Melatonin antagonizes ABA action to promote seed germination by regulating Ca\textsuperscript{2+} efflux and H\textsubscript{2}O\textsubscript{2} accumulation

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\textbf{A B S T R A C T}

Seed germination is a vital stage in the plant life-cycle that greatly contributes to plant establishment. Melatonin has been shown to promote seed germination under various environmental stresses; however, the mechanism remains largely underexplored. Here, we reported that melatonin antagonized abscisic acid (ABA) to promote seed germination by regulating ABA and gibberellic acid (GA\textsubscript{3}) balance. Transcriptomic analysis revealed that such a role of melatonin was associated with Ca\textsuperscript{2+} and redox signaling. Melatonin pretreatment induced Ca\textsuperscript{2+} efflux accompanied by an up-regulation of vacuolar H\textsuperscript{+}/Ca\textsuperscript{2+} antiporter 3 (CAX3). \textit{AtCAX3} deletion in \textit{Arabidopsis} exhibited reduced Ca\textsuperscript{2+} efflux. Inhibition of Ca\textsuperscript{2+} efflux in the seeds of melon and \textit{Arabidopsis} mutant \textit{AtCAX3} compromised melatonin-induced germination under ABA stress. Melatonin increased \textsubscript{H}\textsubscript{2}\textsubscript{O}\textsubscript{2} accumulation, and \textsubscript{H}\textsubscript{2}\textsubscript{O}\textsubscript{2} pretreatment decreased ABA/GA\textsubscript{3} ratio and promoted seed germination under ABA stress. However, complete inhibition of \textsubscript{H}\textsubscript{2}\textsubscript{O}\textsubscript{2} accumulation abolished melatonin-induced ABA and GA\textsubscript{3} balance and seed germination. Our study reveals a novel regulatory mechanism in which melatonin counteracts ABA to induce seed germination that essentially involves CAX3-mediated Ca\textsuperscript{2+} efflux and \textsubscript{H}\textsubscript{2}\textsubscript{O}\textsubscript{2} accumulation, which, in turn, regulate ABA and GA\textsubscript{3} balance by promoting ABA catabolism and/or GA\textsubscript{3} biosynthesis.

\section{1. Introduction}

Seed germination is the first step toward the establishment of seed plants, which has immense significance in agricultural crop production [1]. This physiological process integrates multiple events that start with the absorption of water by the quiescent dry seeds and end with the emergence of the embryonic axis [2]. As a complex process, seed germination is sensitive to and controlled by many environmental cues such as temperature, light, oxygen, and moisture, as well as endogenous factors such as plant hormones [3]. Unfavorable sowing conditions or poor quality of seeds result in adverse impacts on seed germination and crop establishment, leading to ultimate yield losses [4]. The phytohormone abscisic acid (ABA) is thought to be central to the transduction of environmental information and control of seed dormancy [5]. Over-expression of genes encoding ABA biosynthetic enzymes or mutation of genes relating to ABA catabolic enzymes enhances endogenous ABA content in seeds, thereby prolonging the dormancy or delaying the germination of \textit{Arabidopsis} seeds [2,6].

Melatonin (N-acetyl-5-methoxytryptamine), an extremely conserved biomolecule, is ubiquitous in animals, plants, and many other organisms [7]. Starting from the discovery of phytomelatonin in 1995 [8,9], a large number of studies have explored the roles of melatonin in plant growth, development, and responses to environmental stresses [10,11]. Increasing studies have demonstrated that melatonin improves seed germination under favorable as well as adverse conditions such as chilling, drought, salinity, and heavy metal stress [12–14]. Some evidence supports that melatonin-induced germination is closely associated with the regulation of ABA catabolism and GA anabolism [15,16]. However, the mechanisms by which melatonin and ABA antagonistically regulate seed germination are still poorly understood.

In plants, Ca\textsuperscript{2+} acts as a versatile intracellular signal that can regulate multiple cellular processes [17,18]. Ca\textsuperscript{2+} signal functions by eliciting...
characteristic transient cytoplasmic Ca\(^{2+}\) fluctuations through Ca\(^{2+}\)-permeable channels-mediated influx (thermodynamically passive) and membrane transporters-mediated efflux (thermodynamically active) activities [19]. Activated Ca\(^{2+}\) influx systems induce a transient increase in cytoplasmic Ca\(^{2+}\) and elicit Ca\(^{2+}\) signal, while Ca\(^{2+}\) efflux systems have the potential to dampen the magnitude and duration of a Ca\(^{2+}\) signal [20]. In the absence of Ca\(^{2+}\) efflux systems, a Ca\(^{2+}\) transient can morph into a “cell-death Ca\(^{2+}\) signature”. Similar to Ca\(^{2+}\) influx systems, Ca\(^{2+}\) efflux systems play important roles in plant growth, development, and tolerance to environmental stresses. For example, a Ca\(^{2+}\) efflux-mediated efflux (thermodynamically passive) during seed imbibition can trigger direct oxidation of various cellular biomolecules such as proteins, nucleic acids, and mRNAs, leading to adequate cell functions that drive seed germination [26]. The complex interaction of ROS and plant hormones such as ABA and GA in regulating seed germination is well recognized [27]. ROS homeostasis not only regulates ABA catabolism and GA biosynthesis but also affects their signaling pathways [28].

Despite the abundant literature on the individual role of melatonin, Ca\(^{2+}\) and H\(_2\)O\(_2\) in seed germination, their potential interactions in mediating ABA and GA balance, and subsequent germination of seeds remain largely unaddressed. Recently, Wei et al. [29] demonstrated that melatonin-induced stomatal closure is dependent on Ca\(^{2+}\) and H\(_2\)O\(_2\)-dependent signaling cascade. These findings motivated us to examine whether Ca\(^{2+}\) and H\(_2\)O\(_2\) signaling are also involved in melatonin-regulated seed germination. Our results reveal that Ca\(^{2+}\) and H\(_2\)O\(_2\) act as a key node to integrate the melatonin and ABA signaling pathways, thus providing new insights into the signal transduction of melatonin during seed germination.

2. Materials and methods

2.1. Plant materials

Melon (Cucumis melo L. cv. SSMA) seeds used in this study were obtained from the Watermelon and Melon Research Group of Northwest A&F University, Yangling, Shaanxi, China. Seeds of wild-type Arabidopsis thaliana (Columbia ecotype, Col-0) and the T-DNA insertion lines AtCAX3 (SALK_094565C), AtCML5 (SALK_065778), AtRBOHD (SALK_120299) and AtRBOHF (SALK_034674) in the var. Columbia background were obtained from the SALK collection and the seed stock center of the Arabidopsis Biological Resource Center (ABRC, https://www.arabidopsis.org/).

2.2. Low temperature and salt treatment

Melon seeds were immersed in double-distilled water for 7 h. Pre-soaked seeds were placed in Petri dishes (9 × 9 cm) containing three filter papers moistened with 12 mL double distilled water and the Petri dishes were covered with Petri dish covers. Then, the seeds were incubated at the temperature of 30 °C (as control) or 20 °C (low temperature) for 7 d in a germinator in the dark. Seeds were considered germinated when the radicle emerged (2 mm). For salt treatment, seeds were soaked in NaCl (300 mM) for 7 h followed by rinsing with the double distilled water for the five times. Then those seeds were incubated under conditions identical to the control. Each treatment included three Petri dishes with 30 seeds per Petri dish. The seeds were sampled at 24 h after imbibition from each treatment. All samples were rapidly frozen in liquid nitrogen and stored at −80 °C before analysis. The seed germination was counted after incubation for 1, 2, 3, 5, and 7 d.

2.3. Chemical treatment

To investigate the mechanisms by which melatonin counteracts ABA to regulate seed germination, the seeds of melon or Arabidopsis were presoaked in different chemical solutions including melatonin (MT, Sigma-Aldrich, St. Louis, MO, USA), abscisic acid (ABA, Sigma-Aldrich), gibberellic acid (GA\(_3\), Sigma-Aldrich), H\(_2\)O\(_2\), dimethylthiourea (DMTU; an H\(_2\)O\(_2\) scavenger) [30], diphenylethionium (DPI; it inhibits NADPH oxidases and related oxidative burst, which produces H\(_2\)O\(_2\)) [30,31], eosin Y (EY, an inhibitor of the Ca\(^{2+}\)-ATPase that blocks the Ca\(^{2+}\) efflux) [32,33], LaCl\(_3\) (a Ca\(^{2+}\) channel blocker) [34], or double distilled water for 7 h. After rinsing with double distilled water, melon seeds were incubated for 7 d under control conditions (at 30 °C) as described in the previous section 2.2, while Arabidopsis seeds were placed in Petri dishes (3 × 3 cm) containing three filter papers soaked with 2 mL double distilled water in a 21 °C growth chamber under continuous light at 100 μmol m\(^{-2}\) s\(^{-1}\) for 7 d. Each treatment included three Petri dishes with 30 seeds per Petri dish. The seeds were sampled after imbibition for 0, 0.5, 1, 3, and 7 d. The seed germination rate was counted after incubation for 7 d.

Test solutions used for treating melon seeds were as follows: ABA (0.2, 0.4, 0.6, 0.8, or 1 mM), mixture of ABA (1 mM) and MT (0.5, 1, 5, 10, or 30 mM), mixture of ABA (1 mM) and GA\(_3\) (0.1, 0.3, 0.5, 1, or 3 mM), mixture of ABA (1 mM) and LaCl\(_3\) (0.1, 0.5, or 1 mM, Table S1), mixture of ABA (1 mM) and H\(_2\)O\(_2\) (1, 5, 10, or 30 mM), mixture of ABA (1 mM) and DPI (2, 5, or 10 μM, Table S1), mixture of ABA (1 mM) and DMTU (0.5, 1, or 5 mM, Table S1), mixture of AMT (1 mM ABA + 10 mM MT) and EY (20, 100, or 500 μM, Table S1), mixture of AMT and GA (1 mM), mixture of AMT and DPI (5 or 10 μM), mixture of AMT and DMTU (1 or 5 mM). To continuously block Ca\(^{2+}\) influx and efflux, EY was added in the respective incubation medium. Test solutions used for treating Arabidopsis seeds included ABA (0.05, 0.1, 0.2, or 0.5 mM), mixture of ABA (0.2 mM) and MT (0.05, 0.1, 0.2, or 0.5 mM), mixture of ABA and H\(_2\)O\(_2\) (1, 5, or 10 mM).

From the physiological point of view, the concentrations of exogenous chemicals used in this study were higher than the endogenous levels of these biomolecules. Nonetheless, such high concentrations of melatonin (10 mM) [35], GA\(_3\) (2.88 mM) [36], H\(_2\)O\(_2\) (10 mM) [30], and DMTU (5 mM) [30] were also used in various plant species to assess their functions in previous studies. In the present study, the concentrations of chemicals were sufficiently justified by using a range of concentrations and the most effective concentration was chosen (Table S1). The seeds were presoaked in test solutions for 7 h, followed by rinsing with the double-distilled water. Due to the presence of the seed coat as a barrier, the ABA and GA\(_3\) content of melon seeds increased from 16.8 ng g\(^{-1}\) and 1.88 ng g\(^{-1}\) to 133.6 ng g\(^{-1}\) and 22.37 ng g\(^{-1}\), after presoaking in 1 mM ABA and 1 mM GA\(_3\) for 7 h, respectively, suggesting a remarkable difference between exogenous treatment dose and endogenous tissue concentrations of ABA and GA\(_3\). Then, the presoaked seeds were incubated under normal conditions to observe the effects of melatonin and other chemicals on ABA catabolism and other physiological changes. Such treatments can avoid the complex influence of external environments during seed germination.

2.4. Measurement of net Ca\(^{2+}\) flux

The net Ca\(^{2+}\) flux (the difference between Ca\(^{2+}\) efflux from plant cell and Ca\(^{2+}\) influx into plant cell) was measured with a Noninvasive Microtest Technology (NMT) Physiolizer (NMT Physiolizer, Xuyue (Beijing) Science and Technology Company Limited, Beijing, China) at the points
Measurements, the ready-to-measure seeds were equilibrated in Petri dishes that contained a 10 mL of liquid medium (0.1 mM CaCl\(_2\), 0.2 mM NaSO\(_4\), 0.3 mM MES, pH 6.0) for 20–30 min. Pre-pulled and silanized microelectrodes (0.4–5 μm, XY-CGQ-01, YoungerUSA) were first backfilled with Ca\(^{2+}\) solution (100 mM CaCl\(_2\)) to one centimeter (approx.) from the tip and then front-filled with 25–30 μm columns of specific Liquid Ion exchange (LIX; XY-SJ-Ca; YoungerUSA). In the course of the measurements, a stepper motor controlled by a computer moved the electrodes in a gentle (six seconds) square-wave cycle from one position to another, close to (5 μm) the seed surface. Then the steady-state ion fluxes were directly recorded using imFluxes software V2.0 (YoungerUSA LLC, Amherst, MA 01002, USA). The flux unit is pmol cm\(^{-2}\) s\(^{-1}\) and the positive and negative values represent efflux and influx, respectively.

2.5. \(\text{H}_2\text{O}_2\) quantification

\(\text{H}_2\text{O}_2\) contents in seed samples were quantified following the method of Willekens et al. [39]. Briefly, \(\text{H}_2\text{O}_2\) was extracted from 0.2 g seed samples in 3 mL of HClO\(_2\) (1 M) at 4 °C. After centrifugation, the pH of supernatants was adjusted between 6.0 and 7.0 by adding 4 M KOH. The supernatants were centrifuged again, and passed through an AG1 × 8 prepacked column (Bio-Rad, Hercules, CA, USA), and then eluted with 4 mL double distilled water, and an aliquot of sample (800 μL) was combined with 400 μL reaction buffer consisting of 100 mM potassium acetate at pH 4.4, 4 mM 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid), 0.25 U of horseradish peroxidase, and 400 mL deionized water. \(\text{H}_2\text{O}_2\) content was quantified by recording the absorbance at OD\(_{412}\). The histochemical staining of \(\text{H}_2\text{O}_2\) was performed according to the protocol described previously [40]. Peeled melon seeds were immersed in a 3, 3’-diaminobenzidine (DAB) solution (1 mg DAB in 1 mL of 50 mM Trisacetate, pH 3.8) and incubated under light at 200 μM m\(^{-2}\) s\(^{-1}\) for 5 h.

2.6. RNA-seq sequencing and data analysis

Melon seeds pretreated with ABA (1 mM) or AMT (1 mM ABA + 10 mM MT) were collected as samples on the 2nd day of incubation. For each treatment, two biological replicates were used to sequence four independent mRNA libraries with the technical supports of Novogene Co. (Beijing, China). After extracting total RNA, mRNA libraries were constructed by using NEBNext Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). Notably, to each sample, index codes were added to attribute sequences. We used the Agilent Bioanalyzer 2100 system to assess the library quality. Finally, sequencing was done by using Illumina HiSeq 4000. The sequences generated in the study were deposited into the NCBI Sequence Read Archive database (SRA accession: PRJNA5899501).

Firstly, raw data (sequenced reads) were processed with in-house Perl scripts to acquire clean reads. The clean reads were then mapped to the melon reference genome using a mapping software TopHat v2.0.12. To generate fragments per kilobase of exon per million mapped fragments (FPKM), we calculated and normalized the expression level of each unigene. The differentially expressed genes (DEGs) were demarcated as the genes with a specific fold change (≥1.5) and a P-value (≤0.05). To analyze the functions of DEGs, their functional assignments were performed based on KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) and Gene Ontology (GO) databases (http://www.geneontology.org/).

2.7. Quantification of ABA and GA3

Extraction and quantification of ABA and GA3 were carried out as described previously [15,41]. In brief, 0.5 g of frozen seed tissues was ground into powder in liquid nitrogen. ABA and GA were extracted by overnight incubation in 5 mL of 1-propanol/H\(_2\)O/concentrated HCl (2/1/0.002, v/v/v). Then, 5 mL of dichloromethane was added and the mixture was shaken for 30 min at 4 °C. After centrifugation at 15,000 g for 5 min at 4 °C, two phases were clarified and the lower phase was collected and dried under the flow of N\(_2\) (gas). The dried samples were dissolved in methanol. During extraction, seed samples were spiked with D\(_0\)-ABA and (\(\_2\)H\(_2\)) gibberellic acid (GA\(_3\)) as internal standards. ABA and GA\(_3\) were analyzed using an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionization source, operated in the negative ion or positive multiple-reaction monitoring mode. The separation was performed on an Agilent SB-C18 column (4.6 × 50 mm; 1.8 μm; USA) at a temperature of 40 °C. The mobile phases consisted of acetonitrile (solvent A) and 0.1
% acetic acid in water (v/v, solvent B). The flow rate of the mobile phase was 0.8 mL min\(^{-1}\) and at a linear gradient as follows: 0 min 10 % A, 0.5 min 10 % A, 5 min 95 % A, 5.1 min 10 % A, and 8 min 10 % A.

### 2.8. Extraction of antioxidant enzymes and activity assay

The activity of the antioxidant enzyme was assayed following spectrophotometric methods. Seed samples (0.2 g each) were homogenized with 3 mL ice-cold 25 mM HEPES buffer (2 mM AsA, 0.2 mM EDTA, 2 % PVP, pH 7.8). The obtained homogenates were then centrifuged at 4 °C for 20 min at 12,000 g. The supernatants (extracts) were used for the measurement of enzymatic activity. Ascorbate peroxidase (APX) activity was measured according to the method of Nakano and Asada [42]. Peroxidase (POD) activity was analyzed following the protocol of Cakmak and Marschner [43]. Glutathione S-transferase (GST) activity was measured using an assay kit (Solarbio Life Sciences, Beijing, China) in accordance with the manufacturer’s instructions. Absorbance (colorimetric readings) at OD\(_{340}\) was recorded with a Multimode Plate Reader M200 pro (Tecan, Männedorf, Switzerland).

### 2.9. Glutathione content assay

Total glutathione content was analyzed following the method as described by Rao et al. [44]. Briefly, seed samples (0.2 g each) were ground with 2 mL of 5 % metaphosphoric acid comprising 2 mM EDTA and centrifuged at 12,000 g for 10 min at 4 °C. For the total glutathione assay, the supernatant (0.1 mL) was added to a reaction mixture comprising 100 mM phosphate buffer (pH 7.5), 0.2 mM NADPH, 0.6 mM 5,5′-dithio-bis (2-nitrobenzoic acid), and 5 mM EDTA. The reaction was started through the addition of glutathione reductase (3 U) and the activity was recorded by measuring the changes at A\(_{412}\) for 1 min. For the oxidized glutathione (GSSG) assay, reduced glutathione (GSH) was masked by adding 20 μL of 2-vinylpyridine for 1 h at 25 °C. The GSH concentration was obtained by subtracting the GSSG concentration from the total concentration.

### 2.10. RNA isolation and qRT-PCR analysis

Total RNA from seeds was isolated using an RNA extraction kit
To remove residual DNA from the total RNA, we used a DNase Mini Kit (Qiagen, Hilden, Germany). One microgram (1 μg) total RNA was used for reverse transcription using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer’s recommendations. The gene-specific primer pairs were designed on the basis of EST sequences and used for amplification (Table S2). The expression stability of 13 candidate reference genes during seed germination was evaluated by using the geNorm and NormFinder software. The sequences and relative primers of these 13 genes were obtained from melon literature [45], NCBI, or Melon Unigene (http://www.icugi.org, Table S2). The gene actin was identified as the most stable gene and thus was used as an internal control (Fig. S1).

2.11. Statistical analysis

The experimental layout was a completely randomized design with three independent biological replicates. Each replicate contained 30 seeds. Experimental data were subject to analysis of variance (ANOVA), and significant differences among treatments were analyzed using Tukey’s test at P < 0.05. Significant differences between treatment means are designated by different letters.

3. Results

3.1. Melatonin counteracts ABA to promote seed germination

In the present study, low temperature (20 °C) and salt stress (300 mM NaCl) remarkably delayed the time required for germination and decreased the germination rate of melon seeds (Fig. 1a). Compared to the control, the ABA content and the ABA/GA3 ratio were significantly increased by both low temperature and NaCl, while GA3 content was only increased by low temperature (Fig. 1b–d). Similar to the effects of low temperature and salt stress, exogenous ABA treatment severely inhibited seed germination, and the germination rate decreased with the increase in ABA concentration (Fig. 1e). However, the application of melatonin at appropriate concentrations (0.5–30 mM) attenuated the ABA-caused inhibition on seed germination, and the most effective concentration was 10 mM (Fig. 1f, g). For instance, the germination rates of melon seeds pre-treated with a combination of 1 mM ABA and 10 mM melatonin (AMT) was 87.7 %, far higher than that (24.4 %) of ABA-pretreated seeds. Under normal conditions, the seed germination was promoted slightly, but not significantly, by presoaking with 10 mM melatonin (Fig. S2).

3.2. Transcriptomic analysis

To assess the potential signaling pathways involved in melatonin-promoted seed germination, mRNA sequencing-based transcriptomic analysis was performed in melon seeds pre-treated with ABA and AMT. A total of 20.05 million mapped reads were obtained by mapping all unique sequences to the melon genome (Table S3). A total of 55 and 15
differentially expressed genes (DEGs) were significantly up- and down-regulated, respectively, in the comparison between AMT and ABA (Fig. 2a). A strong correlation ($R^2 \geq 0.98$) between the 2 biological replicates for each treatment and the similar trends of expression changes in 14 key DEGs between by qRT-PCR assay and RNA-seq indicated the high reliability of the RNA-seq data (Fig. 2b, Fig. S3). According to annotation, a set of DEGs were involved in Ca$^{2+}$ transport (i.e. vacuolar H$^+$/Ca$^{2+}$ antiporter 3 (CAX3) and calmodulin-like protein 5 (CML5)) during seed germination. Seeds of melon were presoaked in double distilled water or test solutions for 7 h and then the seeds were incubated under normal conditions. Data are presented as the means of three replicates ($\pm$SD). Means denoted with different letters differed significantly at $P < 0.05$.

involved in melatonin counteracting ABA to promote seed germination.

3.3. Melatonin decreases ABA content but increases GA3 content under ABA stress

During seed imbibition for one day under normal condition (control), the ABA level decreased, but the GA3 level increased, and finally, the ABA and GA3 ratio decreased (Fig. 3a). However, the levels of both ABA and GA3 in control seeds increased from the 1st day and then decreased from the 3rd day after imbibition. After presoaking treatment with ABA (1 mM), the ABA content of melon seeds increased from 16.8 ng g$^{-1}$ to 133.6 ng g$^{-1}$. Compared with ABA, both AMT and AGA decreased ABA content but increased GA3 content, and subsequently decreased the ABA and GA3 ratio during seed incubation. Compared to ABA alone, AMT increased the transcript levels of GA biosynthesis gene (ent-kaurenoic acid oxidase 1, KAO1) and ABA catabolism gene (ABA 8'-hydroxylase 2, CYP707A2) during seed incubation (Fig. 3b, c). Similar to melatonin, AGA also increased the transcript levels of CYP707A2. The application of GA3 (0.1–3 mM) attenuated the ABA-caused inhibition on seed germination, and the most effective concentration of GA3 was 1 mM (Fig. 3d). Melatonin further promoted the germination rate of seeds treated with a combination of ABA and GA (Fig. S4)

3.4. Ca$^{2+}$ efflux-elicited signaling mediates melatonin-induced seed germination

To determine whether Ca$^{2+}$ signal is involved in melatonin-regulated seed germination under ABA stress, we first measured Ca$^{2+}$ flux at the site of the embryo using an NMT technology. After water presoaking, the seeds exhibited Ca$^{2+}$ influx at 0 h, but Ca$^{2+}$ efflux appeared approximately from 12 h, and the rate of such efflux rapidly increased with increasing incubation time (Fig. 4). Notably, ABA induced continuous Ca$^{2+}$ influx during seed incubation. On the contrary, AMT induced Ca$^{2+}$ efflux at 0 h after imbibition, but the efflux rate decreased with increasing incubation time. CAXs are endomembrane Ca$^{2+}$ efflux transporters that load Ca$^{2+}$ into vacuole [47]. Previous studies have demonstrated that CAX transporters can alter the activities of plasma-membrane H$^+$/Ca$^{2+}$ ATPase pumps, suggesting that CAXs may indirectly regulate H$^+$/Ca$^{2+}$ flux across the plasma membrane [48, 49]. CMLs are Ca$^{2+}$-binding proteins and act as Ca$^{2+}$ signal sensors [50]. Being consistent with the changes in Ca$^{2+}$ flux, CAX3 transcripts increased but CML5 transcripts decreased with increasing incubation time in control seeds. ABA inhibited the increase and decrease of CAX3 and CML5 transcripts, respectively; however, such inhibition was alleviated by melatonin.

Application of LaCl$_3$ (a Ca$^{2+}$ channel blocker, 0.1 mM) alleviated ABA-caused inhibition on seed germination of melon (Fig. 5a). However, EY (a Ca$^{2+}$ efflux blocker) at 0.1 mM completely abolished the melatonin-induced seed germination under ABA stress. These results suggested that Ca$^{2+}$ efflux-elicited signaling might mediate melatonin-induced seed germination under ABA stress. Due to the difficulty of transgenic technology of melon and the lack of melon mutants, Arabidopsis mutants AtCAX3 and AtCML5 were used to further explore the role of Ca$^{2+}$ signal in seed germination as the consequence of the antagonistic effect of melatonin on ABA action. Pretreatment with melatonin at appropriate concentrations (0.05–0.5 mM) also attenuated the ABA-caused inhibition on germination of wild-type Arabidopsis seeds, and the most effective concentration of melatonin was 0.2 mM (Fig. S5). AtCAX3 deletion in Arabidopsis increased seed sensitivity to ABA and attenuated the induction of seed germination by AMT. The seeds with AtCML5 deletion exhibited less sensitivity to ABA and melatonin (Fig. 5b). La$^{3+}$ significantly reduced Ca$^{2+}$ influx under ABA stress at 0 h after imbibition, while EY attenuated the melatonin-induced Ca$^{2+}$ efflux (Fig. 5c). As observed in melon seeds, ABA induced Ca$^{2+}$ influx while
AMT (0.2 mM ABA + 0.2 mM melatonin) induced Ca\(^{2+}\) efflux in wild-type Arabidopsis seeds (Fig. 5d). AtCAX3 deletion in Arabidopsis decreased Ca\(^{2+}\) efflux (Fig. 5d), indicating that the expression of CAX3 is positively related with Ca\(^{2+}\) efflux. AtCAX3 deletion promoted Ca\(^{2+}\) influx under normal condition and ABA stress but comprised AMT-induced Ca\(^{2+}\) efflux. Compared to ABA, the combination of ABA and LaCl\(_3\) (ALa) induced ABA decrease and GA\(_3\) increase, while EY abolished the AMT-induced ABA decrease and GA\(_3\) increase (Fig. 5e). Finally, ALa decreased the ABA to GA\(_3\) ratio, while EY completely abolished the AMT-induced decrease of ABA to GA\(_3\) ratio (Fig. 5f). These results strongly suggest that the role of melatonin in alleviating ABA-induced inhibition of seed germination is largely dependent on Ca\(^{2+}\) efflux-elicited Ca\(^{2+}\) signaling.

3.5. \(H_2O_2\) is involved in melatonin-induced seed germination under ABA stress

To examine the role of \(H_2O_2\) in melatonin-induced seed germination under ABA stress, the accumulation of \(H_2O_2\) was firstly analyzed using spectrophotometric methods and histochemical staining. During seed incubation under normal condition, \(H_2O_2\) production rapidly increased after imbibition, reaching a peak at 12 h, which subsequently declined to lower levels (Fig. 6a). \(H_2O_2\) rise was delayed and repressed by ABA; however, such delay and repression were alleviated by melatonin. For instance, \(H_2O_2\) content in AMT-treated seeds was 104.3 nmol g\(^{-1}\) on the 3rd day after imbibition, higher than that (80.7 nmol g\(^{-1}\)) in ABA-treated seeds. \(H_2O_2\) can be produced and modulated through various pathways such as the activity of plasma membrane-localized NADPH oxidase, respiratory activity (mitochondria), and ROS-scavenging activity [25]. The changes in the transcripts of respiratory burst oxidase homolog (RBOH) D and RBOH F that encode NADPH oxidase, showed similar trends with \(H_2O_2\) during seed incubation under normal condition (Fig. 6c). ABA enhanced the increases of the transcript levels of \(RBOHD\) and \(RBOHF\) at 0 h after imbibition. ABA-induced increase of \(RBOHD\) transcripts was promoted by melatonin. For instance, after pre soaking, the expression of \(RBOHD\) was up-regulated a 9.3 fold by ABA, far higher than that (3.3 fold) by ABA. However, ABA-induced increase of \(RBOHF\) transcripts was reduced by melatonin, suggesting that \(RBOHD\) but not \(RBOHF\) is involved in melatonin-mediated \(H_2O_2\) accumulation under ABA stress. In plants, antioxidant systems, composed of antioxidant enzymes as well as nonenzymatic antioxidants, play a critical role in ROS metabolism. In comparison to ABA, AMT decreased GSH content and GSH/GSSG ratio, but increased the activities of GST and APX on the 3rd day after imbibition (Fig. 6d, e). However, the GSSG content and POD activity was almost unchanged by AMT, when compared to ABA. \(H_2O_2\) at appropriate concentration promoted seed germination under ABA stress, and the most effective concentration of \(H_2O_2\) for melon and Arabidopsis seeds was 10 mM and 5 mM, respectively (Fig. 7a, b). Unexpectedly, lower doses of DMTU (an \(H_2O_2\) scavenger, 1 mM, DMTU-L) and DPI (an inhibitor of NADPH oxidase and oxidative burst, 5 \(\mu\)M, DPI-L) also alleviated the inhibitory effects of ABA on seed germination of melon (Fig. 7c, Table S1). Higher, however, doses of DMTU (5 mM, DMTU-H) and DPI (10 \(\mu\)M, DPI-H) did not affect ABA-induced inhibition on germination but abolished AMT-induced germination. In Arabidopsis seeds, \(AtRB1\) and \(AtRB5\) mutation showed less sensitivity to ABA, while exogenous \(H_2O_2\) increased sensitivity of \(AtRB1\) mutant seeds to ABA (Fig. 7d). In comparison to ABA, a combination of ABA and \(H_2O_2\) (ABH) decreased the ABA level, but it increased the GA\(_3\) level (Fig. 7e). DPI-H and DMTU-H inhibited the AMT-induced decrease in ABA and increase in GA\(_3\). Consequently, ABH decreased ABA and GA\(_3\) ratio, while DPI-H and DMTU-H completely abolished AMT-decreased ABA and GA\(_3\) ratio (Fig. 7f).
4. Discussion

4.1. Melatonin counteracts ABA to promote seed germination via regulating ABA degradation and GA₃ biosynthesis

ABA and GA are well-recognized as the two major signaling molecules involved in sensing environmental changes, and they function antagonistically to regulate seed germination [5]. In the present study, ABA levels were increased, but GA₃ levels were not decreased by low temperature and salt stress, which repressed seed germination, suggesting that the increase in ABA is the main factor that disturbs ABA/GA₃ balance and consequently impairs seed germination under stresses (Fig. 1). Increasing studies have revealed that melatonin plays an important role in promoting seed germination, which is associated with the regulation of the balance between ABA and GA [15, 16]. Consistently, we found that presoaking with the mixture of melatonin and ABA decreased ABA content but increased GA₃ content in melon seeds and thus decreased ABA/GA₃ ratio and alleviated ABA-mediated inhibition on germination (Figs. 1 and 3). These results provided strong evidence on the antagonistic effect of melatonin on ABA in regulating seed germination. Such an interaction between melatonin and ABA in regulating seed germination seems to be contrary to the synergistic interaction of melatonin and ABA in promoting stomatal closure [29]. Therefore, we speculate that this is related to the specificity of melatonin action in regulating different physiological processes.

4.2. Melatonin-induced seed germination under ABA stress is dependent on Ca²⁺ efflux-elicited signaling

Many studies have indicated that melatonin has a critical role in modulating the expression of a wide array of genes related to various regulatory pathways such as signal perception, hormone signaling, secondary signaling, and transcriptional regulation, reflecting its pleiotropic physiological roles in plant growth and development [51, 52]. Based on the RNA-Seq analysis, we showed that melatonin changed the expression of a subset of genes involved in Ca²⁺ (i.e. CAX3 and CML5) and redox (i.e. PODs and GSTs) signaling, during ABA-inhibited seed germination (Fig. 2; Table S4). Recent studies have revealed that Ca²⁺ and ROS as important signals were involved in melatonin signaling during ABA-mediated germination.

Accumulating evidence has shown that Ca²⁺ signal is a critical factor for seed germination, which functions synergistically or antagonistically with ABA signaling. Loss-of-function mutations of Ca²⁺ channel- and signaling-related genes, such as two-pore channel 1, CML39, and CDPKs, result in insensitivity to ABA during seed germination, suggesting that Ca²⁺ signal is positively involved in ABA-regulated seed germination [53–55]. However, Pandey et al. [56, 57] found that double mutation of CaM B-like protein 9 (CBL9, a Ca²⁺ sensor) and its target kinase encoding gene CIPK3 showed the hypersensitive response to ABA during germination, suggesting that Ca²⁺ signal plays a negative role in ABA-regulated seed germination. Here, we showed that ABA induced...
Contrary to ABA, AMT induced Ca\(^{2+}\) efflux and reduced the transcript levels of CML5 (Fig. 4). Furthermore, pretreatment with EY, which inhibits the Ca\(^{2+}\)-ATPase to block the transport of Ca\(^{2+}\) [32,33], abolished the AMT-induced Ca\(^{2+}\) efflux, ABA decrease, and subsequently seed germination (Fig. 5a, c, e). Therefore, the role of melatonin in relieving the negative effects of ABA on germination is largely dependent on Ca\(^{2+}\) efflux-induced attenuation of Ca\(^{2+}\) signaling. Ca\(^{2+}\) exchanger antiporters (CAXs) and plasma membrane-type Ca\(^{2+}\)-ATPase pumps provide two types of energized transport systems that move Ca\(^{2+}\) out from the cytoplasm [20]. CAXs are endomembrane transporters that load Ca\(^{2+}\) into vacuole [47]. Interestingly, AtCAX3 deletion in Arabidopsis decreased Ca\(^{2+}\) efflux (Fig. 5d), indicating that AtCAX3 may indirectly regulate Ca\(^{2+}\) efflux across the plasma membrane. In accordance with a previous study [48], we found that AtCAX3 deletion in Arabidopsis increased sensitivity to ABA during seed germination (Fig. 5b). Melatonin increased the transcript levels of CAX3 in melon seeds (Fig. 4), while AtCAX3 deletion in Arabidopsis compromised melatonin-induced Ca\(^{2+}\) efflux and seed germination under ABA stress (Fig. 5b, d), suggesting that CAX3 mediated melatonin-induced Ca\(^{2+}\) efflux and subsequent germination.

4.3. H\(_2\)O\(_2\) plays a vital role in melatonin-induced alleviation of the inhibitory effects of ABA on germination

Similar to the Ca\(^{2+}\) signal, ROS as essential signal molecules play a crucial role in regulating seed germination, and such a role of ROS is concentration-dependent and controversial as well [25]. For instance, Leymarie et al. [58] reported that inhibition of H\(_2\)O\(_2\) by DPI (an NADPH oxidase inhibitor) or mutation of AtRBOHD suppressed seed germination; however, the results of Kwak et al. [59] showed that AtRBOHD/F deficiency promoted seed germination by impairing ABA signaling. In the current study, the transient increase of H\(_2\)O\(_2\) during seed germination under normal conditions suggests that transient oxidative status is required for driving cell functions towards germination (Fig. 6a) [26].
Notably, ABA delayed and attenuated H$_2$O$_2$ increase during seed incubation. However, germination of DPI-1 or DMTU-L pretreated melon seeds and Arabidopsis AtRBOHD and AtRBOHF mutant seeds exhibited less sensitivity to ABA (Fig. 7c, d). These results indicate that a lower H$_2$O$_2$ level is involved in ABA-induced inhibition of germination. Melatonin increased H$_2$O$_2$ accumulation under ABA stress, and such an increase was associated with the up-regulated expression of RBOHD and decreased GSH/GSSG ratio (Fig. 6). Consistent with the previous study on Arabidopsis [60], exogenous H$_2$O$_2$ counteracted ABA-retarded seed germination by decreasing and increasing the contents of ABA and GA$_3$, respectively (Fig. 7). Moreover, inhibition of H$_2$O$_2$ accumulation by higher doses of DMTU (DMTU-H) or DPI (DPI-H) prevented the melatonin-regulated ABA and GA$_3$ balance and germination under ABA stress. Taken together, increased H$_2$O$_2$ levels played a vital role in melatonin-ABA antagonism to promote seed germination. This is consistent with earlier observations that H$_2$O$_2$ as a key signaling molecule mediates melatonin-induced lateral root formation as well as tolerance to environmental stresses [61–63].

To date, the mechanisms underlying melatonin offsets ABA to promote seed germination are largely unknown. Here we show that melatonin-induced antagonism to ABA during seed germination essentially involves CA3-mediated Ca$^{2+}$ influx and H$_2$O$_2$ accumulation, which, in turn, regulate ABA and GA$_3$ balance by promoting ABA catabolism and/or GA$_3$ biosynthesis (Fig. 8). To our knowledge, this is the first study of its kind to provide evidence for the role of Ca$^{2+}$ and ROS signaling in seed germination by the antagonistic effect of melatonin on ABA action.

CRediT authorship contribution statement

Hao Li: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Review & editing. Yanliang Guo: Data curation, Investigation, Methodology, Software, Visualization, Writing - original draft. Zhixiang Lan: Data curation, Investigation, Software, Visualization. Xizhang Zhang: Data curation, Investigation, Software, Visualization. Golan Jalal Ahamed: Conceptualization, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. Jingjing Chang: Investigation, Methodology, Resources. Yong Zhang: Conceptualization, Resources, Validation. Chunhua Wei: Conceptualization, Validation. Xian Zhang: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declared that they have no conflicts of interest to this work.

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Appendix A. Supplementary data

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