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Cloning, Sequencing and Expression Analysis of a cDNA Encoding Glutamate Dehydrogenase Gene in Broccoli During Postharvest Senescence

Dewoowoogen Porras Baclayon, Toshiyuki Matsui, Haruo Suzuki and Yusuke Kosugi
Department of Bioresource Production, Faculty of Agriculture,
Kagawa University, Miki, Kagawa 761-0795, Japan

Abstract: Quality of broccoli deteriorates rapidly after harvest due to major physio-biochemical changes. In this study, the changes in ammonia content and activity and gene expression of glutamate dehydrogenase (GDH; EC 1.4.1.2) during storage at 20°C for five days were investigated. The branchlets were separated from the florets at the end of each storage period. Ammonia assay showed that the level in the branchlet portion was almost constant while in the floret portion increased rapidly after three days of storage to about seven times the content at harvest. Enzymatic analysis, on the other hand, revealed that GDH, in both amination and deamination activities, decreased in the branchlets while a gradual increase was observed in the floret portion as storage progressed. To better understand these biochemical changes, a cDNA encoding GDH in broccoli was isolated, cloned and sequenced. The partial cDNA clone referred to as BoGDH (*Brassica oleracea* glutamate dehydrogenase gene; AB212934) encodes an mRNA of 781 bp. The deduced amino acid sequence showed highest similarity with the GDH gene from *Arabidopsis* associated with stress. Although the transcript was not consistent with enzyme activity, RNA gel blot analysis revealed that BoGDH was present in both branchlet and floret portions throughout the storage duration. The results suggest that GDH plays an essential role during postharvest senescence. Furthermore, it is likely that its expression is controlled by multigenes and regulated either transcriptionally or posttranscriptionally.

Key words: Ammonium, broccoli, cDNA, glutamate dehydrogenase, gene expression

INTRODUCTION

Perishable commodities usually produce a considerable amount of ammonia after few days from harvest. High levels of ammonia would cause toxicity to the cells thereby enhancing quality deterioration in harvested products. Moreover, the activity of glutamine synthetase (GS; EC 6.3.1.2), an enzyme responsible for the assimilation of ammonia into amino acids (Suárez *et al*., 2002), has been reported to decrease during senescence (Enriquez *et al*., 2001; Matsui *et al*., 2004). In addition, the disruption of continuous supply of nutrients after harvest will eventually result to cell starvation. The harvested product is dependent on the energy it has accumulated before harvest, thus after being severed from the mother plant, it senesces progressively with time. In broccoli, a decline in sucrose within the first 6 h of storage to about half the level present at harvest has been reported (Downs *et al*., 1997). As a survival mechanism to detoxify the cells from NH_4^+ and replenish the decreasing C supply, plant tissues undergo appropriate modification in their metabolism. Considering the metabolic action of glutamate dehydrogenase (GDH; EC 1.4.1.2), which catalyzes both the amination of 2-oxoglutarate, with NAD(P)H as the electron donor and the deamination of glutamate to ammonia and 2-oxoglutarate, in a reaction that is coupled with the conversion of $NAD(P)^+$ to NAD(P)H (Sakakibara *et al*.,1995), it may have a possible role during senescence and stress conditions (Bechtold *et al*., 1998). Additionally, a catabolic activity of the enzyme to replenish the TCA cycle when the cells are deprived of carbon sources has also been considered (Maestri *et al*., 1991; Robinson *et al*., 1992; Lancien *et al*., 2000). However, besides several studies to characterize the enzyme physiologically and/or biochemically in different plant species (Robinson *et al*., 1992; Stewart and Rhodes, 1978; Dubois *et al*., 2003), its specific role remains a matter of debate. At the molecular level, at least two genes encoding GDH have been identified namely GDH1(A) and GDH2(B) in *Arabidopsis thaliana* (Melo-Oliveira *et al*., 1996; Turano *et al*., 1997), *Asparagus officinalis* (Pavesi *et al*., 2000) and *Nicotiana plumbaginifolia* (Restivo, 2004). In maize, the existence of gene(s) encoding NADH-GDH subunits has also been suggested (Sakakibara *et al*., 1995). The presence of multigenes encoding GDH further complicates its role in the growth, development and senescence of plants.

Corresponding Author: Dewoowoogen Porras Baclayon, Postharvest Horticulture Laboratory, Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki, Kagawa 761-0795, Japan
Tel: 81 0903 780 6691 Fax: 81 87 891 3070

To improve understanding of GDH regulation in harvested broccoli head, cDNA encoding GDH was isolated, cloned and sequenced. The pattern of GDH gene expression in relation to enzyme activity during postharvest senescence was also investigated.

MATERIALS AND METHODS

Plant material: Broccoli (Hartland) heads grown in winter of 2004 were harvested before noon time from a farmer's commercial field in Miki, Kagawa, Japan. Right after harvest, the heads were packed in a box with crushed ice and transported to the laboratory. Each head was trimmed, enclosed with a perforated plastic sheet and held at 20°C for 5 days. At the end of each storage period, the florets were separated from the branchlets and immediately kept at -30 and -80°C until enzyme analysis and RNA extraction were performed, respectively.

Extraction and assay of GDH activity: Enzyme extraction was performed using a procedure by Hurst and Clark (1993). GDH activity was determined in both aminating and deaminating directions as described in our previous report (Baclayon *et al*., 2004). Protein concentration was determined following the method of Lowry *et al.* (1951).

Ammonia assay: Two-gram of fresh-weight sample each of the floret and branchlet portions of the broccoli head was extracted with 10% TCAA at 0°C (ice bath) and centrifuged at 12,000 x g at 2°C for 10 min. Ammonia content was assayed using the procedure of Kun and Kearney (1974).

RNA isolation: RNA isolation was carried out following the hot borate method of Wan and Wilkins (1994) with few modifications.

Amplification of poly $(A)^+$ RNA by RT-PCR: The first strand cDNA was synthesized using 5 μg of total RNA and Reverse Transcriptase (RT) with oligo-(dT) primer following the instructions of SUPERSCRIPT™ First Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invetrogen Life Technologies, USA). The PCR mixture (25 μL) contained 1 μL of the first strand cDNA product, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 1 U Taq DNA polymerase (Promega, USA) and 0.4 μM of each primer. The primers $5'$-GATGAGGTAAACGCTTTGGC-$3'$ (upstream) and $5'$-TTCTGNACCCACTCRAAGTA-$3'$ (downstream) were designed and synthesized on the basis of the amino acid domains (DEVNALA and FEWVQNI, respectively) conserved from various GDH genes in the database. The PCR procedure was performed in an ASTEC Program Temperature Control System PC-700 under the following conditions: initial denaturation at 95°C for 10 min followed by 35 cycles of 40 sec at 95°C, 40 sec at 50°C, 40 sec at 72°C and 5 min at 72°C. The PCR products were confirmed by gel electrophoresis using 1% agarose gel stained with ethidium bromide and visualized under UV light.

Cloning and sequencing of cDNA: The amplified cDNA was ligated to the plasmid pT7Blue vector (Novagen, Inc., USA) using DNA Ligation Kit v2.1 (TaKaRa Bio, Inc., Japan) and cloned into *E. coli* (DH5á) (Invitrogen Life Technologies, USA). The cloned cDNA was sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI 3100 Genetic Analyzer (Applied Biosystems, USA).

Sequence data analysis: Nucleotide sequencing, homology analysis and phylogenetic tree construction were performed using computer software BioEdit (Hall, 1999).

Preparation of the dioxigenin (DIG)-dUTP labeled PCR probe: The DIG-labeled PCR probe was prepared following the instructions in the PCR DIG Labeling Mix (Roche, Germany) using cloned GDH cDNA as template.

RNA gel blot analysis: RNA gel blot analysis was performed following the instructions of ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham Biosciences, UK). The levels of transcripts were measured using computer software Image J (Abramoff *et al*., 2004.)

Gene bank accession number: The nucleotide sequence of partial cDNA was submitted to the DDBJ/EMBL/GenBank nucleotide sequence database and was designated as BoGDH with accession number AB212934.

RESULTS

Ammonia content: A rapid increase in ammonia content in the floret portion was found after third day from harvest (Fig. 1). At the end of the 5 days experimental period, the increase accounted for more than seven times the initial content. In the branchlet portion, the concentration of ammonia did not change remarkably throughout the whole storage duration.

Glutamate dehydrogenase activity: Initially, higher enzyme activity was observed in the branchlet than in the floret portion of the broccoli head. However, after 24 h

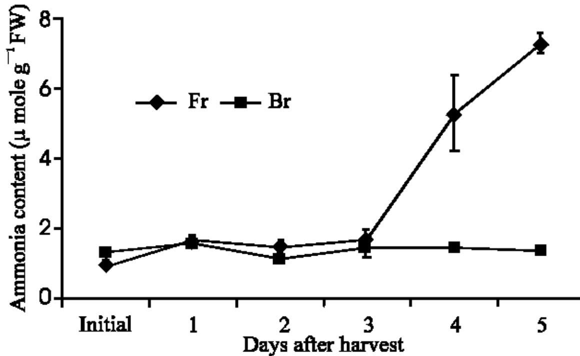

Fig. 1: Ammonia content in the floret and branchlet portions of broccoli head held at 20°C for 5 days after harvest. Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Fr-Florets; Br-Branchlets

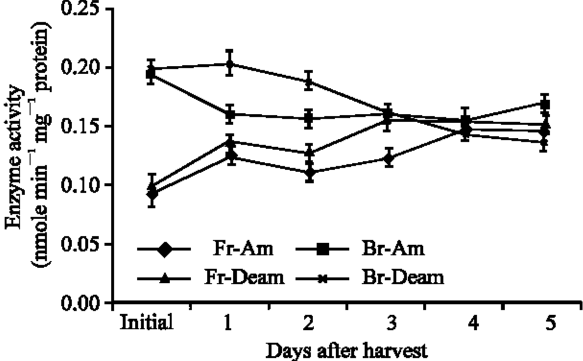

Fig. 2: Changes in the activities of GDH-amination and deamination in the floret and branchlet portions of broccoli head held at 20°C for 5 days after harvest. Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Fr-florets; Br-branchlets; Am-amination and Deam-deamination

from harvest, GDH-aminating activity in the branchlets dropped and remained at almost constant level until the fourth day. GDH-deaminating activity in the branchlet, on the other hand, decreased continuously as senescence progressed. In the floret portion, both aminating and deaminating activities increased gradually until the fourth and third day after harvest, respectively (Fig. 2).

Isolation and identification of the cDNA clone encoding GDH and phylogenetic tree analysis: The cloned partial cDNA designated as BoGDH (*B. oleracea* glutamate dehydrogenase, AB212934) contained 781 nucleotides

Table 1: Percent homology of nucleotide and deduced amino acid sequences between GDH from broccoli and other plant species in the database

Plant	Accession No.	Nucleotide	Amino acid
Arabidopsis thaliana	ATU53527	90	95
Brassica napus	AB066298	84	76
Glycine max	AJ879895	81	89
Oryza sativa	AY332470	81	83
Lupinus luteus	AY681352	82	85
Asparagus officinalis	AJ011096	81	83
Lycopersicon esculentum	AF403178	81	90
Zea mays	ZMU93561	80	81
Nicotiana plumbaginifolia	Y08293	79	88
Vitis vinifera	X86924	79	76
Nicotiana tabacum	AJ420266	78	88

Brassica oleracea (AB212934) is calculated as 100%

(Fig. 3). The cDNA exhibited high homology with GDH genes from other plant species in the database (Table 1). It showed highest identity at nucleotide level with *Arabidopsis thaliana* (90%) and at least 80% identity with *Brassica napus, Glycine max, Oryza sativa, Lupinus luteus, Asparagus officinalis, Lycopersicon esculentum* and *Zea mays.* At amino acid level, BoGDH has also the highest identity with *Arabidopsis thaliana* (95%) and *Lycopersicon esculentum* (90%) (Table 1).

The phylogenetic tree derived from deduced amino acid sequences and analyzed by multi-alignment analysis using BioEdit software revealed that BoGDH from *B. oleracea* (AB212934) and GDH1 from *A. thaliana* (ATU53527) are strongly clustered in a subgroup belonging to the *Brassicaceae* family and having closest relationship with GDH2 and NAD(H)-GDH genes from *B. napus* (AB066298) and *V. vinifera* (X86924), respectively; all are dicotyledonous plants (Fig. 4).

GDH gene expression: Gene expression of the partial clone BoGDH encoding GDH transcript from broccoli head was examined by RNA gel blot analysis of total RNA isolated from branchlets and florets tissues. The DIG-labeled cDNA was used as a probe for the analysis of transcript levels of GDH. The transcripts were detected in both portions from harvest until the end of the 5-day storage period (Fig. 5). However, the expression was not consistent with enzyme activity.

DISCUSSION

Broccoli like other perishable vegetables such as asparagus spears (Enriquez *et al*., 2001) and bamboo shoots (Matsui *et al*., 2004) produces a remarkable amount of ammonia (Fig. 1) and loses about 50% of sucrose (Downs *et al*., 1997; Pramanik *et al*., 2004) few days after harvest. These conditions trigger plant tissues to undergo appropriate modification in their metabolism to survive. Considering the metabolic action of GDH, it is likely that the enzyme plays an essential role during

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1     CAG CTC ATG ACA TGG AAA ACA GCA GTG GCT AAC ATT CCG TAC GGA   46
1      Q   L   M   T   W   K   T   A   V   A   N   I   P   Y   G    15

47    GGA GCC AAA GGA GGC ATT GGC TGT GAT CCG AGC AAG CTC AGC ATC   91
16     G   A   K   G   G   I   G   C   D   P   S   K   L   S   I    30

92    TCT GAG CTT GAG AGA TTG ACT AGA GTT TTC ACT CAG AAG ATT CAT   136
31     S   E   L   E   R   L   T   R   V   F   T   Q   K   I   H    45

137   GAT CTC ATC GGG ATA CAC ACT GAT GTT CCA GCT CCT GAT ATG GGC   181
46     D   L   I   G   I   H   T   D   V   P   A   P   D   M   G    60

182   ACT GGT CCT CAG ACA ATG GCT TGG ATT CTT GAC GAG TAC TCT AAG   226
61     T   G   P   Q   T   M   A   W   I   L   D   E   Y   S   K    75

227   TTC CAT GGA TAC TCG CCT GCA GTT GTC ACT GGA AAA CCT ATT GAT   271
76     F   H   G   Y   S   P   A   V   V   T   G   K   P   I   D    90

272   CTT GGT GGA TCA CTC GGG AGA GAC GCT GCC ACT GGA AGA GGA GTG   316
91     L   G   G   S   L   G   R   D   A   A   T   G   R   G   V    105

317   ATG TTT GCA ACT GAA GCT TTG CTT AAT GAG CAC GGC AAG AGC ATT   361
106    M   F   A   T   E   A   L   L   N   E   H   G   K   S   I    120

362   TCA GGC CAA CGT TTT GTC ATC CAG GGG TTT GGG AAT GTG GGC TCA   406
121    S   G   Q   R   F   V   I   Q   G   F   G   N   V   G   S    135

407   TGG GCG GCG AAG CTG ATA AGT GAA CAA GGT GGG AAG ATA GTT GCG   451
136    W   A   A   K   L   I   S   E   Q   G   G   K   I   V   A    150

452   GTG AGT GAT ATT ACC GGA GCC ATC AAG AAC AAG GAC GGT ATC GAT   496
151    V   S   D   I   T   G   A   I   K   N   K   D   G   I   D    165

497   ATC GAG AGC TTG CTC AAC TAT ACC AAA GAA CAC AGA GGT GTT AAA   541
166    I   E   S   L   L   N   Y   T   K   E   H   R   G   V   K    180

542   GGG TTT GAT GGT GCG CAT CCG ATC GAT GCA AAC TCG ATA CTG GTC   586
181    G   F   D   G   A   H   P   I   D   A   N   S   I   L   V    195

587   GAG GAT TGT GAT ATC CTC ATC CCT GCT GCA CTT GGT GGT GTC ATC   631
196    E   D   C   D   I   L   I   P   A   A   L   G   G   V   I    210

632   AAC AGG GAG AAT GCG AAT GAG ATT AAA GCA AAG TTC ATC ATT GAA   676
211    N   R   E   N   A   N   E   I   K   A   K   F   I   I   E    225

677   GCT GCT AAC CAT CCA ACT GAT CCC GAT GCT GAT GAG ATC TTG AGT   721
226    A   A   N   H   P   T   D   P   D   A   D   E   I   L   S    240

722   AAG AAA GGT GTG GTC ATT CTC CCA GAC ATA TAT GCA AAT TCT GGA   766
241    K   K   G   V   V   I   L   P   D   I   Y   A   N   S   G    255

767   GGA GTT ACT GTC AGC   781
256    G   V   T   V   S
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Fig. 3: Nucleotide and deduced amino acid sequences of the cDNA clone corresponding to BoGDH. The predicted amino acid sequence is given in single letter code for each amino acid. The arrows indicate the position of degenerate primers (sense →, anti-sense ←) used for RT-PCR. Numbering refers to total nucleotide (upper) and amino acid (lower) residues on each line

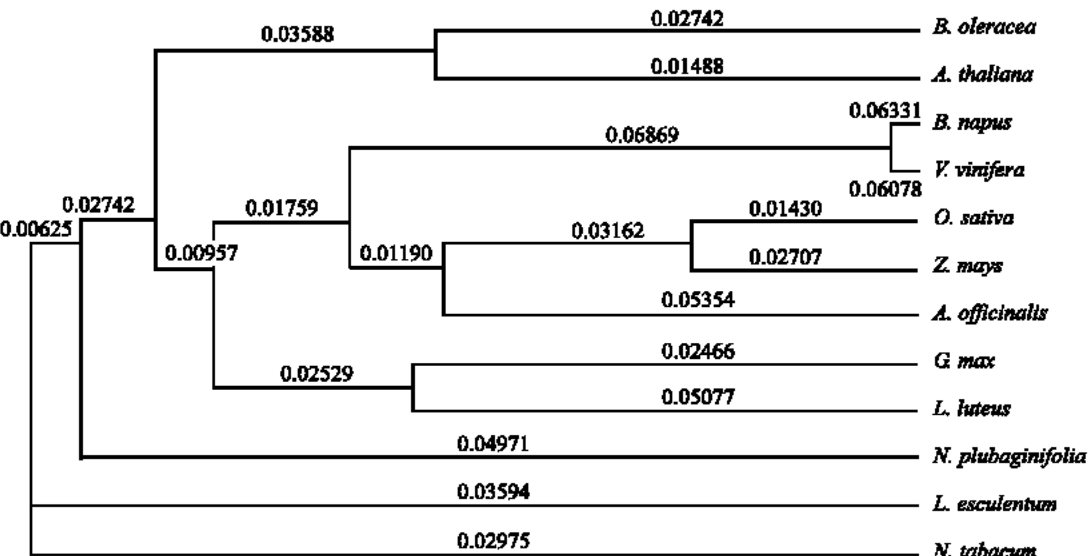

Fig. 4: Phylogenetic tree of the alignment of BoGDH deduced amino acid sequence with other GDH amino acid sequences in the database. Amino acid sequences were aligned using UPGMA and phylogenetic tree was constructed using BioEdit software

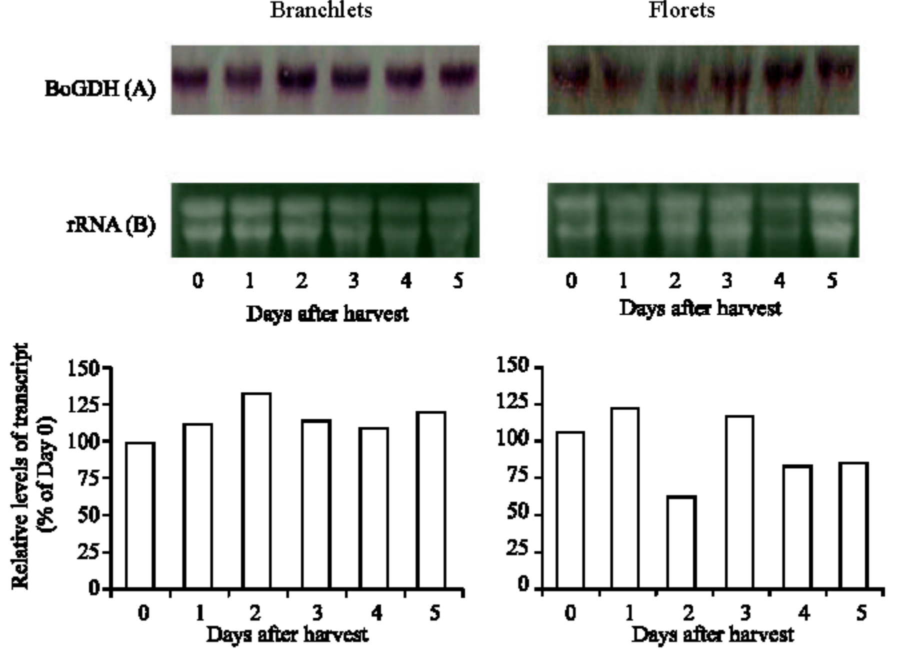

Fig. 5: RNA gel blot analysis of BoGDH transcript. The upper panels (A) are gel blots of total RNA (each lane containing 10 μg) isolated from broccoli branchlets and florets and separated by electrophoresis on 1% agarose gel containing 20x MOPS and 37% formaldehyde while the bottom panels (B) are representatives from ethidium bromide stained gels showing rRNA. Relative level of transcripts (% of day 0) of each band was measured from color precipitation of NBT and BCIP appeared on the membrane using Image software

postharvest senescence. In this study, it was observed that GDH activity (both amination and deamination) has changed during storage. The enzyme activity in the branchlet portion decreased while in the floret portion increased gradually with time (Fig. 2). The changes in the branchlet portion may imply that the enzyme may have only an alternative role due to the almost constant ammonia level (Fig. 1) and higher sugar content (Pramanik *et al.*, 2004) of this tissue than the floret. The level of ammonia may not have reached a repressive level for GS/GOGAT and the high sugar content increases the energy level of the tissue, thereby favoring the activity of other ammonia-assimilating enzymes rather than GDH. The increasing activity in the floret portions may suggest the detoxification and energy generation roles of GDH.

To understand the molecular basis of induction of GDH during postharvest senescence, a cDNA for GDH was isolated from broccoli head. The 781 bp partial clone cDNA (Fig. 3) BoGDH gene was found to have highest homology at nucleotide and amino acid levels (Table 1) with *A. thaliana* GDH gene associated with stress, e.g., sucrose-starved condition and/or application of exogenous supply of ammonia (Melo-Oliveira *et al.*, 1996). Phylogenetic analysis of GDH sequences revealed the existence of two clusters that contain characteristics of conserved amino acid sequences from monocotyledonous and dicotyledonous species (Fig. 4); most of them have stress related functions. Although the level of transcripts was not consistent with enzyme activity, the expression was found in both portions throughout the experimental period (Fig. 5). The results indicate that the enzyme is ubiquitously present in the tissues. However, the inconsistency between the enzyme activity and gene expression could be due to multiple levels of regulation (transcriptional or posttranscriptional) as observed in *N. plumbaginifolia* (Restivo, 2004) and/or encoded by multigenes as found in ACS/ACO in broccoli florets (Nishikawa *et al.*, 2005). In maize, NADH-GDH was encoded by GDH1 and GDH2 and is located in two independent loci (Pryor, 1979; Goodman *et al.*, 1980).

CONCLUSIONS

This study suggests that GDH may have played a significant role during senescence of broccoli florets in response to stress imposed by harvest and storage. The results provide additional basis for further comprehensive molecular studies on the regulation of its function in broccoli after harvest as well as, other nitrogen and sugar-assimilatory enzymes.

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