Plant Omics Journal POJ

POJ 6(1): 13-17 (2013) ISSN:1836-3644

The effects of glutamate decarboxylase (*GAD*) RNAi knockout in tissue cultured transgenic tomato (*Solanum lycopersicum*)

Bee Lynn Chew[1*], Graham B. Seymour[2]

[1]School of Biological Sciences, Universiti Sains Malaysia, Minden, 11800, Georgetown, Penang, Malaysia
[2]University of Notttingham, Plant and Crop Science Division, Sutton Bonington, Loughborough, LE12 5RD, United Kingdom

***Corresponding author: beelynnchew@usm.my**

Abstract

The amino acid glutamate has commonly been associated with the umami or brothy taste which is classified as the fifth sensation other than sweet, sour, salty and bitter. The production of glutamate in plants is linked to the tricarboxylic acid cycle (TCA) and amino acid metabolism. In this study, the effects of down-regulating the tomato glutamate decarboxylase gene (*GAD*) using an RNAi construct under the control of the 35S promoter are reported. The aim of the experiment was to enhance the levels of glutamate in transgenic tomato plants. The *GAD* gene sequence was isolated from Micro Tom fruit. Tomato cotyledons were transformed with the *GAD* gene fragment using *Agrobacterium tumefaciens* and transformants were observed for the regeneration of transformed plantlets. PCR was performed to confirm the presence of the inserted gene. The infected cotyledons failed to regenerate transformed plantlets using the *GAD* RNAi transgene. A possible explanation is that the construct resulted in altered levels of γ-aminobutyric acid (GABA) and other amino acids known to be essential for plant survival. This work provides further support for the central role of this *GAD* gene in tomato.

Keywords: Gateway cloning. Glutamate. Glutamate decarboxylase. Micro Tom. *Solanum lycopersicum.*
Abbreviations: GABA Gamma-aminobutyric acid; *GAD* Glutamate decarboxylase; MSG Monosodium glutamate; TCA Tricarboxylic acid cycle.

Introduction

Palatability determines human acceptance, preference and consumption of foods. The human tongue is sensitive towards the four taste modalities, sweet, salty, sour and bitter. However, recently a fifth sensation 'umami' or the savoury flavour has been recognized (Ikeda, 1908; Yamaguchi, 1991). Kurihara (2009) reported that the chemical basis of this taste is the salt form of the amino acid glutamate, usually monosodium glutamate (MSG), which produces the umami taste. The umami flavour is mainly influenced by the presence of glutamate, but is potentiated by ribonucleotides such as inosinate and guanylate (Yamaguchi and Ninomiya 2000). The role of this taste modality is thought to be to signal the presence of amino acid rich food necessary for human nutrition and to promote healthy growth and development. Food proteins in meat, seafood and vegetables are main sources of glutamate which is available naturally in the form of glutamic acid (Ninomiya, 1998). Ripe tomato fruit contain high amounts of free glutamic acid with up to 246 mg/100g, one of the highest levels found in fruits and vegetables (Ninomiya, 1998). Umami is an important flavour component in processed food but this is often due to the addition of MSG. This has cost implications for food companies and negative consumer impact as there is a general preference for food without additives. It is therefore important to find strategies to harness the natural bioavailable sources of glutamate in food. The glutamate pathway is summarized in Fig 1. Glutamate decarboxylase (*GAD*) catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA). GABA was first reported in plants by Steward et al. (1949) in potato tubers. Tomato was described as containing high levels of GABA and glutamate (Inaba et al., 1980; Rolin et al., 2000). Rolin et al. (2000) and Carrari et al. (2006) reported levels of glutamate in tomato were closely related to the levels of GABA. Glutamate and GABA are linked with the tricarboxylic acid (TCA) cycle and essential for the synthesis of other amino acids in tomato and other organisms. Several reports have been published relating to the successful manipulation of glutamate levels in transgenic plants. Lightfoot et al. (1999) managed to increase glutamate levels by 1.3 to 1.4 fold in roots of tobacco and corn respectively through transformation with a glutamate dehydrogenase gene. Kisaka et al. (2006) reported transgenic tomatoes with enhanced levels of glutamate through silencing of *GAD* using an antisense transgene obtained from the roots of young tomato plants. These transgenic tomato fruits contained elevated levels of total free amino acids where glutamate was found to be more than 2 times higher compared to the wild type variety. Sorrequieta et al. (2010) also revealed a significant decrease in the levels of γ-aminobutyric acid (GABA) which could be a result of decreased activity of glutamate decarboxylase (*GAD*) that converts glutamate into GABA in tomato. However, a further tomato fruit related *GAD* gene (Gallego et al., 1995) in addition to that used in the experiments of Kisaka et al. (2006) is also known. The *GAD* gene isolated by Kisaka et al. (2006) had only 85% homology to the tomato *GAD* gene

previously discovered by Gallego et al. (1995). The current study investigated the effects of silencing the tomato fruit *GAD* gene sequence isolated previously by Gallego et al. (1995), with the aim of markedly increasing the levels of glutamate in fruits.

Results

Isolation of the tomato GAD gene and cloning into gateway vectors

The tomato fruit *GAD* gene with a sequence identical to that previously published by Gallego et al. (1995) was isolated. The primers used for the amplification of the PCR product at the size of 345 bp were designed with additional *attB*1 and *attB*2 sites to anneal to only a small region of the *GAD* gene at the C-Terminus. This 345 bp sequence was designed to function as an RNAi construct. Amplification of the *GAD* gene fragment by PCR yielded a product of the predicted size and this amplicon had a sequence with 99% homology at the nucleotide level to that reported by Gallego et al. (1995) (Fig 2). The *GAD* fragment was then used in a suitable Gateway vector for plant transformation. The *GAD* gene fragment captured in an entry clone (pENTR-*GAD*) was transferred in a single LR clonase reaction into destination vector pK7GW1WG2(I). Fig 3 describes the LR reaction for the incorporation of the *GAD* fragment into pK7GW1WG2(I). The successful insertion of the *GAD* gene fragment was confirmed by the survival of the bacterial clones and PCR using primers specific to the *GAD* fragment as used for the amplification of the *GAD* sequence. The presence of the PCR product with the size of 345 bp re affirmed the insertion of the *GAD* gene fragment in the expression vector. This gateway expression vector was transformed into *Agrobacterium tumefaciens* strain C58 and then into tomato cotyledons.

Observation of plantlet regeneration and confirmation of transgene

Transformed cotyledons produced small white callus after three to four weeks and only a few shoots emerged towards week 5. Growth was very slow compared to wild type MicroTom cotyledons infected with an empty vector control (Fig 4). For the *GAD* transgenic treatment, very few cotyledons produced shoots (in the ration of 1:40). Several attempts to repeat the whole plant transformation process to increase transformation efficiency were made. However all shoots showed signs of bleaching. About 3000 to 4000 cotyledons were used in these experiments, but they failed to yield healthy plantlets with green unbleached shoots. Leaf tissues from shoots that survived up to week 10 were collected and after DNA extraction, PCR was used to confirm the presence of the RNAi-*GAD* construct in the plant DNA. Primers used were designed to amplify across the 3' end of the 35S promoter and into the *GAD* gene fragment. The positive control used was the expression vector containing the gene (pEXPR-RNAi-*GAD*). None of the plantlets from the transgenic experiments yielded amplicons, indicating an absence of the transgene in all cases (Fig 5). These results indicate that the *GAD* gene used in our experiments are essential for normal metabolism in tomato and RNAi suppression of this gene was lethal to the regeneration of plantlets in our transgenic experiments. The expression of the RNAi construct to knockout the internal tomato gene may have caused imbalances in various metabolites and compounds essential for plant growth and one of them is the

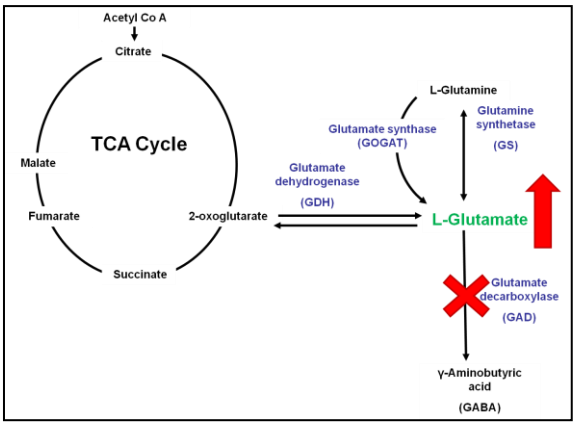

Fig 1. The glutamate pathway and the tricarboxylic acid cycle. Silencing of the glutamate decarboxylase (*GAD*) gene would be predicted to enhance glutamate levels at the expense of GABA.

depletion of GABA itself. GABA is linked to the TCA cycle and also to the metabolism of amino acid in plants. Knockout of the gene and the unsuccessful regeneration of the transformed plantlets further imply the central role of the enzyme in amino acid metabolism in tomato plants and the TCA cycle.

Discussion

There are several possible factors that may explain why the generation of the knockout *GAD* transgenic plants was unsuccessful. In the TCA cycle, 2-oxoglutarate is converted into glutamate by enzyme glutamate dehydrogenase as part of the nitrogen assimilation pathway. Glutamate can then be converted into GABA by *GAD* and GABA will then undergo transamination to obtain succinic semialdehyde catalysed by GABA transaminase (GABA-T). Succinic semialdehyde can be converted into succinic by enzyme succinic semialdehyde dehydrogenase and enters the TCA cycle again as part of the GABA shunt (Busch and Fromm, 1999). Therefore the *GAD* enzyme may have a central role in primary metabolism and significant suppression of its expression could be predicted to be lethal. Additionally GABA was previously reported in plants to function in response to various stress factors such as regulation of the pH in the cytosol of the cell (Snedden et al., 1995) and oxidative stress protection (Bouchè et al., 2003). Levels of GABA were reported to be elevated under these conditions and this indicates the importance of GABA in the cell system. Lack of GABA due to suppression of the *GAD* gene could explain the failure to regenerate low transgenic plants, because in tissue culture the cells harboring the RNAi construct at the early stages of rapid cell growth may not be able to survive in conditions where GABA is present at low levels. Previous works have reported the generation of the transgenic tomato plants with reduced *GAD* expression (Kisaka et al., 2006). However they reported problems in the germination of the transgenic seeds and also growth abnormalities in the T1 generation. This clearly suggested that the knockout might have impacted on plant survival. Furthermore, even though the transgenic tomato fruits contained higher levels of amino acids including glutamate, it was noted that these levels were not significant when compared to the wild type control (Kisaka et al., 2006). This indicated that their *GAD* antisense gene construct produced limited effects. The experiment reported by Kisaka et al. (2006) utilized the tomato root *GAD* sequence at 85%

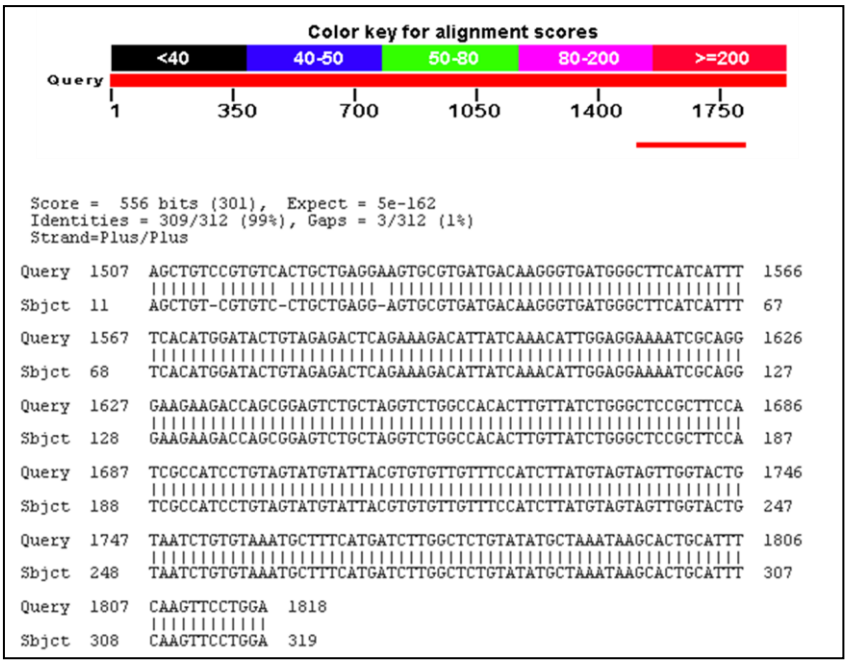

Fig 2. Comparison of nucleotide sequences of published tomato sequence (X80840) with that cloned in the present study. BLAST results indicated that the *GAD* gene fragment isolated in this study has 99% homology to the published *GAD* gene in the region used for the RNAi construct.

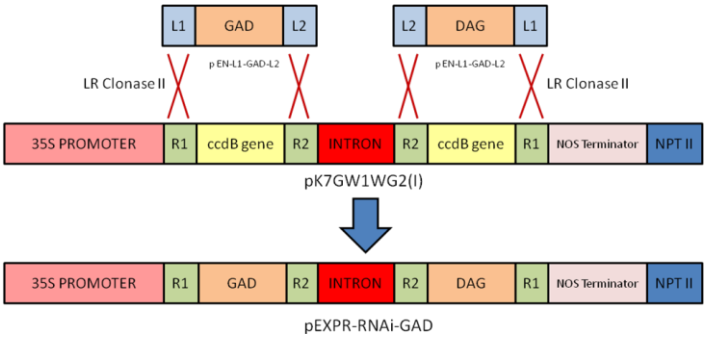

Fig 3. An illustration on the LR reaction to incorporate the *GAD* gene fragment into pK7GW1WG2. Both gateway cassettes are separated by the intron spacer in order to form the hairpin RNA (hpRNA) for silencing on the targeted gene.

homology to the actual tomato fruit *GAD* gene and its detrimental effects are obvious when its expression was suppressed. In this study, an RNAi *GAD* construct using the tomato gene and driven by the 35S promoter is lethal and targeting *GAD* levels in tomato fruits will likely require a promoter that is only active in fruit tissues.

Materials and methods

Plant materials

Tomato MicroTom wild type variety were grown in M3 compost and in heated glasshouse under standard cultural practices with regular supplementary nitrogen, phosphorus and potassium fertilizers. Lighting was supplied to the plants when necessary. The glasshouse temperature, humidity and radiation were regulated at different levels during day and night. The average temperature for daytime was maintained at 19ºC with humidity at 92%. The average temperature for night was regulated at 16ºC with humidity at 94%. Fruits were harvested at breaker and breaker+7 for RNA extractions.

Cloning of the GAD gene into the binary vector

RNA extractions were performed on these tissues using the RNAeasy Mini Kit (Qiagen). The synthesis of the first strand of cDNA from tomato fruit mRNA was performed as described in the manufacturers protocol (Invitrogen, UK). The first–strand cDNA generated was used as a template for Polymerase Chain Reaction (PCR) to amplify the tomato *GAD* gene. This PCR was performed using Phusion master mix (Finnzymes, New England Biolabs, UK) and incubation time and temperatures are as follows; initial denaturation at 98°C for 30 seconds; 30 cycles at 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. The forward and reverse primers used were designed to amplify the 3' end fragment of the *GAD* gene yielding a product with the size of 345 base pairs and was fused with the gateway *attB*1 and *attB*2 sites. They are GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTTGG ACACACAGCCTCCT and GGGGACCACTTTGTACAA-GAAAGCTGGGTCCAGGAACTTGAAATGCAGTG, respectively. The PCR product was purified using QIAquick

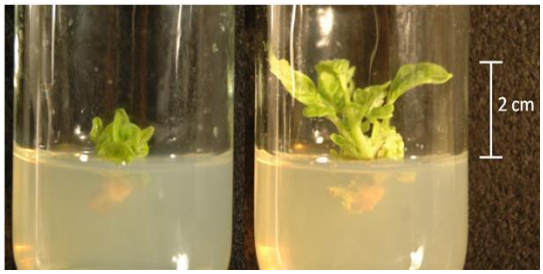

Fig 4. *GAD* transformed plantlet (left) and wild type (right) 7 weeks after transformation of cotyledons. Slow growth with no apical shoot for transformed plantlet in comparison to healthy growth with visible apical shoot for the control.

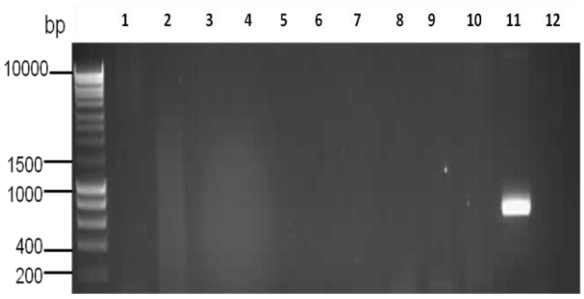

Fig 5. Gel image of PCR product from transgenic leaf tissue harbouring the RNAi *GAD* construct downstream of the 35S promoter. None of the plantlet leaf DNA samples exhibited amplicon to demonstrate presence of the transgene. Only the positive control (pEXPR-RNAi-*GAD*) showed the presence of amplified product. Lanes 1 through 10, transformed plants; lane 11, pEXPR-RNAi-*GAD*; lane 12, wild type MicroTom.

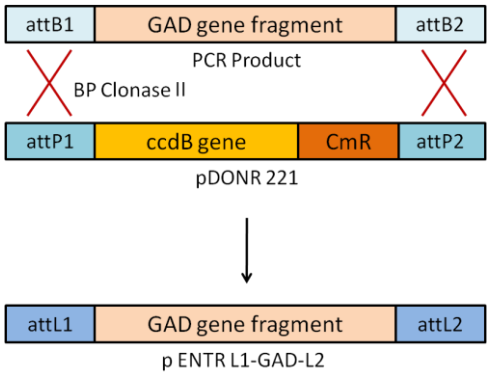

Fig 6. Description on the BP reaction to incorporate the *GAD* gene into the donor vector pDONR 221.

Gel Extraction Kit (Qiagen, UK) and cloned into the donor vector pDONR 221 (Invitrogen, UK) using BP Clonase II Enzyme Mix (Invitrogen, UK) following manufacturer's guidelines. The BP reaction mix was transformed into *E. coli* DH5α and plated on LB agar containing 100mg/ml kanamycin. The BP cloning strategy is summarised in Fig 6. Plasmids obtained from colonies emerged were purified using GeneJET Plasmid Miniprep Kit (Fermentas, UK). Entry vectors with the *GAD* 3' end gene fragment were sent for sequencing for confirmation and correct entry vectors were used for the LR reaction to generate expression clones. The LR reaction was performed using LR Clonase II Enzyme Mix (Invitrogen, UK) following manufacturers guidelines with destination vector pk7GW1WG2(I) (Karimi et al., 2002). Plasmids of the destination vector containing the gene of interest were purified and transformed into *Agrobacterium tumefaciens* strain C58 through electroporation at 2.5V, 25 µFD and 400 OHMS. Transformed *Agrobacterium tumefaciens* was selected on Luria Bertani (LB) agar supplemented with 50µL mg/ml rifampicin and 100 mg/ml spectinomycin. Colonies that were confirmed to have the correct gene of interest were used for plant transformation.

Tomato transformation

The tomato transformation protocol used for Micro Tom following that of Sun et al. (2006) with minor modifications. Seeds were surface sterilized with 70% (v/v) ethanol and 50% (v/v) sodium hypochlorite before rinsing with sterile water. Seeds were sown on 50% Murashige and Skoog (MS) medium and left to grow at 25ºC in the tissue culture room with 16 hours of light per day. The cotyledons were cut at both extremities at 7 days old and placed on solid KCMS medium supplemented with 0.9 g/L thiamine and 100 µM acetosyringone. Cotyledons were incubated at 25ºC in the tissue culture room for 24 hours. Prior to the transformation, the *Agrobacterium* culture was prepared in LB medium supplemented incubated in the 28ºC shaker (250rpm) for 48 hours. The *Agrobacterium* suspension was diluted to a final optical density of 0.05 in liquid KCMS media supplemented with 100 µg/L kinetin and 200 µg/L 2,4-Dichlorophenoxyacetic acid (2,4-D). The cotyledons recovered were soaked in the bacterial suspension for 30 minutes with shaking, dried on sterile filter paper, placed on solid KCMS medium and incubated for 48 hours. Transformed cotyledons were then cultured on 2Z medium supplemented with 150 mg/L of timentin and 75 ml/L kanamycin for shoot regeneration.

Confirmation of the presence of the introduced gene

Leaves regenerated from the cotyledons were excised for DNA extraction using the DNeasy Plant Mini Kit (Qiagen, UK). DNA samples of the leaves were screened for the transgene by PCR using the forward primer amplifying the 3' end of the 35S promoter and the reverse primer of the *GAD* gene as stated previously. The forward primer annealing to the 35S promoter was CACAATCCCACTATCCTTCGCAAGAC generating an amplicon of 345 bp. The PCR reaction are as follows; 30 cycles of incubation at 94°C for 30 seconds, at 55°C for 1 minute and 68°C for 1 minute; with the final extension at 72°C for 7 minutes. The PCR products obtained were confirmed from electrophoresis on 1% agarose gel stained with ethidium bromide.

Acknowledgements

Funding and facilities provided by the University of Nottingham and Universiti Sains Malaysia are gratefully appreciated. I would also like to thank Dr. Yu Pan and Dr. Sreeramanan Subramaniam for their kind assistance.

References

Bouché N, Fait A, Bouchez D, Møller SG and Fromm H (2003) Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. Proc Natl Acad Sci USA 100, 6843-6848.

Busch KB and Fromm H (1999) Plant succinic semialdehyde dehydrogenase. cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. Plant Physiol 121, 589–598.

Carrari F, Baxter C, Usadel B, Urbanczyk-Wochniak E, Zanor MI, Nunes-Nesi A, Nikiforova V, Centero D, Ratzka A, Pauly M, Sweetlove LJ and Fernie AR (2006) Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and highlight regulatory aspects of metabolic network behavior. Plant Physiol 142, 1380-1396.

Gallego PP, Whotton L, Picton S, Grierson D and Gray JE (1995) A role for glutamate decarboxylase during tomato ripening: the characterisation of a cDNA encoding a putative glutamate decarboxylase with a calmodulin-binding site. Plant Mol Biol 27, 1143-1151.

Ikeda K (1908) Japanese Patent, vol. 4805. Japan.

Inaba A, Yamamoto T, Ito T and Nakamura R (1980) Changes in the concentration of free amino acids and soluble nucleotides in attached and detached tomato fruits during ripening. J Jpn Soc Hortic Sci 49, 435–441.

Kisaka H, Kida T and Miwa T (2006) Antisense suppression of glutamate decarboxylase in tomato (*Lycopersicon esculentum*) results in accumulation of glutamate in transgenic tomato fruits. Plant Biotechnol 23, 267–274.

Kurihara K (2009) Glutamate: from discovery as a food flavor to role as a basic taste (umami). Am J Clin Nutr 90, 719S-722S.

Lightfoot DA, Long LM and Lightfoot MEV (1999) Plants containing a bacterial gdhA gene and methods of use thereof, vol. US005998700A (ed. U. S. Patent). United States: The Board of Trustees of Southern Illinois University.

Ninomiya K (1998) Umami: natural occurence. Food Rev 14, 177-211.

Rolin D, Baldet P, Just D, Chevalier C, Biran M and Raymond P (2000) NMR study of low subcellular pH during the development of cherry tomato fruit. Aust J Plant Physiol 27, 61 - 69

Snedden WA, Arazi T, Fromm H and Shelp BJ (1995) Calcium/calmodulin activation of soybean glutamate decarboxylase. Plant Physiol 108, 543-549

Sorrequieta A, Ferraro G, Boggio SB and Valle EM (2010) Free amino acid production during tomato fruit ripening: a focus on L-glutamate. Amino Acids 38, 1523-1532.

Steward F, Thompson J and Dent C (1949) γ-Aminobutyric acid: a constituent of potato tubers?. Science 110, 439-440.

Sun H-J, Uchii S, Watanabe S and Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol 47, 426–431.

Yamaguchi S and Kobori I (1991) Nucleotides as umami substances or umami enhancers. In 25th Japanese Symposium on Taste and Smell (JASTS XXV), vol. 17, pp. 85-116. Matsumoto Dental College, Shiojiri, Japan: Chemical Senses.

Yamaguchi S. and Ninomiya K (2000) Umami and food palatability. J Nutr 130, 921S–926S.