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Accumulation of γ-aminobutyric acid and transcription of glutamate decarboxylase in *Brassica juncea* (L.) Czern.

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Abstract

Mustard leaf (*Brassica juncea* (L.) Czern.) using as material of a traditional fermented vegetable food (Kimchi) in Korea, is one of the important vegetables. Two cultivars of mustard (red and green) were grown in the experimental farm of Chungnam National University and allowed them to grow until flowering stage. In this study, the variability of γ-aminobutyric acid (GABA) content and mRNA transcript levels of genes involved in GABA biosynthesis were investigated in 2 cultivars (green and red mustard) of *Brassica juncea.* We observed that the transcript levels of glutamate decarboxylase (*GAD) 1*, *2*, and *4a* in roots were the highest, whereas those in seeds were the lowest compared with other organs harvested at flowering stage in green mustard. The transcript levels in green and red mustards displayed similar transcription patterns among a variety of organs, except for those of *BjGAD2* and *4a* in roots. In red mustard, the GABA content in flower buds was the highest (179.8 mg 100 g^{-1} FW), whereas roots contained the lowest amount (1.77 mg 100 g^{-1} FW). In green mustard, the GABA content in young leaves was the highest (97.76 mg 100 g^{-1} FW), whereas seeds had the lowest (1.23 mg 100 g^{-1} FW). Green and red mustard contained varying amounts of GABA in different organs. Specially, because green and red mustard leaf has high GABA content compared with other vegetables, it can be used as a good vegetable for healthy.

Keywords: *Brassica juncea*; glutamate decarboxylase; γ-aminobutyric acid; transcript level.
Abbreviations: GAD_glutamate decarboxylase; GABA_ γ-aminobutyric acid; HPLC_ high-performance liquid chromatography.

Introduction

Brassica species include agronomically important vegetables. To date, *Brassica* crops are globally the third most important sources of vegetable oil after soybean and groundnut (Warwick et al., 2006). Among them, *Brassica juncea* (L.) Czern., an important vegetable of the *Brassicaceae* family, has been cultivated in Asia and Europe for thousands of years. The seeds are pressed to make mustard oil, and the edible leaves can be eaten as salads or mixed with other salad greens (Zohary and Hopf, 2000). Specially, mustard leaf has been used as material of a traditional fermented vegetable food (Kimchi) in Korea. Many Korean people have been consuming red and green mustard leaves for their mustard Kimchi in Korea. Therefore, mustard is important vegetable in Korea. The mustard leaves are large and soft. Red mustard leaves are red color, whereas green mustard leaves are light green color and has a special flavor. Mustard leaf is rich in many nutritional compounds including antioxidnats, proteins and minerals. Specially, very recently, Kim et al. (2011) reported that the anticancer activity of red mustard leaves was higher than that of green mustard leaves. In red and green mustard leaves, sinigrin was the most abundant glucosinolate and its content was determined as 4.71 and 3.06 mg g^{-1} dry wt., respectively. γ-aminobutyric acid (GABA) is a 4-carbon non-protein amino acid conserved from bacteria to plants and vertebrates. It is highly soluble in water and can assume several conformations in solution, including a cyclic structure that is similar to proline (Christensen et al., 1994). GABA is mainly metabolized via a short pathway composed of 3 enzymes, glutamate decarboxylase (GAD), GABA transaminase (GABA-T), and succinic semialdehyde dehydrogenase (SSADH) in plants and animals (Bouché and Fromm, 2004; Snedden and Fromm, 1999) (Fig 1). Schousboe and Waagepetersen (2007) reported that GABA is the major neurotransmitter inhibitor in the central nervous system that prevents anxiety and stress-related messages from reaching the motor centers of the brain in vertebrates. Interestingly, GABA is also rapidly induced in a variety of plant tissues under several abiotic environmental conditions that contain mechanical stimulation, damage, cold or heat shock, hypoxia, cytosolic acidification, darkness, water stresses, and hormonal changes (Bouché and Fromm, 2004; Snedden and Fromm, 1999). Very recently, Karladee and Suriyong (2012) described that GABA content in different varieties of brown rice during germination. Additionally, Zhao et al. (2011) demonstrated GABA content in pu-erh and other types of Chinese tea. GAD is an enzyme that catalyzes the conversion of L-glutamate to GABA (Steward et al., 1949) and the

Table 1. List of primers used in this study.

Primer Name	Sequence (5’ → 3’)	Amplicon (base pair)	GenBank Access No.
BjGAD1-RT(F)	ATGGTGCTCTCTCACGCCGC	170	AY559321
BjGAD1-RT(R)	CTTGGATTACCGTCAAGCATCAACTC		
BjGAD2-RT(F)	CTTCACGAGCTCGACACCTTGC	165	AY559318
BjGAD2-RT(R)	TCAACACACACCATTCATCTTCTTCC		
BjGAD4a-RT(F)	CTCCACGAGCTCGATACGCTTC	162	AY559319
BjGAD4a-RT(R)	TTAGCAAATCCTGTTCTTGTTACTCTTCTTTG		
BjGAD4b-RT(F)	CTTCACGAGCTCGATACACTTCCG	162	AY559320
BjGAD4b-RT(R)	TTAGCAAATCTTGTTCTGGTTAGTCTTCTTTG		
BjActin (F)	CCGACCGTATGAGCAAGGAAATC	190	HM565958
BjActin (R)	TTCCTGTGGACAATGGATGGAC		

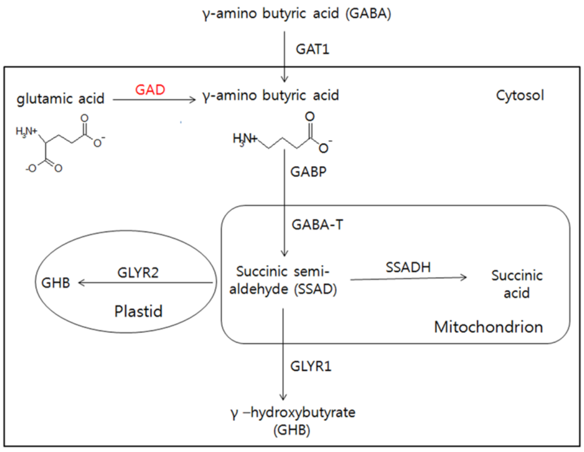

Fig 1. Proposed biosynthetic pathway and metabolism of GABA (redrawn from Shelp et al. 2012). The gene used in this study is colored red. Abbreviations: GAD, glutamate decarboxylase; GABP, GABA permease; GABA-T, GABA transaminase; GAT1, GABA transporter 1; SSADH, succinic semialdehde dehydrogenase; GLYR1 and GLYR2, glyoxylate reductase isoforms 1 and 2.

calmodulin-mediated modulation of GAD activity affects GABA metabolism and normal development in plants (Baum et al. 1996). *In vitro* GAD activity has been characterized in crude extracts from many plant species and tissues (Bown and Shelp, 1989, 1997; Satyanarayan and Nair, 1990). The subcellular location of GAD is in the cytosol and GABA-T and SSADH are localized in mitochondria, and thus implicates the transport of GABA across the mitochondrial membranes (Clark et al., 2009) (Fig 1). Several cDNA clones encoding Ca^{2+}/calmodulin-dependent GAD have been identifiedfrom a variety of dicotyledonous species, such as *Petunia* (Baum et al., 1993), tomato (Gallego et al., 1995), tobacco (Yu and Oh, 1998), *Arabidopsis* (Turano and Fang, 1998; Zik et al., 1998), faba bean (Ling et al., 1994), and monocotylendonous species such as rice (Akama et al. 2001; Oh et al., 2005; Xu et al., 2010), corn (Zhuang et al., 2010). Up to now, many studies about GABA have been reported in several plants, but there has been no research in red and green mustards. Therefore, we analyzed GABA composition and investigated transcript level of *BjGAD* from different organs of red and green mustards. In addition, we investigated the relationship between GABA accumulation and the *BjGAD* gene expression in different organs of red and green mustards.

Results and discussion

Expression patterns of BjGAD genes among different organs of 2 mustard cultivars

The transcript levels of *B. juncea* GAD (*BjGAD*) genes involved in GABA biosynthesis among different organs of 2 mustard cultivars, red and green mustards, were examined by the quantitative RT-PCR analysis (Fig. 2). There were full-length BjGAD2, 4a, and 4b, except for BjGAD1 in GenBank of NCBI and the deduced BjGAD2 shared 82%,81%, 92%, 82%, and 82% identities with BjGAD4a and GAD4b, *Arabidopsis thaliana* GAD2, *Nicotiana tabacum* GAD2, and *Vitis vinifera* GAD1, respectively (Data not shown). In green mustard, the transcript levels of *BjGAD1*, *2*, and *4a*, except for *BjGAD4b* were the highest in roots and the lowest in seeds, while red mustard showed the highest level in 10 days after sowing (DAS). In the case of *BjGAD1*, the transcript levels of red mustard among different organs (except for roots) were similar or somewhat higher than those of green mustard. The transcript levels of seeds and 10 DAS in red mustard were 5.7- and 3.5-fold higher, respectively, compared with green mustard in *BjGAD1*. In green mustard, the transcript level of *BjGAD1* in roots was 6.7 times higher than that of flowers. Since the sequencing analysis of the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2002), 5 *GAD* genes have been studied by sequence comparisons (Shelp et al., 1999). It was reported that *AtGAD1* and *AtGAD2* differ in their organ distribution; *AtGAD1* is induced predominantly in roots, whereas *AtGAD2* is expressed in *Arabidopsis* all organs (Zik et al., 1998; Turano and Fang, 1998). Bouché and Fromm (2004) pointed out that the different *GAD* isoforms are expressed in a tissue-dependent manner and might have specific functions. Unlike *Arabidopsis*, in this study, *BjGAD1* and *BjGAD2* were the highest expressed in roots of green mustard, whereas the transcription of *BjGAD1* and *BjGAD2* in red mustard showed the highest levels in 10 DAS. In red mustard, the transcript levels of *BjGAD1, 2,* and *4a* were increased between 3 and 10

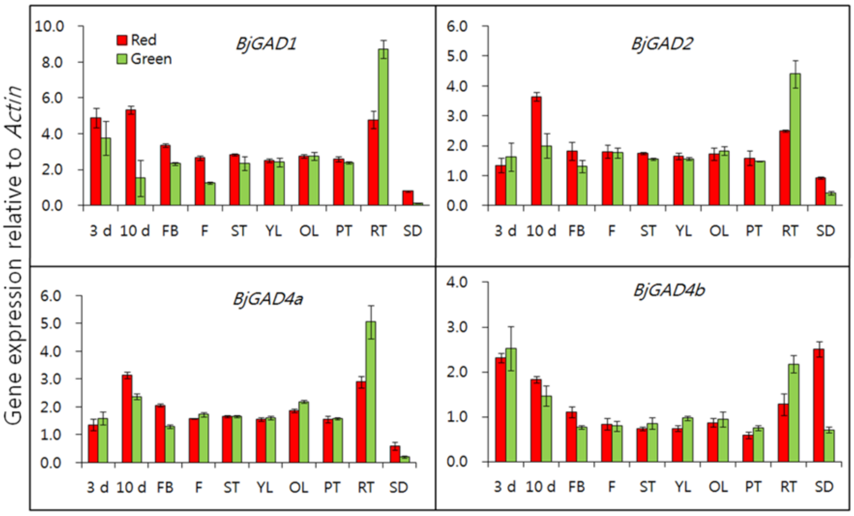

Fig 2. Expression of *BjGAD* isoforms involved in GABA biosynthesis in green and red mustards. Each value represents the mean of 3 replicates, and error bars indicate standard deviation. 3 d and 10 d, days after sowing; FB, flower bud; F, flower; ST, stem; PT, petiole; YL, young leaf; OL, old leaf; RT, root; SD, seed.

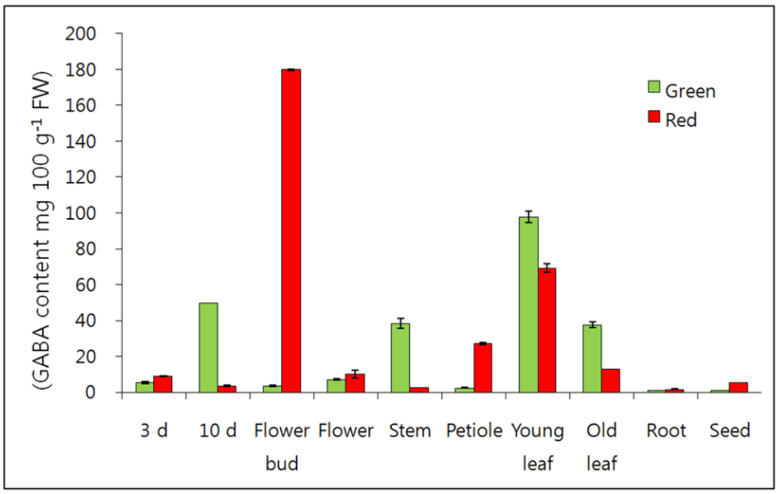

Fig 3. GABA contents in different organs of green and red mustards (mg 100 g^{-1} fresh weight). Each value represents the mean of 3 replicates, and error bars indicate standard deviation.

DAS, while *BjGAD4b* was decreased. Moreover, the transcript level of *BjGAD4b* in seeds of red mustard was the highest unlike *BjGAD1*, *2*, and *4a*. Bouché and Fromm (2004) reported that the importance of GAD in regulating glutamate levels in wild-type plants remains to be clarified. Interestingly, Oh et al. (2005) reported that *RicGAD* was expressed strongly in rice roots obtained from rice seedlings grown under phosphorus deprivation conditions, as well as in non-germinated brown rice. They also suggested that the enhanced expression of *RicGAD* in the phosphorus-deficient roots can be attributed to the growth condition of the seedlings for mRNA, which was prepared from roots grown under phosphorus deprivation conditions.

Analysis of GABA in different organs of 2 mustard cultivars

The GABA content in various organs of 2 mustard cultivars was analyzed by HPLC (Fig. 3). In red mustard, the GABA content was the highest in flower buds [179.8 mg 100 g^{-1} fresh weight (FW)] and the lowest in roots (1.77 mg 100 g^{-1} FW). In green mustard, the highest GABA content was in young leaves (97.76 mg 100 g^{-1} FW) and the lowest in seeds (1.23 mg 100 g^{-1} FW). The GABA levels in flower buds and petioles of red mustard were 45- and 13-fold higher than those of green mustard, respectively; the levels 10 DAS, in stems, and old leaves of green mustard were 12.5-, 12.6-, and 2.9 times higher, respectively, compared with those of red mustard. Overall, the GABA levels in various sites were ranked in descending order as follows: flower buds > young leaves > petioles > old leaves > flowers > 3 DAS > seeds > 10 DAS > stems > roots in red mustard; and in green mustard, young leaves > 10 DAS > stems > old leaves > flowers > 3 DAS > flower buds > petioles > roots > seeds. Moreover, the total GABA content (322.17 mg 100 g^{-1} FW) in red mustard was somewhat higher than that in green mustard (244.81 mg 100 g^{-1} FW). It is also noted that the GABA levels in young leaves of green and red mustards were 2.5- and 5.3-fold higher than those in old leaves, respectively. Therefore, young leaves might be better choices when we eat mustard leaves. Previously, our group has also investigated the GABA content in *Momordica charantia* fruits. We showed that Philippines cultivar Galaxy had the highest GABA content (19.3 mg 100 g^{-1} FW), whereas Peacock cultivar contained the lowest level (3.5 mg 100 g^{-1} FW) (Kim et al., 2009). Zhao et al. (2011) also reported that the GABA content in white tea (45.7

mg 100 g^{-1} FW) was found to be significantly higher than that of green tea, black tea, and oolong tea. In addition, the purple rice variety Kum Doi Saket from Thailand (23.48 mg 100 g^{-1} DW) exhibited the highest GABA content of all 21 rice varieties (Karladee and Suriyong, 2012. The red and green mustard had abundant amount of GABA compared with other plants. The GABA accumulation in plants tend to be enhanced during high stress conditions, such as hypoxia, darkness, drought, low temperature, and wounding (Shelp et al., 1999; Bouché and Fromm, 2004). Mae et al. (2012) also demonstrated that upregulation of GAD activity and downregulation of GABA-T activity caused GABA accumulation in tomatoes stored under low O_2 conditions. In addition, Shelp et al. (1995) reported that GABA accumulation might also result from decreased import of GABA either to mitochondria or out of the cell under different stress conditions. Our results in this study indicate that green and red mustards exhibit varying GABA content in different organs and especially in the young leaf contained higher amount, which can be used as a good source of GABA for human being.

Materials and methods

Plant materials

B. juncea plants were grown in the experimental farm of Chungnam National University (Daejeon, Korea) in April 2012. Each organ (flowers, stems, leaves, and roots) was harvested at flowering stage and frozen in liquid nitrogen upon collection and stored at –80°C. All the samples were freeze-dried at –80°C for at least 72 h and then ground into a fine powder using a mortar and pestle. They were used for RNA isolation and high performance liquid chromatography (HPLC) analysis.

RNA extraction and Real-time PCR

Total RNA was isolated from *B. juncea* different organs using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and genomic DNA was removed by RNase-free DNase (Sigma-Aldrich, St. Louis, MO, USA). The first-strand cDNA was synthesized from the total RNA (1 µg) with ReverTra Ace-α- (Toyobo, Osaka, Japan) Kit and oligo $(dT)_{20}$ primer according to the manufacturer's protocol. Gene specific primers (0.5 µM each) were designed using an online program (http://frodo.wi.mit.edu/primer3) (Table 1). Diluted cDNA (1:10) was used as templates in the following PCR amplification program: hot start at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 72°C for 20 s, and annealing temperature at 55°C. Reactions were performed in triplicate on the CFX96 Real Time System (Bio-Rad, Hercules, CA, USA). The actin gene was used as a housekeeping gene because actin gene exhibited expression stability in different organs. The actin gene has also been used as a reference gene in several studies (Yuan et al., 2009; Wang et al., 2010; Wei et al., 2011; Tuan et al. 2012).

Chemicals

Trichloroacetic acid (TCA, 99.0%) was obtained from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea), and GABA standard and sodium phosphate monobasic monohydrate (Na_2HPO_4) were purchased from the Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were supplied by J. T. Baker (Phillipsburg, NJ, USA). Ultrapure water having a resistivity of 18.2 MΩ/cm was produced by a PureLab Option from ELGA Labwater (Model LA 621, Marlow, UK).

Extraction and HPLC analysis of GABA

One hundred mg of freeze-dried plant powder was weighted into a 2.0 mL-eppendorf tube, and then added 1.2 ml of 5% (v/v) trichloroacetic acid (TCA) solution. After vortexing, the mixture was allowed to stand for at least one hour at room temperature and then centrifuged at 15,000 rpm, at 4°C for 15 minutes. The supernatant was filtered through 0.45 µm PTFE hydrophilic syringe filter (Ø 13 mm) into the HPLC-vial. HPLC analysis of GABA was conducted according to the 'Rapid, accurate, sensitive, and reproducible HPLC analysis of amino acids analysis using Zorbax Eclipse-AAA Columns and the Agilent 1100 HPLC (http://www.chem.agilent.com/Library/chromatograms/59801193.pdf). Briefly, GABA was determined by using Agilent Technologies 1200 series HPLC system equipped with Zorbax Eclipse AAA analytical column (150 × 4.6 mm i.d., particle size 5 µm) and guard column (12.5 × 4.6 mm i.d., particle size 5 µm). The HPLC conditions were set at 338 nm of wavelength, 40°C of oven temperature and 2.0 ml/min of flow rate. The mobile phase consisted to 40 mM Na_2HPO_4 pH 7.8 (solvent A) and ACN: MeOH: water (45:45:10, v/v/v) (solvent A). The gradient programs were as follows: a linear step from 0 to 57% of solvent B from 1.9 to 21.1 min, and from 57 to 100% of solvent B to 21.6 min, and then isocratic conditions with 100% solvent B to 25.0 min, followed by a rapid drop to 0% solvent B at 25.1 min, and then isocratic conditions with 0% B to 30.0 min (total 40 min). The quantification was performed using 50 pmol μL^{-1} (0.05 mM) of GABA solution.

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