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Article

GABA Enhances Thermotolerance of Seeds Germination by Attenuating the ROS Damage in Arabidopsis

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Abstract: Seeds germination is strictly controlled by environment factor such as high temperature (HT) through altering the balance between gibberellin acid (GA) and abscisic acid (ABA). Gama-aminobutyric acid (GABA) is a small molecule with four-carbon amino acid, which plays a crucial role during plant physiological process associated with pollination, wounding or abiotic stress, but its role in seeds germination under HT remains elusive. In this study we found that HT induced the overaccumulation of ROS, mainly H_2O_2 and O_2^-, to suppress seeds germination, meanwhile, HT also activated the enzyme activity of GAD for the rapid accumulation of GABA, hinting the regulatory function of GABA in controlling seeds germination against HT stress. Applying GABA directly attenuated HT-induced ROS accumulation, upregulated GA biosynthesis and downregulated ABA biosynthesis, ultimately enhanced seeds germination. Consistently, genetic analysis using the *gad1/2* mutant defective in GABA biosynthesis, or *pop2-5* mutant with high endogenous GABA content supported the potential function of GABA in improving seeds germination tolerance to HT through scavenging ROS overaccumulation. Based on these data, we propose that GABA acts as a novel signal to enhance thermotolerance of seeds germination through alleviating the ROS damage to seeds viability.

Keywords: GABA; thermotolerance; ROS damage; seeds germination

1 Introduction

Seed germination at suitable time is crucial for plant survival against unfavorable environmental conditions, also avoid pre-harvest sprouting (PHR) for crops, which causes substantial losses in grain yield and quality [1–3]. Understanding the fundamental mechanisms that control seed germination and dormancy is helpful for us to the development of sustainable agriculture. The timing of germination is strictly controlled by a combination of both genetic and environment cues [4,5]. The phytohormones gibberellin acid (GA) and abscisic acid (ABA) are regarded as the two main regulators that antagonistically control the shift between germination and dormancy. GA promotes germination and represses dormancy, whereas ABA establishes and maintains dormancy, thus inhibiting germination. Genetic screening with either ABA mutants such as *aba1, aba2, nced6, nced9* and *cyp707a2*, or GA

mutants such as *ga1*, *ga2, ga3, ga4, ga5*, and *ga2ox2*, shows that these mutant present germination defect, demonstrating the significance of the GA/ABA pathway in germination. Mutations in the components associated with ABA signaling transduction (transcriptional factors ABI3, ABI4 and ABI5) or inactivation of DELLAs (negative regulators of GA signal) also influences germination or dormancy frequency. These factors also mutually modulate seed germination. For example, RGL2, the main component of DELLAs, stimulates ABA biosynthesis and ABI5 activity to suppress germination [1,2,6]. Other phytohormones (ethylene, auxin, BR, etc.,) also control germination and dormancy via being directly or indirectly associated with GA/ABA signal. Besides endogenous phytohormone levels [7–9]; Environmental cues, mainly light and temperature, also affect germination. There exist various photoreceptors in *Arabidopsis thaliana* for sensing different spectra of light. Phytochromes perceive red or far-red light; cryptochromes, phototropins, zeitlupes, and URV8 perceive blue or UV light. Among these photoreceptors, phytochrome B (phyB) is the major photoreceptor in dry seeds. Phytochrome A (phyA) predominantly accumulates during the seed imbibition period and is capable of sensing both very low quantities of visible light and prolonged far-red light for germination [10]. In contrast, supraoptimal ambient high temperatures (HT) inhibit germination, or thermoinhibition, which is a common phenomenon that many winter annual and biennial species use to avoid germination during summer months [11]. High temperature induces the biosynthesis of ABA via upregulation of several ABA biosynthetic enzymes like 9-*cis*-epoxycarotenoid dioxygenase 9 (NCED9) or via reducing GA content; both of which suppress germination. Expression of 9-cis-*epoxycarotinoid dioxygenase 4* (NCED4) is essential for the thermoinhibition of lettuce seed germination [11,12]. Consistently, the seeds of ABA-deficient mutant *aba1* or ABA-insensitive mutant *abi3* can germinate at high temperature. The mutants of GA negative regulators, *spy* or *rgl2*, also show germination tolerance at high temperatures, suggesting the important role of GA/ABA in thermoinhibition [12]. It is reported that ROS, mainly H_2O_2 and O_2^-, is necessary for initiating seeds germination, and controlling seeds desiccation tolerance, but the role and regulatory mechanism of ROS in seeds germination under HT need more investigation.

Gama-aminobutyric acid (GABA) is a ubiquitous four-carbon ammonic acid, which acts as an inhibitory neurotransmitter in animals [13–15]. Accumulated evidences demonstrate that GABA is also functional in plant responses to various abiotic stresses such as hypoxia, heat, cold and touch, as well as biotic stresses such as herbivory, wounding and pathogen infection [13,16,17]. In general, GABA signal regulates the balance of C:N or cytosolic pH, it is biosynthesized from glutamate by a decarboxylase (GAD), a Ca^{2+}-calmodulin related enzyme in plants [13,18]. It is degraded in mitochondria through GABA shunt, a metabolic pathway that bypasses two successive steps of the tricarboxylic acid cycle, catalyzed by a-ketoglutarate dehydrogenase and succinyl CoA. During the GABA shunt cycle, the GABA transaminase (GABA-T) converts GABA to succinic semialdehyde, after which succinic-semialdehyde dehydrogenase (SSADH) oxidizes succinic semialdehyde to succinate, a reaction that is coupled with NADH production [18,19]. In Arabidopsis, salt stress induces the transcriptional upregulation of GAD1 and GAD2, leading to a high level of GABA. The loss-of-function mutant *pop2*, which is deficient in GABA-T, is oversensitive to ionic stress but not to osmotic stress, suggesting a specific role in salt tolerance [20]. GABA has also been reported to regulate the antioxidant response against abiotic stress [19].

In this study, we systemically investigated the role of GABA signal in seeds germination during HT stress, and found that HT triggered the enzyme activity of GAD1 for the rapid accumulation of GABA, hinting the its role in controlling seeds germination under HT stress. Furthermore, we found that applying GABA treatment directly enhanced the seeds germination after HT stress, also attenuated HT-induced ROS accumulation. Genetic analysis showed that the seeds germination of mutant defective in GABA biosynthesis was lower, while the mutant with high endogenous GABA content showed higher seeds germination, in the contrast to the wild-type Col seeds subjected to HT stress. Finally we found GABA improved thermotolerance of seeds germination through increasing GA or decreasing ABA biosynthesis.

Thus, our findings reveal a novel function of GABA in modulating thermotolerance of seeds germination through attenuating ROS damage and altering GA/ABA ratio.

2 Materials and Method

2.1 Plant Materials and Growth

All *Arabidopsis* mutant and transgenic lines were generated from a Columbian (Col-0) background. Mutant seeds of *gad1*/2 and *pop2-5* were obtained from the ABRC (Ohio State University, Columbus, USA). The *gad1/2* double mutant was obtained by crossing the *gad1* and *gad2* single mutant. After cold stratification at 4°C for 3 days, the seeds were sterilized and sown on 0.6% agar (pH 5.7) plates. Adult plants were grown under long-day conditions at 22°C in a growth chamber with a 16-h white light (50 μmol 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 prior to the germination assays.

2.2 Germination Measurements

Seeds were harvested after the siliques dried. The seeds stored less than 2 months were used for germination experiment. The analysis of seed germination was performed according to previously reported methods [21,22]. Seeds were surface-sterilized in 5% hypochlorite and 0.02% Triton-X100 solution for about 5 min, and washed for three times with sterile water, the washed seeds was sowed on the MS medium. For HT stress, the seeds were imbibed at a constant temperature (high temperature at 32°C, or different indicated high temperature) under continuous white fluorescent light (60 μmol $m^{-2}s^{-1}$). Germination of the seeds was scored based on radicle protrusion, and germination frequency was calculated. As the control, the seeds were germinated at 22°C. At least three biological replicate experiments were performed for each germination assay.

2.3 RNA Extractions and Quantitative RT-PCR

After different treatments, seeds were collected and total RNA was extracted using TRIzol (Invitrogen) according the manufacturer's recommended method [22]. After DNAase I treatment to remove potential genomic contamination, first-strand cDNA was synthesized using 500 ng of total RNA in a 10-μL system with M-MuLV reverse transcriptase (Fermentas) and oligo$(dT)_{18}$ primers. Transcription levels of target genes were measured via real-time PCR using SYBR Green I master mix and a Roche LightCycler 96 PCR machine (Roche). At least three different biological replicates were used to confirm gene expression patterns, and PP2A or Ubiquitin 10 was used as an internal gene expression control. Primers used are described before.

2.4 GA_4 and ABA Concentration Measurement

The concentration of GA and ABA in imbibed seeds was determined as in previously described methods [21,22]. For GA content analysis, seeds were weighed and ground to fine powder under liquid nitrogen. Internal standards of 1 ng g^{-1} $^2H_2$-GA_4 were added to the samples followed by extraction with 500 μl solvent (methanol/H_2O, 80/20, v/v) at 4°C for 12 h. The supernatants were sequentially passed through the preconditioned tandem solid-phase extraction cartridges containing C18 adsorbent (50 mg) and strong anion exchange adsorbent (200 mg). The strong anion exchange cartridge was then rinsed with 2 ml of 20% methanol (v/v), and the targeted acidic phytohormones were eluted with 3 ml acetonitrile with 1% formic acid (v/v). The eluent was evaporated under mild liquid nitrogen stream at 35°C and dissolved in 100 μl H_2O. The solution was acidified with 10 μl formic acid and extracted with 1 ml ether twice. The combined ether phase was dried under nitrogen gas and reconstituted in 100 μl acetonitrile followed by the addition of 10 μl triethylamine (20 mmol ml^{-1}) and 10 μl 3-bromoactonyltrimethylammonium bromide (20 μmol ml^{-1}). The reaction solution was vortexed at 35°C for 30 min and then evaporated

under nitrogen gas. The samples were dissolved in 200 µl 10% acetonitrile (v/v) and subjected to nano-liquid chromatography–electrospray ionization–quadrupole time-of-flight–mass spectrometry analysis.

For ABA measurements, seeds were ground under liquid nitrogen, and 45 pmol of $^{2}H_2$-ABA internal standard was added to 200 mg of powder. The samples were extracted with 2 ml methanol at 20°C overnight. After spinning at 4°C for 15 min at 18,000 rpm, the supernatant was dried under nitrogen gas and dissolved in 1 ml 5% ammonia solution (v/v). Crude extracts were purified by preconditioned Oasis MAX strong anion-exchange column (Waters), and the samples were eluted with 4 ml methanol containing 5% formic acid. The eluent was dried under nitrogen gas and dissolved in 200 µl 80% methanol (v/v) and subjected to ultra-performance liquid chromatography tandem mass spectrometry analysis.

2.5 Analysis of H_2O_2 and O_2^- Content

H_2O_2 content was determined using xylenol orange [23,24]. In brief, the seeds were collected after different treatments and homogenized in 5 ml 0.2 M $HClO_4$ at 4°C. The extract was held on ice for 5 min and then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant (100 µl) was added to 1 ml reaction buffer containing 0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 1.25 M xylenol orange, and 1 mM sorbitol and kept at room temperature for 1 h. Absorbance was measured at 560 nm, and H_2O_2 levels were calculated based on H_2O_2 standards.

O_2^- content was determined according to a previously published method [24]. The supernatant (100 µl) was added to a 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM hydroxylamine hydrochloride, 17 mM sulfanilic acid and 7 mM α-naphthyl. The absorbance of the pink phase was measured at 530 nm. O_2^- content was calculated by comparing with a standard curve.

2.6 Analysis of GABA Content and GAD Enzyme Activity

Measurement of GABA content from leaf extracts was performed as previously described [25]. In brief, a powdered seeds sample (about 100 mg) was placed in a falcon tube, extracted with 10 mL 80% (v/v) ethanol and shaken on a vortex mixer for 5 min, the sample was then centrifuged at 500 rpm for 10 min at 4°C and the supernatant was filtered using Millipore filter paper. The extraction step was repeated twice, the resultant combined extract was dried on a Rotary Evaporator until the ethanol completely evaporated, after which the dried residue was dissolved in 1 mL water for GABA derivatization. 1 mL of 2-hydroxynaphthaldehyde (2.5% w/v) in methanol was added to the dissolved GABA (1 mL) for derivatization treatment, followed by addition of 0.5 mL boric acid-NaOH (pH 8.5) in a 5 mL volumetric flask. The resultant mixture was heated at 85°C for 15 min in a water bath, and the solution was allowed to cool to room temperature. The volume was adjusted to 5 mL with methanol and the sample kept at 4°C until analysis. HPLC analysis was performed on an Agilent 1200 HPLC System. The solution (5 mL) was injected on a reverse phase SB-C18 column (2.1 50 mm) with a methanol (B) and water (A) gradient system (at 0 min, solvent B 50%; at 2 min, solvent B was 60%; at 5 min, B was 70%; at 8 min, B was 80%; at 10 min, B was 90%; and at 12 min, B was 50%) with a flow rate of 0.8 mL/min and UV detection via photodiode array at 254 nm. Identification of each peak was based on the comparison of retention time of the standard.

GAD activity was determined by measuring the conversion rate of the substrate glutamate to GABA [26]. The reaction mixture consisted of 10 L of 80 mM sodium phosphate, pH 5.6, 100 mM L-glutamate and 1 g powdered plant tissues. The reaction solution was incubated at 40°C for 60 min, then terminated by incubating at 90°C for 5 min. After centrifugation (3000 rpm, 10 min), the GABA in the supernatant was assayed. One unit of GAD activity was defined as the release of 1 nmol of GABA produced from glutamate per 30 min at 40°C. The GAD activity of plant tissue is defined as units of GAD activity per gram of plant tissue.

3 Results

3.1 HT Induced the Production of H_2O_2 to Suppress Seeds Germination

Our previous study showed that ambient temperature over 32°C completely suppressed the seeds germination [21,22]. To determine whether HT induced the over-accumulation of ROS to suppress seeds germination, we measured the endogenous H_2O_2 content in the Col seeds during imbibition under 32°C, and found HT induced the transient and strong accumulation of H_2O_2, reaching the peak level after 12 h of HT treatment, and sustained high level during the following 24 h. As the control, the H_2O_2 level in the imbibition Col seeds under 22°C was slightly increased, but not as strong as that under HT stress (Fig. 1A). Similarly, HT also induced the quick and strongly accumulation of O_2^- (Fig. 1B). These data

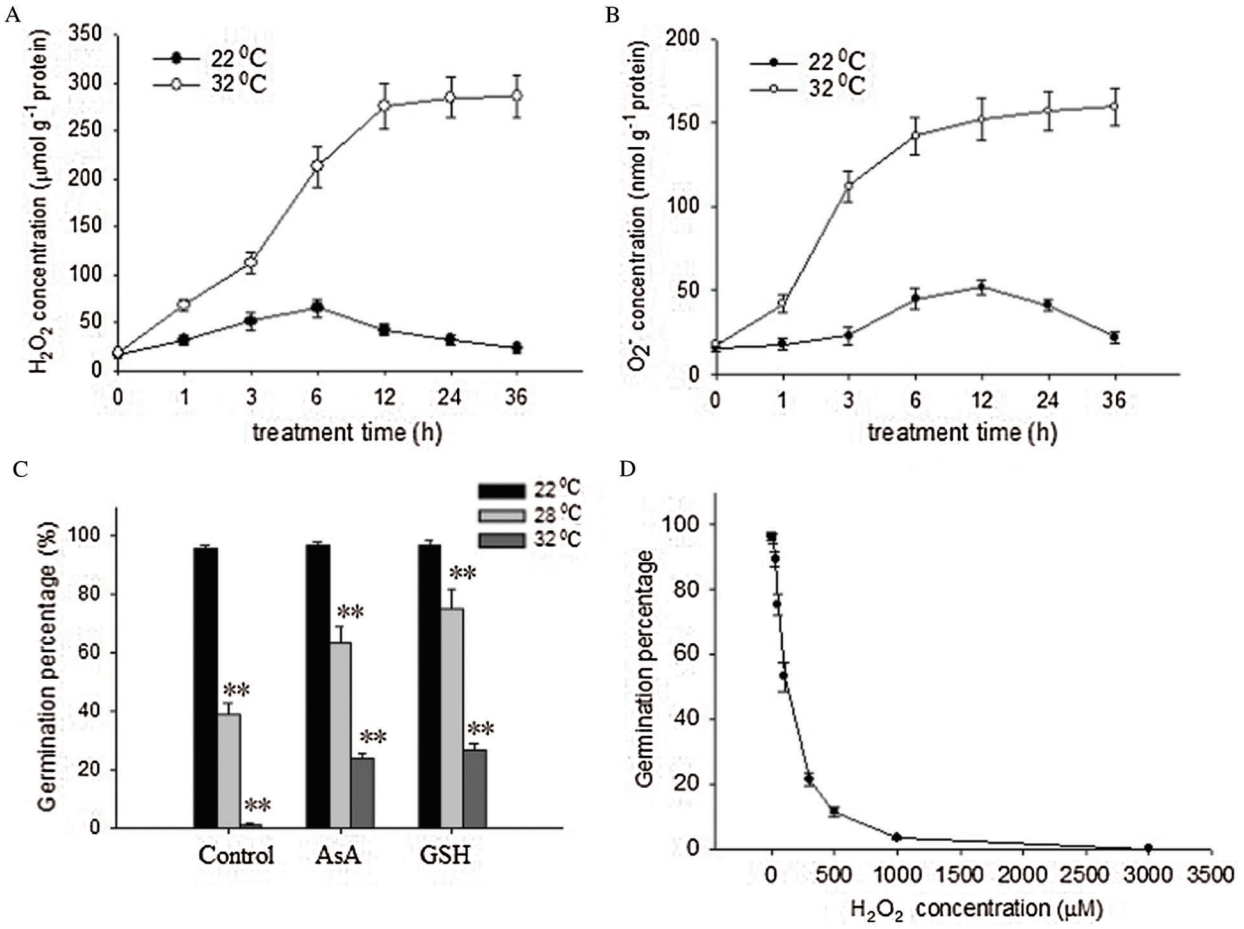

Figure 1: HT induced the generation of ROS in the imbibition Col seeds. Values are means ± SD. Error bars indicate SD of three biological repeated experiments. The bar means ±SE of three independent batches of seeds experiments. The statistical difference was analyzed by *t*-test (double stars means $p < 0.01$) (A and B) Time-course effect of HT on the generation of H_2O_2 and O_2^- in the imbibition Col seeds. The wild-type Col seeds was imbibed under normal temperature at 22°C or HT at 32°C for indicated time, and the content of H_2O_2 (A) and O_2^- (B) was measured. (C) Effect of ROS and antioxidant on the seeds germination. The wild-type Col seeds was imbibed under normal temperature at 22°C or HT at 32°C with or without antioxidant AsA (1mM) or GSH (100 μM), and the content of H_2O_2 and O_2^- was measured after 12 h of incubation. (D) The dose effect of on the seeds germination. The wild-type Col seeds were imbibed under normal temperature at 22°C with gradient H_2O_2 treatment, and the seeds germination rate was recorded

suggest that over-accumulation of ROS, mainly H_2O_2 and O_2^-, suppresses the seeds viability. To confirm whether HT-induced over-accumulation of ROS is response for the lower seeds germination, we pretreated the Col seeds with the ROS scavenger ascorbic acid (AsA) and glutathione (GSH) following with HT stress, and found that AsA or GSH pretreatment obviously attenuated the inhibitory effect of HT on seeds germination (Fig. 1C), as the control, the used concentration of AsA or GSH treatment at normal condition did not obviously accelerate the seeds germination under normal condition (Fig. 1C), possibly the Col seeds already showed high germination percentage under 22°C, which conceal the promoting effect of AsA or GSH on seeds germination. Gradient exogenous H_2O_2 treatment directly also gradually suppressed the seeds germination (Fig. 1D). Based on these data, we propose that relatively high ROS is the main reason to suppress the seeds germination viability under HT stress.

3.2 Application with GABA Enhances Seeds Germination under HT Stress by Attenuating ROS Accumulation

It is reported the ROS derived from plant mitochondria plays the critical role for seeds germination [27], and the small molecular amino acid GABA through mitochondrial SSDH-mediated GABA shunt pathway is required for restrict ROS level during environment stress [19]. Thus we want to know the role of GABA during seeds germination under HT stress, and treated the imbibition seeds under HT condition with or without additional GABA, and found that GABA treatment increased the seeds germination under HT stress compared with seed without GABA treatment (Fig. 2A), specially, GABA concentration at 1 μM was obvious. Meanwhile, GABA treatment reduced HT-induced accumulation of ROS, mainly H_2O_2 and O_2^{-1} (Fig. 2B). This data hints the positive function of GABA in enhancing seeds germination tolerance to HT stress via reducing the over-accumulation of ROS.

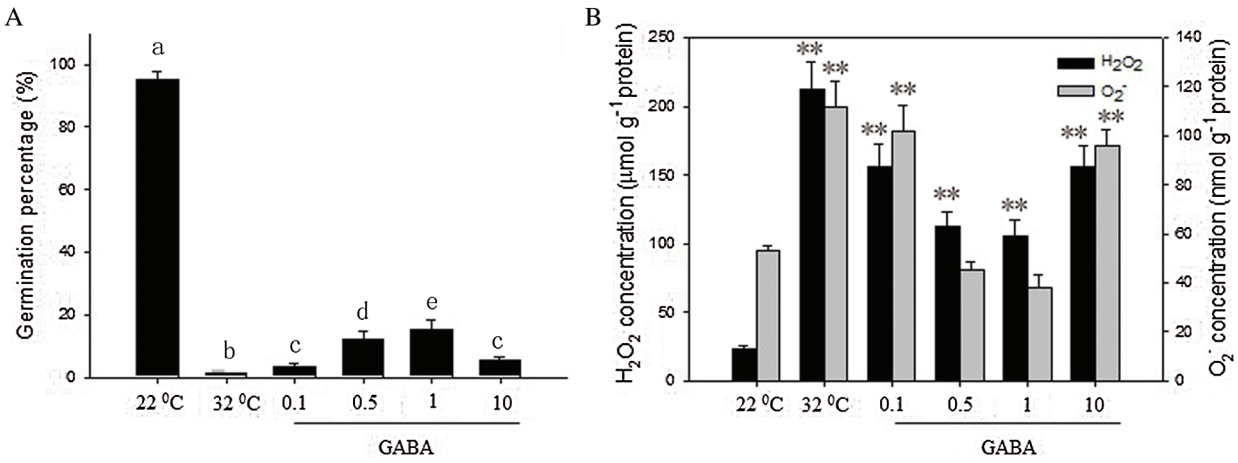

Figure 2: GABA enhances seeds germination tolerance to HT through ROS (A) Dose effect of GABA on enhancing thermotolerance of seeds germination. The wild-type Col seeds was imbibed under normal temperature at 22°C, or HT at different concentration of GABA for 3 days, and the seeds germination percentage was recorded. The bar means ±SE of three independent batches of seeds experiments. Bars with different letters are significantly different at $p < 0.05$ (Tukey's test). (B) Dose effect of GABA on the generation of ROS. The wild-type Col seeds were imbibed under normal temperature at 22°C, or HT at different concentration of GABA for 12 h, and the content of H_2O_2 and O_2^- was measured. The bars mean ±SE of three independent batches of seeds experiments. The statistical difference was analyzed by t-test (double stars means $p < 0.01$)

3.3 GABA Signal Alters the GA and ABA Metabolism under HT Stress

The balance of GA/ABA determines the status of seed germination or dormancy [4,28]. We want to know if GABA regulates seeds germination through altering GA/ABA content, thus we measured the endogenous GA and ABA content with or without HT or GABA treatment. In agreement with previous result, HT treatment suppressed the bioactive GA_4 content and increased endogenous ABA content, however GABA treatment obviously retarded the effect of HT on upregulating ABA content and down-regulating GA_4 content (Fig. 3A). Like GABA, the ROS scavenger AsA and GSH treatment also reduced ABA content and increased GA_4 content under HT stress.

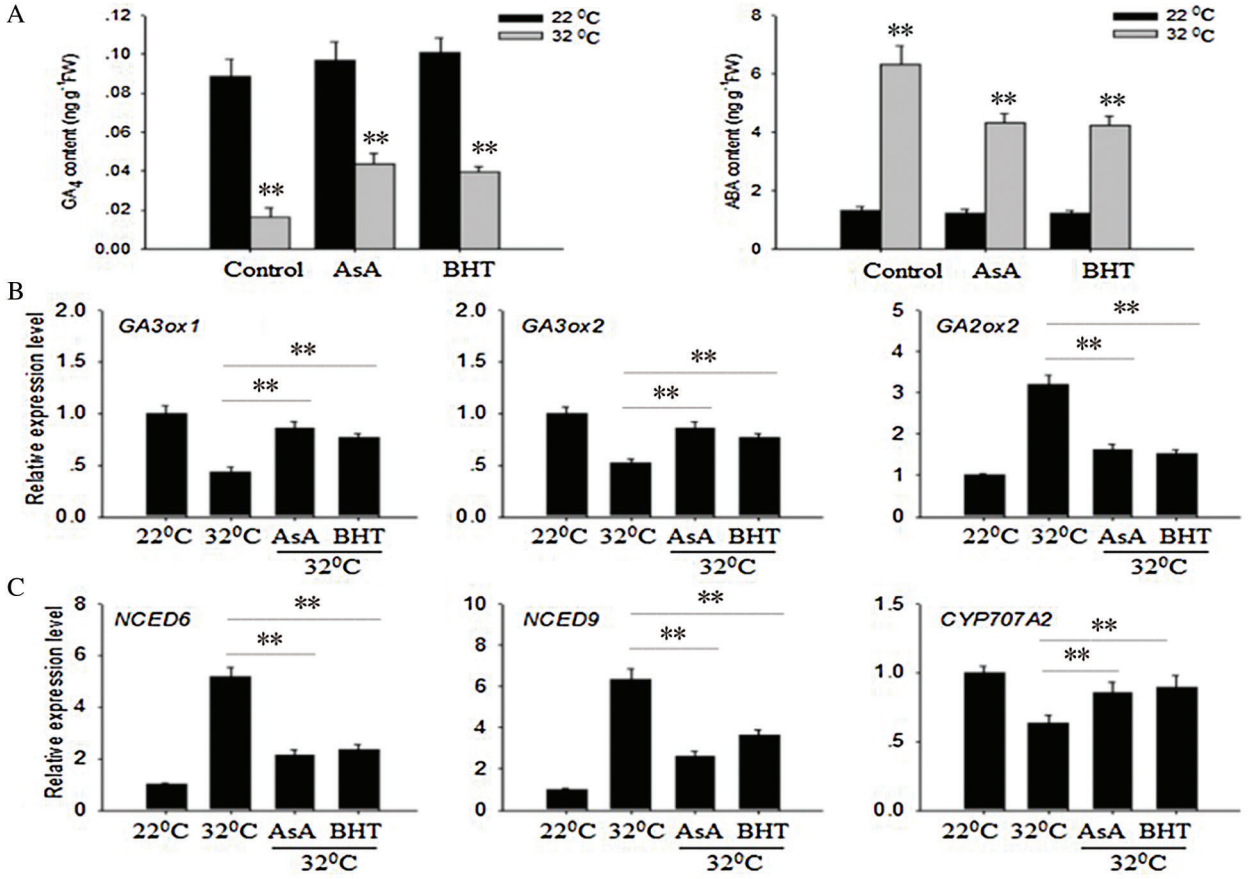

Figure 3: HT-induced ROS alters the ABA/GA metabolism to suppress seeds germination. (A and B) Effect of HT and antioxidant on GA and ABA content in the imbibition Col seeds. The wild-type Col seeds was imbibed under normal temperature at 22°C or HT at 32°C with or without antioxidant AsA or GSH, and the content of GA_4 and ABA was measured after 3 days of incubation. The bars mean ±SE of three independent batches of seeds experiments. The statistical difference was analyzed by *t*-test (double stars means $p < 0.01$). (C and D) Effect of HT and antioxidant on the transcriptional level of genes associated with GA and ABA metabolism. The wild-type Col seeds was imbibed under normal temperature at 22°C or HT at 32°C with or without antioxidant AsA or GSH for 12 h, and the transcriptional level of genes associated with GA anabolic gene *GA3ox1, GA2ox1* and *GA2ox2* (A), and the gens associated with ABA catabolic *NCDE3, NCDE6* and anabolic gene *CYP707A2* (B) was measured by RT-qPCR analysis. The *IPP2A* gene was used as the internal control. The bar means ±SE of three independent batches of seeds experiments. The statistical difference was analyzed by *t*-test (double stars means $p < 0.01$)

In Arabidopsis seeds, GA biosynthesis is regulated by GA anabolic gene including *GA3ox1* and *GA3ox2*, and catabolic gene such as *GA2ox2*, and ABA biosynthesis is regulated by ABA anabolic genes including *NCED6* and *NCED9*, and ABA catabolic gene *CYP707A2* [29]. We also checked the effect of HT and antioxidant on the transcriptional levels of these above genes. As shown in Figs. 3B and 3C, HT treatment reduced the expression of *GA3ox1*, *GA3ox2* and *CYP707A2*, but increased the expression of *GA2ox2, NCED6*, and *NCED9*. By contrast, HT-induced increasing of *GA2ox2, NCED6*, and *NCED9* expressions after AsA or GSH treatment was reduced, whereas HT-induced decreasing of *GA3ox1*, *GA3ox2* and *CYP707A2* was reversed after AsA or GSH treatment. Thus these data suggest that HT-induced ROS alters the expressions of gens associate with GA/ABA metabolism to suppress seeds germination.

3.4 HT Induced the Transient Accumulation of GABA through Activating GAD Enzyme Activity

As GABA is important for seeds germination tolerance to HT stress, we then measured the content of endogenous GABA in the imbibition seeds response to HT stress, and found that HT treatment induced the rapid accumulation of GABA at the first 6 h and reached the peak level after 12 h of HT treatment, and then the content of GABA dropped down during the following 48 h of treatment (Fig. 4A). In Arabidopsis, GAD is main enzyme responsible for the biosynthesis of GABA, we thus measured the enzyme activity of GAD in the imbibition wild type seeds after HT stress. Similar to GABA pattern, HT treatment indeed induced the GAD enzyme activity during the first 12 h of HT treatment, and then decreased after prolonging the HT treatment (Fig. 4B). As the control, the GAD enzyme activity in the imbibition seeds was not apparently change under the normal condition at 22°C.

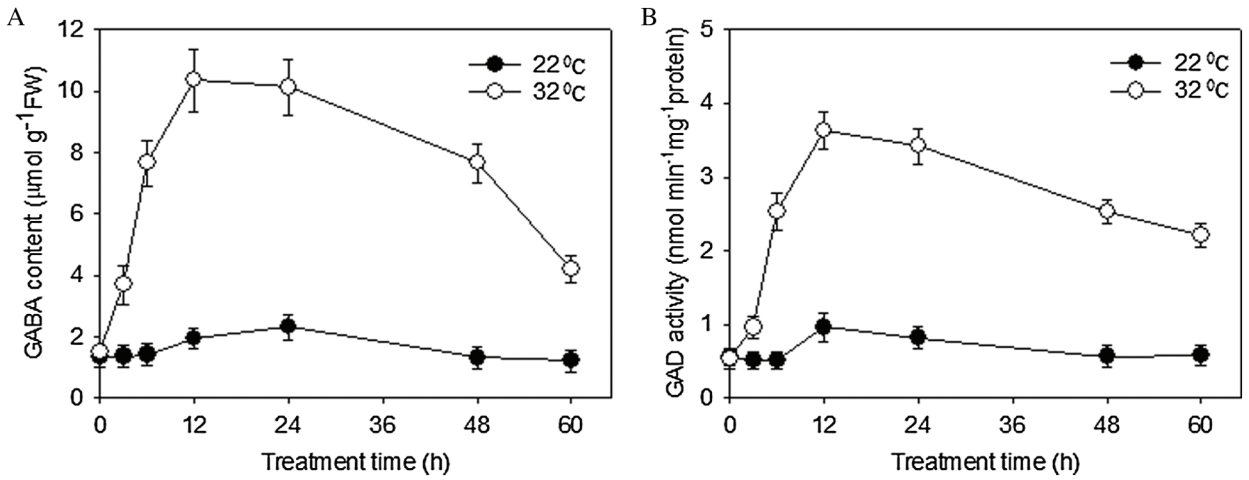

Figure 4: HT induced the rapid accumulation of GABA and transiently increased GAD enzyme activity. The wild-type Col seeds was imbibed under normal temperature at 22°C or HT at 32°C for indicated time, the content of GABA (A) and the enzyme activity of GAD (B) was measured. The bars mean ±SE of three independent batches of seeds experiments

3.5 The Mutants Deficiency in GABA Shunt Show Different Seeds Germination Capability under HT Stress

GABA is bio-synthesized through GABA shunt in plant mitochondria, GAD1/2 is responsible for GABA accumulation while GABA transaminase coverts GABA to succinic semialdehyde [19]. The double mutant of *gad1/2* contains lower level of endogenous GABA and show sensitive to salt stress, while the *pop2-1* mutant deficiency in GABA transaminase (GABA-T) contains relative higher level of

GABA [14,17]. We compared the seed germination percentage of *gad1/2* and *pop2-5* mutant with the wild type Col seeds under HT stress, both of Col and *gad1/2* seeds did not germinate under HT stress at 32°C, but about 19.6% seeds of *pop2-5* germinated under such condition (Fig. 5A), this data showed that the seeds germination of *pop2-5* shows tolerance to high temperature stress (Fig. 5A). Meanwhile, we also compared their seeds germination after 28°C stress, and found about 42.3% of Col seed and 63.6% of *pop2-5* seeds germinated, but only about 21.3% of *gad1/2* seeds germination, which is lower than that of Col and *pop2-5* seeds, suggesting that *gad1/2* seeds germination show sensitive to HT stress (Fig. 5A), thus these genetic data suggest that GABA acts as the endogenous signal to positively regulate seeds germination under HT stress.

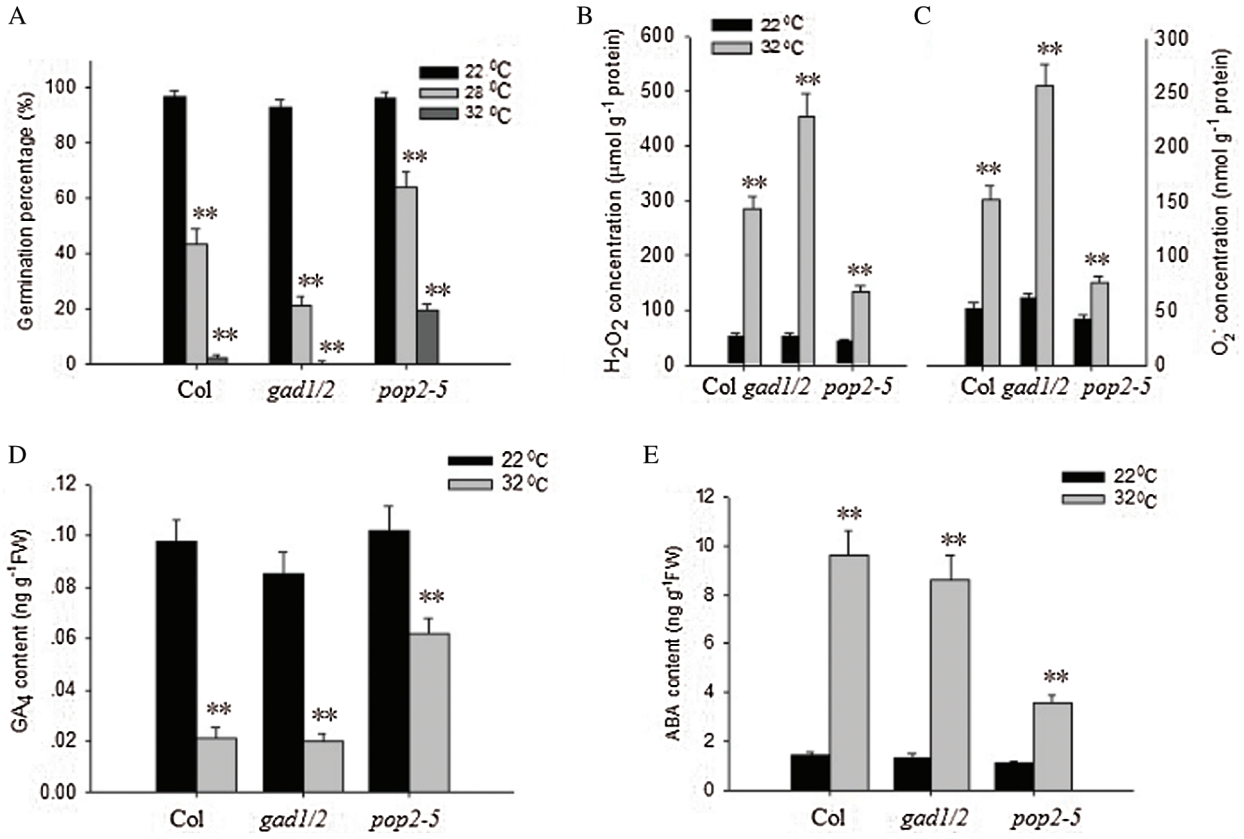

Figure 5: GABA signal enhances seeds germination tolerance to HT through attenuating ROS accumulation and change endogenous GA/ABA level. The bar means ±SE of three independent batches of seeds experiments. The statistical difference was analyzed by *t*-test (double stars means $p < 0.01$). (A and B) Different seeds germination of *gad1/2* and *pop2-5* in response to HT. The Col, *gad1/2* and *pop2-5* was incubated under at 22°C, 28°C or HT at 32°C for 3 days, and the seeds germination percentage was recorded. (C and D) Different ROS and GA/ABA content of *gad1/2* and *pop2-5* in response to HT. The Col, *gad1/2* and *pop2-5* was incubated under at 22°C, or HT at 32°C for 12 h, the content of H_2O_2 (B) and O_2^- (C) was recorded. The content of GA_4 (D) and ABA (E) was also measured after 3 days of incubation

We also compared the ROS level among Col, *gad1/2* and *pop2-5* seed under HT stress, and observed that HT increased the accumulation of H_2O_2 and O_2^- in wild-type Col seeds, but HT-induced H_2O_2 and O_2^- was aggravated in *gad1/2* mutant but attenuated in *pop2-5* mutant seeds (Figs. 5B and 5C), suggesting endogenous GABA acts as the ROS scavenger to reduce HT-induced ROS accumulation. Furthermore,

we measured the GA_4 and ABA content in *gad1/2* and *pop2-5* mutant seeds before or after HT stress. As shown in Figs. 5D and 5E, HT suppressed the accumulation of GA_4 but increased ABA accumulation, but such effect was more obvious in *gad1/2* mutant seeds, but a little weaker in *pop2-5* seeds. Consistent with the GA/ABA content pattern, HT treatment downregulated the expression of expression of *GA3ox1*, *GA3ox2* and *CYP707A2*, and upregulated the expression of *GA2ox2, NCED6*, and *NCED9* in Col seeds

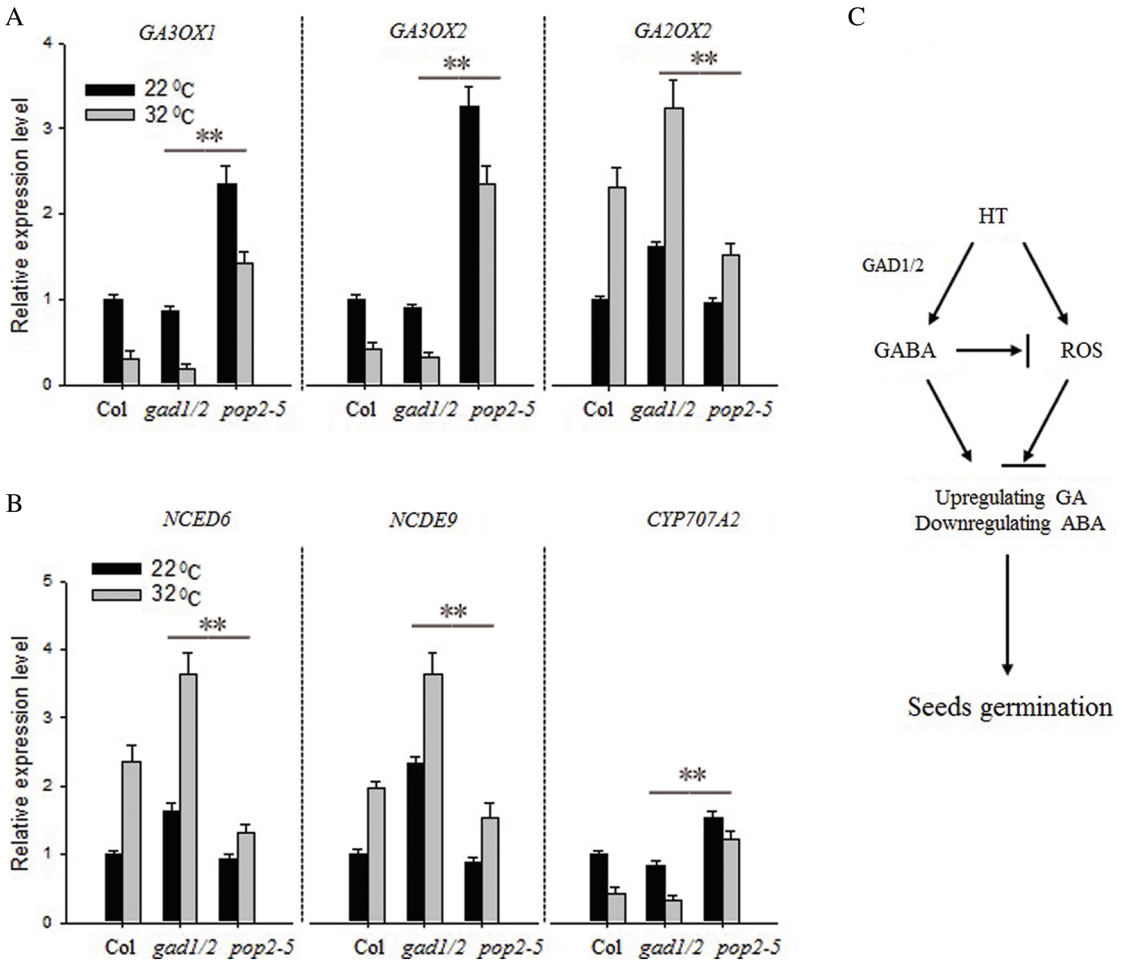

Figure 6: GABA and HT stress affect the transcriptional levels of genes associated with GA/ABA metabolism. (A and B) Effect of HT on the transcriptional level of genes associated with GA/ABA metabolism. The Col, *gad1/2* and *pop2-5* seeds was imbibed under normal temperature at 22°C or HT at 32°C for 12h, and the transcriptional level of genes associated with GA anabolic gene *GA3ox1, GA2ox1* and *GA2ox2* (A), and the gens associated with ABA catabolic *NCDE3, NCDE6* and anabolic gene *CYP707A2* (B) was measured by RT-qPCR analysis. The *IPP2A* gene was used as the internal control. The bar means ±SE of three independent batches of seeds experiments. The statistical difference was analyzed by t-test (double stars means $p < 0.01$). (C) The propose model to illustrate the role of GABA signal in enhancing thermotolerance of seeds germination through attenuating ROS damage and altering GA/ABA metabolism. HT stress induces the overaccumulation of ROS which upregulates ABA and downregulates GA to suppress seeds germination, simultaneously, HT induces the transient increase of GABA through GAD enzyme activity to attenuate ROS damage, but HT-induced GABA is inadequate, therefore, additional appropriate GABA treatment is an efficient strategy to enhance seeds germination potential under HT stress

(Figs. 6A and B), we found that the expression level of *GA3ox1*, *GA3ox2* or *CYP707A2* was lower in *gad1/2*, but higher in *pop2-5*. Conversely, the expression level of *GA2ox2, NCED6* or *NCED9* was higher in *gad1/2*, but lower in *pop2-5*. It is possible that GABA reduced the HT-induced ROS level to increase GA biosynthesis and decrease ABA biosynthesis.

4 Discussion

ROS play the important role to regulate plant growth and development, Ma et al. [27] reported that temperature-dependent ROS generation from mitochondria is prerequisite for cotton seeds germination. However, environment stress also induced the over-accumulation of ROS which damage plant viability, for most recalcitrant seeds such as *Baccaurea ramiflora* or *Antiaris toxicaria*, desiccation treatment causes the over-accumulation of ROS to damage seeds viability [30,31]. As a result, plant evolved the specific mechanism to scavenge ROS damage. For example, mitochondria AOX are alternative pathway to fine-turn endogenous ROS level, in order to avoid the ROS toxin. Our previous study also reported that the desiccation treatment induced the overaccumulation of ROS in the seeds to suppress its germination [27], while the antioxidant chemicals, such as AsA treatment attenuated the inhibitory effect of desiccation treatment on seed germination [30,31]. Ambient high temperature stress triggers the seeds secondary dormancy [21,22,32]. In this study, we found increasing environment temperature gradually induced seeds dormancy with lower seeds germination rate. HT treatment also induced the overaccumulation of ROS (Figs. 1A and 1B), which might be the main reason leading to low seeds germination, because scavenging HT-induced ROS generation by antioxidant AsA or GSH increased seeds germination (Fig. 1C), while high level of H_2O_2 treatment obvious suppressed seed germination (Fig. 1D). Meanwhile, we observed that high ROS altered the metabolism of GA an ABA, by decreasing GA_4 content through up-regulating the expressions of GA anabolic genes and down-regulating the expressions of GA catabolic genes (Figs. 3A–3C), or by increasing ABA content through up-regulating the expressions of ABA anabolic genes or down-regulating the expressions of ABA catabolic genes (Figs. 3A–3C). On the contrary, the antioxidant AsA or GSH treatment increased GA content and decreased ABA content to antagonize the inhibitory effect of ROS on seeds germination. Thus these data support the opinion that HT treatment results into the overaccumulation of ROS that reduced the ratio of GA to ABA, ultimately suppress seeds germination.

Not just as an inhibitory neurotransmitter in animals, the widely physiological function of GABA in plants was also reported, particularly, GABA is reported to act as the antioxidant. GAD is the main enzyme responsible for GABA biosynthesis [19]. In this study we fist found that GABA efficiently scavenge HT-induced ROS accumulation to increase seeds germination under HT stress (Figs. 2A and 2B). HT treatment quickly activated the GAD activity in the imbibition seeds, and rapid accumulation of GABA (Figs. 4A and 4B). In agreement with it, HT did not efficiently accumulate GABA in the imbibed seeds of *gad1/2* mutant defective in GAD enzyme activity. We further found that the mutant seeds of *gad1/2* showed lower germination rate in comparison with the wild-type Col seeds under HT stress, while the *pop2-5* mutant seed with high endogenous GABA showed high seeds germination rate (Fig. 5A), which support our conclusion that GABA act as the novel signal to enhance thermotolerance of seeds germination to HT stress. We also noticed the endogenous ROS level in *pop2-5* mutant was lower whereas the endogenous ROS level in *gad1/2* was higher under HT (Figs. 5B and 5C), which coincide with our above result that GABA enhances thermotolerance of seeds germination through scavenger HT-induced ROS overaccumulation. Supporting our conclusion, we found the endogenous GA level was lower, but ABA level was higher, in the *pop2-5* mutant seeds exposure to HT stress, correspondingly, the GA level was higher, but ABA level was lower, in the *gad1/2* mutant seeds after HT stress (Figs. 5D, 5E and 6A, 6B). Thus these combined genetic and biochemical data further propose that GABA acts as a novel regulator to enhance seeds germination against HT stress through attenuating ROS damage and altering GA/ABA metabolism.

5 Conclusion

We agree with previous study that ROS is the critical signal for seeds germination, as we observed the increasement of ROS during the seeds germination under normal condition, however, HT triggers the overaccumulation of ROS which alter the GA/ABA metabolism and raised the ratio of ABA to GA, leading to the seeds thermoinhibition. In this study we identified GABA acts as the novel signal to enhance seeds germination rate against HT stress, and propose a model to illustrate the role of GABA in seeds germination under HT. As shown in Fig. 6C, HT induced the activity of GAD to generate GABA, which efficiently scavenge HT-induced accumulation of ROS to reduce ROS damage, subsequently increasing GA and decreasing ABA content for seeds germination under HT stress. Thus our finding uncovers a novel function of GABA in plant and broadens the application of GABA in seeds biology.

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