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Full Length Article

Characterization of Genes Involved in γ-Aminobutyric Acid Metabolic Pathways Response to Metabolites Accumulation in Embryos during Barley Germination

Mengyuan Jin[1], Qin Wu[1], Peiwen Mao[1], Zhiwei Zhou[1,3], Weiran Cong[2], Juan Lin[3] and Xuanwei Zhou[1*]
[1]*School of Agriculture and Biology, Engineering Research Center of Cell & Therapeutic Antibody, Ministry of Education, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China*
[2]*Xuncaofang (Shenyang) Biological Technology Co., Ltd., Benxi 117004, People's Republic of China*
[3]*School of Life Sciences, Fudan University, Shanghai 200439, People's Republic of China*
[*]For Correspondence: xuanweizhou@sjtu.edu.cn; xuanweizhou@163.com
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Abstract

To reveal the key enzyme genes involved in γ-aminobutyric acid (GABA) metabolic pathways response to elevated metabolite storage in embryos during barley germination, this study investigated the GABA content, cloned GABA metabolic pathway genes and analyzed their expression levels, respectively. In barley embryos, GABA content continued to rise during the soaking process and then decreased after the germination. Three genes including glutamic acid decarboxylase (GAD), GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) involved in the GABA pathway were cloned and characterized from the barley embryos, respectively. Before the germination, the expression of GAD gene was up-regulated, while GABA-T gene expression was down-regulated. After the germination, GAD gene expression was lowered, but GABA-T gene expression was rapidly increased. The SSADH gene expression remained stable after soaking of 4 h, and then down-regulated. There is evidence that the high GABA content in germinating barley seeds is parallel with the upregulation of the GAD *gene*, and down-regulation of *GABA-T* gene. These results indicate that the expression level of the genes involved in GABA pathway is a crucial factor in GABA accumulation during soaking and germination. This study is beneficial for the development of GABA-rich barley products by germination. © 2021 Friends Science Publishers

Keywords: Barley grains; Germination; Embryos; γ-aminobutyric acid; Gene expression

Introduction

Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid isolated from potato tubers in plants (Steward 1949). It has a variety of bioactivity such as inhibitory neurotransmitter (Carmans *et al.* 2013), lower blood pressure (Kajimoto *et al.* 2004), replenish the brain, treat mental illness, and improve immunity (Zhang *et al.* 2002; Diana *et al.* 2014). Because of the multiple effects on human health, the development of GABA-rich products has attracted widespread interest among researchers. In Japan, GABA-rich tea, Gabaron, was brought to the market as a functional food with blood pressure lowering effect (Wang *et al.* 2013). GABA has been identified as a new resource food, and extensively used in foods such as soft drinks, condiments, and dairy products by the Chinese Ministry of Health (Liang *et al.* 2013). However, the GABA content is maintained at a low level in an organism. The content ranges of 0.03~2.58 mg/g (FW) in plant tissues under normal growth conditions (Shelp 2012). For example, the GABA content is about 0.06~0.09 mg/g (DW) in brown rice, lower in refined rice (Zhang *et al.* 2002). Germination is an effective process for improving the nutritional quality and functionality of cereals. The content of GABA in germinated grains was higher than in the un-germinated grains (Gangopadhyay *et al.* 2015). Therefore, developing GABA-rich cereal food with lowering blood pressure function can largely and effectively alleviate the symptoms of hypertension in many individuals and reduce the mental stress and economic burden in the hypertensive population.

Accumulation of GABA content in plant is related to its anabolic and catabolism pathways. Metabolic pathway of GABA is a metabolic bypass of tricarboxylic acid (TCA) in the plant that start with TCA cycle in which α-ketoglutarate is catalyzed by glutamate dehydrogenase (GHD) to produce glutamate (Glu) (Ling *et al.* 1994; Schultz and Coruzzi 1995). When Glu is transported from mitochondria across the mitochondrial membranes into the cytoplasm, it is catalyzed by glutamate decarboxylase (GAD) to generate GABA through an irreversible reaction, and then

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transported into mitochondria. GABA produces the succinic semialdehyde (SSA) through GABA-T action (Shelp *et al.* 1999; Van Cauwenberghe and Shelp 1999). Succinic semialdehyde dehydrogenase (SSADH) catalyzes a reaction through SSA to generate succinate involved in the TCA cycle. Furthermore, the reaction catalyzed by SSADH is an irreversible reaction (Bouche and Fromm 2004; Akçay *et al.* 2012). Otherwise, SSA generates a γ-hydroxybutyrate (GHB) (Fig. 1). According to which, the synthesis of GABA by activating GAD or inhibiting GABA-T or SSADH enzyme activity has become a research hotspot. GAD is one of the first key enzymes during the germination of plant seeds (Lamkin *et al.* 1983). GAD enzyme activity in germination of soybean seed is correlated with the content of GABA (Xu and Hu 2014). GAD enzyme activity after soaking in rice is positively correlated with the accumulation of GABA, which indicate that the accumulation of GABA in embryo after soaking is related to the catalytic reaction of GAD (Liu *et al.* 2005). GABA-T is a key enzyme in the GABA degradation pathway. In previous studies, such as rice and tomato using RNA interference technique to decrease the expression of *GABA-T* can increase GABA content (Koike *et al.* 2013; Zhou *et al.* 2015). Therefore, it is very important to regulate the gene expression of GABA synthesis and degradation pathway to obtain a rich-GABA food.

Hordeum vulgare Linn. var. nudum Hook. f., mainly distributed in the Qinghai-Tibet Plateau, is a typical nutrition-balanced crop, with high content of dietary fiber, vitamins and mineral elements, moderate protein, and low sugar and fat (Lin *et al.* 2016). In addition, barley grains are also rich in GABA, β-glucan and polyphenols, *etc.* (Yang *et al.* 2015). The rich bioactive ingredient makes it become a valuable raw material for the development of functional food (Zhou *et al.* 2018). However, the studies were based on barley grains, but the accumulation of GABA was mainly occurred in embryo and its molecular mechanisms were not reported. In the present study, first time, the changes of GABA content in embryos of the barley grains were analyzed during the whole germination process, then the relative gene involved in GABA metabolisms pathway were cloned, while the gene expression of GABA metabolic pathway during the soaking and germination were investigated. Results will provide a theoretical basis for the development of germinating products and the molecular breeding of barley grains through studying of the accumulation of GABA and their molecular regulation mechanism during the germination.

Materials and Methods

Seeds soaking, germination and embryos collection

The barley grains were provided by Tibet Shenglong Industry Co., Ltd. The barley seeds, were soaked and germinating barley seeds were used further as previously described (Zhang and Zhou 2014), subsequently the embryos were isolated carefully from barley seed using a scalpel and forceps, which frozen in liquid nitrogen for further use.

Extraction and determination of GABA

For this purpose, principal method was adapted as reported earlier (Liu *et al.* 2018). The collected embryos sample was inactivated at 85°C for 30 min, dried at 65°C to constant weight, and then grounded into powder. The powder 40 mg and 5 mL distilled water were added into a 15 mL centrifuge tube, treated for 4 h at 200 rpm and 60°C on a constant temperature oscillator, cycled for three times, and collected the extracting solution. The extracting solution was mixed together, and then centrifuged at 4000 rpm for 10 min, and returned to the supernatant for determination of GABA. The GABA content from the extraction was carried out by the colorimetric method (Luo *et al.* 2014) at 645 nm wavelength.

RNA extraction

Total RNA was extracted using RNA pure Plant Kit (CWBIO, Beijing, China). The quality and concentration of isolated RNA were checked by agarose gel electrophoresis using a spectrophotometer (WFZUV-2100, Unico™ Instruments Inc.). Total RNA was treated with RNase-free DNAase I (Promega, Madison, W.I., U.S.A.) to remove contaminating genomic DNA.

Cloning of GABA branch genes and bioinformatic analysis

A Takara reverse transcription PCR kit was used for synthesis of first-strand cDNA from about 1μg RNA. According to three putative GABA branch gene sequences searched from the National Center for Biotechnology Information (NCBI) (AK355055, AK249113, AK248458), primers listed in Table 1 were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, C.A., U.S.A.). After PCR and following A-tailing procedure, the purified target fragment was inserted into a pMD 19-T Vector (Takara, Bio Inc., Shiga, Japan) and transferred into *E. coli* DH-5α (Vazyme Biotech Co., Ltd., Nanjing, China). Positive clones were sequenced by Shanghai Ruidi Biotech Company (Shanghai, China).

Quantitative reverse transcription-PCR (qRT-PCR)

The relative gene expression analysis was performed with Thermo real-time PCR (RT-PCR) for 8 embryos from different soaking and germination periods of barley grains. Total RNA was extracted using a CWBIO kit (Beijing China) and cDNA synthesis was performed by reverse transcription kit (Takara, Dalian, China) according to the

Table 1: Primers used in this study

Names	Sequences	Target fragment
Primers used in the gene cloning		
GAD-F	5'-CAGAGCCAAGAGCGAGTAGC-3'	1549 bp
GAD-R	5'-GCTTGGATTTTGGACGCTG-3'	
GABAT-F	5'-ATGACGATGATTGCCCGCGGC-3'	1631bp
GABAT-R	5'-CACGGTGTTATTACTGGCATTTG-3'	
SSADH-F	5'-ATGGGCAGCGTGGACGCGG-3'	1595bp
SSADH-R	5'-CCATCTTACACCTTACGCCT-3'	
Primers used in the qRT-PCR		
qGAD-F	5'-TCGACATCGACACCGTCATGG-3'	170 bp
qGAD-R	5'-CTTCTTGGCGAGCACAAACTC-3'	
qGABAT-F	5'-ACGTGGCCTGGGATTGATAC-3'	138 bp
qGABAT-R	5'-CCTGCGACCCTGATAAGCAT-3'	
qSSADH-F	5'-AGCCAACTGTGGTAGGGAAC-3'	137 bp
qSSADH-R	5'-TAAGCCTGCATTGGTGTCGT-3'	
qactin-F	5'-CCAGGTATCGCTGACCGTAT-3'	139 bp
qactin-R	5'-GGAAAGTGCTGAGTGAGGCT-3'	

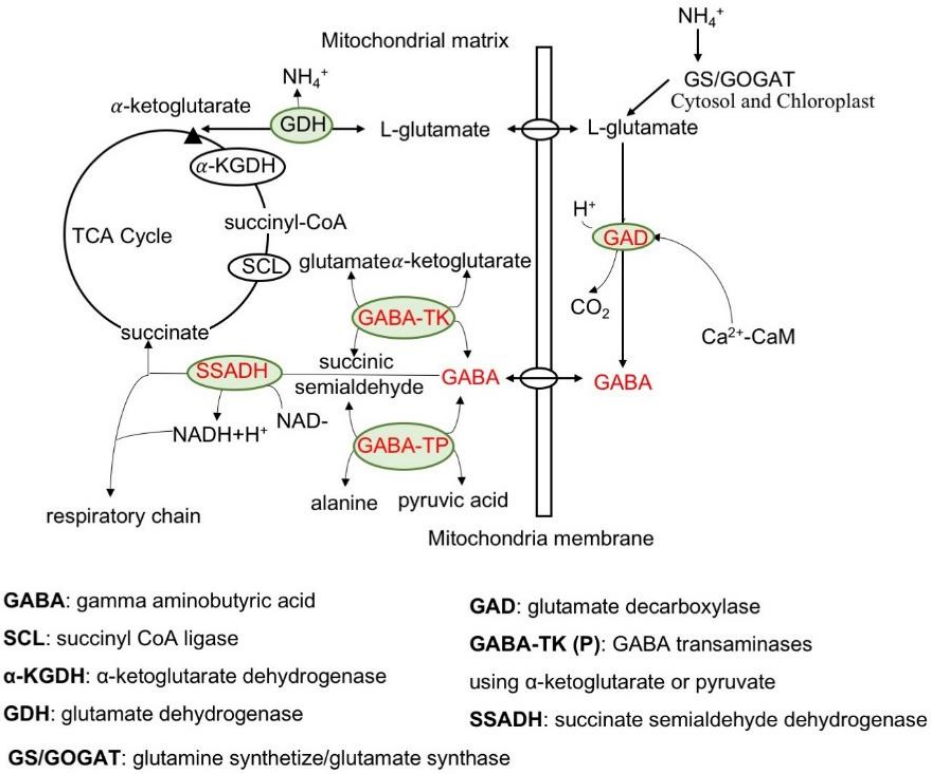

Fig. 1: GABA metabolic pathway and its regulation in plant

manufacturer's instructions. Then qRT-PCR was carried out with Takara SYBR Kit (TaKaRa, Dalian, China) in accordance with the manufacturer's protocols. Using *β-actin* (GeneBank: AY145451.1) from barley as internal reference gene, the specific primers for qRT-PCR were designed according to reference mRNA sequences of GAD, GABAT, SSADH by using NCBI's pick primer software. The program was set as 30 s at 95°C, 40 cycles for 5 s at 95°C and 30 s at 60°C, with a default melting curve stage for 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. The target gene expression change was calculated by the comparative ΔCt method regarding barley β-actin gene as an internal control. All the experiments were conducted for three times repetitions and the primers were listed in Table 1.

Statistical analysis

All experiments contained three parallel tests. Calculations of mean, standard deviation (SD), and *P*-values were performed on triplicate experiments using S.P.S.S. 19.0 software (S.P.S.S. Inc., N.Y., U.S.A.). The Student's t test

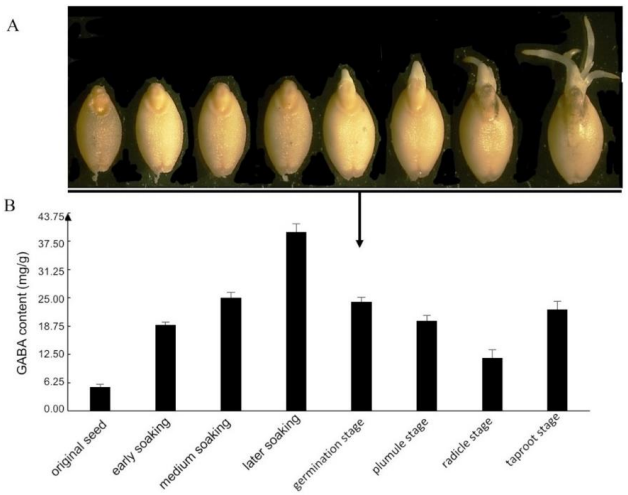

Fig. 2: Changes in GABA content during barley grains germination
A. The process of barley grains germination; **B**. GABA content in embryos from different periods of barley grains germination

was used to calculate *P*-values for comparison. Significant statistics were set at a *P*-value < 0.05.

Results

Collection of samples

The barley embryos were selected from the soaking and germination periods. The soaking period included original seed, early (2 h), middle (4 h) and later (6 h) soaking, and the germination period included germination, plumule, radicle and taproot stages (Fig. 2A). Samples also represented morphological characters of barley seed during soaking and germination.

Changes of GABA content in embryos

Base on the established standard curves of GABA, the regression R^2=0.99 showed that there exists a linear relationship between GABA content and the corresponding absorption wave when the GABA content varied range from 0 to 0.5 mg, so it can be used to determine the GABA content in the sample. The results showed that the GABA content continued to rise during soaking. For soaking duration of 6 h, GABA content was the highest (40 mg/g), about 8 times than in untreated dry seed embryos. However, the GABA content decreased after germination (Fig. 2B).

Cloning and sequence analysis of GABA branch gene

Based on the information related to GABA pathway such as GAD, GABA-T and SSADH, three genes have been isolated from barley grains by using PCR techniques (Fig. 3A–C). The GAD sequence obtained by the open reading frame

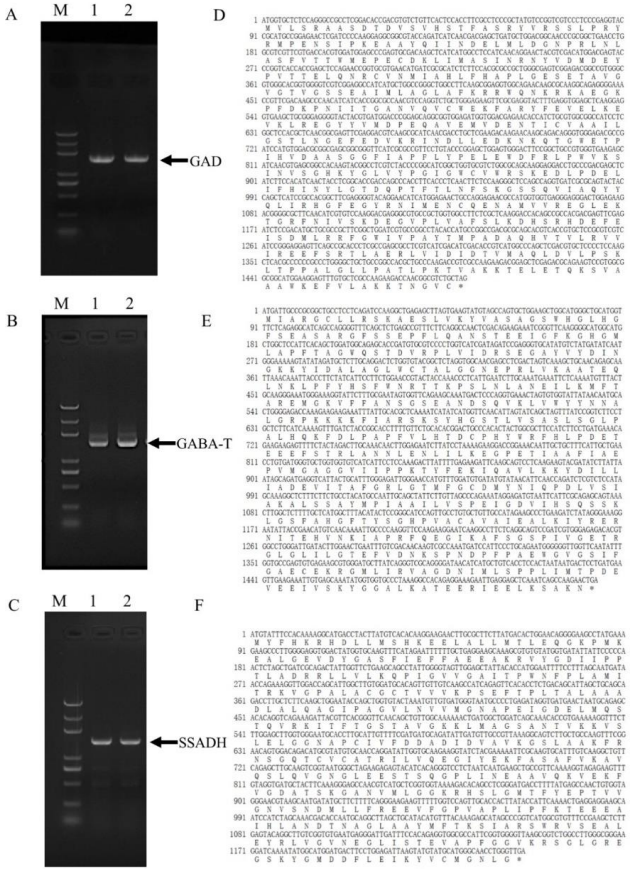

Fig. 3: The cloning of key gene of GABA pathway
A, **B**, **C**. Electrophoretogram of GAD, GABA-T and SSADH cDNA PCR products, respectively; **D**, **E**, **F**. Open reading framework of GAD, GABA-T and SSADH gene sequence and the corresponding amino acids sequence, respectively

(ORF)-Finder of NCBI，harbored a 1491 bp ORF that encoded 496 amino acids (Fig. 3D). The GAD sequence has the highest similarity with *Aegilops tauschii* GAD1-like (XM_020292464.1), which is 95% using BLASTN analysis. And the sequence similarity of other gramineous plant, such as *Brachypodium distachyon* GAD1 (XM_003558361.3), *Oryza sativa* GAD1 (XM_015772426.1), *Setaria italic* GAD1 (XM_004985025.2), *Sorghum bicolor* GAD1 (XM_002468163.2), *Zea mays* GAD1 (NM_001174470.1) is among 90–92% (Fig. 4A). All these conclusions showed that the *GAD* gene has been cloned successfully from barley grains. The sequence of the GABA-T positive clone analyzed by the ORF-Finder of NCBI, harbored a 1524 bp ORF and encoded 507 amino acids (Fig. 3E). The sequence of GABA-T had similarity with *A. tauschii* GABA-T3 (XM_020291859.1), which is 95% using BLASTN analysis. And the sequence similarity of other gramineous plant, such as *B. distachyon* GABA-T3 (XM_010236148.3), *O. sativa* GABA-T3 (XM_015792818.1), *S. bicolor* GABA-T3 (XM_002445162.2), *S. italic* GABA-T3 (XM_004972552.3), *Z. mays* GABA-T1 (XM_008670668.2) is among 81–90% (Fig. 4B). The sequence of the SSADH positive clone analyzed by the ORF-Finder of NCBI，

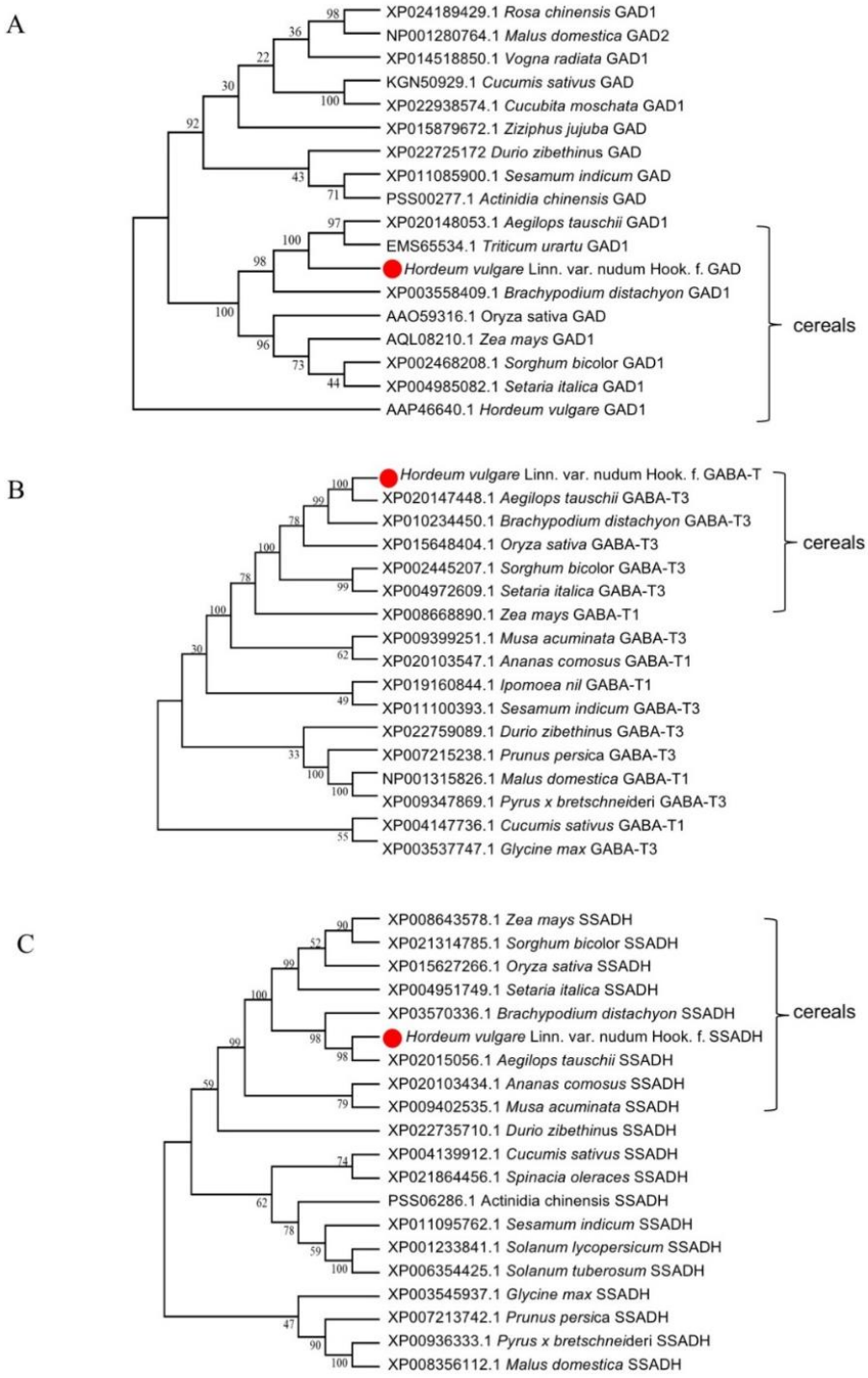

Fig. 4: Phylogenetic tree of key gene of GABA pathway
A, **B**, **C**. Phylogenetic tree of GAD, GABA-T and SSADH amino acids sequence, respectively

harbored a 1236 bp ORF and encoded 411 amino acids (Fig. 3F). The sequence of SSADH was analyzed by BLASTN, and the results showed that the sequence similarity is the highest with *A. tauschii* SSADH (XM_020294976.1), which is 96%. And the sequence similarity of other gramineous plant, such as *B. distachyon* SSADH (XM_003570288.4), *S. bicolor* SSADH (XM_021459110.1), *S. italic* SSADH (XM_004951692.4), *Z. mays* SSADH (NM_001153701.1), *O. sativa* SSADH (XM_015771780.1), is among 88–89% (Fig. 4C). And these conclusions showed that the GAD, GABA-T, SSADH gene of barley grains has been cloned successfully.

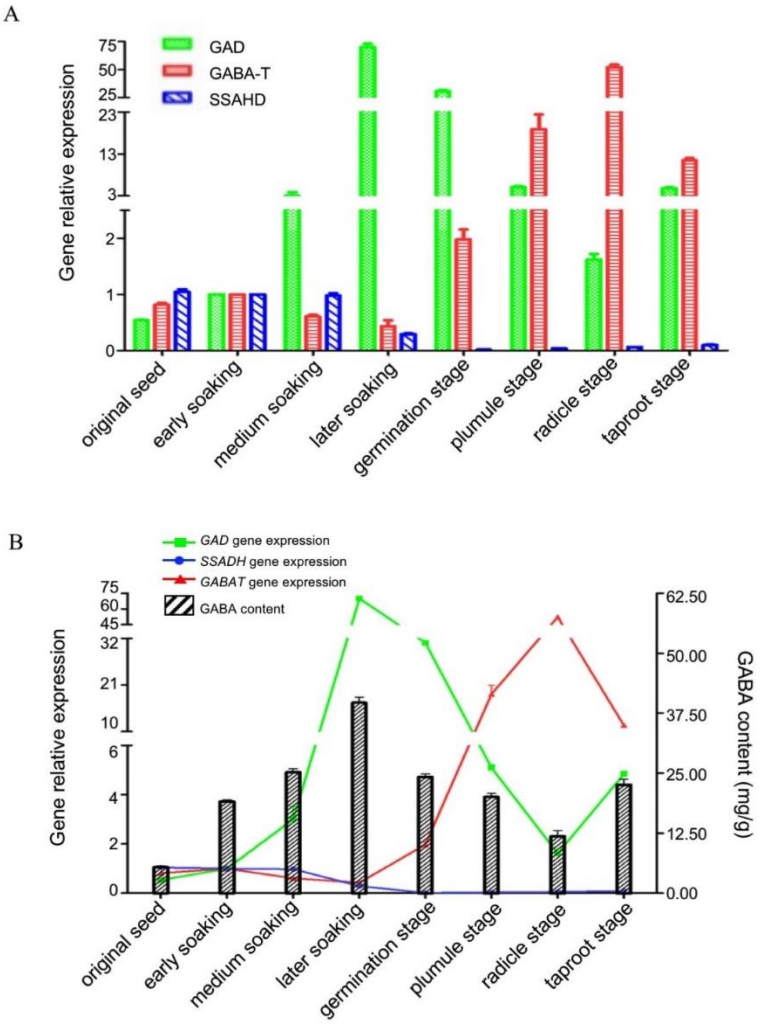

Fig. 5: The Relative expression of key gene in GABA pathway and the comparison of GABA content during grains germination
A. Relative expression of key gene in GABA pathway in barley grains embryo during germination; **B**. Comparison of gene expression in GABA pathway and GABA content during germination of barley grains

Expression analysis of GABA branch gene in soaking and germination

Expression of three genes involved in GABA pathway in the embryo during germination of barley grains was determined using qRT-PCR. The results showed that the expression of *GAD* showed an upward trend before the bud germination period and decreased after germination (Fig. 5A). However, the expression of *GABA-T* was down-regulated before the bud sprouting period, and then increased rapidly. The expression of *SSADH* mainly stayed stable but deceased after soaking for 4 h. From the early stage to the late stage during soaking (from 2 h to 6 h), the expression of *GAD* genes which controlled GABA synthesis was significantly up-regulated, while the expression of *GABA-T* and *SSADH* in the GABA degradation pathway showed a downward trend (Fig. 5B). Thus, it was speculated that the continuous increase of GABA content during this process may be associated with changes in gene expression. From germination to rooting, the *GAD* gene expression decreased rapidly, while the *GABA-T* gene expression which controlled the first step of the GABA degradation pathway increased promptly, and is speculated to cause GABA content to decrease. After rooting, the expression of *GAD* in multiple root stage was higher than its expression in single root stage. Besides, the expression of GABA-T in multiple root stage became lower, and the GABA content also showed an upward trend after rooting. The results also showed that during the entire germination treatment, the expression of the *SSADH* remained basically unchanged, but then its expression was down-regulated after soaking for 4 h.

Discussion

Changes in GABA content were accordant with the

expression level of gene and the key enzyme activity in metabolic pathway in various tissues and at different stages of plant development. For example, there's a lot of GABA in the fruit before the discoloration period of tomato and citrus fruit, then the GABA is quickly broken down. This is highly correlated with the activity of the synthase and catabolism enzyme in the GABA pathway of tomato fruit (Diaz *et al.* 2005; Cercos *et al.* 2006; Akihiro *et al.* 2008). Metabolic pathway of GABA consists of three enzymes: GAD, GABA-T and SSADH. Therefore, it is important to colon the key enzyme genes for analysis of GABA accumulation in germination at the molecular level. The seed embryo is the storage place of GABA in barley grains (Inatomi and Slaughter 1971), therefore, embryo was selected for further study.

It was demonstrated that changes in the gene expression whose mRNA levels and their encoding enzymes are related to GABA content in the various tissues. The GABA content is determined by the key enzyme gene both the synthesis and the decomposition pathways. The *GAD* gene is generally considered to be the key enzyme gene in the GABA synthesis pathway, and the increase of GAD enzyme activity is the main factor leading to GABA accumulation (Baum *et al.* 1996; Akama and Takaiwa 2007; Hyun *et al.* 2013; Xu and Hu 2014). For example, studies in soybeans have shown that the GAD enzyme activity increases with germination, and that the mRNA expression level of GAD maintains a relatively high level 15 to 35 days after flowering (Clark *et al.* 2009; Takahashi *et al.* 2013; Xu and Hu 2014). Prior to the maturity of tomato fruit, GAD, a key enzyme activity in the GABA synthesis pathway is high. This indicates that the GABA accumulation is positively correlated with the activity of GAD enzyme. Decreased the downward trend of GABA-T and SSADH enzyme activity in the GABA decomposition pathways can also lead to GABA accumulation. For example, GABA is rapidly degraded after the ripening stage of tomato fruit. In the same time, the expression of SlGABA-T1 is greatly increased. This indicates that GABA-T plays a key role in the GABA decomposition pathways (Akihiro *et al.* 2008; Koike *et al.* 2013). However, some studies have suggested that there is no significant relationship between changes in the key genes expression of the GABA pathway and GABA content. For example, the GABA content of the frost-resistant barley grains increased 15-fold in response to freeze stress, but without accompanying changes in the expression of *GAD*, *GABA-T* and *SSADH* genes (Mazzucotelli *et al.* 2006). It was found that the changes of GABA concentration did not accompany any change of *GABA-T* transcriptional abundance when Arabidopsis was domesticated at low temperature (Kaplan *et al.* 2007), while it was also found to have no obvious relationship between the change of *GABA-T* and GABA content rice grains (Narsai *et al.* 2009). In the present study, GABA content in the barley seeds increased gradually with soaking and up to the highest at 6 h but decreased after germination. Correspondingly, the expression of GAD gene continued to increase after immersion in water, and reached the highest point in the later period of water swelling (soaking for 6 h), this indicates that the increase of GABA content is indeed related to the activation of GAD gene. From the principle of gene expression regulation products, the increase in gene expression should be earlier than the increase in expression products. These experiments showed that the amount of GABA in the barley grains increased greatly in the early stage of water absorption and swelling. But there was no significant increase in gene expression, which might be related to the increasing GABA content in the embryo at the initial stage of soaking. At this stage, the increase of GABA content is due to the release of bound GABA, rather than the GAD enzymatic response (Liu *et al.* 2005). The enzymatic response of GAD promotes the increase of GABA content mainly in the middle and late stages of grain swelling.

These experimental results showed that, in the later stages of hydration swelling of barley grains, not only the *GAD* expression reached the highest level, but also the amount of *GABA-T* and *SSADH* reached the lowest. Therefore, it is believed that the barley grains are dominated by GABA synthesis in the water-swelling period. After the grains became white, the GABA content began to slowly decline, but reached the lowest level in the single root stage, and then there is a slight rise, and this is consistent with the increasing trend of brown rice after germination (Zheng 2006). During the period from whitening to single root, the expression of *GAD* gradually decreased, and the expression of *GABA-T* gradually increases. Therefore, the decrease of GABA expression results from the decrease in the synthesis and the increase in decomposition. The increase in decomposition is mainly due to the increase in GABA-T enzyme activity, which is consistent with studies on soybean seed (Takahashi *et al.* 2013). Therefore, in order to maintain the amount of GABA in the barley grain embryo, increasing GAD enzyme activity (after barley grain is becoming white) or using the GABA-T inhibitor to inhibit the expression of GABA-T should be tried to use in processing practice. The SSADH is the last key enzyme in the GABA decomposition pathway, but it does not show an increase in expression during the period from whitening to a single root. It is speculated that the decomposition of GABA in grain is mainly regulated by GABA-T. In addition, SSADH gene in plant has multiple copies, such as three copies in *Arabidopsis* and maize, and the numbers of SSADH copies in the barley grains are unknown, and there may be other copies of genes that contribute to GABA degradation during this period.

Conclusion

The evidence indicated that the expression level of the genes involved in GABA pathway is a crucial factor in soaking and germination. The GABA content of barley grains in the treatment process varied with the related gene changes in

gene expression in embryos. It revealed a role that GABA is dominated by anabolic metabolism in soaking, but catabolism in germination. The investigation is beneficial for the development of γ-aminobutyric acid-rich barley products.

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Author Contributions

X-W Z managed the project; X-W Z and J L designed the experiments and provided support for the experiments; M-Y J, QW and P-W M managed the samples and performed the experiments. Z-W Z and W-R C led the data analysis and preparation of the graph, M-Y J and Q W drafted the manuscript; all authors reviewed and approved the manuscript.

Conflict of Interest

There is no conflict of interest among the authors and the institutions where the research has been conducted

Data Availability Declaration

All data related to this article are in the custody of corresponding author and will be available on request

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