METABOLOME ALTERATION OF GABA RICH GREEN TEA PRODUCED BY VAPOR TREATMENT WITH *trans*-2-HEXENAL

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ABSTRACT

GABA (γ-aminobutyric acid) rich green tea produced by vapor treatment of *trans*-2-hexenal was examined for its possible metabolic pathway using metabolome and transcriptome analysis. Metabolome analysis showed enrichment of GABA (15.1 times higher than control) was accompanied with the increased amount of .pyruvic acid (11.9 times higher than control), alanine (8.5 times higher than control), α-ketoglutaric acid (2.4 time g higher than control), as well as decreased amount of succinic acid (0.8 times as control), aspartic acid (0.4 times as control) and glutamine (0.2 times as control). Based on transcriptome analysis, it was determined that glutamate decarboxylase activity was highly activated (4.1 times higher than control), with the decreased activity of GABA transaminase (0.6 times as control), succinic semialdehyde dehydrogenase (0.7 times as control) and glutamate dehydrogenase activity (0.6 times as control), respectively.

Key words: GABA shunt, glutamate decarboxylase, metabolome, transcriptome

INTRODUCTION

GABA (γ-aminobutyric acid) rich products attract the interests of health conscious people both in developed and developing countries, such as Southeast Asian countries, since GABA is known as one of the major inhibitory transmitters of the central nervous system (Bowery and Smart 2006) and has the potential to inhibit diabetic brain abnormality (Huang et al. 2013), promotes sleep (Cheng et al. 2009), and regulates blood pressure (Abe et al. 1995), among others. The GABA rich product market is now expanding all around the world (Horie et al. 2019) including Southeast Asian countries.

A new method was developed to produce GABA rich tea by *trans*-2-hexenal vapor treatment, which succeeded in enriching GABA content almost 10 times higher than when treated in a ventilation system (Uchida et al. 2022). The metabolome alteration caused by *trans*-2-hexenal vapor treatment, however, is not yet clear. In this article, the study sought to clarify the possible physiological mechanism of GABA enrichment, using metabolome and transcriptome analysis, focusing on the GABA shunt.

MATERIALS AND METHODS

Treatment of tea leaves with *trans*-2-hexenal. Fresh leaves of young green tea, Yabukita cultivar of *Camelia sinensis* L., were harvested at Yoshida Cha-en on June 23, 2018 (1181 Ōtsutsumi, Koga,

Ibaraki prefecture). These were treated with 0, 10, 100 ppm *trans*-2-hexenal for 1, 3, or 6 h with two biological replications, and used for the following experiments.

GABA measurement and metabolome analysis with GC-MS. Each sample (0.1 g of frozen tissue powder) was extracted with 250 µl of methanol and chloroform, one after another vigorously and centrifuged at 12000 rpm for 10 min at room temperature. The supernatant fluid (80 µl) of each sample was corrected into a 1.5 ml plastic tube and evaporated to dryness for 3 h in a centrifuge evaporator (CVE-200D, Tokyo Rikakikai Co, Ltd, Japan). The samples were freeze-dried overnight using a lyophilization container (Modulyo 4K, Edwards, USA). For methylation, 40 µl of methoxylamine (20 mg/ml pyridine) was added to the samples and incubated for 90 min at 37℃ using a dry block bath (EB603, AS ONE company, Japan). Trimethylsilylation was performed by adding 50 µl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) solution for 30 min at 37 ℃. Metabolome analysis including GABA was conducted with GCMS-QP2010 Plus (Shimadzu, Japan), using an electron ionization, on a nonpolar phase column (DB-5, Agilent Technologies, USA) according to Yin et al. (2010) and Ijima and Aoki (2009) with some modifications. Helium gas was used as carrier gas at 2.0 mL/min. The initial column oven temperature was set at 100 ℃ for 4 min, then increased by 4 ℃/min until 320℃ for 10 min. Metabolites were identified by comparing fragment patterns and retention indices with those of standard compounds in databases.

Transcriptome analysis

1. RNA isolation, library construction, and sequencing. The total RNA was isolated from fresh green tea leaves treated with 0, 10, 100 ppm *trans*-2-hexenal for 1, 3, or 6 h with two biological replications. The RNA was extracted with a Trizol reagent (Invitrogen). RNase free DNase (Qiagen, Germany) was used to eliminate genomic DNA contamination. To check the purity of the RNA, gel electrophoresis, nanodrop, and the Agilent 2100 bioanalyzer were used. Highly pure Messenger RNA (mRNA) was isolated from the total RNA using oligo (dT) beads. The Illumina TruSeq RNA Library Prep Kit v2 was used to synthesize the second strand cDNAs library. The Illumina HiSeq 2500 platform was used to sequence the constructed cDNA libraries. Sequencing results were obtained as paired-end reads (2 × 100 bp each) in the FASTQ format.

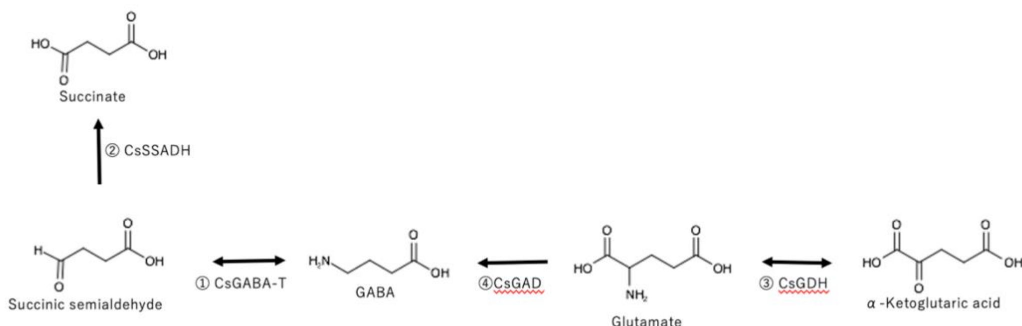
2. *De novo* assembly, ORF detection, and clustering. Raw reads were subjected to quality control by fastaQC (an online tool). Any poor-quality reads and adaptor sequences were filtered by the Trimmomatic and the FASTX-toolkit (Bolger et al. 2014; Gordon et al. 2014). The clean reads were deposited in the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRP128956. The obtained clean reads were assembled into transcriptome, *de novo*, by Bridger (Chang et al. 2015). After the transcriptome was assembled, a TransDecoder was used for the identification of long open reading frames (ORFs) within the transcripts and to score them according to their sequence similarity (Haas et al. 2013). In .order to filter redundancies and to reduce noise in the generated contigs, clustering was performed by the CD-HIT program (Li and Godzik 2006).

3. Gene annotations. Contigs generated by the *de novo* assembly were regarded as the products of tea genes and used as queries for the BLASTX search (Altschul et al. 1990) to examine which protein they encode. For the BLASTX search, non-redundant protein sequences were used as the database. On the basis of the results of the BLASTX search, functional annotations were assigned to the contigs.

4. Differential gene expression and pathway enrichment analysis. To obtain the lists of differentially expressed genes (DEGs) in the control and *trans*-2-hexenal treated green tea, the CLC genomic workbench version 9.5 (QIAGEN) was used. The Empirical Analysis of Differential Gene Expression (EDGE) test was implemented to calculate the p-values and the false discovery rate (FDR). The DEGs were filtered as upregulated and downregulated based on the FC (fold change) ≥ 2 or FC ≤ -2

respectively with FDR-corrected p values < 0.01. KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed on the upregulated and downregulated differentially expressed genes in order to map them with various biological pathways (Ogata et al. 1999; Kanehisa, 2002). The PlantTFcat online tool (http://plantgrn.noble.org/PlantTFcat/) was used to identify genes encoding transcription factors (Dai et al. 2013).

5. Validation of the RNA-Seq results by the real-time PCR. Ten randomly selected DEGs were chosen for validation by qRT-PCR. The reaction was performed using TB Green™ Premix Ex Taq™ II Kit (Tli RNaseH Plus) (TaKaRa, Tokyo, Japan) in a volume of 20 µl containing 10 µl of TB Green premix Ex Taq II (2X), 0.4 µl of ROX Reference Dye II, 200 ng of cDNA template, and 0.4 µM of each of the primers. Amplification was performed as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All the experiments were performed in biological triplicate. The tea gene *Css* and *Csa* were used as a reference gene as previously described (Zhang et al. 2021). Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001). The primers used for real-time PCR study are listed in the following Fig. 1.



① CsGABA-T 1 F 5'-TTCACAGATAACAAGTCACCTAAT-3'
R 5'-TCTCACACTCTGCTCCAA-3'
2 F 5'-TAGTATGTTGGCACCATTCAC-3'
R 5'-ACCATAGACCAGCGAGAG-3'
3 F 5'-CGCAGTAGAAGTAGCAGTTG-3'
R 5'-GTCGGTTGTAAGAGATGTGAAT-3'
② CsSSADH 1 F 5'-CCACCAAGTTCCAGAGATAC-3'
R 5'-GCAAGTCCACAGGTAAGG-3'
2 F 5’-ACATTCGCTATAACTTCACCAT-3'
R 5'-TCCTCTCTCGGCAGATTAG-3'
③ CsGDH 1 F 5'-AGGTGGAGTTACGGTTAGTTA-3'
R 5'-GCACGAGCAACACGATTA-3'
2 F 5'-TTGTCCTCCTATTACCTCCAT-3'
R 5'-GAATCCAAGCCGAGAATGT-3'
④ CsGAD 1 F 5'-AGTGACATCCAGAAAGTCTTGCT-3'
R 5'-CACCATTAGTCTTCTTCCTACTGAG-3'
2 F 5'-TTCGACA TCTGCAAGGTGCTCCA-3'
R 5'-ACTTGTGTTTTCCTAGCCAAGAC-3'
3 F 5'-TTTCACATAACAAATGCAACGTC-3'
R 5'-CTCCCTTTGTCTTACCACCCATA-3'

Fig. 1. Primers of GABA related enzymes used for real time PCR

6. Pathway analysis. GABA pathway assignments were carried out using online KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html).

7. RNA Seq-data submission. The obtained RNA-Seq data were submitted to the DNA Data Bank of Japan (DDBJ) under the following accession numbers:

Submission: DRA013348 (tomohiro-0491_Submission)
BioProject: PRJDB12914 (PSUB016606)
BioSample: SAMD00439333-SAMD00439341 (SSUB020458)
Experiment: DRX327085-DRX327093 (tomohiro-0491_Experiment_0001-0009)
Run: DRR338129-DRR338137 (tomohiro-0491_Run_0001-0009)

RESULTS AND DISCUSSION

The metabolome alteration caused by 10 ppm *trans*-2-hexenal vapor treatment on green tea leaves is shown in Table 1. Three hour vapor applications of *trans*-2-hexenal to green tea leaves in ventilation system enhanced GABA content up to 0.98μmol/g, which was 15.1 times higher than control (0.06μmol/g). Increased amount in pyruvic acid (11.9 times higher than control), alanine (8.5 times higher than control) and α-ketoglutaric acid (2.4 times higher than control), and decreased amount of succinic acid (0.8 times as control), aspartic acid (0.4 times as control) and glutamine (0.2 times as control) were accompanied with GABA increase.

Table 1. Metabolome alteration caused by 10 ppm *trans*-2-hexenal vapor treatment on green tea leaf

	Control (0 h)	Treatment (3 h)	Ratio
GABA	0.06	0.93	15.1
Pyruvic acid	4.85	57.5	11.9
Alanine	0.12	1.02	8.5
α-Ketoglutaric acid	0.45	1.10	2.4
Succinic acid	0.22	0.18	0.8
Aspartic acid	0.53	0.20	0.4
Glutamine	1.62	0.39	0.2

Data are shown in μmol/g and the ratio is calculated by division of treatment value by control value.

As for the transcriptome analysis, the changes in the activities of main enzymes concerning to GABA metabolism are shown in Table 2. The data compares the values of control and 10 ppm *trans*-2-hexenal treatment for 3 h. The increase in GABA could well be attributed to the increased gene expression of glutamate decarboxylase (4.1 times higher than control), and decreased expression of GABA transaminase (0.6 times as control), succinic semialdehyde dehydrogenase (0.7 times as control) and glutamate dehydrogenase (0.6 times as control).

Table 2. Transcriptome alteration caused by 10 ppm *trans*-2-hexenal vapor treatment on green tea leaf.

	Control (0 h)	Treatment (3 h)	Ratio
GABA transaminase①	3.22	1.78	0.6
Succinic Semialdehyde dehydrogenase②	1.34	0.91	0.7
Glutamate dehydrogenase③	0.77	0.50	0.6
Glutamate decarboxylase④	0.93	3.67	4.1

Data are shown in TPM (Transcripts Per Million) and the ratio is calculated by division of treatment value by control value.

The possible physiological mechanism of the enhancement of GABA by *trans*-2-hexenal vapor treatment is based on the results of both metabolome analysis (Table 1) and transcriptome analysis (Table 2) as shown in Figure 2 .

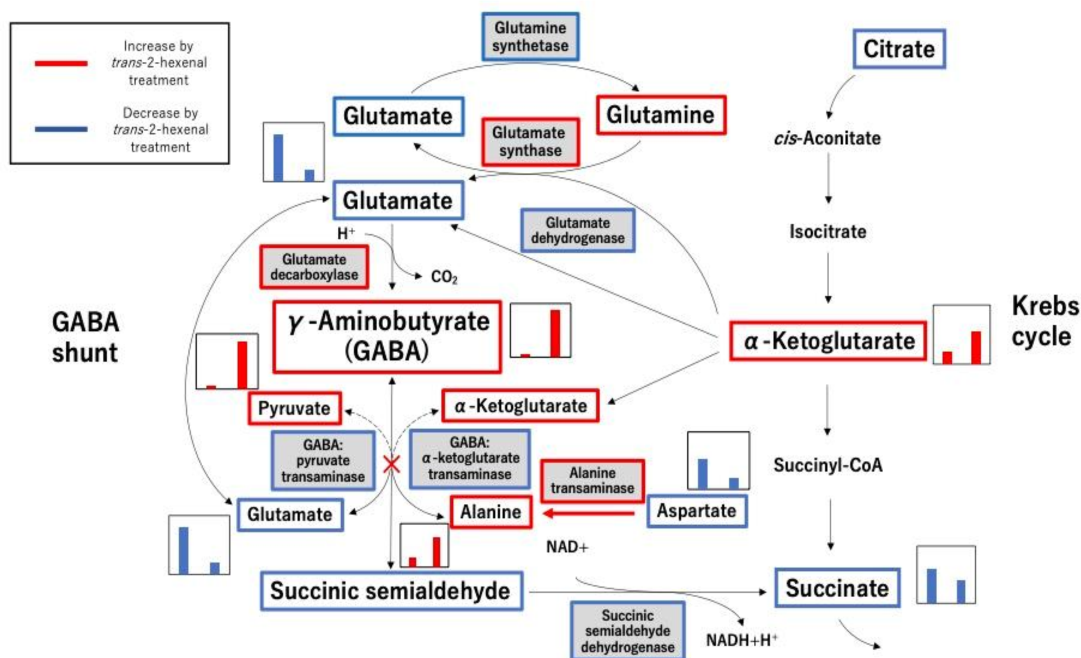


Fig. 2. Possible mechanism of GABA increase in *trans*-2-hexenal vapor treated green tea leaves

Considering the biosynthesis of GABA, the increased GABA content might primarily be derived from the activation of glutamate decarboxylase (4.1 times higher than control) which directly changes glutamate into GABA. This result can be well supported by the reports of Tsushida and Murai (1987), which mentioned the activation of the identical enzyme under anaerobic conditions through the process of Gabaron tea production. The mechanism of stress reaction of tea is different from the one of Arabidopsis, characterized by the accumulation of GABA which is accompanied with the activation of CsGAD1 (tea glutamate decarboxylase 1) through the regulation by calmodulin as well as the accumulated mRNA of CsGAD2 (tea glutamate decarboxylase 2) (Mei et al. 2016). Glutamate, a direct precursor of GABA, could be provided from the enriched amount of glutamine mainly by the activation of glutamate synthase (3.9 times higher than control, although not shown in Table 2), rather than by glutamate dehydrogenase, which was deactivated down to 0.7 times as control. The enriched α-ketoglutaric acid could also serve as a rich precursor of glutamate.

As for the catabolism of GABA, the deactivation of both GABA transaminase (0.6 times as control) and succinic semialdehyde dehydrogenase (0.7 times as control), might well contribute to the higher amount of GABA which remained without catabolism. The activation of glutamate decarboxylase was observed under anaerobic conditions in tomato fruit (Mae et al. 2012), and it was considered to be caused by the activation of glutamate decarboxylase as well as the deactivation of α-ketoglutaric acid dependent GABA transaminase. The present experimental results also support the similar metabolic alteration.

CONCLUSION

A new method to produce GABA rich green tea was recently developed by *trans*-2-hexenal vapor treatment and a possible metabolome alteration concerning the GABA shunt. From the analysis of metabolome and transcriptome, the main reason for enriched GABA by *trans*-2-hexenal vapor treatment could be attributed to the activation of glutamate decarboxylase with decreased activity of GABA transaminase and succinic semialdehyde dehydrogenase.

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