antioxidants

MDPI

Article

H_2O_2 and Ca^{2+} Signaling Crosstalk Counteracts ABA to Induce Seed Germination

Mengjie Cheng [1,†], Yanliang Guo [1,†], Qing Liu [1], Sanwa Nan [1], Yuxing Xue [1], Chunhua Wei [1], Yong Zhang [1], Feishi Luan [2], Xian Zhang [1] and Hao Li [1,*]

[1] College of Horticulture, Northwest A&F University, Yangling 712100, China
[2] College of Horticulture and Landscape Architecture, Northeast Agricultural University, Harbin 150000, China
* Correspondence: lhyy@nwafu.edu.cn
† These authors contributed equally to this work.

Abstract: Seed germination is a critical stage and the first step in the plant's life cycle. H_2O_2 and Ca^{2+} act as important signal molecules in regulating plant growth and development and in providing defense against numerous stresses; however, their crosstalk in modulating seed germination remains largely unaddressed. In the current study, we report that H_2O_2 and Ca^{2+} counteracted abscisic acid (ABA) to induce seed germination in melon and *Arabidopsis* by modulating ABA and gibberellic acid (GA_3) balance. H_2O_2 treatment induced a Ca^{2+} influx in melon seeds accompanied by the upregulation of *cyclic nucleotide-gated ion channel (CNGC) 20*, which encodes a plasma membrane Ca^{2+}-permeable channel. However, the inhibition of cytoplasmic free Ca^{2+} elevation in the melon seeds and *Arabidopsis* mutant *atcngc20* compromised H_2O_2-induced germination under ABA stress. $CaCl_2$ induced H_2O_2 accumulation accompanied by the upregulation of *respiratory burst oxidase homologue (RBOH) D* and *RBOHF* in melon seeds with ABA pretreatment. However, inhibition of H_2O_2 accumulation in the melon seeds and *Arabidopsis* mutant *atrbohd* and *atrbohf* abolished $CaCl_2$-induced germination under ABA stress. The current study reveals a novel mechanism in which H_2O_2 and Ca^{2+} signaling crosstalk offsets ABA to induce seed germination. H_2O_2 induces Ca^{2+} influx, which in turn increases H_2O_2 accumulation, thus forming a reciprocal positive-regulatory loop to maintain a balance between ABA and GA_3 and promote seed germination under ABA stress.

Keywords: hydrogen peroxide; calcium signal; abscisic acid; gibberellic acid; seed germination

Citation: Cheng, M.; Guo, Y.; Liu, Q.; Nan, S.; Xue, Y.; Wei, C.; Zhang, Y.; Luan, F.; Zhang, X.; Li, H. H_2O_2 and Ca^{2+} Signaling Crosstalk Counteracts ABA to Induce Seed Germination. *Antioxidants* **2022**, *11*, 1594. https://doi.org/10.3390/antiox11081594

Academic Editors: Sophie Vriz, Christine Rampon and Stanley Omaye

Received: 24 June 2022
Accepted: 16 August 2022
Published: 17 August 2022

1. Introduction

Seed germination is the first and vital step in a plant's life cycle [1]. This physiological process starts with water absorption by dry seeds and ends with radicle emergence, during which the imbibed seeds shift from a quiescent state to active metabolism [2]. The transition from seed dormancy to germination involves simultaneous seed reserve mobilization and seed coat rupture, which cumulate in radicle emergence and subsequent seedling establishment [3,4]. Seed germination is a complex process that depends on multiple environmental factors, such as temperature, water availability, light, and oxygen, as well as certain intrinsic factors such as phytohormones [5]. Both poor seed quality and sowing conditions adversely affect seed germination and subsequent crop establishment, health, and yield [6].

Abscisic acid (ABA) and gibberellic acid (GA) are two central hormones that transduce environmental information and play antagonistic roles in regulating seed germination [7]. In fact, seed dormancy or germination largely depends on the dynamic balance of ABA and GA [8]. ABA promotes seed dormancy but inhibits seed germination. Knockout of ABA biosynthesis- or signaling-related genes promoted seed germination and reduced seed dormancy [9]. However, the mutation of ABA catabolic enzymes or overexpression of ABA biosynthetic enzymes delays seed germination but prolongs seed dormancy [2,10].

Conversely, GA breaks seed dormancy and promotes seed germination by activating the growth potential of embryos and weakening the embryo surrounding tissues [11].

In plants, reactive oxygen species (ROS), such as hydroxyl radicals ($OH^{\cdot}$), superoxide (O_2^{-}), and hydrogen peroxide (H_2O_2), play important roles in regulating seed dormancy or germination [12,13]. The controlled germination of ROS during seed imbibition can oxidize a subset of biomolecules such as mRNAs, nucleic acids, amino acids, and proteins, resulting in adequate cell functions that trigger seed germination [14]. In particular, H_2O_2 generated by *respiratory burst oxidase homologues* (*RBOHs*)-encoded NADPH oxidase is a critical secondary signaling molecule that regulates ABA and GA balance and affects their signaling pathways in regulating seed germination [15,16].

In addition to ROS, calcium (Ca^{2+}) functions as a vital second messenger and plays an important role in regulating various physiological processes, including seed germination [17,18]. The Ca^{2+} signal functions by eliciting characteristic transient fluctuations of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration through membrane transport proteins-mediated activities of Ca^{2+} influx and efflux [19]. Activated Ca^{2+} influx channels, such as two-pore channels (TPCs), glutamate receptor homolog (GLR) 3.5, and cyclic nucleotide-gated ion channels (CNGCs), induce a transient $[Ca^{2+}]_{cyt}$ elevation and then trigger Ca^{2+} signal, which is decoded and relayed to downstream regulators of gene expression by a set of Ca^{2+} sensor proteins [19]. Abundant evidence suggests that the Ca^{2+} signal plays an important role in regulating seed germination by altering ABA and GA signaling [20,21].

Despite abundant studies on the individual role of H_2O_2 and Ca^{2+} in regulating seed germination, their potential interaction in modulating ABA/GA balance and subsequent seed germination remains largely unknown. The crosstalk of H_2O_2 (a major ROS) and Ca^{2+} regulating plant growth and development and its role as a defense against numerous stresses have been well documented [22]. For example, in plant response to cold exposure, Ca^{2+} signal is rapidly induced and then activates RBOH activity to trigger H_2O_2 production, which in turn induces Ca^{2+} transient influx in plant cells, forming a reciprocal positive-regulatory loop [23–25]. This raises the possibility that H_2O_2 and Ca^{2+} may also interact with each other in modulating seed germination. We demonstrate that the positive crosstalk of H_2O_2 and Ca^{2+} promotes seed germination under ABA stress. H_2O_2 promotes Ca^{2+} influx, which in turn increases H_2O_2 accumulation to regulate ABA and GA balance.

2. Materials and Methods

2.1. Plant Materials

In this study, melon (*Cucumis melo* L. cv. SSMA) seeds were provided by the Watermelon and Melon Research Group of Northwest A&F University, Yangling, China. Seeds of *Arabidopsis* mutant *atrbohd* (SALK_120299), *atrbohf* (SALK_034674), and *atcngc20* (SALK_074919C) with a Columbia genetic background were obtained from the Arabidopsis Biological Resource Center (https://www.arabidopsis.org/, accessed on 1 June 2018).

2.2. Experimental Design

To investigate the role and mechanisms of H_2O_2 and Ca^{2+} in counteracting ABA to promote seed germination, the melon or *Arabidopsis* seeds were soaked in double distilled water (as control) or different test solutions for 7 h and then were rinsed three times with double-distilled water. The rinsed melon seeds were incubated at 30 °C in the dark for 7 days in 9 cm diameter Petri dishes containing double-layered rolled filter paper moistened with distilled water. The rinsed *Arabidopsis* seeds were incubated at 21 °C under continuous light at 100 μM m^{-2} s^{-1} for 7 days in 3 cm Petri dishes containing moistened filter papers. Each treatment comprised three replicates. Each replicate consisted of 30 seeds. Seeds were considered germinated when the radicle emerged (1–2 mm). Germination was scored daily as radicle emergence.

Test solutions used to treat melon seeds included ABA (1 mM), ABA (1 mM) + H_2O_2 (10 mM), ABA (1 mM) + $CaCl_2$ (0.2, 0.5, 1, or 3 mM), ABA (1 mM) + diphenyleneiodonium (DPI, an inhibitor of NADPH oxidases, which produces ROS, 10 μM) [26–28], ABH (1 mM

ABA + 10 mM H_2O_2) + ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, a Ca^{2+} chelator, 5 mM) [18,29], ABH + $LaCl_3$ (a plasma membrane-located Ca^{2+} channel blocker, 5 mM) [18,29], ACa (1 mM ABA + 1 mM $CaCl_2$) + DPI (10 µM). Test solutions used to treat *Arabidopsis* seeds included ABA (0.2 mM), ABA (0.2 mM) + H_2O_2 (5 mM), ABA (0.2 mM) + $CaCl_2$ (0.2, 0.5, or 1 mM). The concentrations of the different chemicals are referred to Li et al. [30].

Notably, the chemical concentrations were higher in this study than those in previous studies. These high concentrations of chemicals were also used to assess their functions in various plant species [27,30]. The chemical concentrations were justified previously by using a range of concentrations, and the optimum concentration was used [30]. Generally, in previous studies, the chemicals such as ABA were added to the incubation medium during seed germination for several days. In the current study, the seeds were soaked with test solutions for 7 h and then rinsed three times with distilled water. Such treatments can increase endogenous chemical content and avoid exogenous chemicals' constant and complex influence during seed incubation. After ABA (1 mM) pretreatment for 7 h, the endogenous ABA in melon seeds increased from 16.8 to 133.6 ng g^{-1} [30]. Therefore, a high concentration of ABA did not completely inhibit seed germination owing to the short treatment time (see Figure 1).

2.3. Net Ca^{2+} Flux Assay

The net Ca^{2+} flux of the seed embryo cells was measured with a Noninvasive Micro-test Technology (NMT) Physiolyzer (Xuyue Science and Technology Company Limited, Beijing, China) following the method of Li et al. [30]. The flux unit is pmol cm^{-2} s^{-1}, and the negative and positive values represent Ca^{2+} influx and efflux, respectively.

2.4. Analysis of H_2O_2

The H_2O_2 content in seeds was measured according to the method described by Willekens et al. [31]. In brief, 0.2 g samples were ground in 3 mL of ice-cold $HClO_4$ (1 M). After centrifugation at 6000× *g* for 5 min at 4 °C, the supernatant pH was adjusted to 6.0–7.0 with KOH (4 M). The supernatants were further centrifuged at 12,000× *g* for 5 min at 4 °C, passed through an AG1-X8 prepacked column (Bio-Rad, Hercules, CA, USA), and then were eluted with 4 mL ddH_2O. The sample extract (800 µL) was combined with 400 µL of 100 mM potassium acetate (pH 4.4) containing horseradish peroxidase (0.25 U), 2,2′-azino-di (3-ethylbenzthiazoline-6-sulfonic acid) (4 mM), and 400 µL deionized water. The H_2O_2 content was measured at OD_{412}.

2.5. Analysis of ABA and GA_3

The plant hormones were extracted as described by Yang et al. [32] with minor modifications. In brief, 0.3 g of frozen seed samples was homogenized in 4 mL of methanol (80%, *v*/*v*) containing 1 mM 2,6-di-*t*-butyl-*p*-cresol as an antioxidant. The homogenates were incubated at 4 °C for 4 h and then were centrifuged at 1000× *g* for 20 min at 4 °C. The obtained supernatants (crude extracts) were filtered through C18 Sep-Park Cartridge (Millipore, Millford, MA, USA), dried under N_2 (gas) flow, and then were dissolved in 5 mL of 50 mM Tris (pH 8.1) containing 10% (*v*/*v*) methanol, 1 mM $MgCl_2$, and 150 mM NaCl. ABA and GA_3 contents were analyzed with an immunoassay kit (China Agricultural University, Beijing, China) following the manufacturer's instructions. Colorimetric readings were conducted using a Multimode Plate Reader M200 pro (Tecan, Männedorf, Switzerland).

2.6. qRT-PCR Analysis

The total RNA were isolated from the seeds using an RNA extraction kit (Axgen, Union City, CA, USA). After extraction, a DNase Mini Kit (Qiagen, Hilden, Germany) was used to remove residual DNA. Then, total RNA (1 µg per sample) was reverse-transcribed to cDNA using a FastKing RT kit (TIANGEN, Beijing, China). qRT-PCR was conducted on an iCycler Iq TM Multicolor PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR®

Premix ExTaq™ II (2×) kit (Takara, Tokyo, Japan). The gene-specific primers were designed according to the EST sequences (http://cucurbitgenomics.org/, accessed on 1 March 2018): 5′-TTGGTGCTGGCGAATTGGTTGA-3′ and 5′-ATGATCTGAGGCAGCGGCAAA-3′ for *CmCNGC20* (MELO3C001941); 5′-AGTGAGTGACAGCCGAGTTCTAAGT-3′ and 5′-CTGCTCTGTGACGGTATTGGATGAA-3′ for *CmRBOHD* (MELO3C026754); 5′-GCACGAGTTGAAGGCTGAGTTGA-3′ and 5′-GGAATCCATCCTTGGCGAGCTTATC′ for *CmRBOHF* (MELO3C005718); and 5′-ATTCTTGCATCTCTAAGTACCTTCC-3′ and 5′-CCAACTAAAGGGAAATAACTCACC-3′ for *CmActin* (MELO3C008032). *CmActin* was used as the internal control genes [30]. The relative expression of mRNA was calculated as described previously [33].

2.7. Statistical Analysis

The experiment was a completely randomized design with three independent replications. Each replicate included 30 seeds. Analysis of variance (ANOVA) was used to test for significance, and significant differences among treatments were determined using Tukey's test at the $p < 0.05$ level.

3. Results

3.1. H_2O_2 and Ca^{2+} Counteract ABA to Induce Seed Germination

Exogenous ABA pretreatment remarkably delayed germinating time and severely reduced germination rates of both melon and *Arabidopsis* seeds (Figure 1). However, exogenous H_2O_2 or $CaCl_2$ pretreatment attenuated the ABA-induced inhibition of seed germination in melon and *Arabidopsis* seeds. The most effective concentration of $CaCl_2$ was 1 mM and 0.5 mM for melon and *Arabidopsis* seeds, respectively. The promoting effect of $CaCl_2$ on seed germination was attenuated with $CaCl_2$ concentrations both lower and higher than the optimum concentration. For example, the germination rates of melon seeds pretreated with ABA (1 mM) + H_2O_2 (10 mM) and ABA (1 mM) + $CaCl_2$ (1 mM) were 53.3% and 74.4%, respectively, far higher than that of seeds with ABA pretreatment.

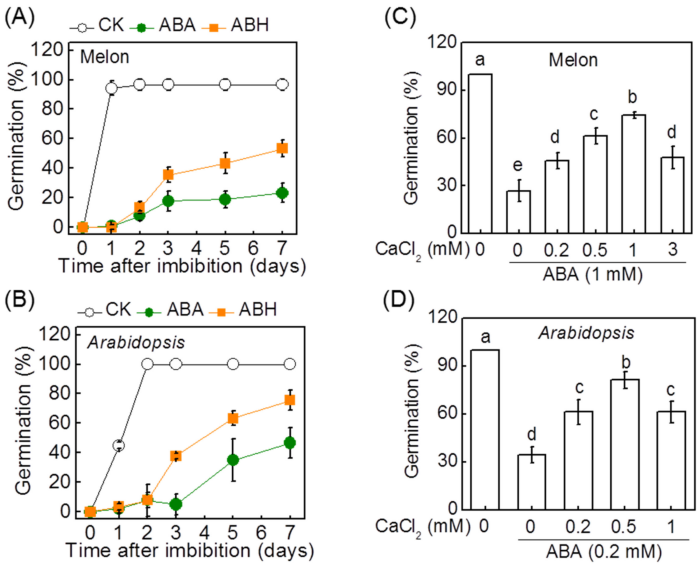

Figure 1. H_2O_2 and Ca^{2+} counteract abscisic acid (ABA) to promote seed germination in melon and *Arabidopsis*. (**A**,**B**) The effects of ABA and the combination of ABA and H_2O_2 (ABH) on the germination of melon and *Arabidopsis* seeds. (**C**,**D**) The effects of ABA and the combination of ABA and $CaCl_2$ (ACa) on the germination of melon and *Arabidopsis* seeds. The melon seeds were presoaked in double-distilled water or test solutions for 7 h and then were incubated at 30 °C for 7 days. In (**C**,**D**), the germination rates were recorded on the seventh day after seed incubation. Data are means ± SD (3 replicates × 30 seeds). The different letters denote a significant difference ($p < 0.05$) according to Tukey's test.

3.2. Ca^{2+} Signal Is Involved in H_2O_2-Induced Seed Germination

To investigate whether the Ca^{2+} signal participates in H_2O_2-induced seed germination under ABA stress, we firstly analyzed the effects of H_2O_2 and its deficiency on Ca^{2+} flux. As shown in Figure 2, the ABA pretreatment had very little effect on the net flux of Ca^{2+} in both melon and *Arabidopsis* seeds. Compared to ABA, ABH (ABA+H_2O_2) pretreatment induced Ca^{2+} influx, accompanied by an increase in the transcript level of *CmCNGC20*, a gene encoding a plasma membrane Ca^{2+}-permeable channel (Figure 2A,B). However, ABA+DPI (an inhibitor of NADPH oxidase-mediated ROS generation, 10 μM) decreased Ca^{2+} influx and downregulated the expression of *CmCNGC20*. Consistently, *atrbohd* and *atrbohf* mutation in *Arabidopsis* seeds inhibited Ca^{2+} influx under both control conditions and ABA stress (Figure 2C). These results suggest that RBOH-dependent ROS mediates Ca^{2+} influx during seed germination under ABA stress.

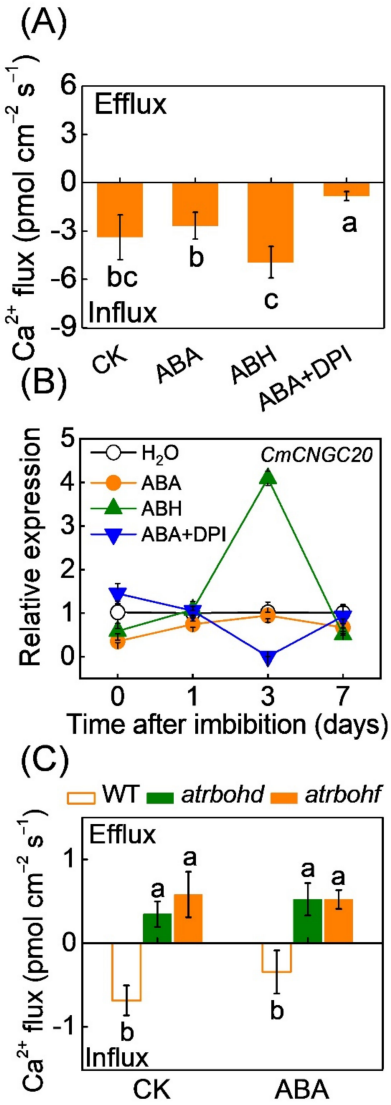

Figure 2. The effects of H_2O_2 on Ca^{2+} flux in seeds under ABA stress. (**A**,**B**) Ca^{2+} flux and *CmCNGC20* expression in melon seeds pre-treated with ABA, a combination of ABA and H_2O_2 (ABH), and a combination of ABA and diphenyleneiodonium (DPI, an inhibitor of NADPH oxidases, which produces H_2O_2). (**C**) Ca^{2+} flux in seeds of wild-type (WT) *Arabidopsis* and *atrbohd* and *atrbohf* mutants under normal conditions and ABA stress. In (**A**,**C**), dry melon seeds or *Arabidopsis* were soaked in double distilled water or test solutions for 7 h and then were used to analyze the changes in Ca^{2+} flux. In (**B**), melon seeds were treated as that in Figure 1. Data are means ± SD (3 replicates × 30 seeds). The different letters denote a significant difference ($p < 0.05$) according to Tukey's test.

To further investigate the role of Ca^{2+} signals in H_2O_2-induced seed germination and ABA/GA_3 balance under ABA stress, EGTA (a Ca^{2+} chelator) and $LaCl_3$ (a plasma membrane-located Ca^{2+} channel blocker) were used to chelate Ca^{2+} and block Ca^{2+} influx, respectively. As shown in Figure 3A, both EGTA and $LaCl_3$ inhibited H_2O_2-induced seed germination under ABA stress. The germination rates of seeds pretreated with ABA, ABA+H_2O_2 (ABH), ABH+EGTA, and ABH+$LaCl_3$ were 36.7%, 90.0%, 58.9%, and 57.8%, respectively. EGTA but not $LaCl_3$ promoted ABH-induced decrease in ABA level (Figure 3B). However, both EGTA and $LaCl_3$ completely compromised the ABH-induced increase of GA level. As a result, $LaCl_3$ but not EGTA abolished ABH-decreased ABA and GA_3 ratio. The ABA/GA_3 ratio in seeds with ABH+$LaCl_3$ pretreatment was 29.0, significantly higher than that (14.7) in seeds with ABH pretreatment. When compared to wild-type *Arabidopsis* seeds, seeds of *atcngc20* mutant showed lower germination rates under ABA stress (Figure 3D). However, *AtCNGC20* deletion attenuated H_2O_2-induced seed germination under ABA stress.

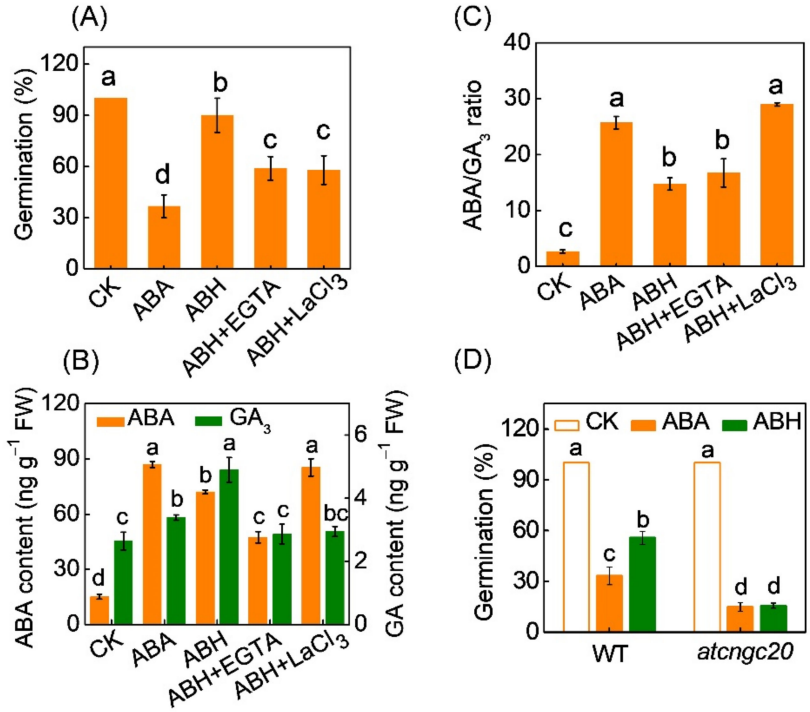

Figure 3. The role of Ca^{2+} in H_2O_2 counteracting ABA to promote seed germination. (**A**) The germination rates of melon seeds treated with ABA, a combination of ABA and H_2O_2 (ABH), a combination of ABH and EGTA (a Ca^{2+} chelator), and a combination of ABH and $LaCl_3$ (a plasma membrane-located Ca^{2+} channel blocker). (**B**,**C**) ABA and GA_3 content and ABA/GA_3 ratio in melon seeds treated with ABA, ABH, ABH+EGTA, and ABH+$LaCl_3$. ABA and GA_3 contents were measured on the third day after seed incubation. (**D**) The effects of ABA and ABH on the germination rates of seeds in wild-type (WT) *Arabidopsis* and *atcngc20* mutant. In both (**A**,**D**), germination rates were recorded on the seventh day after seed incubation. Data are means ± SD (3 replicates × 30 seeds). The different letters denote significant difference ($p < 0.05$) according to Tukey's test.

3.3. H_2O_2 Mediates Ca^{2+}-Induced Seed Germination

To investigate whether H_2O_2 was involved in Ca^{2+}-induced seed germination under ABA stress, we analyzed the effects of $CaCl_2$ on H_2O_2 accumulation under ABA stress. During incubation at 30 °C, the H_2O_2 level in melon seeds increased by ABA pretreatment from the first day, reached their peaks on the third day, and then declined gradually (Figure 4). $CaCl_2$ promoted ABA-induced H_2O_2 accumulation. On the 1st and third day of incubation at 30 °C, H_2O_2 content in melon seeds pretreated with ABA+$CaCl_2$ (ACa) were 115.2% and 25.0% higher, respectively, than that in the seeds pretreated with

ABA alone. The expression of *CmRBOHD* was increased by ABA pretreatment, and this increase was promoted by $CaCl_2$ on the first and seventh days during seed incubation. The expression of *CmRBOHF* was increased by ABA pretreatment from the third day of incubation. $CaCl_2$ upregulated *CmRBOHF* expression in early response to ABA (within one day after seed incubation).

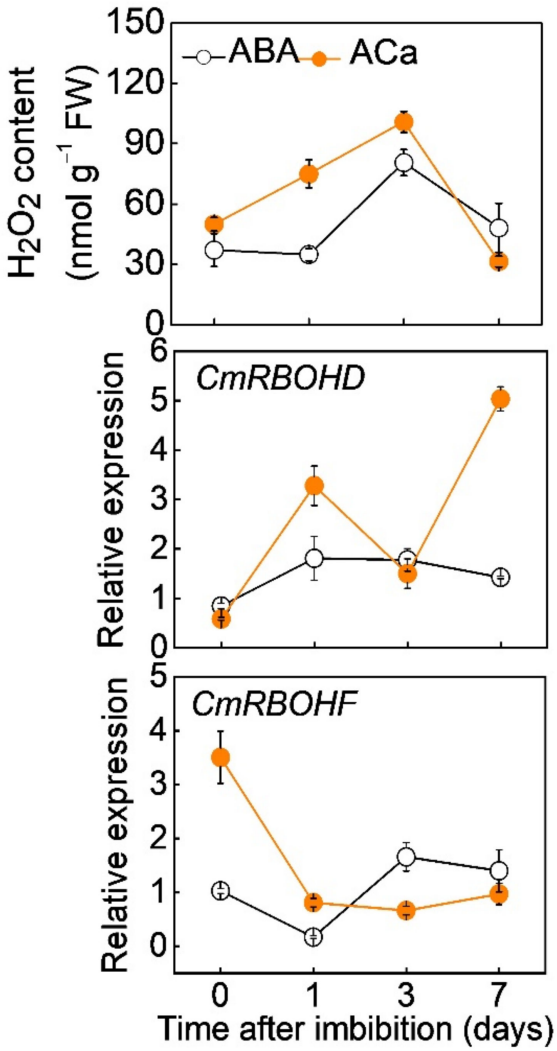

Figure 4. The effects of ABA and combination of ABA and $CaCl_2$ (ACa) on the H_2O_2 accumulation and the relative expression of *CmRBOHD* and *CmRBOHF* during melon seed incubation. Data are means ± SD (3 replicates × 30 seeds).

As well as H_2O_2, $CaCl_2$ induced seed germination, ABA decrease, GA increase, and decrease of ABA/GA ratio under ABA stress (Figure 5). To further investigate the role of H_2O_2 in Ca^{2+}-induced seed germination and ABA/GA_3 balance under ABA stress, DPI, an inhibitor of NADPH oxidases, was used to inhibit H_2O_2 production. DPI attenuated or completely abolished Ca^{2+}-induced seed germination, ABA decrease, GA increase, and decrease of ABA/GA ratio under ABA stress.

When compared to wild-type *Arabidopsis* seeds, seeds of the *atrbohd* and *atrbohf* mutants showed higher germination rates under ABA stress (Figure 6), suggesting that H_2O_2 is involved in ABA-induced inhibition of seed germination. However, the deletion of *AtRBOHD* and *AtRBOHF* completely abolished $CaCl_2$-induced seed germination under ABA stress. Taken together, these results indicated that the Ca^{2+} and H_2O_2 signals interacted with each other, forming a reciprocal positive-regulatory loop, which antagonize ABA to promote seed germination.

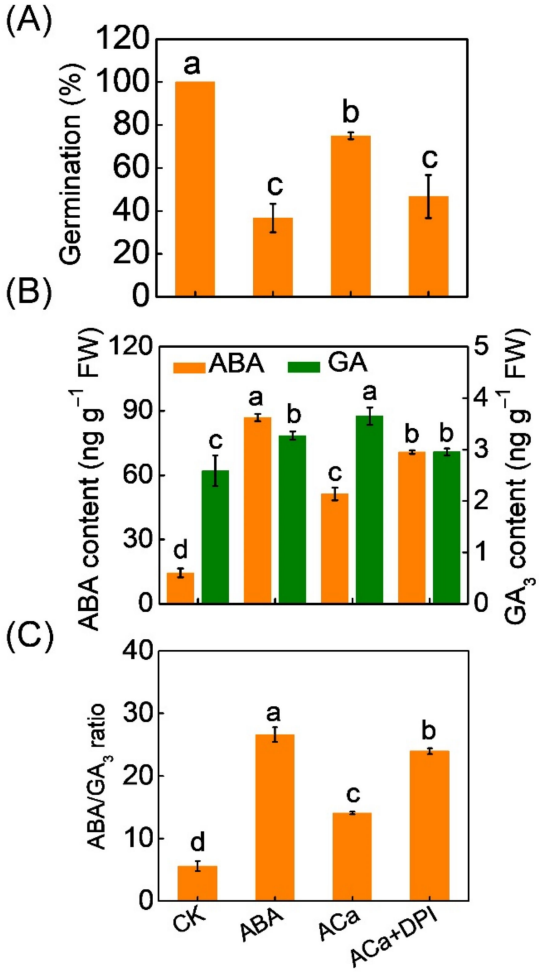

Figure 5. Involvement of H_2O_2 in Ca^{2+} counteracting ABA to promote seed germination. (**A**) The germination rate of melon seeds treated with ABA, a combination of ABA and $CaCl_2$ (ACa), and a combination of ACa and diphenyleneiodonium (DPI, an inhibitor of NADPH oxidases, which produces ROS). Germination rates were recorded on the seventh day after seed incubation. (**B**,**C**) ABA and GA_3 content and ABA/GA_3 ratio in melon seeds treated with ABA, a combination of ABA and $CaCl_2$ (ACa), and a combination of ACa and DPI. ABA and GA_3 contents were measured on the third day after seed incubation. Data are means ± SD (3 replicates × 30 seeds). The different letters denote a significant difference ($p < 0.05$) according to Tukey's test.

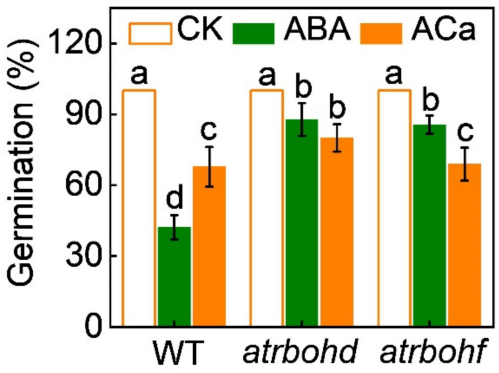

Figure 6. The effects of ABA and combination of ABA and $CaCl_2$ (ACa) on the germination rates of seeds in wild-type (WT) *Arabidopsis* and *atrbohd* and *atrbohf* mutants. Germination rates were recorded on the seventh day after seed incubation. Data are means ± SD (3 replicates × 30 seeds). The different letters denote a significant difference ($p < 0.05$) according to Tukey's test.

4. Discussion

ABA and GA are two critical plant hormones and function antagonistically in regulating seed germination [7]. In the seeds exposed to salt and cold temperature, ABA accumulation was increased but GA_3 accumulation was not decreased, indicating that ABA increase is the principal factor that disturbs the balance between ABA and GA_3 and consequently inhibits seed germination unfavorable conditions [30].

As an important signal molecule, ROS generated by *RBOHs*, which encode NADPH oxidase, plays a dual role in regulating seed germination [30,34]. The H_2O_2 levels transiently increase during seed germination and inhibition of H_2O_2 production by NADPH inhibitor DPI or *AtRBOHD* deletion suppresses seed germination [35], suggesting that transient H_2O_2 generation is required for seed germination [14]. However, *atrbohd* and *atrbohf* double mutations impair ABA-induced promotion of ROS production and inhibition of seed germination, indicating that H_2O_2 was involved in ABA-induced seed dormancy [36].

In the present study, we found that exogenous H_2O_2 alleviated ABA-induced inhibition of seed germination, providing evidence on H_2O_2-ABA antagonism in regulating seed germination (Figures 1 and 3). However, ABA pretreatment increased H_2O_2 accumulation and the transcript levels of *RBOHD* and *RBOHF* in melon seeds and *Arabidopsis* seeds with *AtRBOHD* or *AtRBOHF* deletion showed less sensitivity to ABA, indicating that H_2O_2 is required for ABA-induced seed dormancy (Figures 4 and 6) [36]. In addition, the uncontrolled accumulation of H_2O_2 under stress conditions or during seed aging also inhibits seed germination [35]. Therefore, the occurrence of seed germination progresses is restricted to a critical range of H_2O_2 levels [34]. H_2O_2 at low or high levels would not permit progress toward germination. The previous study by Liu et al. showed that exogenous H_2O_2 promote ABA catabolism and GA biosynthesis during *Arabidopsis* seed imbibition [37]. Consistently, we found that exogenous H_2O_2 decreased the ABA level and increased the GA_3 level during seed germination under ABA stress, suggesting that H_2O_2-induced seed germination was closely associated with its regulatory role in balancing ABA/GA_3 (Figure 3).

Similar to H_2O_2, Ca^{2+} has a critical secondary signaling molecule and functions synergistically or antagonistically with ABA signaling during seed germination. For example, the repression of *Arabidopsis GLR3.5* (*AtGLR3.5*), which encodes a plasma membrane Ca^{2+}-permeable channel, impairs $[Ca^{2+}]_{cyt}$ elevation and enhances seed sensitivity to ABA, whereas *AtGLR3.5* overexpression promotes seed germination but reduce seed sensitivity to ABA by suppressing the expression of *ABSCISIC ACID INSENSITIVE4*, suggestive of a negative role of Ca^{2+} in ABA signaling [38]. However, the deletion of *TPC1* (a vacuolar Ca^{2+} channel gene) and Ca^{2+} signaling-related genes (e.g., *calcium-dependent protein kinase (CPK) 4*, *CPK11*, and the *calmodulin-like protein 39*) results in insensitivity to ABA during seed germination, suggestive of a positive role of the Ca^{2+} signal in ABA signaling [39–41].

In the current study, we found that $CaCl_2$ pretreatment decreased the ABA/GA_3 ratio likely by promoting ABA catabolism and GA_3 biosynthesis and thus promoted seed germination under ABA stress, suggesting that cytoplasmic Ca^{2+} signaling acts as a positive regulator in ABA-regulated seed germination. Therefore, the Ca^{2+} signal plays a dual role in seed germination response to ABA as well as H_2O_2. Extracellular Ca^{2+} enters the cytosol through plasma membrane Ca^{2+}-permeable channels to positively regulate seed germination, whereas the Ca^{2+} release from the vacuole through vacuolar channels is required for ABA signaling that inhibits seed germination.

The crosstalk between the H_2O_2 derived from RBOHs and the Ca^{2+} signal is well documented in various physiological actions [22]. Evidently, the H_2O_2-triggered influx of Ca^{2+} has long been thought to be involved in H_2O_2 sensing and signalling [42]. Ca^{2+} acts as a key downstream component of AtRBOHD and AtRBOHF, transducing ROS signals in plant growth and in responses to various stresses [36,43–45]. For example, in ABA signalling in guard cells, H_2O_2 activates Ca^{2+}-permeable channels and induces influx of Ca^{2+} and increases in $[Ca^{2+}]_{cyt}$ in guard cells, which mediate stomatal closure induced by ABA [46,47]. However, the crosstalk between H_2O_2 and Ca^{2+} in regulating

seed germination remains largely unknown. In the current study, H_2O_2 promoted Ca^{2+} influx in melon seeds under ABA stress, while the H_2O_2 deficiency in both melon and *Arabidopsis* seeds prevented Ca^{2+} influx under ABA stress (Figure 2). Similarly, the *atrbohd* and *atrbohf* mutations impair the hypoxia-elicited Ca^{2+} enhancement *in Arabidopsis* root cells [48]. CNGC20 is an important Ca^{2+} transport system that conducts external Ca^{2+} in the cytoplasm [49]. H_2O_2 induced the upregulation of *CmCNGC20,* suggesting that *CmCNGC20* may be involved in H_2O_2-induced Ca^{2+} influx (Figure 2B). Moreover, blocking of Ca^{2+} influx by $LaCl_3$ or chelation of Ca^{2+} by EGTA inhibited H_2O_2-induced ABA/GA_3 balance and germination in melon seeds pretreated with ABA (Figure 3A–C). *AtCNGC20* deletion in *Arabidopsis* seeds completely abolished H_2O_2-induced seed germination under ABA stress (Figure 3D). These results indicate that a Ca^{2+} signal is involved in H_2O_2-induced ABA/GA_3 balance and subsequent seed germination under ABA stress.

Calcineurin-B-like (CBL) proteins and their interacting protein kinases (CIPKs) have been shown to function in many Ca^{2+}-signaling processes, including seed germination. The mutations of *CBL9* and *CIPK3* exhibited hypersensitivity to ABA during seed germination, suggesting a negative role of Ca^{2+} signals in ABA-inhibited seed germination [50,51]. Interestingly, a calcium signal-activated CBL1/9-CIPK26 module can enhance ROS production via phosphorylation of RBOHF [52]. CPK5 phosphorylates RBOHD and produces ROS to induce systemic defense responses [53]. Here, $CaCl_2$ increased H_2O_2 accumulation under ABA stress, accompanied by the upregulation of *CmRBOHD* and *CmRBOHF* (Figure 4).

Additional experiments showed that DPI prevented the $CaCl_2$-induced ABA/GA_3 balance and subsequent germination of melon seeds under ABA stress (Figure 5). Moreover, *AtRBOHD* and *AtRBOHF* deletion in *Arabidopsis* seeds completely abolished $CaCl_2$-induced seed germination under ABA stress (Figure 6). Therefore, it is apparent that H_2O_2 participates in Ca^{2+}-induced ABA/GA_3 balance and subsequent seed germination under ABA stress. Taken together, the H_2O_2 and Ca^{2+} signals function together in a self-amplifying feedback loop, in which H_2O_2 induces Ca^{2+} influx, and Ca^{2+} subsequently increases H_2O_2 accumulation during seed response to ABA. Such an H_2O_2/Ca^{2+} activation circuit has also been reported to be required for rapid defense signal propagation in plants [53,54].

5. Conclusions

To date, the crosstalk underlying H_2O_2 and Ca^{2+} signals antagonize ABA to promote seed germination are unclear. In this study, we show that H_2O_2 and Ca^{2+} signals interact with each other to regulate ABA/GA_3 balance and seed germination during ABA response. H_2O_2 promotes Ca^{2+} influx, which in turn increases H_2O_2 accumulation, forming a reciprocal positive-regulatory loop, to sustain Ca^{2+} influx-elicited signature and regulate ABA and GA_3 balance. To our knowledge, this is the first study of its kind to provide evidence for the role of ROS and Ca^{2+} signaling in antagonizing ABA to regulate seed germination.

Author Contributions: Conceptualization, H.L.; data curation, M.C., Y.G., Q.L., S.N. and Y.X.; Formal analysis, Y.G. and H.L.; funding acquisition, X.Z. and H.L.; investigation, M.C., Y.G., Q.L., S.N., Y.X. and C.W.; project administration, H.L.; resources, Y.Z., F.L. and X.Z.; supervision, H.L.; writing—original draft, H.L.; writing—review and editing, H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the China Agriculture Research System (CARS-25), the National Natural Science Foundation of China (31972479), the Young Science and Technology Star of Shaanxi (2022KJXX-80), and the Science and Technology Innovation Team of Shaanxi (2021TD-32).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All datasets generated for this study are included in the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Donohue, K.; Rafael, R.D.C.; Burghardt, L.; Kovach, K.; Willis, C.G. Germination, post-germination adaptation, and species ecological ranges. *Annu. Rev. Ecol. Evol. Syst.* **2010**, *41*, 293–319. [CrossRef]
2. Holdsworth, M.J.; Bentsink, L.; Soppe, W.J.J. Molecular networks regulating *Arabidopsis* seed maturation, after ripening, dormancy and germination. *New Phytol.* **2008**, *179*, 33–54. [CrossRef] [PubMed]
3. Finkelstein, R.; Reeves, W.; Ariizumi, T.; Steber, C. Molecular aspects of seed dormancy. *Annu. Rev. Plant Biol.* **2008**, *59*, 387–415. [CrossRef]
4. Rajjou, L.; Duval, M.; Gallardo, K.; Catusse, J.; Bally, J.; Job, C.; Job, D. Seed germination and vigor. *Annu. Rev. Plant Biol.* **2012**, *63*, 507–533. [CrossRef] [PubMed]
5. Barba-Espin, G.; Diaz-Vivancos, P.; Clemente-Moreno, M.J.; Albacete, A.; Faize, L.; Faize, M.; Pérez-Alfocea, F.; Hernández, J.A. Interaction between hydrogen peroxide and plant hormones during germination and the early growth of pea seedlings. *Plant Cell Environ.* **2010**, *33*, 981–994. [CrossRef]
6. Lamichhane, J.R.; Debaeke, P.; Steinberg, C.; You, M.P.; Barbetti, M.J.; Aubertot, J.N. Abiotic and biotic factors affecting crop seed germination and seedling emergence: A conceptual framework. *Plant Soil* **2018**, *432*, 1–28. [CrossRef]
7. Footitt, S.; Douterelo-Soler, I.; Clay, H.; Finch-Savage, W.E. Dormancy cycling in *Arabidopsis* seeds is controlled by seasonally distinct hormone-signaling pathways. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 20236–20241. [CrossRef]
8. Weitbrecht, K.; Müller, K.; Leubner-Metzger, G. First off the mark: Early seed germination. *J. Exp. Bot.* **2011**, *62*, 3289–3309. [CrossRef] [PubMed]
9. Finkelstein, R.R.; Gampala, S.S.L.; Rock, C.D. Abscisic acid signaling in seeds and seedlings. *Plant Cell* **2002**, *14*, S15–S45. [CrossRef]
10. Okamoto, M.; Kuwahara, A.; Seo, M.; Kushiro, T.; Asami, T.; Hirai, N.; Kamiya, H.Y.; Koshiba, T.; Nambara, E. CYP707A1 and CYP707A2, which encode abscisic acid 8′-hydroxylases, are indispensable for proper control of seed dormancy and germination in *Arabidopsis*. *Plant Physiol.* **2006**, *141*, 97–107. [CrossRef]
11. Ogawa, M.; Hanada, A.; Yamauchi, Y.; Kuwahara, A.; Kamiya, Y.; Yamaguchi, S. Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *Plant Cell* **2003**, *15*, 1591–1604. [CrossRef] [PubMed]
12. Bailly, C. Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* **2004**, *14*, 93–107. [CrossRef]
13. Gomes, M.P.; Garcia, Q.S. Reactive oxygen species and seed germination. *Biologia* **2013**, *68*, 351–357. [CrossRef]
14. El-Maarouf-Bouteau, H.; Meimoun, P.; Job, C.; Bailly, C. Role of protein and mRNA oxidation in seed dormancy and germination. *Front. Plant Sci.* **2013**, *4*, 77. [CrossRef] [PubMed]
15. Bahin, E.; Bailly, C.; Sotta, B.; Kranne, I.; Corbineau, F.; Leymarie, J. Crosstalk between reactive oxygen species and hormonal signalling pathways regulates grain dormancy in barley. *Plant Cell Environ.* **2011**, *34*, 980–993. [CrossRef] [PubMed]
16. Bailly, C. The signalling role of ROS in the regulation of seed germination and dormancy. *Biochem. J.* **2019**, *476*, 3019–3032. [CrossRef]
17. Berridge, M.J.; Lipp, P.; Bootman, M.D. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 11–21. [CrossRef]
18. Zhang, Z.; Wu, P.; Zhang, W.; Yang, Z.; Liu, H.; Ahammed, G.J.; Cui, J.X. Calcium is involved in exogenous NO-induced enhancement of photosynthesis in cucumber (*Cucumis sativus* L.) seedlings under low temperature. *Sci. Hortic.* **2020**, *261*, 108953. [CrossRef]
19. Dodd, A.N.; Kudla, J.; Sanders, D. The language of calcium signaling. *Annu. Rev. Plant Biol.* **2010**, *61*, 593–620. [CrossRef]
20. Salahshoor, F.; Kazemi, F. Effect of calcium on reducing salt stress in seed germination and early growth stage of *Festuca ovina* L. *Plant Soil Environ.* **2016**, *62*, 460–466. [CrossRef]
21. Singh, K.L.; Chaudhuri, A.; Kar, R.K. Role of peroxidase activity and Ca^{2+} in axis growth during seed germination. *Planta* **2015**, *242*, 997–1007. [CrossRef]
22. Niu, L.J.; Liao, W.B. Hydrogen peroxide signaling in plant development and abiotic responses: Crosstalk with nitric oxide and calcium. *Front. Plant Sci.* **2016**, *7*, 230. [CrossRef] [PubMed]
23. Kawarazaki, T.; Kimura, S.; Iizuka, A.; Hanamata, S.; Nibori, H.; Michikawa, M.; Imai, A.; Abe, M.; Kaya, H.; Kuchitsu, K. A low temperature-inducible protein AtSRC2 enhances the ROS-producing activity of NADPH oxidase AtRbohF. *BBA-Mol. Cell Res.* **2013**, *1833*, 2775–2780. [CrossRef] [PubMed]
24. Baxter, A.; Mittler, R.; Suzuki, N. ROS as key players in plant stress signalling. *J. Exp. Bot.* **2013**, *65*, 1229–1240. [CrossRef]
25. Yuan, P.; Yang, T.; Poovaiah, B.W. Calcium signaling-mediated plant response to cold stress. *Int. J. Mol. Sci.* **2018**, *19*, 3896. [CrossRef] [PubMed]
26. Miller, G.; Schlauch, K.; Tam, R.; Cortes, D.; Torres, M.A.; Shulaev, V.; Dangl, J.L.; Mittler, R. The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Sci. Signal.* **2009**, *2*, 45. [CrossRef]
27. Xia, X.J.; Wang, Y.J.; Zhou, Y.H.; Tao, Y.; Mao, W.H.; Shi, K.; Asami, T.; Chen, Z.X.; Yu, J.Q. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. *Plant Physiol.* **2009**, *150*, 801–814. [CrossRef]
28. Xia, X.J.; Zhou, Y.H.; Ding, J.; Shi, K.; Asami, T.; Chen, Z.X.; Yu, J.Q. Induction of systemic stress tolerance by brassinosteroid in *Cucumis sativus*. *New Phytol.* **2011**, *191*, 706–720. [CrossRef]
29. Khangembam, L.S.; Anindita, M.; Ru, K.K. Early axis growth during seed germination is gravitropic and mediated by ROS and calcium. *J. Plant Physiol.* **2017**, *216*, 181–187. [CrossRef]

30. Li, H.; Guo, Y.L.; Lan, Z.X.; Zhang, Z.X.; Ahammed, G.J.; Chang, J.J.; Zhang, Y.; Wei, C.H.; Zhang, X. Melatonin antagonizes ABA action to promote seed germination by regulating Ca^{2+} efflux and H_2O_2 accumulation. *Plant Sci.* **2021**, *303*, 110761. [CrossRef]
31. Willekens, H.; Chamnongpol, S.; Davey, M.; Schraudner, M.; Langebartels, C.; Van, M.M.; Inzé, D.; Van, C.W. Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. *EMBO J.* **1997**, *16*, 4806–4816. [CrossRef]
32. Yang, J.; Zhang, J.; Wang, Z.; Zhu, Q.; Wang, W. Hormonal changes in the grains of rice subjected to water stress during grain filling. *Plant Physiol.* **2001**, *127*, 315–323. [CrossRef]
33. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef]
34. Bailly, C.; El-Maarouf-Bouteau, H.; Corbineau, F. From intracellular signaling networks to cell death: The dual role of reactive oxygen species in seed physiology. *CR Biol.* **2008**, *331*, 806–814. [CrossRef] [PubMed]
35. Leymarie, J.; Vitkauskaité, G.; Hoang, H.H.; Gendreau, E.; Chazoule, V.; Meimoun, P.; El-Maarouf-Bouteau, H.; Bailly, C. Role of reactive oxygen species in the regulation of *Arabidopsis* seed dormancy. *Plant Cell Physiol.* **2012**, *53*, 96–106. [CrossRef]
36. Kwak, J.M.; Mori, I.C.; Pei, Z.M.; Leonhardt, N.; Torres, M.A.; Dangl, J.L.; Bloom, R.E.; Bodde, S.; Jones, J.D.; Schroeder, J.I. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J.* **2003**, *22*, 2623–2633. [CrossRef] [PubMed]
37. Liu, Y.; Ye, N.; Liu, R.; Chen, M.; Zhang, J. H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in *Arabidopsis* seed dormancy and germination. *J. Exp. Bot.* **2010**, *61*, 2979–2990. [CrossRef]
38. Kon, D.D.; Ju, C.L.; Parihar, A.; Kim, S.; Cho, D.; Kwak, J.M. *Arabidopsis* glutamate receptor homolog3.5 modulates cytosolic Ca^{2+} level to counteract effect of abscisic acid in seed germination. *Plant Physiol.* **2015**, *167*, 1630–1642. [CrossRef]
39. Peiter, E.; Maathuis, F.J.M.; Mills, L.N.; Knight, H.; Pelloux, J.; Hetherington, A.; Sanders, D. The vacuolar Ca^{2+}-activated channel TPC1 regulates germination and stomatal movement. *Nature* **2005**, *434*, 404–408. [CrossRef]
40. Zhu, S.Y.; Yu, X.C.; Wang, X.J.; Zhao, R.; Zhang, D.P. Two Calcium-Dependent Protein Kinases, CPK4 and CPK11, Regulate abscisic acid signal transduction in *Arabidopsis*. *Plant Cell* **2007**, *19*, 3019–3036. [CrossRef] [PubMed]
41. Ubaid, M.; Ting, M.K.Y.; Teresinski, H.J.; Snedden, W.A. The calmodulin-like protein, CML39, is involved in regulating seed development, germination, and fruit development in *Arabidopsis*. *Plant Mol. Biol.* **2018**, *96*, 375–392. [CrossRef]
42. Wu, F.; Chi, Y.; Jiang, Z.; Xu, Y.Y.; Xie, L.; Huang, F.F.; Wan, D.; Ni, J.; Yuan, F.; Wu, X.M.; et al. Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in *Arabidopsis*. *Nature* **2020**, *578*, 577–581. [CrossRef] [PubMed]
43. Mori, I.C.; Schroeder, J.I. Reactive oxygen species activation of plant Ca^{2+} channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. *Plant Physiol.* **2004**, *135*, 702–708. [CrossRef] [PubMed]
44. Ma, L.Y.; Zhang, H.; Sun, L.R.; Jiao, Y.H.; Zhang, G.Z.; Miao, C.; Hao, F.S. NADPH oxidase AtrbohD and AtrbohF function in ROS dependent regulation of Na^+/K^+ homeostasis in *Arabidopsis* under salt stress. *J. Exp. Bot.* **2012**, *63*, 305–317. [CrossRef]
45. Jiao, Y.H.; Sun, L.R.; Song, Y.L.; Wang, L.M.; Liu, L.P.; Zhang, L.Y.; Liu, B.; Li, N.; Miao, C.; Hao, F.S. AtrbohD and AtrbohF positively regulate abscisic acid inhibited primary root growth by affecting Ca^{2+} signaling and auxin response of roots in *Arabidopsis*. *J. Exp. Bot.* **2013**, *64*, 4183–4192. [CrossRef] [PubMed]
46. Pei, Z.M.; Murata, Y.; Benning, G.; Thomine, S.; Klüsener, B.; Allen, G.J.; Grill, E.; Schroeder, J.I. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **2000**, *406*, 731–734. [CrossRef]
47. Brandt, B.; Munemasa, S.; Wang, C.; Nguyen, D.; Yong, T.M.; Yang, P.G.; Poretsky, E.; Belknap, T.F.; Waadt, R.; Alemán, F.; et al. Correction: Calcium specificity signaling mechanisms in abscisic acid signal transduction in *Arabidopsis* guard cells. *eLife* **2015**, *4*, e03599. [CrossRef]
48. Liu, B.; Sun, L.; Ma, L.; Hao, F.S. Both AtrbohD and AtrbohF are essential for mediating responses to oxygen deficiency in *Arabidopsis*. *Plant Cell Rep.* **2017**, *36*, 947–957. [CrossRef]
49. Spalding, E.P.; Harper, J.F. The ins and outs of cellular Ca^{2+} transport. *Curr. Opin. Plant Bio.* **2011**, *14*, 715–720. [CrossRef]
50. Pandey, G.K.; Cheong, Y.H.; Kyung-Nam, K.; Grant, J.J.; Li, L.G.; Wendy, H.; Cecilia, D.; Stefan, W.; Jörg, K.; Sheng, L. The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. *Plant Cell* **2004**, *16*, 1912–1924. [CrossRef]
51. Pandey, G.K.; Grant, J.J.; Cheong, Y.H.; Beom-Gi, K.; Li, L.G.; Sheng, L. Calcineurin-B-like protein CBL9 interacts with target kinase CIPK3 in the regulation of ABA response in seed germination. *Mol. Plant* **2008**, *1*, 238–248. [CrossRef] [PubMed]
52. Drerup, M.M.; Schlücking, K.; Hashimoto, K.; Manishankar, P.; Steinhorst, L.; Kuchitsu, K.; Kudla, J. The Calcineurin B-like calcium sensors CBL1 and CBL9 together with their interacting protein kinase CIPK26 regulate the *Arabidopsis* NADPH oxidase RBOHF. *Mol. Plant* **2013**, *6*, 559–569. [CrossRef] [PubMed]
53. Dubiella, U.; Seybold, H.; Durian, G.; Komander, E.; Lassig, R.; Witte, C.P.; Schulze, W.X.; Romeis, T. Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8744–8749. [CrossRef] [PubMed]
54. Zhu, J.K. Abiotic stress signaling and responses in plants. *Cell* **2016**, *167*, 313–324. [CrossRef]