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Original article

Accumulating mechanism of γ-aminobutyric acid in soybean (*Glycine max* L.) during germination

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Summary γ-aminobutyric acid (GABA) has been found accumulating significantly in soybean seeds during germination. However, the mechanism of the accumulating process is not clear. Therefore, gene expression, enzyme activity and metabolites associated with GABA shunt in ZH 13 soybean during germination were analysed in this paper. GABA content in 5-day germinated soybean was 0.26 ± 0.016 mg g^{-1} DW, which was equivalent to six times concentration of original soybean. The GAD activity has a positive effect on the accumulation of GABA, as well as the GABA-T activity was found to play a significant role in the degradation of GABA. The expression levels of GmGAD and GmGABA-T may affect the GABA content by regulating the respective enzyme activities. In conclusion, upregulation of GAD and downregulation of GABA-T may cause the accumulation of GABA.

Keywords Germination, glutamate decarboxylase, mechanism, soybean, succinic semialdehyde dehydrogenase, γ-aminobutyric acid, γ-aminobutyric acid transaminase.

Introduction

Soybean [*Glycine max* (L.) Merill] is one of the most important crops in the world and contains rich nutrients (Costa *et al.*, 2017). For the beneficial effects on health, people paid more attention to soybean products in recent years (Messina, 2014). Sprouted soybean is a traditional and popular soybean product in Orient. Significant changes of the biochemical, nutritional and sensory characteristics have occurred in cereals during germination (Sharma *et al.*, 2016; Wang *et al.*, 2017). It is reported that the content of γ-aminobutyric acid (GABA) increases significantly during germination in soybean, suggesting that germinated soybean could be a good source of GABA (Quinhone & Ida, 2015; Wang *et al.*, 2015a; Huang *et al.*, 2017).

γ-Aminobutyric acid (GABA), a type of nonprotein amino acid that is widely distributed in most prokaryotic and eukaryotic organisms, is an inhibitory neurotransmitter in the mammalian central nervous system (François *et al.*, 2017). Food rich in GABA has various biological activities such as regulating blood pressure, improving cerebral function and ameliorating type-II diabetes (Abdou *et al.*, 2006; Yoshimura *et al.*, 2009; Imam *et al.*, 2012). For these reasons, GABA became one of the most valuable components in food.

Plant tissues contain GABA ranging from 0.03 to 2.00 μmol g^{-1}, and the content increased under different stimuli such as hypoxia, hydraulic pressure, salt stress, temperature shocking, germination and other biotic stress (Molina-Rueda *et al.*, 2010; Shelp *et al.*, 2012; Yang *et al.*, 2015). In leguminous plants, GABA is mainly metabolized via a short pathway called GABA shunt, whereby glutamate is converted to succinate. In the pathway, GABA is synthesised from glutamate via glutamate decarboxylase (GAD, EC 4.1.1.15), after that GABA, in turn, is converted to succinic semialdehyde (SSA) by GABA transaminase (GABA-T, EC 2.6.1.19), and then the last step of the shunt pathway is the conversion of SSA to succinate via succinic semialdehyde dehydrogenase (SSADH, EC 1.2.2.16) (Shelp *et al.*, 2012).

Numerous research findings focus on the mechanism of GABA accumulation in other species. For example, the accumulation mechanism of GABA in tomato and tea during storage period has been researched particularly. However, the study of this mechanism during germination in soybean merely focuses on the enzyme activity level and the particular research has not been reported. Whether the accumulating mechanism of GABA in different species under various treatments similar was not clear. The previous study in our

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laboratory has confirmed that GABA content increased significantly in soybean after germination, and ZH 13 soybean was screened out as the best cultivar for the GABA accumulation (Wang *et al*., 2015a). In this study, we aim to clarify the physiological mechanism of the accumulation process. The changes in concentration of metabolites and enzymatic activities associated with the GABA shunt, as well as the mRNA for GmGAD, GmGABA-T and GmSSADH were investigated. The relationship between the measured data, germination time and GABA content was then analysed to clarify the mechanism of GABA accumulation during germination. This study aims to conduct a particular investigation of the accumulation mechanism of GABA in soybean during germination and establishing scientific base for the future research.

Materials and methods

Materials

The soybean cultivar ZH 13 was provided by the Institute of Crop Sciences of Chinese Academy of Agricultural Sciences (ICS, CAAS), Beijing, China. All seeds were harvested in 2015 and immediately stored at −20 °C with ≤50% humidity in the refrigerator.

Seed germination

The germination condition consulted the method optimised in our laboratory before (Wang *et al*., 2015a). Sprouting samples, including the cotyledons and shoots, were collected every 24 h until day 7. The day 0 samples were untreated soybeans. The samples for mRNA expression levels and the enzyme activity test were stored at −80 °C. The samples for determining the concentration of GABA and metabolites were freeze-dried using a vacuum freeze-dryer (Four-ring Science Instrument Plant Beijing Co. Ltd, Beijing, China), ground into powder by high-speed multifunctional grinder (model SL-100, Zhejiang, China), and passed through a sieve of 60 mesh and then stored at −20 °C.

Sample extraction

Samples were extracted by the method reported previously (Phommalth *et al*., 2008). The extraction was filtered through a 0.22 μm PTFE Millex LCR syringe filter (Thermo Fisher Scientific, Waltham, MA, USA), and stored in the dark at 4 °C for the determination of GABA and metabolites.

Determination of γ-aminobutyric acid

A reported method (Khuhawar & Rajper, 2003) was used for the derivatization of samples and GABA standard solutions with concentrations ranging from 0 to 500 μg mL^{-1}. GABA analysis was carried out using a Waters UPLC system with a PAD detector (Acquity™, Waters, Milford, MA, USA) as reported (Wang *et al*., 2015a).

Enzyme extraction and assays of GABA shunt-related enzymes

PBS buffer was used to extract total protein. GAD, GABA-T and SSADH activities were measured using an ELISA kit (TSZ®, USA TSZ biological Trade Co., Ltd, San Francisco, CA, USA). The experimental procedure was carried out according to the introduction attached.

Expression analysis of GABA shunt enzyme genes by Quantitative PCR

The gene sequences of GAD, GABA-T and SSADH were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). Primers were designed using Primer 5 software. All the primers and gene IDs are listed in Table 1. Total RNA from soybean sprouts was extracted using the SGTriEx kit (SinoGene, Beijing, China), and an aliquot of total RNA (1 μg) was reverse-transcribed into cDNA using the Thermo First cDNA Synthesis Kit (SinoGene), according to the manufacturer's instructions. Quantitative real-time PCR was performed by the SYBR Green detection method using the StepOne Plus Rt-PCR system (Applied Biosystems, Carlsbad, CA, USA). Melting curve analysis was conducted after each PCR run to confirm the absence of primer dimers and the specificity of the SYBR Green dye. The comparative ΔΔCT method was used to quantify relative RNA expression levels (Livak & Schmittgen, 2001).

Determination of glutamic acid and succinic acid

Glutamic acid was determined by AB Sciex QTRAP® 5500 LC/MS/MS (AB, USA Applied Biosystems Co.,

Table 1 Primers sequences and gene ID

Gene Name	Gene ID	Primer ID	Sequence (5′-3′)	Size (bp)
SSADH	100808318	Q1870	ctgtaggggttgtaggtgca	100
	100793318	Q1871	atgaccactgtacagccaca	
GAD	547724	Q1872	gtttgctgggagaaatttgc	136
	100796533	Q1873	aggatagcagcaacacaaat	
GABA-T	100815181	Q1874	ggcggtatcatcttccaggt	98
	100792355	Q1875	gtctctggtccctctttca	
Actin	Actin	Q1876	tggaatggtgaaggcaggat	103
		Q1877	gcccataccaaccatcacac	

Ltd) as reported (Zhang *et al.*, 2016). Determination of succinic acid was carried out by a DIONEX ICS-3000 ion exchange chromatography system according to a reported method (Xiang *et al.*, 2015).

Statistical analysis

SPSS Statistics 17.0 was used for data analysis. Results were analysed by one-way analysis of variance (ANOVA), and LSD test was used for variation analysis. Data were presented as mean ± standard deviation (SD) for triplicate analyses. *P*-value less than 0.05 was considered as significant difference.

Results

Changes in GABA content

The changes in GABA concentration during germination are shown in Fig. 1. The GABA content initially increased sharply and began to decrease after reaching the peak value on day 5. GABA content was 0.26 ± 0.016 mg g^{-1} DW at peak, which was a sixfold increase compared to the original soybean (day 0).

Changes in enzymatic activities associated with the GABA shunt

Changes in enzymatic activities associated with the GABA shunt over germination time are presented in Fig. 2. GAD activity increased and reached its peak value on day 5 as 1.97 ± 0.05 U g^{-1} and then decreased during the germination process (Fig. 2a). Changes in GABA-T activity are shown in Fig. 2b. GABA-T activity was 0.59 ± 0.01 U g^{-1} initially and increased slightly until day 5. The GABA-T activity after 5-day germination has a drastic increase and

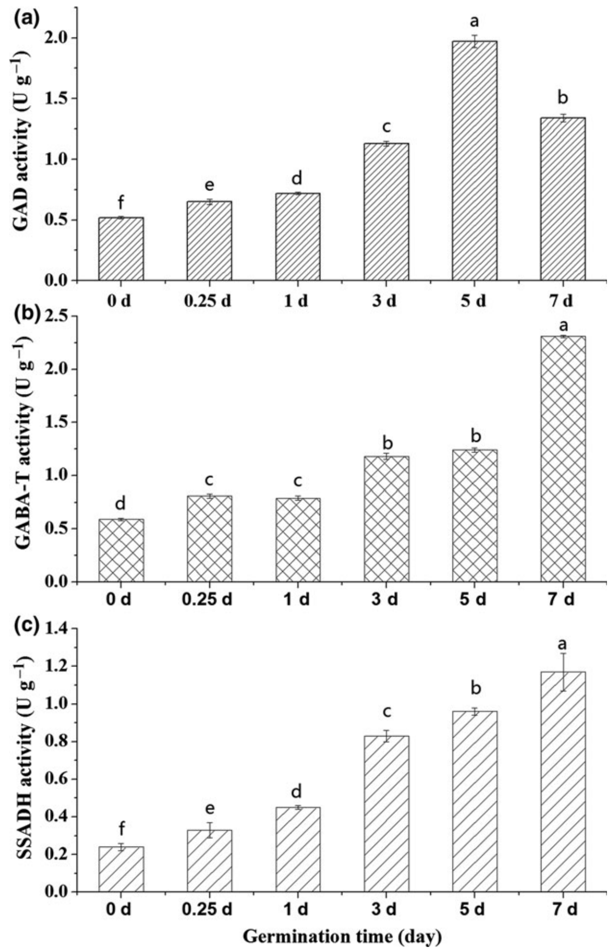

Figure 2 Changes in enzymatic activity associated with the GABA shunt (mean ± SD, $n = 3$). Bars with different letters are significantly different ($P < 0.05$).

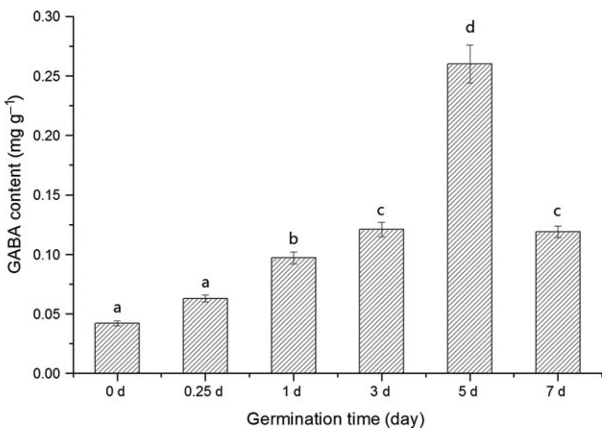

Figure 1 Changes in GABA content during germination (mean ± SD, $n = 3$). Bars with different letters are significantly different ($P < 0.05$).

reached 2.39 ± 0.01 U g^{-1} at day 7. SSADH activity increased from 0.24 ± 0.02 to 1.17 ± 0.1 U g^{-1} placidly during the entire germination process (Fig. 2c).

Changes in expression level of genes for GAD, GABA-T and SSADH

The expression level of genes for GAD, GABA-T and SSADH is shown in Fig. 3. The relative transcript level of the GAD genes increased initially and started to decrease after day 5 during germination (Fig. 3a). The relative transcript level of GABA-T was higher at first and decreased alone with germination. After 3-day germination, the relative transcript level of GABA-T was much lower than beginning (Fig. 3b). The relative transcript level of SSADH increased initially and started to decrease after day 1 during germination (Fig. 3c).

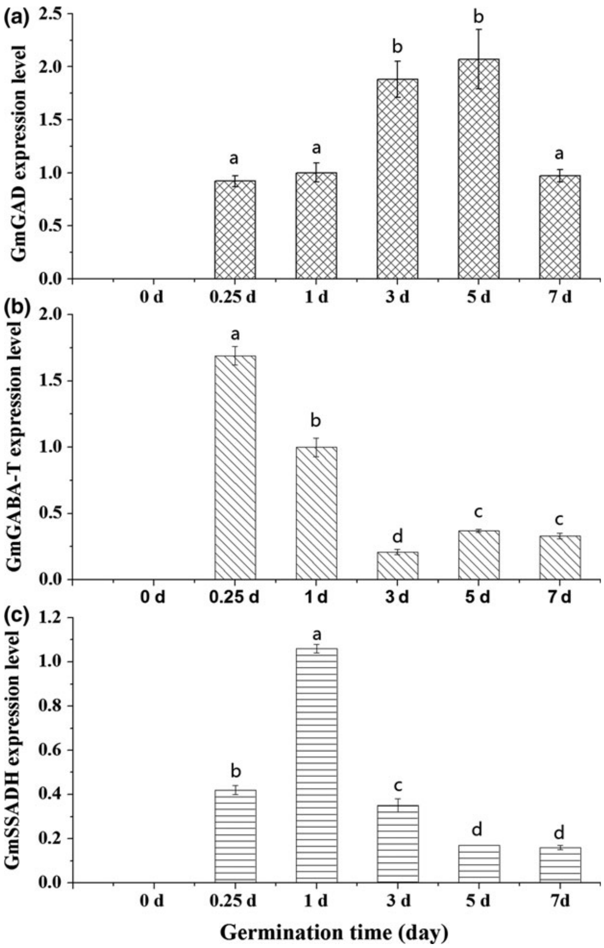

Figure 3 Changes in the expression level of mRNA for GAD, GABA-T and SSADH (mean ± SD, n = 3). Bars with different letters are significantly different (P < 0.05).

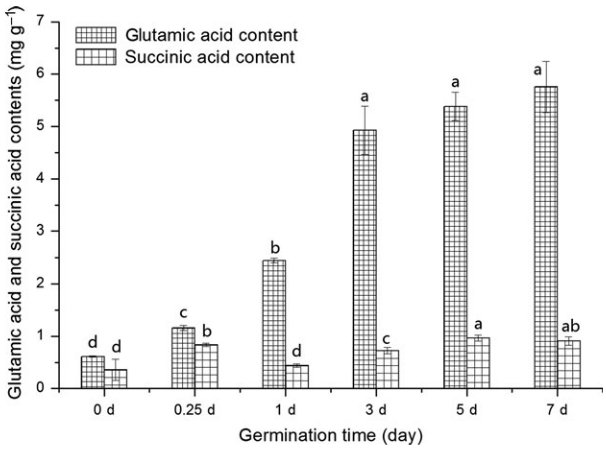

Figure 4 Changes in contents of glutamic acid and succinic acid (mean ± SD, n = 3). Bars with different letters are significantly different (P < 0.05).

The changes in contents of glutamic acid and succinic acid

Changes in contents of glutamic acid and succinic acid are shown in Fig. 4. The content of free glutamic acid increased during germination and leveled off after 3 days, and its content in 7-day germinated soybean was 9.4-fold higher than the dormant seeds. The content of succinic acid did not change appreciably during germination. The content of 7-day germinated soybean was a little higher than the original seeds.

Discussion

The accumulation of GABA during the germination has been observed in various plants, such as brown rice, wheat and mung bean (Bai *et al.*, 2009). During germination, soybean respiration is enhanced, endogenous enzyme systems are activated and proteins are hydrolysed into amino acids,which lead to the accumulation of GABA. After 5-day germination, the enzymes and genes which connected with the degradation of GABA may be activated, that caused a decrease in content of GABA. It is proposed that the activation of enzymes related to the GABA shunt and the upregulation or downregulation in mRNA levels of these enzyme genes in different germination period may play an important role in accumulation of GABA in soybean. Results in this paper are consistent with a previous study which showed GABA content increasing from 3.89- to 6.97- fold by the end of germination (Yang *et al.*, 2013; Xu & Hu, 2014), and also similar to research completed by our team, in which GABA content in ZH 13 was found to reach maximum level on day 5 (Wang *et al.*, 2015a).

Results indicated that GAD activity may have a positive effect on GABA content, which is consistent with previous report (Matsuyama *et al.*, 2009; Hyun *et al.*, 2013; Yang *et al.*, 2015). In the early stage of germination, the increase in GABA-T activity was too slight to offset the increase in GAD activity. Therefore, the content of GABA kept increasing day by day. Decrease in GABA level after 5-day germination may attribute to the drastic increase in GABA-T activity. This indicated that the GABA-T may make an immense contribution in the declining stage of GABA. The content of succinic acid semialdehyde may increase due to the enhancement of GABA-T activity, which directly stimulated the increase in the SSADH activity.

The GABA content has a dramatic accumulation in the postharvest storage period in tomatoes, and GAD and GABA-T may play crucial roles in the accumulation process (Akihiro *et al.*, 2008; Mae *et al.*, 2012;

Mei *et al*., 2016). This conclusion is similar to ours, which suggested that the GAD activity has a positive effect on GABA content, and GABA-T has made an immense contribution in the declining stage of GABA content. Our paper has presented much more detailed contribution of GAD and GABA-T to the GABA accumulation compared to other reports. Although germination and storage are two different processes, the GABA metabolic pathway in plants is similar. Therefore, the mechanism of GABA accumulation may be alike during germination and storage.

To some extent, the enzyme activity depends on the expression level of genes. The increasing relative transcript level of the GAD genes induces the enhancement of the GAD activity. The changes in GmGABA-T genes expression level were different from the GABA-T activity, which may due to the time lag from the GmGABA-T genes expression to the expression of GABA-T activity. We have a conclusion that the GmGAD and GmGABA-T genes expression level may affect the GABA content by determining the respective enzyme activities. Few studies have focused on the role of GmGABA-T genes in GABA accumulation in soybean germination. Our results provided evidence to support a previous study which reported that GABA-T genes may be the essential isoform for GABA accumulation, and suppression of GABA-T induces prominent GABA accumulation in tomatoes (Koike *et al*., 2013). On the contrary, some studies have reported that the mRNA levels of GABA-T genes did not affect GABA content (Akihiro *et al*., 2008; Mae *et al*., 2012). This result is different from ours because the inner link between genes expression and enzyme activity was not be explored.

The proteins were hydrolysed to free amino acids to support the respiration and synthesis of new cell constituents (Cho *et al*., 2009; Wang *et al*., 2015b), increasing free glutamic acid, which is the substrate of GABA synthesis. It is well known that the increasing glutamic acid content could stimulate the GAD activity, followed by the increase in GABA content (Mae *et al*., 2012). As the final product of GABA, succinic acid was estimated to increase along with the decreasing GABA. However, the result was different. Succinic acid may be immediately involved in the tricarboxylic acid cycle as soon as it was produced, so its content kept constant.

Conclusion

In conclusion, there was a significant accumulation of GABA during soybean germination and the GABA content reached its peak value at 5 days. The GAD activity has a positive effect on GABA accumulation, and the GABA-T activity may play an important role in the degradation process of GABA. The mRNA expression level of GmGAD and GmGABA-T may affect GABA content via regulating the expression of respective enzyme activity. The glutamic acid content was increasing during germination accompanied by the advance of GABA level. This study has deeply discussed the inherent relation between the items associated with GABA shunt and provides a preliminary exploration of the accumulation mechanism of GABA in soybeans during germination and lays a foundation for future research.

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Conflict of interest

The authors declare that they have no conflict of interest.

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