# Studies on the Relationship between the Effects of Glutamine and the Gene Expression of Glutamine Synthetase Isoforms during Somatic and Zygotic Embryogenesis in Carrot

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**Abbreviations**

ADP; adenosine diphosphate

α-Ala; α-alanine

ATP; adenosine triphosphate

Glu; L-glutamic acid

Gln; L-glutamine

2,4-D; 2,4-dichlorophenoxyacetic acid

γ-GHA; L-glutamic acid γ-monohydroxamate

GOGAT; glutamate synthase

GS; glutamine synthetase

MS medium; Murashige and Skoog's medium

MS-$NH_4$ medium; MS medium without $NH_4NO_3$

MS+$NH_4$ medium; MS-$NH_4$ medium with 10 mM $NH_4Cl$

MSA medium; MS-$NH_4$ medium with 10 mM α-alanine

MSE medium; MS-$NH_4$ medium with 10 mM L-glutamic acid

MSQ medium; MS-$NH_4$ medium with 10 mM L-glutamine

NiR; nitrite reductase

NR; nitrate reductase

ORF; open reading frame

PCR; polymerase chain reaction

RT; reverse transcription

RACE; rapid amplified cDNA ends

SSC; salt-sodium citrate buffer

TCA; trichloroacetic acid

TE buffer; Tris-EDTA (ethylenediaminetetraacetic acid) buffer {10 mM Tris-HCl (pH;8.0) and 1 mM EDTA}

Tris; 2-amino-2-hydroxymethyl-1,3-propanediol

x*g*; x gravitational field

# Abstract

The addition of L-glutamine, α-alanine or L-glutamic acid strongly stimulates somatic embryo formation in carrot, not only the number of somatic embryos formed but also their development. The effects of the amino acids on somatic embryogenesis were stronger than that of ammonium ion. In particular, L-glutamine strongly stimulates the development of somatic embryos. To clarify the different effects of amino acids and ammonium ion, the activity of glutamine synthetase (GS; EC 6.3.1.2.), a key enzyme involved in nitrogen assimilation, was measured. Its activity decreased during the later stages of embryo development.

Three cDNA clones corresponding to isoforms of carrot GS were isolated and used as probes for an analysis of the expression patterns of the isoform genes during somatic embryogenesis and seed development in carrot. Transcripts corresponding to two of the cDNAs, "CGS102" and "CGS201", were observed during somatic embryogenesis. Their levels were high at the early stage of embryogenesis but low at the late stage of embryogenesis. This pattern of expression is similar to the pattern of changes in GS activity observed during somatic embryogenesis. By contrast, expression of the transcript for another GS isoform gene, corresponding to "CGS103" cDNA, was not observed in somatic embryos.

I also analyzed the levels of the transcripts for "CGS102" and "CGS201" in somatic embryos which had been cultured in medium with either ammonium ions or L-glutamine as the source of nitrogen. The amounts of the transcripts for "CGS102" and "CGS201" fell when L-

glutamine was supplied in the medium, but the decline of the transcripts at the late stage of embryogenesis was observed irrespective of the supply of L-glutamine or ammonium ion. These results indicate that the GS activity was regulated at the transcriptional level and that the expression pattern of GS genes during somatic embryogenesis was regulated by embryogenesis-specific manner.

The expressions pattern of GS genes in developing seeds was similar to the pattern observed in somatic embryogenesis except for the pattern of the expression of the gene for "CGS103" which was weak during zygotic embryogenesis and strong during seed maturation. Expression of the gene for "CGS103" in developing seeds was considered to be in senesced maternal tissues but not in zygotic embryos, because the transcripts for "CGS103" was only observed in scenesced leaves but not in young leaves. Therefore, the expression patterns of GS genes observed in developing seeds except for the gene for "CGS103" are likely to correspond to those in zygotic embryos and the expression patterns of the genes for GS isoforms during somatic embryogenesis reflects their patterns during zygotic embryogenesis. I concluded that somatic embryogenesis and zygotic embryogenesis have common regulatory systems with respect to the expression of the genes for GS isoforms. Finally, I proposed a model by the environment of zygotic embryos in developing seeds.

# General Introduction

In higher plants, a fertilized egg cell develops to a zygotic embryo which has almost all basic structures and organs of an individual plant. Thus, the study on this process is prerequisite to understand the differentiation and development of plant. However, zygotic embryogenesis progresses inside of the mother plant and this feature makes embryogenesis difficult to be analyzed the biochemical and molecular biological changes during embryogenesis. Somatic embryogenesis, an embryogenesis from somatic cells, was first reported by Steward *et al.* (1958) and Reinert (1958). Somatic embryogenesis changes of developing somatic embryos are morphologically and developmentally similar to zygotic embryogenesis. Thus, somatic embryogenesis was used as a model system for understanding the regulation of gene expression required for the earliest development events in the life of a higher plant. However, there are no information about any factors regulating the development and morphogenesis of somatic embryos.

Carrot is known as one of the best plant materials for investigating somatic embryogenesis, because an easy experimental method to induce synchronously numerous somatic embryos had been established (Kamada and Harada 1984a). Thus, the process of somatic embryogenesis in carrot has been extensively investigated in physiological, biochemical and molecular biological aspects (Zimmerman 1993).

It is well known that addition of nitrate ion to a culture medium as a sole source of nitrogen does not promote somatic

embryogenesis in various plant species including carrot, but simultaneous application of reduced nitrogenous compounds with nitrate ion strongly stimulates somatic embryogenesis (Halperin and Wetherell 1965; Kamada and Harada 1979; Reinert and Tazawa 1969; Reinert *et al.* 1967; Walker and Sato 1981). Among various nitrogenous compounds, α-alanine, L-glutamine and L-glutamic acid exhibit strong stimulative effects on somatic embryogenesis in carrot and pollen embryogenesis in tobacco and potato (Kamada and Harada 1979; Harada and Imamura 1983; Harada *et al.* 1982; Wetherell and Dougall 1976; Wetherhead and Henshaw 1979). From these findings, there are some regulatory systems involving in nitrogen metabolism during embryogenesis. However, previous reports only said about the stimulating effects of those amino acids on the number of somatic embryos formed. Thus, in this study, I observed details of somatic embryogenesis when different reduced nitrogenous compounds were supplied and clarified what the effects of those amino acids on somatic embryogenesis were. Then I tried to obtain the reason why those amino acids requirement was occurred during somatic embryogenesis and to show embryogenesis-specific regulation for nitrogen metabolism, especially nitrogen assimilation.

In part I, I re-examined the effect of the three effective amino acids, L-glutamic acid, L-glutamine and α-alanine, and ammonium ion on somatic embryogenesis. When these amino acids were supplied in the medium, I observed not only increasing the number of somatic embryos but also stimulating the development of somatic embryos.

On the other hand, the cause of these effects of each reduced nitrogenous compound has been still unknown. Dougall (1965, 1966) reported that in tobacco cells, α-alanine, L-glutamine, L-glutamic acid, L-asparagine and L-aspartic acid were readily transformed to other amino acids and incorporated into proteins, but L-histidine, L-leucine and some others were not. Thus, Kamada and Harada (1984a) considered that the affect of a particular amino acid, either stimulatory or inhibitory, on somatic embryogenesis may be determined by its availability in protein synthesis.

In higher plants, it is thought that the main route of nitrogen assimilation is as follows (Mifflin and Lea 1977): nitrate ion is metabolized to nitrite ion by nitrate reductase (NR; EC 1.6.6.1); nitrite ion to ammonium ion by nitrite reductase (NiR; EC 1.6.6.4); this ammonium ion is bound with L-glutamic acid to form L-glutamine by glutamine synthetase (GS; EC 6.3.1.2.); a molecule of L-glutamine shares its two amino groups with a molecule of oxo-glutaric acid and forms two molecules of L-glutamic acid by glutamate synthase (GOGAT; EC 1.4.1.13). In this system, NR and GS are thought to be the key enzymes. NR activity decreases in early stages of carrot somatic embryogenesis and the reduction of NR activity results in fewer somatic embryos being formed in media without reduced nitrogenous compounds (Kamada and Harada 1984a).

The most effective amino acids for somatic embryogenesis, L-glutamine, α-alanine and L-glutamic acid, are able to be metabolized as nitrogen sources through L-glutamic acid. L-glutamine and L-

glutamic acid are the molecules involving in nitrogen assimilation cycle called GS-GOGAT cycle (Fig. 1). Exogenously supplied α-Alanine was rapidly transformed to L-glutamic acid. On the other hand, ammonium ion is located upstream of GS-GOGAT cycle and it is not so effective for somatic embryogenesis. From these findings, I assumed that low GS activity results in limited use of nitrogenous compounds located upstream of GS-GOGAT cycle. GOGAT is not limiting for somatic embryogenesis because glutamine which is only utilized about the substance of GOGAT can be also effective for somatic embryogenesis.

From these findings, I measured GS activity during somatic embryogenesis, and determined when the effective amino acids are required for somatic embryogenesis.

Plant GS exists as two isoforms (Wallsgrove *et al.* 1987; Freeman *et al.* 1990; Kamachi *et al.* 1991; Yamaya *et al.* 1992): one located in cytosol (GS1) and the other in plastids, especially in chloroplasts (GS2; Mcnally and Hirel 1983). GS1 acts for the translocation of nitrogen inside of plant, including the run out of nitrogen from senesced tissues, while GS2 involves in re-assimilation of ammonium ion generated from photorespiration. However, these findings only got from the study for maternal tissues, there were no information for embryos.

It is reported that there are multigene families of GS genes in the genomes of various higher plants. In maize, for example, there

are five genes for isoforms of GS1 and one of GS2 (Li *et al.* 1993). The expression patterns of genes for these six isoforms differ and, thus, each isoform is likely to have a different role. Moreover, the existence of so many isoforms indicates that the regulation of expression of genes for GS in plants is very complex. Thus, the study to analyze the expression pattern of each gene for GS isoforms is important for understanding mechanisms controlling GS activity.

In Part II, three cDNA clones for GS isoforms were isolated from carrot, and the expression of the genes for these GS isoforms during somatic embryogenesis in carrot was investigated. The results suggested that GS in somatic embryos is also regulated stage-specifically and embryo-specifically at transcriptional level.

In Part III, I analyzed the expression of the genes for GS isoforms in seed development and discussed the propriety of the measurement in seeds instead of zygotic embryos. Finally, I discussed the physiological meanings of the change of GS activity during embryogenesis.

# Part I

The relation between different effects of several reduced nitrogenous compounds and the change of GS activity in somatic embryogenesis of carrot

# Introduction

It is well known that simultaneous supply of ammonium ion with nitrate ion, is prerequisite for formation of asexual embryos including somatic embryos in various plant species (Noreel and Nitsch 1968, Thomas and Street 1972, Wetherell and Dougall 1976). Concerning with this stimulating effects of ammonium ion on asexual embryogenesis, some researchers examined extensively the effects of other reduced nitrogenous compounds including amino acids in carrot and they clarified that some amino acids such as L-glutamine (Gln), L-glutamic acid (Glu), asparagine, aspartic acid and α-alanine (α-Ala), possessed stimulative effects, but other amino acids are not so effective or inhibitory (Kamada and Harada 1979). These findings showed that the number of somatic embryos formed was different depending on the kind of reduced nitrogenous compounds supplied including amino acids. Especially, the supply of Gln, Glu and α-Ala were more effective for increasing the number of somatic embryos formed than the supply of ammonium ion in carrot (Wetherell and Dougall 1976, Kamada and Harada 1979). However, those reports only said the different effect of reduced nitrogenous compounds on the total number of somatic embryos formed, but did not other effects on somatic embryogenesis.

Thus, in this part, I re-examined the number of somatic embryos formed in the medium containing three effective amino acids, Glu, Gln and α-Ala, and ammonium ion, to know how the supply of these effective amino acids is different from the supply of ammonium ion containing the efficiency of somatic embryogenesis. In this

experiment, I found that the stimulatory effect of these amino acids was mainly caused by the increment of the number of well-developed embryos, especially torpedo-shaped embryos. On the basis of this observation, I discussed the relationship between the different effects of these reduced nitrogenous compounds and the change of GS activity during somatic embryogenesis.

# Materials and Methods

*Plant materials and induction of somatic embryogenesis*

Ten-day-old carrot seedlings (*Daucus carota* L. cv. US-Harumaki-gosun) were surface-sterilized with sodium hypochlorite solution (available chlorine concentration of 1 %) for 15 min and rinsed 3 times with sterile deionized water. Ten-mm-long hypocotyl segments were cut from the seedlings and placed on semi-solidified (0.2% Gellangum) Murashige and Skoog's medium (Murashige and Skoog 1962) (hereafter referred to as MS medium) containing $4.5 \times 10^{-6}$ M of 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic callus was obtained from these segments after one month of culture, and suspended with 100 ml of MS liquid medium containing $4.5 \times 10^{-6}$ M of 2,4-D in 300 ml Erlenmeyer flasks. These suspension cultures were put on a gyratory shaker (70 rpm) at 25°C in darkness and subcultured every 2 weeks.

To obtain small cell clusters, two-week-old cultures were successively sieved through three stainless steel meshes with 1000, 63 and 37 μm in pore size. Cell clusters remaining on the 37 μm mesh were rinsed with 2,4-D-free fresh MS medium, and collected by centrifugation (100 x*g*). The cell clusters were rinsed additional five times with the fresh MS medium, then suspended in 15 ml of test medium at a final density of 1.0 ml packed cell volume (the value represented the volume of pellet after 5 min of centrifugation at 100 x*g*) per liter and cultured in 50 ml Erlenmeyer flasks.

*Effects of reduced nitrogenous compounds*

In the examination to test the effects of some amino acids, it is necessary to avoid the contamination of ammonium ion from previous subculture, thus small cell clusters between 37 and 63 μm were rinsed well with MS-$NH_4$ medium (MS medium without $NH_4NO_3$). Test media used were MS-$NH_4$ medium containing various reduced nitrogenous compounds, as indicated in Table 1. After 2 weeks of culture, the number of somatic embryos at each developmental stage (globular-, heart- or torpedo-stage) (Fig. 2) were counted. Each experiment was repeated at least twice.

*Exchange of culture medium*

To clarify the effective stage of Gln on the development of somatic embryos, the cell clusters cultured in MS+$NH_4$ or MSQ medium for 7 days were collected by centrifugation (100 x*g*), rinsed twice with fresh MS-$NH_4$ medium and resuspended in fresh MS+$NH_4$ or MSQ medium. Two weeks after the transfer to somatic embryo inducing medium, the number of somatic embryos formed were counted as described above.

*Protein extraction and analysis of GS activity*

Protein extraction was performed according to the method described by Kang and Hymowiz (1988). Cell clusters and somatic embryos cultured in growth regulator-free MS medium were collected on a glass filter with suction by aspirator, measured the fresh weight, and stored at -80°C until use. These frozen samples were ground in liquid $N_2$, then homogenized for 5 min at 4°C by a glass-teflon homogenizer in a 4-fold volume of 25 mM Tris-HCl buffer (pH 7.6) containing 1 mM $MgSO_4$, 10 mM β-mercaptoethanol and 1 mM dithiothreitol. These homogenates were centrifuged (20,000 x*g*) for 30 min at 4°C and supernatants were collected. The supernatants were desalted through a PD-10 column (Pharmacia), then used for the GS enzyme assay.

The synthetase activity of GS had been measured according to the methods described by Kang and Titus (1981). The reaction mixture consisted of 653 mM Tris-HCl (pH 7.6) containing 44.4 mM $MgSO_4$, 44.4 mM Glu, 4.4 mM ATP and 44.4 mM of hydroxylamine. The reaction was started by the addition of hydroxylamine. The total reaction volume was 1.125 ml. After 10 min incubation at 35°C, the reaction was stopped by the addition of 0.375 ml of a solution containing 6.7% (w/v) $FeCl_3$, 1.0 M HCl and 4.0% (w/v) TCA. The transferase activity of GS was measured by the method of Shapiro and Stadtman (1970). The 2x reaction mixture consisted of 80 mM imidazole-HCl (pH 7.0),

containing 67 mM Gln, 60 mM $MnCl_2$, 0.8 mM ADP-Na, 40 mM $K_2AsO_4$ and 120 mM hydroxylamine. The reaction was started by the addition of equal volume of enzyme solution. The total reaction volume was 0.4 ml. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 1 ml of a solution with 3.3% (w/v) $FeCl_3$, 2.0% (w/v) TCA and 250 mM HCl. The enzyme activity was measured as the amount of synthesized L-glutamic acid γ-mono-hydroxamate (γ-GHA).

# Results

*Effects of amino acids*

According to the report of Kamada and Harada (1979), three amino acids, Gln, Glu and α-Ala, strongly stimulated the number of somatic embryos formed. Thus I selected these three amino acids for my experiment. Hereafter, the media containing Gln, Glu or α-Ala at 10 mM with 18.7 mM $KNO_3$ are referred to as MSQ, MSE and MSA media, respectively. I also prepared two other MS media containing 18.7 mM $KNO_3$ with or without $NH_4Cl$ (10 mM). The nitrogen composition of these media are summarized in Table 1 and the number of somatic embryos formed in each medium is shown in Fig. 3. Significantly more embryos were formed in MSQ or MSA medium as compared to that of control medium (MS+$NH_4$ medium). Because only Gln has two amino groups in a molecule, I also tested another MS medium containing half concentration (5 mM) of Gln. The number of embryos formed in the medium was similar to that in MSA medium (data not shown). On the other hand, the total number of embryos in MSE medium was slightly higher than that of control medium (MS+$NH_4$ medium), the number of well-developed embryos (torpedo-shaped stage) was apparently increased with the addition of each of the three amino acids as compared to that in control medium (MS+$NH_4$ medium).

The morphological features of somatic embryos formed in each medium are shown in Fig. 4. In MS+$NH_4$ medium, the somatic embryos did not fully develop and many embryos remained in the globular and

heart-shaped stages of development. In the media containing amino acids, well-developed somatic embryos at the torpedo-shaped stage were frequently observed. It is noteworthy that the somatic embryos in MSQ medium were larger than those in other media.

From these observations, the effects of these three amino acids on somatic embryogenesis seems to involve in the development of somatic embryos. Ammonium ion was not so effective to induce highly developed embryos. Ammonium ion is usually assimilated to amino acids by GS activity in plants. From these findings, I supposed that insufficient GS activity in developing somatic embryos cause these differences of utilization of reduced nitrogenous compounds. Thus, I measured GS activity of somatic embryos.

*Estimation of the methods for measurement of GS activity*

There are two kinds of methods for measurement of GS activity; synthetase assay and transferase assay. I examined which method is better for the measurement of GS activity in somatic embryogenesis of carrot. The amount of γ-GHA produced by transferase activity is 10-fold more than the amount of γ-GHA synthesized by synthetase activity (Table 2). Because collecting the sufficient amount of cells in early somatic embryogenesis is difficult, I decided to use transferase assay method for measurement of GS activity in following experiments.

*GS activity during somatic embryogenesis*

GS activity increased during the first 5 days after transfer to 2,4-D-free MS medium, and then decreased during the following 10 days (Fig. 5). This decline of the GS activity in somatic embryos at the late stage of somatic embryogenesis suggested that the requirement of amino acids for their development is caused by the lack of GS activity.

*Effects of glutamine on somatic embryos at different developmental stages*

To determine whether Gln was effective in early or late stages of somatic embryo formation, the culture medium (MSQ or MS+$NH_4$ medium) was exchanged after 7 days of culture (Fig. 6-A). As a control experiment, MSQ or MS+$NH_4$ medium were used during the whole culture period. When MSQ medium was used during the last 7 days, the number of well-developed embryos (torpedo-shaped embryos) was higher (Fig. 6-B), but the number of globular and heart-shaped embryos did not rise apparently. When MSQ medium was used during the first 7 days, the number of globular and heart-shaped embryos increased, but the number of torpedo-shaped embryos did not.

# Discussions

Somatic embryos of carrot are practically induced for two weeks of cultivation. The development of somatic embryos in my experimental conditions are shown in Fig. 2. Globular, heart-shaped and torpedo-shaped embryos had been observed since 5 days, 7 days and 10 days after transfer to the 2,4-D-free MS medium, respectively (Fig. 2). Thus I divided the culture period to the first 7 days as the early stage of somatic embryogenesis and the last 7 days as the late stage of somatic embryogenesis and selected two to three points of both developmental stages to measure GS activity or GS gene expression.

The addition of three amino acids, Gln, α-Ala or Glu did not result in only increasing of the total number of embryos formed but also stimulating the development of embryos (Fig. 3). The addition of Glu did not increase the total number of somatic embryos formed so much as compared to that in the addition of ammonium ion, but stimulated the number of well-developed somatic embryos like torpedo-shaped embryos (Fig. 3-d). Previous studies only claimed the effects of amino acids on the total number of the embryos formed (Wetherell and Dougall 1976, Kamada and Harada 1979), but my observations showed that the common effect of these three amino acids was to increase the number of well-developed embryos. Thus, it is possible that the effects of these amino acids involved in the development of somatic embryos, not in the stimulation of them.

The morphological features of embryos formed were different depending on the nitrogen source used (Fig. 4). Torpedo-shaped

embryos obtained in MSQ medium were bigger than the embryos in other media tested. In contrast to the above case, the size of the embryos in MSA medium was small, though the number and the developmental stages were similar to those in the MSQ medium. Embryos in MSE medium sometimes showed well-developed vascular system and root elongation (data not shown). The reason why these amino acid-dependent morphological differences occur was unknown, but Gln seemed to be one of the most suited reduced nitrogenous compounds to induce somatic embryos of carrot.

The GS activity decreased at the late stage of somatic embryogenesis in carrot (Fig. 5). This result suggest that somatic embryos at the late stage of embryogenesis did not utilize externally supplied ammonium ion well for the low activity of GS. From the feeding experiments, the supply of the three amino acids particularly affected the developmental process from heart-shaped to torpedo-shaped embryos because this process progressed the latter half of the culture period of induction of somatic embryos (Fig. 2). This also supported by the fact that the number of well-developed somatic embryos such as torpedo-shaped embryos increased by the supply of Gln only in the latter half of the culture period (Fig. 6-B). Form these findings, I suppose that the decrease of GS activity specifically affect the development of somatic embryos. In the early stage of somatic embryogenesis, the carrot cells have certain GS activity enough to develop themselves just supplying ammonium ion, but GS activity was decreases in the late stage of

somatic embryogenesis and they cannot utilize ammonium ion to develop torpedo-shaped embryos from heart-shaped embryo. Glu and Gln are the down stream of nitrogen assimilation pathway of the reaction catalyzed by GS. α-Ala, another effective amino acid for somatic embryogenesis is not positioned nitrogen assimilation pathway, but the report which exogenously supplied another effective amino acid, α-Ala, was also rapidly converted into Gln or Glu in the cells of carrot somatic embryos (Kamada and Harada 1984b) supported my explanation.

# Part II

Isolation of cDNA clones for GS isoform in carrot and the analyses of the expression patterns of the genes corresponding to these cDNA clones during somatic embryogenesis

# Introduction

Plant GS exists as two types (Wallsgrove *et al.* 1987, Freeman *et al.* 1990, Kamachi *et al.* 1991, Yamaya *et al.* 1992): one located in the cytosol (GS1) and the other in the plastids, in particular the chloroplasts (GS2; Mcnally and Hirel 1983). Many researchers have investigated the roles of these two isoforms. GS1 regards to be involved in translocation of nitrogenous compounds. Kamachi *et al.* (1992) showed that GS1 distributed in bundle sheath cells in rice leaves. Studies on mutants without GS2 activity in their chloroplasts demonstrate clearly that a major role of GS2 is to facilitate the reassimilation of ammonium ions released during photorespiration (Wallsgrove *et al.* 1987, Freeman *et al.* 1990).

In maize, for example, there are five genes for isoforms of GS1 and one for isoform of GS2 (Li *et al.* 1993). The patterns of expression of genes for these six isoforms differ, and, thus, each isoform is likely to have a different role. Moreover, the existence of so many isoforms indicates that the regulation of expression of genes for GS in plants is very complex. Thus, the study to analyze the expression pattern of each gene for GS isoforms is important for understanding mechanisms controlling GS activity.

In this part, three GS cDNA clones named "CGS102", "CGS103" and "CGS201" were isolated from three distinct cDNA libraries of carrot, then I studied the expression patterns of the genes for GS isoforms to clarify the relation between the expression patterns of the GS genes and developmental status of somatic embryos.

# Materials and Methods

*Isolation of RNA and construction of cDNA libraries*

Total RNA was extracted from cultured cells and several organs by the Tris-SDS-phenol method (Schmidt *et al.* 1981). For the construction of the cDNA libraries, poly(A)$^+$ RNA was separated from the total RNA of leaves, somatic embryos and embryogenic callus on a prepacked oligo(dT) cellulose column (Pharmacia, Uppsala, Sweden). The cDNA libraries were constructed with a cDNA cloning system (Amersham, Buckinghamshire, England) according to the manufacturer's protocol.

*Reverse transcription-polymerase chain reaction (RT-PCR)*

A 1-μg aliquot of total RNA from 7-day-old somatic embryos was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Toyobo Co. Ltd., Tokyo, Japan), according to the manufacturer's instructions, in a total volume of 20 μl. The first PCR was carried out for 30 cycles in 100 μl of a solution composed of 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$ and 2.5 units of AmpliTaq™ DNA polymerase (Perkin Elmer, California, USA), with 20 μl of the solution of the products of reverse transcription. The conditions for PCR were as follows: 40 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. The second PCR was carried out

for 25 cycles under the same reaction conditions as the first PCR with 1 μl of the solution of the products of the first PCR. The products of the second PCR were then precipitated in ethanol, dissolved in 10 μl of TE buffer and subjected to electrophoresis. The primers were 5'-ATATGGATCGGTGGATCAGG-3' as the forward primer for the first and second PCR, and 5'-CCTCATTGCCTTCTCCATAAGC-3' and 5'-GGTGGTNTCNGCGATCATGG-3' as the reverse primers for the first and second PCR, respectively. These primers were designed by reference to a cDNA for rice GS1 (RGS28).

*5' Rapid amplification of cDNA ends (5' RACE)*

I isolated a "CGS102" cDNA clone with lacking 5' region of the cDNA. Then I performed rapid amplification of cDNA ends (5' RACE) according to the protocol supplied with the Amplifinder RACE kit (Clonetech, California, USA) to obtain a 5' fragment with overlapping regions that were identical to the partial cDNA clone for "CGS102". I determined the entire sequence of the "CGS102" cDNA clone, including this 5' fragment.

*Genomic Southern hybridization*

Genomic DNA was extracted from carrot leaves by a modified version of the method described by Wagner *et al.* (1987) and Bousquet

*et al.* (1990). Ten-μg aliquots of carrot genomic DNA were digested separately with *Eco*R I, *Hin*d III and *Xba* I and subjected to electrophoresis on a 0.7% agarose gel. Bands of DNA were blotted onto a charged nylon membrane (Hybond $N^+$; Amersham, Buckinghamshire, England). Procedures for hybridization were those described by the manufacturer of the membrane. For high-stringency conditions, hybridization and washing were performed at 65°C. For low-stringency conditions, these steps were performed at 50°C. Probe DNAs were labelled with [$^{32}$P]-dCTP by the Multiprime-labeling system (Amersham, buckinghamshire, England) and results of all hybridizations were analyzed with a Bio-Imager (BAS2000; Fuji Film Co. Ltd., Tokyo, Japan) or by autoradiography.

*Northern hybridization*

Twenty μg of total RNA from cultured cells and various organs were denatured with 2.2 M formaldehyde and 50% (w/v) formamide, subjected to electrophoresis on a 1.2% (w/v) agarose gel contained 2.2 M formaldehyde and then transferred to a charged nylon membrane (GeneScreen Plus; DuPont/NEN, Massachusetts, USA) with 20x SSC as the transfer buffer. Hybridization was performed according to the instructions from the manufacturer of the membrane with [$^{32}$P]-labeled probe DNAs that had been prepared with the Multiprime-labeling

system (Amersham, Buckinghamshire, England). The results of hybridization were visualized as described above.

# Results

*Isolation of cDNA clones for GS from carrot cDNA libraries*

I designed primers for PCR to amplify a fragment of cDNA for carrot GS and used total RNA as a template for RT-PCR. After PCR, I subcloned the fragment and confirmed that it was a fragment of cDNA for GS by sequencing (hereafter this cDNA is referred to as "CGS101").

The cDNA library constructed from 14-day-old somatic embryos was screened with "CGS101" as a probe. I screened $10^5$ plaques and found 10 positive plaques in the first screening. Then I selected one positive plaque in the 2nd screening based on the size of the cDNA fragments. This cDNA clone lacked a part of its 5' region. Then I performed 5' RACE and obtained the 5' fragment with overlapping regions that were identical to the partial cDNA clone. I determined the entire sequence of this GS cDNA clone of carrot (Fig. 7), including this 5' fragment. The deduced amino acid sequence was 83.3% homologous to lettuce GS1, 82.6% to pea GS1 and 82.4% to maize GS11 (Table 3). Thus, this cDNA clone appeared to encode a GS1 homolog of carrot, called "CGS102".

Two other cDNA clones for GS, "CGS103" and "CGS201", were isolated from cDNA libraries that had been constructed from embryogenic callus and leaves, respectively, with the open reading frame (ORF) of "CGS102" as a probe under low-stringency hybridization conditions (Figs. 8 and 9). "CGS201" was identified as

the cDNA for a GS2 homolog because it encoded a putative transit sequence for protein transport into plastids (60% homology to GS2 of *Arabidopsis* and 58% to *Nicotiana sylvestris* GS2 at the amino acid level) in the 5' region (Fig. 10). "CGS102" and "CGS103" were more similar (about 81-87% at the amino acid level) to GS1 than GS2 (about 73-78% at the amino acid level) of other plant species (Table 3), such as *Arabidopsis*, rice and radish. On the other hand, "CGS201" was very similar to other cDNAs for GS2 (86-90% at the amino acid level). Thus, I determined the three cDNA clones as carrot GS cDNA clones from these sequence similarities. Furthermore, the deduced amino acid sequence of each cDNA clone has an ATP binding domain (Gly-X-Phe-Glu-Asp-Arg) conserved in eukaryotic GS (Figs. 7, 8 and 9; Kim and Rhee 1988). The fact also supported that the three cDNAs encode GS.

*Genomic Southern analysis*

To determine the copy number of genes for GS isoforms, I performed genomic Southern hybridization. Leaf genomic DNA was isolated, digested with the restriction endonucleases, *Eco*R I, *Hin*d III and *Xba* I, and then fragments were allowed to hybridize, after electrophoresis, to each probe (Fig. 11). The PCR-amplified 3'-noncoding regions of the cDNA clones were used as specific probes (Figs. 7, 8 and 9). There were many bands detected on the hybridization with the coding region of "CGS102" as a probe (Fig.

11-a). On the other hand, only one band was observed when hybridization was performed with the specific probes derived from "CGS103" cDNA (Fig. 11-c) and "CGS201" cDNA (Fig. 11-d). The results show that the genes for these two isoforms are single-copy genes in the carrot genome. When the hybridization was performed with the 3'-specific probe derived from "CGS102" cDNA, I observed several bands (Fig. 11-b). Thus, there are at least two copies of the corresponding gene in the carrot genome. I could not distinguish the transcripts by hybridization so I treated all results of Northern analysis with the "CGS102"-specific probe as being indicative of the expression of the "CGS102" family of genes.

*Expression of GS isoforms during somatic embryogenesis*

In order to analyze the patterns of expression of the carrot GS isoforms, I isolated total RNA at 4, 7, 10 and 14 days after transfer of embryogenic callus to somatic embryo-inducing medium. Twenty μg of each RNA were subjected to electrophoresis, blotted onto a membrane and allowed to hybridize with the 3'-noncoding region of the cDNA for each GS isoform as specific probes (Fig. 12). Hybridization with the coding region of "CGS102" as a probe, the signals were strong at 4 and 7 days and weak at 10 and 14 days. The patterns were quite similar to the patterns of changes in GS activity during somatic embryogenesis: a decrease in activity was

also detected at the late stage of embryogenesis (Fig. 5). Signals were obtained when the probes specific for the "CGS102" family and "CGS201" were used. The amounts of transcripts of both isoforms were also high at the early stage (4 and 7 days) and low at the late stage of embryogenesis. Then the amounts increased again slightly in 14-day-old somatic embryos. On the contrary, no signal was observed during somatic embryogenesis after hybridization with the "CGS103"-specific probe. These results show that transcripts from only two of the three genes for GS isoforms were expressed and accumulated during somatic embryogenesis, especially at the early stage.

*Expression of the "CGS102" family of genes and the "CGS201" gene in somatic embryos cultured in medium that contained different reduced nitrogenous compounds*

In radish cotyledons, the expression of cytosolic GS (GS1) is affected by the application of various nitrogenous compounds (Watanabe *et al.* 1994). This findings showed that the expression of GS genes was affected by products or substrate of their enzyme action. In carrot somatic embryogenesis, the supply of different nitrogenous compounds also affected the expression of the "CGS102" family of genes (Fig. 13). In somatic embryos cultured in MSQ medium, the level of "CGS102" and "CGS201" transcripts was significantly low as compared to those in MS+$NH_4$ medium at both 7 days and 14 days after the transfer to 2,4-D-free MSQ or MS+$NH_4$

medium. But in each culture condition, the level of the transcripts for both GS isoforms decreased at 14 days after the transfer. These results suggest that expression of the genes for "CGS102" and "CGS201" was suppressed by the treatment with nitrogenous compounds.

# Discussions

I isolated cDNA clones for three isoforms of GS, "CGS102" (Fig. 6), "CGS103" (Fig. 7) and "CGS201" (Fig. 9), from several tissues of carrot. Both "CGS102" and "CGS103" encoded proteins with considerable similarity to GS1 of other plants (Table 3). "CGS201" encoded a protein with considerable similarity to GS2 of other plants throughout its entire ORF and also exhibited similarity to the 5' regions of the ORFs for GS2 in other plants (Table 3), which encode a transit peptide for protein transport into plastids (Fig. 9). Genomic Southern hybridization revealed that "CGS103" and "CGS201" represented single-copy genes, but the gene corresponding to "CGS102" was present as at least two copies in the carrot genome (Fig. 11-b, c, d). I could not distinguish the members of the "CGS102" family of transcripts because of their strong similarity, and the genomic clones of the "CGS102" family remains to be analyzed. Furthermore, the results of genomic Southern hybridization showed that 3'-noncoding regions of the cDNA clones were not cross-hybridized to each other. Therefore, these probes were used as specific probes to detect the expression of the genes for GS isoforms in carrot.

From the analysis of the expression of transcripts, total amount of transcripts for GS was high at the early stage of embryogenesis and low at the late stage of embryogenesis. The patterns of the expression were similar to the changes in GS activity during somatic embryogenesis (Fig. 5). This result suggested that GS activity was controlled at transcriptional level.

I also found that two among the three types of genes for GS isoforms, the "CGS102" family and "CGS201", were expressed during somatic embryogenesis, but the gene for "CGS103" did not (Fig. 12). The expression patterns of the genes for "CGS102" family and "CGS201" were same as total GS gene expression. Thus, both genes for "CGS102" family and "CGS201" were expressed mainly in somatic embryogenesis in carrot whereas these two isoform genes encoded the different types of the GS, GS1 and GS2, respectively. It had been reported that these two types of the genes and their products were usually regulated in different ways of in adult plant (Kozaki *et al.* 1991, 1992, Kamachi *et al.* 1992, Yamaya *et al.* 1992, Pérez-Rodríguez and Valpuesta 1996). Furthermore, it is also known that the expression of GS genes and GS activity were affected by nitrogenous compounds supplied (Loyola-Vargas *et al.* 1986, Watanabe, *et al.* 1994, Mäck 1995). This effect was also observed in somatic embryogenesis (Fig. 13), but the decline of the amounts of transcripts of the genes for "CGS102" family and "CGS201" at the late stage of embryogenesis was unchangeable by the supply of nitrogenous compounds. It is probable that there was an other regulation mechanism which is dependent on the development of the embryos, but independent of nitrogenous compounds. These observations indicate the existence of embryogenesis-dependent regulations of GS gene expression.

It has been unknown whether the roles of GS1 and GS2 in early stages of somatic embryos are the same or not, and what kinds of

roles they carry out. However, there must be some physiological meanings that both GS isoforms subjected to the same regulation.

# Part III

The expression patterns of the genes for GS isoforms in somatic and zygotic embryogenesis

# Introduction

One of the problem regarding somatic embryos as a model for zygotic embryos is that both embryos are subjected to different circumstances during their embryogenesis. Somatic embryos usually induced in synthetic medium and they do not have any connections to other plant tissues. Under these conditions, there are some abnormal events, for example secondary embryogenesis, were observed (Ammirato 1974). Furthermore, gene expression observed in somatic embryogenesis are sometimes the same as those in zygotic embryogenesis, but sometimes not (Zimmerman 1993). Thus, if the genes for GS isoforms are subjected to embryogenesis-specific regulations, the same expression patterns are observed during zygotic embryogenesis.

In Part I and Part II, I found that the expression of the genes for GS isoforms affects the level of GS activity during somatic embryogenesis of carrot, and the decline of the GS activity caused the requirement of amino acids such as Gln. This pattern of the GS gene expression seems to be specific to embryogenesis, the same pattern of the expression would be observed during zygotic embryogenesis. But it is difficult to collect zygotic embryos at early stages from developing seeds because their size is too small. Felker and Muhitch (1980) showed that the amount of GS was low in endosperm which occupies large part of the seed. However, there is no report indicating the changes of gene expression of GS during zygotic embryogenesis. I thought that whole seeds can be used instead of zygotic embryos, and used carrot seeds to investigate the

expression of the genes for GS isoforms. In this part, I will describe the expression patterns of the genes for GS isoforms in developing seeds which included developing zygotic embryos.

# Materials and Methods

*Plant materials*

Young leaves and naturally senesced leaves were collected from adult plants that had been growing in a greenhouse. Immature seeds were harvested in the Ibaraki Experimental Field of Takii Seed Co. Ltd. (Ibaraki, Japan).

*Northern hybridization*

Twenty μg of total RNA from various organs were denatured with 2.2 M formaldehyde and 50% (w/v) formamide, subjected to electrophoresis on a 1.2% (w/v) agarose gel contained 2.2 M formaldehyde and then transferred to a charged nylon membrane (GeneScreen Plus; DuPont/NEN, Massachusetts, USA) with 20x SSC as the transfer buffer. Hybridization was performed according to the instructions from the manufacturer of the membrane with [$^{32}$P]-labeled probe DNAs that had been prepared with the Multiprime-labeling system (Amersham, Buckinghamshire, England). The results of hybridization were visualized as described in Part II.

# Results

*Expression of GS isoforms in developing seeds*

Shiota *et al.* (1997) showed that the stages of development of carrot zygotic embryos were as follows: the globular stage, 14 days after flowering (DAF); the heart-shaped stage, 20 DAF; and the torpedo-shaped stage, 30 DAF. I examined the gene expression of the "CGS102" family, "CGS103" and "CGS201" during seed development with the same plant materials as Shiota et al. (Fig. 14). The patterns of expression of the corresponding genes were similar to those during somatic embryogenesis. The expression was high in the early stage and decreased gradually to the late stage. The expression of the genes for "CGS102" and "CGS201" were also high in 11 DAF. In somatic embryogenesis, GS gene expression also raised before globular embryos formed (Fig. 12, 4 days after transfer). These observation showed that GS gene expression stimulated at very early stages of embryogenesis, at least before globular embryos formed. The level of the "CGS103" transcripts was low during zygotic embryogenesis but the mRNA accumulated after the development of zygotic embryos was complete.

*Expression of the transcripts for "CGS103" in senesced leaves of carrot*

The pattern of the accumulation of the transcripts for "CGS103" in developing seeds may indicate that "CGS103" is one of the

senescence specific GS isoforms. To confirm this hypothesis, I compared the amount of the transcripts of "CGS102", "CGS103" and "CGS201" in young leaves to that in natural senesced leaves (Fig. 15). Only the transcripts of "CGS103" increased in senesced leaves. This result strongly supports that the gene for "CGS103" is involved in senescence.

# Discussion

I found that both types of genes for GS, GS1 ("CGS102") and GS2 ("CGS201"), were expressed strongly during the first 25 days after pollination of developing carrot seeds, in which the developmental stages of zygotic embryos correspond to the early developmental stages of somatic embryos. The expression of both genes decreased after 25 DAF (Fig. 14). The similar changes in expression of GS during both somatic and zygotic embryogenesis strongly suggest that expression of GS is developmentally controlled.

The mRNA for another GS isoform "CGS103", accumulated faintly during zygotic embryogenesis but not in somatic embryogenesis (Figs. 12 and 14), but it strongly accumulated at the late stage of seed development (Fig. 14). At that time, dramatic changes occur in carrot seeds: the amount of Abscisic acid which is one of the phytohormones increases transiently, the pericarp turns brown and dehydration starts (Shiota et al. 1997). The maturation of embryos and senescence of maternal tissues occur at this stage. The senescence-specific expression of GS1 has been observed in several plant species (Egli *et al.* 1989, Kamachi *et al.* 1991, Bernhard and Matile 1994, Pearson and Ji 1994, Watanabe *et al.* 1994, Pérez-Rodríguez and Valpuesta 1996). The expression of GS isoform genes during natural senescence of leaves indicates that "CGS103" cDNA encodes one of the senescence-specific isoforms of GS1 (Fig. 15). The "CGS102" transcripts, for another isoform of carrot GS1, did not accumulate during senescence (Fig. 15). These observations showed that the gene for "CGS103" was one of the senescence-specific genes

and might express in the maternal tissues of the developing seeds but not in zygotic embryos.

From these observations, I concluded that the expression patterns of the genes for GS in zygotic embryogenesis were similar to those in somatic embryogenesis. This suggested that the expression patterns of the genes for GS in somatic embryogenesis occurred in a development-dependent manner seen in zygotic embryogenesis. It is possible to conclude that somatic and zygotic embryogenesis is identical at least the regulation of the expression of the genes for GS.

# General Conclusions

In Part I, I used four reduced nitrogenous compounds for inducing somatic embryogenesis in carrot and analyzed the effect of each of them on somatic embryogenesis. All reduced nitrogenous compounds tested stimulated somatic embryogenesis, and the addition of three amino acids, Gln, α-Ala or Glu resulted in the increase of the number of well-developed somatic embryos (Fig. 3). These reduced nitrogenous compounds related to nitrogen assimilation system, called GS-GOGAT cycles (Fig. 1), including two types of enzymes. GOGAT (E.C. 1.4.1.13), one of the enzymes, catalyzes the reaction transferring one amino group of Gln to 2-oxo glutaric acid and making two Glu. If this process had the problem during somatic embryogenesis, somatic embryos could not utilize ammonium ion and Gln. However, Gln is the best nitrogen source for induction and development of somatic embryos among all nitrogenous compounds tested (Figs. 3 and 4), so GOGAT acts enough to allow the development of the embryos. GS, another enzyme of the GS-GOGAT cycle, catalyzes the first step to take inorganic nitrogen to organic nitrogen. Lack of this enzyme activity causes plant not to be able to utilize inorganic nitrogenous compounds for nitrogen metabolism necessary for protein synthesis. My results on the stimulatory effects of the reduced nitrogenous compounds including Gln for somatic embryogenesis suggest the possibility of the lack or decrease of GS activity during the developmental process from heart-shaped stage to torpedo-shaped stage (Fig. 4). In fact, the change of GS activity during somatic embryogenesis was supported by this

idea (Fig. 5). This idea was also supported by the observation that the number of well-developed somatic embryos such as torpedo-shaped embryos was increased by the supply of Gln only in latter half of the culture period (Fig. 6).

In Part II, the analyses of the expression of the genes for GS isoforms showed that there were two genes for GS isoforms expressed during somatic embryogenesis and the expression patterns were similar to the change of GS enzyme activity during somatic embryogenesis(Fig. 12). Surprisingly, these two genes were different types of GS. It is generally thought that the gene expression of each GS1 and GS2 is subjected to different regulation in adult plants. My results presented in this thesis show that the expression of the genes for both GS1 and GS2 was controlled during somatic embryogenesis. The roles of both GS, especially GS2 which usually act for photosynthesis in adult plant, have not been clarified yet, but the expression of the gene for GS2 in somatic embryos which harbor undifferentiated plastids, proplastids, may indicate that GS2 has another unknown role.

In Part III, I showed that the expression of the genes for GS during seed development was comparable to that in somatic embryogenesis. This result indicates that the GS gene expression during somatic embryogenesis were not controlled by artificial conditions in cultured cells but the expression was controlled by embryogenesis itself (Fig. 14).

Based on these analyses, I concluded that the expression patterns of the genes for GS in zygotic embryogenesis correspond with those in somatic embryogenesis, and that these patterns of gene expression cause the change of GS activity in somatic embryogenesis.

Why the expression of the genes for GS is suppressed at the late stage of embryogenesis? To answer this question, I propose a model by the environment of zygotic embryos in developing seeds (Fig. 16). At the late stage of somatic embryogenesis, the cell mass and protein contents increased rapidly (data not shown). These changes may also happen in zygotic embryos. For these changes, quick nitrogen assimilation is required. However, nitrogen assimilation pathway needs a large amount of energy to assimilate inorganic nitrogen, and also needs a large amount of organic acid as 2-oxo-glutaric acid. To avoid these energy-consuming events, utilization of Gln is a good choice for the development of zygotic embryos. In fact, a large amount of Gln was supplied from mother tissues and present surrounding the zygotic embryos (Ohkawa and Maeda, 1992). Thus, zygotic embryos can suppress their nitrogen assimilation by the controls of the expression of the genes for GS. Somatic embryos have the same control mechanism for GS gene expression, and so they cannot utilize ammonium ions at the late stage of embryogenesis.

All thorough my experiments, I clarified there are an embryogenesis-specific regulation for nitrogen assimilation caused by the transcriptional regulations of GS genes. These regulation may involve in the developmental program of plant embryogenesis. Thus,

these findings may give the fundamental information to understand the developmental process of embryogenesis. And to study further the details of the nitrogen metabolism in somatic and zygotic embryogenesis lead us to clarify the roles of changes GS activity and the meanings of the patterns of gene expression for GS isoforms during embryogenesis of carrot.

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# Tables and Figures

Table 1. Composition of nitrogenous compounds in culture media used, with concentration in mM.

| Nitrogenous compounds | MS-$NH_4$ | MS+$NH_4$ | MSA | MSE | MSQ | MS |
|---|---|---|---|---|---|---|
| $NH_4NO_3$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 20.5 |
| $KNO_3$ | 18.7 | 18.7 | 18.7 | 18.7 | 18.7 | 18.7 |
| $NH_4Cl$ | 0.0 | 10.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| α-Ala | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 0.0 |
| L-Glu | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 |
| L-Gln | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 |

Table 2. Comparison between two methods for measuring GS activity.

| | Activity | Minimum sample amount necessary for significant detection |
|---|---|---|
| Synthetase assay | 15.4 * | 0.31 (g fw) |
| Transferase assay | 229.6 * | 0.02 (g fw) |

*; γ-GHA μmoles/min/mg protein

fw; fresh weight

Table 3. Homology(%) of carrot GS gene sequences and some GS genes of other plant families at amino acid level.

| | GS1 | | | | GS2 | | | |
|---|---|---|---|---|---|---|---|---|
| | lettuce | pea | radish | maize | *Arabido-psis* | tobacco | rape | maize |
| CGS102 | 83.3 | 82.6 | 81.3 | 82.4 | 74.2 | 73.3 | 75.1 | 73.9 |
| CGS103 | 87.1 | 85.0 | 84.9 | 85.3 | 78.0 | 74.9 | 77.0 | 76.3 |
| CGS201 | 77.5 | 76.2 | 75.3 | 78.3 | 86.4 | 89.8 | 86.0 | 87.6 |

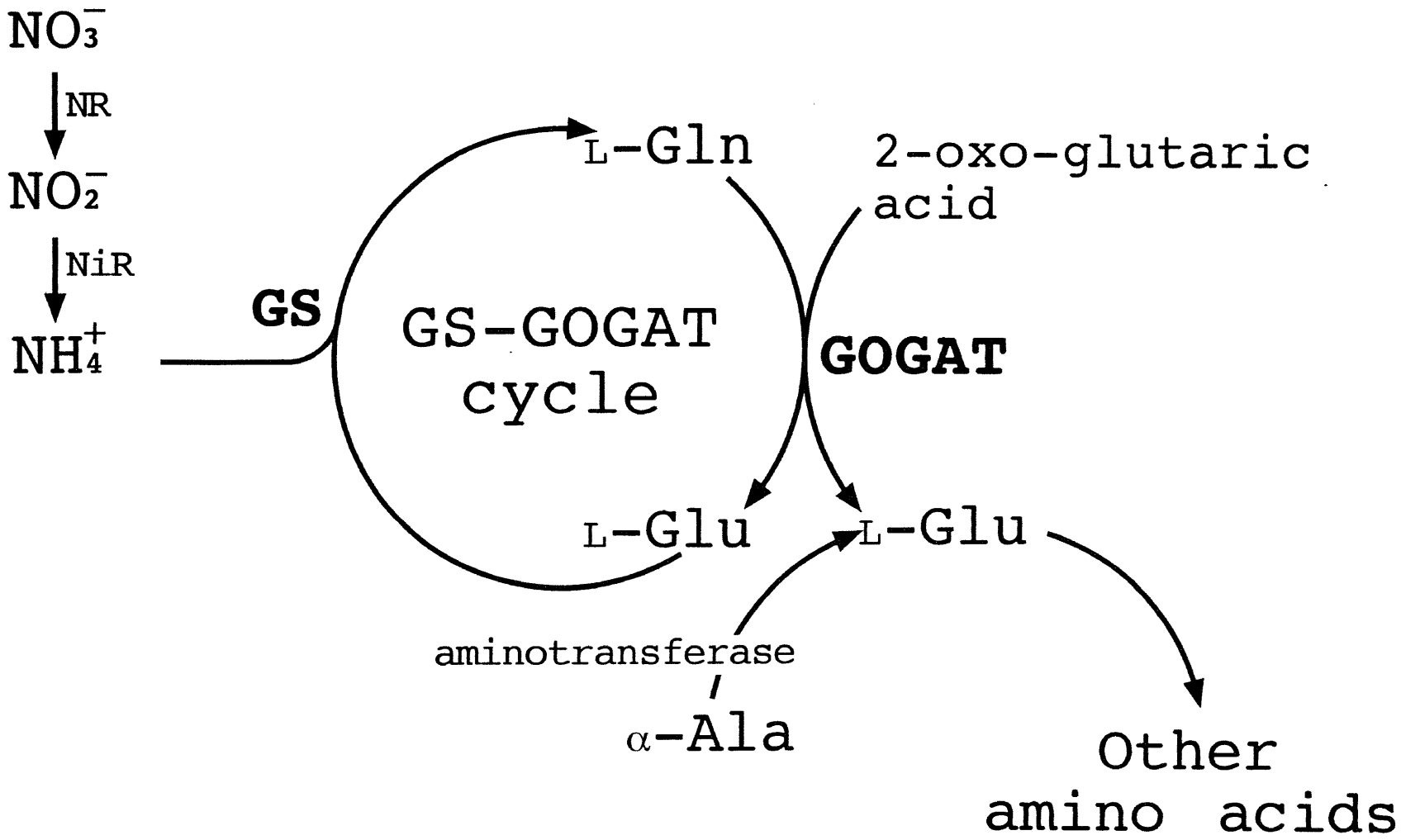



**Figure 1. Nitrogen assimilation pathway in higher plants.**

Inorganic nitrogen is once reduced to ammonium ion, then assimilated into organic nitrogen by GS-GOGAT cycle. This cycle produces an additional glutamic acid and the glutamic acid utilizes in the synthesis of other amino acids.

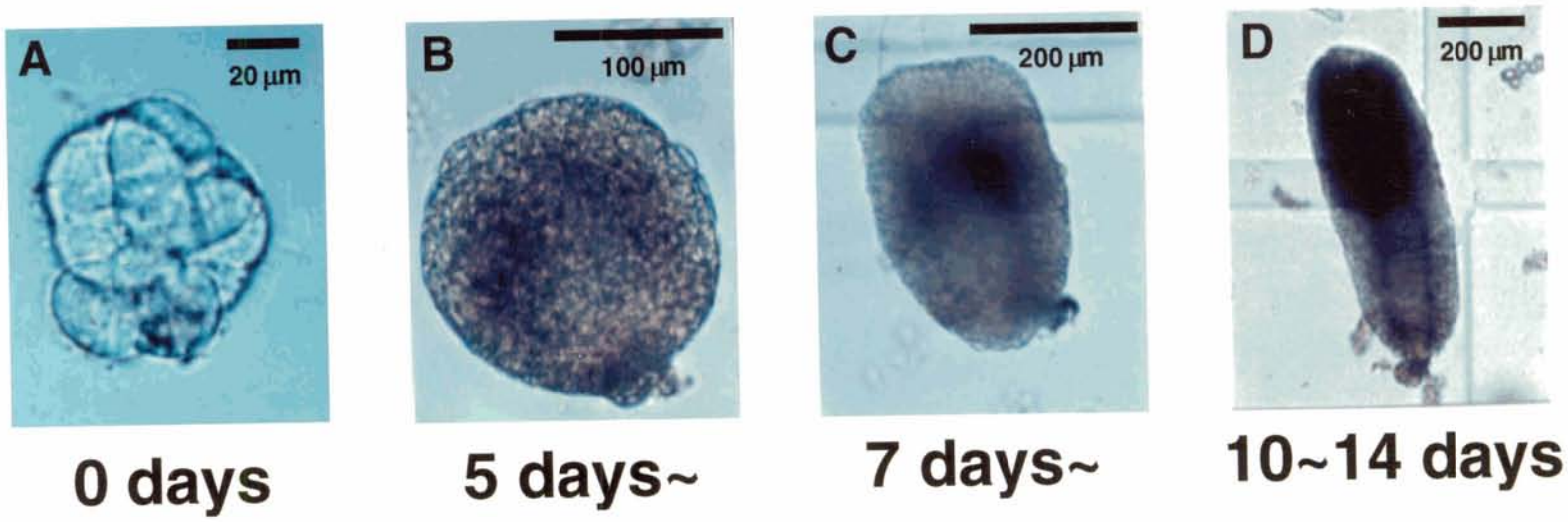



**Figure 2. Developmental changes of carrot somatic embryos.**

**A;** A sieved cell cluster (37~63 μm), **B;** A globular embryo, **C;** A heart-shaped embryo, **D;** A torpedo-shaped embryo.

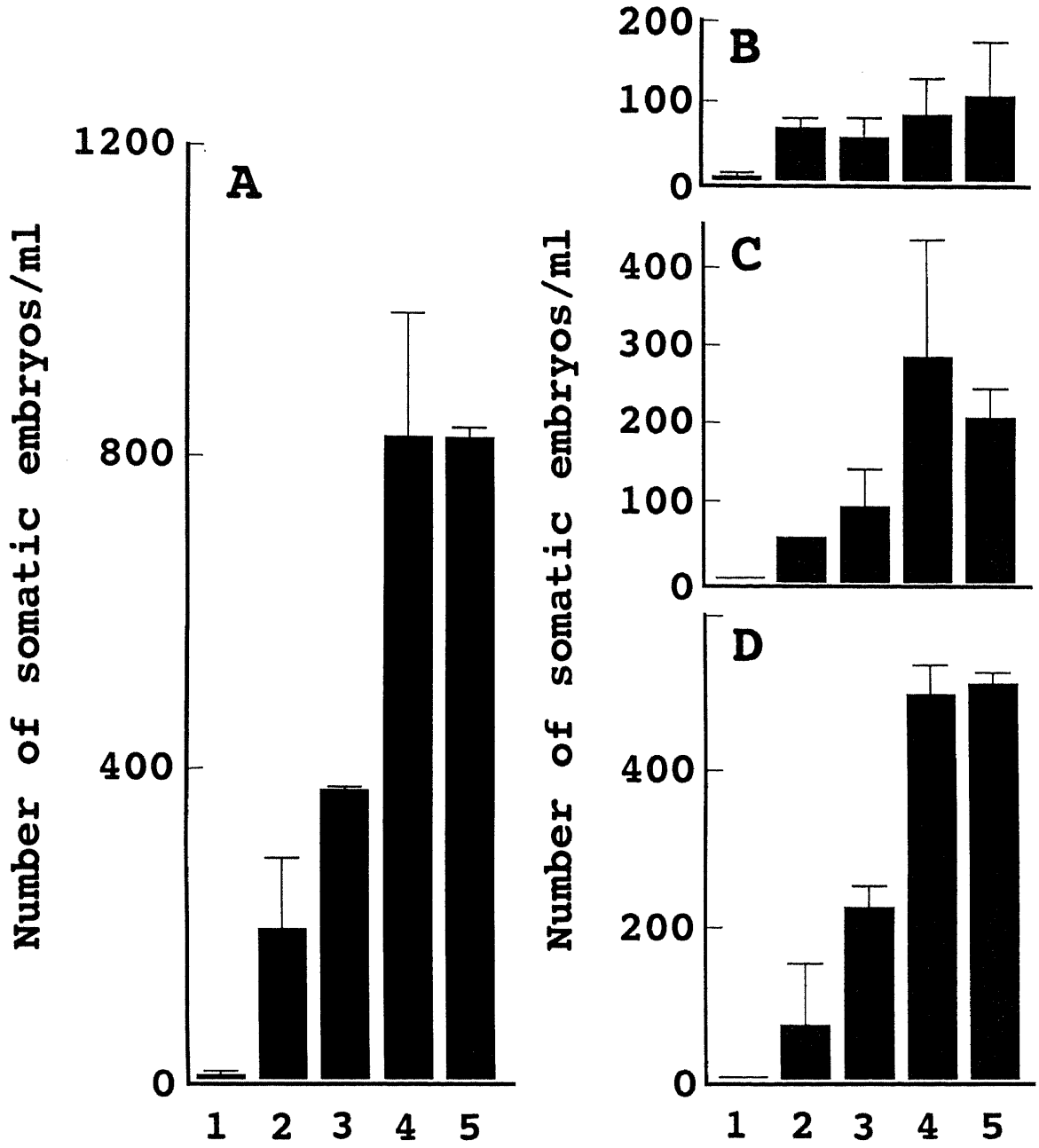



**Figure 3. Effects of 4 reduced nitrogenous compounds on somatic embryo formation.**

**A.** Total number of somatic embryos formed after two weeks of culture in 2,4-D-free test media. **B**, **C** and **D** show the number of globular, heart-shaped and torpedo-shaped embryos in **A**, respectively. **1**; MS-$NH_4$ medium, **2**; MS+$NH_4$ medium, **3**; MSE medium, **4**; MSA medium, **5**; MSQ medium. The error bar represents the SE value (n=2).

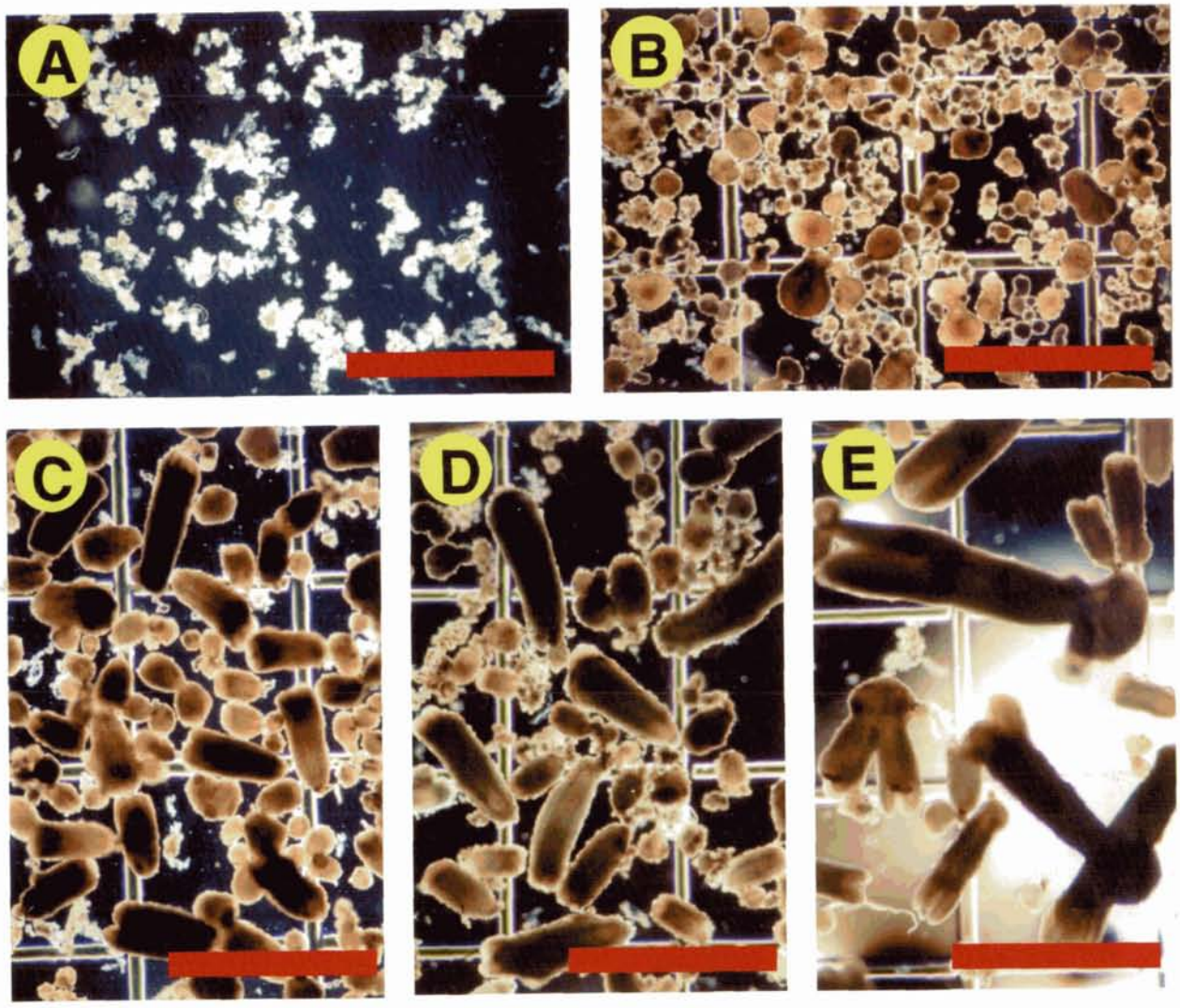



**Figure 4. Morphology of somatic embryos formed after 14 days of culture.**

**A-E** show the somatic embryos formed in MS-$NH_4$, MS+$NH_4$, MSE, MSA and MSQ media, respectively. Bars indicate 1 mm.

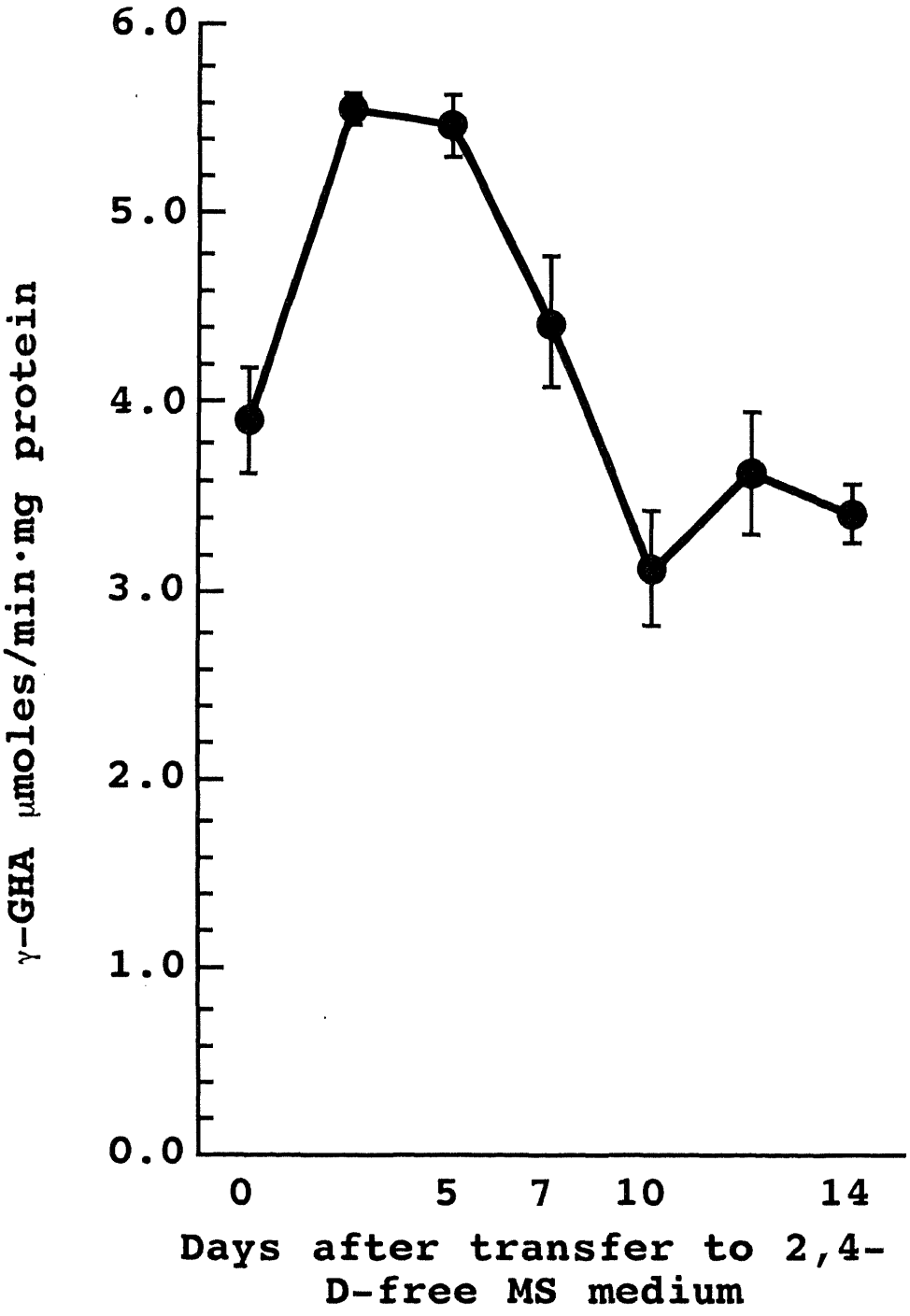



**Figure 5. Time course of the change of GS activity during somatic embryogenesis.**

Somatic embryos formed in 2,4-D-free MS medium were collected at various culture periods and measured the GS activity in the extract. The error bar represents the SE value (n=2).

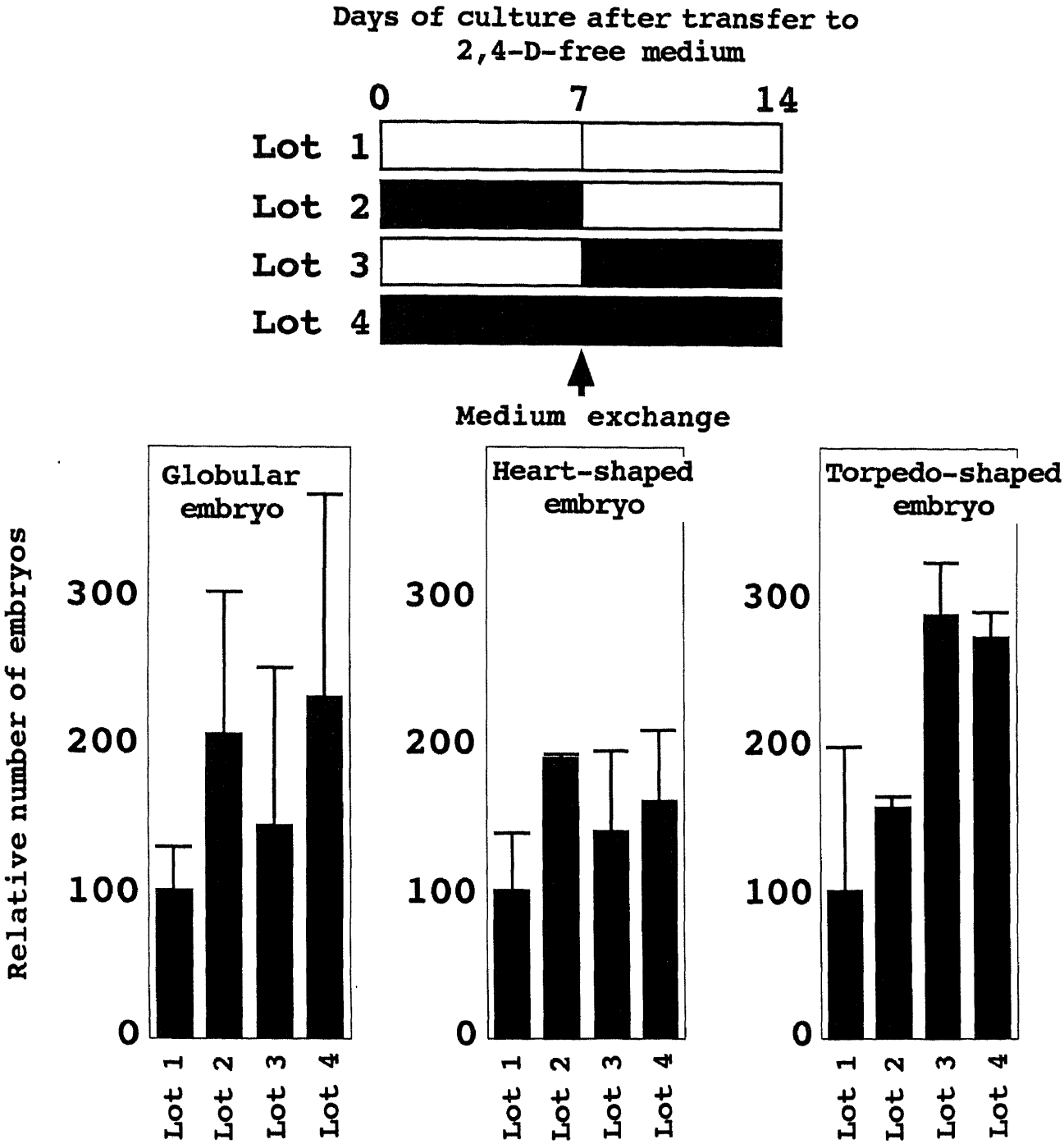



**Figure 6. Effects of Gln at different developmental stages on the development of somatic embryos.**

**A**; Experimental design of the supply of Gln. Black bar and white bar shows the supply of Gln (10 mM) or $NH_4Cl$ (10 mM) in culture period, respectively. In all cases, medium was exchanged to a new one at 7 days of culture. **B**; Relative number of somatic embryos formed in each Lot. The number of the embryos in Lot 1 (the number of globular, heart and torpedo-shaped embryos formed were 20.6, 65.0 and 94.0 per ml of culture medium, respectively) is referred to as 100 in each graph. The error bar represents the SE value (n=2).

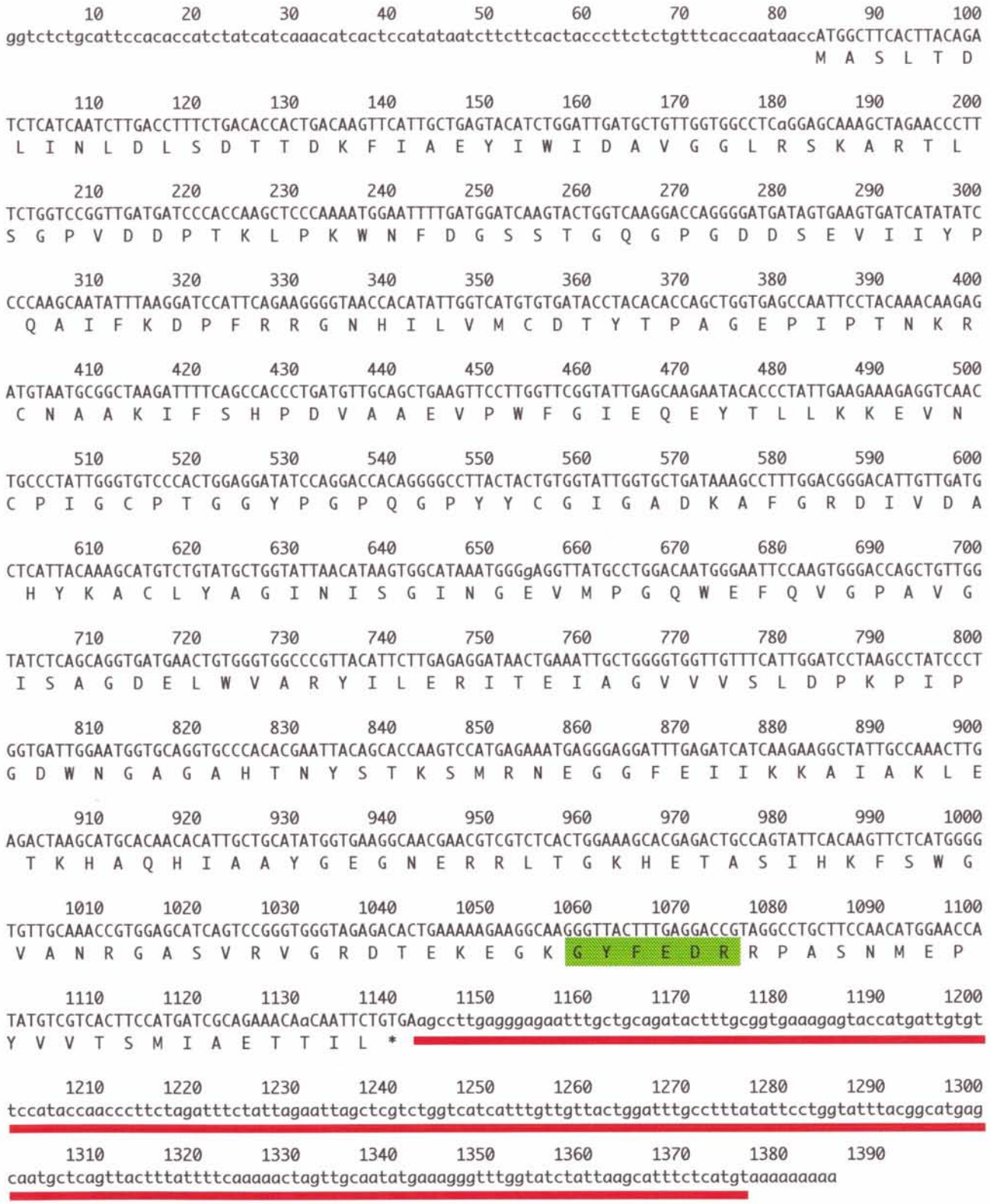



**Figure 7. Nucleotide sequence of cDNA for "CGS102" and the deduced amino acid sequence.**

Specific probe for "CGS102" is underlined. ATP binding site is shown by green box.


**Figure 8. Nucleotide sequence of cDNA for "CGS103" and the deduced amino acid sequence.**

Specific probe for "CGS103" is underlined. ATP binding site is shown by green box.

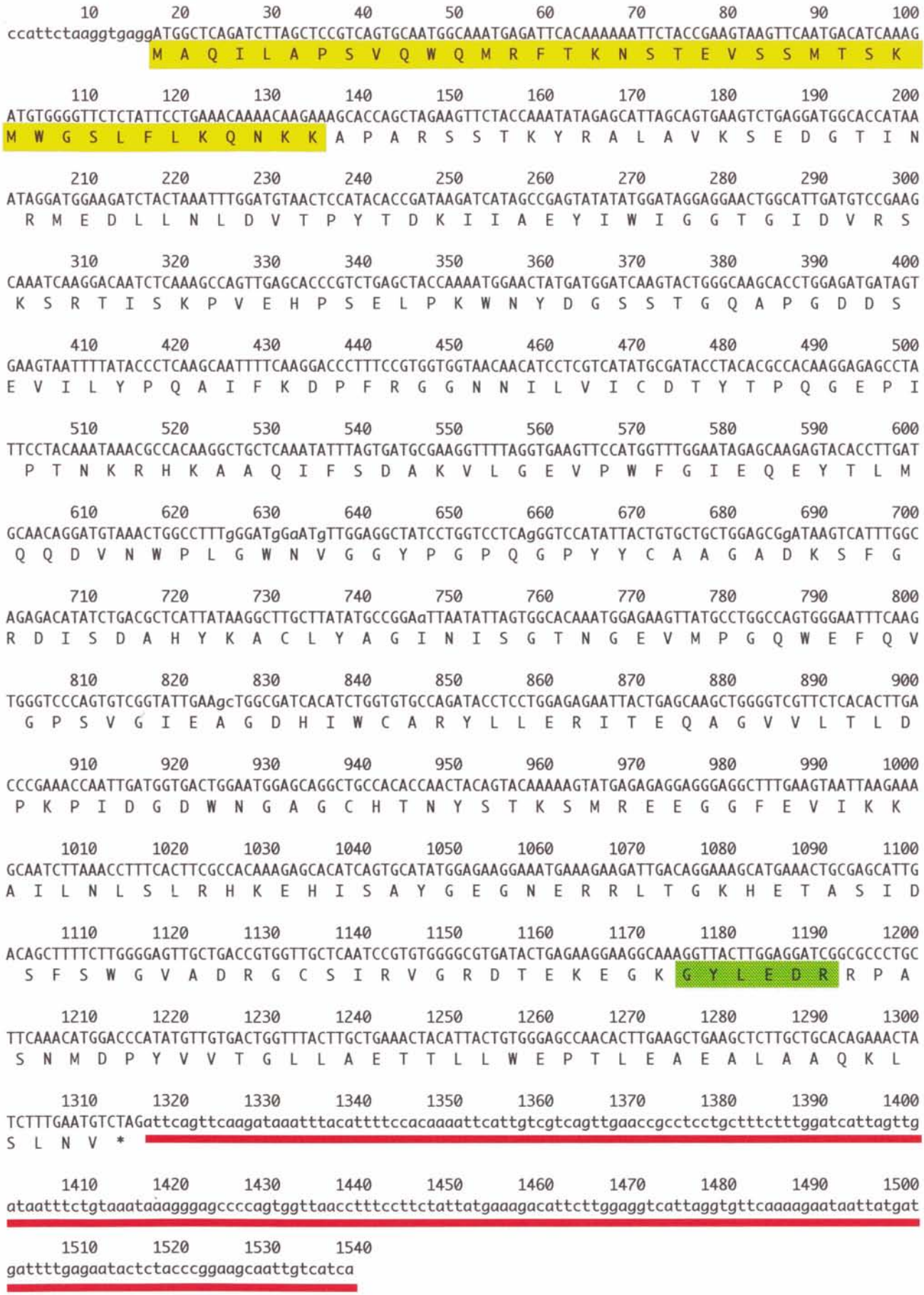



**Figure 9. The nucleotide sequence of cDNA for "CGS201" and deduced amino acid sequence.**

Specific probe for "CGS201" is underlined. Transit peptides and ATP binding site are shown by yellow and green box, respectively.




**Figure 10. Comparison of the amino acid sequences in N terminal region of GS2 from carrot ("CGS201"), *Arabidopsis* and *Nicotiana sylvestris*.**

Comparison of the amino acid sequences of signal peptide region of GS2. Consensus amino acids are shaded. Characters in yellow boxes represent identical amino acids in all the sequences alligned. Amino acidhomologous substitutions are shown by blue box.

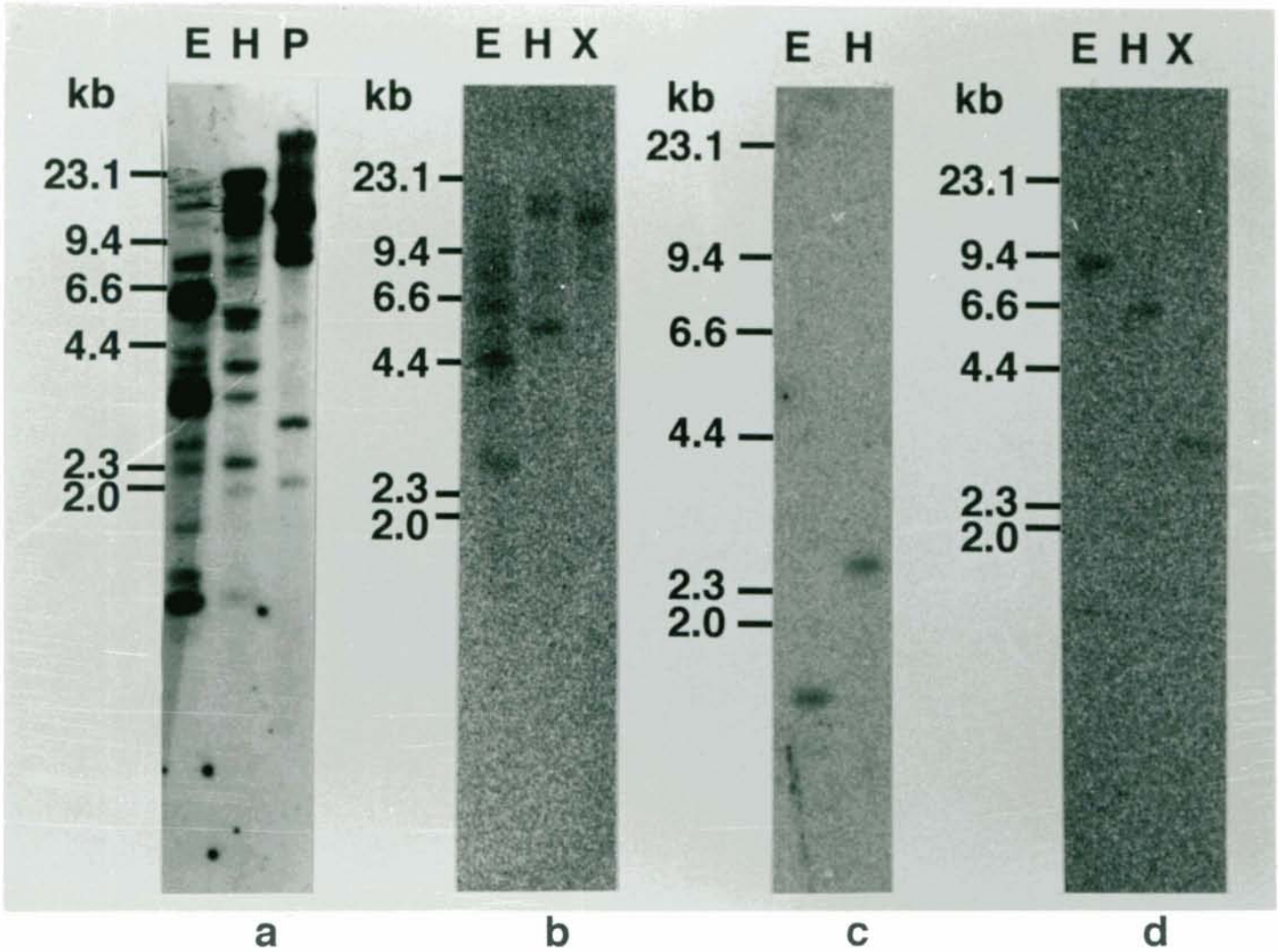



**Figure 11. Analysis by genomic Southern hybridization of the genes for GS in carrot.**

Aliquots of genomic DNA (10 μg) from carrot leaves were digested separately with *Eco*R I, *Hin*d III and *Pst* I (panel a), *Eco*R I, *Hin*d III and *Xba* I (panels b and d) and with *Eco*R I and *Hin*d III (panel c), subjected to electrophoresis on an agarose gel, blotted onto a nylon membrane (Hybond $N^+$) and then allowed to hybridize with probes. The [$^{32}$P]-labeled coding region of "CGS102" cDNA and the 3'-noncoding regions of "CGS102", "CGS103" and "CGS201" cDNAs were used as probes in panels a, b, c and d, respectively. Fragments of *Hin*d III-digested lambda DNA were used as size markers. E, *Eco*R I; H, *Hin*d III; P, *Pst* I; X, *Xba* I.

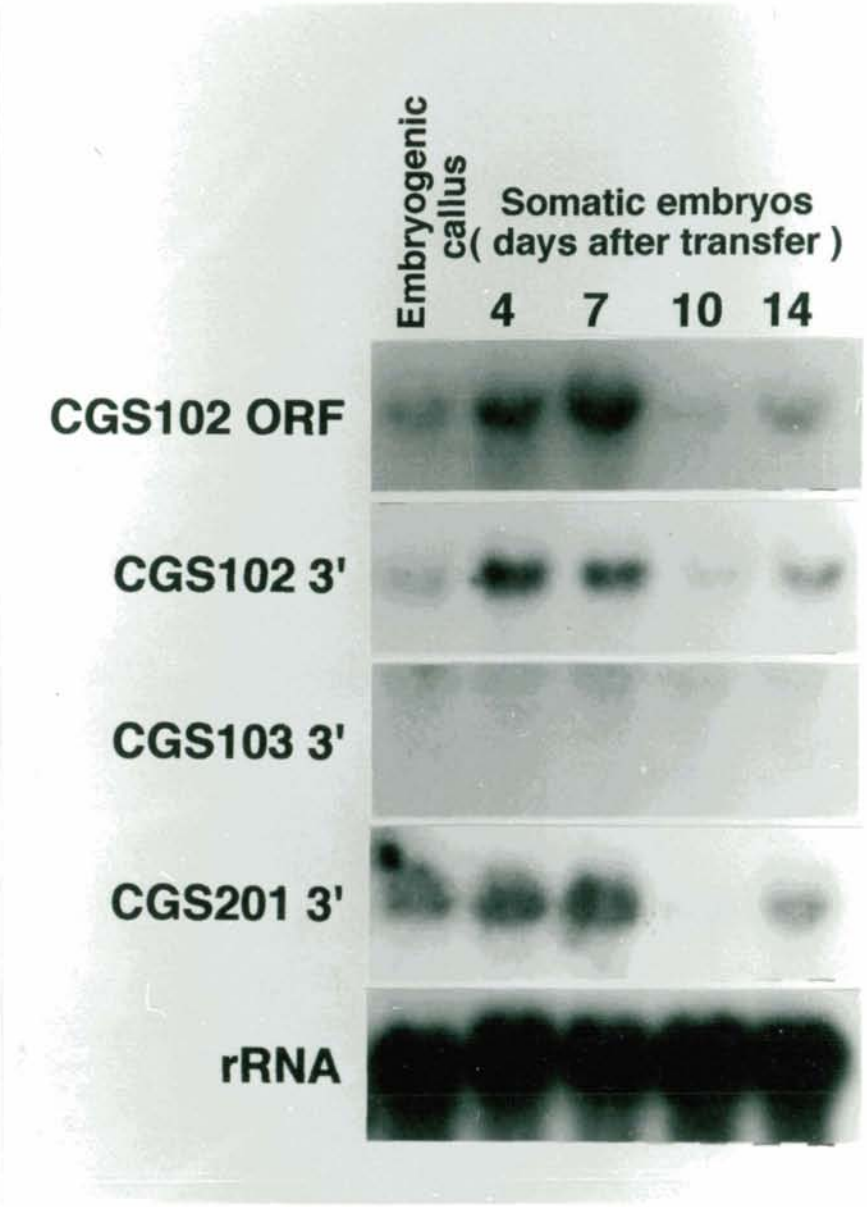



**Figure 12. Accumulation of transcripts of GS isoform genes during somatic embryogenesis.**

Total RNA (20 μg) from carrot somatic embryos prepared 5, 7, 10 and 14 days after transfer to somatic embryo-inducing medium was subjected to electrophoresis on an agarose gel, blotted onto a nylon membrane (GeneScreen Plus) and allowed to hybridize with the probes. The [$^{32}$P]-labeled probes of the coding region of "CGS102" cDNA, the 3'-noncoding regions of "CGS102", "CGS103" and "CGS201" cDNAs and 18S ribosomal RNA were used.

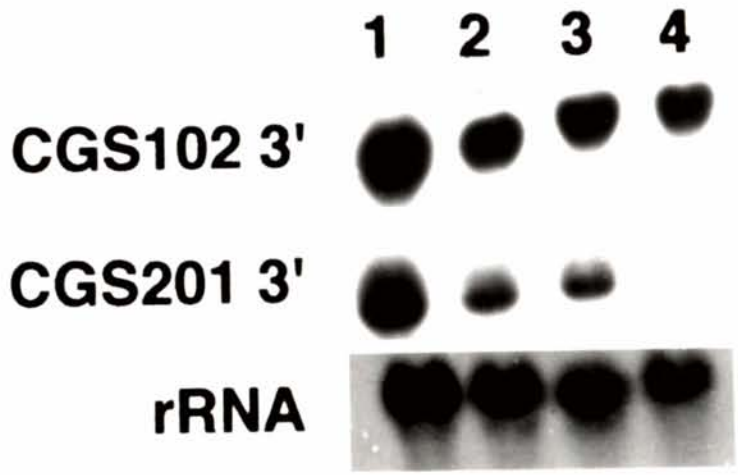



**Figure 13. The effects of exogenously applied nitrogenous compounds on the expression of transcripts of GS isoform genes.**

Total RNA (20 μg) from somatic embryos cultured for 7 days (lanes 1 and 2) or 14 days (lanes 3 and 4) in MS+$NH_4$ medium (lanes 1 and 3) or MSQ medium (lanes 2 and 4), was subjected to electrophoresis on an agarose gel, blotted onto a nylon membrane (GeneScreen Plus) and allowed to hybridize with probes. The [$^{32}$P]-labeled probes of the 3'-noncoding regions of "CGS102" cDNA and "CGS201" cDNA and 18S ribosomal RNA were used.

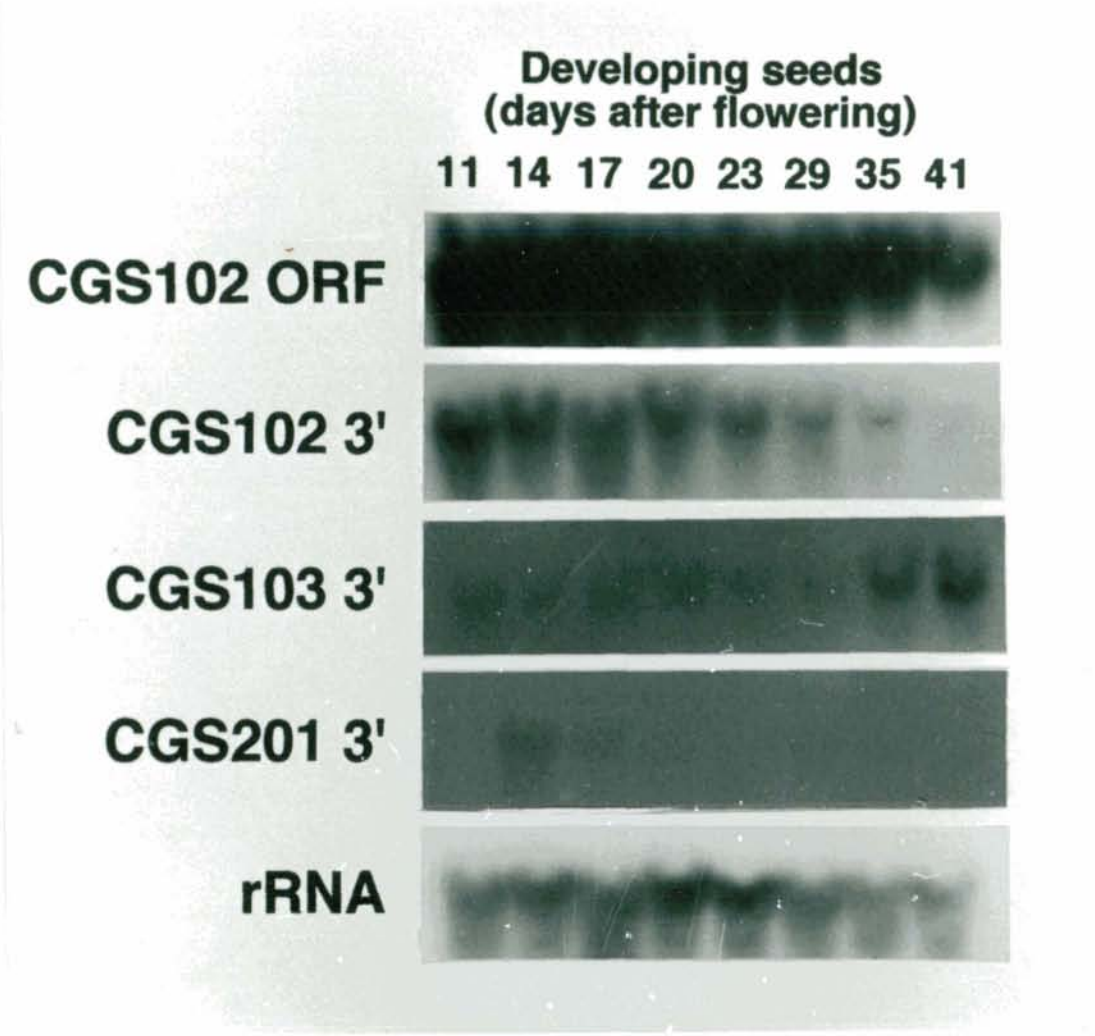



**Figure 14. Accumulation of transcripts of GS isoform genes during seed development.**

Total RNA (20 μg) from carrot immature seeds, collected 11 to 41 days after pollination, was fractionated by electrophoresis on an agarose gel, blotted onto a nylon membrane (GeneScreen Plus) and then allowed to hybridize with probes. The [$^{32}$P]-labeled probes of the coding region of "CGS102" cDNA, the 3'-noncoding regions of "CGS102", "CGS103" and "CGS201" cDNAs and 18S ribosomal RNA were used.

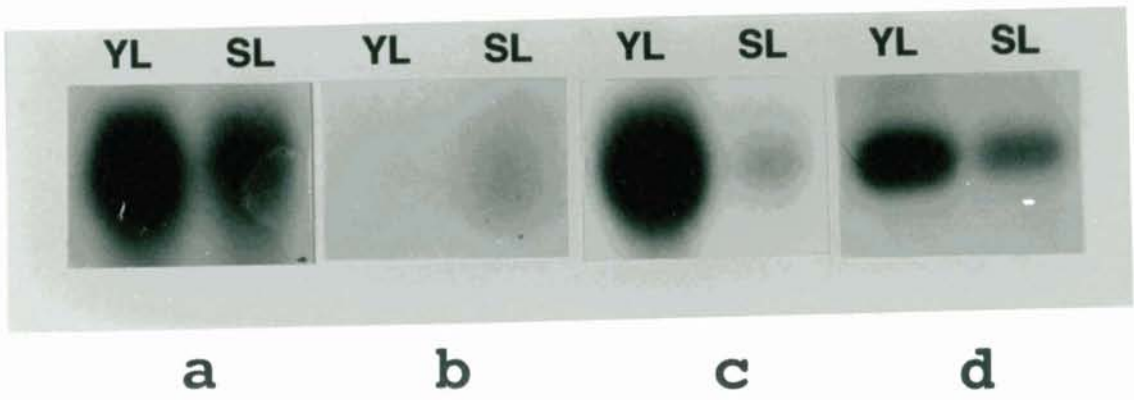
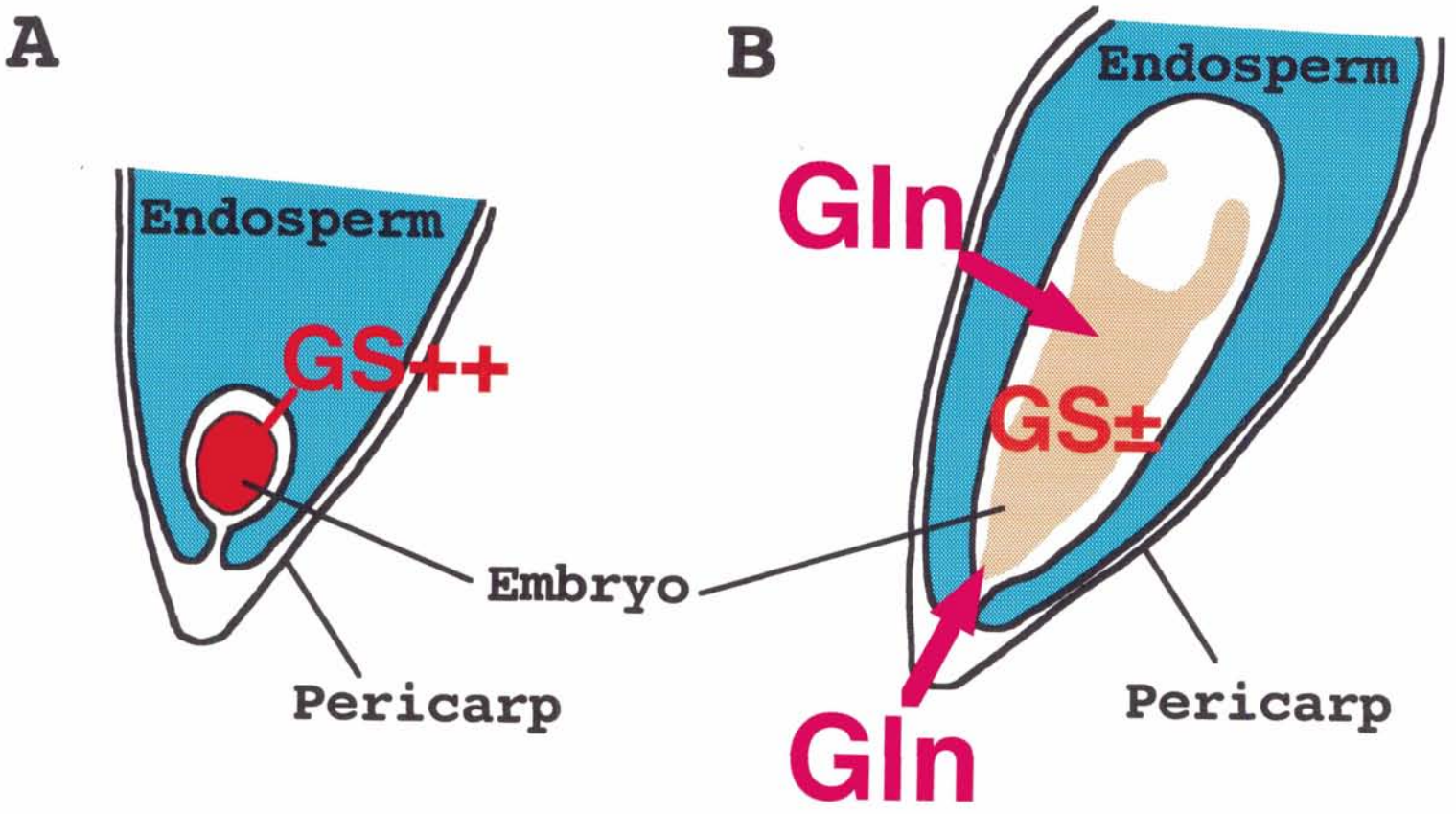



**Figure 15. Accumulation of transcripts of GS isoform genes during natural leaf senescence.**

Total RNA (20 μg) from young green leaves and old yellowish leaves was fractionated by electrophoresis on an agarose gel, blotted onto a nylon membrane (GeneScreen Plus) and allowed to hybridize with probes. The [$^{32}$P]-labeled probes of the 3'-noncoding regions of "CGS102", "CGS103" and "CGS201" cDNAs, and 18S ribosomal RNA were used in panels a, b, c and d, respectively. YL, Young green leaves (fully expanded); SL, Senesced leaves.




(The embryo can utilize some reduced nitorogenous compounds including $NH_4^+$)

(The embryo can utilize Gln supplied from maternal tissues, but not $NH_4^+$)

**Figure 16. Schematic model for nitrogen metabolism during zygotic embryogenesis in carrot.**

**A.** A model for the early stage of zygotic embryogenesis. **B.** A model for the late stage of zygotic embryogenesis.

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