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Original Paper

Antisense suppression of glutamate decarboxylase in tomato (*Lycopersicon esculentum* L.) results in accumulation of glutamate in transgenic tomato fruits

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Abstract Tomato plants were transformed with a plasmid that contained a the gene for glutamate decarboxylase (GAD) from *Lycopersicon esculentum* L. coupled, in the antisense orientation, with the constitutively active 35S promoter from cauliflower mosaic virus. Four independent transformants were obtained. In the fruits of these transgenic plants, the level of expression of GAD mRNA was lower than that in non-transgenic plants. When tomatoes were harvested six weeks after the first flowering, we found that the levels of total free amino acids in transgenic fruits were 1.2 to 3.2 times higher than those in non-transgenic plants. In particular, the level of glutamate in fruits of transgenic plants was about twice that in fruits of non-transgenic plants.

Key words: Antisense regulation, glutamate decarboxylase, *Lycopersicon esculentum*, ripening fruits.

Glutamate is one of the α-amino acids that is synthesized during the first step of nitrogen metabolism in higher plants. Glutamine and asparagine, formed from glutamate, are distributed to plant tissues via the phloem and used for the synthesis of other amino acids and proteins. In higher plants, glutamate is present at high concentrations in phloem and it is found at high concentrations in the edible parts of plants. For example, tomato fruits contain 2,800 nmol g^{-1} fresh weight glutamate (17.2% of total free amino acids in cv. Platense; Boggio et al. 2000).

Reports on the biosynthesis of amino acids in transgenic plants include, a report that the level of free lysine was increased 200-fold by the introduction, into tobacco, of a gene for dihydropicolinate synthase from *E. coli* (Glassman et al. 1988); a report that the level of free lysine was increased by introduction, into corn, of a gene for aspartate kinase from *E. coli* (Falco 1993); a report that the level of asparagine was increased 100-fold in seeds by introduction, into tobacco, of a gene for asparagine synthetase from pea (Brears et al. 1993); and a report that the level of tryptophan was increased 90-fold by introduction, into rice, of a gene for anthranilate synthetase from corn (Anderson et al. 1997). However, it is not easy to engineer high concentrations of glutamate in plants because glutamate provides the amino group for the biosynthesis of several amino acids, namely, asparagine, alanine, glycine, serine, proline and γ-aminobutyric acid (GABA), and is also metabolized in various biosynthetic pathways. It has not yet proved possible, to our knowledge, to increase levels of glutamate in edible parts of plants by cross breeding. It was reported that, when the gene for glutamate dehydrogenase (GDH) from *E. coli* was introduced into tobacco and corn to engineer resistance to the herbicide phosphinothricin, the level of glutamate in roots was increased only 1.3- to 1.4-fold, as compared to control plants (Lightfoot and Long 1999). The glutamate content of fruits of transgenic tomato that harbored a gene for NADP-dependent GDH from *Aspergillus nidulans* was approximately twice that of wild-type fruits (Kisaka and Kida 2003).

It is well known that GABA accumulates in storage organs, such as tomato fruits and sugar beet roots. It is also known that the accumulation of GABA is induced by environmental stresses, such as low temperature and heat shock (Streeter and Thompson, 1972; Reggiani et al. 1988; Menegus et al. 1989; Aurisano et al. 1995;

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Abbreviations: GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; IAA, indole-3-acetic acid; MS, Murashige and Skoog; SSADH, succinic semialdehyde dehydrogenase.
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Wallace et al. 1990; Mayer et al. 1995). GABA is synthesized from glutamate in a reaction catalyzed by glutamate decarboxylase (GAD). The activity of GAD is controlled by the intracellular concentration of Ca^{2+} ions and calmodulin (Ling et al. 1994; Snedden et al. 1995, 1996, Arazi et al. 1995). It is likely that changes in the intracellular concentration of Ca^{2+} ions are induced by various types of stress and result in rapid increases in GAD activity. Thus, GABA might be expected to act as a signal-transducing compound in plants, but no details of such a phenomenon have yet been elucidated at the molecular level.

Genes encoding GAD have been isolated from petunia (Baum et al. 1993), tomato (Gallego et al. 1995), *Arabidopsis* (Zik et al. 1998; Turano and Fang 1998), rice (Akama et al. 2001) and tobacco (Yevtushenko et al. 2003). In all cases, a calmodulin-binding site of 30 to 32 amino acids was found at the carboxyl terminus of the gene product. However, the organs expressing the gene were different, depending on the species of plant. In petunia, these organs were the flower petals and flowers and in tomato the organs were fruits. It was also reported that *GAD1* genes were expressed only in *Arabidopsis* roots and *GAD2* genes were expressed in the entire *Arabidopsis* plant.

Baum et al. (1996) introduced a *GAD* gene from petunia into tobacco to investigate the regulatory role of calmodulin in the expression of *GAD* activity. In one set of experiments, they introduced the entire *GAD* gene in the sense orientation and, in another, they introduced the gene in the same manner but removed the sequence that encoded the calmodulin-binding site. In the resultant transformed tobacco plants, levels of GABA increased and those of glutamate decreased in both cases. The extent of the change was greater when the calmodulin-binding site had been removed. Moreover, the plants were shorter than control plants and differences in morphology were apparent.

In the present study, we attempted to increase the level of glutamate in tomato fruits by introducing a gene for glutamate decarboxylase in the antisense orientation.

Materials and methods

Isolation and sub-cloning of a gene for GAD from tomato

A solution of the inorganic salts of MS medium (Murashige and Skoog 1962) and MS vitamins was used for the cultivation of plants from tomato seeds. Tomato seeds were surface-sterilized with 70% (v/v) ethanol (30 sec) and 2% (v/v) sodium hypochlorite (15 min) and placed on hormone-free agar-solidified MS medium. They were cultured at 25°C for one week with 16 h of light daily to generate sterile plants. Total RNA was prepared from the roots of the young plants. A poly(A) Quick mRNA Isolation Kit (Stratagene, La Jolla, CA, USA) was used to purify the mRNA from total RNA and then first-strand cDNA was generated with a First-Strand cDNA Synthesis Kit (Invitrogen, San Diego, CA, USA). The first-strand cDNA was then used as the template for PCR. Amplification by PCR was performed with PCR system 2400 (Perkin Elmer, USA) with incubations as follows: 35 cycles at 94°C for 45 sec, at 59°C for 30 sec, and at 72°C for 90 sec; and then elongation at 72°C for 10 min. The primers used were 5′-CCG AAA GAA GCA GCA TAT CAG AT-3′ and 5′-TCA GTG AGG CTA CGG CTG AAA TCT-3′.The products of PCR were cloned with a TA-Cloning Kit (Invitrogen). Nucleotide sequences were determined for six plasmids that contained inserts of the appropriate length with an automated sequencer (model 377A; Applied Biosystems, Foster City, CA, USA), and the homology between these sequences and a previously sequenced *GAD* gene (Gallego et al. 1995) was examined.

The *GAD* gene derived from tomato, cloned in the pCR2.1 vector, was sub-cloned into a Ti-plasmid (pMAT037; Matsuoka and Nakamura 1991), which was the vector used for plant transformation. A fragment of the *GAD* gene (*GAD-19*) was obtained by cleavage at the *Xba*I and *Hin*dIII sites in PCR2.1 and the fragment was inserted at the *Xba*I and *Hin*dIII sites of pUC18. The resultant pUC18 plasmid was digested with restriction enzymes *Kpn*I and *Hin*dIII. After ligation of the *Kpn*I-*Hin*dIII fragment into the *Kpn*I-*Hin*dIII region of the multicloning site adjacent to the CaMV 35S promoter of pMAT037, *E. coli* DH5α cells were transformed with the newly synthesized construct. The Ti plasmid into which the antisense *GAD* gene had been introduced (pAnti-GAD-19) was transferred into *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993; Figure 2A), which was then used to transform tomato plants.

Production of transgenic plants by infection of tomato cotyledons with Agrobacterium

Tomato seeds (*Lycopersicon esculentum* L.) were surface-sterilized with 70% (v/v) ethanol (30 sec) and 2% (v/v) sodium hypochlorite (15 min) and then placed on hormone-free agar-solidified MS medium. The seeds were cultured at 25°C for one week with 16 h of light daily. Cotyledons were collected from the resultant sterile plants, placed on agar-solidified MS medium that contained 2 mg l^{-1} zeatin and 0.1 mg l^{-1} indole-3-acetic acid (IAA; regeneration medium) in 9-cm petri dishes, and cultured under the same conditions for two days. *Agrobacterium* harboring pAnti-GAD-19 was cultured overnight in YEP medium and used for transformation. The cotyledons that had been cultured for two days were collected in a sterilized petri dish, and a suspension of *Agrobacterium* (10^4 cells ml^{-1} in 10 ml) was added to initiate infection. After 10 min, cotyledons were gently

blotted with sterilized filter paper and a piece of sterilized filter paper was placed on fresh medium in a new set of petri dishes. The infected cotyledons were placed on the filter paper and, after co-culture for 24 h, the cotyledons were transferred to MS regeneration medium (selection medium) that contained 2 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA, 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime for selection of transformants. Regenerated shoots were transferred to fresh selection medium for further selection. Green shoots were cut at the stems and transferred to hormone-free MS medium. Finally, rooted plants were acclimated to soil.

Confirmation of the presence of the introduced gene

Total DNA was extracted as described by Honda and Hirai (1990). Purified DNA was diluted to 0.01 μg/μl and used as the template for genomic PCR. Amplification was performed with two pairs of primers. One pair of primers was designed to yield amplification products of approximately 1.0 kbp and was based on the sequence from the *NPTII* gene to the Nos promoter in the T-DNA (5′-CCC CTC GGT ATC CAA TTA GAG-3′ and 5′-CGG GGG GTG GGC GAA GAA CTC CAG-3′) and the second set of primers was designed to yield amplification products of approximately 1.1 kbp and was based on the sequence of the gene for *GAD* (5′-CCG AAA GAA GCA GCA TAT CAG AT-3′ and 5′-TCA GTG AGG CTA CGG CTG AAA TCT-3′). The reaction conditions were as follows: 35 cycles of incubation at 94°C for 1 min, at 55°C for 1 min and at 72°C for 2 min. The products of PCR were subjected to electrophoresis on a 1% agarose gel and then stained with ethidium bromide.

Northern blotting and analysis by RT-PCR

Total RNA was extracted from the leaves and fruits of each transformed tomato plant by the phenol/SDS method. Twenty μg of total RNA were subjected to electrophoresis on a formaldehyde-agarose gel [1.0% (w/v)] and then bands of RNA were transferred to a nylon membrane. The RNA on the membrane was then subjected to Northern hybridization with the *GAD-19* gene the probe and a DIG-labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). After treatment with DNase, first-strand cDNA was prepared with an RNA PCR Kit (TaKaRa Bio, Japan) using 1 μg of total RNA. Amplification was performed as described above with primers specific for the *GAD* gene (5′-CCG AAA GAA GCA GCA TAT CAG AT-3′ and 5′-TCA GTG AGG CTA CGG CTG AAA TCT-3′) and a pair of tubulin-specific primers (5′-ATG AGA GAA ATT CTT CAC-3′ and 5′-GTT CAT CTA CCT CCT TTG-3′). The products of PCR were subjected to electrophoresis on a 1% agarose gel and then stained with ethidium bromide.

Extraction and quantitation of free amino acids

Amino acids were extracted from three fruits in the first cluster of fruits on each plant for quantitation of amino acids in fruits. The results shown are averages of results from three fruits.

Fruits of transgenic plants were harvested six weeks after flowering and stored at −80°C. Each fruit was weighed, placed in a mortar, frozen with liquid nitrogen and ground into a powder with a pestle. Then 20 ml of 80% (v/v) ethanol were added, and the suspension was transferred to a centrifuge tube and incubated at 80°C for 20 min. After centrifugation at 10,000 rpm for 20 min, the supernatant was transferred into another tube. Twenty ml of 80% (v/v) ethanol were added to each pellet, and the pellets were ground again in the mortar. Then the suspension was transferred to a centrifuge tube and incubated at 80°C for 20 min. After a second centrifugation, the supernatant was combined with the first supernatant. The total volume of the mixture was adjusted to 50 ml with 80% (v/v) ethanol. After mixing, 1 ml of the mixture was evaporated to dryness and the residue was dissolved in 300 μl of sterilized water plus 200 μl of ethyl ether. After mixing, the mixture was centrifuged at 12,000 rpm for 10 min. After the ether layer had been removed, the aqueous layer (200 μl) was transferred to a new tube and evaporated to dryness. The residue was dissolved in 200 μl 0.02 N hydrochloric acid. After filtration through a 0.22-μm filter, the sample was subjected to amino acid analysis with a high-speed amino acid analyzer (L-8800; Hitachi Ltd., Tokyo, Japan).

Results

Isolation of the GAD *gene from tomato*

We found no inserts with nucleotide sequences identical to the reported sequence of a tomato gene for *GAD* (Gallego et a1. 1995). Among the clones that we examined, a gene that we designated *GAD-19* had the strongest homology (85%) and was 1,180 bp long. We decided to use the *GAD-19* gene in this study (Figure 1).

Selection of transgenic plants

We used the binary vector pAnti-GAD-19, in which the gene for glutamate decarboxylase (*GAD-19*; Figure 1) had been inserted in the antisense direction, to produce tomato transformants (T_0) by *Agrobaceterium*-mediated transformation (Figure 2A). We obtained four regenerated T_0 plants, designated GAD-1, GAD-2, GAD-3 and GAD -4. In terms of morphology, the GAD-1 plant was a dwarf plant with thick stems. The morphology of the other transgenic plants was similar to that of non-transgenic plants. These transgenic plants made only a few fruits. Progeny were selected on MS medium supplemented with 250 mg l^{-1} kanamycin and 300 mg l^{-1}

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LeGAD     1 ATGGTGTTAA CAACGACGTC GATAAGAGAT TCAGAAGAGA GCTTGCACTG TACATTTGCA
LeGAD    61 TCAAGATATG TACAGGAACC TTTACCTAAG TTCAAAATGC CTAAAAAATC CATGCCGAAA
GAD-19    1                                                    CCGAAA

LeGAD   121 GAAGCAGCTT ATCAGATTGT AAACGACGAG CTTATGTTGG ATGGTAACCC CAGGTTGAAT
GAD-19    7 GAAGCAGCAT ATCAGATTCT AAATGATGAA CTTATGTTAG ATGGAAATCC AAGGTTGAAT

LeGAD   181 TTAGCTTCCT TTGTTAGCAC ATGGATGGAG CCCGAGTGCG ATAAGCTCAT CATGTCATCC
GAD-19   67 TTGGCATCTT TTGTGACAAC ATGGATGGAA CCAGAATGTG ACAAATTGAT GATGGATTCC

LeGAD   241 ATTAATAAAA ACTATGTCGA CATGGATGAG TATCCTGTCA CCACTGAACT TCAAAATAGA
GAD-19  127 ATTAACAAAA ATTATGTTGA CATGGATGAA TATCCTGTCA CCACTGAGCT TCAGAATCGG

LeGAD   301 TGTGTTAACA TGTTAGCACA TCTTTTCCAT GCCCCGGTTG GTGATGATGA GACTGCAGTT
GAD-19  187 TGTGTAAACA tgatagcgca TTTATTTAAT GCACCACTTG AAGATGGAGA AACTGCAGTT

LeGAD   361 GGAGTTGGTA CAGTGGGTTC ATCAGAGGCA ATAATGCTTG CTGGCCTTGC TTTCAAACGC
GAD-19  247 GGAGTTGGAA CAGTTGGTTC TTCAGAAGCC ATTATGCTTG CTGGATTGGC CTTTAAGAGA

LeGAD   421 AAATGGCAAT CGAAAAGAAA AGCAGAAGGC AAACCTTTCG ATAAGCCTAA TATAGTCACT
GAD-19  307 AAATGGCAAA ACAAAATGAA AGCCCAAGGA AAGCCCTATG ATAAGCCCAA CATTGTTACT

LeGAD   481 GGAGCTAATG TGCAGGTCTG CTGGGAAAAA TTTGCAAGGT ATTTTGAGGT TGAGTTGAAG
GAD-19  367 GGTGCTAATG TCCAGGTGTG TTGGGAAAAA TTTGCAAGGT ATTTTGAAGT TGAGCTAAAA

LeGAD   541 GAGGTGAAAC TAAAAGAAGG ATACTATGTA ATGGACCCTG CCAAAGCAGT AGAGATAGTG
GAD-19  427 GAAGTGAAGT TGACTGATGG ATACTATGTG ATGAGCCCTG AGAAAGCTGT GGAAATGGTT

LeGAD   601 GATGAGAATA CAATATGTGT TGCTGCAATC CTTGGTTCTA CTCTGACTGG GGAGTTTGAG
GAD-19  487 GATGAGAACA CAATTTGTGT AGCTGCTATT TTGGGTTCAA CTCTGAATGG GGAGTTTGAG

LeGAD   661 GATGTGAAGC TCCTAAACGA GCTCCTTACA AAAAAGAACA AGGAAACCGG ATGGGAGACA
GAD-19  547 GAAGTGAAGA AATTGAATGA CCTCCTTATT GAAAAGAACA AGGAAACAGG GTGGGACACT

LeGAD   721 CCGATTCATG TCGATGCTGC GAGTGGAGGA TTTATTGCTC CTTTCCTCTG GCCAGATCTT
GAD-19  607 CCAATTCATG TGGATGCAGC TAGTGGTGGA TTTATTGCAC CATTTATATA TCCAGAACTT

LeGAD   781 GAATGGGATT TCCGTTTGCC TCTTGTGAAA AGTATAAATG TCAGCGGTCA CAAGTATGGC
GAD-19  667 GAATGGGACT TTAGATTGCC ATTAGTTAAA AGTATAAATG TCAGCGGTCA CAAGTATGGC

LeGAD   841 CTTGTATATG CTGGTGTCGG TTGGGTGATA TGGCGGAGCA AGGAAGACTT GCCCGATGAA
GAD-19  727 CTTGTATATG CTGGTGTCGG TTGGGTCATA TGGCGGAGCA AGGAAGACTT GCCCGATGAA

LeGAD   901 CTCGTCTTTC ATATAAACTA CCTTGGGTCT GATCAGCCTA CTTTTACTCT CAACTTCTCT
GAD-19  787 CTTATTTTTC ATATTAATTA CCTTGGGTCT GATCAACCTA CTTTCACCCT CAATTTCTCT

LeGAD   961 AAAGGTTCCT ATCAAATAAT TGCACAGTAT TATCAGTTAA TAAGACTTGG CTTTGAGGGT
GAD-19  847 AAAGGATCAA GTCAAGTAAT TGCTCAATAT TATCAACTAA TTCGTTTTGG TTATGAGGGT

LeGAD  1021 TATAAGAACG TCATGAAGAA TTGCTTATCA AACGCAAAAG TACTAACAGA GGGAATCACA
GAD-19  907 TATAAGAACG TCATGAAGAA TTGCTTATCA AACGCAAGGG TACTAACAGA GGGAATCACA

LeGAD  1081 AAAATGGGGC GGTTCGATAT TGTCTCTAAG GATGTGGGTG TTCCTGTTGT AGCATTTTCT
GAD-19  967 AAAATGGGAA GATTCGAGAT TGTCTCCAAG GAGATTGGTG TTCCCTTGGT TGCATTTTCT

LeGAD  1141 CTCAGGGACA GCAGCAAATA TACGGTATTT GAAGTATCTG AGCATCTCAG AAGATTTGGA
GAD-19 1027 CTTAAAGACA ATAGCAAACA TGATGAGTTT GAAGTATCTG AAACTTTAAG GAGATTTGGA

LeGAD  1201 TGGATCGTCC CTGCATACAC AATGCCACCG GATGCTGAAC ACATTGCTGT ACTGCGGGTT
GAD-19 1087 TGGATTGTTC CAGCATACAC TATGCCACCA GATGCTCAAC ACATCACAGT TCTTAGAGTT

LeGAD  1261 GTCATTAGAG AGGATTTCAG CCACAGCCTA GCTGAGAGAC TTGTTTCTGA CATTGAGAAA
GAD-19 1147 GTTATTAGAG AAGATTTCAG CCACAGCCTA GCTGA

LeGAD  1321 ATTCTGTCAG AGTTGGACAC ACAGCCTCCT CGTTTGCCCA CCAAAGCTGT CCGTGTCACT
LeGAD  1381 GCTGAGGAAG TGCGTGATGA CAAGGGTGAT GGGCTTCATC ATTTTCACAT GGATACTGTA
LeGAD  1441 GAGACTCAGA AAGACATTAT CAAACATTGG AGGAAAATCG CAGGGAAGAA GACCAGCGGA
LeGAD  1501 GTCTGCTAG
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Figure 1. Alignment of the sequences of the *GAD-19* and Le*GAD* genes. Shaded regions are identical, and boxed regions show the primers used for the analysis of genomic PCR and for RT-PCR.

cefotaxime. Only one transgenic plant (GAD-2) was clearly resistant to kanamycin. Moreover, seeds of the other three lines did not germinate. We tried to culture the seeds on MS medium without kanamycin but, again, they failed to germinate.

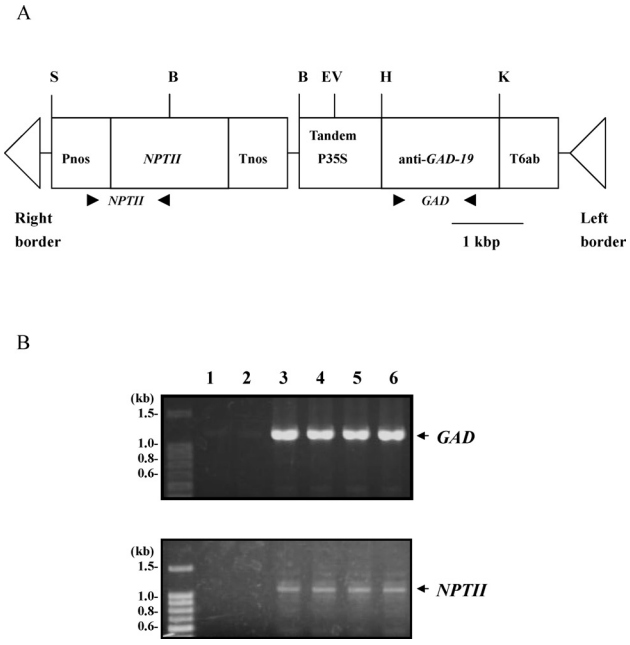

Figure 2. Construction, using the pMAT037 vector, of pAnti-GAD-19, with the *GAD-19* gene inserted in the antisense orientation (A). Abbreviations: Tandem P35S, the promoter of the 35S transcript from cauliflower mosaic virus with a tandemly duplicated enhancer; T6ab, the transcription terminator region of T-DNA transcripts 6a and 6b; *anti-GAD-19*, the gene for glutamate decarboxylase from tomato in the antisense orientation; Pnos, promoter of a gene for nopaline synthase; Tnos, transcription terminator region of a gene for nopaline synthase; *NPTII*, gene for neomycin phosphotransferase; S, *Sal*I; B, *Bam*HI; EV, *Eco*RV, H, *Hin*dIII; and *X*, *Xba*I. Arrows show the positions of primers used for the analysis of genomic DNA. Results of genomic PCR analysis (B). Amplification by PCR was performed with primers specific for the *GAD-19* and *NPTII* genes, as indicated in the text. The target bands (1.1 kbp for the *GAD-19* gene and 1.0 kbp for the *NPTII* gene; indicated by arrows) were observed only in the case of plants transformed with the vector that harbored the antisense *GAD-19* gene. Lanes 1 and 2, Non-transformed plants; lanes 3 through 6, transgenic plants GAD-1, GAD-2, GAD-3 and GAD-4, respectively.

Confirmation of the presence of the GAD-19 *gene*

We analyzed four transgenic plants that had been infected with *Agrobacterium* that harbored pAnti-GAD-19 and two non-transgenic lines. We extracted genomic DNA from leaves and obtained a fragment of approximately 1.1 kbp, the expected size of the *GAD-19* gene, and a fragment of approximately 1.0 kbp, the expected size of the *NPTII* gene, from the four transgenic plants but no such fragments were amplified from non-transgenic plants (Figure 2B).

Northern blotting and analysis by RT-PCR

We examined the expression of the *GAD-19* gene in leaves of four T_0 transgenic plants by RT-PCR and in fruits of two T_0 transgenic plants (GAD-1 and GAD-2) by Northern blotting. The results of RT-PCR revealed that the level of transcript in transgenic tomato leaves was lower than that in non-transgenic leaves (Figure 3A). Northern blotting analysis of steady-state levels of *GAD*

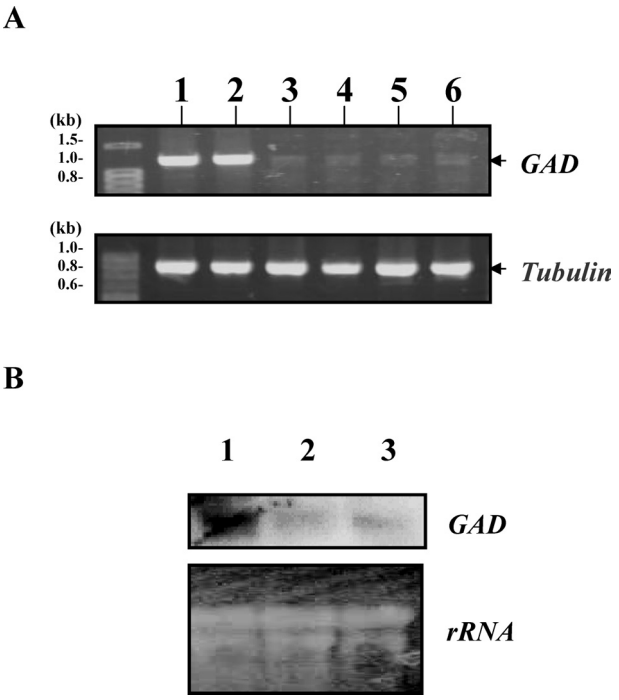

Figure 3. Results of analysis by RT-PCR (A). Amplification by PCR was performed with primers specific for the *GAD-19* gene and a gene for tubulin. Total RNA was extracted from leaves. After treatment with DNase, first-strand cDNA was generated using 1 μg of total RNA. Amplification was performed as described in the text with primers specific for the *GAD-19* gene and for a gene for tubulin. The products of PCR were subjected to electrophoresis on a 1% agarose gel and then stained with ethidium bromide. Lanes 1 and 2, Non-transformed plants; lanes 3 through 6, transgenic plants GAD-1, GAD-2, GAD-3 and GAD-4, respectively. Results of Northern blotting analysis (B). Total RNA samples (20 μg), extracted from fruits, was fractionated and subjected to Northern blotting analysis with the *GAD-19* gene as probe. Lane 1, Non-transformed plant; lane 2, transgenic plant GAD-1; and lane 3, transgenic plant GAD-2. rRNA was used as a control to confirm the loading of equal amounts of RNA in each lane. The arrow indicates *GAD-19* mRNA.

transcripts in green fruits from four weeks after flowering revealed that the level of the transcript was low in transgenic fruits but high in the fruits of non-transgenic plants (Figure 3B).

Amino acid analysis

We analyzed the fruits of non-transgenic plants to determine the stage at which the levels of amino acids were relatively stable. We examined levels of amino acids in fruits in the first-fruit cluster at different stages of ripening. In four-week-old fruits, which were green, γ-aminobutyric acid (GABA) was the predominant amino acid. During ripening, the color of fruits changed from green to orange and auxesis ceased five weeks after flowering. In six-week-old fruits, which were red, the levels of GABA were lower and those of glutamate were significantly higher than at earlier stages. The epidermis of seven-week-old fruits was broken but the levels of amino acids were similar to those in six-week-old fruits

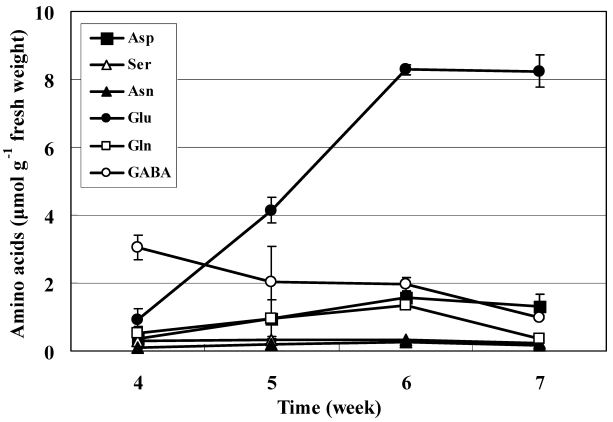

Figure 4. Levels of amino acids in non-transgenic tomato fruits from 4 to 7 weeks after flowering (n=3).

(Figure 4). Therefore, we used six-week-old fruits for comparisons of levels of amino acids between transgenic and non-transgenic plants.

The total amount of free amino acids in four T_1 transgenic tomato fruits, direct descendants of the GAD-2 T_0 plant, was 1.2 to 3.2 times higher than that in non-transgenic tomato fruits. Levels of GABA in GAD2-1, GAD2-2 and GAD2-4 fruits were lower than the levels in fruits of non-transgenic plants. However, the fruits of the GAD2-3 plant contained very high levels of GABA and other free amino acids. In addition, the levels of asparagine, alanine and glutamate in transgenic tomato fruits were much higher than those in non-transgenic tomato fruits (Table 1).

Discussion

Glutamate decarboxylase (GAD) catalyzes the conversion of glutamate to GABA. Baum et al. (1996) introduced *GAD* genes from petunia into tobacco and found that, in both the leaves and stems of transformants, levels of GABA were elevated and those of glutamate were reduced. Moreover, plants were shorter than controls and differed morphologically from them. Therefore, we tried to raise levels of glutamate by controlling the conversion of glutamate to GABA. We generated transgenic tomato plants that expressed a gene for glutamate decarboxylase (*GAD*) in the antisense orientation, in an effort to increase the glutamate content of tomato fruits (Figure 5). We obtained four transgenic tomato plants. However, the seeds of the T_1 generation that had been obtained from the GAD-1, GAD-3 and GAD-4 transformed tomato plants did not germinate. In addition, the GAD-1 plant was morphologically abnormal, being shorter than non-transgenic tomato plants. We measured the levels of free amino acids in fruits of four T_1 transgenic plants obtained from the GAD-2 transformant. As shown in Table 1, the levels of GABA in GAD2-1, GAD2-2 and GAD2-4 were lower

Table 1. The levels of amino acids in fruits of tomato plants transformed with antisense *GAD-19* gene (T_1 generation; nmol g^{-1} fresh weight)

	Asp	Thr	Ser	Asn	Glu	Gln	Gly	Ala	Val	Met
Control	1093	100	220	184	6416	443	14	287	85	19
	(±484)	(±33)	(±51)	(±91)	(±1156)	(±331)	(±8)	(±111)	(±54)	(±9)
GAD2-1	1471	131	324*	396**	8442	104	22	555	135	24
	(±311)	(±9)	(±46)	(±52)	(±1389)	(±98)	(±5)	(±226)	(±35)	(±6)
GAD2-2	2224**	177**	394**	181	11745**	451	35*	800**	130	26
	(±282)	(±11)	(±59)	(±171)	(±2087)	(±482)	(±11)	(±248)	(±10)	(±2)
GAD2-3	3960**	311**	743**	542**	19674**	1108**	71**	1146**	134	44**
	(±561)	(±30)	(±86)	(±76)	(±3566)	(±624)	(±16)	(±458)	(±28)	(±6)
GAD2-4	1877*	153*	412**	385**	11747**	36	28*	684**	117	25
	(±259)	(±11)	(±54)	(±342)	(±2089)	(±62)	(±5)	(±11)	(±16)	(±5)
	Ile	Leu	Tyr	Phe	GABA	Lys	His	Arg	Pro	Total
Control	38	42	47	87	961	43	127	167	42	10363
	(±18)	(±9)	(±5)	(±36)	(±108)	(±16)	(±41)	(±118)	(±38)	(±4235)
GAD2-1	53	44	36	104	432	56	173	33	48	12569
	(±5)	(±5)	(±15)	(±12)	(±254)	(±12)	(±56)	(±19)	(±43)	(±2174)
GAD2-2	96**	81**	56	155	891	85**	251**	44	58	17851*
	(±16)	(±17)	(±7)	(±37)	(±237)	(±7)	(±30)	(±26)	(±24)	(±1916)
GAD2-3	178**	152**	87**	427**	3956**	204**	363**	85	227**	33374**
	(±47)	(±19)	(±7)	(±74)	(±1182)	(±23)	(±48)	(±49)	(±44)	(±4381)
GAD2-4	70**	63**	44	91	880	64	253**	58	58	16964*
	(±1)	(±3)	(±7)	(±11)	(±65)	(±25)	(±56)	(±24)	(±22)	(±2469)

Values in parentheses are standard errors (n=3). *P<0.1, **P<0.05, versus controls.

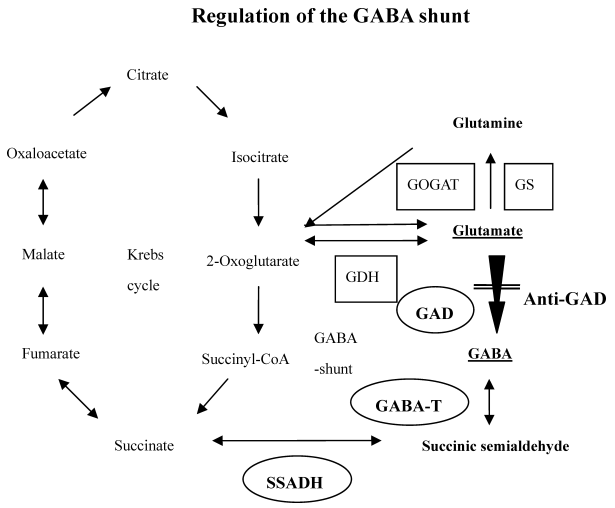

Figure 5. Model for the regulation of the GABA shunt in plants. The three enzymes in the GABA shunt, namely GAD (glutamate decarboxylase), GABA-T (GABA transaminase) and SSADH (succinic semialdehyde dehydrogenase), are indicated in bold letters inside ellipses. Three enzymes involved in nitrogen fixation, namely, GS (glutamine synthetase), GOGAT (glutamate synthase) and GDH (glutamate dehydrogenase), are enclosed by rectangles.

than that in non-transgenic tomato. This result suggests the possible inhibition of GAD activity by antisense regulation. However, the difference between transformants and the wild type was not significant. The fruits of the GAD2-3 plant contained very high levels of GABA and other free amino acids, perhaps as a result of some metabolic abnormality in the GAD2-3 plant. Levels of total free amino acids were much higher (double or more) in transgenic tomato fruits than in non-transgenic tomato fruits at the later stage of ripening. In particular, the level of glutamate was two to three times higher than that in non-transgenic plants. Our results indicate that GAD is very important in the metabolism of amino acids.

In non-transgenic tomato fruit, GABA was predominant at the earlier stages of ripening but the level of glutamate subsequently increased to close to 60% of the total level of free amino acids, with a decrease in the level of GABA, at a later stage (six to seven weeks after flowering). These data are similar to those reported for cv. Cherry by Boggio et al. (2000). GABA undergoes transamination to yield succinic semialdehyde, which is converted, in turn, to succinate. These reactions are catalyzed by GABA transaminase and succinic semialdehyde dehydrogenase, which, together with GAD, make up the so-called GABA shunt (Bown and Shelp 1989; Satyanarayan and Nair 1990; Busch and Fromm 1999). Therefore, GABA is a metabolite on the pathway from glutamate to succinate and the Krebs cycle (Figure 5; Tuin and Shelp 1994). GABA might be a nitrogen-storage compound in fruit cells (Satyanarayan and Nair 1990). GABA that accumulates in green tomato fruits is metabolized to succinate via the Krebs cycle and might be a substrate for the synthesis of glutamate in ripening tomato fruits. The metabolism of glutamate to GABA might have been modified in our transgenic plants because the synthesis of GAD had been modified and, thus, glutamate accumulated at abnormally high levels. We also observed significant increases in levels of aspartic acid, serine and alanine, which belong to the glutamate family, in our transgenic tomato fruits (Table

1). This result was probably due to the use of glutamate as a substrate for the synthesis of other amino acids as a consequence of the considerable increase in the amount of glutamate in ripening tomato fruits. Increases in the levels of asparagine, aspartic acid, alanine, and GABA were found in the ripening fruits of tomato plants that harbored a gene for glutamate dehydrogenase from *Aspergillus nidulans* that we described previously (Kisaka and Kida 2003).

In conclusion, our present experiments demonstrated that fruits of tomato plants that had been transformed with the gene for glutamate decarboxylase from *Lycopersicon esculentum* L., in the antisense orientation, accumulated elevated levels of glutamate.

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References

Akama K, Akihiro T, Kitagawa M, Takaiwa F (2001) Rice (*Oryza sativa*) contains a novel isoform of glutamate decarboxylase that lacks an authentic calumodulin-binding domain at the C-terminus. *Biochim Biophys Acta* 1522: 143–150

Anderson PC, Chomet PS, Griffor MC (1997) Anthranilate synthase gene and use thereof. Patent: WO 9726366

Arazi T, Baum G, Snedden WA, Shelp BJ, Fromm H (1995) Molecular and biochemical analysis of calmodulin interactions with the calmodulin-binding domain of plant glutamate decarboxylase. *Plant Physiol* 108: 551–561

Aurisano N, Bertani A, Reggiani R (1995) Involvement of calcium and calmodulin in protein and amino-acid metabolism in rice root under anoxia. *Plant Cell Physiol* 36: 1525–1529

Baum G, Chen Y, Arazi T, Takatuji H, Fromm H (1993) A plant glutamate decarboxylase containing a CaM-binding domain: cloning, sequence and functional analysis. *J Biol Chem* 268: 19610–19617

Baum G, Lev-Yadun S, Fridmann Y, Arazi T, Katsnelson H, Zik M, Fromm H (1996) Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. *EMBO J* 15: 2988–2996

Boggio SB, Palatnik JF, Heldt HW, Valle EM (2000) Changes in amino acid composition and nitrogen-metabolizing enzymes in ripening fruits of *Lycopersicon esculentum* Mill. *Plant Sci* 159: 125–133

Bown A, Shelp BJ (1989) The metabolism and physiological roles of 4-aminobutyric acid. *Biochem Life Sci Adv* 8: 21–25

Brears T, Liu C, Knight TJ, Coruzzi GM (1993) Ecotopic overexpression of asparagine synthetase in transgenic tobacco. *Plant Physiol* 103: 1285–1290

Busch KB, Fromm H (1999) Plant succinic semialdehyde dehydrogenase: cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. *Plant Physiol* 121: 589–597

Falco SC (1993) Nucleic acid fragments and methods for increasing the lysine and threonine content of the seeds of plants. Patent: WO 9319190

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

Glassman KF, Barnes LJ, Pilacinski WP (1988) Molecular genetics research and development limited partnership. Patent: US 5258300

Honda H, Hirai A (1990) A simple and efficient method for identification of hybrids using non-radioactive rDNA as probe. *Breeding Sci* 40: 339–348

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgen Res* 2: 208–218

Kisaka H, Kida T (2003) Transgenic tomato plant carrying a gene for NADP-dependent glutamate dehydrogenase (*gdh*A) from *Aspergillus nidulans*. *Plant Sci* 164: 35–42

Lightfoot DA, Long LM (1999) Plants containing a bacterial *gdh*A gene and methods of use thereof. Patent: US 5998700

Ling V, Snedden WA, Shelp BJ, Assmann SM (1944) Analysis of a soluble calmodulin-binding protein from fava bean roots: identification of glutamate decarboxylase as a calmodulin-activated enzyme. *Plant Cell* 6: 1135–1143

Matsuoka K, Nakamura K (1991) Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. *Proc Nati Acad Sci USA* 88: 834–838

Mayer R, Cherry J, Rhodes D (1990) Effect of heat shock on amino acid metabolism of cowpea cells. *Plant Physiol* 94: 796–810

Menegus F, Cattaruzza L, Chersi A, Fronza G (1989) Differences in the anaerobic lactate-succinate production and in the changes of cell sap pH for plants with high and low resistance to anoxia. *Plant Physiol* 29: 29–32

Murashige T, Skoog G (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473–497

Reggiani R, Cantu C, Brambilla A, Bertani A (1988) Accumulation and interconversion of amino acids in rice roots under anoxia. *Plant Cell Physiol* 29: 981–987

Satyanarayan V, Nair PM (1990) Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochem* 29: 367–375

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

Snedden WA, Koutsia N, Baum G, Fromm H (1996) Activation of a recombinant petunia glutamate decarboxylase by calcium/calmodulin-binding domain. *J Biol Chem* 271: 4148–4153

Streeter JG, Thompson JF (1972) Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus*). *Plant Physiol* 49: 572–578

Tuin LG, Shelp AW (1994) *In situ* [^{14}C] metabolism by developing soybean cotyledons. I. Metabolic routes. *J Plant Physiol* 143: 1–7

Turano FJ, Fang TK (1998) Characterization of two glutamate decarboxylase cDNA clones from *Arabidopsis*. *Plant Physiol* 117: 1411–1421

Yevtushenko DP, McLael MD, Peiris S, Cauwenberghe OR, Shelp BJ (2003) Calcium/calmodulin activation of two divergent glutamate decarboxylases from tobacco. *J Exp Bot* 54: 2001–2002

Wallace W, Secor J, Schrader LE (1984) 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* 75: 170–175

Zik M, Arazi T, Snedden WA, Fromm H (1998) Two isoforms of glutamate decarboxylase in *Arabidopsis* are regulated by calcium/calmodulin and differ in organ distribution. *Plant Mol Biol* 37: 967–975