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Enhanced total flavonoid accumulation and alleviated growth inhibition of germinating soybeans by GABA under UV-B stress†

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Germination of soybeans under ultraviolet-B (UV-B) treatment is a simple and effective way to enrich soybean isoflavones, but its mechanism of action is not yet clear. G-Aminobutyric acid (GABA) is a signaling molecule that is involved in the accumulation of secondary metabolites as well as the regulation of plant development and metabolism. In this study, the effects of exogenous GABA and its inhibitors on the physiological and biochemical, antioxidant systems, total flavonoid content, activity and gene expression of isoflavone metabolism related enzyme in germinating soybeans under UV-B treatment were investigated. Compared to UV-B treatment alone, soybean treated with GABA (5 mM) in combination with UV-B significantly increased sprout length, fresh weight, Ca^{2+} inward flow and peroxidase and catalase activities, and decreased malondialdehyde and H_2O_2 and $O_2^{\bullet-}$ fluorescence intensity, while soybean treated with GABA inhibitor showed the opposite trend. Meanwhile, total flavonoid content increased by 11.2% and 6.7%, respectively, in 2- and 4 day-old soybeans under UV-B treatment, compared to UV-B treatment alone. Moreover, the application of GABA under UV treatment significantly increased the activity of phenylalanine ammonia-lyase and cinnamic acid-4-hydroxylase, with values increasing by 43.6% and 18.5%, respectively, in four-day-old soybean compared to UV treatment alone, which also increased the relative expression of key genes involved in isoflavone metabolism. The GABA inhibitor 3-mercaptopropionic acid blocked these occurrences. According to this research, GABA could operate as a signaling molecule to mediate isoflavone accumulation in soybean sprouts under UV radiation and stimulate soybean sprout growth.

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1. Introduction

Soybean (*Glycine max* L.) is a significant source of vegetable protein and one of the most important food and oil crops for the global food sector.[1,2] Isoflavones, the major phenolic molecule in soybeans, are a secondary metabolite that can prevent cardiovascular disease, menopausal syndrome, reduce osteoporosis and certain cancers,[3–5] making the development of isoflavone-rich meals popular all over the world. According to research, germination under abiotic stress is a simple and effective approach to enrich isoflavones as internal enzymes like protease and amylase are activated during the germination process,[6] leading to the breakdown of macromolecules and inducing the synthesis of life-giving substances such as isoflavones.[7] Simultaneously, ultraviolet-B (UV-B) radiation has been repeatedly shown to promote the production of secondary metabolites in various plants[8–12] such as soybean, barley and tomato, including some reports on soy isoflavones.[13–17] Soybean sprouts have been observed to accumulate large quantities of phenolic chemicals[18] when exposed to UV-B radiation.

G-Aminobutyric acid (GABA) is a four-carbon non-protein amino acid[19] that plays various physiological roles in plants and animals, including pH regulation, development and growth, carbon and nitrogen nutritional balance, and stress response.[20,21] In recent years, its dual physiological roles as a signaling molecule for secondary metabolite accumulation[22] and plant resistance to abiotic stresses[23] have been widely described. It has been demonstrated that GABA can be involved in abiotic stresses as a plant stress tolerance agent to scavenge free radicals produced in the plant and that GABA treatment increases the phenolic acid content of tomato plants under both stress and non-stress conditions.[24] Previously, research revealed that GABA is a key signaling molecule in the metabolism of phenolic buildup in sprouting soybean seedlings[22] and sprouting barley[25] under salt stress. GABA mediated the accumulation of phenolic compounds and strengthened the antioxidant

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system in sprouting barley under salt stress according to Ma *et al.*,[25] and similar conclusions were obtained by Xie *et al.*[26] Although GABA has been proven to be a signaling molecule for phenolic component enrichment in growing soybeans when exposed to salt, it is unknown if GABA has the same impact when exposed to UV-B radiation. It is therefore necessary to explore whether GABA can act as a signaling molecule for isoflavone enrichment in germinating soybean under UV-B radiation and its exact mechanism of action.

To address this issue, the effects of GABA and its inhibitor 3-mercaptopropionic acid (3-MP) on total flavonoid content, key enzyme activities, antioxidant enzyme activities and their gene expression in germinating soybean seedlings under UV-B radiation treatment were investigated in this study, thus exploring the effects of GABA on total flavonoid accumulation and growth metabolism in germinating soybean seedlings under UV-B radiation.

2. Materials and methods

2.1. Materials and chemicals

Soybean seeds (*Glycine max* L. cultivar Dongsheng No. 1) were provided by the Nanjing Agricultural University (Nanjing, China) in 2018, and stored at −20 °C until usage. GABA, 3-MP were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals and reagents used (analytical grade) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Seeds germination and experimental design

100 g soybean seeds were selected without damage and disinfected in 1% (v/v) sodium hypochlorite solution for 15 min. The disinfected seeds were washed with deionized water to neutral pH and then immersed for 6 h in distilled water at 30 °C. Soaked soybeans were divided into numerous germination trays, then placed in a soybean germinator and germinated for four days at 30 °C in a dark incubator. (1) CK: The soybeans were sprayed with deionized water; (2) GABA: the germination was conducted according to (1), and sprayed with 5 mM GABA; (3) 3-MP: the germination was conducted according to (1), and sprayed with 0.2 mM 3 MP; (4) UV-B: during germination, a 15 W UV-B light bulb was put 1 ft above the sprouter in the chamber. This was under 9/15 h light/dark photoperiod, and sprayed with distilled water; (5) UV-B + GABA: the germination was conducted according to (4), and sprayed with 5 mM GABA; (6) UV-B + 3 MP: the germination was conducted according to (4), and sprayed with 0.2 mM 3-MP. The (1)–(6) were marked as CK, G, M, UV, UG, UM respectively. A narrowband UV-B lamp tube with a 313 nm central wavelength (15 W, ZW20S19Y, Beijing Electronic Resource, Inc., China) was utilized for UV-B treatment. To prevent wavelengths less than 280 nm from passing through the UV-B tube, 0.13 mm thick cellulose diacetate filters were used. Every 12 h during germination, 40 mL of different culture solution was sprayed. Biochemical studies were performed on samples obtained at 2 and 4 days after germination (ESI Fig. S1†). The pre-experiment determined the optimal UV irradiation time and GABA concentration (ESI Fig. S2 and S3†).

2.3. Determination of sprout length, fresh weight, dry weight and malondialdehyde

Sprout length of 30 randomly selected samples were measured with vernier calipers, in mm. Fresh weight and dry weight were established by properly weighing 30 fresh soybean sprouts (fresh weight) and then baking them in an oven (100°C ± 5 °C, 1 MPa) to a constant weight before measuring (dry weight), constant weight being defined as a difference of no more than 2 mg between the first and second weighing. The content of malondialdehyde (MDA) was determined using the method of Zhuang *et al.*[27] 1.0 g of fresh sprouts were added to 5.0 mL 5% trichloroacetic acid and ground into homogenate and centrifuged for 8000*g* for 10 min. Add 2.0 mL 0.76% thiobarbituric acid solution to the supernatant and stir for 30 min in a boiling water bath. At last, the supernatant was taken and the absorbance values at 450 nm, 532 nm and 600 nm were measured.

2.4. Determination of total flavonoid content

Determination of the total flavonoid content using the method of Djeridane *et al.*[28] A standard curve was made with rutin. The diluted samples were mixed 1 : 1 with 2% ethanolic solution of aluminium chloride respectively. Measure the absorbance of the reaction mixture at OD_{430nm}.

2.5. Determination of intracellular free calcium, H_2O_2 and $O^{2\cdot-}$

The intracellular free calcium was measured using the method of Cheng *et al.*[29] The H_2O_2 and $O^{2\cdot-}$ fluorescence staining we referred to the method of Zhang *et al.*[30] Soybean root tips were washed with deionized water and cut to 3 mm length in 300 μL of HBSS staining solution (containing 0.02% Pluronic F-127, 10 μM Fluo-4AM, pH 7.2) and incubate in the dark at 4 °C for 2 h, then wash three times with hanks balanced salt buffer solution (HBSS) and incubate in the dark at 25 °C for 2 h. The incubated samples were placed on dry slides and observed using an LSM 880 NLO confocal laser scanning microscope (Leica Excitation Technology Co., Ltd., Germany).

2.6. Determination of antioxidant enzyme activity

Peroxidase (POD) and Catalase (CAT) activities were measured as described by Wang *et al.*[31] 1.0 g of fresh sprout were added to 5 mL of acetate buffer (pH 7.0, 50 mM) containing soluble polyvinyl chloride pyrrolidine (4%) and Triton X-100 (1%), ground on ice and homogenized at 4 °C. Centrifuge at 12 000*g* and centrifuge for 25 min. A unit of POD activity (U) is defined as a change of 0.01 per min in OD_{470nm}. 1.0 g of fresh sprouts were added to 5 mL of phosphate buffer solution (pH 7.0, 50 mM) including soluble polyvinyl pyrrolidine (1%) and ground on ice and homogenized at 4 °C. Centrifuge at 12 000*g* and centrifuge for 25 min. A unit of CAT activity (U) was defined as an increase of 0.01 per min in OD_{240nm}.

2.7. Determination of isoflavone metabolism-related enzyme activity

To determine phenylalanine ammonia-lyase (PAL) activities, we referred to the method by Assis *et al.*[32] 1.0 g of fresh sprouts were added to 5 mL of boric acid–borax buffer solution (pH 7.0, 50 mM) containing soluble polyvinyl pyrrolidine (0.5%) and mercaptoethanol (5 mM) and ground on ice and homogenized at 4 °C. Centrifuge at 12 000*g* and centrifuge for 25 min. The unit (U) of PAL activity was defined as 0.01 change in absorbance per gram of bean sprouts (fresh weight) enzymatic reaction system per hour at OD_{290nm}.

Cinnamic acid 4-hydroxylase (C4H) activities were measured according to Lamb and Rubery.[33] 1.0 g of fresh sprouts were added to 5 mL of Tris–HCl buffer (pH 8.9, 0.1 M) and ground on ice and homogenised by centrifugation at 12 000*g* for 25 min at 4 °C. The unit (U) of C4H activity was defined as 0.01 change in absorbance per gram of bean sprouts (fresh weight) enzymatic reaction system per hour at OD_{340nm}.

2.8. Ribonucleic acid extraction and quantitative real-time polymerase chain reaction analysis

Total Ribonucleic Acid (RNA) was isolated from frozen soybeans powdered with liquid nitrogen using the Plant RNA Extraction Kit (R6827-01, OMEGA, USA) according to the manufacturer's instructions. The PrimeScript™ RT Master Mix Kit (RR036A, Takara, Japan) was used to reverse transcribe RNA samples into cDNA. Three replicates of each cDNA were quantified using SYBRR Premix EX-TaqTM (RR420A, Takara, Japan) and the ABI 7500 Sequence Detection System, according to the manufacturer's procedure (Applied Biosystems, USA). Table S1† listed the oligonucleotide primer sequences utilized in this study for quantitative real-time polymerase chain reaction (qRT-PCR). The $2^{-\Delta\Delta Ct}$ method[34] was used to calculate relative gene expression levels.

2.9. Data processing and statistical analysis

Three biological and technical replicates were used in the experiment, and the data were expressed as mean ± standard deviation. DPS software was used to statistically analyze the test results, and Tukey's multiple comparisons with a significance test at the 0.05 level ($p < 0.05$) were used to compare average.

3. Results

3.1. Effects of GABA on physiological and biochemical indexes of soybean germination under UV-B treatment

Morphological plots of soybean under different treatments are shown in Fig. 1I. Compared to the control (CK) treatment, 4 day-old soybeans under UV-B treatment showed a significant decrease in fresh weight (Fig. 1III). Compared to the CK treatment, the application of GABA alone resulted in a significant increase in sprout length ($p < 0.05$), with 61.2% and 36.1% increase in sprout length for 2- and 4 day-old soybeans, respectively (Fig. 1II), while the addition of 3 MP resulted in a significant decrease in sprout length ($p < 0.05$); similarly, under UV-B radiation, the addition of GABA resulted in a significant increase in sprout length compared to the UV treatment alone ($p < 0.05$). The addition of GABA and 3 MP also had similar effects on the fresh and dry weights of germinating soybeans (Fig. 1III and IV). In addition, malondialdehyde (MDA) is one of the common indicators of oxidative stress and reflects the extent of membrane lipid peroxidation in plants, as shown in Fig. 1IV, the addition of GABA reduced the extent of membrane damage and significantly reduced the MDA content compared to UV-B treatment ($p < 0.05$), while the addition of 3-MP increased the MDA content. These results suggest that UV-B treatment has a stressful effect on soybean growth, and that exogenous GABA effectively promotes the growth of sprouts, while the inhibitor further inhibits the growth and development of soybean.

3.2. Effects of GABA on total flavonoid content in germinated soybeans under UV-B treatment

The effect of different treatments on the total flavonoids content of germinating soybeans was investigated. As shown in Fig. 2, the UV-B plus GABA treatment resulted in the highest total flavonoids content, with 1.81 and 1.46 times more total flavonoids in 2- and 4 day-old soybeans than in the CK group. Under non-radiation conditions, the addition of GABA alone increased the total flavonoid content of 2- and 4 day-old soybeans by 12.3% and 9.2%, respectively, compared to CK, whereas the addition of 3-MP decreased the total flavonoid content of 2- and 4 day-old soybeans by 4.9% and 9.9%, respectively, compared to CK. Similarly, under UV-B treatment, the addition of GABA increased the total flavonoid content of 2- and 4 day-old soybean by 11.2% and 6.7%, respectively, while the addition of 3-MP decreased the total flavonoid content of 2- and 4 day-old soybean by 7.9% and 9.3%, respectively, compared to UV-B treatment. We can thus conclude that GABA plays an important role in the accumulation of total flavonoids in the sprouts.

3.3. Effect of GABA on intracellular free calcium, H_2O_2 and $O_2^{\bullet-}$ fluorescence in soybean sprouts under UV-B treatment

In each treatment, the root tips of 4 day-old germinating soybeans were stained with free calcium, H_2O_2, and $O_2^{\bullet-}$ fluorescence as shown in Fig. 3. The more red, blue and green areas were stained in root tips, the more H_2O_2, $O_2^{\bullet-}$ and intracellular free calcium were present. Under UV-B radiation, compared with the UV-B treatment, the UV-B plus GABA treatment increased the fluorescence intensity of green (intracellular free calcium), but UV-B plus 3-MP treatment decreased it. A similar phenomenon was observed under non-radiation conditions. The strongest fluorescence intensity was observed under the GABA plus UV-B treatment (Fig. 3III). In this study, ROS were characterised by measuring the distribution of superoxide anion radicals and hydrogen peroxide in the root tips of soybean sprouts., UV-B treatment caused the accumulation of H_2O_2 and $O_2^{\bullet-}$ in the root tips of germinating soybeans, the addition of GABA alleviated this effect, while 3 MP treatment made the situation worse (Fig. 3I and II). These results showed that UV-B with GABA treatment lowered H_2O_2 content and $O_2^{\bullet-}$

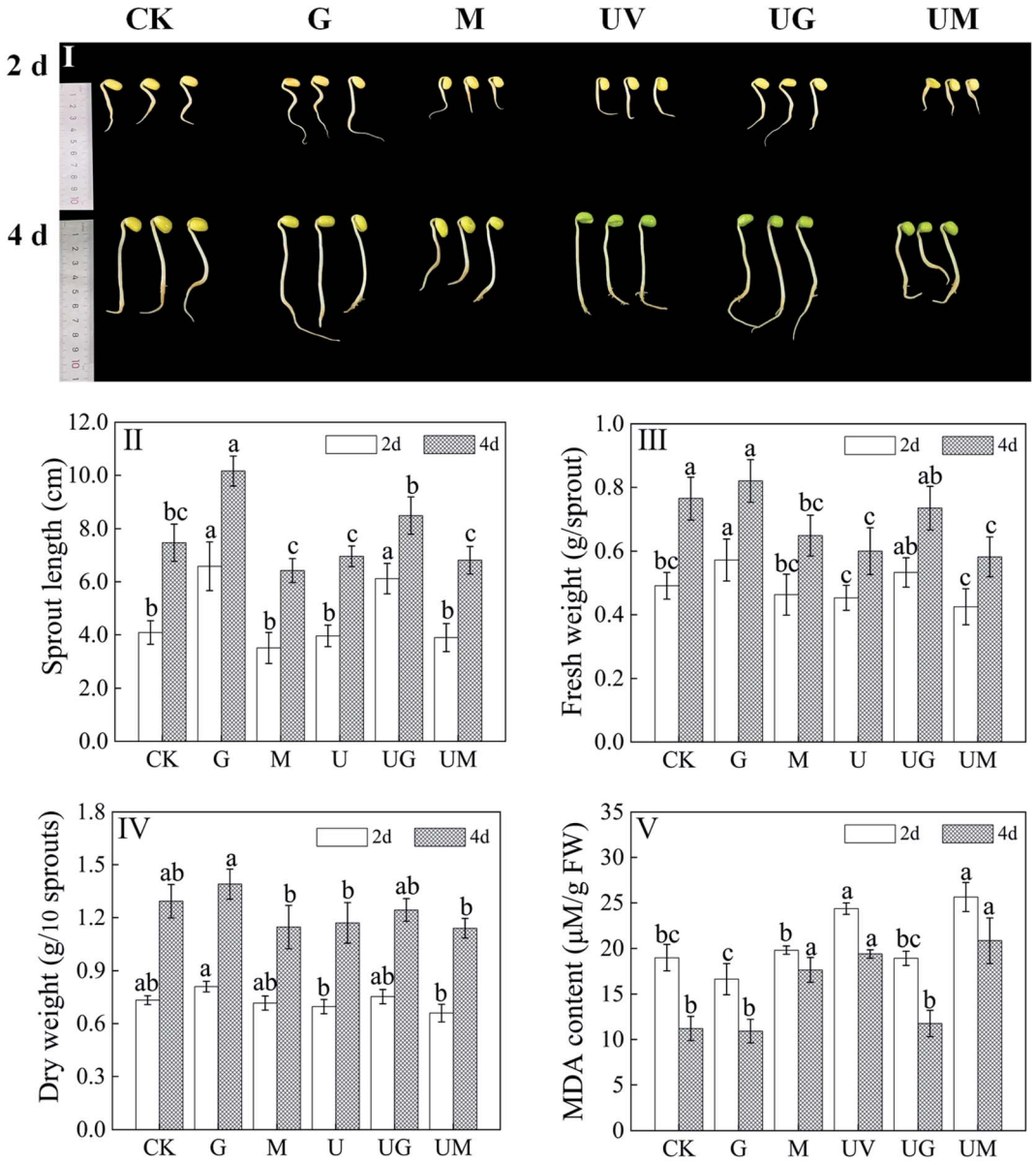

Fig. 1 The effects of GABA on growth state (I), sprout length (II), fresh weight (III), dry weight (IV), MDA content (V) in 2- and 4 day-old germinated soybeans under UV-B treatment. Each data point represents the average of three independent biological replications (mean ± SD). Different letters indicate a significance difference in indexes among treatments at the same germination time according to ANOVA and Tukey's test ($p < 0.05$). CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3 MP; UV: 9 h/d UV-B; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3 MP.

generation rate, as well as reduced damage to sprouting soybeans.

3.4. Effect of GABA on antioxidant enzyme in soybean sprouts under UV-B treatment

Measurement of antioxidant enzymes can respond to changes in metabolism in soybeans during a certain period of time. Compared to the CK, UV-B treatment significantly increased POD and CAT activity in soybean sprouts during germination (Fig. 4I and II). Meanwhile, compared to the UV-B treatment, the addition of GABA significantly increased POD activity ($p < 0.05$), but had no significant effect on CAT activity ($p > 0.05$). But the addition of 3-MP significantly reduced POD and CAT activities ($p < 0.05$). The POD activity of 2- and 4 day-old soybean sprouts treated with GABA plus UV-B treatment was 1.18 and 1.07 times higher than that of UV-B radiation-treated alone soybean sprouts, respectively (Fig. 4I).

3.5. Effect of GABA on isoflavone metabolism-related enzyme activity in soybean sprouts under UV-B treatment

PAL and 4CL are the rate-limiting enzymes for isoflavone biosynthesis. In soybean sprouts treated with UV-B radiation, the activity levels of isoflavone metabolism-related enzymes (PAL, C4H) were significantly higher than CK (Fig. 5). When

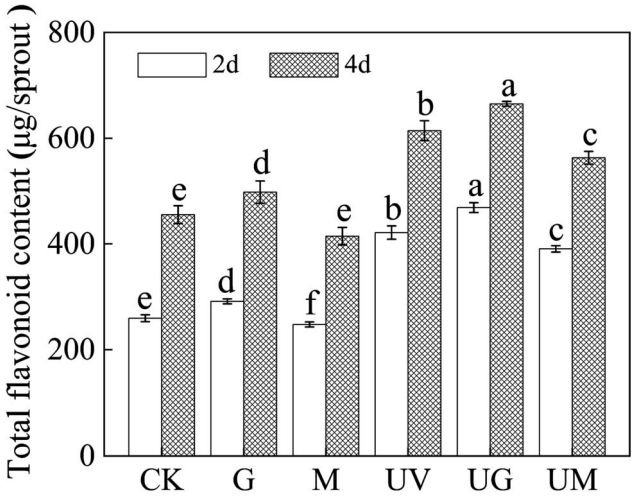

Fig. 2 Effects of GABA on contents of total flavonoid in 2- and 4 day-old germinated soybeans under UV-B treatment. Studies were performed on samples obtained at 2 and 4 days after germination. Each data point represents the average of three independent biological replications (mean ± SD). Different letters indicate a significance difference in indexes among treatments at the same germination time according to ANOVA and Tukey's test ($p < 0.05$). CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3 MP; UV: 9 h/d UV-B; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3 MP.

compared to the UV-B treatment, the use of GABA under UV-B radiation had a significant increase in PAL and C4H activity during germination. However, 3-MP could have the opposite effect (Fig. 5I and II). Meanwhile, under 3-MP treatment alone, PAL and C4H activity in germinated soybeans decreased significantly (Fig. 5I and II) compared with CK.

3.6. Effect of GABA on the relative expression of antioxidant enzyme genes in soybean sprouts under UV-B treatment

To evaluate the impact of GABA treatment on the antioxidant enzyme system, the antioxidant enzyme genes (POD, CAT, SOD and APX) were chosen (Fig. 6). Under UV-B radiation during germination, the relative expression levels of *POD*, *CAT*, *SOD*, and *APX* were considerably up-regulated compared to the control ($p < 0.05$). Meanwhile, after 2 and 4 days of UV-B radiation, the addition of GABA considerably boosted relative expression of the four genes, whereas the addition of 3-MP greatly decreased them (Fig. 6). In 4 day-old sprouts added with GABA and UV-B, relative expression of *POD*, *CAT*, *SOD*, and *APX* increased by 2.93, 3.83, 3.66, and 3.42 times, respectively, compared to CK. It should be observed that in 4 day-old sprouts treated with GABA, the relative expression levels of practically all identified genes were considerably higher ($p < 0.05$) than in CK.

3.7. Effect of GABA on the relative expression of isoflavone metabolism-related genes in soybean sprouts under UV-B treatment

The effects of GABA treatment on germinated soybean were further studied by isoflavone biosynthesis genes (*PAL1*, *4CL*, *IFR*, *CHR*, *CHS*, and *C4H*). The relative expression levels of *PAL1*, *4CL*, *IFR*, *CHR*, *CHS*, and *C4H* were significantly up-regulated under UV-B radiation compared to the control ($p < 0.05$) (Fig. 7). Meanwhile, following 2 and 4 days of UV-B exposure, GABA significantly increased relative expression of these genes, with the exception of *4CL* ($p < 0.05$). Except for *IFR* and *4CL*, 3 MP significantly reduced relative expression of these genes ($p < 0.05$). After 4 days of germination, GABA plus UV-B treatment increased the expression of *PAL*1, *IFR*, *CHR*, *CHS*, and *C4H* by

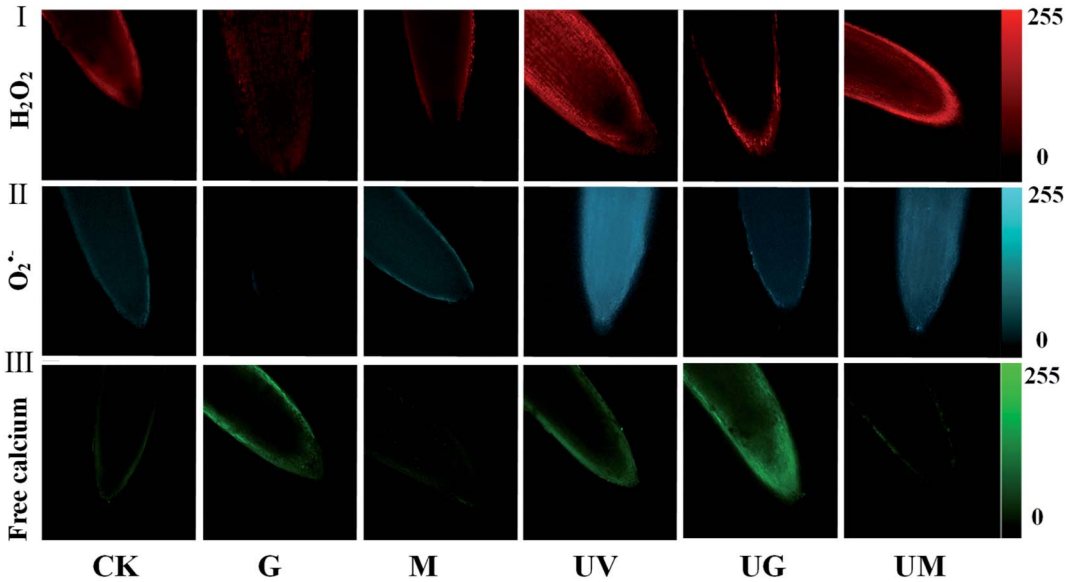

Fig. 3 The effect of GABA on germinating soybean root tip staining in 4 day-old germinated soybeans under UV-B treatment. The horizontal axis represents treatment, while the vertical axes represent intracellular free calcium (green), H_2O_2 (red), and $O_2^{\bullet-}$ (blue), with a scale length of 25 μm. CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3 MP; UV: 9 h/d UV-B; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3 MP.

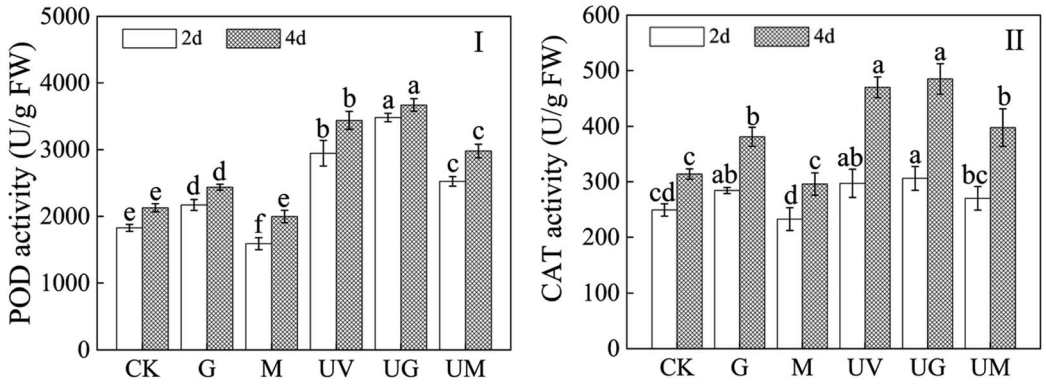

Fig. 4 Effects of GABA treatment on peroxidase (POD; I) and catalase (CAT; II) in 2- and 4 day-old germinated soybeans under UV-B treatment. Each data point represents the average of three independent biological replications (mean ± SD). Different letters indicate a significance difference in indexes among treatments at the same germination time according to ANOVA and Tukey's test ($p < 0.05$). CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3 MP; UV: 9 h/d UV-B; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3 MP.

1.15, 1.94, 1.86, 1.19, and 1.25 times, respectively, compared to UV-B treatment alone. UV-B treatment alone enhanced the relative expression of 4CL on day 2 of germination ($p < 0.05$), but GABA plus UV-B treatment decreased its gene expression. Furthermore, in 2- and 4 day-old soybeans, GABA treatment alone significantly elevated gene expression of *PAL*1, *4CL*, *CHS*, and *C4H* in non-radiation conditions ($p < 0.05$).

4. Discussion

As the functional food industry has grown worldwide in recent years, soy isoflavones have become increasingly popular because of their disease prevention and health promoting properties. Several studies have demonstrated that UV-B treatment is an effective method for enhancing soy isoflavones. The significant increase in total flavonoid content under UV-B treatment compared to the CK group (Fig. 2) confirms the feasibility of treating germinated soybeans with UV-B radiation for isoflavone production. However, UV-B radiation in this study significantly reduced the fresh weight of germinating soybeans, increased MDA content (Fig. 1) and increased H_2O_2 and $O_2^{\cdot-}$ fluorescence intensity (Fig. 3), these results suggest that UV-B radiation has an inhibiting effect on the growth and biomass of germinating soybeans although the total flavonoid content increased significantly. Therefore, ensuring the biomass of soybean sprouts while accumulating isoflavones during soybean germination is a critical issue to be addressed.

Recently, the role of GABA as a signaling molecule regulating the accumulation of phenolic substances in higher plants has been repeatedly demonstrated.[22,23,26] Furthermore, it has also been reported that GABA is a signaling molecule that regulates gene expression in plants under salt stress.[35] In the pre-experiment (Fig. S3†), it was found that low concentrations of GABA (5 mM) increased soybean sprout biomass and stimulated the synthesis of isoflavones, while high concentrations of GABA (>5 mM) had the opposite effect. In order to further increase the accumulation of isoflavones, it is essential to understand the mechanism of the effect of GABA on isoflavones synthesis under UV-B radiation, so this experiment added an inhibitor of GABA synthesis (3-MP) under UV-B radiation. It was found that the

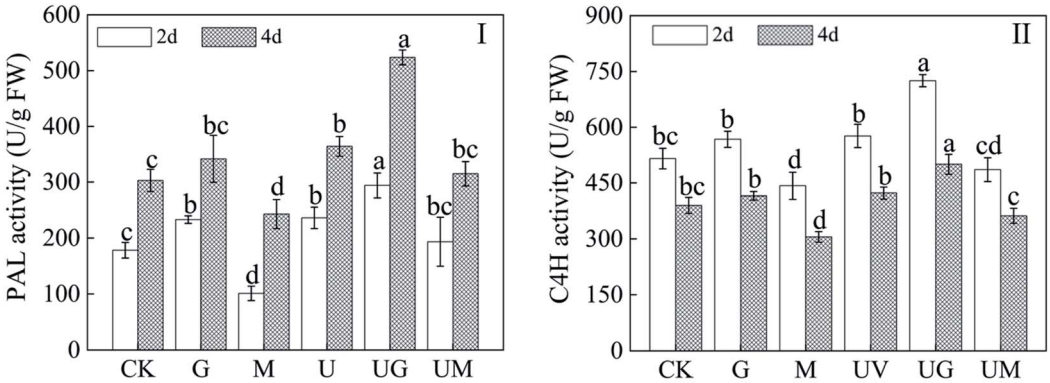

Fig. 5 Effects of GABA treatment on phenylalanine ammonia lyase (PAL; I), cinnamic acid 4-hydroxylase (C4H; II) in 2- and 4 day-old germinated soybeans under UV-B treatment. Each data point represents the average of three independent biological replications (mean ± SD). Different letters indicate a significance difference in indexes among treatments at the same germination time according to ANOVA and Tukey's test ($p < 0.05$). CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3 MP; UV: 9 h/d UV-B; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3 MP.

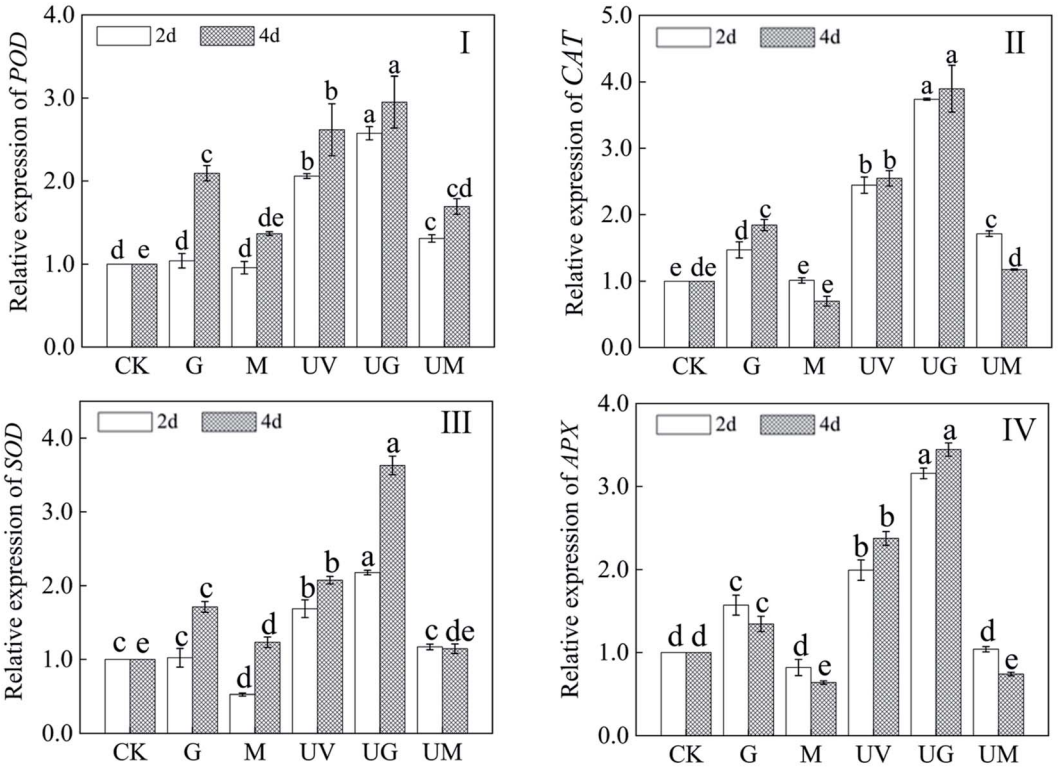

Fig. 6 The effects of GABA on gene expression of POD (I), CAT (II), SOD (III) and APX (IV) in 2- and 4 day-old germinated soybeans under UV-B treatment. The gene expression in germinated soybean under water treatment was used as the control. Each data point represents the average of three independent biological replications (mean ± SD). Different letters indicate a significance difference in indexes among treatments at the same germination time according to ANOVA and Tukey's test ($p < 0.05$). CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3 MP; UV: 9 h/d UV-B; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3 MP.

addition of GABA increased the antioxidant properties and total flavonoid content of soybean sprouts under UV-B radiation, while 3-MP treatment had the opposite effect. Therefore, we can deduce that GABA is engaged in the growth metabolism and production of isoflavones in soybean sprouts exposed to UV-B radiation. This study showed that under UV-B radiation, The application of GABA increased sprout length and biomass, decreased MDA content (Fig. 1) and reduced H_2O_2 and $O_2^{\bullet-}$ fluorescence intensity (Fig. 3) in germinating soybean. The addition of the GABA synthesis inhibitor 3-MP made things worse. These findings imply that GABA eased the inhibitory effect of UV-B radiation on sprouting soybean growth and biomass, which is consistent with the findings that exogenous GABA regulates the growth and development of germinating soybean under salt stress.[22] Moreover, GABA modulates the growth and development of barley shoot seedlings under stress, according to Ma *et al.*,[36] and they argued that GABA treatment in the root tip cells of barley seedlings alleviates stress by causing a substantial and persistent net Ca^{2+} influx into the root tip cells of barley seedlings, which is similar with the root tip free calcium results in this experiment (Fig. 3III).

In the present study, the intracellular free calcium and Ca^{2+} migration fluorescence intensity was the strongest in GABA plus UV-B treatment compared to CK and UV-B treatments (Fig. 3III). Ca^{2+}, as an essential nutrient, is not only involved in regulating physiological metabolic processes during plant growth and development, but it also acts as a second messenger in plant signal transduction, coupling extracellular signals with intracellular physiological responses, while it is also involved in the response of higher plants to abiotic stresses. Under abiotic stress, the antioxidant enzyme activities of various plants such as broccoli[37] and soybean[38] were significantly increased by exogenous calcium treatment, while the antioxidant system was weakened in calcium-deficient plants, which is consistent with the findings of this paper that the addition of exogenous GABA increased the content of endogenous Ca^{2+} and thus enhanced the antioxidant enzyme activities. In addition, several studies[39] have shown that the increase in Ca^{2+} level facilitates the accumulation of secondary metabolites in higher plants, which is consistent with the increased total flavonoid content in the present study. In future studies, we will measure changes in endogenous Ca^{2+} levels and distribution to further explore the association between GABA and Ca^{2+} and the possible role on antioxidant systems and total flavonoid accumulation.

This study also investigated the effect of GABA on the activity of some antioxidant enzymes (POD, CAT) and the expression of antioxidant enzyme genes (*POD*, *CAT*, *SOD*, *APX*). In agreement with Rezaei *et al.*,[40] exogenous GABA significantly increased the CAT activity of black cumin under water deficit stress and alleviated the stress to some extent. And interestingly, trends in

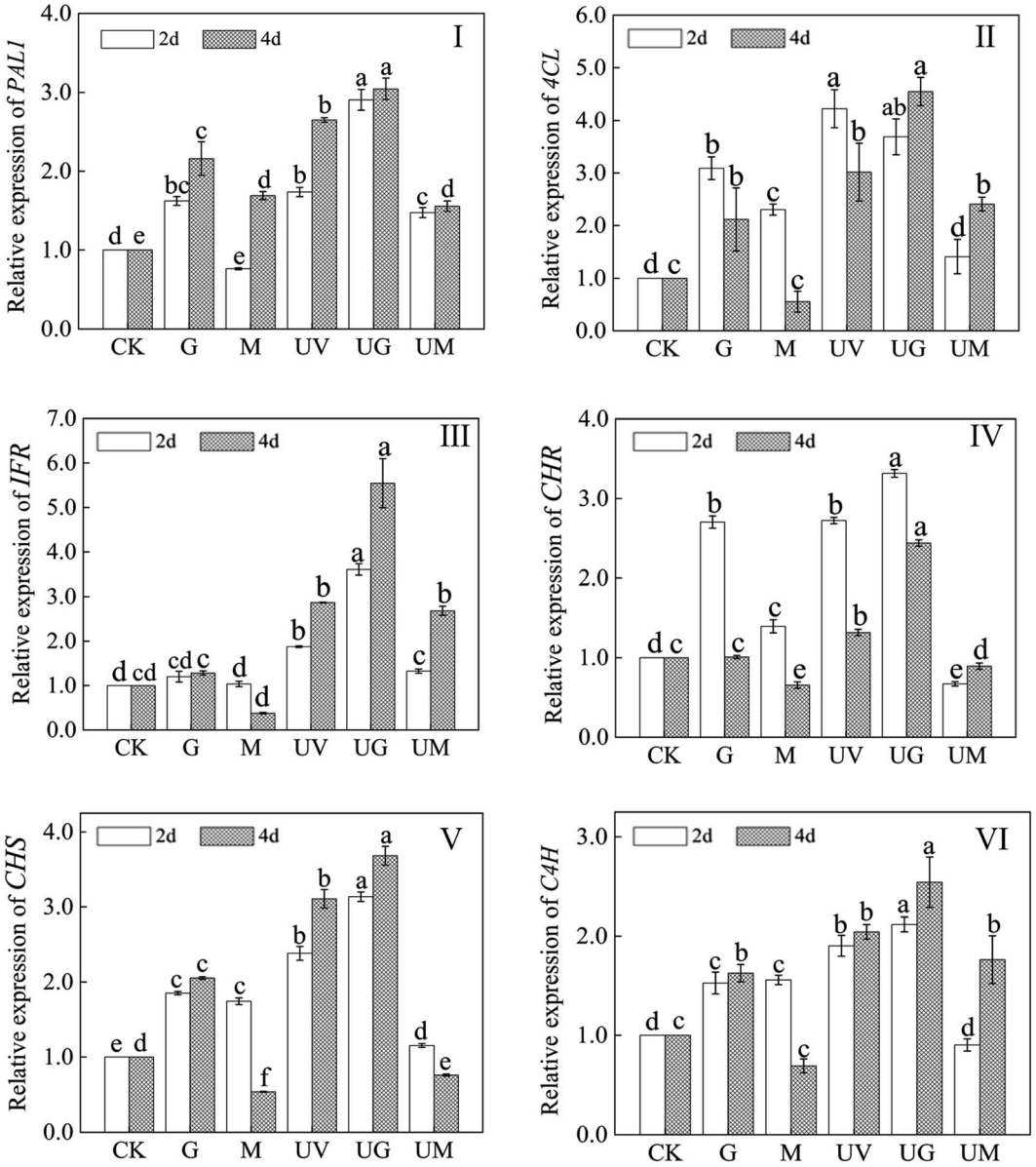

Fig. 7 The effects of GABA on gene expression of PAL1 (I), 4CL (II), IFR (III), CHR (IV), CHS (V) and C4H (VI) in 2- and 4-day-old germinated soybeans under UV-B treatment. The gene expression in germinated soybean under water treatment was used as the control. Each data point represents the average of three independent biological replications (mean ± SD). Different letters indicate a significance difference in indexes among treatments at the same germination time according to ANOVA and Tukey's test ($p < 0.05$). CK: deionized water; G: 5 mM GABA; M: 0.2 mM 3-MP; UV: 9 h/d UVB; UG: 9 h/d UV-B + 5 mM GABA; UM: 9 h/d UV-B + 0.2 mM 3-MP.

antioxidant enzyme activity and related gene expression were generally consistent with the total flavonoid content. As soybean sprouts acquire substantial amounts of phenolic compounds to protect themselves from oxidative damage produced by high levels of reactive oxygen species (ROS),[18] we can expect that the antioxidant capacity of UV-B treated soybean sprouts is positively linked with total flavonoid content.

PAL and C4H are two enzymes required for the metabolic core reactions of isoflavone biosynthesis.[41] The activities of PAL and C4H in soybean sprouts under UV-B radiation were significantly increased by GABA treatment, whereas 3-MP markedly inhibited their activities, suggesting that GABA participates in the regulation of key enzyme activities for isoflavones in soybean sprouts under UV-B radiation. Furthermore, in terms of isoflavone metabolism-related genes' relative expression, the relative expression of *PAL1*, *4CL*, *IFR*, *CHR*, *CHS* and *C4H* involved in the flavonoid biosynthesis pathway were significantly up-regulated during seed germination under UV-B radiation compared to the CK, which is consistent with previous findings. Wei[42] suggested that UV-B radiation boosted the formation of *CHS*, *F4H*, *FLS* in the phenolic acid, flavonoid and anthocyanin biosynthetic pathways. The formation of transcripts for enzymes such as CHS, F4H and FLS in the phenolic acid, flavonoid and anthocyanin biochemical activities were

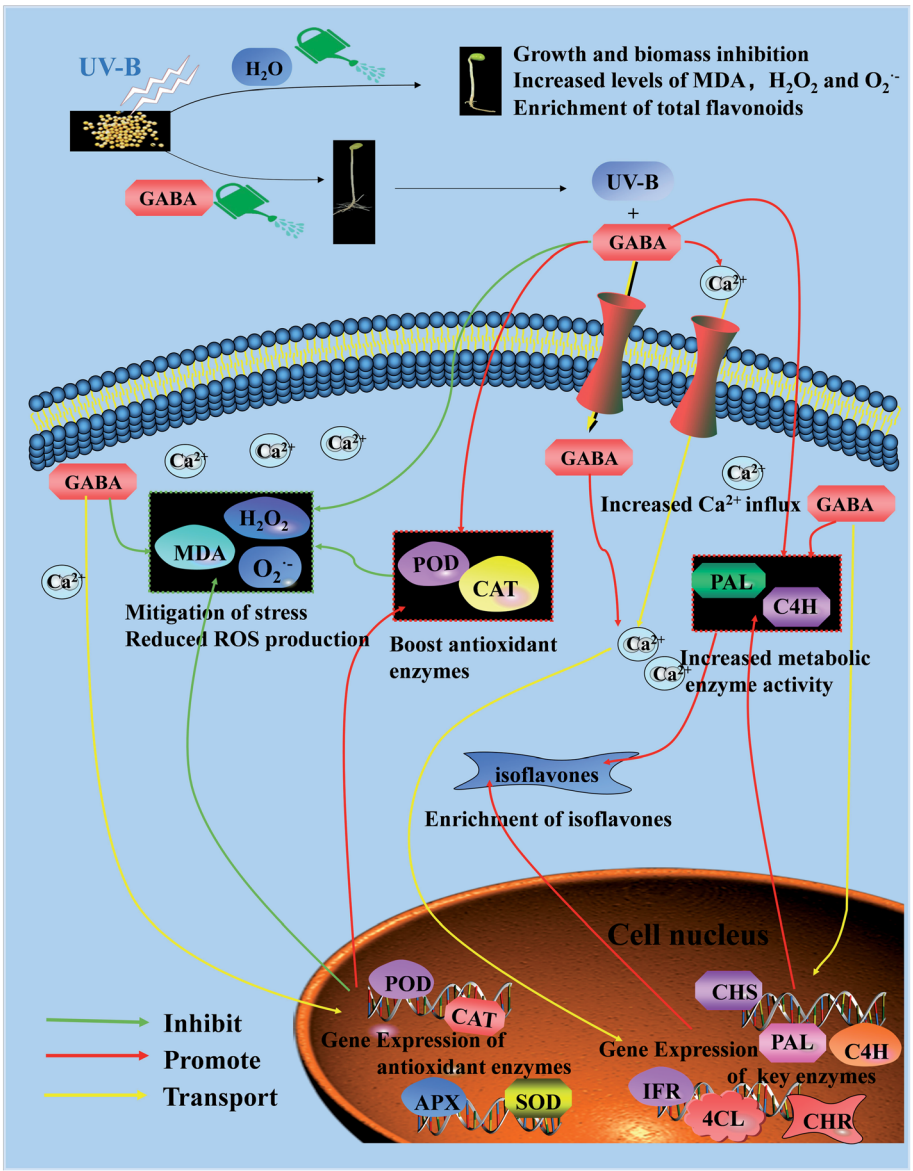

Fig. 8 A putative molecular mechanism for the stimulation of germinating soybean isoflavone biosynthesis by GABA signaling molecules under UV-B treatment. MDA: malondialdehyde; H_2O_2: hydrogen peroxide; $O_2^{\cdot-}$ superoxide radical; GABA: γ-aminobutyric acid; CAT: catalase; POD: peroxidase; SOD: superoxide dismutase; APX: ascorbate peroxidase; 4CL: 4-coumarate coenzyme A ligase; PAL. Phenylalanine ammonia lyase; C4H: cinnamic acid 4-hydroxylase; PAL1: phenylalanine ammonia lyase 1; IFR: isoflavone reductase gene; CHS: chalcone synthase gene; CHR: chalcone reductase gene.

stimulated by UV-B radiation. The relative expression of *PAL*1, *4CL*, *CHS* and *C4H* was significantly up-regulated by the addition of GABA compared to the CK, while the addition of GABA inhibitors had the opposite effect. In this study, GABA plus UV-B treatment significantly induced a net inward flow of Ca^{2+} in soybean root cells (Fig. 3), resulting in a significant increase in *CHS* expression. This is consistent with the study that increased Ca^{2+} in Arabidopsis cell culture under UV-B treatment increased *CHS* expression.[43] Meanwhile, the addition of GABA under UV-B treatment resulted in the highest relative expression of all genes, and the relative expression levels of these genes were consistent with the total flavonoid content. These results suggest that GABA promotes isoflavone biosynthesis by up-regulating the expression of these genes, which is consistent with previous findings.

As a result, we can identify GABA as the signaling molecule that mediates the synthesis of isoflavone in the presence of UV-B. Exogenous GABA enhanced endogenous GABA signaling,[44] which stimulated the activity of key enzymes engaged in isoflavone synthesis in soybean sprouts (Fig. 5) and promoted isoflavone synthesis. GABA was signaling positively regulated the synthesis of isoflavone in soybean sprouts during UV-B exposure. GABA metabolism is required for isoflavone production in soybean sprouts, and exogenous GABA increases isoflavone levels by increasing the activity and gene expression of critical phenylpropane pathway enzymes.[45,46] Based on these

results, we obtained a schematic diagram of the molecular mechanism of a hypothetical GABA as a signaling molecule to stimulate isoflavone synthesis in soybean during germination under UV-B radiation (Fig. 8).

5. Conclusion

This study show that soybean germinating under GABA combined with UV-B treatment would be an effective way to accumulate total flavonoids. The application of exogenous GABA increased the total flavonoids content in 2- and 4 day-old soybean sprouts under UV-B stress, with 80.7% and 46.1% increase compared to CK treatment, respectively.

GABA (5 mM) could further increase the growth of soybean sprouts under UV-B radiation by increasing the activity of antioxidant enzymes, minimizing the formation of reactive oxygen species and maintaining membrane integrity during soybean germination. Meanwhile, the application of exogenous GABA under UV-B stress induced total flavonoids accumulation by increasing activity and relative gene expressions of the flavonoid biosynthesis-relate enzymes. These findings add to our new knowledge of how isoflavones accumulate in soybean sprouts when exposed to UV-B radiation and will allow us to better understand the processes of isoflavones accumulation in soybean seedlings under abiotic stress, paving the way for further studies on their mechanisms of action. In addition, this study will also provide technical support for the industrial production of isoflavone-rich soybean sprouts.

Author contributions

Minglang Gu, Xin Tian and Yongqi Yin are responsible for experimental related work, writing – original manuscript preparation, and writing – review and editing preparation. Jia Yang, Jinpeng Xu and Weiming Fang are responsible for methodological investigation and analysis of results.

Conflicts of interest

There are no conflicts of interest to declare.

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