Research Article

Effects of exogenous γ-Aminobutyric acid on the regulation of respiration and protein expression in germinating seeds of mungbean (*Vigna radiata*) under salt conditions

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ABSTRACT

Background: γ-Aminobutyric acid (GABA) bypasses the TCA cycle via GABA shunt, suggesting a relationship with respiration. However, little is known about its role in seed germination under salt conditions.
Results: In this study, exogenous GABA was shown to have almost no influence on mungbean seed germination, except 0.1 mM at 10 h, while it completely alleviated the inhibition of germination by salt treatment. Seed respiration was significantly inhibited by 0.1 and 0.5 mM GABA, but was evidently enhanced under salt treatment, whereas both were promoted by 1 mM GABA alone or with salt treatment. Mitochondrial respiration also showed a similar trend at 0.1 mM GABA. Moreover, proteomic analysis further showed that 43 annotated proteins were affected by exogenous GABA, even 0.1 mM under salt treatment, including complexes of the mitochondrial respiratory chain.
Conclusions: Our study provides new evidence that GABA may act as a signal molecule in regulating respiration of mungbean seed germination in response to salt stress.
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1. Introduction

γ-Aminobutyric acid (GABA) is a nonprotein amino acid that is produced in the mitochondria through the GABA shunt, a metabolic pathway that bypasses two successive steps of the tricarboxylic acid (TCA) cycle in the mitochondria [1]. In plants, GABA plays crucial roles in biotic and abiotic stresses [2], carbon–nitrogen metabolism [1,3], and development processes, such as pollen tube growth [4] and seed maturation [5]. In particular, the aluminum-activated malate transporter (ALMT) as a plant GABA receptor, providing definite evidence regarding the metabolic and signaling functions of GABA in plants [6].

Mitochondria, the primary organelles of respiration, are the main target for oxidative damage to proteins under stress conditions [7,8,9], and represent the major source of reactive oxygen species (ROS) [8], which may result from the inhibition of the mitochondrial respiratory chain complexes [9]. In *Arabidopsis*, GABA shunt deficiencies result in the accumulation of H_2O_2 when exposed to UV-B irradiation or low light intensity [3,10]. Furthermore, GABA shunt can be activated by salt treatment to overcome the salt-induced inhibition of TCA cycle activity [7]. This may suggest a close relationship between GABA metabolism and mitochondrial respiratory chain under stress conditions.

Notably, in previous studies, the GABA content has been shown to significantly increase during the germination process [11]. It has been

reported that endogenous GABA accumulates in seeds when seeds are developing and germinating [5,12]. Moreover, exogenous GABA has been shown to affect seed germination and respiration during the seed germination of *Haloxylon ammodendron* [13], and regulates the production of H_2O_2 in the legume shrub [14]. Generally, seed germination is most sensitive to salinity; low and moderate concentrations of salt treatment delay germination, while a high concentration inhibits germination [15]. Priming with GABA could be an effective technique to alleviate salinity and osmotic stresses causing inhibition of rice seed vigor [16]. Recent work showed that GABA can mitigate salt damage by enhancing starch catabolism and the utilization of sugars and amino acids [17] or by enhancing the antioxidant system and accumulation of phenolic compounds during seed germination [18]. However, the regulatory mechanism of GABA on seed germination under salt conditions is still little understood.

Given that GABA has a close relationship with mitochondria and seed germination, in this study, the effects of exogenous GABA on seed germination, including respiration and protein expression, were investigated under salt conditions, which involve a rapid acceleration in oxygen consumption and respiratory activity [19], as well as under normal conditions. The study aimed to decipher the roles of exogenous GABA in respiratory control and protein expression during seed germination of mungbean (*Vigna radiata*), which not only has a great economic value in agriculture for human food but also has a scientific research value as a legume model plant.

2. Materials and methods

2.1. Plant material and treatments

Mungbean (*Vigna radiata*) seeds, which were purchased from the local supermarket, Haidian, Beijing, China, were washed 5 times with distilled water. Fifty seeds for each biological replicate were placed on three sheets of a filter paper in glass Petri dishes, which were added of different concentrations of exogenous GABA (Sigma-Aldrich, St. Louis, MO, USA) (0, 0.1, 0.5, 1.0, and 10 mM), or NaCl (0, 50, 100, 150, and 200 mM), or a combination of NaCl (100 mM) and different GABA levels and imbibed for 6, 8, 10, 12, or 14 h in the dark at 25°C in a constant temperature box. Distilled water was used as control solution (without NaCl and GABA). Germination included the process from 6 h imbibition to the emergence of the radicle (2–3 mm) through the outer coat of the seeds. The designated samples were used to observe germination and measure respiration, and were subjected to a proteomic analysis.

Besides, 50 seeds for each biological replicate as above were treated with control solution, and 100 mM NaCl plus different solutions of exogenous GABA (0, 0.1, 0.5, and 1.0 mM) for 10 h. The samples were then harvested for the verification of differentially expressed proteins by qRT-PCR.

2.2. Seed germination test

For the germination tests, seeds were treated as above, and radicle protrusion was used as a criterion for judging germination. Germinated seeds were counted at the designated treatment times, and each test was repeated three to six times.

2.3. Isolation of mitochondria

Fresh materials (10 g) were homogenized in 15 mL of cold grinding medium [400 mM sucrose, 75 mM 3-(N-Morpholino) propanesulfonic acid (MOPS)/KOH, pH 7.2, 4 mM EDTA, 0.2% (w/v) polyvinylpyrrolidone 40 (PVP-40), 8 mM cysteine, and 0.2% (w/v) bovine serum albumin (BSA)]. The homogenate was filtered through four layers of Miracloth (Sigma-Aldrich) and centrifuged at 3000 g for 10 min. The supernatant was further centrifuged at 16,000 g for 10

min. The washed mitochondria were then suspended in 1 mL of medium [10 mM MOPS pH 7.2, 300 mM sucrose, 1 mM EDTA, 0.1% (w/v) BSA] and used for respiratory measurements [20,21].

2.4. Measurement of respiration

The oxygen consumption rates of germinated seeds and mitochondria were monitored with an oxygen electrode system (Hansatech, King's Lynn, Norfolk, UK) [13]. The oxygen consumption rates of 15 germinated seeds and four separate replicates were measured in the gas phase. The oxygen consumption of 100 μL mitochondria in 1 mL of reaction medium was measured in the liquid phase [0.3 M sucrose, 30 mM MOPS, pH 6.8, 1 mM EDTA, and 0.6% (w/v) BSA] at 25°C. Three separate replicates were measured.

2.5. Determination of H_2O_2

Fresh materials (~0.5 g) were homogenized in 5 mL 0.1% cold trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min. The endogenous H_2O_2 was measured spectrophotometrically at 390 nm by reaction with 1 M KI [17], and three to six separate replicates were determined.
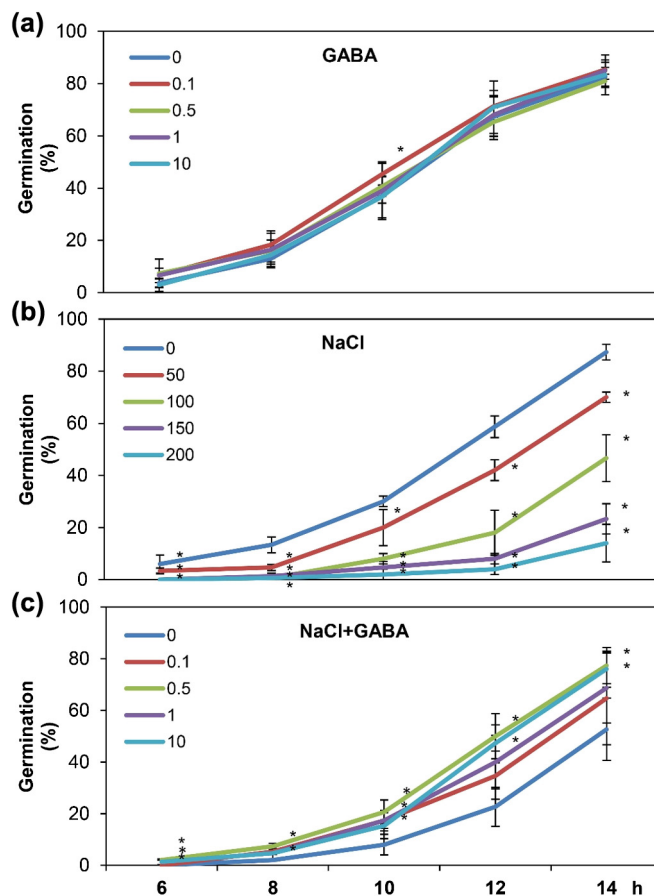
Fig. 1. Effects of exogenous GABA on germination of seeds in mungbean during germination under control and salt treatments. (**a**) 0, 0.1, 0.5, 1.0, and 10 mM GABA; (**b**) 0, 50, 100, 150, and 200 mM NaCl; (**c**) 100 mM NaCl plus 0, 0.1, 0.5, 1.0, and 10 mM GABA. Control treatment: 0 mM NaCl. The vertical bars represent mean ± standard deviation for n = 3–6 (representative of 3–6 separate replicates). * indicates statistically significant differences in the designated treatment compared to the corresponding control at $p < 0.05$.

2.6. Proteomic analysis of germinated seed proteins

Proteins of germinated seeds were extracted following treatment with 0, 0.1, 0.5, and 1.0 mM GABA in 100 mM NaCl separately. A total of 0.25 g of seeds were ground in 2 mL of precooled homogenization buffer [20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM ethylene glycol-bis(b-aminoethylether)-N,N,N0,N0-tetraacetic acid (EGTA), 1 mM PMSF, 1 mM DTT, and 1% Triton X-100]. The homogenate was centrifuged at 15,000 *g* for 15 min at 4°C. The supernatant was then mixed with one-fourth volume 50% cold trichloroacetic acid (TCA) and kept in an ice bath for 30 min. Then, it was centrifuged at 15,000 *g* for 15 min at 4°C, and the pellet was washed with cold acetone three times. After centrifugation, the pellet was vacuum-dried.

The dried powder was solubilized in a sample buffer [7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholine (pH 3.5–10), and 20 mM DTT]. The protein concentration of each treatment was quantified using the Bradford method [22]. Then, equal amounts of proteins were used for two-dimensional gel electrophoresis (2-DGE) analysis in conjunction with mass spectrometry to reveal the changes in proteins of germinating seeds according to our group's method [23]. The proteins were successfully identified based on 95% or higher confidence intervals of their scores, and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search for homologs of the spotted proteins. The patterns of protein expression were calculated by the ratio of each treatment compared with the control. A fold change of >1.5 was regarded as upregulation of protein expression in at least one treatment; otherwise, it was regarded as downregulation.

2.7. Genes expression analysis

Total RNA was extracted with RNAprep Pure Plant Kit (TIANGEN, Beijing, China), and cDNA was synthesized from 1.0-μg total RNA digested by DNase I, and the specific primers (Table S1) were designed using Primer3 software (http://primer3.ut.ee/) according the mRNA sequences searched by accession numbers of the selected proteins in GenBank (Table S1). qRT-PCR analyses were performed using SYBR Premix Ex Taq II (Takara, Otsu, Japan) on a Roche light Cycler 480 (Roche, Penzberg, Germany) according to the manufacturer's instructions. The $2^{-\triangle Ct}$ method was used to analyze the qRT-PCR results, where $\triangle Ct = Ct_{target} - Ct_{actin}$. Values shown were representative of three biological experiments with two technical repeats.

2.8. Statistical analysis

Statistical analysis of the data including physiological parameters and spot intensity was performed by SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences were identified as significant at the $p < 0.05$ level.

3. Results

3.1. The effects of GABA on seed germination under normal and salt conditions

Under normal conditions, during the period from 6 h to 14 h, the germination rate was significantly enhanced only by 0.1 mM GABA at 10 h (Fig. 1a). However, under salt conditions, significant dose-dependent inhibition on germination was shown during this period (Fig. 1b), and the germination rate was reduced by 73.3% and 93.3%, respectively, at 10 h following treatment with 100 mM and 200 mM NaCl. Therefore, we selected the 100 mM NaCl treatment to study the effects of GABA on germination. We found that the application of GABA clearly increased the germination rate, especially at concentrations of 0.1, 0.5, and 1.0 mM from 6 to 10 h, under salt conditions (Fig. 1c), which demonstrated a clear positive effect of GABA on germination under salt treatment. This result demonstrated that exogenous GABA can significantly regulate the germination process and alleviate the repression of salt treatment.
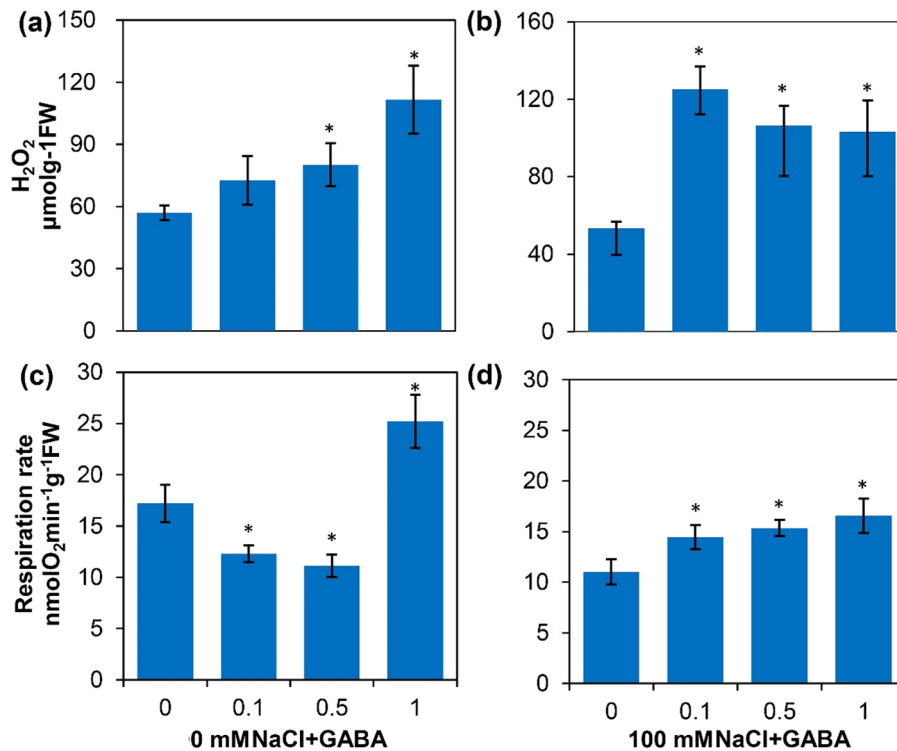
Fig. 2. Effects of exogenous GABA on hydrogen peroxide contents and respiration rates in the germinated seeds of the mungbean at 10 h under control and salt treatments. (**a, c**) 0, 0.1, 0.5, and 1.0 mM GABA; (**b, d**) 100 mM NaCl plus 0, 0.1, 0.5, and 1.0 mM GABA. Control treatment: 0 mM NaCl. The vertical bars represent mean ± standard deviation for n = 3–6 (representative of 3–6 separate replicates). * indicates statistically significant differences in the designated concentrations of GABA compared to the control at $p < 0.05$.

3.2. GABA promotes H_2O_2 production of germinating seeds under normal and salt conditions

Exogenous GABA was shown to significantly promote the production of H_2O_2 at concentrations of 0.5 and 1.0 mM, but not at 0.1 mM, under normal imbibition conditions in the absence of NaCl treatment (Fig. 2a), while 0.1 mM GABA greatly promoted H_2O_2 production by 135.2% compared with the control (0 mM GABA) in seeds treated with 100 mM NaCl (Fig. 2b). These promotive effects were lost to some extent at higher GABA concentrations, but H_2O_2 production was still higher than that in the control (Fig. 2b). This result indicates that GABA functions as a signal molecule in the salt-stress response of seed germination by activating H_2O_2 production, which might participate in the regulation of respiration during seed germination.

3.3. Effects of GABA on respiration during seed germination under normal and salt conditions

Seed respiration significantly increased with the extension of germination time under normal conditions without GABA (Fig. S1); however, this was affected by the application of GABA (Fig. 2c). After 10 h of germination, the respiration rate was significantly inhibited at GABA concentrations of 0.1 and 0.5 mM by 28.5% and 35.3%, respectively, contrary to the changes of H_2O_2 at these two concentrations (Fig. 2a). But a significant increase (46.5%) in respiration rate was observed at higher concentrations (1.0 mM) compared with the control (Fig. 2c), which was similar to the trend of H_2O_2 change (Fig. 2a). Respiration in the NaCl-treated seeds decreased significantly with increasing NaCl concentrations (Fig. S2), whereas the application of GABA blocked the respiration inhibition of salt-only treated seeds (Fig. 2d), which was similar to the trend of H_2O_2 changes (Fig. 2b). We also found that even 0.1 mM GABA was associated with the restoration of respiration, with significant increases of 31.2% to 50.4% compared with the control (Fig. 2d). Our findings suggest that the application of GABA at lower concentrations might have a different effect on the regulation of seed respiration under salt and normal conditions compared to that at higher concentrations.

3.4. Effects of GABA on mitochondrial respiration during seed germination

Under normal conditions, the addition of GABA significantly inhibited mitochondrial respiration (Fig. 3a). In the presence of NaCl, however, GABA induced a marked increase in mitochondrial respiration when compared with NaCl-treated alone (Fig. 3a). These results demonstrate that exogenous GABA can enhance or rescue the respiration of germinated seeds during exposure to salt stress.

The role of GABA's isomers (AABA and BABA) in mitochondrial respiration was further investigated in the absence of NaCl treatment. Respiration rates were significantly inhibited in the mitochondria of radicles and cotyledons when 0.1 mM GABA was added (Fig. 3b), while these effects were not observed with 0.1 mM AABA or BABA. These results not only suggest that GABA, rather than its isomers, has a specific role in respirations, but also that the inhibitory effects of exogenous GABA on germinating seeds (Fig. 2c) might be associated with the inhibition of mitochondrial respiration.

3.5. Does GABA function in mitochondrial ETC during seed germination?

Respiration was enhanced after the addition of 0.1 mM NADH, the substrate of NADH dehydrogenase (Complex I), but this effect was significantly weakened when GABA was applied (Fig. 4a), which indicates that GABA acts downstream of Complex I, otherwise respiration cannot decrease so rapidly. The application of 0.1 mM succinate, the substrate of succinate dehydrogenase (Complex II),
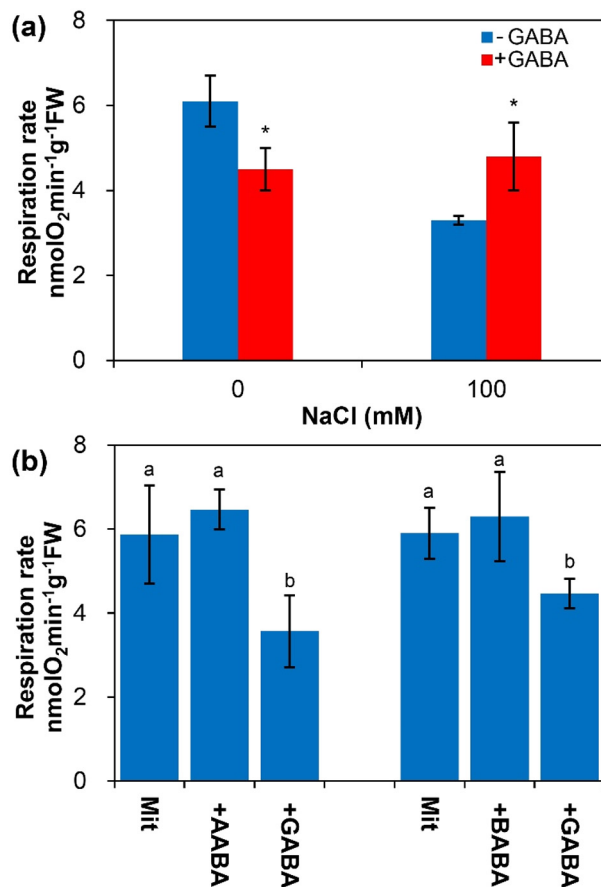
Fig. 3. Respiration rates in the germinated seeds of mungbean at 10 h under different treatments. (**a**) Addition of 0 and 0.1 mM GABA under 0 or 100 mM NaCl treatments; (**b**) Addition of GABA (0.1 mM) and its isomers, AABA (0.1 mM) and BABA (0.1 mM) under control treatment without NaCl. The vertical bars represent mean ± standard deviation for n = 3 (representative of 3 separate replicates). * in (**a**) indicates statistically significant differences between two treatments at $p < 0.05$, and the lowercase letters in (**b**) indicate statistically significant differences between treatments at $p < 0.05$.

followed by GABA gave a similar result (Fig. 4b), which suggests that GABA acts downstream of Complex II. Thus, GABA might act on Complex III or Complex IV. Subsequently, 0.1 mM GABA was added first, which inhibited respiration, as shown in Fig. 2c, and then respiration was rescued to some extent when 0.1 mM cytochrome c (Cytc) was applied, although the inhibitory effect was not completely restored by Cytc (Fig. 4c). This result suggests that exogenous GABA might exert its influence between Complex III and IV of the mitochondrial ETC.

3.6. GABA regulates the expression of seed proteins during seed germination under salt stress

A total of 43 differentially expressed candidate proteins were identified by 2-DGE analysis (Fig. S3), and the threshold was set to |fold changes| > 1.5 and $p < 0.05$, following at least one treatment of 0.1, 0.5, or 0.5 mM compared with the control (0 mM) under 100 mM NaCl (Table S2). Among them, 36 candidate proteins, including 15 upregulated and 21 downregulated, were annotated with the known functions in plants (Table 1). These proteins were mainly involved in metabolism (13), regulation (11), developmental and stress response (6), and storage (6) (Table 1). Of which, the expression patterns of the twelve selected genes had almost similar changing trends with the expression of corresponding proteins respectively (Fig. 5; Table 1), although there were some differences at the designated concentration
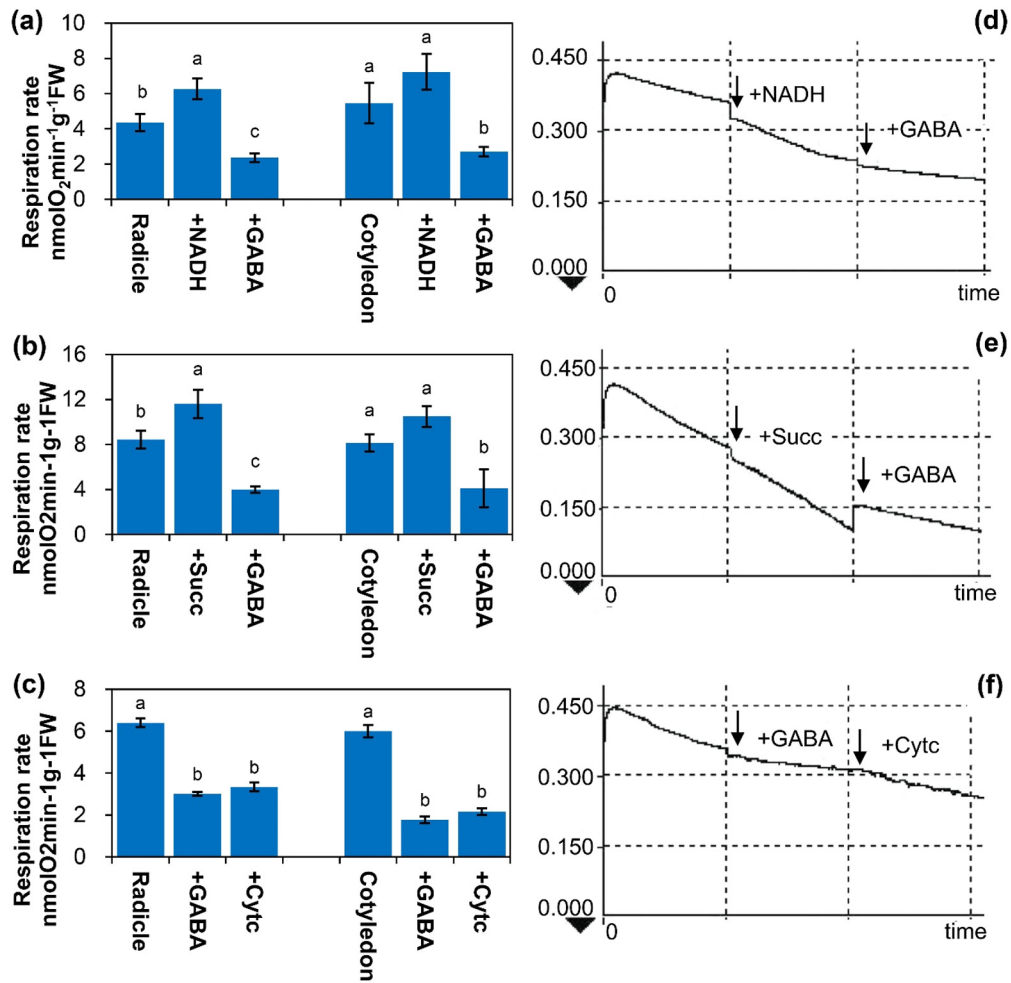
Fig. 4. Effects of exogenous GABA on the mitochondrial respiratory chain of mungbean germinated seeds in control treatment. (**a**) NADH and GABA; (**b**) Succinate (Succ) and GABA; (**c**) GABA and cytochrome c (Cytc). All the substrates were added at a concentration of 0.1 mM. (**d**, **e**, **f**) show the measuring processes of the radicles' mitochondria. Control treatment: 0 mM NaCl. The arrows represent the addition of the substrate during the measurement. The vertical bars represent the mean ± standard deviation for n = 3 (representative of 3 separate replicates). The lowercase letters indicate statistically significant differences among the treatments ($p < 0.05$).

for a few genes, which suggested that our result was reliable at the proteomic level.

During the process of seed germination, the expression of 13 proteins, involved in metabolism, were changed by GABA under salt stress—nine were upregulated and four downregulated (Table 1). Of the three proteins involved in energy metabolism, NADH dehydrogenase 1/Mitochondrial Complex I (Spot 219) and ATP synthase alpha chain/Mitochondrial Complex V (Spot 1473) were found to be up-regulated by GABA—by 2.6-fold at 0.1 mM and 3.3-fold at 0.5 mM, respectively (Table 1a). On the other hand, protoporphyrinogen oxidase (Spot 609), which is a component of cytochrome *c* and is responsible for heme biosynthesis, was shown to be downregulated by GABA (Table 1b). Additionally, two carbon metabolism-related proteins, phosphoenolpyruvate carboxylase (Spot 535) and glyceraldehyde 3-phosphate dehydrogenase (Spot 1328), showed decreases of more than 2–3 folds (Table 1b). However, four proteins (Spot 520, 1010, 1439, and 1537) involved in amino acid and polyamine metabolism demonstrated diverse upregulation trends with the addition of different concentrations of GABA under salt stress (Table 1a). Furthermore, three secondary metabolite related proteins were found to be affected by GABA. Carotenoid isomerase (Spot 1681) was not detected in 0.1 mM GABA, while it increased by 3.3-fold at 0.5 mM and by 6.3-fold in 1.0 mM GABA; isopentenyl-diphosphate delta-isomerase I (Spot

1312) increased by 4.7-fold in 0.1 mM GABA, but decreased to low levels in GABA concentrations of 0.5 and 1.0 mM (Table 1a). However, the cinnamyl alcohol dehydrogenase family protein (Spot 645) was downregulated to low levels compared to the control (Table 1b). Additionally, a high expression level of Nudix hydrolase homolog 21 (Spot 1440), which is involved in nucleoside phosphate metabolism, was induced in response to GABA addition under salt stress (Table 1a).

Eleven regulation-related proteins including one (Spot 1536) involved in DNA repair, three (Spot 1673, 1466, and 1147) in mRNA processing, two (Spot 825 and 1158) in transposition, and five (Spot 1041, 599, 187, 1340, and 981) in protein folding and metabolism, were almost inhibited by the application of GABA (Table 1b). In the process of development and stress response, dynamin-like protein (Spot 390), kinesin-like calmodulin binding protein (Spot 1588), and metallothionein-like protein 4B (Spot 2132) were induced (Table 1a), while other three proteins were almost inhibited by the designated GABA concentrations under salt stress (Table 1b). Similarly, six 8S globin proteins also showed different expression trends, and half of them (Spot 162, 232, and 1021) increased their expression (Table 1a), while the remaining (Spot 166, 208, and 959) showed the opposite response (Table 1b).

The above analysis of protein expression indicates that exogenous GABA affects the expression of seed proteins, including those
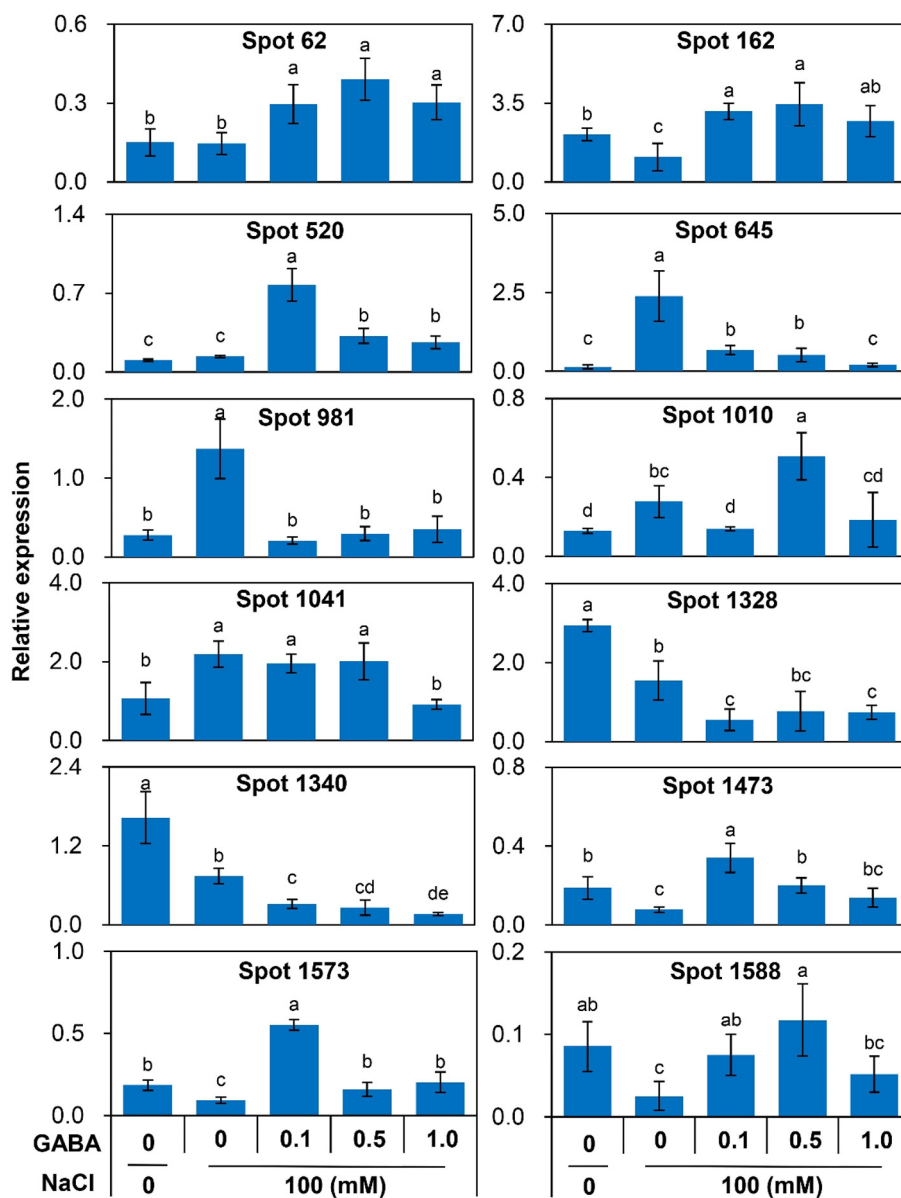
Fig. 5. Expression changes of genes encoding the selected differentially proteins under control and 100 mM NaCl with the different concentrations of GABA by qRT-PCR. Control: 0 mM NaCl; GABA: 0, 0.1, 0.5, and 1.0 mM. Vertical bars represent the mean ± standard deviation for n = 3 (representative of 3 separate replicates). The lowercase letters indicate statistically significant differences among the treatments ($p < 0.05$).

participating in the glycolytic process and mitochondrial respiratory chain, and regulates the respiration of germinating seeds under salt conditions.

4. Discussion

During seed germination, carbon and nitrogen metabolites are closely associated with germination and seedling establishment [24]. GABA, however, is considered to be located at the central position of the interface between plant carbon and nitrogen metabolism because it bypasses two steps of the TCA cycle [1], which has a close relationship with respiration. The maintenance of respiratory homeostasis is essential for rapid adaptation to environmental fluctuations in animals and plants [7,8,25]. The GABA signaling pathway has been implicated in respiratory control and in the adaptation to stresses in animals [25]. However, in plants, few studies have reported the effects of GABA on respiration, except for one report suggesting that the GABA shunt might affect mitochondrial respiration [10].

Our previous study primarily found that exogenous GABA affects the germination and respiration of seeds in the desert shrub [13]. In the current study, we further confirmed that the application of GABA significantly increased the germination rate of mungbean seeds, especially under salt treatment conditions (Fig. 1), which involves rapid initiation of respiratory activity [19]. As known to us, seed respiration significantly increases in germinating seeds under normal conditions (Fig. S1) [26]. The added GABA, however, obviously affected the changes in seed respiration in two opposite ways: a higher concentration (1.0 mM) increased respiration; but lower concentrations (0.1 and 0.5 mM) clearly inhibited it (Fig. 2c). These results are identical to those of our previous study on seeds of *H. ammodendron* [13]. However, salt treatment experiment yielded a surprising result, whereby GABA, even at 0.1 mM, was able to restore the respiration of germinating seeds (Fig. 2d) inhibited by NaCl treatment (Fig. S2) [26]. Moreover, the inhibitory effects on mitochondrial respiration of germinating seeds were found to be specific to the addition of GABA but not to its isomers (Fig. 3b). However, it is unknown whether GABA signaling plays a role in

Table 1

Expression patterns of candidate proteins involved in the seed germination of mungbean under NaCl with the different concentrations of GABA for 10 h. (**a**) Upregulation; (**b**) Downregulation. NaCl: 100 mM; GABA: 0, 0.1, 0.5, and 1.0 mM. Similar results were observed by at least three separate experiments.

Spot no.	Protein annotation[a]	Biological process	Accession	Expression pattern[b]			
				0	0.1	0.5	1.0
(a) Upregulated expression							
Metabolism (9)							
219	NADH dehydrogenase 1/Mitochondrial complex I	Energy metabolism	gi|28372495	1	2.6	1.2	1.2
520	Mitochondrial serine hydroxymethyltransferase	Amino acid metabolism	gi|134142067	1	1.4	0.9	1.2
1010	Methionine synthase	Amino acid metabolism	gi|33325957	1	0	11	4.7
1312	Isopentenyl-diphosphate delta-isomerase I	Isoprene biosynthesis	gi|6225525	1	4.5	0.6	0.2
1439	Spermidine synthase-related	Polyamine metabolism	gi|18414620	1	1.6	1.2	0.8
1440	Nudix hydrolase homolog 21	Nucleoside phosphate metabolism	gi|22330598	1	2.4	1.4	1.3
1473	ATP synthase alpha chain/Mitochondrial complex V	Energy metabolism	gi|5305369	1	1.3	3.3	0
1537	Cysteine synthase	Amino acid metabolism	gi|18252506	1	2	1.6	0.6
1681	Carotenoid isomerase	Carotenoid biosynthesis	gi|226491846	1	0	3.3	6.3
Development/Stress response (3)							
390	Dynamin-like	Cell division	gi|57899504	1	1.2	1.6	0.8
1588	Kinesin-like calmodulin binding protein	Mitochondrial fission	gi|308809065	1	0	2.7	0.3
2132	Metallothionein-like protein 4B	Zinc ion transport in seeds	gi|119372012	1	1.9	0.7	0.8
Storage proteins (3)							
162	8S globulin alpha' isoform precursor	Storage protein	gi|108743974	1	0.5	1.4	0.8
232	8S globulin alpha subunit	Storage protein	gi|108743972	1	1.8	1.8	0.9
1021	8S globulin alpha isoform proprotein	Storage protein	gi|108743972	1	4	1.5	2
(b) Downregulated expression							
Metabolism (4)							
535	Phosphoenolpyruvate carboxylase	Carbon metabolism	gi|154146738	1	0.5	0.5	0.3
609	Protoporphyrinogen oxidase	Energy metabolism	gi|3093412	1	0.5	0.6	0.7
645	Cinnamyl alcohol dehydrogenase family protein	Phenylpropanoid biosynthesis	gi|38568021	1	0.2	0.2	0.2
1328	Glyceraldehyde 3-phosphate dehydrogenase	Carbon metabolism	gi|166864034	1	0	0.5	0.3
Regulatory proteins (11)							
187	Ribosomal protein S7e	Protein biosynthesis	gi|255632936	1	1.2	0.8	0.5
599	Peptide chain release factor	Protein biosynthesis	gi|255556733	1	1	0.7	0.5
825	Plant transposase	Transposition	gi|242081031	1	0.6	0.6	0.5
981	Cyclophilin, putative	Protein folding	gi|255538956	1	0.4	0.2	0.4
1041	LOS1; copper ion binding	Protein biosynthesis	gi|30696056	1	0.2	0.9	0.4
1147	RNA-binding proteins	mRNA processing	gi|255539479	1	0.1	0.1	0.1
1158	Retrotransposon protein, putative	Transposition	gi|78707932	1	0.3	0.3	1.1
1340	26S protease regulatory subunit 8	Protein degradation	gi|149391017	1	0.1	1	0.5
1466	Proline-rich nuclear receptor coactivator	mRNA catabolic process	gi|115436680	1	0.5	0.9	0
1536	DNA mismatch repair protein Mlh1	DNA repair/seed germination	gi|222615900	1	0.4	0.2	0
1673	Maturase K	mRNA processing	gi|32816575	1	0.6	0.4	0.3
Development/Stress response (3)							
376	Metallothionein-like protein 4B	Zinc ion transport	gi|119372012	1	0.3	1	0.9
513	Stress-related protein	Stress response	gi|226491594	1	0.5	0.7	0.4
559	Late-embryogenesis abundant protein	Seed germination	gi|255644372	1	0.5	0.5	0.3
Storage proteins (3)							
166	8S globulin alpha subunit	Storage protein	gi|108743972	1	0.5	1.1	0.9
208	8S globulin alpha' isoform precursor	Storage protein	gi|108743974	1	0.6	1.1	1
959	8S globulin alpha subunit	Storage protein	gi|108743972	1	0.2	0.3	0.5

[a] Protein functions were classified by searching KEGG and related documents.
[b] Fold changes were calculated by the ratio of treatments with control.

respiration control, like in animals [25]. We hypothesized that lower concentrations of GABA may play a positive role in respiration by regulating mitochondrial proteins during seed germination under salt treatments, which might contribute to the adaptation to saline conditions.

Salt stress can induce osmotic and oxidative stresses, and affects the protein composition and function in the mitochondria [8], ultimately affecting mitochondrial respiration. Exogenous GABA might have a close relationship with the respiration of seed mitochondria, because the respiratory inhibition of mitochondria was rescued by exogenous GABA when the germinating seeds were subjected to salt treatment (Fig. 3a). This possibly occurs through the enhancement or rescue of the expression of respiratory proteins damaged by stresses [7,27] by GABA. Some genes encoding mitochondrial enzymes were found to be positively regulated by exogenous GABA in *C. intermedia* under NaCl treatment [14]. For example, 3-methyl-2-oxobutanoate dehydrogenase, which is upregulated by exogenous GABA [14], has been reported to be reduced at the mRNA level by oxidative stress [27], whereas its upregulation can promote respiration in

mitochondria [28]. Our hypothesis was further confirmed by the proteomic result, which demonstrated that some key proteins involved in the complex of the mitochondrial respiratory chain, such as Complex I and ATP synthase alpha chain (Complex V), were induced at higher expression levels by exogenous GABA, even at concentrations of 0.1 or 0.5 mM, under 100 mM NaCl treatment (Table 1).

Previous studies have indicated that mitochondria have reduced protein levels in complexes of the respiratory chain, repressing respiratory functionality under stress conditions [29]. Thus, the enhanced expression of mitochondrial respiratory proteins by exogenous GABA likely explains the GABA-induced restoration of respiration under salt conditions. These results indicated that the regulatory role of exogenous GABA on respiration might involve the induction of mitochondrial proteins. One study also showed that antioxidants could protect the salinity damaged Complex I [9]. It was then shown, through the experiment of adding different substrates (Fig. 4), that exogenous GABA might exert its effects on the complexes of the mitochondrial respiratory chain, which had a strong link with

plant stress tolerance [8]. Therefore, we concluded that GABA has a protective role on the mitochondrial respiratory chain under salt conditions.

Additionally, we found that exogenous GABA changed the expression of six proteins (Table 1) involved in the metabolism of carbohydrates, amino acids, and polyamines, in germinating seeds under salt treatment, which are essential for seed germination [24]. Among them, two carbon metabolism-related proteins decreased, whereas all four-nitrogen metabolism-related proteins increased. For example, it has been proven that glyceraldehyde 3-phosphate dehydrogenase, the key enzyme of the sixth step in glycolysis, acts as a GABAA receptor kinase that links glycolysis to neuronal inhibition in animals [30]. This protein can be specifically induced in germinating seeds [31], while in the present study, it (Spot 1328) showed an evident decrease following GABA treatment, although the reason for this needs to be further tested. Spermidine can promote the germination of seeds [32]. In our study, spermidine synthase-related protein (Spot 1439), which is involved in the synthesis of spermidine in polyamine metabolism and is also a potential source of GABA in plants exposed to abiotic stress [33], was significantly induced by GABA under salt stress. There is a close relationship between polyamines and GABA under stress, wherein 25%–39% of GABA accumulation under stress is derived from the polyamine degradation pathway in soybean, fava bean, and tea [33]. Interestingly, free polyamines, such as putrescine, spermidine and spermine, are increased by exogenous GABA to relieve short-term hypoxia stress [34]. Additionally, mitochondrial serine hydroxymethyl transferase (Spot 520), methionine synthase (Spot 1010) and cysteine synthase (Spot 1537), involved in the synthesis/degradation of cysteine metabolism, were also significantly induced by GABA under salt treatment. It is reported that cysteine occupies a central position in plant metabolism [35]. For instance, cysteine is closely linked to serine and methionine, and influences the production of the hormone ethylene, which is involved in seed germination [35]. Hence, one of the reasons for exogenous GABA alleviating salt stress damage may be the activation of cysteine metabolism. These results indicated that exogenous GABA could affect the metabolism of carbon and nitrogen, which might contribute to the alleviation of salt damage-related inhibition during seed germination.

Furthermore, the mitochondrial respiratory chain can be inhibited by stress conditions, leading to increased electron leakage and subsequently, excess production of H_2O_2 [8,9]. This might also result from the resurgence of mitochondrial respiration in the seed after imbibition [36]. As shown above, exogenous GABA could regulate respiration and protein expression in germinating seeds under salt stress, especially when applied at a relatively low concentration of 0.1 mM (Table 1). Considering the signaling role of H_2O_2 during seed germination, which induces seed storage protein carbonylation and alters the homeostasis of ABA and GA [36], we found that 0.1 mM GABA could promote significant H_2O_2 production in NaCl-treated germinating seeds (Fig. 2b). This was inconsistent with the results of Cheng [17], which shows that 1 μM GABA inhibits the production of H_2O_2 under salt stress. This may be due to the differences in plant species or GABA concentrations used. Regardless, further evidence that GABA might function as a signal molecule [2,14,37] has been provided by the recent identification of its receptor [6] and its participation in the regulation of seed respiration in salt-stress responses, suggesting that it might have similar functions in controlling respiration as in animals subjected to stress conditions.

5. Conclusions

Exogenous GABA showed significant inhibition effects on seed germination of mungbean under salt treatment but almost no effects under normal condition. Moreover, low concentrations of GABA had an inhibitory effect on seed respiration under normal condition, whereas they had an opposite role under salt treatment. Besides, exogenous GABA (0.1 mM) could restore NaCl-inhibited mitochondrial respiration, which might exert its effects via the mitochondrial respiratory chain. Proteomic analysis further proved that exogenous GABA affected the expression of seed proteins including complexes of the mitochondrial respiratory chain under salt treatment. The data presented here provide new evidence that GABA may act as a signal molecule in regulating respiration of mungbean seeds during germination in response to salt stress.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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