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BIOCHEMICAL AND MOLECULAR ANALYSIS OF GLUTAMINE SYNTHETASE GENE IN *BRASSICA NAPUS* L.

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ABSTRACT

Leaf senescence represents a developmental process that recycles valuable nutrient elements for other parts of the plants. The combined action of several internal and external signals may be involved in the induction of senescence. From a practical viewpoint, delaying senescence may result in major economic benefits. Macromolecular changes that occur during leaf senescence in *Brassica napus* L. were investigated as a first step in studying this process. In this study, the changes in the level of glutamine synthetase (GS) enzyme in *Brassica napus* leaves during development were measured. Total GS activity was measured in soluble proteins isolated from leaves at different developmental stages. The level of GS activity in the leaf decreased during senescence. The behavior of the cytosolic (GS1) and chloroplastic (GS2) isoforms of glutamine synthetase was examined in the naturally senescing leaves of *Brassica napus* by western blotting. In the senescence stages the amount of GS1 appeared greater than GS2, indicating that the

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GS2 isoform declined during senescence and the GS1 increased. The level of GS1 expression increased in *Brassica napus* leaves during plant development and reached maximum levels, during senescence at the SS3 stage. To study the GS1 encoded by LSC460 at the molecular and biochemical level, double-stranded sequence was obtained from the LSC460 cDNA clone and compared with sequences in the databases. A search in available GenBank database revealed high sequence similarities of LSC460 to families of glutamine synthetase enzymes (especially with GS1). We conclude, that the GS gene expressed during leaf senescence of *Brassica napus* encoded by LSC460 is a GS1 gene. These results show the importance of the GS1 gene in senescence of the *Brassica napus* leaves.

Key words: *Brassica napus* L., Glutamine synthetase, Leaf senescence, Plant development.

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به ترتیب دانشجوی پیشین دکترا (اکنون استادیار بخش اصلاح نباتات دانشگاه تربیت مدرس صندوق پستی ۴۸۳۸-۱۴۱۵۵ تهران، جمهوری اسلامی ایران)، استادیار و استاد زیست شناسی مولکولی ، پژوهش های بین المللی باغبانی، ولزبورن، انگلستان.

چکیده

پیری برگ یکی از مراحل رشد گیاه است که باعث بازیابی عناصر غذایی مورد نیاز از برگ های پیر به سایر قسمت های گیاه می شود. ترکیبی از پیام های درونی وبرونی ممکن است در ایجاد پیری مؤثرباشند. به تاخیر انداختن پیری می تواند فواید اقتصادی مهمی همراه داشته باشد. به

عنوان اولین مرحله از مطالعه پدیده پیری، تغییراتی که در سطح ملکول های حیاتی در مراحل پیری برگ کلزا رخ می دهد بررسی شد . در این بررسی، میزان تغییرات آنزیم گلوتامین سنتتاز در مراحل رشد برگ کلزا اندازه گیری شد. میزان فعالیت آنزیم گلوتامین سنتتاز در پروتیین محلول که از برگ ها در مراحل مختلف رشد استخراج شده بود اندازه گیری شد. میزان فعالیت آنزیم گلوتامین سنتتاز در طی مراحل پیری برگ کاهش نشان داد. میزان تغییر در سطح ایزوفرم کلروپلاستی (GS2) و سیتوپلاستی (GSI) گلوتامین سنتتاز در برگ های پیر با استفاده از فن وسترن بلاتینگ (Western blotting) آزمایش شد. در مراحل پیری برگ، میزان GS1 بیشتر از GS2 بود که نشانگر کاهش میزان ایزوفرم GS2 و افزایش GS1 در طی مراحل پیری برگ است. میزان بیان GS1 با افزایش رشد برگ های گیاه کلزا افزایش نشان داد تا در مرحله آخر پیری (SS3) به حداکثر رسید. برای مطالعه GS1 در سطح ملکولی و بیوشیمیایی که توسط کلون LSC460 در کلزا کد می شد توالی اسیدهای نوکلئیک در هر دورشته cDNA از کلونLSC460 تعیین و با توالی های موجود در بانک های اطلاعاتی مقایسه شد. مقایسه این زنجیره DNA با داده های موجود در بانک ژن (GenBank) نشان داد که شباهت خیلی زیادی بین این زنجیره cDNA با خانواده آنزیم گلوتامین سنتتاز وجود دارد. بنابراین، با توجه به میزان تشابه خیلی زیاد آن با ایزوفرم GS1 نتیجه گیری شد که آنزیم گلوتامین سنتتازی کهبه وسیله کلون LSC460 کد می شود از نوع ایزوفرم GS1 است. این یافته ها نشان داد که ژن GS1 نقش بسیار مهمی در پیری برگ کلزا داد.

INTRODUCTION

Leaf senescence marks a switch in metabolism net assimilation to degradation. This switch is associated with the visible loss of chlorophyll and the degradation of macromolecules, specifically proteins. Once degraded these nutrients are converted to a transportable form and translocated to the developing sinks.

Glutamine synthetase (GS) an enzyme that catalyses the amination of glutamate is of fundamental importance in the maintenance of the nitrogen

balance of a cell. Gs has two isoforms, a chloroplastic isoform (GS2) that is involved in the re-assimilation of ammonium ions liberated during photorespiration and a cytosolic form (GS1) that is involved the re-assimilation of ammonium released during proteolysis (24). This paper describes the isolation and molecular characterization of a GS1 gene from *Brassica napus* that shows enhanced expression during leaf senescence.

The genetic control of senescence is reflected in the fact that genotypic variation within species exists and has been used in crop breeding. Stay green mutants, showing delayed or inoperative senescence, exist for many important crop species (e.g. maize, sorghum, rice, wheat, soybean) and studies with these have confirmed the importance of delayed senescence in increasing yields (25).

The aim of molecular studies on leaf senescence is, firstly, to identify and clone genes encoding enzymes that are involved in the senescence process and secondly to characterize these genes and to determine their function in order to formulate an overall view of the enzymatic changes that take place during senescence. The objective of this research was to analyse the function of a senescence-induced gene in rape seed (*Brassica napus*).

Leaf senescence has been investigated at the molecular level by isolation of a number of genes that are expressed during senescence. Several cDNA libraries have been constructed from mRNA isolated from senescent leaves of plants such as *Arabidopsis* (13, 21, 26), barley (2), *Brassica napus* (6), maize (23), and tomato (9). In order to isolate genes that are involved in the senescence process, a variety of different techniques could be utilized. The initial step for isolating genes, that are associated with a particular developmental process, is the isolation of mRNA that is transcribed during that developmental phase (e.g. senescence) followed by cDNA library construction.

Two successful approaches in the selection of senescence-associated cDNA clones, are subtractive and differential library screening. Both approaches have been used to identify senescence-related cDNAs from senesceing leaf tissue in *Brassica napus* (6, 7). A number of different genes

expressed at increased levels during senescence. Several types of genes have been identified by sequence analysis of these cDNAs. These genes included metallothioneine, ferritin, an antifungal protein, catalase, ATP sulphurylase, two different proteases and glutamine synthetase.

The next step involves the identification of the isolated cDNAs by the analysis of DNA sequence and its comparison to sequences of genes in databases. Similar sequences in the databases can give an indication of the role of the gene in senescence.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of *Brassica napus* L. were sown in the soil (consisting of four parts peat compost containing macroelements and one part vermiculite) in a 22×17×5 cm tras and incubated in the glasshouse under 14 h photoperiod and 21±4° C temperature. Two weeks after germination, plants were repotted into 20 cm pots with the same soil mixture as before. The plants were grown in a growth chamber (75% average relative humidity) for 2-3 weeks at 25° C, 16 h light and 8 h dark photoperiods. After 3 weeks the plants were transferred to the greenhouse to flower (self-pollination) and set seed.

Northern Blotting

All RNA samples analyzed by electrophoresis contained 10 µg of RNA (5µl). To each sample 12 µl of sample buffer and 2 µl of 0.5 mg ml^{-1} ethidium bromide were added and the RNA was denatured at 65° C for 2 min, then chilled on ice before being loaded onto the agarose gel. Gels were run at 100 volts for 3 h. A nylon membrane, the same size as the gel, was soaked in a 10x SSC solution as the transfer solution for 10 min. The RNA gel was placed upside down on the wicks ensuring that no air bubbles were present. The nylon membrane was placed on top of the gel and any air bubbles were removed to ensure uniform transfer of RNA. Five sheets of 3 MM filter paper were pre-wetted in 10x SSC and placed on top of the membrane. The RNA was transferred to the nylon membrane using 10× SSC

membrane. The RNA was transferred to the nylon membrane using 10× SSC as the transfer solution. Transfer was allowed to proceed for 16 h at room temperature. Following transfer, the membrane was rinsed briefly with 2× SSC and blotted dry. The RNA was fixed to the nylon membrane using the UV transilluminator for 2 min.

Pre-hybridization of membrane blots was done in a large volume of 7% SDS, 250 mM Na-phosphate buffer (pH 7.2). The 32P-labeled DNA probe was denatured in a boiling water-bath for 4 min and placed briefly on ice. The membrane and the prehybridization solution were removed from the box and replaced with a minimal volume of 7% SDS, 250 mM Na-phosphate buffer (pH 7.2) prewarmed to 65° C. The denatured probe was added to the hybridization solution and mixed thoroughly before applying to the filters. Hybridization was carried out overnight at 65° C with shaking. The hybridization solution was then removed and the filters rinsed in 2x SSC/1% SDS followed by two 15-min washes in 2× SSC/0.1% SDS, at 65° C. Filters were wrapped in Saran wrap and were dried briefly by exposing to X-Ray film in a cassette at -80° C.

Protein and Enzyme Analysis

Soluble protein extracts were prepared by homogenizing 1 g leaf tissue with 3 ml of ice-cold 50 mM Tris-Cl pH 7.5, containing 2 mM EDTA and 0.04% (v/v) mercaptoethanol. The homogenate was centrifuged at 4° C for 20 min at 13,000 g in a microcentrifuge. The crude protein extract was spin-desalted on a Sephadex G-25 column equilibrated with 10 mM Tris-Cl pH 7.5 as described by Thomas and Feller (11). Protein aliquots of crude and spin-desalted extracts were precipitated with an equal volume of 20% (w/v) TCA in acetone containing 0.14% (v/v) mercaptoethanol at -20° C overnight.

Protein concentration was determined by a calorimetric method based on that described by Bradford (5) using a commercially available reagent (Bio-Rad Protein Assay dye reagent). Glutamine synthetase assay based on glutamyl hydroxamate synthesis was carried out according to Canovas *et al.* (8).

Western Immunoblotting of Total Soluble Protein Extracts

Total protein extracts were separated by SDS-PAGE as outlined above and electrophoretically transferred to nitro-cellulose using a Protein mini-blot apparatus (Bio-Rad). The GS1 antibodies used in this study were provided by Dr. Histoshi Sakakibara (University of Nagoya, Japan) and the blots were developed according to the method of Blake *et al.* (4).

DNA Sequencing

Plasmid DNA was prepared by the boiling miniprep method and 18 µl DNA was treated with 2 µl RNAse A (10 mg ml^{-1}) at 37°C, for 20 min. 5 µl 1M NaOH 1^{-1} mM EDTA was added and incubated for 15 min to denature the DNA strands. The denatured DNA was purified by spin dialysis on mini-columns of Sepharose CL-6B to remove the NaOH. Eight µl of this DNA was added to 2 µl 5x reaction buffer and 1 µl sequencing primer (10 µg ml^{-1}). The mixture was incubated at 37° C for 15 min. Two and four tenth µl aliquots of the primed template were placed into four tubes labelled A, C, G and T. Two µl of labelling mix were added to each tube and the mixtures incubated at room temperature for 3 min. This generates short oligonucleotide chains with incorporated 35S radioactive label as 35S-dATP. 2 µl of A, C, G and T-termination mixes were added to the corresponding tubes containing the templates and the mixture incubated at 37-45° C for 5 min. The reactions were stopped with 4 µl of stop solution. The reactions were loaded directly on a sequencing gel.

The sequencing reactions were resolved on a denaturing polyacrylamide gel. The plates were clamped together using strips of Whatman 3 mm paper (approximately 0.4 mm thick), as spacers. 300 µl 10% ammonium persulphate (prepared fresh) and 50 µl TEMED were added to 50 ml of a 6 % sequencing gel mixed by gently swirling and the gel poured between the plates. The flat edge of a shark's tooth comb was inserted at the top of the gel and clamped. The gel was allowed to set overnight at room temperature. Wells were formed in the sequencing gel by inverting the shark's tooth comb so that the teeth just pierced the surface of the gel. The

gel set up was placed in an electrophoresis tank and 1x sequencing TBE poured into the gel tank reservoirs. The sequencing reactions were heated at 90°C for 2 min and 2.0 µl of each reaction mixture (A, C, G and T), were loaded into adjoining wells. The gel was run at a constant power of 35 W (1500-2000 V), for 3 h (short run). Following electrophoresis, gels were fixed in 10 % acetic acid, 10 % methanol in distilled water for 15 min, transferred to Whatman 3 MM paper and dried at 70° C, on a gel drier. Dried gels were exposed to X-Ray film to reveal the sequencing ladder.

As an alternative to the previous method, sequencing analysis was carried out using an automatic sequencer system (ABI 310). Sequences were analyzed using the DNA STAR sequence analysis package, including programs Editseq; Mapdraw; Align; Megalign; Seqman and Protean.

RESULTS AND DISCUSSION

Gene Expression and Northern Blotting

The expression of the gene represented by LSC460, in senescing *Brassica napus* leaves, was examined by hybridization of fractionated total RNA, isolated from leaves at different stages of senescence, using a 32P labelled LSC460 cDNA probe. Changes in the amounts of specific transcripts during senescence may not be proportional to the decline in total RNA since the majority of the RNA loss is due to rRNA degradation. Therefore, it would be expected that equal loading of total RNA for fractionation would result in elevated levels of senescence-enhanced transcripts during senescence and so equal loading of total RNA does not permit quantitative assessment of changes in specific gene expression during senescence.

Aliquots of 10µg of total RNA were fractionated on formaldehyde gels. rRNA bands were visible under UV light after staining with ethidium bromide (Fig. 1A). The RNA was then blotted to nylon membrane and hybridized to 32P labelled LSC460 cDNA (as described in materials and methods).

Northern blot analysis was used to determine the expression of the gene represented by LS460 in *Brassica napus* in different tissues (e.g. roots,

flowers). In total RNA extracted from roots and flowers, mRNA homologous to LSC460 was at very low levels. No LSC460 transcripts were detected in roots and only a low level was detected in flowers (Fig. 1B). Northern hybridization with LSC460 showed a single band of 1.4 KB which was expressed at very low levels in green leaves (MG1) of *Brassica napus* (Fig. 1). Expression levels began to increase at the early senescence stage (SS1) and reached to a maximum level at SS3.

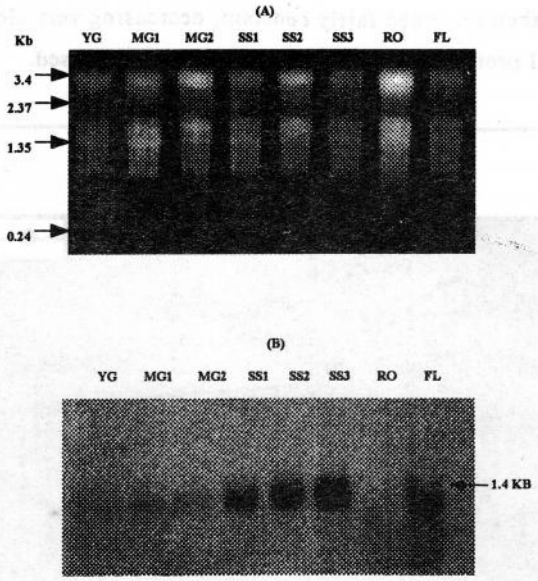


Fig. 1. Analysis of mRNA levels of LSC460 in *Brassica napus*. Northern blots carrying total RNA isolated from roots (RO), flower (FL) and leaves at different developmental stages (young green leaves (YG), mature green leaves (MG1), presenescence mature green leaves (MG2) and senescenct leaves showing 0-25% yellowing (SS1), 25-50% (SS2) 50-75% (SS3). (A) Denatured RNA samples isolated from *Brassica napus* were fractionated on a formaldehyde-agarose gel (1.2% agarose). rRNA bands were visible under UV-light after staining with ethidium bromide (part A). Ten micrograms of RNA were loaded in each track. (B) The same RNA samples (10 μg) were blotted to nylon membrane and hybridised with a 32P-labelled LSC460 probe.

Investigation of The Glutamine Synthetase Enzyme

The same protein extracts were used to determine GS activites. Total GS activity was measured in soluble proteins isolated from leaves at different developmental stages according the assay reported by Canovas *et al.* (8) (deta shown in Table 3). The total GS activity increased a little from the YG to the SS1 stage and after this decreased sharply to the MG2 stage. The level of GS activity then remained fairly constant, decreasing very slowly. During senescence, total protein and GS activity in the leaf decreased.

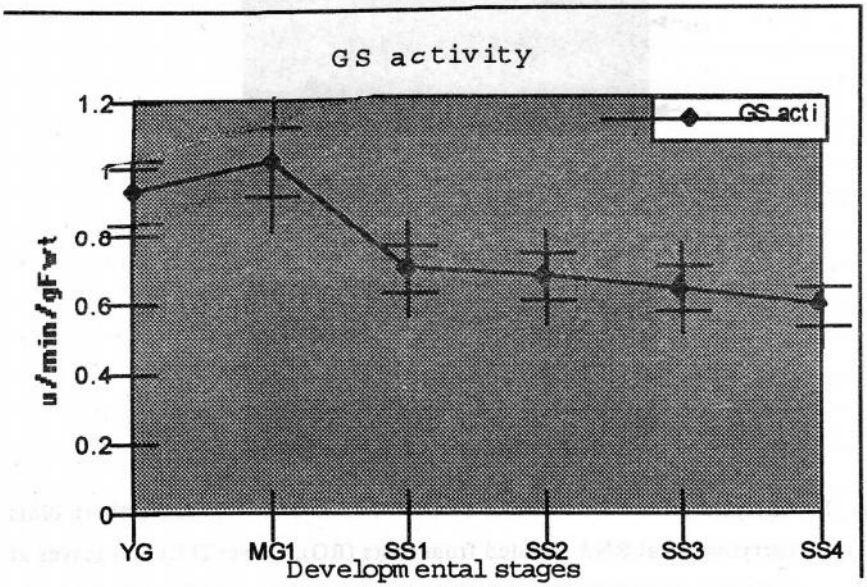


Fig. 2. Glutamine synthetase (GS) assay.

Glutamine synthetase (GS) activity during development: YG, young green; MG1, mature green stage 1; SS1, SS2, SS3 and SS4, senescent leaves during stages 1, 2, 3 and 4, respectively.

The decline in protein content was faster than the decline in GS activity; therefore, GS activity was expressed as units per gram fresh weight

per minute (U g FW^{-1} min^{-1}). The activities of the two isoforms can not be measured individually and it was of interest to know which isoform was responsible for the decrease in total activity. Therefore, we looked for a change in the relative amount of the isoform proteins by detecting them using an immunoblotting technique (western blotting).

Fig. 3A shows the western blot analysis of soluble proteins extracted from *Brassica napus* leaves during different developmental stages. Two bands were observed, the upper band of 44 KDa was thought to represented the GS2 isoform and the lower band, 41 KDa, related to the GS1 isoform.

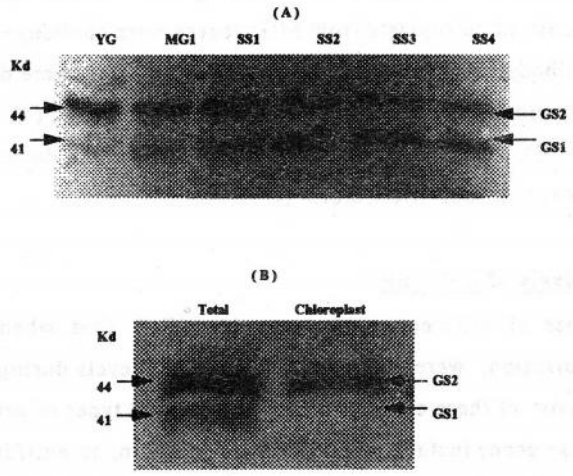


Fig. 3 Western blotting analysis of GS1 and GS2 proteins.(A) Aliquots of 5 mg of soluble protein, were extracted in triplicate from leaves at different developmental stages: young green (YG), mature green stage one (MG1) and senescence stage one, two, three and four (SS1, SS2, SS3 and SS4), were size-fractionated on SDS-PAGE. Proteins were detected by western blotting with specific antibody (raised against the maize GS1 protein).

(A) Aliquots of 5 mg of protein were extracted and separated by SDS-PAGE and electroblotted onto nitro-cellulose. GS isoforms (GS1 and GS2) in total soluble protein and in protein extracted from isolated

chlroplasts from MG1 *Brassica napus* leaves were identified using the GS1 antibody. The GS2 isoform (upper band) showed a decrease with the progress of senescence (especially at the SS3 and SS4 stages). However, levels of the GS1 isoform increased from SS1 with the progress of senescence and reached a maximum level at SS3. The minimum amount of GS1 was observed at the YG stage and the maximum level at SS3. The amount of GS2 polypeptide rapidly decreased during senescence (Fig. 5A) but the amount of the GS1 polypeptide increased. Therefore, the majority of the GS activity measurable in senescence must be due to the GS1 isoform (Fig. 3).

To study the location of the GS proteins in the cell, chlroplasts were isolated from *Brassica napus* leaves at MG1 stage. Total soluble protein and protein from isolated chlroplasts from MG1 leaves were challenged with the maize GS1 antibody. Fig. 3B shows that both isoforms were detected in total soluble protein but in isolated chloroplasts only one band (44 KDa) was observed (Fig. 3B). This confirmed that the 44 KD protein detected by the GS1 antibody is the chlroplastic isoform (GS2).

Sequence Analysis of LSC460

A number of different genes were identified that, when analyzed by northern hybridization, were expressed at increased levels during senescence. Sequence analysis of these cDNAs showed that several types of genes had been identified. These genes include metallothionein, ferritin, an antifungal protein, catalase, ATP sulphurylase, two different proteases and glutamine synthetase. The LSC460 clone is one of these genes and this was the gene investigated during this study.

Preliminary DNA sequence generated from the end of the LSC460 clone was used to perform a DNA database search (BLAST) to determine whether the LSC460 cDNA showed any similarity to existing sequences. The clone showed a high degree of sequence similarity to cytosolic glutamine synthetase (GS1) of other plants especially *Brassica napus* and *Litus japonica* (30).

The second step in characterising LSC460 was to fully sequence the cDNA. The initial sequencing employed the SK and KS flanking primer sites of

the pBluescript vector to sequence the ends of the clone (Fig. 3) using the automatic sequencer (ABI 310).

The subcloning strategy did not allow sequencing of the entire LSC460 clone to be completed on both strands using SK and KS primers without the need for novel oligonuclotide primers. Four oligonuclotide primers were generated and by using those primers and also SK and KS primers, the sequencing of both strands of LSC460 was completed.

LSC460 cDNA was sequenced using an ABI 310 automatic sequencer. The complete nucleotide sequence of the LSC460 cDNA is 1275 bp with a deduced open reading frame of 356 amino acids (Fig. 4). Bold letters in the nucleotide

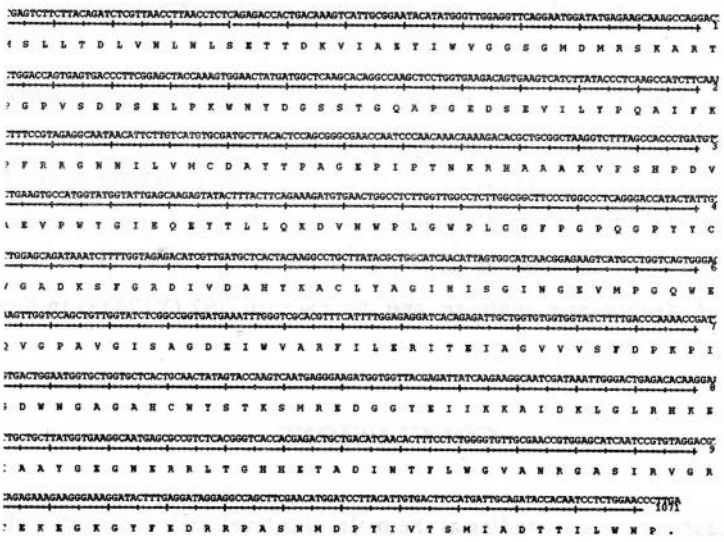


Fig. 4. Translated DNA sequence of LSC460 cDNA with standard genetic code. The glutamine synthetase enzyme is encoded by the LSC460 cDNA and consists of 356 amino acids. The total number of bases translated is 1068 and the molecular weight of this polypeptide is 39125 Daltons.

sequence (Fig. 3) indicate the start codon ATG (1-3) and the stop codon TGA (1068-1071). The sequence has A+T and C+G contents of 51.73% and 48.27%,

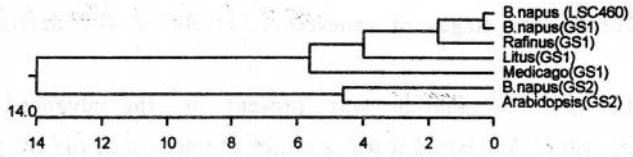
respectively. The protein sequence deduced from the DNA sequence indicates a protein of 356 amino acids and molecular weight 39.12 kD (Fig. 4). The nucleotide sequence and the predicated amino acid sequence for LSC460 clone were used to search the Gen Bank, EMBL, DDBJ and PDB databases for similar sequences. Searches were carried out using the BLAST Network service (National Center for Biological Information, NCBI). The LSC460 clone DNA and sequences exhibited a high degree of similarity to a number of cytosolic glutamine synthetases from a range of plant species (Fig. 3). Based on these observations, this would confirm that the LSC460 protein is glutamine synthetase (GS). In a phylogenetic tree (Fig. 5A), the LSC460 protein (GS1) is clearly distinguished from GS2 proteins. The amino acid sequence of LSC460 clone has greater than 90% similarity to the GS1 proteins of different plants (Fig. 5B) and only 64% similarity to the GS2 proteins of *Brassica napus* (Fig. 5B).

The similarity of LSC460 to other GS1 proteins was very high: 99% between the polypeptides encoded by LSC460 and GS1 (X82997) of *Brassica napus*, 93.2% with GS1 (Y12859) of *Litus japonica* (Thykjaer *et al.*, 1997), 96.9% with GS1 (D25324) of *Raphanus sativus*, 90.6% with GS1 (X04001) of *Medicago sativa*. Similarity to GS2 protein was much lower: 63.7% with GS2 (S18602) of *Arabidopsis thaliana* and 64.2% with GS2 (Y12458) in *Brassica napus*.

CONCLUSIONS

Leaf senescence studies at the molecular level have shown that changes in gene expression occur during senescence. The levels of mRNAs encoding proteins involved in photosynthesis decrease during senescence (1, 13). However, mRNA levels of genes encoding proteins thought to be involved in the senescence program increase (12, 13, 15, 26). A number of senescence-related genes have been identified in *Brassica napus* (6, 7). The LSC460 clone is one of those genes that were investigated during this study.

(A)



(B)

Percent Similarity

	1	2	3	4	5	6	7		
1		99.0	93.2	90.6	96.9	64.2	63.7	1	B.napus (LSC460)
2	0.6		93.7	91.5	97.3	67.1	66.7	2	B.napus(GS1)
3	8.1	7.4		90.6	93.0	67.1	66.9	3	Litus(GS1)
4	11.6	10.4	11.9		90.3	66.2	66.4	4	Medicago(GS1)
5	3.7	3.0	8.4	11.9		66.2	66.2	5	Rafinus(GS1)
6	27.6	30.6	26.8	28.4	28.4		90.9	6	B.napus(GS2)
7	27.6	31.0	26.8	27.6	27.6	9.2		7	Arabidopsis(GS2)
	1	2	3	4	5	6	7		

Fig. 5. Phylogenetic tree of LSC460 proteins, GS1 and GS2. Comparison of LSC460 *Brassica napus* and other glutamine synthetase (GS1 and GS2) genes at the amino acid level. (A) The phylogenetic tree was generated from the multiple alignment. (A) Horizontal branch lengths represent similarities between different glutamine synthetase proteins. (B) Amino acid sequence similarities and divergence are expressed as percentages. GS1 (X82997) in *Brassica napus*, GS1 (Y12859) in *Litus japonica* (27), GS1 (D25324) in *Raphanus sativus*, GS1 (X04001) in *Medicago sativa*, GS2 (S18602) in *Arabidopsis thaliana* and GS2 (Y12458) in *Brassica napus*.

The pattern of expression of LSC460 gene was examined in different tissues of *Brassica napus:* in roots, flowers and also during senescence of leaves. A large amount of transcript from LSC460 accumulated in senescing leaves indicated there was no detectable transcription in roots and very little in flowers. The level of GS1 expression increased in *Brassica napus* leaves during plant development and reached maximum level during senescence, in *Brassica napus* at the SS3 stage (Fig. 1). This indicates that the GS1 protein may play an important role during senescence. This activation is likely to be at the

transcription level and is presumably regulated by some senescence-specific signal. During late stages of senescence, levels of GS1 declined SS4 in *B. napus*.

The GS1 polypeptide was present at the advanced stages of senescence, (Fig. 5A) while it was a minor componant of the GS proteins in green leaves. Therefore, it was concluded that the most of the measurable GS activity in green leaves probably corresponds to the chloroplastic isoenzyme GS2 (Fig. 5B). It was also apparent that amount of GS2 polypeptide declined during senescence. It should be indicated that, during this period, the total soluble protein in the leaf decreased (Fig. 2 and 3) and the GS activity also decreased (Fig. 4). Therefore, it can be concluded that during natural senescence of *Brassica napus* leaves, a GS gene encoding a cytosolic enzyme GS1 is highly expressed. This has also been reported during natural senescence of leaf tissue in other species (3, 15). These facts taken together may suggest that the GS1 and GS2 expression pattern reported here in *Brassica napus* leaves are likely to be a reflection of the leaf senescence pattern. The amount of GS2 polypeptide decreased relative to the progress of senescence but the relation level of GS1 polypeptide increased.

The chloroplastic GS2 is responsible for ammonia assimilation during photorespiration (19, 27), Cytosolic GS (GS1) is involved in assimilation of ammonia produced by nitrogen fixation and amino acid catabolism (14, 29). Results determined so far have indicated the important of the GS1 gene (LSC460) in senescence of *Brassica napus* leaves and methods have been identified to measure levels of GS protein and activity.

The LSC460 cDNA codes for glutamine synthetase, an enzyme that is probably involved in nitrogen remobilisation. Cytosolic GS is involved in assimilation of ammonia produced by nitrogen fixation and amino acid catabolism (14, 16, 31). A role of GS1 in the long distance intercellular transport of nitrogen has been suggested by the phloem-specific expression of GUS, directed by a cytosolic GS1 promoter from *Pisum sativum*, in transgenic tobacco (10).

None of the amino acids are transportable from one part of the plant to another. In senescing leaves, amino acids are converted to transport

compounds by intermediary metabolism (11). It has been proposed that during leaf senescence some amino acids are catabolised and converted into glutamine by the enzyme glutamine synthetase (GS). Glutamine and asparagine can be transported easily during germination and/or senescence, whilst other amino acids can be converted to these transportable amino acids by aminotransferase enzymes.

We conclude, therefore, that the GS gene of *Brassica napus,* encoded by LSC460, is a GS1 gene and this gene plays an important role during leaf senescence. The manipulation of senescence-related genes, such as LSC460, using plant genetic engineering could be of great help in the study of leaf senescence mechanisms. From a practical view point, delaying senescence could result in great economic benefits.

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