

MOLECULAR CLONING AND CHARACTERIZATION OF GLUTAMATE DECARBOXYLASE CDNA FROM THE GIANT-EMBRYO *ORYZA SATIVA*

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Abstract - A full-length cDNA encoding glutamate decarboxylase (designated as OsGAD3), which catalyzes the conversion of glutamate to gamma-aminobutyric acid (GABA), was isolated from the GABA-rich giant-embryo *Oryza sativa* (Shangshi Jing 315). The full-length cDNA of OsGAD3 (SSJ315) has a 1479 bp open reading frame (ORF) encoding a protein of 492 amino acid residues. The deduced protein had an isoelectric point (pI) of 5.72 and a calculated molecular weight of 56.1 kD. Sequence comparison showed that OsGAD3 (SSJ315) matches the glutamate decarboxylases of other plant species reported previously. Analysis of the structural features of the C-terminal portions of plant GADs revealed that OsGAD3 (SSJ315) has the typical CaM-binding domain (CaMBD) in the C-terminal region as most other plant GADs. Evolution analysis showed that plant GADs are conserved in the process of evolution. The cloning and characterization of the OsGAD3 (SSJ315) gene will enable us to use OsGAD3 to enhance GABA production in *O. sativa* (SSJ315) by metabolic engineering in the near future.

Key words: Cloning, RT-PCR, OsGAD3, *Oryza sativa*

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INTRODUCTION

Hypertension is a common chronic disease and is regarded as one of the most critical risk factors causing serious damage to human health (Liu et al., 2005; Akama et al., 2009). Reports indicate that nearly one billion people are now suffering from hypertension in the world (Kearney et al., 2005). Up to now, patients with severe hypertension mainly depend on different kinds of blood pressure-lowering drugs (Liu et al., 2005; Akama et al., 2009), but they have several shortcomings, including the high cost of drugs for long-term treatment, development of drug dependence in patients and side effects (Liu et al., 2005; Akama et al., 2009). Therefore, it is necessary to develop a new effective way for the treatment of hypertension.

Recently, foods or diets that contain bioactive substances with anti-hypertensive effects have received more attention (Akama et al., 2009). Gamma-aminobutyric acid (GABA), as non-protein amino acid that is a well-characterized inhibitory

neurotransmitter for animals with hypotensive and analgesic properties (Erlander et al., 1991; Hayakawa et al., 2004). Therefore, food-based interventions to increase the availability of GABA-rich foods and their consumption have been suggested as an alternative way for the treatment of hypertension (Tang et al., 2009). For example, several giant-embryo rice varieties or lines which are rich in GABA (which has the function of reducing blood pressure) such as Haiminori, Giant-embryo No.1, 6601, W025, Gama-1, TgeA and 98-14geA, have been selected by mutant or cross introgression recently (Zhang et al., 2007).

More recently, the development of plant metabolic engineering provides a possibility for the further improvement of GABA content by transferring the key genes involved in the GABA biosynthetic pathway into GABA-rich giant-embryo rice. However, this significantly relies on the detailed understanding of the pathway for GABA biosynthesis, the enzymes catalyzing the reaction chain, especially the rate-limiting steps and the genes encoding these proteins.

GABA is metabolized mainly via a short, three-enzyme pathway known as the GABA-shunt because it bypasses two successive steps of the tricarboxylic acid (TCA) cycle. Glutamate decarboxylase (GAD, EC 4.1.1.15) catalyzes the conversion of glutamate to GABA; then GABA transaminase (GABA-T) catalyzes the conversion of GABA to succinic semialdehyde (SSA), which is then oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH). Thus, the carbon skeleton of GABA enters the TCA cycle (Wu et al., 2006). Among the three enzymes, GAD, which catalyzes the irreversible decarboxylation of glutamate in the presence of the cofactor pyridoxal 5'-phosphate (PLP), is considered to be the key enzyme for the biosynthesis of GABA (Huang et al., 1990). Data from the rice genome project show that there exist five GAD genes in rice (Project IRGS 2005). In the past 10 years, *OsGAD1* and *OsGAD2* have been intensively investigated (Akama et al., 2009; Akama et al., 2001; Akama et al., 2007). However, little is known about other family members such as *OsGAD3*.

Here we report on the cloning and characterization of the *OsGAD3* gene from the GABA-rich giant-embryo of rice (Shangshi jing 315, SSJ315). Homologous alignment was performed to characterize the catalytic function of *OsGAD3* (SSJ315). Structural feature analysis of the C-terminal portions of GADs and the evolution profiles of *OsGAD3* (SSJ315) were also investigated. This work should provide further understanding of the important step involved in GABA biosynthesis in the GABA-rich giant-embryo rice at the molecular level.

MATERIALS AND METHODS

Materials

Seeds of *O. sativa* (SSJ315), which were the GABA-rich giant-embryo rice and the hybrid offspring from "Haiminori" and "Xiushui 110", obtained from Prof. Dong (Shanghai Normal University), were grown in pots in the greenhouse of our laboratory under 30°C with 12 h light period (white fluorescent tubes: irradiance of 350 μmol m⁻²s⁻¹)

and relative air humidity of 60%. The pMD-18T vector and one Step RNA PCR Kit was purchased from TaKaRa Biotechnology Co., Ltd. Primers' synthesis and DNA sequencing was performed by the Shanghai Sangon Biotechnological Company, China. All the other chemicals used were of analytical grade.

RNA extraction

The leaves (0.3 g) were excised from two-week-old *O. sativa* (SSJ315), pulverized in liquid nitrogen with mortar and pestle, and the total cellular RNA was extracted using the method reported previously. The quality and concentration of the extracted RNA were checked and stored as previously described (Kai et al., 2006). The RNA samples were stored at -80°C until use.

Isolation of the full-length cDNA of GAD3 from Oryza sativa (SSJ315)

The cDNA was synthesized using the TaKaRa RNA PCR Kit (TaKaRa, Japan). Using cDNA as a template, RT-PCR was carried out in order to obtain *OsGAD3*. The reverse transcription reactions were performed by the sequential addition of the following reagents: 50 ng cDNA template, 25 mM MgCl₂, 1 μl 10×RT buffer, 10 mM dNTPs, 0.25 μl RNase inhibitor, 0.5 μl AMV reverse transcriptase and 0.5 μl primers. The amplified product was purified and cloned into a pMD-18T vector and transformed into *E. coli* DH5α. Based on the color reaction using the Xgal-IPTG System and PCR identification, the positive clones were picked out and sequenced by ABI 3730 Sequencer (Perkin-Elmer, USA).

Bioinformatics analysis

Several websites and bioinformatics softwares were used for the analyses of *OsGAD3*. The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) were analyzed, and the sequence comparison was conducted through a database search using the BLAST program (NCBI, National Center for Biotechnology Services, <http://www.ncbi.nlm.nih.gov>). SOPMA analyses

1 **a**tgggtctccaaggccgtccgagagtgcacatgtccgtcactccaccttcgcctcc
 M V L S K A V S E S D M S V H S T F A S
 61 cgctacgtccgcgcctcccccaggatccggatgccgagaactcgatcccgaaggag
 R Y V R A S L P R Y R M P E N S I P K E
 121 gccgcgtaccagatcatacaacgcgagctgtggacggcaacccggcgctgaacctg
 A A Y Q I I N D E L M L D G N P R L N L
 181 gcgtcgttcgtcaccacgtggatggagcccgatgcgcacaagctcatatggccgcac
 A S F V T T W M E P E C D K L I M A I
 241 aacaagaactacgtgcacatggacgagatccccgtcaccaccggagctccagaacccgg
 N K N Y V D M D E Y P V T T E L Q N R C
 301 gtgaacatgtcgccacccgttccacgcgcgcgtcgggaggacgagacggcggtggc
 V N M I A H L F H A P L G E D E T A V G
 361 gtggcacggtgatgtcgaggccatcatgtggccggctggccitcaagcggcgg
 V G T V S S S E A I M L A G L A F K R R
 421 tggcagaacaagcgcacggcgagggaagccgtcgacaagccaaacatcatcaccggc
 W Q N K R K A E G K P F D K P N I I T G
 481 gccaacgtgcagggtgtcgggagaagtcgcggctacttcgagggtggagctcaaggag
 A N V Q V C W E K F A R Y F E V E L K E
 541 gtgaagctccgcacggctactacgtcatggaccccgagaaggccgtcgacatggtaac
 V K L R D G Y Y V M D P E K A V D M V N
 601 gagaacaccatctcgctgcgcgcacccctcgacccctcaacggcgagttcgaggac
 E N T I C V A A I L G S T L N G E F E D
 661 gtcaagctactcaacgcacctctcgacaagaacaagaaggagactgggtggagacggc
 V K L L N D L L D K K N K E T G W E T P
 721 atccacgtggacgcggcgagcggcggtcatcgccgttccgttacccggagctggag
 I H V D A A S G G F I A P F L Y P E L E
 781 tggactccggctccgtggtaagagcatcaacgtgagcggcacaagtacggc
 W D F R L P W V K S I N V S G H K Y G L
 841 gtctacggccatcggtgtgcattggcaacaaggaggacgtccggaggagct
 V Y A G I G W C I W R N K E D L P E E L
 901 atctccacatcaactacccggcaccgaccggccacccctcaacttccaag
 I F H I N Y L G T D Q P T F T L N F S K
 961 ggctccaggccatcgcccgactaccatcgccgttccgttac
 G S S Q V I A Q Y Y Q L I R H G F E G Y
 1021 aggaacatcatggagaactgccacgagaacgcgttgtgaagggctggtaag
 R N I M E N C H E N A M V L K E G L V K
 1081 acggggagggtcgacatcggttccaaaggacgaagggtgcggctggcgatcgct
 T G R F D I V S K D E G V P L V A F S L
 1141 aaggaccggagccggcaccgcgaggatcgagatccgcacatgcgcggcttcggc
 K D R S R H D E F E I S D M L R R F G W
 1201 atcgtccggcgatccatcgccggcaccatcgccggcaccgcgttccgcgtggc
 I V P A Y T M P P D A Q H V T V L R V V
 1261 atccggaggaggtcgccgcaccctcgccgagcgcctcgatcgacatcgagaagg
 I R E E F S R T L A E R L V L D I E K V
 1321 atgtaccatcgacgcgtccctccaggatcgatgcggccgtccgcggcgcgt
 M Y Q L D A L P S R L M P P V P P A P L
 1381 ctgggtcgccaagaagtccggagctcgagacgcgcggcggtgcacggaggcg
 L V V A K K S E L E T Q R S V T E A W K
 1441 aaattcggtcgccaaggaggaccaacggcgctgc*tag* 1479
 K F V L A K R T N G V C *

Fig. 1. The full-length cDNA sequence and deduced amino acid sequence of *OsGAD3* (SSJ315). The start codon (atg) is boxed, the stop codon (*tag*) is underlined italicized.

were performed online (<http://www.expasy.org>). *OsGAD3* and other *GADs* retrieved from GenBank were aligned with CLUSTAL W using default parameters. A phylogenetic tree was constructed using MEGA 2 (Kumar et al., 2001) combined with CLUSTAL W alignments.

RESULTS AND DISCUSSION

Cloning and sequencing of the ORF of OsGAD3 (SSJ315)

The *OsGAD3* was cloned using the primers that were designed according to the *OsGAD3* (*O. sativa japonica* cultivar-group) sequence (AY187941). The ORF of *OsGAD3* was 1479 bp and shared a homology with *OsGAD3* (AY187941) of 99% with BLAST Search in GenBank database (<http://www.ncbi.nih.gov>).

Bioinformatics analysis

The *OsGAD3* from *O. sativa* (SSJ315) contains a 1479 bp ORF encoding a protein of 492 amino acid residues with a calculated molecular mass of 56.1 kDa and a calculated isoelectric point (pI) of 5.72, which is similar to other plant GADs (Akama et al., 2001; Liu et al., 2004).

The results of sequence alignment and BlastP Search in the GenBank database (<http://www.ncbi.nih.gov>) showed that *OsGAD3* (SSJ) had a high homology with many other plant GADs such as *O. sativa* GAD3 (japonica cultivar-group) [OsGAD3 (JCG), AY187941], *Zea mays* (ZmGAD, EU302127), *Citrus sinensis* GAD (CsGAD, DQ001727), *Brassica juncea* GAD4a (BjGAD4a, AY559319), *Brassica juncea* GAD2 (BjGAD2, AY559318), *Nicotiana tabacum* GAD3 (NtGAD3, AF353615), *Arabidopsis thaliana* GAD3 (AtGAD3, NM_126261), *Petunia hybrida* GAD (PhGAD, L16797), suggesting that *OsGAD3* (SSJ) belongs to the plant GAD superfamily. On the amino acid scale, *OsGAD3* (SSJ) shared 99%, 83%, 78%, 78%, 80%, 84%, 85%, 77% sequences with *OsGAD3* (JCG), BjGAD4a, BjGAD2, NtGAD3,

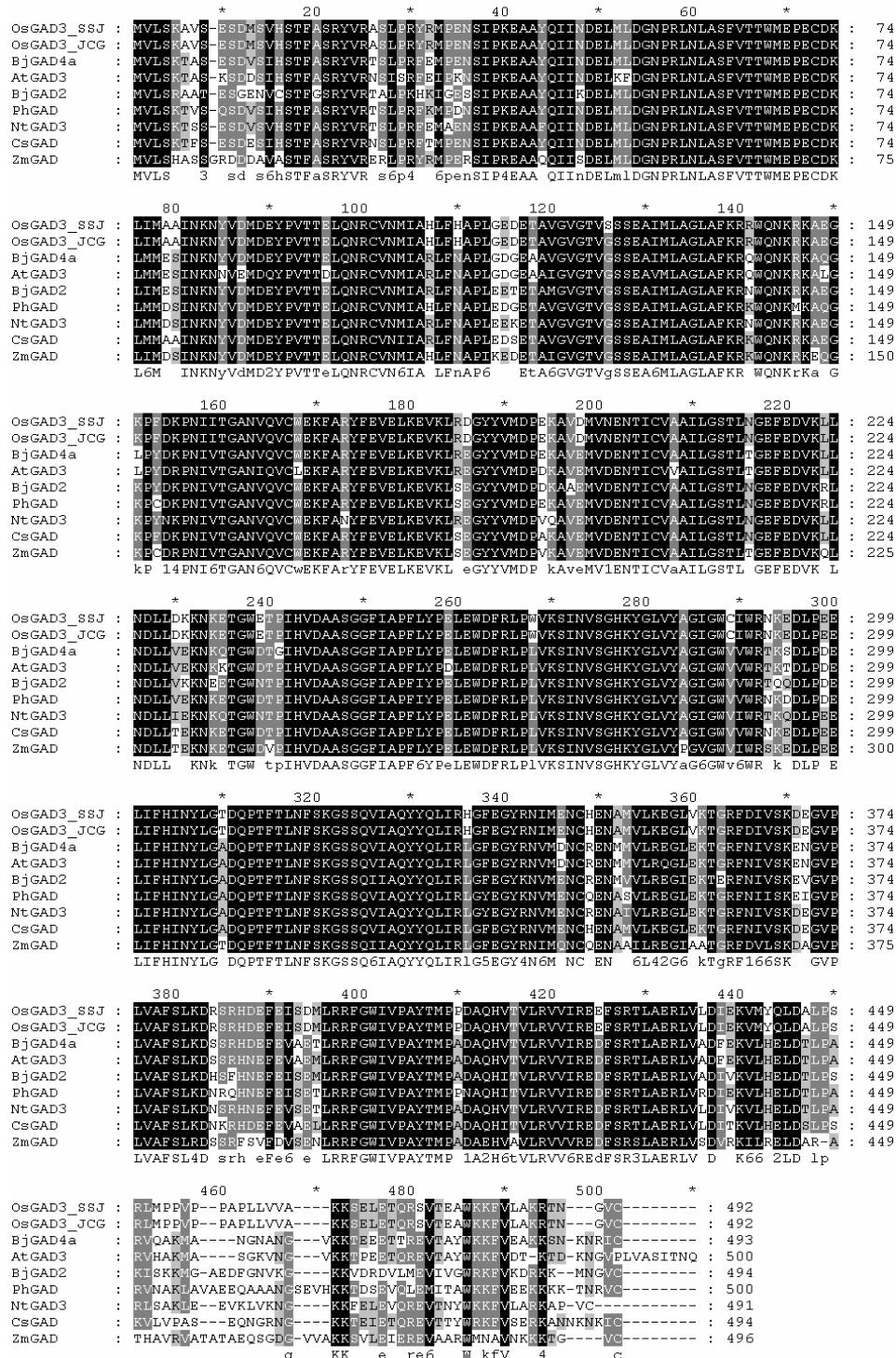
AtGAD3 and PhGAD, respectively, and was 99%, 90%, 88%, 87%, 89%, 91%, 92%, 86% similar to *OsGAD3* (JCG), BjGAD4a, BjGAD2, NtGAD3, AtGAD3 and PhGAD, respectively (Fig. 2), implying that GADs were highly conserved in the plants based on their sequence characteristics and similar roles.

It is well known that a CaM-binding domain (CaMBD) exists in the C-terminal region of most plant GADs (Akama et al., 2001; Akama et al., 2007; Baum et al., 1993). In order to identify whether *OsGAD3* (SSJ315) also contains the typical CaMBD or not, the C-terminal amino acid sequences (the last 32 amino acids) from various plant GADs were compared. As shown in Fig. 3, a Trp (W) residue at the center of this region and a lysine (K) cluster at the C-proximal position (indicated with a box in Fig. 3), which contribute to hydrophobic and electrostatic interactions respectively, were also found in *OsGAD3* (SSJ315). These results reveal that the typical features in *OsGAD3* (SSJ315) are conserved in all the compared plant GAD isoforms.

To investigate the evolutionary relationship of GADs, the phylogenetic tree was constructed based on the deduced amino acid sequences of *OsGAD3* (SSJ315) and other GADs from different organisms including plants, bacteria and animals. The result revealed that GADs are derived from a common ancestor that evolved into three groups; *OsGAD3* (SSJ315) belongs to the plant group and has the closest relationship with the GAD from *Z. mays*, which also belongs to the species of monocotyledonous plants as *O. sativa* (Fig. 4). This suggests that plant GADs are conserved in the process of evolution and GADs from the same species (like monocotyledonous plants) have a closer relationship in the process of evolution.

CONCLUSION

The molecular cloning and characterization of *OsGAD3* (SSJ315) showed that it was very similar to other glutamate decarboxylases, indicating that *OsGAD3* (SSJ315) belongs to a glutamate decarbo-



The completely identical, highly similar and similar amino acids are indicated with “*”, “.” and “.” respectively. The black boxes indicate identical residues; the grey boxes indicate identical residues for at least three of the sequences.

Fig. 2. Multiple alignments of OsGAD3 (SSJ) with other plant GADs.

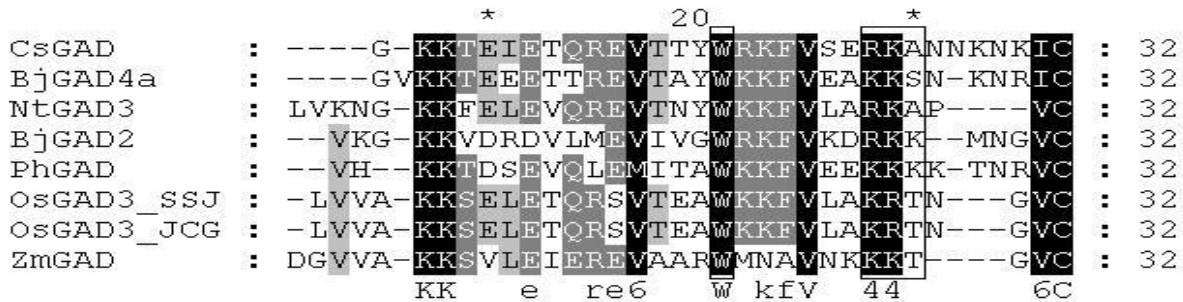


Fig. 3. Structural features of the C-terminal portions of GADs from several plants.

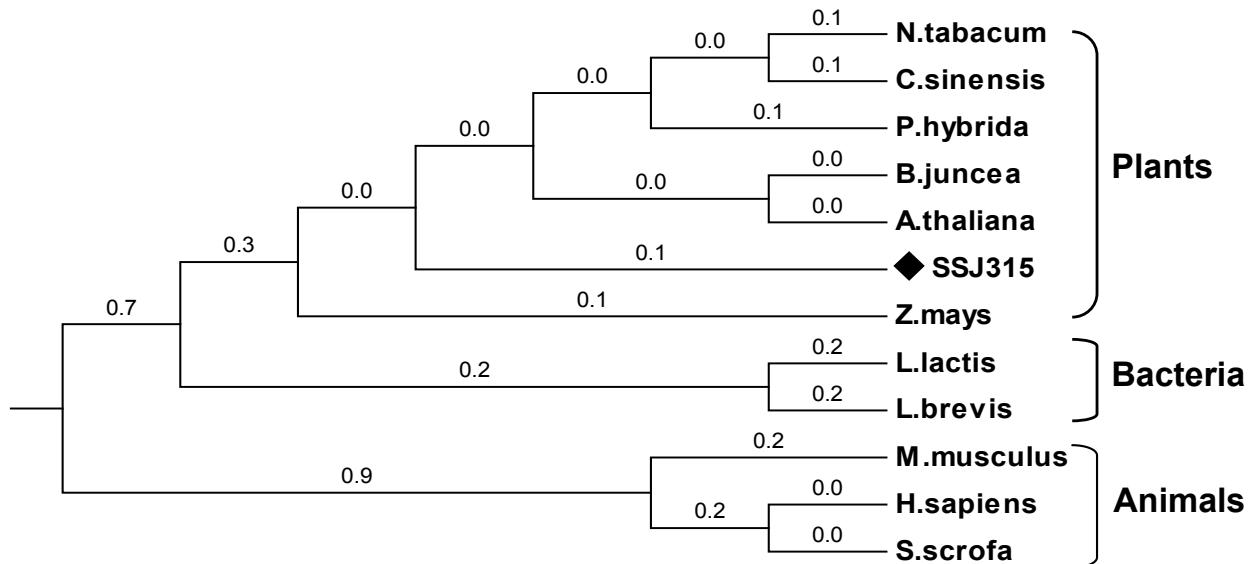


Fig. 4. Structural features of the C-terminal portions of GADs from several plants.

xylase superfamily. OsGAD3 (SSJ315) also possesses the typical CaMBD in the C-terminal region like most other plant GADs. Evolution analysis revealed that OsGAD3 (SSJ315) belongs to the plant group and plant GADs are conserved in the process of evolution. This work should be useful to further understand the important step involved in GABA biosynthesis in GABA-rich giant-embryo rice at the molecular level in the near future.

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REFERENCES

Akama, K., Akihiro, T., Kitagawa, M., and F. Takaiwa (2001). Rice (*Oryza sativa*) contains a novel isoform of

- glutamate decarboxylase that lacks an authentic calmodulin-binding domain at the C-terminus. *Biochimica et Biophysica Acta*, **1522**(3), 143 – 150.
- Akama, K., and F. Takaiwa (2007). C-terminal extension of rice glutamate decarboxylase (OsGAD2) functions as an autoinhibitory domain and overexpression of a truncated mutant results in the accumulation of extremely high levels of GABA in the plant cells. *J Exp Bot*, **58**(10), 2699 – 2707.
- Akama, K., Kanetou, J., Shimosaki, S., Kawakami, K., Tsuchikura, S., and T. Fumio (2009). Seed-specific expression of truncated OsGAD2 produces GABA-enriched rice grains that influence a decrease in blood pressure in spontaneously hypertensive rats. *Transgenic Res*, **18**(6), 865 – 876.
- Baum, G., Chen, Y.L., Arazi, T., Takatsuki, H., and H. Fromm (1993). A plant glutamate decarboxylase containing a calmodulin binding domain. *J Biol Chem*, **268**(26), 19610 – 19617.
- Erlander, M.J., and A.J. Tobin (1991). The structural and functional heterogeneity of glutamic acid decarboxylase. *Neurochem Res*, **1991**, **16**(3), 215 – 226.
- Hayakawa, K., Kimura, M., Kasaha, K., Matsumoto, K., Sansawa, K., and Y. Yamori (2004). Effect of a gamma-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Br J Nutr*, **92**(3), 411 – 417.
- Huang, W.M., Lori, R.F., Wu, E., and J.Y. Wu (1990). Molecular cloning and amino acid sequence of brain L-glutamate decarboxylase. *Proc Natl Acad Sci USA*, **87**(21), 8491 – 8495.
- Kai, G.Y., Miao, Z.Q., Zhang, L., Zhao, D.L., Liao, Z.H., Sun, X.F., Zhao, L.X., and K.X. Tang (2006). Molecular cloning and expression analyses of a new gene encoding 3-hydroxy-3-methylglutaryl -CoA synthase from *Taxus media*. *Biologia Plantarum*, **50**(3), 359 – 366.
- Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K., and J. He (2005). Global burden of hypertension: analysis of worldwide data. *Lancet*, **365**(9455), 217 – 223.
- Kumar, S., Tamura, K., Jakobsen, I.B., and M. Nei (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**(12), 1244 – 1245.
- Liu, L.L., Jiang, L., Liu, S.J., Zhou, S.R., Zhang, W.W., Wang, C.M., Chen, L.M., Zhai, H.Q., and J.M. Wan (2005). Accumulation of the gamma-aminobutyric Acid in Brown Rice-A New Rice Strain W025 with Giant-embryo during Water Soaking. *Acta Agronomica Sinica*, **31**(10), 1265 – 1270.
- Liu, L.L., Zhao, L., Li, Q., Jiang, L., Wang, C.M., Zhang, W.W., Liu, S.J., Chen, L.M., Zhai, H.Q., and J.M. Wan (2004). Molecular cloning and expression of a novel glutamate decarboxylase gene in rice. *Rice Genetics Newsletter*, **21**(16), 39 – 41.
- Tang, G.W., Qin, J., Dolnikowski, G.G., Russell, R.M., and M.A. Grusak (2009). Golden Rice is an effective source of vitamin A. *Am J Clin Nutr*, **89**(6), 1776 – 1783.
- Wu, C.X., Zhou, S.Y., Zhang, Q., Zhao, W.S., and Y.L. Peng (2006). Molecular cloning and differential expression of an gamma-aminobutyrate transaminase gene, OsGABA-T, in rice (*Oryza sativa*) leaves infected with blast fungus. *J Plant Res*, **119**(6), 663 – 669.
- Zhang, X.X., Yuan, L.F., Liu, K., Wei, B.H., and G.L. Luo (2007). Research Progress in Giant-embryo Functional Rice Rich in Gamma-amino Butyric Acid (GABA). *Acta Agriculturae Jiangxi*, **19**(1), 36 – 39.

