JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Influence of Light on the Free Amino Acid Content and γ-Aminobutyric Acid Synthesis in *Brassica juncea* Seedlings

Xiaohua Li,[†] Yeon Bok Kim,[†] Md Romij Uddin,[†] Sanghyun Lee,[§] Sun-Ju Kim,*[,‡] and Sang Un Park*[,†]

[†]Department of Crop Science and [‡]Department of Bio-Environmental Chemistry, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, Republic of Korea

[§]Department of Integrative Plant Science, Chung-Ang University, Anseong 456-756, Republic of Korea

Supporting Information

ABSTRACT: Glutamate decarboxylase (GAD; EC 4.1.1.15) is an important enzyme in γ-aminobutyric acid (GABA) biosynthesis. Here we report the influence of light on amino acid accumulation and investigate the molecular mechanism by which light influences GABA biosynthesis at the seedling stage of two mustard (*Brassica juncea*) cultivars (green-leaf and purple-leaf). Gene expression profiles of four GAD-encoding genes (*GAD1*, *GAD2*, *GAD4a*, and *GAD4b*) and their impact on GABA biosynthesis were analyzed. Light exerted an obvious influence on amino acid accumulation in mustard seedlings. *GAD* gene expression was also significantly regulated by light/dark or dark treatment, which differentially regulated GABA biosynthesis in *B. juncea* seedlings. High-performance liquid chromatography (HPLC) revealed that the seeds of purple cultivars contain a higher amount of free amino acids and GABA than do the seeds of green cultivars. After seed germination, however, the accumulation of free amino acids peaked in dark-treated seedlings on day 9 in both cultivars, whereas GABA synthesis peaked at 9 days under light conditions. This study may provide a foundation for understanding the effect of light on amino acids, particularly GABA biosynthesis in *Brassica* plants.

KEYWORDS: *Brassica juncea, free amino acids, GABA, GAD, gene expression*

INTRODUCTION

Amino acids play an important role in human health; most essential amino acids are obtained in food, because humans have a limited ability to synthesize adequate essential amino acids. The production and distribution of free amino acids in plants could be affected by environmental factors[1−3] or by artificial treatment.[4,5] Seed germination is considered an efficient target for nutritional modification of cereals. The nutritional and protein profile has been improved in germinated Australian Sweet Lupin.[6] The content of nutrients and antinutrients in legume seeds has also been modified by germination processing,[7] and phenolic compounds and antioxidant activity have been enhanced in germinated sprouts.[8,9]

The mustard *Brassica juncea* (L.) is an important oilseed crop. Like other Brassicaceae species, *B. juncea* contains various bioactive compounds such as glucosinolates and phenolics, which contribute to its nutritional value.[10−12] *B. juncea* is usually used as a condiment, and its green leaves are used as a vegetable on the Indian subcontinent and in other Asian countries. *B. juncea* is a saline- and drought-tolerant plant[13] and is sensitive to environmental heavy metal stresses.[14−16] The stress response genes and biochemical pathways in *B. juncea* have been investigated and reported.[14,15,17]

γ-aminobutyric acid (GABA) is a nonprotein amino acid and a significant component of the free amino acid pool. The GABA metabolic pathway was reported previously and is known as the GABA shunt.[18] Glutamate decarboxylase (GAD; EC 4.1.1.15), a pyridoxal 5′-phosphate (PLP), plays an important role in GABA biosynthesis. GAD catalyzes L-glutamic acid to GABA and carbon dioxide through an irreversible α-decarboxylation reaction. GAD is a calcium/calmodulin-dependent enzyme in GABA synthesis.[19−22] Another GABA synthesis pathway is related to polyamine degradation, in which diamine oxidase (DAO; EC 1.4.3.6) is the rate-limiting enzyme for GABA synthesis.[23−25]

In recent years, GABA has been considered a bioactive plant component and GABA-enriched foods have become popular for their health benefits. GABA is an effective pain reliever and anxiolytic,[26] regulates blood pressure,[27] and alleviates chronic alcohol-related symptoms.[28] GABA could be rapidly and largely accumulated by regulating GAD or DAO activities in response to stress factors such as hypoxia, cold, heat, and water stress.[18,29−33] However, the GABA synthetic pathway under varying light conditions has not been reported in *B. juncea*. To investigate the effect of light on amino acid accumulation, especially GABA biosynthesis, in *B. juncea*, two cultivars (purple- and green-leaf mustard) were investigated under light/dark or dark conditions. High-performance liquid chromatography (HPLC) or quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate the light elicitor effect on amino acid content and GABA biosynthesis in *B. juncea*. The association between the transcription of four GAD isoforms and GABA accumulation under light/dark or dark treatment is also discussed.

Received: May 4, 2013
Revised: July 29, 2013
Accepted: August 4, 2013
Published: August 5, 2013

ACS Publications © 2013 American Chemical Society

MATERIALS AND METHODS

Plant Materials and Culture Conditions. Two *B. juncea* (L.) cultivars (purple- and green-leaf mustard) were procured from the Rural Development Administration (RDA), Suwon, Korea. The seeds were surface-sterilized with 70% ethanol for 30 s and 2% (v/v) bleach solution for 10 min and then rinsed several times in sterile water. The seeds were placed on sucrose-free one-quarter sterilized Murashige–Skoog (1/4 MS) medium solidified with 0.8% agar. The seeds were germinated at 25°C in a growth chamber with approximately 60% humidity under light/dark (16 h/8 h) or totally dark conditions. Samples were collected after 3, 6, 9, and 12 days by cutting off the roots with scissors and then rapidly checking the seedling length and fresh weight. All samples were frozen in liquid nitrogen and stored at −80°C until analysis.

Total RNA Extraction and Quantification of Gene Expression. Total RNA was isolated from different *B. juncea* samples using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). For qRT-PCR, 1 μg of total RNA was reverse-transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen, Carlsbad, CA). Transcription levels were analyzed by real-time PCR. The gene-specific primer sets were designed for real-time PCR on the basis of the published genes in the GenBank (accession numbers of *GAD 1*, *GAD 2*, *GAD4a*, and *GAD4b* are AY559321, AY559318, AY559319, and AY559320, respectively). Gene expression was normalized to that of the house-keeping gene actin (accession number HM565958). Real-time PCR reactions were performed in triplicate with a MiniOpticon system (Bio-Rad Laboratories, Hercules, CA) with the Quantitect SYBR Green PCR Kit (Qiagen). The PCR protocol was as follows: denaturation for 5 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 56°C, and elongation for 20 s at 72°C. PCR results were calculated as the mean of three replicated treatments. Statistical differences between treatments were evaluated by standard deviation.

Extraction of Amino Acids from *B. juncea*. The samples were freeze-dried and then ground to powder, 0.10 g of powdered sample was extracted with 1.2 mL of water containing trichloroacetic acid (TCA) solution [5% (v/v)], followed by vortexing for 15 s, and then the sample was stored for 1 h at room temperature and centrifuged at 15 000 rpm and 4°C for 15 min. After centrifugation, the supernatant was filtered through a 0.45 μm PTFE hydrophilic syringe filter (Ø 13 mm) into an HPLC-vial and then used for HPLC analysis.

Quantitative Analysis of Amino Acids and GABA by HPLC. The external standards for free amino acid standards were provided from the Agilent Technologies (Waldbronn, Germany). GABA [γ(gamma)-aminobutyric acid] and sodium phosphate monobasic monohydrate ($Na_2HPO_4 \cdot H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid (TCA, 99.0%) was obtained from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea), and HPLC-grade acetonitrile (CH_3CN) and MeOH were purchased from J. T. Baker (Phillipsburg, NJ). The detection of amino acids is available with automated derivatization using *o*-phthalaldehyde (OPA) and online analyzed by reversed-phase HPLC (RP-HPLC) with ultraviolet–visible (UV–Vis) detection, according to a method published in an Agilent application note.[34] Briefly, filtered samples were determined using a Zorbax Eclipse AAA analytical column (150 × 4.6 mm i.d., 5 μm) and an Agilent 1200 high-performance liquid chromatograph with a UV–Vis detector and autosampler. The analysis was monitored at 338 nm, and the column was maintained at 40°C. The mobile phase was a gradient prepared from mixtures of 40 mM $NaH_2PO_4 \cdot H_2O$ (solvent A, pH 7.8) and CH_3CN:MeOH:water (45:45:10, v/v/v) (solvent B). The flow rate was set at 2.0 mL/min, and the injection volume was 20 μL. The gradient programs were as follows: 0–1.9 min, 0% solvent B; 1.9–21.1 min, 57% B; 21.1–21.6 min, 100% B; 21.6–25 min, 100% B; 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 30 min). Quantification of the different compounds was based on peak areas and calculated as equivalents of representative standard compounds. All contents are expressed as milligrams per 100 g of fresh weight. Each result shown in the figures and tables is the mean of three replicated treatments.

Statistical Analysis. Each result shown in the figures is the mean of three replicated treatments. Standard deviations are provided to indicate the variations associated with the particular mean values. Significant differences between treatments were evaluated statistically using Statistical Analysis Software 8.2 (Student's *t* test). Values of $p < 0.05$ are considered statistically significant.

RESULTS

Effect of Light on the Biomass of *B. juncea* Sprouts. In 3 day hypocotyls, the purple-leaf cultivar showed a slightly deeper purple color than the green-leaf cultivar (Figure 1). The length and fresh weight of both *B. juncea* cultivars were measured from 3 to 12 days after germination. The length and fresh weight in both *B. juncea* cultivars increased over time (Figure 2). Sprouts were 2–4-fold taller in the dark than in light/dark conditions. The green-leaf cultivar showed slightly higher fresh weight than the purple-leaf cultivar, especially in dark culture. The length and fresh weight of both cultivars did not increase significantly after 9 days of culture (Figure 2).

Figure 1. Development of *B. juncea* sprouts under light/dark and dark conditions: G-L3, green cultivar, 3 days light/dark; G-L6, green cultivar, 6 days light/dark; G-D3, green cultivar, 3 days dark; G-D6, green cultivar, 6 days dark; P-L3, purple cultivar, 3 days light/dark; P-L6, purple cultivar, 6 days light/dark; P-D3, purple cultivar, 3 days dark; P-D6, purple cultivar, 6 days dark. The bar represents 1 cm.

Amino Acid Content in *B. juncea* Sprouts. In this study, 9 essential or semiessential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and histidine) and 12 nonessential amino acids (arginine, alanine, asparagine, aspartate, cystine, glutamine, glycine, GABA, glutamate, serine, tyrosine, and vitamin U (Supporting Information, Supplementary Figures 1 and 2) were detected in *B. juncea* sprouts. The amino acid content was much higher in the sprouts of green and purple mustard than in the seeds. The content of all amino acids was elevated in the sprouts of both *B. juncea* cultivars under light/dark and dark conditions. Light conditions inhibited synthesis of arginine, glutamine, cystine, histidine, methionine, tryptophan, phenylalanine, leucine, and lysine during sprout development in both cultivars. The accumulation of these amino acids increased at 6, 9, or 12 days under dark conditions (Tables 1 and 2), whereas the contents of glycine, arginine, and isoleucine, which were up-regulated by light conditions, reached the maximum after 9 or 12 days. On the basis of the total amino acid content, both cultivars have approximately double the amount of amino acids in dark-treated sprouts. At 9 days, dark-treated purple-leaf seedlings contained the highest amount of amino acids (746 mg/100 g of fresh weight) (Table 1) than at any other day in either cultivar.

dx.doi.org/10.1021/jf401956v | J. Agric. Food Chem. 2013, 61, 8624–8631

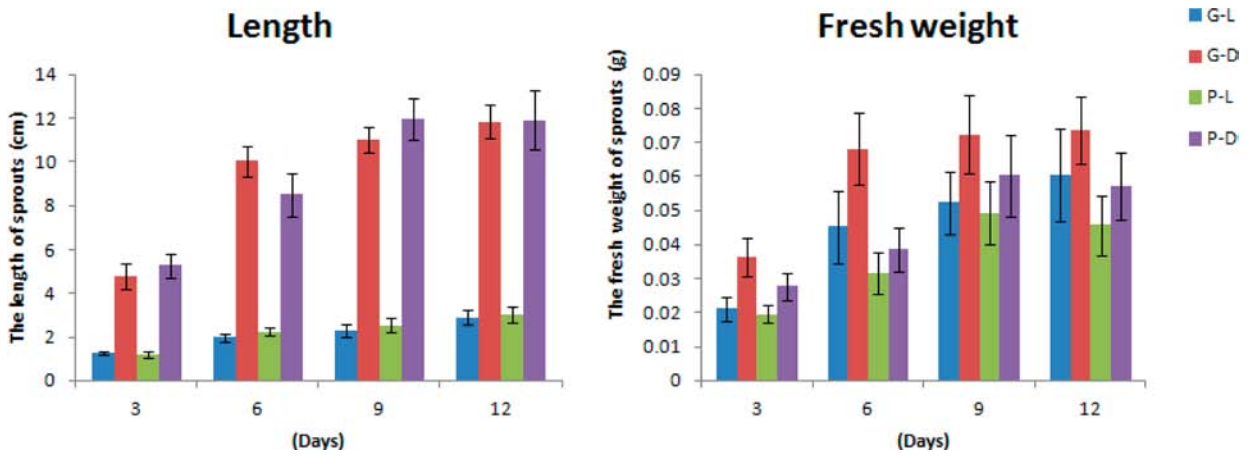

Figure 2. Length and fresh weight of green- and purple-leaf *B. juncea* sprouts under light/dark or dark conditions. G-L and G-D indicate green cultivar in light/dark conditions and green cultivar in dark conditions; P-L and P-D indicate purple cultivar in light/dark conditions and purple cultivar in dark conditions. Each value represents the mean of three replicates, and error bars indicate the standard deviation.

The Effect of Light on the Expression of *GAD* Genes in *B. juncea*. Expression of GABA biosynthesis genes *BjGAD1*, *BjGAD2*, *BjGAD4a*, and *BjGAD4b* were detected in the light/dark- and dark-treated mustard sprouts by qRT-PCR (Figure 3). Transcript levels of *BjGAD1*, *BjGAD4a*, and *BjGAD4b* were markedly reduced after germination in both cultivars. The seeds of purple-leaf mustard showed 2−4-fold higher expression than the seeds of green-leaf mustard. Both green and purple cultivars showed higher *GAD* gene expression in dark than in light conditions during the seedling stage, indicating the dark environment could induce higher *GAD* gene expression than the light elicitor. Transcript levels of *BjGAD1*, *BjGAD4a*, and *BjGAD4b* were 2−3 times higher at 6 or 9 days in purple-leaf mustard than in green-leaf mustard. After 9 days, expression of the *GAD2* gene started to decrease in both green- and purple-leaf mustard sprouts. Compared to other *GAD* genes, *GAD2* was more active during seedling development and was particularly higher under dark culture conditions.

Effect of Light on the GABA Accumulation in *B. juncea*. Accumulation of GABA in the sprouts of both *B. juncea* cultivars differed from the amino acid content and gene expression in response to light/dark or dark treatment. Previously, we found that amino acid accumulation and *GAD* gene expression were higher in the dark-cultured sprouts of both *B. juncea* cultivars, but the GABA content was higher in both cultivars under light conditions (Figure 4). Purple-leaf mustard seeds accumulated 4-fold more GABA than did the green cultivar seeds. The GABA content decreased markedly at the beginning of germination and was almost similar in all sprout developmental stages (3−12 days) in the purple-leaf mustard cultivar. In contrast, the GABA content in the seed of the green mustard cultivar was lower than the GABA content in the sprouts. In this cultivar, GABA accumulation increased from the beginning of germination to 9 days and then started to decline. The highest amount of GABA was detected at 9 days in light-treated green- and purple-leaf mustard sprouts. These results indicated that *GAD* gene expression was inhibited by light; GABA accumulation did not decrease under light conditions, especially in the green-leaf mustard.

DISCUSSION

Light is an important factor affecting biomass and secondary metabolite biosynthesis (e.g., flavonoids, anthocyanins, and terpenoids) in plants.[35−40] In the present study, the effects of light on the biomass and amino acids, especially GABA biosynthesis in two mustard cultivars, were investigated. Our results indicate that light/dark and dark treatments have significant effects on mustard sprouts. The length and fresh weight of two mustard cultivars increased in the dark condition and achieved stable levels after a 9 day culture. The total amino acid content in *B. juncea* green- and purple-leaf mustard cultivars increased with the culture time, within 9 days of germination. In particular, the amino acids notably increased in both *B. juncea* mustard cultivars under dark conditions by 9 days. Our results also proved that the light/dark treatment and germination time affected the accumulation of amino acids.[3,41] When considering biomass and total amino acid accumulation, our results suggest that the sprouts of green- and purple-leaf mustard are suitable for harvesting increased amounts of biomass and dietary amino acids after 9 days of culture under the dark condition. Therefore, green- and purple-leaf mustard sprouts could be marketable as a commercial sprout vegetable that is rich in amino acids.

Several cDNAs of the *GAD* gene have been cloned from plants, such as petunias,[20] *Arabidopsis*,[42,43] rice,[44] and tomatoes.[31] Akama et al. reported that the expressions of *OsGAD1* and *OsGAD2* were differentially regulated, especially in the roots and mature seeds of rice, even though both were present in all tissues.[44] Five GAD isoenzymes have been identified and characterized from *Arabidopsis* (L.) Heynh.[42,45] The expression of these five *GAD* genes was obviously different in the organs and in response to hypoxic conditions. In comparison to other isoforms, however, GAD2 was predominantly more highly expressed in the whole plant and was considered to play a unique role in nitrogen metabolism.[42] In our present study, four GAD isoforms from mustard were investigated. The four GAD isoforms indicated different transcription levels during the seedling stage. In comparison to other isoforms, GAD2 showed higher expression levels, which may indicate a higher contribution to GABA biosynthesis in germinating mustard sprouts.

The accumulation of GABA synthesis could be regulated by different factors. Environmental factors such as salt stress,[46] temperature and darkness,[41] hypoxia,[31,47] and water stress[3,48] have been shown to influence GABA synthesis in plants. Recently, lactic acid fermentation was shown to produce new

Table 1. Free Amino Acid Contents (mg/100 g of fresh weight) in Green-Leaf Mustard[a]

no.	name	seeds	L3	D3	L6	D6	L9	D9	L12	D12
1	aspartate	3.56 ± 0.03	16.36 ± 0.05	10.41 ± 0.25	24.65 ± 0.27	19.28 ± 0.06	16.83 ± 0.42	24.08 ± 0.25	8.62 ± 0.09	20.89 ± 0.53
2	glutamate	3.25 ± 0.02	17.19 ± 0.04	15.60 ± 0.12	10.44 ± 0.18	12.81 ± 0.25	12.14 ± 0.21	10.95 ± 0.38	4.63 ± 0.05	6.42 ± 0.14
3	asparagine	5.88 ± 0.010	18.20 ± 0.25	34.39 ± 0.69	23.75 ± 0.32	139.88 ± 0.43	27.17 ± 0.74	202.18 ± 3.24	17.57 ± 0.10	114.75 ± 2.37
4	serine	1.01 ± 0.02	8.85 ± 0.10	12.19 ± 0.22	14.73 ± 0.25	16.53 ± 0.02	29.12 ± 0.91	13.48 ± 0.24	10.29 ± 0.11	6.85 ± 0.13
5	vitamin U	ND[b]	3.29 ± 0.29	3.30 ± 0.04	9.13 ± 0.33	13.82 ± 0.03	14.51 ± 0.35	17.86 ± 0.31	10.94 ± 0.12	12.46 ± 0.26
6	glutamine	2.59 ± 0.05	45.04 ± 1.29	69.79 ± 1.27	20.85 ± 0.83	86.22 ± 0.72	29.61 ± 0.70	74.51 ± 1.76	7.64 ± 0.18	19.84 ± 0.33
7	histidine	0.58 ± 0.01	17.23 ± 0.20	19.65 ± 0.33	13.70 ± 0.27	37.81 ± 0.04	18.31 ± 0.46	37.96 ± 0.74	12.60 ± 0.09	18.72 ± 0.32
8	glycine	0.82 ± 0.01	18.26 ± 0.10	18.06 ± 0.38	20.81 ± 0.17	26.30 ± 0.07	39.53 ± 1.18	24.40 ± 0.39	23.03 ± 0.24	15.82 ± 0.30
9	threonine	0.84 ± 0.02	6.34 ± 0.13	9.62 ± 0.19	7.94 ± 0.14	14.97 ± 0.03	13.19 ± 0.36	12.12 ± 0.18	4.14 ± 0.04	5.76 ± 0.12
10	arginine	3.17 ± 0.27	32.17 ± 0.11	35.88 ± 0.76	127.16 ± 0.70	113.00 ± 0.20	186.95 ± 4.77	148.29 ± 2.86	195.56 ± 2.33	86.33 ± 1.32
11	alanine	1.99 ± 0.03	6.34 ± 0.06	15.99 ± 0.31	5.31 ± 0.08	14.16 ± 0.01	7.57 ± 0.23	5.90 ± 0.32	2.37 ± 0.03	2.59 ± 0.06
12	GABA	1.46 ± 0.03	2.81 ± 0.13	1.92 ± 0.03	2.93 ± 0.12	2.68 ± 0.02	4.73 ± 0.13	2.95 ± 0.06	1.75 ± 0.11	1.52 ± 0.04
13	tyrosine	0.46 ± 0.01	2.64 ± 0.28	5.42 ± 0.09	2.77 ± 0.18	7.21 ± 0.02	5.78 ± 0.13	6.76 ± 0.12	2.64 ± 0.04	3.19 ± 0.09
14	cystine	2.18 ± 0.04	10.07 ± 0.30	9.91 ± 0.19	8.90 ± 0.28	19.88 ± 0.14	10.32 ± 0.33	21.83 ± 0.33	6.44 ± 0.16	12.62 ± 0.34
15	valine	0.95 ± 0.03	5.43 ± 0.12	6.85 ± 0.16	7.33 ± 0.13	11.41 ± 0.06	15.45 ± 0.40	12.02 ± 0.14	4.87 ± 0.05	6.60 ± 0.18
16	methionine	0.49 ± 0.05	5.56 ± 0.55	7.19 ± 0.22	3.85 ± 0.22	30.58 ± 0.28	5.46 ± 0.29	35.33 ± 0.35	3.08 ± 0.04	24.57 ± 0.87
17	norvaline	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	tryptophan	1.04 ± 0.08	11.32 ± 0.44	9.25 ± 0.13	7.05 ± 0.86	13.77 ± 1.42	9.61 ± 0.24	11.18 ± 0.22	6.88 ± 0.03	8.24 ± 2.10
19	phenylalanine	1.22 ± 0.03	2.41 ± 0.15	3.79 ± 0.06	4.80 ± 0.12	10.92 ± 0.03	12.06 ± 0.19	20.13 ± 0.25	12.15 ± 0.24	16.73 ± 0.30
20	isoleucine	0.58 ± 0.02	3.31 ± 0.14	5.82 ± 0.11	6.03 ± 0.14	5.45 ± 0.01	9.94 ± 0.13	3.96 ± 0.06	3.62 ± 0.04	1.76 ± 0.04
21	leucine	0.67 ± 0.02	4.38 ± 1.90	4.29 ± 0.05	6.32 ± 0.17	14.44 ± 0.07	7.79 ± 0.09	14.22 ± 0.30	4.52 ± 0.04	7.10 ± 0.14
22	lysine	0.65 ± 0.01	35.7 ± 0.07	7.34 ± 0.08	11.97 ± 0.07	26.91 ± 0.05	16.69 ± 0.45	33.39 ± 0.66	14.65 ± 0.14	14.68 ± 0.14
total		33.39 ± 0.47	240.77 ± 4.05	306.64 ± 5.52	340.40 ± 4.21	638.04 ± 2.32	492.76 ± 12.52	733.50 ± 12.03	357.98 ± 3.96	407.45 ± 7.51

[a]Each value represents the mean of three replicates ± standard deviation. Key: L3, light/dark for 3 days; L6, light/dark for 6 days; L9, light/dark for 9 days; L12, light/dark for 12 days; D3, dark for 3 days; D6, dark for 6 days; D9, dark for 9 days; D12, dark for 12 days. [b]ND = not detected.

dx.doi.org/10.1021/jf401956v | J. Agric. Food Chem. 2013, 61, 8624−8631

Table 2. Free Amino Acid Contents (mg/100 g of fresh weight) in Purple-Leaf Mustard[a]

no.	name	seeds	L3	D3	L6	D6	L9	D9	L12	D12
1	aspartate	9.76 ± 0.08	14.42 ± 0.55	6.77 ± 0.42	14.39 ± 0.32	6.77 ± 0.42	12.84 ± 0.01	22.64 ± 0.61	10.03 ± 0.22	22.37 ± 0.07
2	glutamate	0.91 ± 0.00	12.46 ± 0.36	10.75 ± 0.49	7.20 ± 0.14	8.68 ± 0.27	10.83 ± 0.08	7.82 ± 0.15	6.64 ± 0.14	6.80 ± 0.06
3	asparagine	8.76 ± 0.08	11.96 ± 0.46	37.34 ± 2.22	20.29 ± 0.41	115.56 ± 2.30	29.79 ± 0.06	212.26 ± 2.60	18.49 ± 0.40	203.95 ± 1.86
4	serine	0.49 ± 0.02	8.01 ± 0.32	18.02 ± 1.20	25.86 ± 0.43	21.61 ± 0.48	36.46 ± 0.10	14.71 ± 0.14	9.11 ± 0.19	12.95 ± 0.14
5	vitamin U	0.20 ± 0.04	2.90 ± 0.11	6.93 ± 0.34	11.69 ± 0.19	16.83 ± 0.32	17.22 ± 0.06	21.81 ± 0.28	9.73 ± 0.26	23.34 ± 0.26
6	glutamine	3.07 ± 0.41	29.62 ± 0.95	88.71 ± 4.98	23.19 ± 0.45	78.93 ± 11.04	34.16 ± 0.05	62.46 ± 5.68	13.34 ± 0.61	35.81 ± 0.60
7	histidine	0.73 ± 0.05	10.71 ± 0.36	23.65 ± 1.29	14.16 ± 0.21	34.94 ± 0.65	19.19 ± 0.05	38.86 ± 0.30	10.91 ± 0.24	39.75 ± 0.45
8	glycine	1.17 ± 0.01	15.55 ± 0.59	23.47 ± 1.32	35.98 ± 0.60	31.28 ± 0.56	67.19 ± 0.21	14.13 ± 0.51	6.85 ± 0.12	29.26 ± 0.36
9	threonine	0.59 ± 0.01	4.82 ± 0.17	17.19 ± 1.00	9.95 ± 0.19	16.35 ± 0.32	14.39 ± 0.90	10.91 ± 0.11	5.40 ± 0.16	9.12 ± 0.07
10	arginine	3.32 ± 0.01	30.19 ± 1.09	39.30 ± 1.95	131.85 ± 2.10	105.01 ± 2.23	212.40 ± 0.62	158.95 ± 0.90	153.16 ± 3.09	169.22 ± 1.85
11	alanine	ND[b]	6.87 ± 0.26	18.13 ± 1.08	7.53 ± 0.16	15.70 ± 0.33	9.54 ± 0.87	5.64 ± 0.02	3.21 ± 0.17	1.06 ± 0.02
12	GABA	6.63 ± 0.06	2.47 ± 0.09	2.05 ± 0.12	2.46 ± 0.04	2.74 ± 0.06	3.84 ± 0.13	2.21 ± 0.04	2.12 ± 0.28	2.56 ± 0.04
13	tyrosine	64.25 ± 0.28	1.90 ± 0.10	7.58 ± 0.42	5.04 ± 0.11	7.00 ± 0.15	7.66 ± 0.05	5.65 ± 0.06	2.25 ± 0.35	5.20 ± 0.05
14	cystine	4.02 ± 0.04	10.88 ± 0.40	13.44 ± 0.84	8.01 ± 0.10	20.71 ± 0.58	11.28 ± 0.05	21.85 ± 0.09	5.67 ± 0.62	23.68 ± 0.29
15	valine	0.76 ± 0.00	4.26 ± 0.18	11.16 ± 0.66	13.31 ± 0.31	12.95 ± 0.31	19.01 ± 0.03	12.80 ± 0.05	5.27 ± 0.31	11.00 ± 0.10
16	methionine	1.22 ± 0.01	3.98 ± 0.19	11.03 ± 1.09	4.85 ± 0.12	35.22 ± 1.18	5.62 ± 0.08	40.17 ± 0.25	2.10 ± 0.45	42.74 ± 0.40
17	norvaline	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	tryptophan	3.93 ± 0.35	11.86 ± 0.44	12.46 ± 0.54	10.45 ± 0.12	16.20 ± 0.32	11.65 ± 0.20	14.17 ± 0.29	5.41 ± 0.81	11.02 ± 1.78
19	phenylalanine	0.98 ± 0.06	1.97 ± 0.06	5.43 ± 0.19	7.93 ± 0.14	15.95 ± 0.23	15.99 ± 0.30	26.89 ± 0.05	9.66 ± 0.54	37.00 ± 0.32
20	isoleucine	0.44 ± 0.01	2.36 ± 0.10	7.97 ± 0.36	8.85 ± 0.18	4.41 ± 0.07	12.95 ± 0.04	3.30 ± 0.09	4.26 ± 0.28	2.86 ± 0.04
21	leucine	0.32 ± 0.06	7.20 ± 0.22	11.41 ± 0.48	2.76 ± 0.06	15.50 ± 0.49	5.54 ± 3.62	17.78 ± 0.36	4.20 ± 0.26	16.41 ± 0.23
22	lysine	0.78 ± 0.02	2.61 ± 0.08	15.97 ± 0.78	12.52 ± 0.15	25.82 ± 0.57	19.80 ± 0.10	30.95 ± 0.11	11.49 ± 0.22	23.95 ± 0.29
total		111.55 ± 1.26	197.02 ± 7.01	388.76 ± 21.68	379.26 ± 6.47	615.68 ± 19.99	577.33 ± 3.50	746.00 ± 3.95	299.30 ± 8.07	730.05 ± 8.66

[a]Each value represents the mean of three replicates ± standard deviation. Key: L3, light/dark for 3 days; L6, light/dark for 6 days; L9, light/dark for 9 days; L12, light/dark for 12 days; D3, dark for 3 days; D6, dark for 6 days; D9, dark for 9 days; D12, dark for 12 days. [b]ND = not detected.

dx.doi.org/10.1021/jf401956v | J. Agric. Food Chem. 2013, 61, 8624−8631

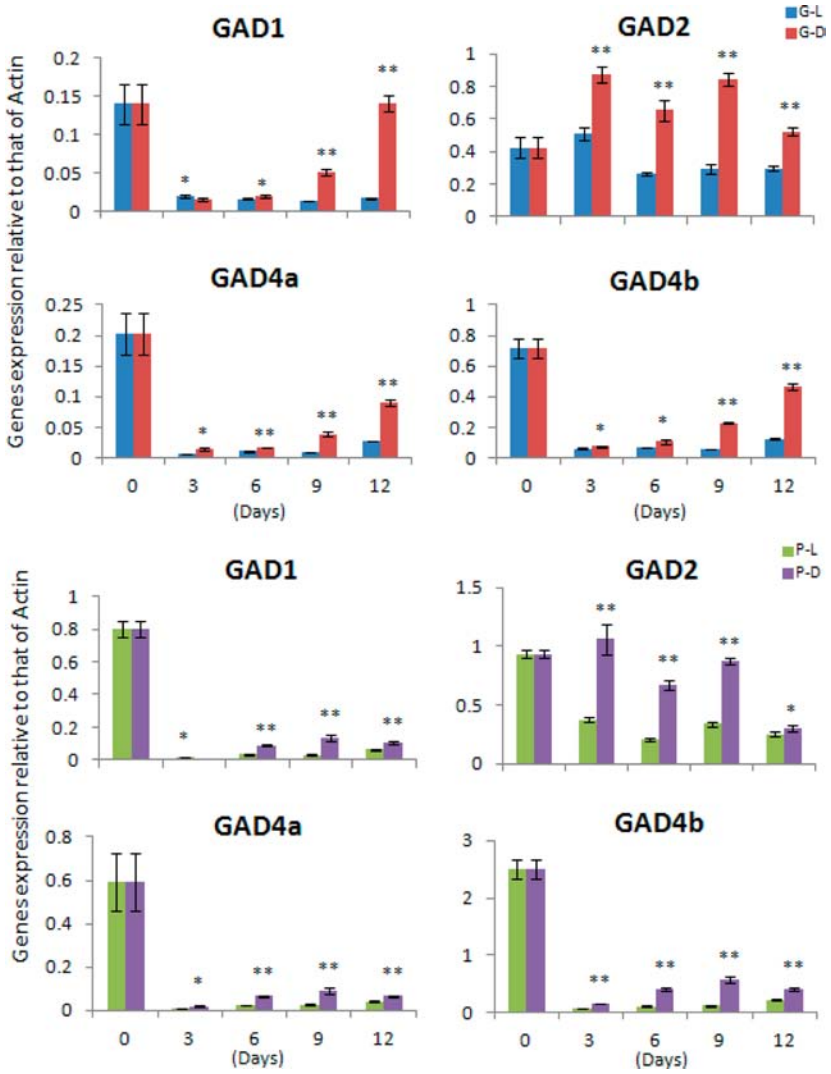

Figure 3. Expression of *GAD* genes involved in GABA biosynthesis during the development of mustard sprouts under light/dark or dark growth conditions. G-L and G-D indicate green cultivar in light/dark conditions and green cultivar in dark conditions; P-L and P-D indicate purple cultivar in light/dark conditions and purple cultivar in dark conditions. Each value represents the mean of three replicates, and error bars indicate the standard deviation. Asterisks indicate significant differences between light/dark-treated and dark-treated samples, as assessed using a Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

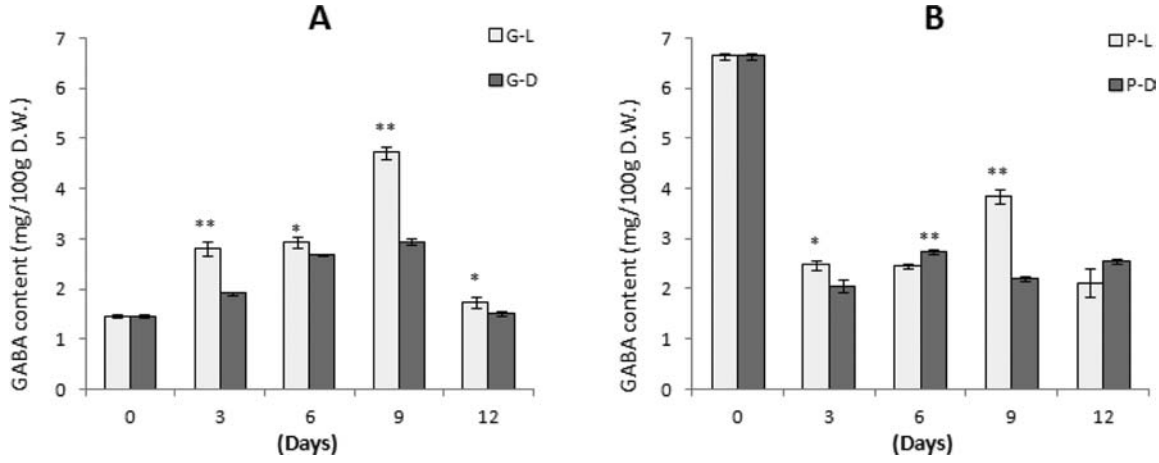

Figure 4. GABA contents in sprouts of green- and purple-leaf mustards (mg/100 g of fresh weight). G-L and G-D indicate green cultivar in light/dark conditions and green cultivar in dark conditions; P-L and P-D indicate purple cultivar in light/dark conditions and purple cultivar in dark conditions. Each value represents the mean of three replicates, and error bars indicate the standard deviation. Asterisks indicate significant differences between light/dark-treated and dark-treated samples, as assessed using a Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

dx.doi.org/10.1021/jf401956v | *J. Agric. Food Chem.* 2013, 61, 8624−8631

and highly potent antihypertensive peptides, with increases in active compounds and GABA content.[49,50] Steeping and germination processes have been reported to effectively accumulate GABA in two oat cultivars,[48] buckwheat[51] and rice.[52] Our data suggest that *GAD* genes are sensitive to a light elicitor (Figure 3), because GABA biosynthesis differed in green- and purple-leaf mustard under light/dark or dark treatments. Interestingly, the correlation between GABA accumulation and transcription levels of the *GAD* genes was not detected in mustard time-course sprouts. Our results are in agreement with those in previous studies on *Arabidopsis* (L.) Heynh, in which the *GAD2* gene did not show a correlation between the transcription level and GABA accumulation in flower stalks and flowers, even though it appeared to be correlated with this activity in vitro.[42]

GABA accumulation is affected by the induction of GAD activity and a number of other factors.[43,46,53] The amount of GABA was shown to induce discrepancy in the GAD activity of *Nicotiana sylvestris* cytoplasmic male sterile II (CMSII) mutants during long-term salt stress,[46] which might be because of the ability of the cells to import GABA or by the induction of the polyamine degradation pathway.[43,54] In addition, in *Arabidopsis thaliana pop2-1* mutants, it was revealed that increased GABA accumulation could be because of the involvement of GABA transaminase in salt stress by the reverse activity of GABA transaminase instead of GAD.[46,55] A similar result was also reported by Yang et al.,[56] who showed that GAD activity was regulated by hypoxia but did not significantly contribute to GABA synthesis in germinating fava bean, and the activity of GABA transaminase was considered to be one of the reasons.[18,56] Moreover, DAO activity, which catalyzes the GABA polyamine degradation pathway, is affected by spectra and varies in different species.[25] The varying GABA content in both the cultivars may be caused by genetic differences, as previously shown for rice and fava beans.[57,58] We may speculate that GABA transaminase or other enzymes may influence GABA synthesis in germinating mustard sprouts. Further studies are required to understand the influence of environmental stresses on GABA biosynthesis in mustard.

■ ASSOCIATED CONTENT

Supporting Information

HPLC chromatograms of amino acid standards and free amino acids under light/dark or dark culture in green- and purple-leaf mustard sprouts. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +82 42 821 5730 (S.U.P.); +82 42 821 6738 (S.-J.K.). Fax: +82 42 822 2631 (S.U.P.); +82 42 822 7142 (S.-J.K.). E-mail: supark@cnu.ac.kr (S.U.P.); kimsunju@cnu.ac.kr (S.-J.K.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Research Group of Vegetable Seed in Golden Seed Project funded by the Korean government.

■ REFERENCES

(1) Labanauskas, C. K.; Stolzy, L. H.; Handy, M. F. Protein and free amino acids in wheat grain as affected by soil types and salinity levels in irrigation water. *Plant Soil* **1981**, *59*, 299−316.

(2) Lorenz, H. Free amino acids in tomato plants in relation to form and concentration of nitrogen in the rooting medium. *Plant Soil* **1976**, *45*, 163−168.

(3) Shen, L.; Foster, J. G.; Orcutt, D. M. Composition and distribution of free amino acids in flatpea (*Lathyrus sylvestris* L.) as influenced by water deficit and plant age. *J. Exp. Bot.* **1989**, *40*, 71−79.

(4) Ghiasi, H.; Paech, C.; Dybing, C. D. Free amino acid content and metabolic activities of setting and aborting soybean ovaries. *Plant Physiol.* **1987**, *85*, 91−95.

(5) Khalil, A. H.; Mansour, E. H. The effect of cooking, autoclaving and germination on the nutritional quality of faba beans. *Food Chem.* **1995**, *54*, 177−182.

(6) Rumiyati, R.; James, A.; Jayasena, V. Effect of germination on the nutritional and protein profile of australian sweet lupin (*Lupinus angustifolius* L.). *Food Nutr. Sci.* **2012**, *3*, 621−626.

(7) Sangronis, E.; Machado, C. J. Influence of germination on the nutritional quality of phaseolus vulgaris and cajanus cajan. *LWT—Food Sci. Technol.* **2007**, *40*, 116−120.

(8) Abderrahim, F.; Huanatico, E.; Repo-Carrasco-Valencia, R.; Arribas, S. M.; Gonzalez, M. C.; Condezo-Hoyos, L. Effect of germination on total phenolic compounds, total antioxidant capacity, maillard reaction products and oxidative stress markers in canihua (*Chenopodium pallidicaule*). *J. Cereal Sci.* **2012**, *56*, 410−417.

(9) Cevallos-Casals, B. A.; Cisneros-Zevallos, L. Impact of germination on phenolic content and antioxidant activity of 13 edible seed species. *Food Chem.* **2010**, *119*, 1485−1490.

(10) Baenas, N.; Moreno, D. A.; Garcia-Viguera, C. Selecting sprouts of Brassicaceae for optimum phytochemical composition. *J. Agric. Food Chem.* **2012**, *60*, 11409−11420.

(11) Cartea, M. E.; Francisco, M.; Soengas, P.; Velasco, P. Phenolic compounds in brassica vegetables. *Molecules* **2010**, *16*, 251−280.

(12) Podsędek, A. Natural antioxidants and antioxidant capacity of brassica vegetables: A review. *LWT —Food Sci. Technol.* **2007**, *40*, 1−11.

(13) Bauddh, K.; Singh, R. P. Growth, tolerance efficiency and phytoremediation potential of *Ricinus communis* (L.) and *Brassica juncea* (L.) in salinity and drought affected cadmium contaminated soil. *Ecotoxicol. Environ. Saf.* **2012**, *85*, 13−22.

(14) Fusco, N.; Micheletto, L.; Dal Corso, G.; Borgato, L.; Furini, A. Identification of cadmium-regulated genes by cDNA-AFLP in the heavy metal accumulator *Brassica juncea* L. *J. Exp. Bot.* **2005**, *56*, 3017−3027.

(15) Minglin, L.; Yuxiu, Z.; Tuanyao, C. Identification of genes up-regulated in response to Cd exposure in *Brassica juncea* L. *Gene* **2005**, *363*, 151−158.

(16) Singh, S.; Sinha, S. Accumulation of metals and its effects in *Brassica juncea* (L.) Czern. (cv. Rohini) grown on various amendments of tannery waste. *Ecotoxicol. Environ. Saf.* **2005**, *62*, 118−27.

(17) Wu, X.-F.; Wang, C.-L.; Xie, E.-B.; Gao, Y.; Fan, Y.-L.; Liu, P.-Q.; Zhao, K.-J. Molecular cloning and characterization of the promoter for the multiple stress-inducible gene *BjCHI1* from *Brassica juncea*. *Planta* **2009**, *229*, 1231−1242.

(18) Bown, A. W.; Shelp, B. J. The metabolism and functions of γ-aminobutyric acid. *Plant Physiol.* **1997**, *115*, 1−5.

(19) Bai, Q.; Chai, M.; Gu, Z.; Cao, X.; Li, Y.; Liu, K. Effects of components in culture medium on glutamate decarboxylase activity and γ-aminobutyric acid accumulation in foxtail millet (*Setaria italica* L.) during germination. *Food Chem.* **2009**, *116*, 152−157.

(20) Baum, G.; Chen, Y.; Arazi, T.; Takatsuji, H.; Fromm, H. A plant glutamate decarboxylase containing a calmodulin binding domain. Cloning, sequence, and functional analysis. *J. Biol. Chem.* **1993**, *268*, 19610−19617.

(21) Knight, M. R.; Campbell, A. K.; Smith, S. M.; Trewavas, A. J. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **1991**, *352*, 524−526.

(22) Snedden, W. A.; Koutsia, N.; Baum, G.; Fromm, H. Activation of a recombinant petunia glutamate decarboxylase by calcium/calmodulin or by a monoclonal antibody which recognizes the calmodulin binding domain. *J. Biol. Chem.* **1996**, *271*, 4148−4153.
(23) Hardt, J.; Larsson, L.-I.; Hougaard, D. M. Immunocytochemical evidence suggesting that diamine oxidase catalyzes biosynthesis of γ-aminobutyric acid in antropyloric gastrin cells. *J. Histochem. Cytochem.* **2000**, *48*, 839−846.
(24) Wakte, K.; Kad, T.; Zanan, R.; Nadaf, A. Mechanism of 2-acetyl-1-pyrroline biosynthesis in *Bassia latifolia* Roxb. flowers. *Physiol. Mol. Biol. Plants* **2011**, *17*, 231−237.
(25) Yang, R.; Chen, H.; Gu, Z. Factors influencing diamine oxidase activity and γ-aminobutyric acid content of fava bean (*Vicia faba* L.) during germination. *J. Agric. Food Chem.* **2011**, *59*, 11616−11620.
(26) Mody, I.; De, K. Y.; Otis, T. S.; Soltesz, I. Bridging the cleft at GABA synapses in the brain. *Trends Neurosci.* **1994**, *17*, 517−525.
(27) Omori, M.; Yano, T.; Okamoto, J.; Tsushida, T.; Murai, T.; Higuchi, M. Effect of anaerobically treated tea (gabaron tea) on blood pressure of spontaneously hypertensive rats. *J. Agric. Chem. Soc. Jpn.* **1987**, *61*, 1449−1451.
(28) Oh, S.-H.; Soh, J.-R.; Cha, Y.-S. Germinated brown rice extract shows a nutraceutical effect in the recovery of chronic alcohol-related symptoms. *J. Med. Food* **2003**, *6*, 115−121.
(29) Bytof, G.; Knopp, S.-E.; Schieberle, P.; Teutsch, I.; Selmar, D. Influence of processing on the generation of γ-aminobutyric acid in green coffee beans. *Eur. Food Res. Technol.* **2005**, *220*, 245−250.
(30) Liu, C.; Zhao, L.; Yu, G. The dominant glutamic acid metabolic flux to produce γ-amino butyric acid over proline in *Nicotiana tabacum* leaves under water stress relates to its significant role in antioxidant activity. *J. Integr. Plant Biol.* **2011**, *53*, 608−618.
(31) Mae, N.; Makino, Y.; Oshita, S.; Kawagoe, Y.; Tanaka, A.; Aoki, K.; Kurabayashi, A.; Akihiro, T.; Akama, K.; Koike, S.; Takayama, M.; Matsukura, C.; Ezura, H. Accumulation mechanism of γ-aminobutyric acid in tomatoes (*Solanum lycopersicum* L.) under low O_2 with and without CO_2. *J. Agric. Food Chem.* **2011**, *60*, 1013−1019.
(32) Xing, S. G.; Jun, Y. B.; Hau, Z. W.; Liang, L. Y. Higher accumulation of γ-aminobutyric acid induced by salt stress through stimulating the activity of diamine oxidases in *Glycine max* (L.) Merr. roots. *Plant Physiol. Biochem.* **2007**, *45*, 560−566.
(33) Youn, Y.-S.; Park, J.-K.; Jang, H.-D.; Rhee, Y.-W. Sequential hydration with anaerobic and heat treatment increases GABA (γ-aminobutyric acid) content in wheat. *Food Chem.* **2011**, *129*, 1631−1635.
(34) Henderson, J. W.; Ricker, R. D.; Bidlingmeyer, B. A.; Woodward, C. *Rapid, Accurate, Sensitive, and Reproducible HPLC Analysis of Amino Acids*; Agilent Technical Note 5980-1193E; Agilent Technologies: Santa Clara, CA, 2000.
(35) Gerhardt, K. E.; Lampi, M. A.; Greenberg, B. M. The effects of far-red light on plant growth and flavonoid accumulation in *Brassica napus* in the presence of ultraviolet B radiation. *Photochem. Photobiol.* **2008**, *84*, 1445−1454.
(36) Han, L.; Dong, B.-c.; Yang, X.-j.; Huang, C.-b.; Wang, X.-d.; Wu, X.-j. Effect of light on flavonoids biosynthesis in red rice *Rdh*. *Agric. Sci. China* **2009**, *8*, 746−752.
(37) Koyama, K.; Ikeda, H.; Poudel, P. R.; Goto-Yamamoto, N. Light quality affects flavonoid biosynthesis in young berries of Cabernet Sauvignon grape. *Phytochemistry* **2012**, *78*, 54−64.
(38) Wang, Y.; Gao, L.; Shan, Y.; Liu, Y.; Tian, Y.; Xia, T. Influence of shade on flavonoid biosynthesis in tea (*Camellia sinensis* (L.) O. Kuntze). *Sci. Hortic. (Amsterdam, Neth.)* **2012**, *141*, 7−16.
(39) Vogt, T.; Ibdah, M.; Schmidt, J.; Wray, V.; Nimtz, M.; Strack, D. Light-induced betacyanin and flavonol accumulation in bladder cells of *Mesembryanthemum crystallinum*. *Phytochemistry* **1999**, *52*, 583−592.
(40) Kawoosa, T.; Singh, H.; Kumar, A.; Sharma, S.; Devi, K.; Dutt, S.; Vats, S.; Sharma, M.; Ahuja, P.; Kumar, S. Light and temperature regulated terpene biosynthesis: Hepatoprotective monoterpene picroside accumulation in *Picrorhiza kurroa*. *Funct. Integr. Genomics* **2010**, *10*, 393−404.
(41) Wallace, W.; Secor, J.; Schrader, L. E. Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiol.* **1984**, *75*, 170−175.
(42) Turano, F. J.; Fang, T. K. Characterization of two glutamate decarboxylase cDNA clones from arabidopsis. *Plant Physiol.* **1998**, *117*, 1411−1421.
(43) Shelp, B. J.; Bown, A. W.; McLean, M. D. Metabolism and functions of γ-aminobutyric acid. *Trends Plant Sci.* **1999**, *4*, 446−452.
(44) Akama, K.; Akihiro, T.; Kitagawa, M.; Takaiwa, F. Rice (*Oryza sativa*) contains a novel isoform of glutamate decarboxylase that lacks an authentic calmodulin-binding domain at the C-terminus. *Biochim. Biophys. Acta, Gene Struct. Expression* **2001**, *1522*, 143−150.
(45) Miyashita, Y.; Good, A. G. Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. *Plant Cell Physiol.* **2008**, *49*, 92−102.
(46) Akçay, N.; Bor, M.; Karabudak, T.; Özdemir, F.; Türkan, İ. Contribution of gamma amino butyric acid (GABA) to salt stress responses of *Nicotiana sylvestris* CMSII mutant and wild type plants. *J. Plant Physiol.* **2012**, *169*, 452−458.
(47) Makino, Y.; Soga, N.; Oshita, S.; Kawagoe, Y.; Tanaka, A. Stimulation of γ-aminobutyric acid production in vine-ripe tomato (*Lycopersicon esculentum* Mill.) fruits under modified atmospheres. *J. Agric. Food Chem.* **2008**, *56*, 7189−7193.
(48) Xu, J. G.; Hu, Q. P.; Duan, J. L.; Tian, C. R. Dynamic changes in γ-aminobutyric acid and glutamate decarboxylase activity in oats (*Avena nuda* L.) during steeping and germination. *J. Agric. Food Chem.* **2010**, *58*, 9759−9763.
(49) Koyama, M.; Naramoto, K.; Nakajima, T.; Aoyama, T.; Watanabe, M.; Nakamura, K. Purification and identification of antihypertensive peptides from fermented buckwheat sprouts. *J. Agric. Food Chem.* **2013**, *61*, 3013−3021.
(50) Komatsuzaki, N.; Shima, J.; Kawamoto, S.; Momose, H.; Kimura, T. Production of -aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol.* **2005**, *22*, 497−504.
(51) Lin, L.-Y.; Peng, C.-C.; Yang, Y.-L.; Peng, R. Y. Optimization of bioactive compounds in buckwheat sprouts and their effect on blood cholesterol in hamsters. *J. Agric. Food Chem.* **2008**, *56*, 1216−1223.
(52) Shu, X.-L.; Frank, T.; Shu, Q.-Y.; Engel, K.-H. Metabolite profiling of germinating rice seeds. *J. Agric. Food Chem.* **2008**, *56*, 11612−11620.
(53) Fait, A.; Yellin, A.; Fromm, H. GABA shunt deficiencies and accumulation of reactive oxygen intermediates: Insight from *Arabidopsis* mutants. *FEBS Lett.* **2005**, *579*, 415−420.
(54) Bhatnagar, P.; Glasheen, B. M.; Bains, S. K.; Long, S. L.; Minocha, R.; Walter, C.; Minocha, S. C. Transgenic manipulation of the metabolism of polyamines in poplar cells. *Plant Physiol.* **2001**, *125*, 2139−2153.
(55) Renault, H.; Roussel, V.; El Amrani, A.; Arzel, M.; Renault, D.; Bouchereau, A.; Deleu, C. The *Arabidopsis pop2-1* mutant reveals the involvement of GABA transaminase in salt stress tolerance. *BMC Plant Biol* **2010**, *10*, 1−16.
(56) Yang, R.; Guo, Q.; Gu, Z. GABA shunt and polyamine degradation pathway on γ-aminobutyric acid accumulation in germinating fava bean (*Vicia faba* L.) under hypoxia. *Food Chem.* **2013**, *136*, 152−159.
(57) Komatsuzaki, N.; Tsukahara, K.; Toyoshima, H.; Suzuki, T.; Shimizu, N.; Kimura, T. Effect of soaking and gaseous treatment on GABA content in germinated brown rice. *J. Food Eng.* **2007**, *78*, 556−560.
(58) Li, Y.; Bai, Q.; Jin, X.; Wen, H.; Gu, Z. Effects of cultivar and culture conditions on γ-aminobutyric acid accumulation in germinated fava beans (*Vicia faba* L.). *J. Sci. Food Agric.* **2010**, *90*, 52−57.