

ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)



Bio Technology



ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Molecular Cloning of Glutamine Synthetase cDNA from *Lactuca sativa*: Sequence Analysis and Gene Expression during Storage

¹Dulal Chandra, ¹Toshiyuki Matsui, ¹Haruo Suzuki, ¹Yusuke Kosugi and ²Koichi Fujimura
¹Department of Bioresource Production Science, Faculty of Agriculture, Kagawa University,
Miki-Cho, Ikenobe 2393, Kagawa 761-0795, Japan

²Kagawa Prefectural Agricultural Experiment Station, Busshouzan, Takamatsu, Kagawa 761-8078, Japan

Abstract: In order to understand the factors contributing to postharvest deterioration of lettuce, the changes in ammonia content as well as activity and gene expression of GS were investigated in the outer and inner leaves of lettuce head during storage at 20°C. About two times higher ammonia content out of its initial content was found in the outer leaf portion after the end of 96 h storage period. GS activity in this portion declined to about 27% of the initial level by 24 h of storage, while activity in the inner leaf portion almost unchanged throughout the storage. To understand clearly these biochemical changes, a cDNA encoding GS was isolated, cloned and sequenced from lettuce leaves. The partial cDNA clone referred to as *LsGS* (*Lactuca sativa* Glutamine Synthetase; AB440673) consisted of 799 nucleotides which showed more than 80% similarity for both nucleotide and amino acid level with the GS genes of other dicotyledonous plant. Northern blot analysis showed that the level of transcript of GS decreased in the outer-leaf portion after 24 h of storage which well correlates with the enzyme activity of this portion. Although, we found no specific trend in GS activity in the inner-leaf portion, the transcript level gradually increased until the end of storage period. The inconsistency between enzyme activity and gene expression may suggest that GS expression in lettuce is controlled by multiple levels of regulations in a tissue-specific manner.

Key words: Ammonium, cDNA, glutamine synthetase, gene expression, *Lactuca sativa*, storage

INTRODUCTION

Vegetables that are harvested at immature stage before cease their growth are subjected to considerable stress due to sudden disruption in energy, nutrient and hormone supplies (Huber, 1987). Consequently, many physio-biochemical changes such as bleaching of green color, deterioration of cellular structure and losses of nutritional content are occurred revealing shorter shelf-life of the produce. In lettuce, a gradual decline in sugar content along with overall quality deterioration has been reported during storage at ambient temperature (Suthumchai *et al.*, 2006). This declining energy level, especially in leafy vegetables like lettuce, favors a preponderance of catabolism and the disappearance of high molecular proteins (Schwerdtfeger, 1978). They are proteolytically split to low-molecular compounds like amino acids and the accumulating amino acids are then transformed to nitrogen-storage compounds which subsequently deaminated with the liberation of free ammonia (Schwerdtfeger, 1978). However, excessive concentration of ammonia or ammonium ions may cause toxic effect to plant cells (Givan, 1979) thereby enhancing

quality deterioration in harvested leafy-green vegetables. For this reason, it needs to be rapidly assimilated. Current evidence indicates that ammonium assimilation is carried out by the concerted action of two enzymes, Glutamine Synthetase (GS) and glutamate synthase (GOGAT). Glutamine synthetase (GS; E.C. 6.3.1.2) catalyzes the conversion of inorganic nitrogen (ammonium) into organic form (glutamine) and for that reason, is a good candidate to be a critical and possibly rate-limiting enzyme in ammonium assimilation (Oliveira *et al.*, 2002). One particular important characteristic of GS is its high affinity for ammonia and thus its ability to incorporate ammonia efficiently into organic combination. Therefore, the accumulation of ammonium in senescing leaves has shown some promise coincided with the disappearance of GS activity (Peeters and Van Laere, 1992). Treatment of green asparagus with phosphinothricin (PPT), a potent inhibitor of GS, results in a rapid accumulation of ammonia in the spear tips and a reduction of shelf-life (Hurst *et al.*, 1993).

GS activity in ammonium assimilation is fulfilled by GS isoforms expressed in specific organ and developmental stages (Mifflin and Habash, 2002). Besides

the subcellular localization, two different classes of GS have been reported, GS₁ in the cytosol and GS₂ in the chloroplasts. In the leaves of many plants, GS₂ is the predominant isoform, where, it is located in mesophyll photosynthetic cells, whereas GS₁ is generally less abundant in photosynthetic cells and a predominant isoform in roots and other non-photosynthetic tissues (Ireland and Lea, 1999). The complete cDNAs of cytosolic and chloroplastic GS have also been isolated from many plants including major crops (Cren and Hirel, 1999; Ireland and Lea, 1999). GS₂ is encoded by a single nuclear gene and its functional role is the assimilation of ammonium derived from the reduction of nitrate and from photorespiration (Lam *et al.*, 1996). Cytosolic GS, on the other hand, exists as a variable number of isoforms which are encoded by a small gene family and the physiological role of individual genes are unclear. However, some report indicated that GS₁ may be involved in a variety of processes such as N recycling and translocation between source and sink tissues as well as reassimilation of N mobilized during senescence (Cren and Hirel, 1999). Although, both the GS₁ and GS₂ cDNA clones have become available from the vegetative parts of many mono and dicotyledonous species, they have not been yet isolated from lettuce. Hence, a partial cDNA encoding GS was isolated, cloned and sequenced from lettuce leaf. The objective of this study was to improve understanding of GS activity and gene expression in the outer (green) and inner (white) leaves of lettuce. The cDNA clone showed high homology with other GS in the database and was used as a probe for northern blot analysis of total GS without separation of cytosolic and chloroplastic forms.

MATERIALS AND METHODS

Plant materials: Iceberg lettuce heads (*Lactuca sativa* L., cv. Takii-242) were harvested from the Kagawa Prefectural Agricultural Experiment Station, Busshouzan, Kagawa, Japan on November 2007. Harvesting was done at commercial maturity stage when the heads were well formed and solid. The wrapper leaves were removed from the harvested heads leaving the first tightly held leaf attached. Then the heads were packed in a box with crushed ice and immediately transported to the laboratory and stored at 20°C in perforated plastic bag for up to 4 days in dark condition. After each storage treatment, heads were weighed and three outer green leaves were separated from each head and designated as outer-leaf portion. The next three white-green leaves were not used and the following three white leaves were collected as inner-leaf portion. Leaves were cut into small

pieces excluding the midrib portion and immediately kept at -80°C until needed for GS and ammonia assay and for total RNA extraction.

Enzyme extraction: Enzyme extraction was done following the procedure described by Hurst and Clark (1993). Briefly, approximately 5 g lettuce sample from the outer- and inner-leaf portion was homogenized with 1% polyvinylpyrrolidone (PVPP) proportional to the sample weight, 1 g sea sand and 5 mL buffer A solution using a cooled mortar and pestle. Buffer A contained 50 mM Tris HCl (pH 7.6), 10 mM MgSO₄·7H₂O, 1 mM EDTA, 1 mM dithiothreitol (DTT), 12 mM 2-mercaptoethanol, 5 mM L-glutamate and 100 mL glycerol per liter. The homogenate was filtered through four layers of cotton cloth. The residual tissues were re-extracted with an additional 5 mL of the same buffer and the filtrate was centrifuged at 11,000 x g at 2°C for 10 min. The resulting supernatant was used for enzyme assay. All the activities were carried out under ice cold condition (0-4°C). All chemicals to prepare buffers and to conduct other biochemical activities were purchased from Wako Pure Chemical Industries Ltd., Japan unless stated otherwise.

GS activity and ammonia assay: GS activity was measured using a double beam spectrophotometer (Model UV-150-02, Shimadzu Corp., Kyoto, Japan) at 540 nm. Along with the enzyme solution, the 1 mL assay mixture contained 80 mM Na-L glutamate, 100 mM tricine-KOH buffer (pH 7.0), 6 mM hydroxylammonium chloride (HONH₂Cl), 20 mM MgSO₄·7H₂O, 1 mM diethylenetriamine pentaacetic acid (DTPA), 8 mM ATP and 8 mM mercaptoethanol. After incubating the assay mixture at 35°C for 8 min, 1 mL ferric chloride reagent that contains 0.37 M FeCl₃, 0.67 N HCl and 0.2 M trichloroacetic acid (TCAA) was added to the mixture to stop the reaction. Soluble protein contents were determined following the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

To assess ammonia content, 2 g sample from each of outer and inner leaves were extracted with 10% trichloroacetic acid at 1:10 ratio (w/v) in an ice bath (0-4°C) and centrifuged at 11,000 x g at 2°C for 10 min. Ammonia content of supernatant was assayed in a triplicate measurement as described by Kun and Kearney (1974), where 1 mL assay mixture contained 200 µL 0.5 M Tris-HCl buffer (pH 8.0), 100 µL 0.1 M 2-oxoglutarate solution (pH 7.4), 30 µL 8 mM β-NADH solution, 20 µL GIDH (10 mg mL⁻¹), 150 µL distilled water and 500 µL of neutral extract sample. The decrease in NADH, as determined by the change of extinction at 365 nm was recorded against a reagent blank.

RNA isolation: Total RNA was isolated according to the hot borate methods of Wan and Wilkins (1994) with few modifications. Briefly, three gram frozen-leaf samples (outer and inner leaf separately) were ground to fine powder in liquid nitrogen and transferred to a 50 mL sterilized plastic tube followed by the addition of 10.5 mL hot borate extraction buffer consisting of 0.2 M sodium borate, 30 mM EGTA, 1% (w/v) Sodium Dodecyl Sulphate (SDS), 1% (w/v) deoxycholic acid sodium salt, 10 mM dithiothreitol (DTT), 1% (v/v) nonidet P-40 and 2% PVP-40. Immediately after gentle vortexing, 315 μ L proteinase K (20 mg mL⁻¹) was added to the homogenate and incubated at 42°C with gentle shaking for 1.5 h and then 840 μ L 2 M KCl was added and incubated in ice for 1 h. After centrifugation at 12,000 x g at 4°C for 20 min, the supernatant was collected and one third (of supernatant) volume of cold 8 M LiCl was added and incubated at 4°C for 16 h. The RNA was pelleted by centrifugation at the same condition, washed twice with 5 mL cold 2 M LiCl by resuspension and centrifugation at the same condition. Finally, the pellets were dissolved in 2 mL of 10 mM Tris-HCl buffer (pH 7.5) and extracted with 100 μ L of K-acetate (pH 5.5). After centrifugation, RNA was precipitated with 100% cold ethanol and incubated at -80°C for overnight. RNA pellets were then precipitated by centrifugation at 10,000 x g at 4°C for 30 min and washed with 70% cold ethanol. Finally, pellets were dissolved in sterilized distilled water and preserved at -80°C.

Amplification of poly (A⁺) RNA by RT-PCR: The first strand cDNA was synthesized from 5 μ g of the total RNA by reverse transcriptase with Oligo-(dT) primer following the instructions of SUPERScript™ First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, USA). The 25 μ L PCR mixture contained 1 μ L of the first strand cDNA reaction product, 1 \times PCR buffer (TaKaRa Bio Inc., Japan), 1.5 mM MgCl₂ (TaKaRa Bio Inc, Japan), 0.2 mM each of dNTP (Roche, Germany), 1 U Taq DNA polymerase (TaKaRa Bio Inc, Japan) and 2 μ M of each primer. The primers 5'-TAYGATGGITCIAGYACHGGICAAGC-3' (upstream) and 5'-CCATGTTIGAAGCHGGICKBCKRTCCTC-3' (downstream) were designed and synthesized on the basis of amino acid domains (YDGSSTGQA and EDRRPASNMD, respectively) conserved from various GS genes in the database. The PCR procedure started with 4 min at 95°C followed by 30 cycles of 40 sec at 95°C, 40 sec at 55°C, 40 sec at 72°C and ended with 5 min at 72°C in a Program Temperature Control System (PC-700, ASTEC, Japan). The PCR products were confirmed by 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 v 2.1 (TaKaRa Bio. Inc., Japan) and cloned into *E. coli* (DH5 α competent cell) (TaKaRa Bio. Inc., Japan). Transformed colonies were screened from LB agar containing X-gal, IPTG and ampicillin sodium. The plasmid containing the cDNA clone was isolated using QIAprep® Spin Miniprep Kit (QIAGEN Sciences, USA). The cloned cDNA was sequenced using BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI 3100 Genetic Analyzer (Applied Biosystems, USA).

Sequence data analysis: Nucleotide sequence and homology analysis were performed using computer software BioEdit (Hall, 1999). Database search were performed using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) website. The radial phylogenetic tree was constructed by using computer software BioEdit with TreeView ver. 1.6.6 and EMBL-EBI/ ClustalW softwares.

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

Northern blot analysis: Ten microgram of total RNA in each lane was subjected to electrophoresis on 1% agarose gel (Type II: Medium EEO, Sigma, USA) containing 1 \times MOPS and 2% formaldehyde. RNA was visualized with ethidium bromide under UV light to confirm equal loading of RNA in each lane. RNA gel blot analysis was performed following the instructions of ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences, UK). Briefly, RNA was transferred to a positively charged nylon membrane Hybond™ N⁺ by capillary action with 20 \times SSC for overnight. The RNA was fixed to the membrane by baking at 80°C for 2 h. The membrane was prehybridized at 47°C with 5 \times SSPE, 5 \times Denhardt's solution, formamide, 0.5% SDS and 1 mg mL⁻¹ denatured salmon sperm DNA. Hybridization was performed with the same condition and buffer using the gene specific DIG labeled PCR probe for about 21 h. The blot was washed twice with 2 \times SSPE containing 0.1% SDS for 10 min at room temperature, then at 65°C with 1 \times SSPE containing 0.1% SDS for 15 min and finally with 0.1 \times SSPE containing 0.1% SDS at 65°C for 10 min. For anti-DIG antibody conjugation, the blot was also washed with maleic acid buffer with polyoxyethylene sorbitan monolaurate (Tween 20) and then with 10% blocking reagent in maleic acid buffer for 30 min. Subsequently, the

membrane was incubated in blocking buffer with Anti-DIG-AP (Roche, Germany) for 30 min. Hybridization signals were detected by color reaction using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitroblue-tetrazolium chloride (NBT) (Roche, Germany) as the substrate.

Gene bank accession number: The isolated nucleotide sequence of partial cDNA was submitted to the DDBJ/EMBL/GeneBank nucleotide sequence database and was designated as *LsGS* with the accession number AB440673.

RESULTS

Glutamine synthetase activity: Figure 1a shows GS activity in the outer and inner leaf portions of lettuce head during storage at 20°C. In the outer leaf portion, GS activity declined to about 27% to its initial level by 24 h of storage and after that almost unchanged with a slight increase at 96 h of storage. However, GS activity in the inner leaf portion did not show any consistent trend except for a slight increase at the end of storage. Higher GS activity was found in the outer leaf portion than in the inner leaf portion.

Ammonia content: A gradual increase in ammonia content was observed after 24 and 48 h of harvest in the outer and inner leaf portions, respectively (Fig. 2). At the end of 96 h storage period, the increase in ammonia contents were accounted for about two and one and half times to their initial level in the outer and inner leaf portion, respectively.

Isolation and identification of cDNA clone: The isolated cDNA clone termed as *LsGS* (*Lactuca sativa* Glutamine Synthetase; AB440673) is a partial clone consisted of 799 nucleotides (Fig. 3, 4) and the deduced amino acid sequence was highly homologous to the amino acid sequences of other plants in the database (Fig. 5). It showed the highest identity at nucleotide level with *Raphanus sativus* (83.6%) and at least 81% identity with *Lycopersicon esculentum*, *Medicago sativa*, *Brassica rapa*, *Solanum tuberosum*, *Cucumis melo* and *Pisum sativum* (Table 1). At deduced amino acid level, *LsGS* showed the highest similarity with *Lycopersicon esculentum* (93.2%) followed by *Medicago sativa* (92.1%).

The radial phylogenetic tree generated from the alignment of the deduced amino acid sequences showed that *LsGS* from *Lactuca sativa* (AB440673) clustered separately under the subgroup that contained *GS₂* of *Z. mays* (X65931) and *O. sativa* (X14246), having closest

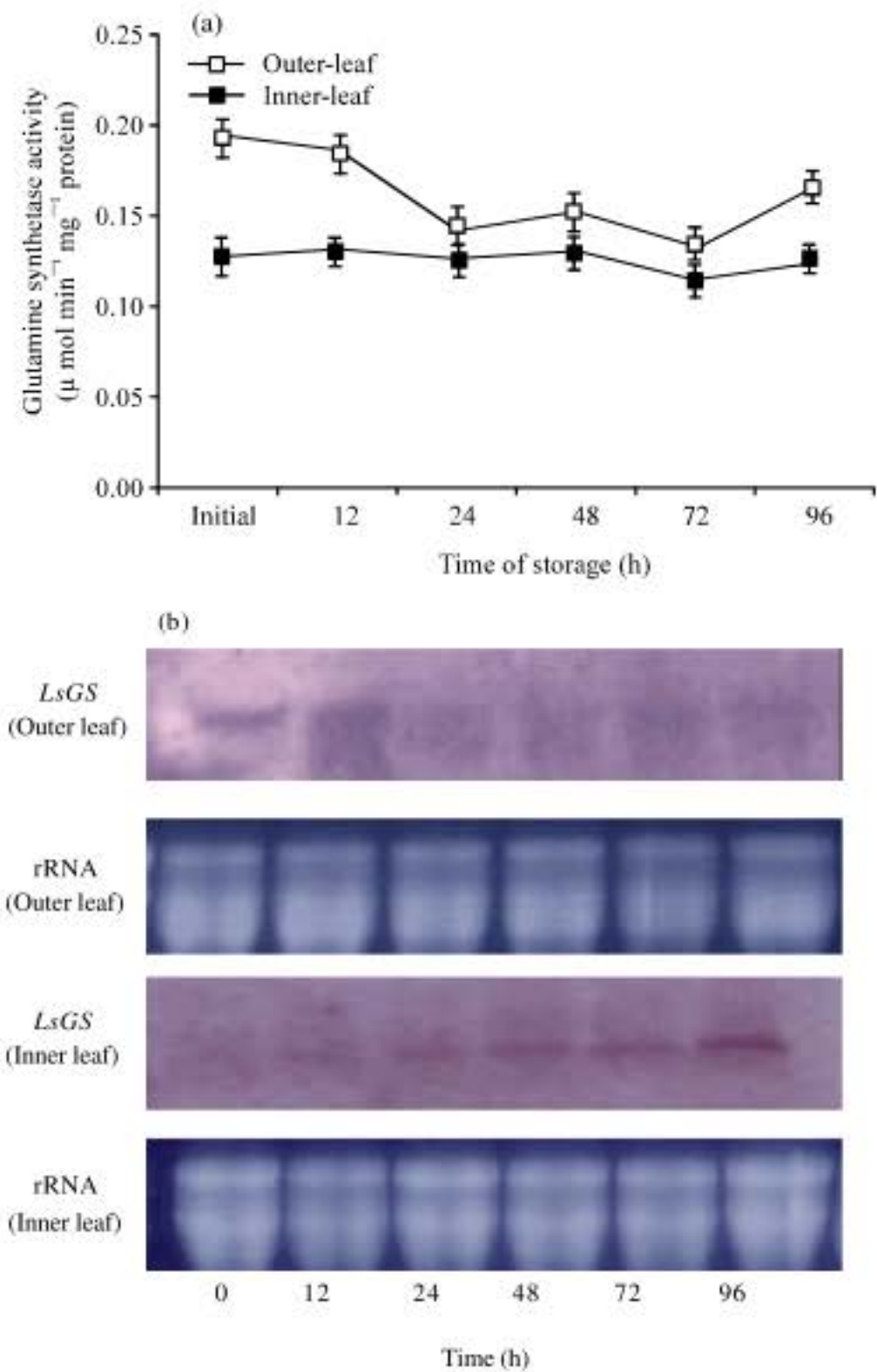


Fig. 1: Changes in glutamine synthetase activity and expression of *LsGS* gene in the outer and inner leaf of lettuce head. (a) Changes in glutamine synthetase activity. Each point represents the mean of three replicates and bars show SE. (b) Northern blot analysis for glutamine synthetase gene. Equal loading of RNA was confirmed by staining a gel with ethidium bromide

relationships with *GS₁* and *GS₂* genes from *R. sativus* (D25326) and *B. rapa* (EU239243), respectively (Fig. 6).

GS gene expression: The partial clone *LsGS* was used as a probe for northern blot analysis to determine the level of transcripts for GS. The level of transcript for GS declined after 24 h of harvest in the outer leaf portion (Fig. 1b) and correlated well with enzyme activity in this portion (Fig. 1a). After that transcript level slightly increased at the end of storage. Although, much lower activity was found in inner leaf portion showing no consistent trend of changes, the transcript level gradually increased until the 96 h storage period.

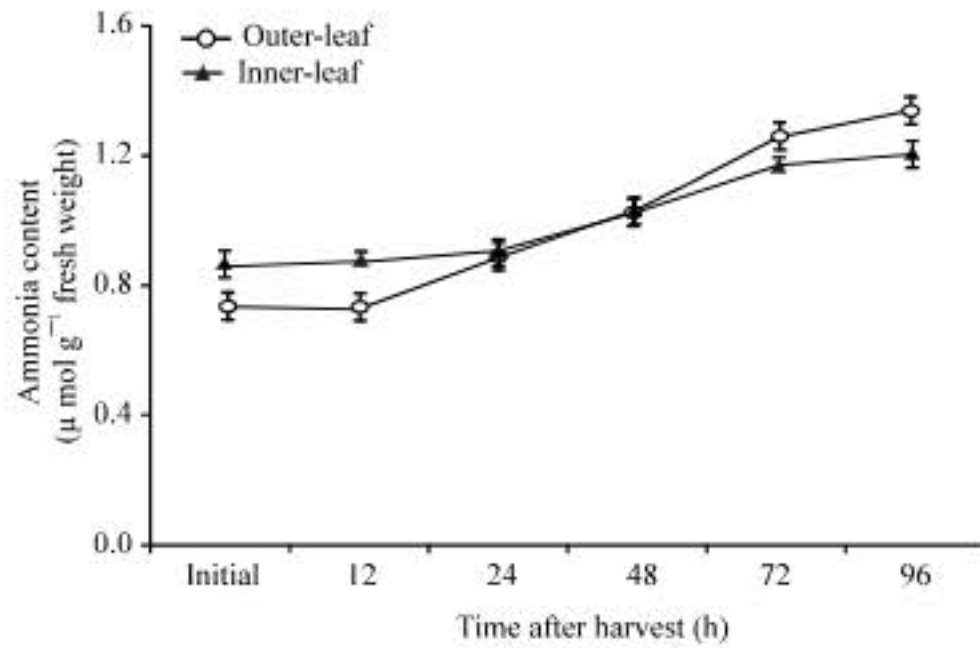


Fig. 2: Ammonia content in the outer and inner leaves of lettuce head during storage at 20°C. Each point represents the mean of three replicates and bar show SE

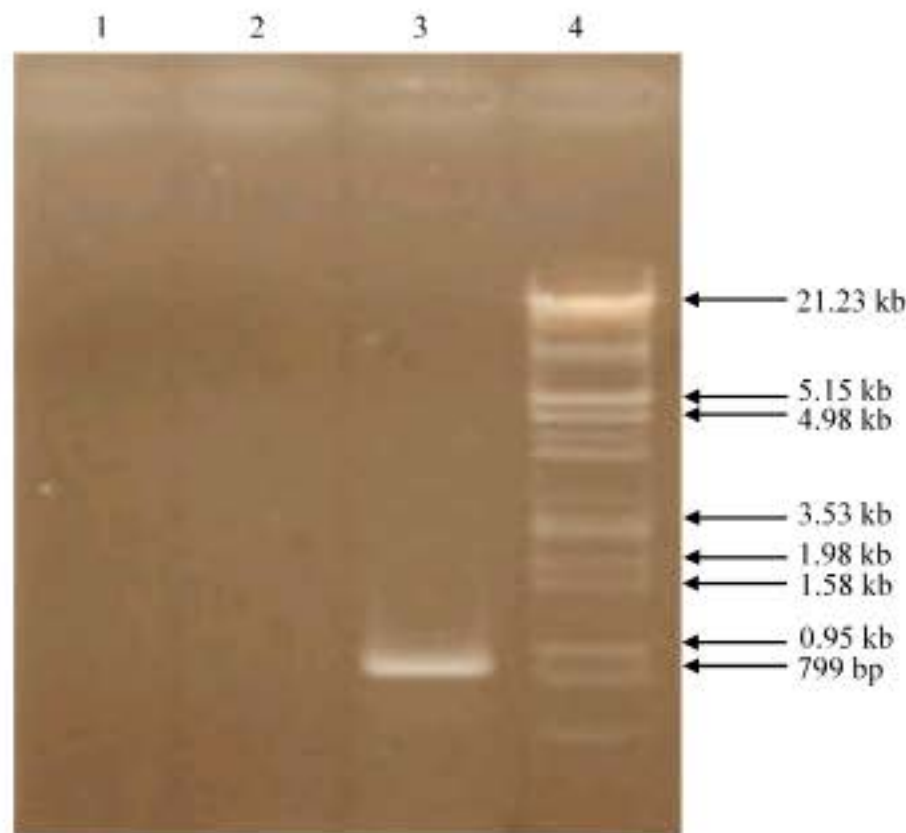


Fig. 3: Estimated length of *LsGS* RT-PCR product. Lane 1: PCR product of GS using only upstream primer; Lane 2: PCR product of GS using only downstream primer; Lane 3: PCR product of GS using both upstream and downstream primer and Lane 4: Standard *Hind III-EcoRI* digested λ -DNA

DISCUSSION

Like many other green vegetables, iceberg lettuce is harvested at immature stage and subjected to sudden disruption in carbohydrate, nutrient and growth regulator supplies (Huber, 1987; King *et al.*, 1990). Storage of the harvested head is some times necessary to ensure a continuous supply of this produce for the consumers. However, storage life is greatly influenced by many physio-biochemical activities associated with senescence

Table 1: Percentage of nucleic acid and deduced amino acid identity between glutamine synthetase from lettuce and other plant species in the database

| Plants | Accession No. | Nucleic acid (%) | Amino acid (%) |
|--------------------------------|---------------|------------------|----------------|
| <i>Raphanus sativus</i> | D25326 | 83.6 | 82.3 |
| <i>Lycopersicon esculentum</i> | BT013265 | 83.1 | 93.2 |
| <i>Medicago sativa</i> | AF124244 | 82.6 | 92.1 |
| <i>Brassica rapa</i> | EU239243 | 82.3 | 90.2 |
| <i>Solanum tuberosum</i> | AF302113 | 81.8 | 91.3 |
| <i>Cucumis melo</i> | AY773090 | 81.8 | 90.9 |
| <i>Pisum sativum</i> | M20664 | 81.8 | 89.8 |
| <i>Spinacia oleracea</i> | EF143582 | 80.9 | 90.2 |
| <i>Glycine max</i> | AF353620 | 80.7 | 91.3 |
| <i>Beta vulgaris</i> | AY026353 | 80.4 | 91.3 |
| <i>Zea mays</i> | X65931 | 79.2 | 89.4 |
| <i>Oryza sativa</i> | X14246 | 78.5 | 89.4 |

Lactuca sativa (AB440673) is calculated as 100%

and quality deterioration. Among them the depletion of carbon sources and the accumulation of ammonia which occurred by some enzymatic actions has been reported by Enriquez *et al.* (2001), Matsui *et al.* (2004) and Pramanik *et al.* (2005). In this study, we also observed an increase in ammonia content during storage of lettuce at 20°C (Fig. 2). The higher accumulation of ammonia was observed in the outer leaf portion compared with inner leaf portion. GS activity, on the other hand, was markedly distinguishable between these two portions; the former did not show any remarkable change, while a considerable decrease was observed in the later (Fig. 1a). The decrease in GS activity in the outer leaf portion might be the reason for the higher accumulation of ammonia. It was also reported that the accumulation of ammonia in senescing leaves has been shown to coincide with almost complete disappearance of GS (Peeters and Van Laere, 1992). It appeared that senescence occurred rapidly in the outer leaves of the lettuce head. However, we found different expression pattern of GS gene in these two types of tissues. Although, the transcript of *LsGS* was well correlated with the enzyme activity in the outer leaf portion, the transcript level in the inner leaf portion increased gradually during storage (Fig. 1). This inconsistency between enzyme activity and gene expression might be due to the multiple levels of regulatory mechanism of GS as observed in leaves of *Phaseolus vulgaris* (Cock *et al.*, 1991) and/or encoded with other distinct isoforms of GS genes as well as expressed in a tissue-specific manner (Ochs *et al.*, 1999). Although, two isoforms of GS gene have been reported in many plant species, Hirel *et al.* (1982) reported that spinach leaf contain only GS₂ (chloroplastic), whereas other C₃ plants like rice, pea and barley showed a minor GS₁ component which contribute only up to 20% of the total GS enzyme activity (McNally *et al.*, 1983). Therefore,

| | | | | | | | | | | | | | | | | | |
|-----|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1 | A | CCA | GGA | GAA | GAC | AGT | GAA | GTT | ATC | TTA | TAC | CCT | CAG | GCG | ATC | TTT | 46 |
| 1 | | P | G | E | D | S | E | V | I | L | Y | P | Q | A | I | F | 15 |
| 47 | | AAG | GAC | CCT | TTC | CGT | GGT | GGC | AAC | AAC | ATC | TTG | GTA | ATC | TGT | GAT | 91 |
| 16 | | K | D | P | F | R | G | G | N | N | I | L | V | I | C | D | 30 |
| 92 | | ACA | TAC | ACT | CCA | CAA | GGA | GTT | CCT | ATC | CCT | ACA | AAC | AAA | CGT | GCA | 136 |
| 31 | | T | Y | T | P | Q | G | V | P | I | P | T | N | K | R | A | 45 |
| 137 | | AAG | GCT | GCT | GAG | ATT | TTC | AGT | GAT | CCT | AAA | GTT | GTA | GCA | CAA | GTT | 181 |
| 46 | | K | A | A | E | I | F | S | D | P | K | V | V | A | Q | V | 60 |
| 182 | | CCC | TGG | TTT | GGA | ATT | GAG | CAA | GAG | TAC | ACT | TTG | CTT | CAA | CAA | GAT | 226 |
| 61 | | P | W | F | G | I | E | Q | E | Y | T | L | L | Q | Q | D | 75 |
| 227 | | GTG | AAG | TGG | CCT | TTG | GGT | TGG | CCT | GTT | GGA | GGG | TAC | CCT | GGT | CCT | 271 |
| 76 | | V | K | W | P | L | G | W | P | V | G | G | Y | P | G | P | 90 |
| 272 | | CAG | GGT | CCA | TAC | TAC | TGT | GGT | GCG | GGG | GCA | GAT | AAG | TCA | TTT | GGA | 316 |
| 91 | | Q | G | P | Y | Y | C | G | A | G | A | D | K | S | F | G | 105 |
| 317 | | AGG | GAC | ATA | TCT | GAT | GCA | CAT | TAC | AAG | GCT | TGT | TTA | TAC | GCT | GGA | 361 |
| 106 | | R | D | I | S | D | A | H | Y | K | A | C | L | Y | A | G | 120 |
| 362 | | ATC | AAC | ATC | AGT | GGA | ACC | AAT | GGT | GAA | GTC | ATG | CCA | GGA | CAG | TGG | 406 |
| 121 | | I | N | I | S | G | T | N | G | E | V | M | P | G | Q | W | 135 |
| 407 | | GAA | TTC | CAA | GTG | GGT | CCC | AGC | GTT | GGA | ATC | GAA | GCC | GGT | GAC | CAT | 451 |
| 136 | | E | F | Q | V | G | P | S | V | G | I | E | A | G | D | H | 150 |
| 452 | | ATC | TGG | TGT | GCC | CGA | TAC | CTC | CTT | GAG | AGA | ATT | ACT | GAA | CAA | GCT | 496 |
| 151 | | I | W | C | A | R | Y | L | L | E | R | I | T | E | Q | A | 165 |
| 497 | | GGT | GTT | GTT | TTG | ACA | CTT | GAC | CCT | AAG | CCA | ATT | GAG | GGA | GAC | TGG | 541 |
| 166 | | G | V | V | L | T | L | D | P | K | P | I | E | G | D | W | 180 |
| 542 | | AAT | GGA | GCA | GGA | TGC | CAC | ACT | AAC | TAC | AGC | ACA | TTA | AGC | ATG | AGA | 586 |
| 181 | | N | G | A | G | C | H | T | N | Y | S | T | L | S | M | R | 195 |
| 587 | | GAA | GAA | GGT | GGA | TTT | GAA | GTA | ATC | AAA | GAG | GCG | ATT | CTG | AAC | CTT | 631 |
| 196 | | E | E | G | G | F | E | V | I | K | E | A | I | L | N | L | 210 |
| 632 | | TCC | CTT | CGC | CAC | GCA | GAC | CAC | ATC | AGT | GCT | TAC | GGT | GAA | GGA | AAT | 676 |
| 211 | | S | L | R | H | A | D | H | I | S | A | Y | G | E | G | N | 225 |
| 677 | | GAA | AGA | AGA | TTG | ACA | GGA | AAA | CAC | GAA | ACT | GCC | AGC | ATC | AAC | ACA | 721 |
| 226 | | E | R | R | L | T | G | K | H | E | T | A | S | I | N | T | 240 |
| 722 | | TTT | TCA | TGG | GGT | GTT | GCT | AAC | CGT | GGT | TGC | TCC | ATC | CGT | GTG | GGG | 766 |
| 241 | | F | S | W | G | V | A | N | R | G | C | S | I | R | V | G | 255 |
| 767 | | CGT | GAC | ACT | GAG | AAG | GCA | GGC | AAA | GGT | TAT | TTG | | | | 799 | |
| 256 | | R | D | T | E | K | A | G | K | G | Y | L | | | | | |

Fig. 4: Nucleotide and deduced amino acid sequences of the cDNA clone correspond to *LsGS*. The predicted amino acid sequence is given in single-letter code for each amino acid. The arrows indicate the positions of degenerated primers (sense →, anti-sense ←) used for RT-PCR. Numbering refers to total nucleotide (upper) and amino acid (lower) residues on each line

apart from the separate determination and isolation of GS_1 and GS_2 , it may likely that inner leaf portion contain very low GS_2 which is largely contributing in the total GS activity in the measurement. Present results are in agreement with the findings of Brugiere *et al.* (2000) who found that chloroplastic GS gene expression and/or protein content decrease in phloem companion cells, whereas cytosolic GS gene expression and protein increase or remain stable in the mesophyll cytosol in tobacco leaves.

The isolated 799 bp partial cDNA clone *LsGS* showed high homology both at nucleotide and amino acid level (Table 1, Fig. 5) with the GS genes of other plants in the database. A radial phylogenetic analysis of GS sequences (Fig. 6) revealed that lettuce GS clustered separately under the subgroup consisting of *Z. mays* and *O. sativa* and was closer to *R. sativus* and *B. rapa* which showed more than 82% identity both for nucleotide and amino acid (Table 1). Lettuce was the only plant in the phylogenetic tree that came from Asteraceae family. The reason for not

| | | | |
|------------------------|-----|--|-----|
| <i>L. sativa</i> GS | 1 | -----P GEDSEV ILYPQA I FKDF FRGGNN ILV ICDTYTPQGV I P TNKRKAAE I FSDP KVVAVPWFV GIE QEYTL LQKDV | 76 |
| <i>R. sativus</i> GS | 40 | LSGP YDTP SLPKWN YDGSS TGQAP GEDSEV ILYPQA I FKDF FRRGNH ILVMCDTYTPAGEPI P TNKRHAAA QI FSHP DVVAEVPWY GIE QEYTL LQKDV | 139 |
| <i>G. max</i> GS | 40 | LP GP YSDP SE LPKWN YDGSS TGQAP GEDSEV ILYPQA I FRDP FRRGN ILV ICDAYTPAGEPI P TNKRHAAA KVFSHP DVVAEVPWY GIE QEYTL LQKDI | 139 |
| <i>Z. mays</i> GS | 91 | ISKP YEDP SE LPKWN YDGSS TGQAP GEDSEV ILYPQA I FKDF FRGGNN VLV ICDTYTPQGEPL P TNKRHAAA QI FSDP KVGQV PWFV GIE QEYTL LQKDV | 190 |
| <i>S. oleracea</i> GS | 99 | LSKP VEHP SE LPKWN YDGSS TGQAP QDSEV ILYPQA I FKDF FRGGNN ILV ICDAYTPAGEPI P TNKRHAAA E I FNNQKVASEVPWFV GIE QEYTL LQPNV | 198 |
| <i>S. tuberosum</i> GS | 100 | ISKP VKDA SE LPKWN YDGSS TGQAP GEDSEV ILYPQA I FKDF FRGGNN ILV ICDTYTPAGEPI P TNKRHAAA QI FSDP KVASQVPWFV GIE QEYTL LQPNV | 199 |
| <i>M. sativa</i> GS | 96 | ISKP VEHP SE LPKWN YDGSS TGQAP GEDSEV ILYPQA I FKDF FRGGNN ILV ICDAYTPQGEPI P TNKRHAAA E I FSNPKVEAE IPWY GIE QEYTL LQTDV | 195 |
| | | *:*****:**** *:***:***** * :***** *:***. . . * :*:***** :* | |
| <i>L. sativa</i> GS | 77 | KWPL GWP VGG YPGPQG PYYCGA GADK SFGRI SD AHYKAC LYAGI NISGTNGEVMPGQWE FQVGP SVG IEAGDH IWCARYL LER I TE QAGVVL TLDPKPI | 176 |
| <i>R. sativus</i> GS | 140 | KWPL GWP IGG FPGPQG PYYC SVGADK SFGRI VDAHYKAC LYAGI NISGINGEVMPGQWE FQVGP SVG ISAADEVWIARY ILER I TE IAGVVVS FDPKPI | 239 |
| <i>G. max</i> GS | 140 | QWPL GWP VGG FPGPQG PYYCGV GADK AFGRI VDAHYKAC IYAGI NISGINGEVMPGQWE FQVGP SVG ISAGDE IWAARY ILER I TE IAGVVVS FDPKPI | 239 |
| <i>Z. mays</i> GS | 191 | NWPL GWP VGG FPGPQG PYYCAV GADK SFGRI SD AHYKAC LYAGI NISGTNGEVMPGQWE YQVGP SVG IEAGDH IWI SRY ILER I TE QAGVVL TLDPKPI | 290 |
| <i>S. oleracea</i> GS | 199 | SWPL GWP VGA YPGPQG PYYCGV GADK SFGRI VSDAHYKAC LYAGI NISGTNGEVMPGQWE FQVGP SVG IEAGDHVWCARYL LER I TE QIGVVMTL DPKPI | 298 |
| <i>S. tuberosum</i> GS | 200 | NWPL GWP VGG YPGPQG PYYCGA GVE K SFGRI SD AHYKAC LYAGI NISGTNGEVMPGQWE FQVGP SVG IEGGDH IWCARYL LER I TE QAGVVL SLDPKPI | 299 |
| <i>M. sativa</i> GS | 196 | KWPL GWP VGG YPGPQG PYYCAA GADK SFGRI SD AHYKAC LYAGI NISGTNGEVMPGQWE YQVGP SVG IEAGDH IWA SRY ILER I TE QAGVVL TLDPKPI | 295 |
| | | .*:***:*. :*****. . .*:***: *****:***** *****:****** :*:***** *****:***** | |
| <i>L. sativa</i> GS | 177 | EGDWNGA GCHT NYS T L SMREEGGFE VIK EA ILNL SLR HDHI SAYEGNERRL TGKHE TAS INT FSWGV ANR GCS IRVGRDTEK AGKGYL | 266 |
| <i>R. sativus</i> GS | 240 | P GDWNGA GAHT NYS TKSMREEGGYE I I KKA IDKL GLRHKE HI SAYEGNERRL TGHHE TADINT FKWGV ANR GAS IRVGRDTEK EGKGYFEDR RP ASNMD | 339 |
| <i>G. max</i> GS | 240 | K GDWNGA GAHT NYS TKSMREEDG GYE V I KAA IDKL GKKHKE HIAAYEGNERRL TGRHE TADINT FLWGV ANR GAS YRVGRDTEK AGKGYFEDR RP ASNMD | 339 |
| <i>Z. mays</i> GS | 291 | Q GDWNGA GCHT NYS TKTMR EDGGFE E I KRA ILNL SLR HDL HI SAYEGNERRL TGKHE TAS IGT FSWGV ANR GCS IRVGRDTEA KGGKGYL EDR RP ASNMD | 390 |
| <i>S. oleracea</i> GS | 299 | EGDWNGA GCHT NYS TKTMR EEGGFE V I KKA ILNL SLR HKDHI SAYEGNERRL TGKHE TADINT FSWGV ANR GCS IRVGRDTEK EGKGYL EDR RP ASNMD | 398 |
| <i>S. tuberosum</i> GS | 300 | EGDWNGA GCHT NYS T L S IREEGGFE V I KKA ILNL SLR HKGHI SAYEGNERRL TGKHE TAS IDQFSWGV ANR GCS IRVGRYTEKEAR GYL EDR RP ASNMD | 399 |
| <i>M. sativa</i> GS | 296 | EGDWNGA GCHT NYS TKSMREEDG GFE V I KKA ILNL SLR HKVHIE AYEGNERRL TGKHE TAS INT FSWGV ANR GCS IRVGRDTEK NGKGYL EDR RP ASNMD | 395 |
| | | *****:***** :*:***: * * :* . . . * * *****:*****:***** * :***** * :***** * :***** | |

Fig. 5: Comparison of the deduced amino acid sequences for GS from *Lactuca sativa* (AB440673), *Raphanus sativus* (D25326), *Glycine max* (AF353620), *Zea mays* (X65931), *Spinacia oleracea* (EF143582), *Solanum tuberosum* (AF302113) and *Medicago sativa* (AF124244) by multi alignment. The amino acid residues are numbered at the beginning and end of the sequences on each line. Dashes in amino acid sequences represent gaps introduced to maximize alignment of the polypeptides. Below the amino acid sequences, conserved residues are indicated by asterisks while conservative substitutions are indicated by dots

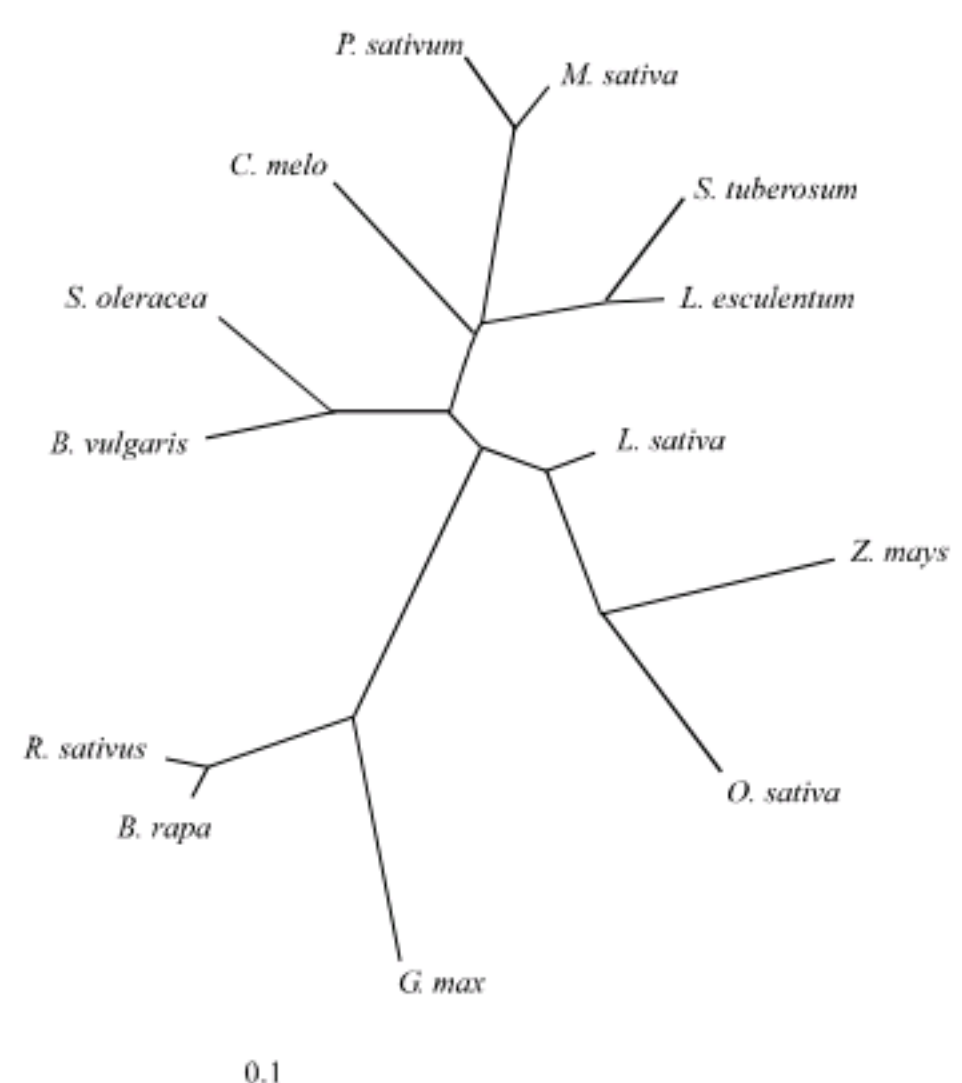


Fig. 6: Radial phylogenetic tree of the alignment of *LsGS* deduced amino acid sequence with other GS amino acid sequences in the database. Amino acid sequences were aligned using UPGMA and phylogenetic tree was conducted using software BioEdit (Hall, 1999) with Tree View ver. 1.6.6 and EMBL-EBI / ClustalW softwares

considering other plants belonged to the same family with lettuce was that very few commercially important plants of Asteraceae family were characterized for GS gene. However, as a dicotyledonous plant, lettuce GS showed more than 80% identity both for nucleotide and amino acid level with all other dicotyledonous plant in this analysis. In conclusion, the increase in ammonia content and decrease in total GS activity in the outer leaves of lettuce was a response to stress imposed by harvest and storage at elevated temperature. Regulation of this enzyme activity during storage would be an important aspect to delay the ammonia accumulation and/or quality deterioration of this perishable produce. The results may provide some basic information for further comprehensive molecular studies in the regulation of GS in lettuce. It would be worthwhile to sequence whole GS genes separately for GS₁ and GS₂ as well as to determine their differential gene expression during postharvest stages of lettuce.

ACKNOWLEDGMENT

The first author gratefully acknowledges the financial support received through the scholarship program for foreign students from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- Brugiere, N., F. Dubois, C. Masclaux, R.S. Sangwan and B. Hirel, 2000. Immunolocalization of glutamine synthetase in senescing tobacco (*Nicotiana tabacum* L.) leaves suggests that ammonia assimilation is progressively shifted to the mesophyll cytosol. *Planta*, 211: 519-527.
- Cock, J.M., I.W. Brock, A.T. Watson, R. Swarup, A.P. Morby and J.V. Cullimore, 1991. Regulation of glutamine synthetase genes in leaves of *Phaseolus vulgaris*. *Plant Mol. Biol.*, 17: 761-771.
- Cren, M. and B. Hirel, 1999. Glutamine synthetase in higher plants: Regulation of gene and protein expression from the organ to the cell. *Plant Cell Physiol.*, 40: 1187-1193.
- Enriquez, F.G., T. Matsui, P.K. Bhowmik, H. Suzuki and K. Kawada, 2001. Postharvest changes in ammonium, glutamine synthetase and glutamate dehydrogenase in asparagus spears during storage at 20°C. *Pak. J. Biol. Sci.*, 4: 293-297.
- Givan, C.V., 1979. Metabolic detoxification of ammonia in tissues of higher plants. *Phytochemistry*, 18: 375-382.
- Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid. Symp. Ser.*, 41: 95-98.
- Hirel, B., C. Perrot-Rechenmann, A. Suzuki, J. Vidal and P. Gadal, 1982. Glutamine synthetase in spinach leaves. Immunological studies and immunocytochemical localization. *Plant Physiol.*, 69: 983-987.
- Huber, D.J., 1987. Postharvest senescence: An introduction to the symposium. *HortScience*, 22: 853-859.
- Hurst, P.L. and C.J. Clark, 1993. Postharvest changes in ammonium, amino acids and enzymes of amino acid metabolism in asparagus spear tips. *J. Sci. Food Agric.*, 63: 465-471.
- Hurst, P.L., G.A. King and W.M. Borst, 1993. Postharvest inhibition of glutamine synthetase activity with phosphinothricin reduces the shelf-life of *Asparagus*. *Postharvest Biol. Technol.*, 3: 327-334.
- Ireland, R.J. and P.J. Lea, 1999. The Enzymes of Glutamine, Glutamate, Asparagines and Aspartate Metabolism. In: *Plant Amino Acids: Biochemistry and Biotechnology*, Singh, B.K. (Ed.). Marcel Dekker, New York, pp: 49-109.
- King, G.A., D.C. Woollard, D.E. Irving and W.M. Borst, 1990. Physiological changes in *Asparagus* tips after harvest. *Physiol. Plant*, 80: 393-400.
- Kun, E. and E.B. Kearney, 1974. *Methods of Enzymatic Analysis*. Vol. 4. 2nd Edn., Verlag Chemie Weinheim, Academic Press, New York.
- Lam, H.M., K.T. Koschigano, I.C. Oliveira, R. Melo-Oliveira and G.M. Coruzzi, 1996. The molecular-genetics of nitrogen assimilation into amino acids in higher plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 47: 569-593.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Matsui, T., P.K. Bhowmik and K. Yokozeki, 2004. Changes in glutamine synthetase activity and gene expression during storage of moso bamboo shoot. *Asian J. Plant Sci.*, 3: 290-294.
- McNally, S.F., B. Hirel, P. Gadal, A.F. Mann and G.R. Stewart, 1983. Glutamine synthetases of higher plants. *Plant Physiol.*, 72: 22-25.
- Miflin, B.J. and D.Z. Habash, 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J. Expt. Bot.*, 53: 979-987.
- Ochs, G., G. Schock, M. Trischler, K. Kosemund and A. Wild, 1999. Complexity and expression of the glutamine synthetase multigene family in the amphidiploid crop *Brassica napus*. *Plant Mol. Biol.*, 39: 395-405.
- Oliveira, I.C., T. Brears, T.J. Knight, A. Clark and G.M. Coruzzi, 2002. Overexpression of cytosolic glutamine synthetase. Relation to nitrogen, light and photorespiration. *Plant Physiol.*, 129: 1170-1180.
- Peeters, K.M.U. and A.J. Van Laere, 1992. Ammonium and amino acid metabolism in excised leaves of wheat (*Triticum aestivum*) senescing in the dark. *Physiol. Plant*, 84: 243-249.
- Pramanik, B.K., T. Matsui, H. Suzuki and Y. Kosugi, 2005. A sucrose synthase gene from broccoli: cDNA cloning, sequencing and its expression during storage. *Biotechnology*, 4: 288-295.
- Schwerdtfeger, E., 1978. Proteolytic changes in leafy vegetables during short-term storage. *Qual. Plant*, 28: 147-163.
- Suthumchai, W., T. Matsui, K. Kawada and Y. Kosugi, 2006. Changes in acid invertase activity and sugar contents in lettuce during storage at ambient temperature. *Asian J. Plant Sci.*, 5: 304-310.
- Wan, C.Y. and T.A. Wilkins, 1994. A modified hot borate method significantly enhances the yield of high quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.*, 223: 7-12.