated with or without CDT were hydrated for 12 h, after which total RNA was extracted for RT–qPCR analysis. *PP2A* was used as the internal control. The experiment was performed in triplicate, and the bars indicate ±SD. Bars with different letters are significantly different at $p < 0.05$ (two-way ANOVA with Tukey's test). (B) Chromatin immunoprecipitation (ChIP)-qPCR analysis of the association of ANAC089 with the *ABI5* promoter *in vivo*. Hydrated Col-0 and *ANAC089-Falg* seeds were used for analysis. Upper panel, diagram of the *ABI5* promoter showing the positions of G-boxes (blue rectangles) and the five regions (the P1, the P2, the P3, P4, and E1) used for ChIP–qPCR amplification, as indicated by black lines. Lower panel, fold enrichment of six amplified fragments, as quantified by qPCR. An anti-FLAG antibody was used for ChIP. *PP2A* served as an internal control, and enrichment values were normalized to the level of input DNA. The values are shown as the means ± standard deviation of triplicate experiments. Asterisks indicate significant differences according to the student's *t*-test (**$p < 0.01$). (C) EMSA assay of the direct binding of the ANAC089-GST protein to probes with a G-box elements *in vitro*. A biotin-labeled probe with a G-box motif. Such probe sequence information is provided on top, highlighted codons with yellow color indicate the binding motif by ANAC089, while the red color indicates the mutated sequence by replacing the CTCGTG with AAAAAA; cold probe means unlabeled probe (un-labeled cold probe at 10 fold or 50 fold was loaded to compete for the specific binding between the labeled probe and the G-box elements); labeled mProbe means biotin-labeled probe with a mutated G-box (highlighted in red); cold mProbe means unlabeled mutated probe. The black arrows point to DNA/protein complexes and free probes, as indicated

To test this hypothesis, we first performed ChIP to test the ability of *ANAC089* to bind to the *ABI5* promoter. Using the imbibed seeds of the *ANAC089-Flag* line and anti-Flag, we found that the P3 fragment containing the NAC-recognizing G-box motif (CACGTG) could be specifically enriched by *ANAC089-Flag*. As a control, other fragments within the reference gene locus could not be efficiently enriched (Fig. 3B). Furthermore, we used probes containing such motifs as probes and performed EMSAs to test the ability of the ANAC089 protein to bind to these probes. The EMSA results showed that ANAC089 indeed strongly bound to this labeled probe, while the unlabeled cold probe could compete with the bind labeled probe to ANAC089, reducing the accumulation of DNA-protein complex (Fig. 3C). As a control, the mutated cold probe could bind the ANAC089 protein to this probe. These data suggest that ANAC089 also specifically recognizes the G-box within the *ABI5* promoter.

3.4 ANAC089 Negatively Regulates ABI5 Expression to Modulate H_2O_2 Accumulation and Seed Viability

Because ANAC089 can bind to the promoter of *ABI5*, we next wanted to determine the relationship between *ANAC089* and *ABI5*. Here, we performed transient protoplast transformation to test the effect of ANAC089 on the expression of *ABI5*. We constructed a reporter vector containing the luciferase gene driven by the *ABI5* promoter (*ABI5p: Luc*) and an effector containing the coding region sequence of *ANAC089* (*35S: ANAC089*, abbreviated as *ANAC089*) and then co-transformed the effector and reporter into the isolated protoplast. The empty effector was used as the control. Compared with the protoplasts co-expressing the empty vector, the protoplasts co-expressing the *ANAC089* effector suppressed the expression of LUC in the *ABI5p: Luc* reporter. We also constructed a mutated *ABI5p: Luc* reporter (*mABI5p: Luc*) by deleting the G-box within the promoter of ABI5 to abolish the binding of *ANAC089* to this promoter. As expected, ANAC089 did not efficiently suppress the expression of LUC in the *mABI5p: Luc* reporter (Fig. 4A). Based on these data, we propose that ANAC089 suppresses the expression of *ABI5* by recognizing and binding the G-box within the promoter of *ABI5*.

Given that ANAC089 affected the accumulation of H_2O_2 during the aging process, we wanted to determine the effect of ABI5 on the accumulation of H_2O_2. Compared with that in the Col line, CDT decreased the H_2O_2 content in the *abi5* mutant but increased H_2O_2 accumulation in the *ABI5-MYC* line, suggesting that *ABI5* negatively regulates the accumulation of H_2O_2 during the aging process (Fig. 4B). Accordingly, the seed germination of the *abi5* mutant was greater than that of the *ABI5-MYC* transgenic line and the Col line after aging treatment (Fig. 4C). Furthermore, we crossed the *abi5* mutant with the *anac089* mutant or transgenic *ANAC089*-overexpressing line and found that both *anac089/abi5* and *ANAC089-Flag/abi5* showed lower H_2O_2 accumulation and a greater percentage of seed germination, similar to the *abi5* mutant (Figs. 4B and 4C). These results are consistent with our findings that ANAC089 targets and binds to the promoter of *ABI5* to negatively regulate *ABI5* expression and indicate that the ability of *ANAC089* to control H_2O_2 accumulation and seed germination after aging treatment is dependent on *ABI5*.

3.5 ABI5 Targets VTC2 to Control H_2O_2 Metabolism and Seed Viability

As our above results showed that the *vtc2* mutant showed lower seed viability after CDT treatment because of increased accumulation of H_2O_2, we speculate that *VTC2* is involved in the seed aging process and that *VTC2* modulates redox status to sustain seed viability during the seed conservation process. To test this hypothesis, we generated a transgenic line overexpressing *VTC2* (overexpressing the *VTC2-Flag* fusion gene under the control of the *35S* promoter, *35S: VTC2-Flag*, abbreviated *VTC2-Flag*) and compared the viability of the seeds after CDT treatment. As shown in Fig. 5A, compared with that in

the Col line, CDT induced lower H_2O_2 accumulation in the *VTC2-Flag* line but more H_2O_2 accumulation in the *vtc2* mutant line (Fig. 5A). Consistently, the percentage of germinated *VTC2-Flag* seeds was also greater than that of the *vtc2* mutant line after the aging process (Fig. 5B), which suggested that *VTC2* attenuates CDT-induced H_2O_2 accumulation to enhance seed germination.

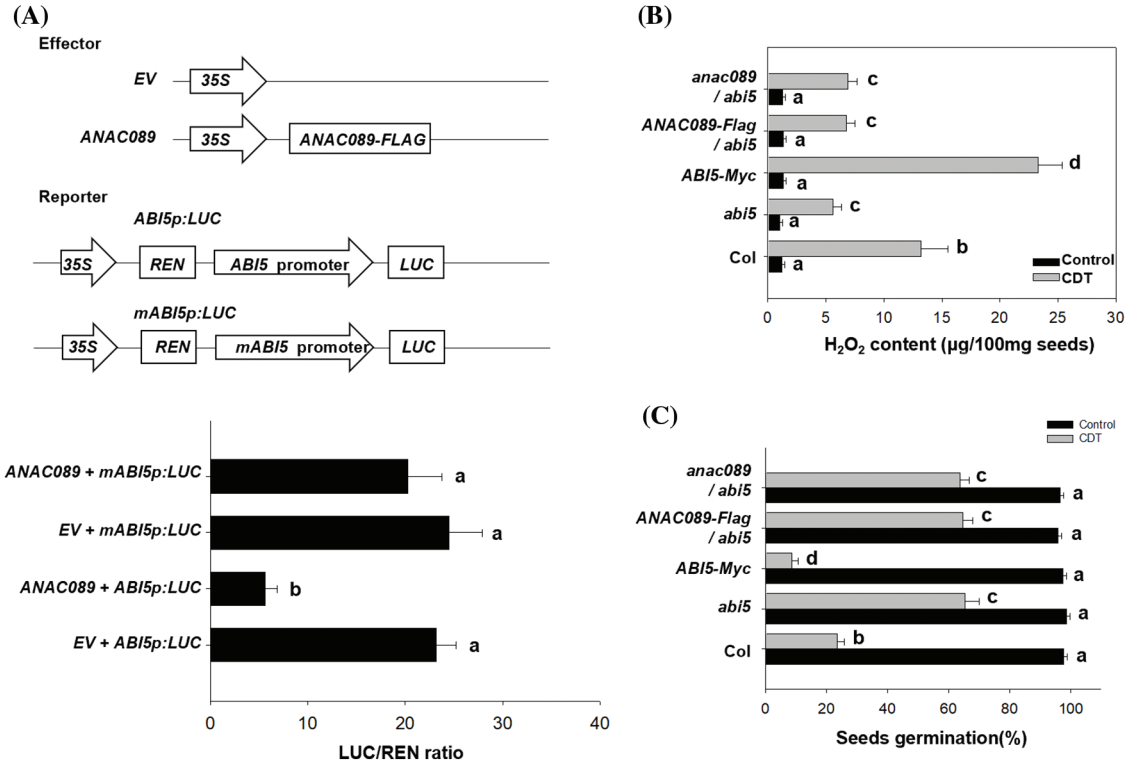

Figure 4: ANAC089 suppresses the expression of *ABI5* to increase seed viability. (A) Transient transformation analysis of the suppressive effect of ANAC089 on *ABI5* expression. The schematic diagram indicates the effector and reporter constructs for the transient transformation assay. The G-boxes are indicated with blue rectangles in the reporter construct, and the reporter with the truncated *ABI5* promoter lacking the G-box is also shown. Full-length or truncated fragment of the *ABI5pro: LUC* reporter was co-expressed with *ANAC089-Flag* effectors overnight, and the firefly luciferase and Renilla luciferase (LUC/REN) ratio was calculated to present the ABI5pro: LUC activity. An empty effector without a promoter fragment was used as the control. The data are presented as the means ± SDs of three biological replicates. Bars with different letters are significantly different at $p < 0.05$ (Tukey's test). (B and C) *ANAC089* and *ABI5* antagonistically control H_2O_2 generation and seed viability during aging treatment. The seeds of Col, *anac089*, *abi5*, *ANAC089-Flag*, and *ABI5-MYC* and their crossed seeds were treated with CDT for 4 days, and the H_2O_2 content in the imbibed seeds was measured (B). The seeds were germinated under normal conditions at 22°C for 5 days, and the percentage of germinated seeds was determined. The experiment was performed in triplicate, and the bars indicate ±SD. Bars with different letters mean significantly different at $p < 0.05$ (two-way ANOVA with Tukey's test)

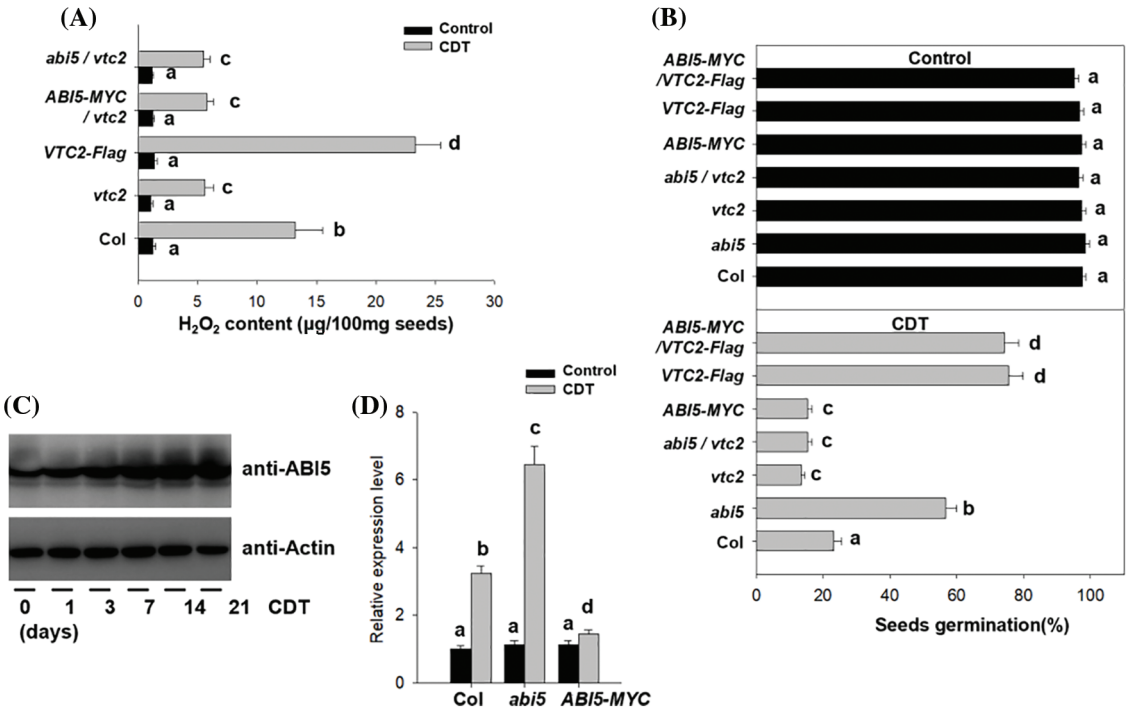

Figure 5: ABI5 affects seed viability through VTC2. (A) The differences in H_2O_2 levels among Col, *ABI5-MYC, VTC2-Flag*, and the double transgenic *ABI5-MYC/VTC2-Flag* line after 4 days of CDT treatment. The experiment was performed in triplicate, and the bars indicate ±SD. The different letters on the top of the bars mean significantly different at $p < 0.05$ (two-way ANOVA following Tukey's test). (B) *ABI5* controls seed germination after aging via *VTC2*. The seeds of Col, *abi5*, *ABI5-MYC*, *vtc2*, *VTC2-Flag*, and their crossed lines were treated with CDT for 4 days, followed by germination under normal conditions at 22°C for 5 days, after which the percentage of germinated seeds was recorded. Seeds not subjected to CDT treatment were used as controls. The experiment was performed in triplicate, and the bars indicate ±SD. The different letters on the top of the bars mean significantly different at $p < 0.05$ (two-way ANOVA following Tukey's test). (C) CDT treatment increased the protein accumulation of ABI5. WT Col seeds were treated with CDT for the indicated times, and the protein abundance of ABI5 was measured by western blotting analysis using an anti-ABI5 antibody. Anti-Actin was used as a loading control. (D) ABI5 suppresses the expression of *VTC2*. After the imbibed Col, *abi5*, and *ABI5-MYC* were treated with CDT for 4 days, the transcriptional level of *VTC2* was measured by RT−qPCR analysis. Seeds not subjected to CDT treatment were used as controls. *PP2A* was used as the internal control. The experiment was performed in triplicate, and the bars indicate ±SD. The different letters on the top of the bars mean significantly different at $p < 0.05$ (two-way ANOVA following Tukey's test)

To understand the relationship between *ABI5* and *VTC2*, we first compared the different transcriptional levels of *VTC2* between the *abi5* mutant and *ABI5-MYC* lines before or after aging treatment by RT–qPCR analysis. As shown in Fig. 5D, overexpressing *ABI5* suppressed the transcription of *VTC2*, whereas the loss-of-function *abi5* mutant contained more *VTC2* transcripts after aging, suggesting that *ABI5* negatively regulates the expression of *VTC2* after aging. Moreover, we found that aging increased the protein abundance of ABI5 in the *ABI5-MYC* line (Fig. 5C). A high level of ABI5 may suppress the expression of *VTC2*, thus increasing H_2O_2 accumulation to decrease seed viability. We then crossed the *ABI5-MYC* or *abi5* mutant with the *vtc2* mutant to obtain *ABI5-MYC/vtc2* and *abi5/vtc2*. Genetic analysis revealed

that both *ABI5-MYC/vtc2* and *abi5/vtc2* contained higher levels of H_2O_2 after aging treatment, similar to the *vtc2* mutant (Fig. 5A). Similarly, the seed germination of *ABI5-MYC/vtc2* and *abi5/vtc2* was also lower than that of Col, similar to that of *vtc2* (Fig. 5B). These data suggest that *ABI5* controls H_2O_2 accumulation and seed viability through *VTC2* during aging treatment.

4 Discussion

4.1 ANAC089 Acts as a New Modulator to Control Seed Viability through H_2O_2 Signaling

A previous study showed that *ANAC089* controls the cytosolic redox status to regulate ABA signaling and control seed germination [25,26]. Additionally, *ANAC089* accelerated the expression of *FLC* to delay flowering [28]. Here, we reported that *ANAC089* controls seed viability. Using the artificial CDT method to simulate the process of seed deterioration, we observed a rapid increase in the *ANAC089* transcript during the early stage of CDT, after which the *ANAC089* transcript decreased quickly during the subsequent long-term treatment with CDT (Fig. 2A), suggesting that *ANAC089* may function during the seed aging process. Furthermore, overexpressing *ANAC089* enhanced seed viability, whereas silencing *ANAC089* reduced seed viability, as determined by tetrazolium staining analysis (Figs. 1D and 1E). A previous study also confirmed that a higher level of ROS, mainly H_2O_2, promoted seed deterioration and decreased seed germination after long-term conservation [14,21,22]. Our previous study also showed that high accumulation of H_2O_2 impaired seed viability during the desiccation process for recalcitrant seeds [35,36]. Here, we also revealed that *ANAC089* affects the biosynthesis of H_2O_2 after CDT treatment. In agreement with the seed germination phenotype, the H_2O_2 content decreased in the transgenic *ANAC089-Flag* line but increased in the *anac089* mutant line (Figs. 1B and 1C), which suggested that increased H_2O_2 may be the main factor affecting the viability of *anac089* seeds during CDT. As vitamin C, or ascorbic acid (AsA), is an efficient antioxidant for removing H_2O_2, *VTC2* is the critical enzyme for AsA biosynthesis, and the *vtc2* mutant cannot scavenge H_2O_2 after stress treatment. Here, we also found a high H_2O_2 content and decreased seed germination of *vtc2* after CDT treatment. Moreover, direct treatment of wild-type Col seeds with H_2O_2 also suppressed seed germination, while the addition of AsA to the *vtc2* mutant partially rescued the decreased seed germination of *vtc2* after CDT treatment (Fig. S2). These data support the hypothesis that a high concentration of H_2O_2 is the main factor affecting seed viability after CDT. Further genetic analysis revealed that the *ANAC089-Flag/vtc2* crossed line presented greater H_2O_2 content and lower seed germination than did the *ANAC089* line, which presented relatively lower H_2O_2 content and greater seed germination (Figs. 2B and 2C). Moreover, compared with low H_2O_2, additional AsA partially increased the germination of *ANAC089-Flag/vtc2* seeds after CDT treatment (Fig. S3). Based on these results, we propose that *ANAC089* enhances the deterioration of seed viability after long-term storage by attenuating the toxic effects of H_2O_2.

4.2 The ABI5-VTC2 Module Mediates ANAC089-Mediated Regulation of ROS Signaling during the Deterioration of Seed Viability

A previous study demonstrated that *ANAC060* can bind the G-box within the promoter of *ABI5* to suppress the transcription of *ABI5* [34]. Here, we further elucidated the relationship between *ANAC089* and *ABI5* and found that *ANAC089*, which is homologous to *ANAC060*, can also negatively regulate the expression of *ABI5* during CDT, as the transcriptional level of *ABI5* decreased in the *ANAC089-Flag* line but increased in the *abi5* mutant line after CDT treatment (Fig. 3A). Furthermore, ChIP and EMSA confirmed that *ANAC089* can recognize the G-box within the promoter of *ABI5* to suppress the expression of *ABI5* (Figs. 3B and 3C). Protoplast transient transformation experiments showed that *ANAC089* obviously suppressed the expression of *ABI5*, but deletion of the G-box within the promoter of *ABI5* dramatically reduced this inhibitory effect (Fig. 4A), suggesting that *ANAC089* specifically binds the G-box within the promoter of *ABI5* to suppress the expression of *ABI5*. Genetic experiments showed that both *ANAC089-Flag/vtc2* and *anac089/vtc2* showed lower seed viability and lower seed germination

after CDT treatment, similar to *vtc2* (Fig. 4C), suggesting that *ANAC089* required *VTC2* to control the deterioration of seed viability during long-term storage.

ABI5 acts as an important regulator to control ABA signaling [37]. Our previous results also showed that ABI5 interacts with its partner protein AFP2 to control seed germination under high-temperature treatment by binding to the *SOMNUS* gene [38]. In this study, we found that simulating the seed aging process using the CDT method induced the accumulation of the *ABI5* protein in the stored seeds (Fig. 5C). Accordingly, the *abi5* mutant showed high seed germination, but overexpressing *ABI5* reduced seed germination after CDT (Fig. 4C), suggesting that *ABI5* negatively regulates seed viability during the seed aging process. In agreement with our above discussion, the level of H_2O_2 was also greater in the *ABI5*-overexpressing line but lower in the *abi5* mutant line (Fig. 4B), suggesting that *ABI5* controls seed viability through ROS metabolism. Previous studies reported that *ABI4* can bind to the promoter of *VTC2* to control its expression during saline stress. As both *ABI4* and *ABI5* are important transmitters of ABA signaling [39], we also found that *ABI5* affects the expression of *VTC2* during CDT treatment, and the transcriptional level of *VTC2* was greater in the *abi5* mutant but lower in the *ABI5*-overexpressing line after CDT (Fig. 5D), suggesting that ABI5 negatively regulates the transcriptional level of *VTC2*, similar to *ABI4*, after environmental stress. This result also coincides with our above results showing the accumulation pattern of H_2O_2 and seed viability in the *abi5* or *ABI5-MYC* lines after CDT and suggests that *ABI5* affects the expression of *VTC2* to control H_2O_2 levels and seed viability during the seed aging process. Moreover, unlike in the *anac089* mutant line, which had a relatively high H_2O_2 content after CDT, the H_2O_2 level in both the *ANAC089-Flag* and *abi5* lines was relatively lower than that in the transgenic *ABI5-Myc* line after CDT. These data further confirm our above conclusion and suggest that *ANAC089* enhances seed viability during seed aging by suppressing *ABI5*, thereby upregulating *VTC2* levels to scavenge ROS.

5 Conclusion

In summary, we reported the novel function of *ANAC089* in controlling seed viability. A series of physiological, biochemical, and genetic studies showed that *ANAC089* can specifically bind the G-box region within the promoter of *ABI5* to negatively regulate the expression of *ABI5*, while *ABI5* further negatively regulates the expression of *VTC2*, thereby scavenging H_2O_2 during the seed aging process. As a result, *ANAC089* suppresses H_2O_2 signaling to enhance seed viability or longevity through the *ABI5-VTC2* module. Thus, our findings reveal the mechanism by which the *ANAC089-ABI5-VTC2* axis controls seed viability through ROS metabolism, providing a possible strategy for the genetic engineering of *ANAC089* to increase seed viability and prolong seed conservation in the field of seed biology.

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Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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