

Ca²⁺ and ABA on the Accumulation of GABA and Flavonoids in Germinated *Salicornia bigelovii* Torr. under NaCl Stress

Dujun Wang¹, Jie Wang¹, Jingying Zheng¹, Yueling Shang¹, Fengwei Li¹,
Runqiang Yang², Chunyin Zhang³, Yangjie Lu⁴, Xiaohong Yu^{1,*}

¹School of Marine and Bioengineering, YanCheng Institute of Technology, Yancheng 224051, China

²College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

³Yancheng Green Garden Saline Soil Agriculture Technology Co., Ltd., Yancheng 224001, China

⁴Yancheng Huijia Aquatic Products Technology Co., Ltd., Yancheng 224300, China

*Corresponding author: yxh1127@ycit.edu.cn

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Abstract The effects of CaCl₂ and abscisic acid (ABA) on the sprouts growth, γ -aminobutyric acid (GABA) and flavonoids accumulation in germinated *Salicornia bigelovii* Torr. were investigated under NaCl stress. Results showed that the content of GABA and flavonoids increased by 1.20 and 1.19 times under 5 mM CaCl₂ treatment compared with the control group. The key enzymes including glutamate decarboxylase (GAD) and diamine oxidase (DAO), phenylalanine ammonolyses (PAL), cinnamic acid-4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) were increased significantly ($p < 0.05$). After being treated with 50 μ M ABA under 7.5 g·L⁻¹ NaCl, *S. bigelovii* had the higher content of GABA (398.26 mg·100g⁻¹) and flavonoids (36.90 mg·g⁻¹) than control group ($p < 0.05$). The mRNA levels of key enzymes were consistent with GABA and flavonoids accumulation. Our results provided the basic for further studies key enzyme genes affect the GABA and flavonoids accumulation and laid the foundation for high value utilization of *S. bigelovii*.

Keywords: *Salicornia bigelovii* Torr, combined stress, γ -aminobutyric acid, flavonoids, key enzyme gene

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1. Introduction

Salicornia bigelovii Torr (*S. bigelovii*) is a kind of salty plant belonging to salicornia of chenopodiaceae and widely distributed along coastal and inland salt marshes around the world [1]. It has a strong salt tolerance and could withstand concentrations of more than 1,000 mM NaCl [2]. *S. bigelovii* plants are rich in mineral elements, dietary fiber, fat, protein, the total amount of vitamins and amino acids [3] with seafood flavor and crisp taste. Therefore, *S. bigelovii* is recognized as a green organic vegetable. In certain countries, it is regarded as a traditional herb. Emerging evidence indicated that *S. bigelovii* has numerous essential bioactivities and health benefits. These includes removal of toxins, promotion of diuresis, reduction of weight [4], anti-oxidation [5], antitumor [6], anti-inflammatory [7], immune regulation [8], blood lipid lowering [9] and others. Owing to its strong salt and alkali resistivity, it is usually used to improve the coastal ecological environment and development of seawater irrigated agriculture. The seed

yield per unit area of *S. bigelovii* can reach 209 kg, and the vegetables of *S. bigelovii* can be harvested at a time of 2333 kg yield per unit area. Therefore, it has a very broad development prospect as a dual-use resource for medicine and food.

Germination is a method of changing plant nutrients and anti-nutritional factors. During seed germination, a series of physiological metabolism changes occur, which are mainly manifested in the recovery of cell physiological activities and complex biochemical metabolisms, thus leading to significant changes in the nutritional composition of seeds [10]. γ -aminobutyric acid (GABA) is a non-protein amino acid widely found in living organisms, and it has been approved by the China Ministry of Health as a new resource food. With the increase of age and the mental stress, the amount of GABA production in the human body gradually decreases. Supplementing GABA from food will be important for human health. However, the GABA content in higher plant tissues ranges from 0.3-32.5 μ mol·g⁻¹, which does not meet the physiological needs of the human body. After germination of plant seeds, endogenous enzymes become activated leading to an increased GABA content.

Especially under the conditions of hypoxia, heat shock and salt stress, the GABA content in germinated kernels was doubled or even tens of times increased [11]. Research has shown that the content of GABA in soybean increased during the germination period [12]. After germinating treatment, the GABA and other functional components of brown rice increased obviously, and the health care function was enhanced [13,14].

Studies have demonstrated that the plant seeds germinate under adverse conditions such as hypoxia and salt (NaCl) stress with a significant increase in the content of GABA [15]. AL-Quraan [16] studied the effect of salt stress on the characterization of GABA in wheat seedlings and found that the content of GABA could be affected by salt stress. Notably, the main pathway for the synthesis of GABA by plants are the GABA shunt and polyamine degradation pathway. In GABA branch, glutamate (Glu) is decarboxylated under the catalysis of glutamate decarboxylase (GAD) to form GABA, next GABA is catalyzed by GABA transaminase to form succinic semialdehyde (SSA), and subsequent oxidation of SSA to succinic acid by semialdehyde dehydrogenase into the tricarboxylic acid (TCA) cycle. In polyamine degradation pathway, diamine or polyamine are catalyzed by diamine oxidase (DAO, EC:1.4.3.6) and polyamine oxidase (PAO, EC:1.5.3.3) to produce 4-aminobutyric aldehyde, and then dehydrogenated by 4-aminobutyraldehyde dehydrogenase to generate GABA, which is involved in TCA cycle metabolism [17,18]. Under adverse conditions, the plant seeds could produce rapid induction by regulating the expression of the gene, and then promote the activity changes of related enzymes. At the same time, the content of osmotic adjustment substances such as GABA, Glu, PAs and proline (Pro) will be increased due to adaptations to environmental stress. It has been reported that GABA enrichment in macroembryonic rice during soaking as a result of GAD transcription [19]. Several researches have indicated soybeans GABA level increases during soaking, for instance, Western Blot analysis result showed an increment in the level of GAD protein [20]. GABA was enriched in wheat seedlings under NaCl stress, and the GAD protein level was increased significantly.

Flavonoids are polyphenolic secondary metabolic compounds, universally distributed in green plant kingdom. Flavonoids play important roles in plants, animals, and bacteria. In plants, flavonoids are responsible for colour, aroma of flowers, fruit to attract pollinators, fruit dispersion and help in seed, spore germination, growth and development of seedling [21]. Biosynthesis of flavonoids occur via the phenylpropanoid pathway with Phenylalanine ammonolyses (PAL), Cinnamicacid-4-hydroxylase (C4H) and 4-coumaric acid CoA ligase (4CL) identified as the key enzymes in the synthesis of flavonoids [22]. When plants are exposed to stress such as cold, heat, mechanical damage, environmental stress and microbial infections, the defense system and phenylpropanoid metabolism could be activated, resulting in the increase of key enzyme activities and content of flavonoids [11]. The study showed that the content of flavonoids in germination treatment of tartary buckwheat seeds was much higher than tartary buckwheat seeds [23]. However,

to the best of our knowledge, no studies have examined of the contents of GABA and flavonoids in *S. bigelovii* seed germination under salt stress.

S. bigelovii seed has a higher protein content, after the seeds germinate, the protein hydrolyzes, and the content of glutamic acid and polyamine increases. This provides sufficient nitrogen-containing substances for the enrichment of GABA and can be used to develop GABA-rich *S. bigelovii* foods. However, there have been no reports on the active substance germination and enrichment of *S. bigelovii*. Therefore, in the present study, *S. bigelovii* was used to investigate the changes of bud length, GABA, flavonoids and the key enzymes during germination by CaCl_2 , and ABA treatment under NaCl stress. Our results provide a foundation for further studies on improving the nutritional value of *S. bigelovii*, and applying it to the functional food industry.

2. Materials and Methods

2.1. Materials and Reagents

S. bigelovii was obtained by Yancheng Luyuan Salt soil Agricultural Technology Co., Ltd.; Trizol RNA extraction kit was purchased from Sangon (Sangon Biotech, Shanghai, China); A Revert Aid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate cDNA for subsequent experiments; The real-time PCR experiments were performed using a Fast Start Essential DNA Green Master kit and a Light Cycle 96 Real-Time PCR system (Roche, Basel, Switzerland).

2.2. NaCl Stress Culture Experiment

The full and even seeds of *S. bigelovii* were selected and washed with deionized water. The germination conditions of *S. bigelovii* were selected with $7.5 \text{ g}\cdot\text{L}^{-1}$ NaCl and pH was 8.0 base on the reference report [24]. The seeds were sterilized with 1% sodium hypochlorite for 30 min, and washed with deionized water. Then, the seeds were soaked in deionized water at 25°C for 8 h and placed in a petri dish covered with two layers of filter paper and distributed uniformly. Next, the seeds were cultured at 24°C for 48 h, the seeds moisture control was by spraying with $7.5 \text{ g}\cdot\text{L}^{-1}$ NaCl medium (pH 8.0) every 8 h.

2.3. CaCl_2 Treatment Experiment

The neatly and consistently sprouted *S. bigelovii* were selected to do the CaCl_2 treatment experiment. Briefly, $7.5 \text{ g}\cdot\text{L}^{-1}$ NaCl medium was added with different concentrations of CaCl_2 for 48 h. The concentrations of CaCl_2 were 0, 0.5, 1.0, 2.5, 5 and $10 \text{ mmol}\cdot\text{L}^{-1}$ respectively. After 96 hours of $7.5 \text{ g}\cdot\text{L}^{-1}$ NaCl and $5 \text{ mmol}\cdot\text{L}^{-1}$ CaCl_2 treatment simultaneously, the samples were collected every 24 hours. All samples were cleaned with deionized water, and the surface moisture was absorbed by absorbent paper, and then stored in the refrigerator at 4°C until they were detected.

2.4. ABA Treatment Experiment

Briefly, 7.5 g·L⁻¹ NaCl medium was added with different concentrations of ABA for 48 h. The concentrations of ABA were 0, 25, 50, 75, 100 and 125 μmol·L⁻¹. After treatment with 50 μmol·L⁻¹ ABA for 96 h, the samples were sampled every 24 h. After 96 hours of 7.5 g·L⁻¹ NaCl and 50 μmol·L⁻¹ CaCl₂ treatment, the samples were collected every 24 hours. All samples were cleaned with deionized water, and the surface moisture was absorbed by absorbent paper, and then stored in the refrigerator at 4°C until they were detected.

2.5. mRNA Expression of Key Enzymes in Germinated *S. bigelovii*

The total RNA was extracted with Trizol reagent after germination for 0, 24, 48, 72 and 96 h. The first chain of cDNA was synthesized according to cDNA Synthesis kit, and the reverse transcription was performed at 42°C for 60 min, followed by 70°C for 5 min, according to the manufacturer’s instructions. According to the enzyme gene sequence obtained by transcriptome sequencing [25]. The primers (Table 1) were obtained from Sangon Biotech (Shanghai, China). The real-time PCR (RT-qPCR) program: 2 × AceQ Universal SYBR qPCR Master Mix 10 μL, Primer-F (10 μM) 0.4 μL, Primer-R (10 μM) 0.4 μL, cDNA 2 μL, add ddH₂O to 20 μL. The RT-qPCR results were analyzed by 2^{-ΔΔCt} method, using β-actin as the reference gene. All the determinations were performed in triplicate.

2.6. Seed Sprout Length and Determination of Key Enzyme Activity

In this experiment, *S. bigelovii* seed sprout length was measured using the vernier calipers, the flavone content was detected by sodium nitrite-aluminum nitrate colorimetric method. Determination of the content of GABA was measured as described by the report [26]. Briefly, 0.2 g dried powder of sprouted *S. bigelovii* was dissolved in 8% trichloroacetic acid solution for 5 mL, then centrifuged at 40°C for 2 h. The supernatant was filtered by 0.45 μm filter membrane as samples to be determined. The reversed-phase high performance liquid chromatography (RP-HPLC) with pre-column OPA (o-phthalaldehyde) derivatization was used to measure the content of GABA. An Agilent 1290 Infinity HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA) with a C18 ODS reversed phase column (250 mm×4.6 mm,

5 μm), the column was maintained at 25°C, the mobile phase ratio was 0.1 mol·L⁻¹ phosphate buffer (pH 6.0) 52%, methanol 46%, tetrahydrofuran 2%. The mobile phase was filtered by 0.22 μm microporous membrane and degassed by ultrasonic wave. The flow rate of mobile phase was 1 mL·min⁻¹.

GAD activity was measured according to the literature report [27]. Briefly, 0.5 g *S. bigelovii* seed buds were homogenized with 70 mmol·L⁻¹ potassium phosphate buffer (pH 5.8, 2 mmol·L⁻¹ β-mercaptoethanol, 2 mmol·L⁻¹ EDTA, 0.2 mmol·L⁻¹ PLP and 10% glycerol) and centrifuged at 4°C, 1000 rpm for 20 min, the supernatant as the enzyme extract. After 200 μL substrate (1% glutamic acid, pH 5.8) was reacted at 40°C for 2 h, the enzyme was inactivated in 90°C water bath for 10 min. The supernatant was centrifuged to determine the content of GABA, and 1 μmol GABA was produced per hour as an enzyme activity unit (U).

DAO activity was measured according to the literature report [28]. Briefly, 0.5 g *S. bigelovii* sprouts to be measured were treated with 4 mL buffer (70 mmol·L⁻¹ potassium phosphate buffer pH 6.5%, 10% glycerol), then ground to homogenate in ice bath and centrifuged at 4°C, 10 000 rpm for 15 min. The crude DAO enzyme extract was obtained. The reaction mixture for determination of DAO activity was composed of 2 mL potassium phosphate buffer, 0.2 mL guaiacol (25 mmol·L⁻¹), 0.1 mL peroxidase solution (250 U·mL⁻¹) and 0.5 mL DAO extract. The mixture was incubated in water bath at 30°C for 5 min and then added with putrescine (Put, 50 mmol·L⁻¹) for 0.1 mL to initiate the reaction. The change of OD₄₇₀ per minute was 0.01 units as a unit of enzyme activity (U).

The key enzyme activity of PAL was measured according to the literature report [29]. Briefly, the *S. bigelovii* sprouts 0.2 g, adding 0.05 mol·L⁻¹ pH 8.8 boric acid buffer (containing β-mercaptoethanol 5 mmol·L⁻¹) 6 mL, a small amount of polyvinylpyrrolidone (PVP), in ice bath grinding homogenate, homogenate at 4°C, 10 000 rpm centrifugation 15 min, supernatant used for enzyme activity detection. The reaction solution consisted of supernatant 0.2 mL, 0.02 mol·L⁻¹ phenylalanine 1 mL, 0.05 mol·L⁻¹ boric acid buffer 3.8 mL, and total volume 5 mL. The control group was treated with boric acid buffer solution for 0.2 mL without adding enzyme solution. The reaction was carried out in 30°C constant temperature water bath for 30 min, and 6 mol·L⁻¹ hydrochloric acid solution was added for 0.5 mL to terminate the reaction. The change of OD₂₉₀ per hour was 0.1 as an enzyme activity unit (U).

Table 1. The primers of RT-PCR

Gene	F primer	R primer
β-actin	GCCATCTTTGATTGGAATGG	GGTGCCACAACCTTGATCTT
GAD	CAGAGGCAGCAGTTGGAGTC	TTGGGCTTGTCTAGGGTAA
DAO	CTCGAGATGGCTTCCACCACCA	CTCTTCTCCAAACCAGCC
PAL	CTACAGCCTTGGAGTTGGAC	TTGAACGCAGGAGTATTTGG
C4H	GGAAGATTCAACATACCGACTA	CAACCCATAATATCATCACAGC
4CL	GTGCTTGATGTTGCCTCG	GTATGGGATGACGGAGGC

GAD: Glutamate decarboxylase; DAO: diamine oxidase; PAL: phenylalanine aminolyase; C4H: cinnamicacid-4-hydroxylase; 4CL: 4-coumarate: CoA ligase.

C4H activity was measured according to the literature report [30]. Briefly, 0.2 g *S. bigelovii* sprouts, quickly rinse the surface impurities with distilled water, absorb dry water with absorbent paper. Adding 0.1 mol·L⁻¹ pH 7.6 phosphate buffer (0.25 mol·L⁻¹ sucrose, 0.5 mmol·L⁻¹ EDTA, 2 mmol·L⁻¹ mercaptoethanol) precooled at 5 mL 4°C, the homogenate was rapidly ground in ice bath. The supernatant was centrifuged at 4°C, 12000 rpm for 30 min. The assay system consisted of enzyme solution 0.2 mL, 50 mmol·L⁻¹ cinnamic acid 0.2 mL, 0.4 g·L⁻¹ NADPH 3 mL and 0.1 mol·L⁻¹ phosphate buffer (pH 7.6) 3 mL. No enzyme solution was added as the control, and phosphate buffer was used instead. The reaction solution was incubated at 30°C for 30 min, and 6 mol·L⁻¹ hydrochloric acid 0.2 mL was added to terminate the reaction. The supernatant was adjusted to pH 11 with NaOH. The change of OD₃₄₀ value per minute was 0.01 as an enzyme activity unit (U).

4CL activity was measured according to the literature the report [31]. Briefly, 0.5 g *S. bigelovii* sprouts, add liquid nitrogen and grind it into powder, put into 5 mL 0.2 mol·L⁻¹ pH 8.0 Tris-HCl buffer (containing 15 mmol·L⁻¹ mercaptoethanol and 10% glycerol). The supernatant was centrifuged at 4°C, 10 000 rpm for 20 min, to determine the enzyme activity. The reaction system consisted of 3 mL 0.1 mol·L⁻¹ pH 8.0 Tris-HCl buffer (containing 0.2 mmol·L⁻¹ for coumaric acid, 5 mmol·L⁻¹ ATP, 0.3 mmol·L⁻¹ CoA, 5 mmol·L⁻¹ MgSO₄ 7H₂O) and 150 µL crude enzyme solution. No CoA was added to the control. After 10 min reaction at 40°C, 0.5 mL 6 mol·L⁻¹ hydrochloric acid was added to terminate the reaction. The OD₃₃₃ value was determined at 333 nm. The change of OD₃₃₃ value per minute was 0.01 as an enzyme activity unit (U).

2.7. Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Data were evaluated for statistically significant

difference by using Student's *t*-test. SPSS 13.0 (IBM SPSS, Armonk, NY, USA) was used for analysis, and *p* < 0.05 was considered statistically significant. Correlation analysis was conducted using GraphPad Prism 5.0 software (San Diego, CA).

3. Result and Discussion

3.1. Effects of CaCl₂ on the Content of GABA, Flavonoids and Key Enzymes in Germinated *S. bigelovii* under NaCl Stress

S. bigelovii was grown and distributed along the coastal zone in saline-alkaline soil reach in calcium ions. Of note, the calcium ions promote the growth and development of *S. bigelovii*. Our results showed variation in sprout length of *S. bigelovii* as the concentration of CaCl₂ increases. The maximum sprout length, 1.15 times greater than that of the control group was recorded at 5 mM concentration of CaCl₂ (Figure 1 A). As shown in figure1 B, the content of GABA increased from 0.0 mM to 5 mM and then decreased at concentrations beyond 5 mM. The maximum amount of GABA recorded was 376.53 mg·100g⁻¹ at 5 mM CaCl₂, which was 1.20 times higher than that of the control group. There was no significant difference between the change in trends of key enzymes, GAD and DAO activities and the GABA content, which were 1.04 and 1.28 times higher than that of the control groups respectively (Figure 1 C and D). Study has demonstrated that the CaCl₂ affect the GABA content because of its regulation of GAD and DAO activities [32]. Therefore, our results corroborate with the report that CaCl₂ could affect GAD and DAO activities, and promote the accumulation of GABA content.

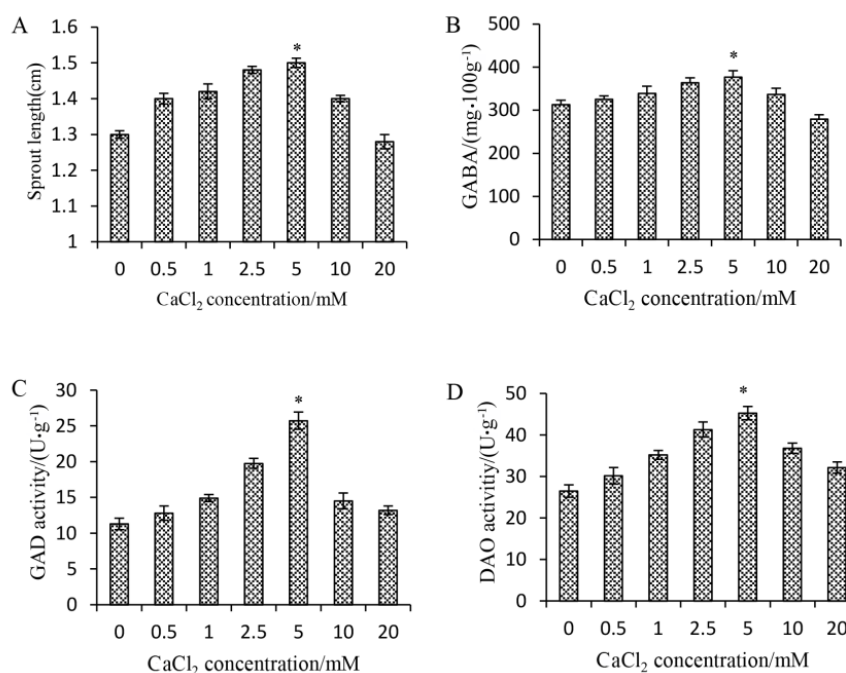


Figure 1. Effect of different CaCl₂ concentration on sprout length (A), GABA content (B), GAD activity (C) and DAO activity (D) in germinated *S. bigelovii* under 7.5 g·L⁻¹ NaCl stress. $\bar{x} \pm s$, **p* < 0.05, compared with the control group

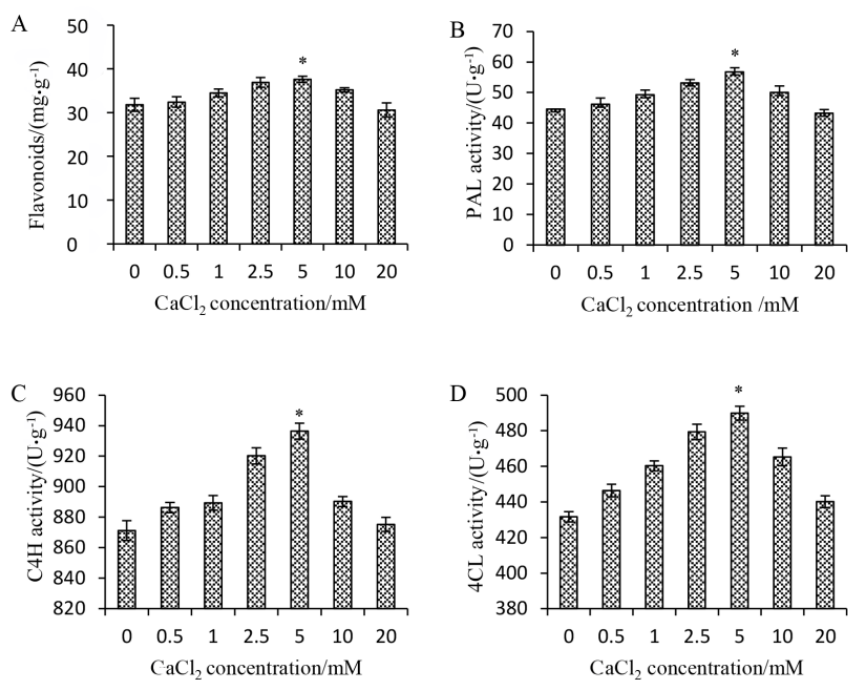


Figure 2. Effect of different CaCl_2 concentration on flavonoids content(A), PAL activity (B), C4H activity (C) and 4CL activity (D) of geminated *S. bigelovii* under 7.5 g·L⁻¹ NaCl stress. $\bar{x} \pm s$, * $p < 0.05$, compared with the control group

As shown in Figure 2 A, the content of flavonoids was increased at first and then decreased, with the concentration of CaCl_2 ranged from 0 to 20 mM. When the concentration of CaCl_2 was 5 mM, the highest (37.60 mg·g⁻¹) flavonoids content was measured, which was 1.19 times of the control group ($p < 0.05$). Simultaneously,

Figure 2 B, C and D showed that the change in trends of key enzyme PAL, C4H and 4CL activities were found to be the same as the flavonoids content, and the activities of PAL, C4H and 4CL reached the maximum value at 5 mM CaCl_2 , which were 1.28, 1.08, 1.14 times higher than the control groups respectively.

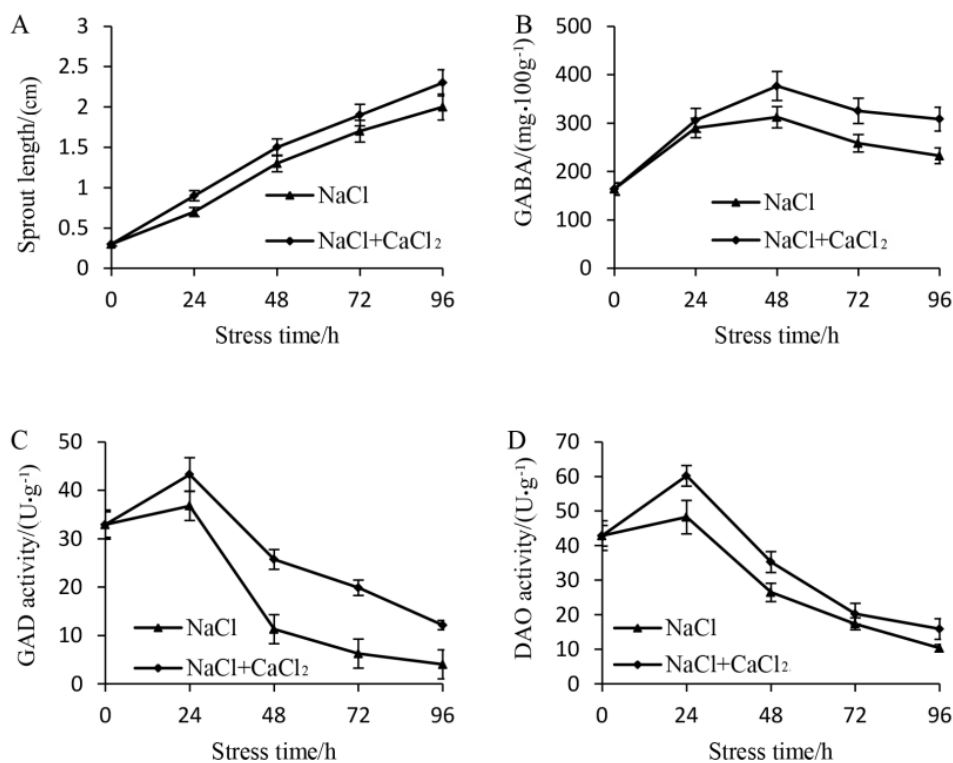


Figure 3. Changes of sprout length(A), GABA content(B), GAD activity(C) and DAO activity(D) of geminated *S. bigelovii* under combined stress of NaCl and CaCl_2

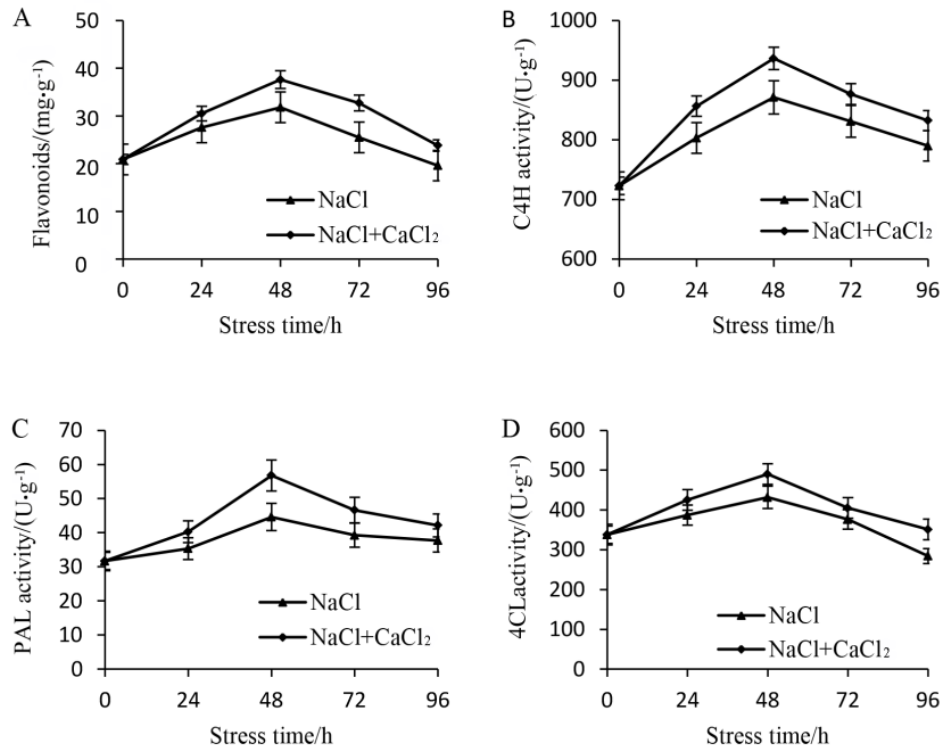


Figure 4. Changes of flavonoids content(A), C4H activity(B), PAL activity(C) and 4CL activity(D) of geminated *S. bigelovii* under combined stress of NaCl and CaCl₂

3.2. The Change of Sprout Length, GABA, Flavonoids Content and Key Enzymes Activity under Combined Stress of NaCl and CaCl₂.

We studied the effects of combination treatment of CaCl₂ and NaCl on *S. bigelovii* sprouts, the content of GABA and flavonoids, the key enzyme activities. The results showed that compared with the NaCl alone treatment group, the sprout length of *S. bigelovii* reached 2.3 ± 0.12 cm, which was 1.15 times longer than NaCl alone treatment group after 96 h treatment (Figure 3 A). The result of this study was consistent with the report that addition of Ca²⁺ can alleviate the inhibition of salt stress caused by NaCl treatment, and provide the necessary trace element Ca²⁺ for the growth of *S. bigelovii* [33]. As shown in Figure 3 B, the content of GABA was $376.53 \text{ mg} \cdot 100\text{g}^{-1}$, reached the highest level at 48 h, which was 1.20 times of that treated with NaCl alone ($p < 0.05$). In Figure 3 C and D, the key enzyme activities of GAD and DAO in combination treatment were 1.18 times and 1.25 times higher than those in NaCl treatment respectively. After 24 h treatment, the enzyme activities of GAD and DAO reached the highest level and then decreased rapidly. The same change trends were found between the GABA content and key enzyme activities. However, the activities of GAD and DAO have the highest activity at 24 h and the content of GABA was highest at 48 h, it may be that GAD and DAO need a certain reaction conversion time to promote the conversion of glutamate to γ -aminobutyric acid. Therefore, the combined treatment of CaCl₂ and NaCl increased the activities of GAD and DAO, and promoted the accumulation of GABA.

Figure 4 A showed that the content of flavonoids increased at first and then decreased under the treatment of CaCl₂ and NaCl. After treated for 48 h, the content of flavonoids was $37.60 \text{ mg} \cdot \text{g}^{-1}$ and reached the highest level, which was 1.19 times that of treated NaCl alone ($p < 0.05$). The same change trends were found in key enzyme activities of PAL, C4H and 4CL (Fig. 4 B, C, D). After 48 h of treatment, the enzymatic activities of PAL, C4H and 4CL were 1.27, 1.07, 1.13 times that of the treated NaCl alone. It can be seen that the changes of flavonoids content and the activities of PAL, C4H and 4CL have similar change under the combined treatment of CaCl₂ and NaCl. The result consistent with the study that the salt ion was beneficial to the synthesis of flavonoids of *Ginkgo biloba*.

3.3. Effects of ABA on the Content of GABA and Flavonoids and Key Enzymes Activity under Salt Stress

As shown in Figure 5 A, with the increase of concentration of ABA treatment, the sprout length of *S. bigelovii* showed a downward trend. When the concentration of ABA was $125 \mu\text{M}$, the sprout length was 0.5 cm, which was only 38.46% of the control group. The results in Figure 5 B, C and D showed that the activity of GAD and DAO in germinated *S. bigelovii* have the same trend with the content of GABA. When the concentration of ABA was $75 \mu\text{M}$, the activities of GAD and DAO were the highest, which were 3.11 and 1.33 times higher than the control group respectively. At the same time, the content of GABA reach the highest was $398.26 \text{ mg} \cdot 100\text{g}^{-1}$, which was 1.28 times significantly higher than the control group ($p < 0.05$). This result consistent with report that the

accumulation of GABA in root of wheat seedlings induced by ABA, that was caused by the increase of L-Glu content and GAD activity [34]. What's more, the content of polyamines in germinated seeds induced by exogenous ABA was increased, and polyamines were one of the precursors of GABA synthesis [35]. Figure 6 A, B, C, D showed that the content of flavonoids and the key enzyme activities of PAL, C4H and 4CL increased at first and then decreased with the increase of ABA concentration. When

the concentration of ABA was 50 μM , the activities of PAL, C4H and 4CL were the highest, which were 1.28, 1.08, 1.14 times higher than the control group, respectively. At the same time, the content of flavonoids in the germinated *S. bigelovii* reached the maximum level, which was 1.16 times higher than the control group ($p < 0.05$). This indicated that ABA stimulated the activities of PAL, C4H and 4CL during germination, and promoted the accumulation of flavonoids to a certain extent.

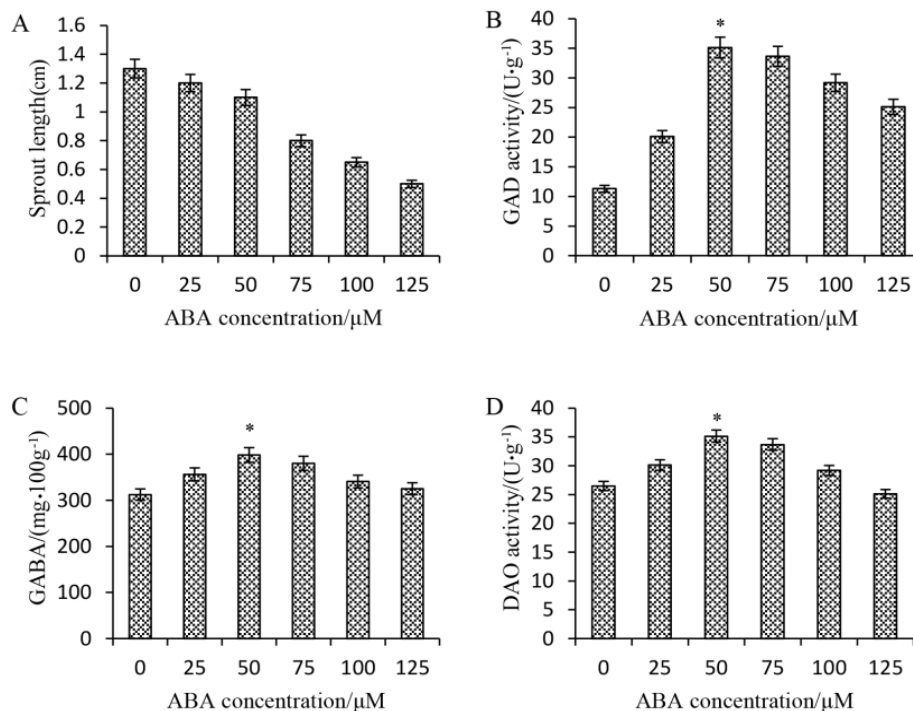


Figure 5. Effect of different ABA concentration on sprout length(A), GAD activity(B), GABA content(C) and DAO activity(D) of germinated *S. bigelovii* under $7.5\text{g}\cdot\text{L}^{-1}$ NaCl stress. $\bar{x} \pm s$, * $p < 0.05$, compared with the control group

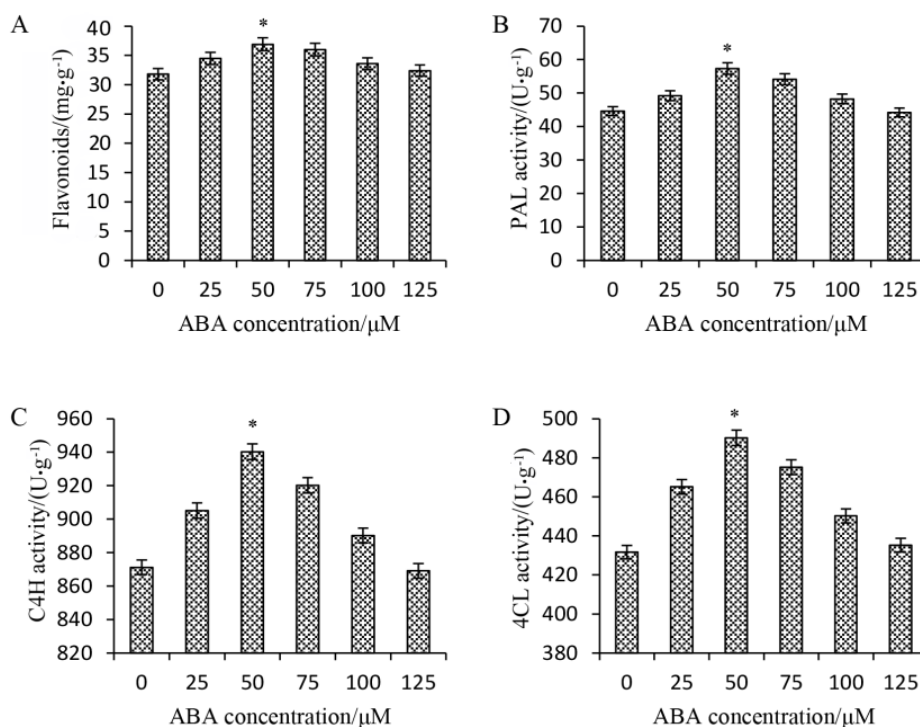


Figure 6. Effect of different ABA concentration on flavonoids content(A), PAL activity(B), C4H activity(C) and 4CL activity(D) of germinated *Salicornia* under $7.5\text{g}\cdot\text{L}^{-1}$ NaCl stress. $\bar{x} \pm s$, * $p < 0.05$, compared with the control group.

3.4. The Change of Sprout Length, GABA, Flavonoids Content and Key Enzymes Activity under Combined Stress of NaCl and ABA.

Figure 7 A showed that the growth rate of *S. bigelovii* treated with ABA, ABA combination with NaCl decreased compared with that treated with NaCl alone. However, there was no significant difference between ABA treatment and ABA combination with NaCl treatment. Both of them had an inhibitory effect on the growth of *S. bigelovii*. The enzymatic activities of GAD and DAO were increased by ABA treatment, ABA combination with NaCl treatment (Figure 7 C and D), GAD and DAO

enzyme activities treated with ABA were higher than NaCl treatment, but lower than ABA combination with NaCl treatment. The results showed that the enzyme activities of GAD and DAO were increased by the combined treatment of ABA and NaCl. After 48 h of treatment, the content of GABA was $398.26 \text{ mg}\cdot 100\text{g}^{-1}$ and reached the highest level, which was 1.08 times of that of ABA alone and 1.27 times of that of NaCl. When plants were exposed to environmental stress, plant defense systems will defend by regulating related metabolic substances, such as ABA. ABA was an important messenger of plant response to stress, and increase the accumulation of ABA will promote the content of GABA.

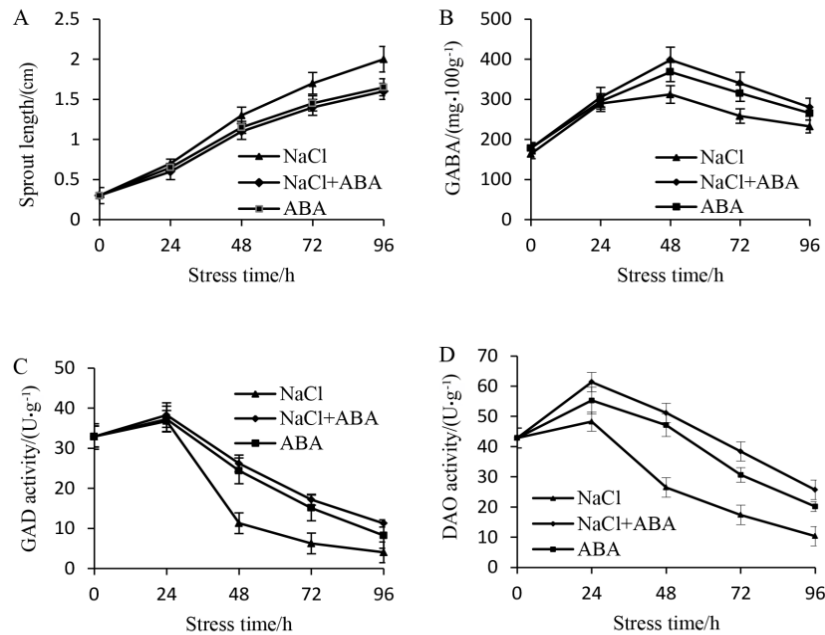


Figure 7. The effects of different processing time on sprout length (A), GABA content (B), GAD activity (C) and DAO activity (D) in germinated *S. bigelovii* under NaCl and ABA treatment

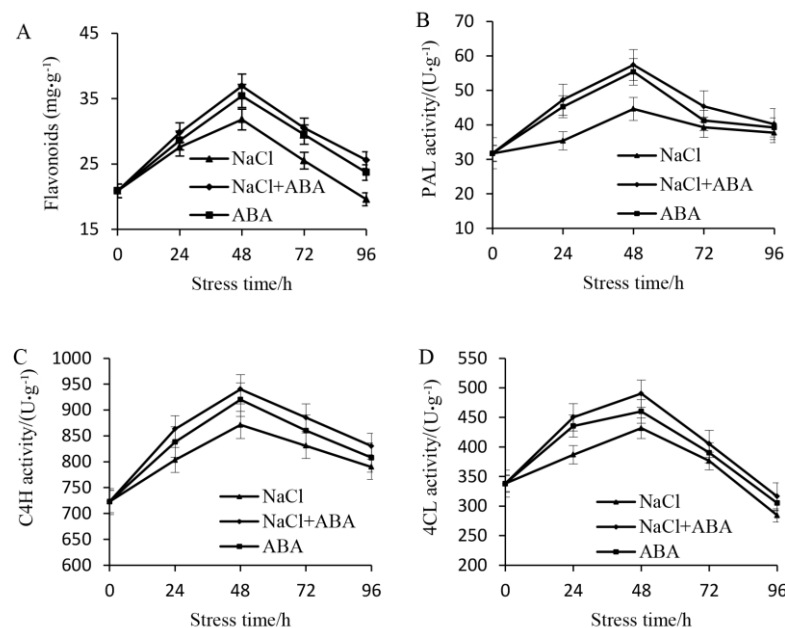


Figure 8. The effects of different processing time on flavones content (A), PAL activity (B), C4H activity (C) and 4CL activity (D) in germinated *S. bigelovii* under NaCl and ABA treatment

Figure 8 A, B, C and D showed that the flavonoids content and the activities of PAL, C4H and 4CL increased at first and then decreased with the delay of treatment time. The content of flavonoids and the activities of PAL, C4H and 4CL in the treatment of ABA combination with NaCl were slightly higher than those in the treatment of ABA, but much higher than those in the treatment of NaCl alone. After 48 h of treatment, the content of flavonoids in combination treatment of ABA and NaCl was $36.90 \text{ mg} \cdot \text{g}^{-1}$ and reached the highest level, which was 1.04 times of that of ABA treatment and 1.11 times of that of NaCl treatment alone. The results showed that the activities of PAL, C4H and 4CL were significantly increased by the combination of ABA and NaCl, and the accumulation of flavonoids was also promoted.

3.5. The Effects of CaCl_2 and ABA on the mRNA Expression Levels of the Key Enzymes

As shown in Figure 9, *S. bigelovii* were treated with 5 mM CaCl_2 under $7.5 \text{ g} \cdot \text{L}^{-1}$ NaCl, during the germination of *S. bigelovii*, the gene expression of GAD and DAO increased first and then decreased, the expression of GAD and DAO was the highest at 48 h, with their expression levels increasing by 3.03 ± 0.12 and 2.53 ± 0.20 fold ($p < 0.05$), respectively (Figure 9 A and B), which correlated with the change of GABA. In Figure 9 C, D and E, the gene expression of PAL, C4H and 4CL increased at first and then decreased, they reached the highest level also at the 48 h, with their expression levels increasing by 5.37 ± 0.43 ($p < 0.01$), 1.44 ± 0.27 ($p < 0.05$) and 5.43 ± 0.56 fold ($p < 0.01$), significantly higher than the control group respectively, which were correlated with the change of flavonoids. Our results provide insight into the molecular mechanism on the accumulation of GABA and

flavonoids in germinated *S. bigelovii*. As shown in Figure 10, *S. bigelovii* were treated with $50 \mu\text{M}$ ABA under $7.5 \text{ g} \cdot \text{L}^{-1}$ NaCl, the expression of GAD and DAO were the highest at 48 h, with their expression levels increasing by 2.94 ± 0.57 and 2.51 ± 0.31 fold ($p < 0.05$), respectively (Fig. 10 A and B), which correlated with the change of GABA. In Figure 10 C, D and E, the gene expression of PAL, C4H and 4CL reached the highest level also at the 48 h, with their expression levels increasing by 5.11 ± 0.52 ($p < 0.01$), 1.65 ± 0.49 ($p < 0.05$) and 5.74 ± 0.38 fold ($p < 0.01$), significantly higher than the control group respectively, which were correlated with the change of flavonoids.

Enriching active ingredients through seed germination has become an important means. During the germination process, endogenous enzymes are activated, especially under adverse conditions such as hypoxia, heat shock and salt stress [36]. Therefore, the method of enriching active ingredients through germination under adverse conditions has received much attention from scholars. In this study, we explored the factors that affect the GABA and flavonoids content during the germination of *S. bigelovii*. In fact, when the sprouting method was utilized to enrich the active ingredients, the buds tend to rot and reduce the accumulation of biomass. Obviously, we found that when the seeds are soaked in a low-concentration NaCl solution to germinate, the sprouts rot were reduced, whereas the accumulation of endogenous enzymes and active ingredients were inhibited in a certain extent. In contrast, the addition of CaCl_2 alleviated this phenomenon and promoted the increase of endogenous enzyme activity and the accumulation of active ingredients such as GABA and flavonoids. The results of this study are consistent with other reports and speculate that CaCl_2 can reduce the damage caused by NaCl to cells by changing the membrane structure [37].

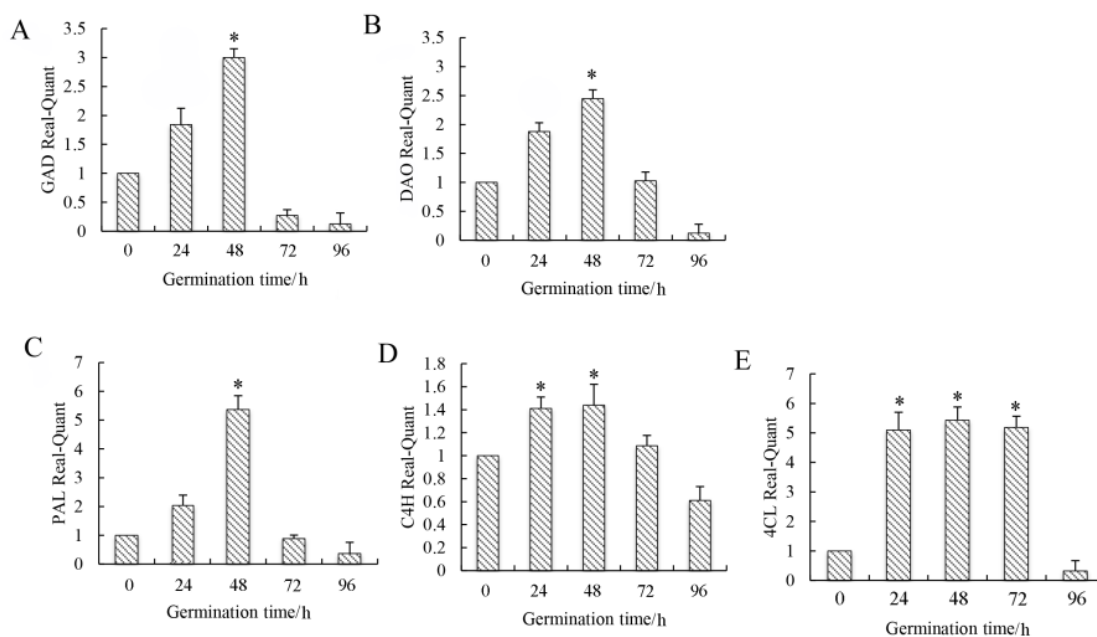


Figure 9. Effects of CaCl_2 on the mRNA expression levels of the key enzyme GAD (A), DAO (B), PAL (C), C4H (D) and 4CL (E). $\bar{x} \pm s$, * $p < 0.05$ and ** $p < 0.01$, compared with the control group.

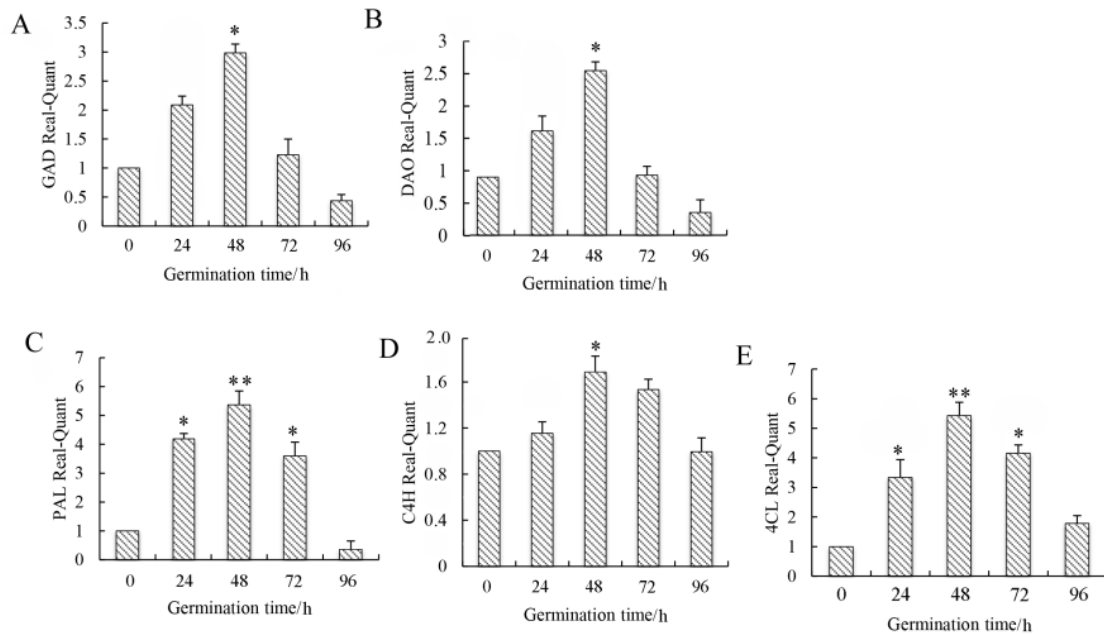


Figure 10. Effects of ABA on the mRNA expression levels of the key enzyme GAD (A), DAO (B), PAL (C), C4H (D) and 4CL (E). $\bar{x} \pm s$, * $p < 0.05$ and ** $p < 0.01$, compared with the control group

Studies have shown that the content of GABA in plants affect the content of ABA. As the plant stress hormone, ABA plays a critical role in a variety of signal transduction pathways, such as chemical signals in plant environmental stress. Herein, we found that adding 50 μ M ABA can significantly increase the content of GABA and flavonoids under NaCl stress. Under stress conditions, the accumulation of protein will accelerate the degradation rate due to the accumulation of ABA, which will increase the content of free amino acids and Glu in the cell, and the concentration of CaCl_2 will increase correspondingly. Consequently, the activity of GAD enhanced significantly, thereby promoting the synthesis of GABA [38]. Indeed, studies have shown that salt stress can promote the synthesis of ABA. The stimulation of the second messenger in the body increases the intracellular calcium ion concentration, which further promotes the accumulation of GABA and flavonoids. In this study, the factors affecting the accumulation of GABA and flavonoids were explored. The results showed that under NaCl stress, the addition of calcium ions and growth hormone ABA can promote the accumulation of GABA and flavonoids. The enrichment of active ingredients provides a reference and has important application value.

4. Conclusion

Collectively, the effects of CaCl_2 and ABA on the content of GABA and flavonoids were firstly analyzed in germinated *S. bigelovii*. The study indicated that CaCl_2 not only promote the sprout length, but also improve the content of GABA and flavonoids. However, the ABA has no significant effect on promotion of sprout length, it can improve the contents of GABA and flavonoids in germinated *S. bigelovii*. Meanwhile, the study showed that the expression of key enzyme GAD, DAO, PAL, C4H and 4CL were consistent with the change of GABA and

flavonoids content. In summary, our results provide insight into the molecular mechanism and optimal germination condition on the accumulation of GABA and flavonoids, it also promote the development of functional foods of in *S. bigelovii*.

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Conflict of Interest

The authors have declared no conflicts of interest for this article.

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