BMC Plant Biology

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Gamma-aminobutyric acid (GABA) releases seed dormancy by orchestrating abscisic acid and gibberellin metabolism and signaling

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Abstract

Background Seed dormancy release, regulated by abscisic acid (ABA) and gibberellin (GA), is essential for plant development and yield. Gamma-aminobutyric acid (GABA) is known to mediate plant stress responses; however, its role in seed dormancy release remains elusive.

Results Here, we reported that seed dormancy release was impaired in *GAD2*-deficient seeds lacking GABA biosynthesis while enhanced in *GABA-T*-deficient seeds with impaired GABA catabolism, indicating a positive regulatory role of GABA in seed dormancy release. Further, ABA antagonized GABA-facilitated seed dormancy release, and GABA failed to alleviate seed dormancy in the GA-deficient mutant *ga1-t*. Analysis of mutants defective in ABA and GA biosynthesis revealed that seed dormancy release, characterized as decreased ABA content and increased GA_3 content, was suppressed in *gad2* while enhanced in *gaba-t1-1* mutant. Mechanistically, GABA promoted GA_3 biosynthesis by upregulating *GA20ox1* expression, while inhibited ABA biosynthesis by downregulating *NCED6* and upregulating *CYP707A2* transcription. GABA also amplified GA signaling and suppressed ABA signaling by downregulating genes expression of *DELLAs* and *ABI3*. Further, GABA-mediated seed dormancy alleviation was confirmed in seeds of pear, apple, and cherry, highlighting its broad applicability across diverse plant species.

Conclusion Our findings not only advance our understanding on the mechanism of GABA-mediated seed dormancy release via coordinated regulation of abscisic acid and gibberellin pathways, but also highlight the potential of GABA as a natural alternative to synthetic chemicals for promoting sustainable and environmentally friendly agricultural practices.

Keywords ABA, GA, GABA-T, GAD, Gamma-aminobutyric acid, Seed dormancy

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Background

Seed dormancy represents a pivotal adaptive trait evolved by seed plants to endure seasonal fluctuations and adverse environmental conditions [1–3]. Defined as the inability of viable seeds to germinate under favorable conditions, seed dormancy necessitates dormancy release for successful germination [4]. The precise regulation of dormancy release is critical for ensuring uniform seed germination, which directly affects crop growth and yield. Environmental cues such as light, temperature, and reactive oxygen species, as well as phytohormones, play key roles in governing seed dormancy [5–7]. Among these factors, abscisic acid (ABA) and gibberellins (GA) are the principal hormones modulating seed dormancy [8–10]. It is generally accepted that the balance between ABA and GA determines seed dormancy status: a high ratio promotes dormancy, whereas a low ratio facilitates seed germination [11].

ABA plays a critical physiological role in promoting seed dormancy [12]. Oxidative cleavage of 9-cis-epoxycarotenoids (NCEDs) are key enzymes involving in ABA biosynthesis, and regulation of *NCEDs* gene transcription modulates the ABA synthesis rate in plants [13]. In Arabidopsis, the *NCED* gene family includes *NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9* [14]. Several *NCED* genes exhibit seed-specific expression, enabling stage-specific control of ABA synthesis and, consequently, precise regulation of dormancy and germination [15, 16]. Moreover, *NCED3* transcription is regulated by multiple transcription factors, which in turn control the synthesis of ABA [17, 18]. Upon completion of its function, ABA is degraded by CYP707As (Cytochrome P450, family 707, subfamily A) [19]. Seeds of *cyp707a1* and *cyp707a2* mutants accumulate elevated ABA levels, resulting in enhanced dormancy [20]. Upon binding to its receptors, ABA forms a complex that activates SNF1-related protein kinases 2, which in turn phosphorylates downstream transcription factors, such as ABA-INSENSITIVE 3 (*ABI3*), ABI4, and ABI5, thereby initiating ABA signaling pathway [21–23]. During seed maturation, ABI3 regulates ABA synthesis through feedback mechanism to respond to ABA signaling [24]. Genetic analysis indicates that *ABI3* loss-of-function leads to reduced ABA levels and premature germination [25]. Notably, ABI4 binds to CCAC or CACCG *cis*-acting elements to directly repress *CYP707A1* and *CYP707A2* expression, thus reinforcing dormancy [26]. Under stress conditions, ABA-activated *ABI5* interacts with various proteins to regulate ABA signal transduction and thereby modulate dormancy and germination [27–30].

In contrast to ABA, GA acts antagonistically by promoting dormancy release and facilitating germination. Ent-ent-copalyl diphosphate synthase, encoded by *GA1*, is a key enzyme for GA biosynthesis [31]. Due to GA deficiency, *ga1-t* mutants fail to germinate without exogenous GA treatment [32]. GA biosynthesis also requires GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) enzymes [33]. In tomato, mutations in *GA20ox1* and *GA20ox2* result in decreased gibberellin (GA) content, which further deepens seed dormancy [34]. Low GA levels lead to the stabilization of DELLA proteins, which not only inhibit GA signaling by modulating downstream targets but also suppress the transcription of *GA20ox2*, *GA3ox1*, and *GID1*, thereby maintaining dormancy [35]. GA signaling initiates when GA binds to the GIBBERELLIN INSENSITIVE DWARF 1 (GID1) receptor, forming the GA-GID1-DELLA complex [36]. Then the complex interacts with the SCFSLY1/GID2 E3 ubiquitin ligase to degrade DELLA proteins, thereby activating downstream GA-responsive genes expression [37–39]. Five DELLA proteins have been identified in Arabidopsis, including GA-INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE 1 (RGL1), RGL2, and RGL3 [40]. Notably, RGL2 forms a complex with ABI4, functioning as a transcription factor complex that mediates ABA/GA antagonism and finely regulates the transition from dormancy to germination [41].

Gamma-aminobutyric acid (GABA), a four-carbon non-proteinogenic amino acid, is widely present in microorganisms, animals, and plants, functioning as a key intermediate in nitrogen metabolism and amino acid biosynthesis [42]. In plants, GABA is primarily synthesized via two major pathways: the GABA shunt and polyamine degradation, with the GABA shunt being the predominant route [43]. In the GABA shunt pathway, glutamate undergoes decarboxylation catalyzed by glutamate decarboxylase (GAD) to form GABA in the cytoplasm, which is then transported to the mitochondria. In the mitochondria, GABA is subsequently converted to succinic acid semialdehyde by GABA-transaminase (GABA-T), which is then catalyzed by succinic semialdehy dehydrogenase(SSADH) to form succinic acid that enters the tricarboxylic acid cycle. Thus, GAD facilitates GABA synthesis, while GABA-T catalyzes its degradation [44]. For instance, *gad2* mutants exhibit significantly reduced GABA levels in shoots and leaves [45, 46]. Another pathway for GABA biosynthesis involves polyamine degradation. In this pathway, polyamines like putrescine (Put), spermine (Spd), and spermidine (Spm) are metabolized to produce GABA [44]. Put is oxidized to 4-aminobutyraldehyde (GABald) by diamine oxidase (DAO), while Spd and Spm are oxidized to GABald by polyamine oxidase (PAO). GABald is then converted to GABA by betaine aldehyde dehydrogenase [44]. Additionally, 3-mercaptopropionic acid (3-MP), an inhibitor of GABA biosynthesis, effectively blocks GABA production [47].

Growing evidence supports the essential role of γ-aminobutyric acid (GABA) in seed germination. In giant embryo rice, germination is associated with upregulation of *OsGAD* and downregulation of *OsGABA-T3*, resulting in elevated GABA levels [48]. Exogenous application of GABA has also been shown to promote germination in barley [49]. Additionally, GABA levels steadily increase during germination in both oats and wheat [50, 51]. While these findings indicate a positive role for GABA in germination, the underlying mechanism in seed dormancy regulation remains largely unknown. It is known that the establishment and maintenance of seed dormancy depend on the balance between ABA and GA [52–54]. Although germination and dormancy represent interconnected yet distinct physiological processes, further investigation is warranted to determine whether GABA participates in dormancy release through modulation of ABA-GA homeostasis.

In this study, we provide evidence that GABA promotes GA_3 biosynthesis by upregulating *GA20ox1* expression and inhibits ABA biosynthesis by downregulating *NCED6* transcription, while concurrently upregulating *CYP707A2* to increases GA_3/ABA ratio. Moreover, downregulation of *GAI*, *RGA*, *RGL1*, *RGL2*, *RGL3*, and *ABI3* genes by GABA enhances GA signaling while suppresses ABA signaling, thus alleviating seed dormancy. Notably, the dormancy-alleviating effect of GABA has also been validated in the seeds of several fruit tree species, including 'Duli' (*Pyrus betulifolia* Bunge.), mahlab cherry (*Prunus mahaleb* L.), and 'Hongmanao' apple (*Malus domestica* Borkh.). Our study not only advances our understanding on the mechanism of GABA-mediated seed dormancy release but also underscores the potential of GABA as a natural alternative to chemical reagents, contributing to sustainable and environmentally friendly agricultural practices.

Results

Exogenous GABA treatment relieves seed dormancy

After 4 weeks of storage, the Col-0 seeds germinated completely (Fig. 1A), confirming the normal vitality of these seeds. To investigate the effect of GABA on seed dormancy release, freshly harvested Arabidopsis wild-type (WT) Col-0 seeds were incubated at 22℃ for 3 days on 1/2 MS medium supplemented with varying concentrations of GABA. Compared with untreated seeds, GABA-treated seeds exhibited alleviated seed dormancy, as indicated by increased germination rates in a concentration-dependent manner. Although all tested concentrations promoted seed germination, a germination rate of 50% was indicative of dormancy release. At 0.5 mM GABA, the germination rate reached 57.35%, identifying this as the optimal effective concentration (Fig. 1B). These results demonstrate that exogenous GABA effectively alleviates seed dormancy.

To further assess the role of GABA in seed dormancy release, WT seeds were cultured on 1/2 MS medium containing varying concentrations of 3-MP, a GABA biosynthesis inhibitor [47]. Germination rates were significantly reduced under 1.0, 1.5, and 2.0 mM 3-MP treatments in a concentration-dependent manner, with germination rates of 26.13%, 22.34% and 17.53% respectively (Fig. 1C). These findings suggest that inhibition of GABA biosynthesis by 3-MP enhances seed dormancy by lowering GABA levels.

Arabidopsis seeds exhibit physiological dormancy post-maturity, which can be alleviated through post-ripening or low-temperature stratification treatments [2]. We next investigated the dynamic changes in endogenous GABA levels during dormancy release in WT seeds subjected to various post-harvest durations and low-temperature treatments. As the post-harvest duration prolongs, the GABA content gradually increased after harvest, peaking at 2 days; Moreover, low-temperature stratification treatment also significantly elevated the GABA content (Fig. 1D). These findings indicated that dormant seeds initially possess low GABA levels, which increase as dormancy release, further underscoring that increased GABA level during seed ripening plays a pivotal role in releasing seed dormancy.

GABA-mediated seed dormancy release requires GABA biosynthesis primarily from GABA shunt pathway

Given the pivotal roles of GAD, GABA-T, and SSADH for GABA metabolism in the GABA shunt pathway [55, 56], *GAD1-5*, *GABA-T*, and *SSADH* expression profiles were examined during seed dormancy release. *GAD1*, *GAD2*, and *GAD3*, key genes for GABA biosynthesis, were significantly upregulated, whereas *GABA-T*, responsible for GABA degradation, was notably downregulated (Fig. 2A and Supplementary Fig. 1A). As an alternative route, the polyamine degradation pathway also contributes to GABA production, with DAO and PAO serving as key enzymes [44]. During seed dormancy release, transcript levels of *DAO2*, *PAO3*, and *PAO4* were significantly upregulated (Fig. 2B, Supplementary Fig. 1A). We further assessed GAD and PAO enzyme activities in seeds undergoing dormancy release induced by low temperature. The enzyme activities of DAO and PAO significantly enhanced after 6 h of stratification (Fig. 2C), implying that the heightened activities of GAD and PAO enzymes might primarily account for the elevation in GABA content. In total, our results suggested that *GAD1-3*, *GABA-T*, *PAO3*, *PAO4*, and *DAO2* played crucial roles in GABA accumulation during seed dormancy release.

To further validate the significance of *GAD1*, *GAD2*, and *GABA-T*, we next investigated their roles in seed

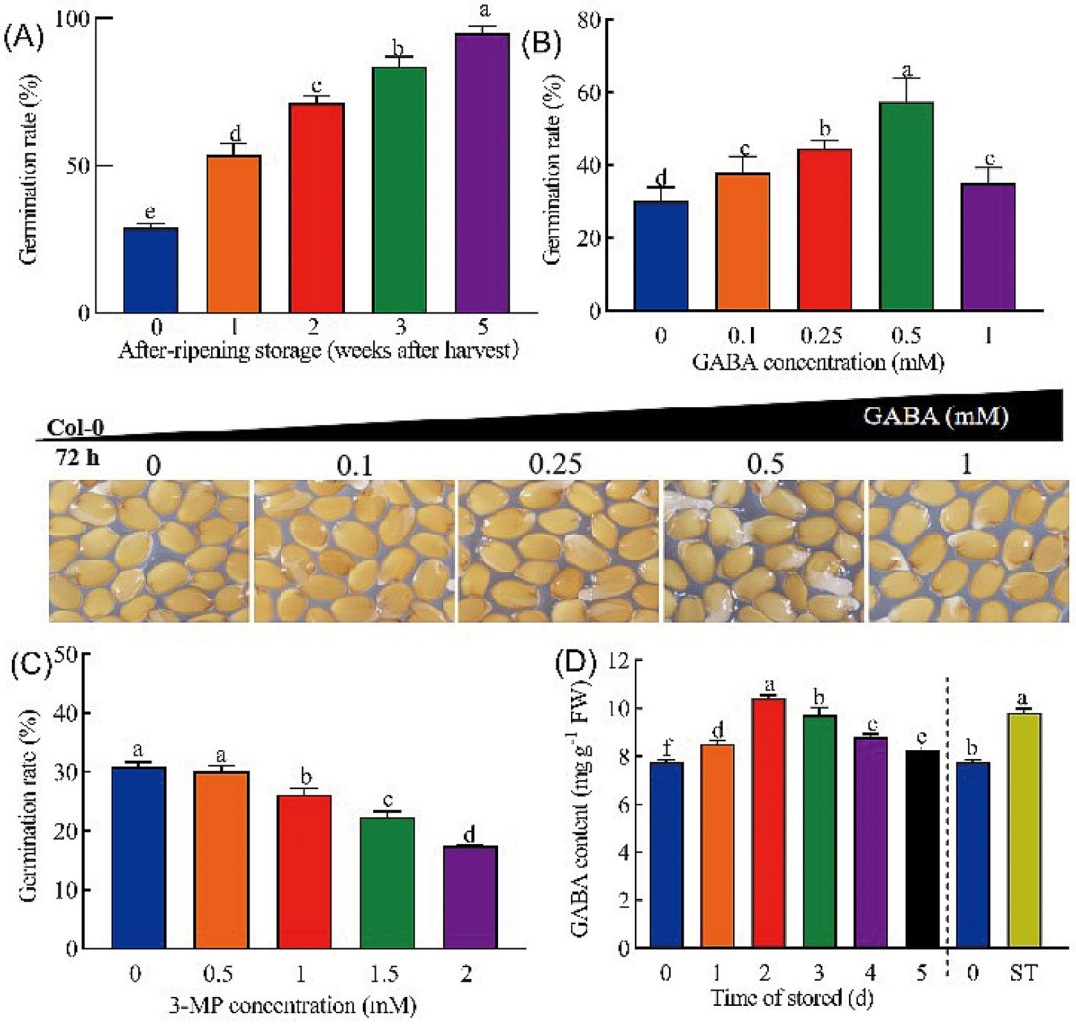

Fig. 1 Exogenous GABA relieves Arabidopsis seed dormancy. (**A**) Germination rates of Col-0 grown for three days after 0, 1, 2, 4 weeks of post-ripening. (**B**) The effects of, 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM GABA on the germination rate of freshly harvested (dormant) Col-0 seeds grown for three days as compared with the control (0 mM GABA). (**C**) Dormancy release of freshly harvested Col-0 seeds by 0.1 mM, 0.5 mM, 1 mM, 1.5 mM, and 2 mM 3-MP as compared with the control (0 mM 3-MP). (**D**) GABA contents of freshly harvested Col-0 seeds after 0, 1, 2, 3, 4 or 5 days of stratification for 2 days at 4 ℃ (ST). All data represent the mean ± SE of three biological replicates (> 50 seeds per replicate). Similar results were obtained among all seed batches. Different letters indicate statistically significant differences between the data ($p < 0.05$)

dormancy release using several loss-of-function mutants. After 4 weeks of storage, mutant seeds of *gad1*, *gad2*, *gaba-t1-1*, *pao4*, and *dao2* germinated completely (Supplementary Fig. 1B), confirming seed viability. Following 3 days of imbibition, WT seeds exhibited a germination rate of 30.79%, whereas *gad1* and *gad2* mutants exhibited slightly reduced rates of 30.53% and 24.30%, respectively. In contrast, germination rate of *gaba-t1-1* seeds displayed a significantly higher germination rate of 86.15% (Fig. 2D). However, *pao4* and *dao2* mutants did not show substantial differences in germination compared to WT (Fig. 2D). These findings suggested a correlation between GABA levels and seed dormancy release. To verify this association, GABA content was measured in *gaba-t1-1* and *gad2* seeds during dormancy. In *gad2* mutants, GABA content was 2.35-fold lower than in WT seeds after 72 h of imbibition (Fig. 2E). Additionally, exogenous GABA restored the germination rate of *gad2* seeds to WT levels (Fig. 2F). Conversely, *gaba-t1-1* mutants accumulated more GABA, an effect that was reversed by 3-MP (Fig. 2F). These results indicated that GAD deficiency reduces GABA levels, resulting in deeper seed dormancy, while loss of *GABA-T* impeded GABA degradation, resulting in weak dormancy.

GABA alleviates seed dormancy by repressing ABA biosynthesis and signaling

ABA plays a crucial role in promoting seed dormancy and inhibiting seed germination. To investigate ABA involvement in GABA-induced seed dormancy release,

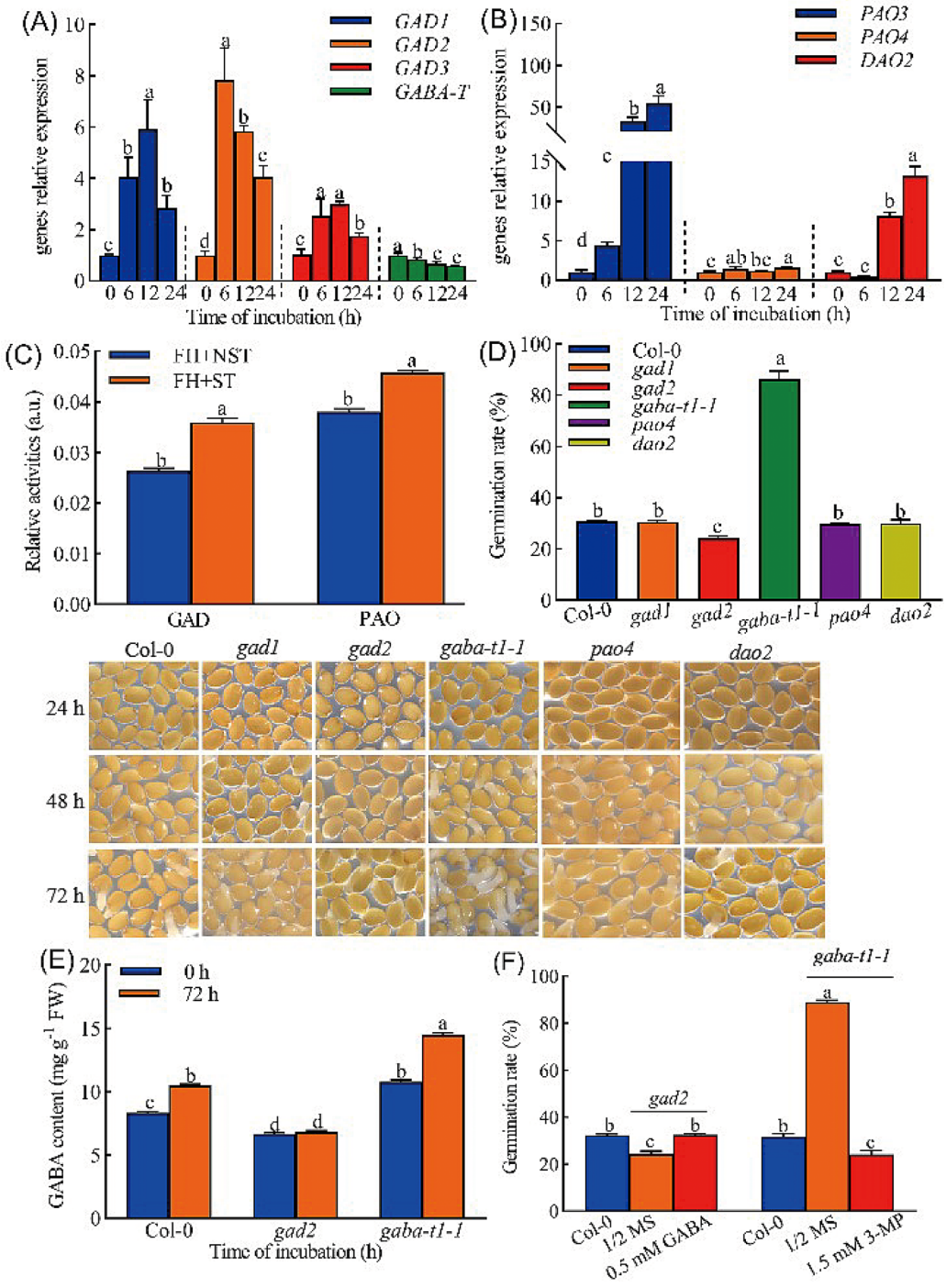

Fig. 2 GABA-mediated seed dormancy release requires GABA biosynthesis. qRT-PCR analyses of seed transcript abundances for key genes in (**A**) GABA shunt pathway and (**B**) polyamine degradation pathway for GABA biosynthesis in Col-0 seeds. Freshly harvested Col-0 seeds were grown on filter paper soaked in H_2O for 0, 6, 12, or 24 h. (**C**) Enzyme activities of GAD and PAO in FH + NST and FH + ST seeds. (**D**) Germination responses of freshly harvested *gad1*, *gad2*, *gaba-t1-1*, *pao4*, and *dao2* mutant seeds compared with Col-0. (**E**) GABA contents of freshly harvested Col-0, *gad2*, and *gaba-t1-1* seeds after 24 h of imbibition. (**F**) Germination rates of freshly harvested Col-0, *gad2*, and *gaba-t1-1* seeds treated with 0.5 mM GABA, or 1.5 mM 3-MP grown for three days, respectively. Relative gene expressions are presented as values relative to *ACTIN2* at 0 h. All data represent the mean ± SE of three biological replicates (> 50 seeds per replicate). Similar results were obtained from three different seed lots. For all panels, different letters indicate significant differences between the data ($p < 0.05$) using one-way ANOVA with Dunnett's test

we analyzed the germination rate of dormant WT seeds treated with a combination of ABA and GABA against treated with ABA alone. The germination rate of the ABA + GABA-treated seeds were significantly higher than those treated with ABA alone, suggesting that GABA mitigated ABA-mediated seed dormancy (Fig. 3A). We further examined the expression of genes involved in ABA biosynthesis (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*), ABA signaling (*ABI3*, *ABI4*, and *ABI5*), and ABA degradation (*CYP707A1* and *CYP707A2*) during GABA-induced seed dormancy release. Specifically, *NCED5*, *NCED6*, and *ABI3* were significantly downregulated under GABA treatment and markedly upregulated under 3-MP treatment, while *CYP707A2* exhibited contrasting expression patterns (Fig. 3B, Supplementary Fig. 1C). Our results suggested that GABA might alleviate seed dormancy by inhibiting ABA biosynthesis and signaling, while enhancing ABA degradation.

Other than exogenous application of GABA, we further genetically examined the impact of endogenous GABA on ABA biosynthesis and signaling using *gad2* and *gaba-t1-1* mutants. Notably, the expression levels of *NCED5*, *NCED6*, and *ABI3* were significantly upregulated in *gad2* mutants and downregulated in *gaba-t1-1* mutants, exhibiting contrasting trends compared to *CYP707A2* expression (Fig. 3C, D). In addition, ABA contents in *gad2* and *gaba-t1-1* mutant seeds were approximately 1.32 times and 0.80 times that of Col-0 after 24-h imbibition, respectively. Meanwhile, ABA levels in *gad2* and *gaba-t1-1* imbibed seeds were 1.65 times and 0.45 times that of dry seeds, respectively (Fig. 3E). Given the upregulation of *NCED6* expression in the *gad2* mutant (Fig. 3C), it suggested that GABA was likely to alleviate seed dormancy by reducing ABA accumulation. To test this hypothesis, we thus generated the *gad2/nced6* double mutant and the vitality of seeds was confirmed (Supplementary Fig. 1B). As expected, the germination rate increased in *nced6* seeds but decreased in *gad2* seeds compared to Col-0, while the germination rate of the *gad2/nced6* double mutant was closer to *nced6*, significantly higher than that of *gad2* and Col-0 (Fig. 3F), indicating that GABA pathway was upstream of ABA pathway and might alleviate seed dormancy by negatively regulating *NCED6* expression. Taken together, these results illustrated that GABA overcame seed dormancy by regulating ABA metabolism and signaling.

GABA relieves seed dormancy by enhancing GA biosynthesis and increasing GA/ABA ratio

GA serves as a key signal for seed dormancy release, and the degree of dormancy is closely linked to the balance of GA and ABA [19]. To investigate the interplay between GA and GABA, we treated dormant WT seeds with a combined treatment of GABA and the gibberellin synthesis inhibitor paclobutrazol (PAC) [57]. We found that PAC inhibited GABA-induced dormancy release in a concentration-dependent manner (Fig. 4A), suggesting that a potential dependence of GABA's effects on GA. To validate this hypothesis, we next examined the impact of GA_3, GABA, and GA_3 + GABA on the *ga1-t* mutant, which blocks GA synthesis and inactivates downstream signaling pathways [26]. GABA treatment had no effect on dormancy release in *ga1-t* seeds. However, the germination rate of *ga1-t* seeds treated with combined GABA and GA_3 was significantly higher than that of GA_3 alone (Fig. 4B), suggesting that GABA function depended on GA and might potentially amplify GA signaling during seed dormancy release.

Given that the effect of GABA in relieving seed dormancy depended on GA, it was plausible that GABA could modulate GA synthesis. To elucidate whether GABA impacted GA synthesis, we scrutinized the expression of GA biosynthetic genes in seeds with 3-MP or GABA treatment. GABA significantly promoted expression levels of *GA20ox1*, *GA20ox3*, and *GA3ox1*, while 3-MP notably repressed their expression (Fig. 4C). In addition, *GA20ox1* expression in *gad2* mutants exhibited down-regulation at 12-h and 24-h imbibition, significantly lower than that in WT seeds (Fig. 4D). Conversely, *GA20ox1* expression was up-regulated in the *gaba-t1-1* mutant during imbibition, markedly higher than that of WT seeds (Fig. 4D). To confirm the involvement of *GA20ox1*, we generated the *gaba-t1-1/ga20ox1-3* double mutant. Its germination rate was markedly lower than *gaba-t1-1*, similar to *ga20ox1-3*, and below WT levels (Fig. 4E), supporting a role for *GA20ox1* in GABA-induced dormancy release.

To analyze the regulatory effect of GABA on GA synthesis, GA_3 content was determined in dormant seeds of *gad2* and *gaba-t1-1* mutants. After 72-h imbibition, we observed a reduced GA_3 response in *gad2* mutants and an elevated response in *gaba-t1-1* mutants (Fig. 4F), suggesting that the GA content regulated by endogenous GABA might serve as a pivotal factor in seed dormancy release.

Considering that seed dormancy release depends on the balance of GA and ABA [19], we further investigated the GA_3/ABA ratio. The GA_3/ABA ratio in *gad2* and *gaba-t1-1* mutant seeds was 0.21 times and 1.83 times that of Col-0 after 72-h imbibition, respectively (Fig. 4G). These results clearly indicated that GABA potentially regulated seed dormancy release by adjusting the GA to ABA ratio.

GABA negatively regulates *DELLAs* expression during seed dormancy release

The DELLA proteins GAI, RGA, RGL1, RGL2, and RGL3 are key repressors in the GA signaling pathway and are

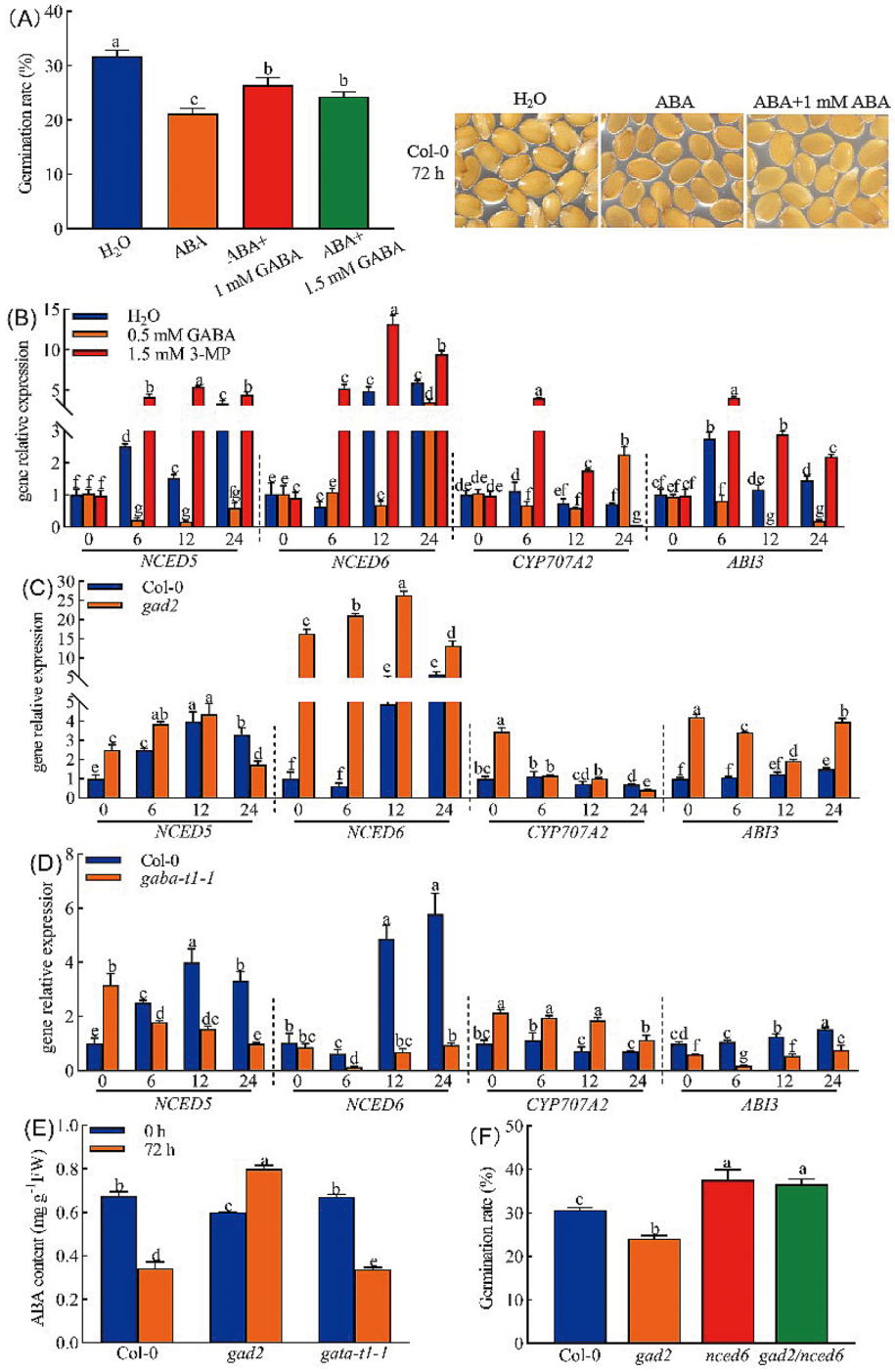

Fig. 3 GABA alleviates seed dormancy by regulating ABA metabolism and signaling. (**A**) Germination percentage of freshly harvested Col-0 seeds grown for three days in the presence of 0.1 μM ABA, or with 1.0 or 1.5 mM GABA compared with control (H_2O) (**B**) Reverse transcription quantitative polymerase chain reaction (qRT-PCR) quantification of *NCED5*, *NCED6*, *CYP707A2*, and *ABI3* in Col-0 seeds. Freshly harvested Col-0 seeds were grown on filter paper soaked in H_2O, 0.5 mM GABA, or 1.5 mM 3-MP for 0, 6, 12, or 24 h. (**C**, **D**) Reverse transcription quantitative polymerase chain reaction quantification of *NCED5*, *NCED6*, *CYP707A2*, and *ABI3* mRNA levels in Col-0, *gad2*, and *gaba-t1-1* seeds. Freshly harvested seeds were grown on filter paper soaked in H_2O for 0, 6, 12, and 24 h. Seeds used were a pool of three biological replicates and average values and SEs for three replicates relative to *ACTIN2* levels are shown. (**E**) ABA content in *gad2* and *gaba-t1-1* seeds after 72 h of imbibition. (**F**) Germination rate of freshly harvested wild-type Col-0 and single and double mutants grown for three days. All data represent the mean ± SE of three biological replicates (> 50 seeds per replicate). Gene expression was normalized to ACTIN2. Different letters indicate significant differences ($p < 0.05$)

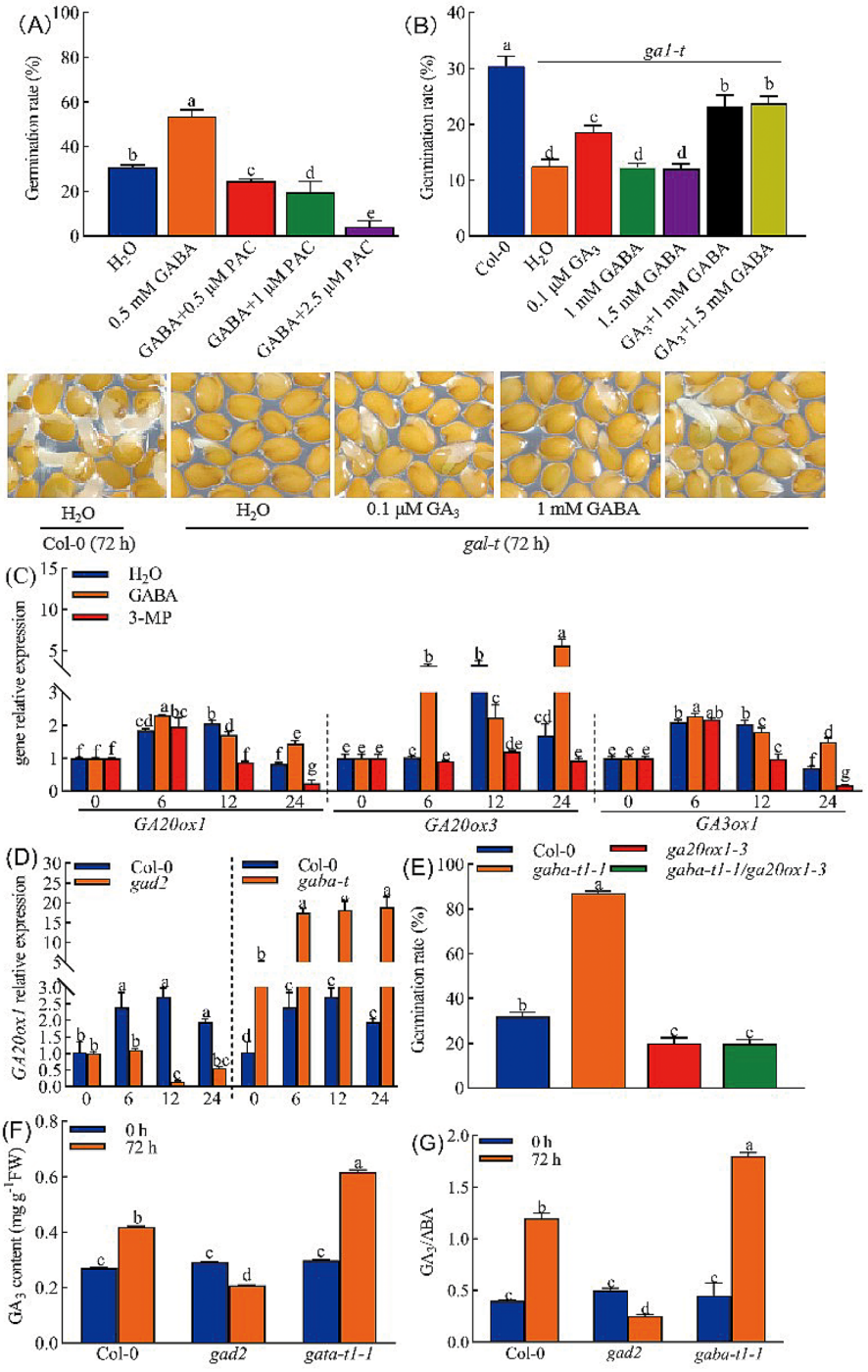

Fig. 4 GABA relieves seed dormancy by regulating GA synthesis. (**A**) Germination assays of Col-0 seeds imbibed in water containing the indicated concentrations of GA or GABA + PAC. (**B**) Germination rates of *gal-t* seeds under H_2O, 0.1 μM GA_3, (1, 1.5) mM GABA, or 0.1 μM GA_3 + (1, 1.5) mM GABA treatments grown for three days compared with Col-0. (**C**) qRT-PCR of *GA20ox1*, *GA20ox3*, and *GA3ox1* in freshly harvested Col-0 seeds grown on filter paper soaked in H_2O, 0.5 mM GABA, or 1.5 mM 3-MP for 0, 6, 12, and 24 h. (**D**) Expression of *GA20ox1* in Col-0, *gad2*, and *gaba-t1-1* seeds. Seeds used were a pool of three biological replicates and average values and SEs for three replicates relative to *ACTIN2* levels are shown. (**E**) Germination assays with seeds freshly harvested from Col-0, *gaba-t1-1*, *ga20ox1-3*, or *gaba-t1-1/ga20ox1-3* lines. (**F**) GA_3 content in Col-0, *gad2* and *gaba-t1-1* seeds after 72 h of imbibition. (**G**) The GA_3/ABA ratio in Col-0, *gad2* and *gaba-t1-1* seeds. All data represent the mean ± SE of three biological replicates (> 50 seeds per replicate). Gene expression was normalized to ACTIN2. Different letters indicate significant differences ($p < 0.05$) determined by one-way ANOVA with Dunnett's test

actively expressed during seed germination [58, 59]. To examine the impact of GABA on DELLA expression, we analyzed transcript levels of *GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3* in seeds treated with GABA or the GABA biosynthesis inhibitor 3-MP. GABA treatment significantly downregulated all five DELLA genes, while 3-MP treatment upregulated their expression (Fig. 5A). Consistently, DELLA gene expression was markedly increased in the *gad2* mutant and reduced in the *gaba-t1-1* mutant (Fig. 5B, C).

To genetically validate that GABA modulates GA signaling by reducing *DELLA* gene expression, we generated the *gad4-2/gai2* double mutant. The germination rate of *gad4-2/gai2* seeds was markedly higher than that of *gad4-2* seeds but lower than that of *gai2* seeds during imbibition (Fig. 5D). After 72-h imbibition, the germination rates were 19.94% for *gad4-2* and 41.15% for *gai2*, while the *gad4-2/gai2* double mutant exhibited a germination rate of 29.9%, closely resembling the germination rate of WT seeds 28.79% (Fig. 5D). These results suggested that the enhanced dormancy observed in the GABA-deficient

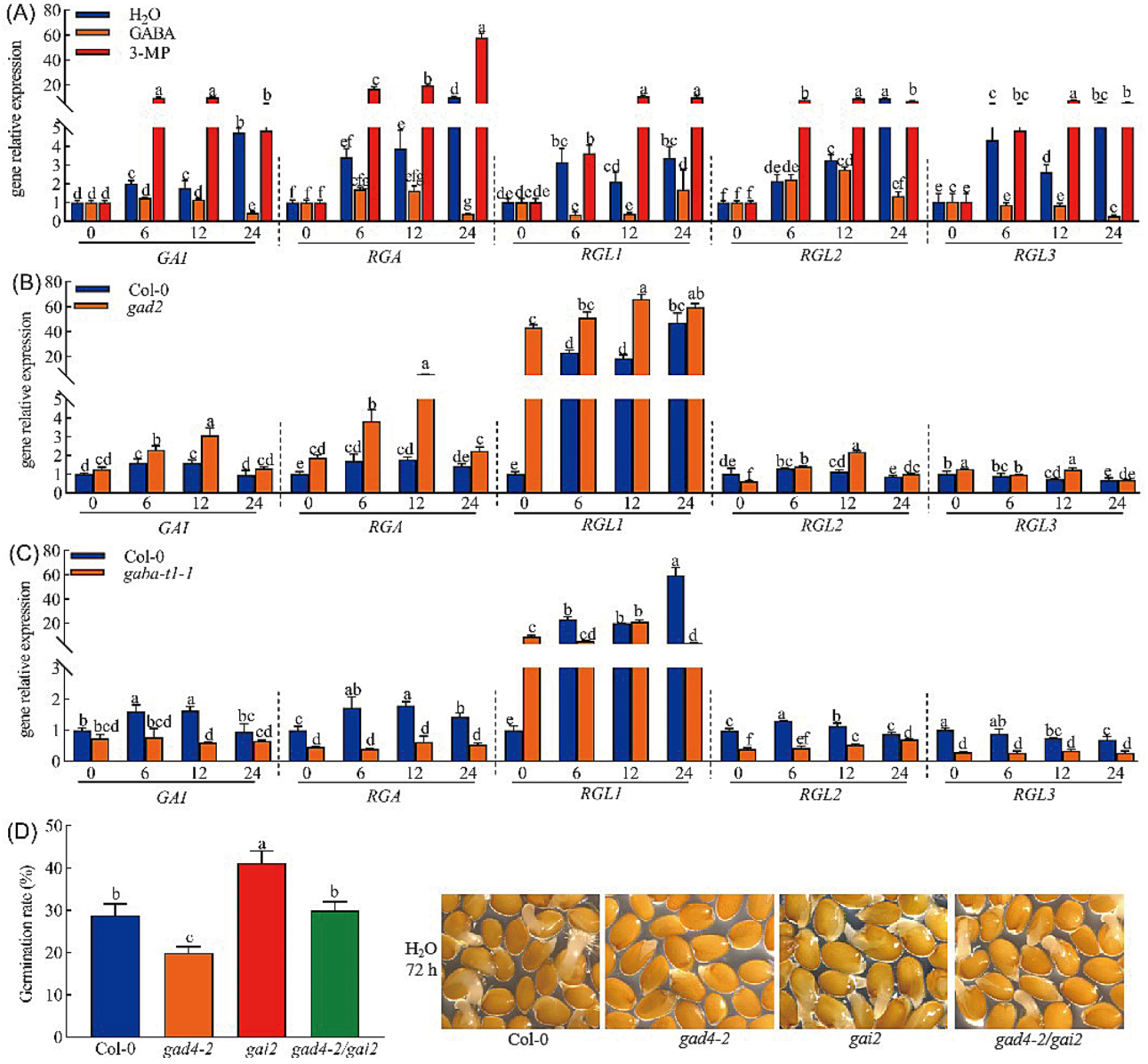

Fig. 5 GABA negatively regulates DELLAs in the GA signaling pathway during seed dormancy release. (**A**) Expression of *GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3* in Col-0 seeds. Freshly harvested seeds were treated with 0.5 mM GABA, or 1.5 mM 3-MP compared with the control (H_2O). (**B**, **C**) Expression of *GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3* in Col-0, *gad2*, and *gaba-t1-1* seeds. Freshly harvested seeds were grown on filter paper soaked in water for 0, 6, 12, or 24 h. (**D**) Germination rates of freshly harvested Col-0, *gad4-2*, *gai2*, and *gad4-2/gai2* seeds grown for three days. All data represent the mean ± SE of three biological replicates (> 50 seeds per replicate). Different letters indicate significant differences ($p < 0.05$) determined by one-way ANOVA with Dunnett's test

gad4-2 mutant can be partially rescued by the *gai2* mutation, supporting a model in which GABA alleviates dormancy by negatively regulating DELLA expression within the GA signaling pathway.

Exogenous GABA promotes seed dormancy release in several fruit tree species

To evaluate the broader utility of GABA in promoting dormancy release, we tested its effects on dormant seeds from three fruit tree species: pear, apple, and cherry. After 10 weeks of storage, all seed lots showed full germination capacity (Supplementary Fig. 1D), confirming their viability. 'Duli' (*Pyrus betulifolia* Bunge), an important pear rootstock and greening tree, features seeds with weak dormancy. In *Pyrus betulifolia* seeds, germination rates under different GABA concentrations were higher than untreated seeds, which had a generation rate of 32.2%. Notably, 5 mmol/L GABA resulted in the most substantial difference, yielding a germination rate of 64.4% (Fig. 6A). The 'Hongmanao' apple (*Malus domestica* Borkh.), a pollination variety for apples, possesses seeds with deep dormancy. In *Malus domestica* cv Hongmanao seeds, a concentration of 125 mmol/L GABA exerted the most pronounced impact, resulting

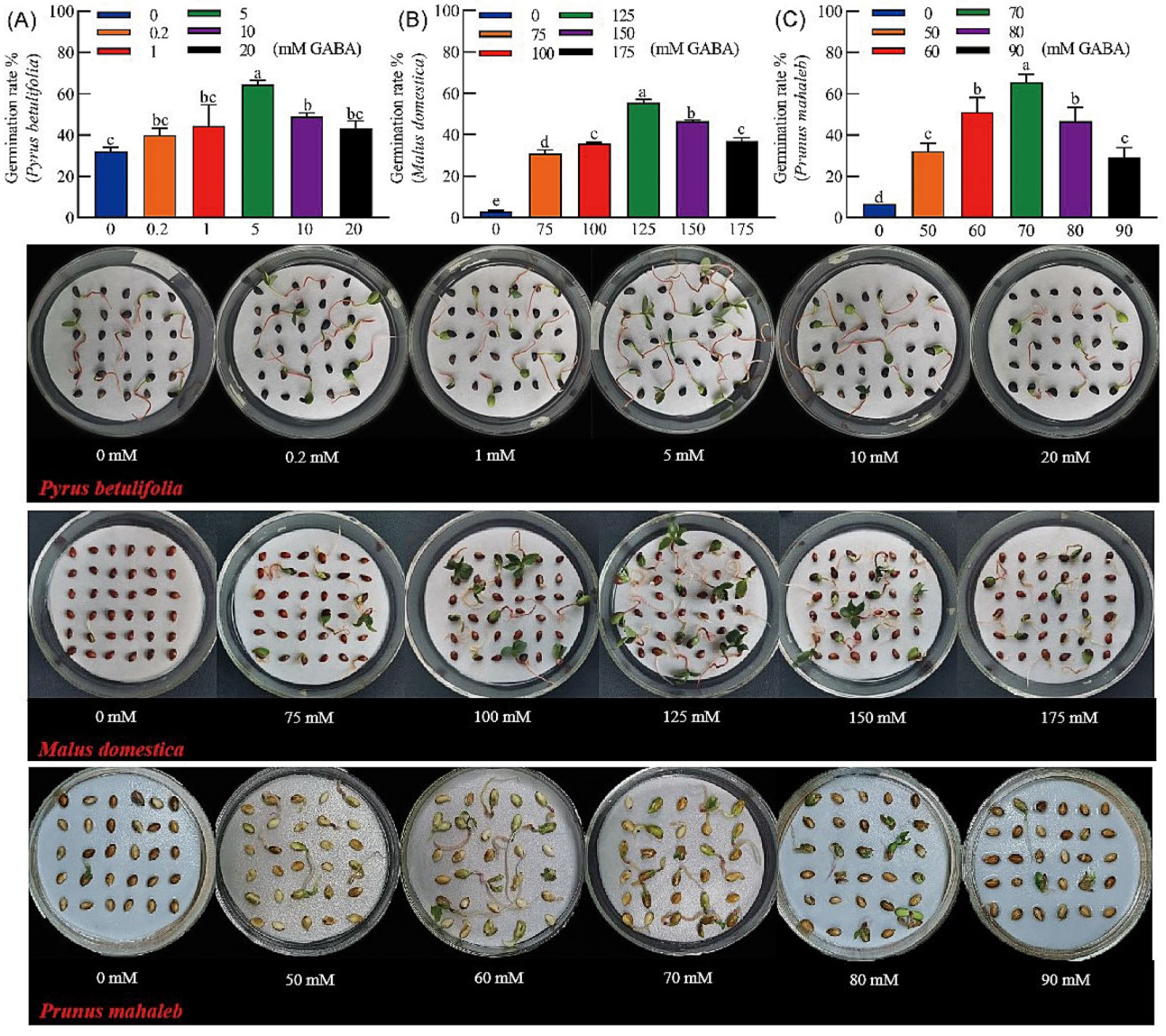

Fig. 6 Exogenous GABA promotes seed dormancy release in *Pyrus betulifolia*, *Malus domestica*, and *Prunus mahaleb* L. (**A**) Germination rate of freshly harvested *Pyrus betulifolia* seeds grown for 13 days in the presence of 0 mM, 0.2 mM, 1 mM, 5 mM, 10 mM, or 20 mM GABA. (**B**) Germination rates of freshly harvested *Malus domestica* seeds grown for 13 days after stratification with 0 mM, 75 mM, 100 mM, 125 mM, 150 mM, or 175 mM GABA. (**C**) Germination rates of freshly harvested *Prunus mahaleb* L. seeds grown for 13 days in the presence of 0 mM, 50 mM, 60 mM, 70 mM, 80 mM, or 90 mM GABA. All data represent the mean ± SE of three biological replicates (> 50 seeds per replicate). Different letters indicate significant differences ($p < 0.05$) determined by one-way ANOVA with Dunnett's test

in a germination rate of 62.04%, compared to untreated seeds with a generation rate of 3.7% (Fig. 6B). The mahlab cherry (*Prunus mahaleb* L.), a primary rootstock for sweet cherries, harbors seeds with exceptionally deep dormancy, rendering it an ideal material for validating the efficacy of GABA in seed dormancy release. The control seeds exhibited only 6.67% germination rate, while 70 mmol/L GABA elevated the germination rate to 65.56%. These results demonstrate that GABA significantly promotes seed dormancy release across diverse species, particularly in seeds that have not undergone post-harvest after-ripening.

Discussion

GABA functions as a key regulator of seed dormancy release through the GABA shunt metabolic pathway

GABA is widely present in plant tissues and organs, playing various roles in plant growth and development as well as stress adaptation. Although GABA has been reported in seed germination [49–51], whether GABA relieves seed dormancy is unclear. Here, we show that exogenous GABA effectively relieved Arabidopsis seed dormancy, and endogenous GABA accumulation was observed during seed dormancy release induced by low temperature stratification (Fig. 1). These findings establish a clear link between GABA accumulation and dormancy alleviation, emphasizing GABA's role as a signaling molecule in this process. Given the substantial elevation of GABA levels during dormancy release, we investigated the metabolic origins of this increase. Our data reveal a marked reduction in germination in the *gad2* mutant, in contrast to a significantly enhanced germination rate in the *gaba-t1-1* mutant. Although *pao4* mutants did not exhibit a pronounced difference in germination rate (Fig. 2D), the potential functional redundancy among PAO family members may account for this observation [60–62]. Collectively, these findings underscore the critical role of the GABA shunt pathway in mediating GABA accumulation and promoting seed dormancy release.

GABA regulates seed dormancy release by precisely modulating ABA and GA metabolism and signaling pathways

The establishment and maintenance of physiological dormancy rely on the balance between ABA and GA [52–54]. Previous studies have demonstrated that loss of *NCED6* or *NCED9* significantly reduces ABA content and seed dormancy, while *NCED6* overexpression increases ABA content and enhances seed dormancy [63–65]. In this context, we show that GABA reduces ABA content by downregulating *NCED6* expression (Fig. 3). Additionally, we find that GABA enhances ABA metabolism through upregulating *CYP707A2* expression and inhibits ABA signal transduction by downregulating *ABI3* expression (Fig. 3). Notably, the ABA receptor complex, primarily composed of PYR/PYL proteins, PP2C phosphatases, and SnRK2 kinases, undergoes conformational changes upon ABA binding to initiate signaling cascades [66]. There may be a potential crosstalk between GABA signaling and core ABA receptor components. As such, GABA reduces ABA level and signaling to counteract the seed dormancy. In addition, we show that GABA promotes GA accumulation by upregulating *GA20ox1* (Fig. 4), a key rate-limiting enzyme in the GA synthesis pathway [57]. Thus, regarding hormonal regulation, GABA is able to increase GA content while decreasing ABA content. Consistent with this, GA_3/ABA ratio decreases in the *gad2* mutant but increases in the *gaba-t1-1* mutant (Fig. 3G), further indicating that GABA facilitates seed dormancy release by modulating the relative levels of GA_3 and ABA. Notably, GABA appears to release seed dormancy by amplifying GA signal, as evidenced by the significantly higher germination rate of *ga1-t* seeds under GABA + GA_3 treatment compared to GA alone (Fig. 4B). Concomitantly, our results also indicate that GABA regulates GA signaling pathway (Fig. 5), thus effectively promoting seed dormancy release.

While our findings position ABA and GA signaling pathways downstream of GABA, hormonal networks often employ feedback loops to maintain homeostasis. Therefore, it is essential to examine the expression dynamics and enzyme activities of GABA metabolism under ABA and GA treatments, as well as GABA accumulation patterns in ABA and GA biosynthesis and signaling mutants. Such investigations will determine whether the GABA-ABA/GA relationship forms a bidirectional regulatory module, potentially uncovering new layers of control in seed dormancy. In addition, studies show that DOG1 enhances ABA signaling by inhibiting AHG1/AHG3 phosphatases while simultaneously modulating GA metabolic genes in a temperature-dependent manner. The *dog1* mutants exhibit decreased ABA and increased GA levels [67, 68], mirroring the hormonal profile observed in GABA-treated seeds. This parallel suggests that GABA may interface with dormancy networks through modulation of the DOG1 pathway.

GABA serves as a universal and eco-friendly dormancy-breaking agent with significant agricultural applications

Low temperature stratification is the most commonly used approach in agricultural production to relieve the physiological dormancy of embryos, particularly in seeds that have developed dormancy due to germination-inhibiting substances. However, this method is time-consuming, and seeds are prone to mold and rot during the stratification process, causing production delays and economic losses. The Rosaceae family, which includes economically important fruit trees such as pears, apples,

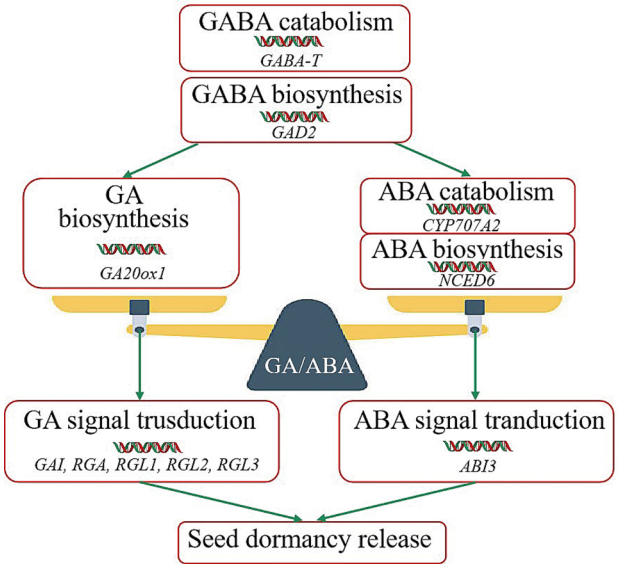

Fig. 7 Model of GABA-mediated seed dormancy release through orchestrating ABA and gibberellin metabolism and signaling. Upregulation of *GAD2* expression and suppression of *GABA-T* expression leads to increased GABA levels. Subsequently, accumulated GABA induces *GA20ox1* while inhibits *NCED6* and *CYP707A2* expression to adjust GA_3/ABA ratio. GABA amplified GA signaling and inhibited ABA signaling by downregulating genes expression of *GAI*, *RGA*, *RGL1*, *RGL2*, *RGL3*, and *ABI3*, ultimately leading to the alleviation of seed dormancy

and cherries, plays a crucial role in global fruit production. Notably, we further confirm the efficacy of GABA in significantly relieving seed dormancy in 'Duli' pear (*Pyrus betulifolia* Bunge), mahlab cherry (*Prunus mahaleb* L.), and 'Hongmanao' apple (*Malus domestica* Borkh.) (Fig. 6). Our findings offer tangible solutions to longstanding agricultural challenges. By demonstrating GABA's efficacy across three economically important Rosaceae species, we provide new strategies for managing seed dormancy in perennial crops. Compared to traditional stratification methods, the performance of GABA treatment, in terms of efficiency, reliability, and seed health, positions it as a game-changing technology for the fruit tree industry. From an implementation perspective, GABA's natural occurrence and safety profile make it particularly attractive for sustainable agriculture. Its effectiveness in overcoming physiological dormancy caused by germination inhibitors suggests potential applications in other difficult-to-germinate species. These findings open exciting possibilities for developing GABA-based seed priming protocols that could transform nursery practices and shorten breeding cycles, ultimately contributing to global food security.

Conclusions

Our study supports a regulatory model in which GABA relieves seed dormancy through orchestrating the balance between ABA and GAs. Specifically, GABA accumulation is initiated by the upregulation of *GAD2* expression and the suppression of *GABA-T* expression, leading to increased GABA levels. The resultant higher content of GABA induces *GA20ox1* while inhibiting *NCED6* and *CYP707A2* expression, thereby adjusting the GA_3/ABA ratio. Furthermore, GABA enhances the GA signaling pathway and suppresses the ABA signaling pathway by downregulating the expression of key genes, ultimately leading to the alleviation of seed dormancy (Fig. 7).

Materials and methods

Plant materials and growth conditions

Arabidopsis ecotype Col-0 was used as the wild type in this study. The T-DNA insertion lines of *gad1* (SALK_111024C), *pao4* (SALK_133599C), *gaba-t1-1* (SALK_007661C), *gai2* (SALK_089146C), and *gad4-2* (SALK_033307C) were obtained from the Arabidopsis Biological Resource Center. The *gad2* (SALK_067677C), *dao2* (SALK_205223C), *ga20ox1-3* (SALK_016701C), and *nced6* (SALK_209085C) were obtained from the AraShare. The *ga1-t* mutant was described previously [32]. The double mutants *gad2/nced6*, *gaba-t1-1/ga20ox1-3*, and *gad4-2/gai2* were generated by genetic crossing in this study. Primers used for genotyping are listed in Supplementary Table S1.

Arabidopsis seeds were surface sterilized with 10% sodium hypochlorite solution and washed ten times with sterilized ultrapure water. Then sterile seeds were sown onto Petri dishes containing 1/2 strength Murashige & Skoog (MS) medium buffered with 2 mM MES, pH 5.7, 1% (w/v) sucrose, and 0.7% (w/v) agar. KOH was used to adjust the pH of the MS media before autoclaving. GABA and 3-MP were dissolved in sterilized ultrapure water and ABA, GA or PAC were dissolved in ethanol to form stock solutions, and then diluted to working concentrations as needed to add to the autoclaved MS. After 3 days at 4 °C, plates were placed in a growth chamber under a 16-h light/18-h dark cycle (22 °C/ 20 °C) with 60% humidity with 100 μmol m^{-2} s^{-1} light intensity.

Freshly harvested seeds were collected from every plant when the youngest siliques started to brown and were immediately used for germination experiments within three days to maintain dormancy. Seed dormancy levels were evaluated by measuring germination rates under suitable conditions [69]. Photos were taken under a stereomicroscope every 12 h to record germination rate. The breakthrough of the embryonic root through seed coat was defined as germination. Treatments were repeated at least three times and at least fifty seeds for each biological replicate were used.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from seeds using the HiPure Plant RNA Mini Kit (Magen, China) according to the manufacturer's instructions. RNA samples treated with DNase I to remove residual genomic DNA were used as templates for reverse transcription using the HiScript®IIReverse Transcriptase (Vazyme, China). The qRT-PCR was performed using ChamQ™ SYBR qPCR Master Mix (Vazyme, China) on an CFX96 real-time system (Bio-Rad Laboratories, USA). The relative expression quantification of each gene was analyzed using the $2^{-\Delta\Delta Ct}$ method with *ACTIN2* as an internal reference [70, 71]. The primer sequences for qRT-PCR are listed in Supplementary Table S2.

The GABA content measurement

High Performance Liquid Chromatography (HPLC) was used to determine GABA concentration as previously reported [46]. Briefly, GABA was extracted using 10 mM sodium acetate, and then the samples were derivatized with the AccQ Tag Ultra Derivatization Kit (Waters). Chromatographic analysis of GABA was performed by Venusil XBP C18 column (5 μm, 4.6 × 250 mm) using gradient protocol for amino acids analysis.

The PAO activity assay

As reported previously [72], seeds (0.5 g) were sampled and immediately fully ground in liquid nitrogen in 1.0 mL of 0.1 M sodium phosphate (pH 6.5). The homogenates were centrifuged at 15,000 g and 4 °C for 15 min. Supernatants were transferred to new tubes and were ready for the PAO activity assay. The reaction solution (1 mL) containing 0.2 mL of a crude enzyme extract, 0.2 M sodium phosphate buffer (pH 6.5), 0.06 mg horseradish peroxidase, and 2 mM spermidine was incubated at 30 °C for 30 min. Then a spectrophotometer was used to measure the PAO activity at 515 nm. The enzyme activities were presented as international units (1 unit is the amount of enzyme that catalyzes the oxidation of 1 μmol substrate per min).

The GAD activity assay

Following a previous report [73], seed samples (each weighs 0.05 g) were frozen in liquid nitrogen. The 500 μL buffer containing 70 mM phosphate buffer (pH 5.8), 0.2 mM pyridoxal 5′-phosphate, and 2 mM EDTA was added to each sample. Crude enzyme solutions were obtained by collecting the supernatant (4000 r/min, 30 min, 4 ℃). Mixing 100 μL of crude enzyme solution with 50 μL of 50 mM Glu-^{15}N solution at 40 °C for 30 min initiates the reaction, and then incubate at 90 °C for 10 min to stop the enzymatic reaction. Supernatant was collected by centrifugation (4 °C, 10,000 rpm, and 10 min). 100 μL of the supernatant was used to separate lipid components in addition to 300 μL of methanol and 20 μL of internal standard (20 mM d4-DL-alanine solution in 50% MeO-Haq). Protein was removed by using ultrapure water and chloroform and 100 μL of product diluted 5-fold was dispensed into a vial with a 250 μL insert and analyzed. Chromatographic separation was conducted using a crownpak CR-I (+) column (3.0 mm i.d., 150 mm, 5 mm; Daicel CPI, Japan). The relative GAD activity was determined by calculating the area of the GABA-^{15}N peak divided by the area of the d_4-DL-alanine peak.

The GA content measurement

The GA content was measured as previously reported with modifications [74]. Approximately 0.5 g of seeds were fully ground in liquid N_2, mixing with 80 mL of methanol (chromatographically pure) to extract with ultrasound for 20 min, then dilute to 100 mL with methanol. 50 mL mixture was performed rotary evaporation (dissolved in 10 mL of water at 40 ℃) to extract with 50 mL of ethyl acetate (chromatographically pure). The ethyl acetate layer was used to perform rotary evaporation (dissolved in 1 mL of methanol at 40 ℃). The solution passed through a 0.45-μm filter membrane was analyzed using HPLC-MS/MS with a Venusil XBP C18 column (5 μm, 4.6 × 250 mm). Different GA_3 standard solutions (0.1, 0.2, 0.5, 2, 5, 20, 50, and 200 ng/mL) were prepared using methanol (0.1% formic acid) as the solvent.

The ABA content measurement

The ABA measurement was performed as described [75]. Sample of 0.2 g was added to 500 μL of ABA extraction buffer (volume ratio of methanol, isopropanol, and acetic acid at 20:79:1) and was then ground to homogenate. The homogenate was swirled for 5 min and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was carefully transferred to a clean tube and filtered through a 0.22 μM filter membrane. A HPLC-MS/MS equipped with a Venusil XBP C18 column (4.6 mm×250 mm×5 μm) with a column temperature set to 30 °C was used to analyze ABA abundance. Nanopure water and acetonitrile with 0.05% acetic acid were used as solvents. Samples were eluted with a linear 15-min gradient from 10 to 90% acetonitrile.

Statistical analysis

All data were analyzed using SPSS 16.0. Differences among treatments were evaluated by one-way ANOVA followed by post hoc multiple comparison tests. Student's t-test was used for pairwise comparisons. A p-value of < 0.05 was considered statistically significant.

Abbreviations

ABA	Abscisic acid
GA	Gibberellin
GAD	Glutamate decarboxylase

GABA-T	GABA-transaminase
GABA	γ-aminobutyric acid
PAC	Paclobutrazol
PAO	Polyamine oxidase
3-MP	3-mercaptopropionic acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06707-3.

Supplementary Material 1

Acknowledgements
Authors are grateful to the teachers and students who were part of this study.

Author contributions
NZ, XW, and XT-W: Formal Analysis; XT-W, and GD-W: Funding Acquisition; NZ, XW and JP-N: Investigation; WH, NZ and XW: Methodology; WH, and XT-W: Project Administration; WH, XT-W, GD-W, and KS: Resources; WH, XT-W, GD-W, and KS: Supervision; NZ, XW, JP-N and XZ: Validation; NZ, XW, XT-W, JP-N and XZ: Visualization; XT-W: Original Draft Preparation; GD-W, XT-W, WH, and KS: Review & Editing. All authors reviewed the manuscript.

Funding
This work was supported partially by the National Natural Science Foundation of China (32370850), Natural Science Foundation of Shaanxi (2020JC-29 and 2023-JCZD-09), and Fundamental Research Funds for the Central Universities (GK202402003) to GD-W; Natural Science Research Plan of Shaanxi Province (No. 2025JC-YBMS-239), Shaanxi Academy of Science Research Funding Project (No. 2024k-19) and Xi'an Innovation Capacity Foundation Strengthening Plan-Agricultural Technology Research and Development Project (No. 21NYYF0045) to XT-W; Project of the Science and Technology Program of Shaanxi Academy of Science (No. 2024k-32) to XZ.

Data availability
All data related to this manuscript can be found within this paper and its supplementary data. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval
Not applicable.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 24 March 2025 / Accepted: 13 May 2025
Published online: 21 May 2025

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