Chloroplast and Cytosolic Glutamine Synthetase Are Encoded by Homologous Nuclear Genes Which Are Differentially Expressed in Vivo*

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We have shown that the individual members of the plant gene family for glutamine synthetase (GS) are differentially expressed in vivo, and each encode distinct GS polypeptides which are targeted to different subcellular compartments (chloroplast or cytosol). At the polypeptide level, chloroplast GS (GS2) and cytosolic GS (GS1 and GS) are distinct and show an organ-specific distribution. We have characterized full length cDNA clones encoding chloroplast or cytosolic GS of pea. In vitro translation products encoded by three different GS cDNA clones, correspond to the mature GS2, GS1, and GS polypeptides present in vivo. pGS185 encodes a precursor to the chloroplast GS2 polypeptide as shown by in vitro chloroplast uptake experiments. The pGS185 translation product is imported into the chloroplast stroma and processed to a polypeptide which corresponds in size and charge to that of mature chloroplast stromal GS2 (44 kDa). The 49 amino terminal amino acids encoded by pGS185 are designated as a chloroplast transit peptide by functionality in vitro, and amino acid homology to other transit peptides. The cytosolic forms of GS (GS1 and GS) are encoded by highly homologous but distinct mRNAs. pGS299 encodes the cytosolic GS1 polypeptide (38 kDa), while pGS341 (Tingey, S. V., Walker, E. L., and Coruzzi, G. M. (1987) EMBO. J. 6, 1–8) encodes a cytosolic GS polypeptide (37 kDa). The homologous nuclear genes for chloroplast and cytosolic GS show different patterns of expression in vivo. GS expression in leaves is modulated by light, at the level of steady state mRNA and protein, while the expression of cytosolic GS is unaffected by light. The light-induced expression of GS2 is due at least in part to a phytochrome mediated response. Nucleotide sequence analysis indicates that chloroplast and cytosolic GS have evolved from a common ancestor and suggest a molecular mechanism for chloroplast evolution.

Higher plant glutamine synthetase (GS, EC 6.3.1.2), the major enzyme which catalyzes the assimilation of ammonia, occurs as distinct isoforms in the chloroplast and cytosol (1). The distinct isoforms of GS function to assimilate ammonia generated by a variety of plant processes. Chloroplast GS, which is present predominantly in leaves, is the major isoform responsible for the reassimilation of photorespiratory ammonia (2). Light has been shown to modulate the level of GS2 activity in etiolated rice seedlings (3). In nitrogen-fixing legumes, there are several distinct isoforms of cytosolic GS (GS1 and GS) (4). In roots, where GS functions to assimilate the ammonia fixed by bacteroids, the levels of certain cytosolic GS isoforms (GS3) are induced concomitantly with nodule development (5).

In the legume Pisum sativum, the subunit polypeptides of chloroplast stromal GS2 and cytosolic GS (GS1 and GS3) are each distinct in size and charge (6). There are at least three different GS mRNAs in pea which are expressed in an organ-specific fashion (6). To determine whether the differential presence of the various GS polypeptides during plant development correlates with differential gene expression, full length cDNA clones encoding the three types of GS polypeptides (GS2, GS1, and GS) have been isolated and characterized. The complete nucleotide sequence of full length GS cDNAs pGS185 and pGS299 are presented. Translation products corresponding to these cDNAs, and a third full length GS cDNA (pGS341) (6), were synthesized in vitro and correlated with the mature GS2, GS1, and GS polypeptides present in vivo. In vitro chloroplast uptake experiments were used to show that the pGS185 translation product is a precursor to chloroplast GS2. The effect of light on the steady state levels of transcripts and polypeptides for chloroplast and cytosolic GS has been examined. The role of the photoreceptor phytochrome in mediating the light response has also been examined. A comparison of the deduced amino acid sequences for chloroplast and cytosolic GS has provided data concerning the molecular evolution of chloroplasts.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions—Seeds of P. sativum (var. "Sparkle") were imbibed and germinated in a Conviron environmental chamber with a day length of 16 h, illumination of 1000 microeinsteins m^-2 s^-1 (1 einstein = 1 mol of photons), at a day/night cycle of 21/18 °C.

1 The abbreviations used are: GS, glutamine synthetase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEpHGE, nonequilibrium pH gradient electrophoresis.
For etiolated samples, pea seeds were imbibed and germinated in the dark, and grown at 22 °C for 7 days in black Lucite boxes within a dark environmental chamber. Light regimes were as follows: 7-day-old etiolated seedlings were irradiated with red light (red fluorescent lamps, General Electric F20T12R) at a fluence of 40 microeinsteins per second per millimeter2 for 4 min or were given a 4 min red flashes followed by 12 min of far-red light (Westlake, FRF700) at the same fluence. After light treatments, plants were returned to the dark for 24 h before RNA extraction. Alternatively, etiolated seedlings were kept in continuous white light for 72 h.

**Protein Isolation and Characterization**—Total soluble protein was extracted from leaves of dark or light grown pea seedlings (see above for growth conditions) as described previously (6). Polypeptides were separated by SDS-PAGE according to Laemmli (7), in gels containing a 7.5–15% gradient of polyacrylamide. Proteins were prepared for non-equilibration pH gradient electrophoresis (NEPHGE) according to O’Farrell (8). Polyacrylamide gels were processed for Western blot analysis according to Towbin (9), using antibody raised against Nicotiana tabacum GS (10).

**Isolation and DNA Sequence Analysis of Full Length GS cDNA Clones**—DNA fragments from the 5' end of the partial GS cDNA clones pGS197 and pGS234 were used to screen Agt11 pea leaf and root cDNA libraries (6). pGS185 and pGS299 are full length cDNA clones which correspond to the partial cDNA clones pGS197 and pGS234, respectively (6). Restriction endonuclease fragments of the GS cDNA inserts were excised from Agt11, subcloned into M13 vectors mp18 and mp19 (11), and the nucleotide sequence of both DNA strands determined by the dideoxy method (12).

**Vitrification/Transcription and Chloroplast Uptake Experiments**—The cDNA inserts of the full length clones pGS185, pGS299, and pGS341 (6) were subcloned into the EcoRI site of pTZ18U (Genescribe, U.S. Biochemicals) and transcripts generated in vitro using purified T7 polymerase (13). A 50-μl transcription reaction mixture contained 5 μg of SnII linearized plasmid DNA, 60 units of T7 RNA polymerase, 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 60 units of human placental RNase inhibitor (Boehringer Mannheim), and 0.5 mM each of ATP, CTP, UTP, and 0.24 mM 7mGpppG. After incubation for 5 min at 37 °C, 0.5 mM GTP was added and incubated for an additional 15 min at 37 °C. Aliquots of the transcription mixture were translated in vitro in a wheat germ translation system, in the presence of [35S]methionine, and displayed on 7.5–15% SDS-PAGE. Alternatively, in vitro translation products were incubated with isolated intact chloroplasts (14) in an in vitro chloroplast uptake reaction (15, 16). Following in vitro uptake, chloroplasts were re-isolated and one-half was treated with ethylenediamine (100 μg/ml) for 15 min at 25 °C. In tissue translation products were displayed on 7.5–15% SDS-PAGE, transferred to nitrocellulose filters, treated with ENHANCE (Du Pont-New England Nuclear) and exposed to x-ray film at −80 °C.

**RESULTS**

**Chloroplast GS Polypeptide Accumulates during Greening of Etiolated Seedlings**—It has been shown that GS2 activity in rice leaves increases upon illumination of etiolated seedlings (3). We have examined the GS polypeptides present in etiolated or greened pea seedlings to examine the effect of light on the steady state level of the chloroplast and cytosolic GS polypeptides. Leaf soluble protein was extracted from pea plants grown 7 days in the dark (D), and from etiolated plants which were transferred to continuous white light for 72 h (L). Protein was separated by SDS-PAGE and either stained with Coomassie Blue (Fig. 1, lanes 1 and 2), or transferred to nitrocellulose and GS polypeptides detected by Western blot analysis with anti-GS, IgG (10) (Fig. 1, lanes 3 and 4).

![Fig. 1. Effect of light on GS polypeptides present in leaves. Leaf soluble protein from dark (D) (lanes 2 and 4) or light (L) (lanes 1 and 3) grown peas was separated on SDS-PAGE and stained with Coomassie Blue (lanes 1 and 2), or transferred to nitrocellulose and GS polypeptides detected by Western blot analysis with anti-GS, IgG (10) (lanes 3 and 4). Sizes of the GS polypeptides are indicated on the right in kilodaltons. Sizes of molecular mass markers are indicated on the left in kilodaltons.](https://www.jbc.org/content/274/46/28138.full.html)
Glutamine Synthetase Gene Family of Pea

Nucleotide sequence of pea GS cDNA clones for chloroplast GS2 (pGS299) and cytosolic GS1 (pGS185).

Pea Glutamine Synthetase Gene Family

were incubated with intact chloroplasts in an in vitro chloroplast uptake system (15, 16). Following uptake, chloroplasts were reisolated in the absence (Fig. 3, lanes 3 and 5), or presence (Fig. 3, lanes 4 and 6) of protease, the soluble proteins displayed on SDS-PAGE, and in the in vitro translation products detected by autoradiography. The in vitro GS polypeptide encoded by pGS185 is processed by the isolated chloroplasts in vitro, to a mature size of 44 kDa (Fig. 3, lane 3). The processed 44-kDa GS polypeptide is imported into the chloroplasts, as evidenced by its resistance to protease treatment following uptake (Fig. 3, lane 4). This size is in excellent agreement with that of mature chloroplast stromal GS2 (6).

In contrast, the 38-kDa cytosolic GS is not processed by the isolated chloroplasts in vitro (Fig. 3, lane 5), and the polypeptide is not imported into chloroplasts as evidenced by its digestion during protease treatment of the chloroplasts following uptake (Fig. 3, lane 6).

These results show that pGS185 encodes a precursor to chloroplast stromal GS2, and establish the presence of a chloroplast "transit" peptide. The amino acid sequence encoded by pGS185 extends 58 amino acids further at the amino terminus than the pGS299 sequence (Fig. 2). The first 49 amino acids of pGS299 are shown below, where identity with amino acids denoted above indicates identity with amino acids denoted in the one-letter code. Nucleotide sequence and deduced amino acid sequence of pGS299 denoted in the one-letter code, where + indicates identity with amino acids denoted above, and * indicates identity with amino acids denoted below for pGS185. Arrowhead indicates the proposed processing site for chloroplast GS1, transit peptide.
transit peptide required for import into chloroplasts. The exact processing site of the chloroplast transit peptide cannot be unequivocally identified because the sequence of the amino terminus of the mature chloroplast GS polypeptide is not known. However, the assignment of the GS2 transit peptide processing site to amino acid residue 49 is based on the following criteria: 1) the relative difference in size of the pGS185 primary translation product and the mature processed form of chloroplast stromal GS, and 2) amino acid homology of the putative pGS185 transit peptide processing site to amino acid residue 49 is based on the pGS185 primary translation product and the mature process by their relative migration on two-dimensional NEPHGE gels (8). cDNA inserts cloned into the EcoRI site of pTZ18U were used as templates in an in vitro transcription reaction. Transcripts corresponding to each clone were translated in vitro in the presence of [35S]methionine, mixed with leaf soluble protein, separated by two-dimensional NEPHGE, transferred to nitrocellulose, and exposed to x-ray film (Fig. 3, compare panels A and C). The other charge variants of the 44 and 37 kDa GS polypeptides seen in vivo may be the products of highly homologous but distinct GS genes, or they may represent post-translational modifications of the primary translation products.

GS2 mRNA Levels Are Induced by Light—We have previously shown that mRNA for GS2 is expressed at high levels in leaves and is below detection in roots (6). In contrast, the mRNAs for cytosolic GS are expressed at constitutive levels in leaves and roots and are induced in nitrogen-fixing root nodules (6). Northern blot analysis of leaf RNA was used to determine whether light affects the steady state level of the mRNAs encoding chloroplast GS2, or cytosolic GS (Fig. 5). A DNA probe specific for GS2 mRNA (5’ end of pGS185 encoding the GS2 transit peptide), was hybridized to total RNA isolated from leaves of pea plants exposed to various light regimes (Fig. 5, lanes 1-4). GS2 mRNA (1.5 kb) is present at low levels in leaves of etiolated pea plants (D) (Fig. 5, lane 1), and at high levels when the etiolated plants are exposed to continuous white light for 72 h (L) (Fig. 5, lane 4). In contrast, a replicate Northern blot hybridized with a DNA probe encoding cytosolic GS1 (pGS299), reveals that the mRNA for
of total RNA separated on 1.5% agarose, transferred to nitrocellulose, and hybridized to DNA probes for pGS185 transit peptide (lanes 1-4) or pGS299 (lanes 5-8). Plants were grown in continuous dark (D) (lanes 1 and 5) and subsequently treated with: a pulse of red light (R) (lanes 2 and 6) followed by a pulse of far-red light (R/FR) (lanes 3 and 7), or continuous white light for 72 h (L) (lanes 4 and 8). Sizes of GS mRNAs are indicated in kilobases (kb), as estimated by migration relative to denatured DNA markers.

cytosolic GS (1.4 kilobases) is present at equal levels in leaves of dark (D) or light (L) grown pea plants (Fig. 5, lanes 5 and 8).

To determine if the photoreceptor phytochrome is involved in this light-induced accumulation of GS2 mRNA, transcripts for GS were examined in etiolated plants which were exposed to light regimes that activate (red light, R) or inactivate (far-red light, FR) the chromophore phytochrome (21). Northern blots of total RNA from etiolated plants treated with a red light pulse (R), and red/far-red (R/FR)-treated plants (see “Experimental Procedures”) were probed with the pGS185 transit peptide probe (Fig. 5, lanes 2 and 3). Levels of GS2 mRNA increase with red-light treatment of etiolated plants (R) (Fig. 5, lane 2), and this effect is reversed by a subsequent pulse of far-red light (R/FR) (Fig. 5, lane 3). In contrast, a replicate Northern blot probed with pGS299 reveals that the level of mRNA for cytosolic GS is unaffected by the red and far-red light treatments of etiolated pea plants (Fig. 5, lanes 6 and 7). These results show that the light-induction of GS2 mRNA expression is mediated, at least in part, through the chromophore phytochrome.

**DISCUSSION**

In eukaryotes, there are many instances in which an enzyme is located within more than one subcellular compartment. In cases where the molecular basis for the different forms of an enzyme have been elucidated, several different mechanisms have been revealed. For the yeast enzyme sucrose invertase, differential transcriptional initiation of a single gene is responsible for the production of cytosolic and secreted forms of the enzyme (22). Similarly, several enzymes which occur in the mitochondria and cytosol of yeast are encoded by a single nuclear gene which is subject to differential transcription and/or differential translation of the mRNA (23). In contrast, the mitochondrial and cytosolic forms of yeast citrate synthetase (24) and alcohol dehydrogenase (25) are encoded by homologous but distinct nuclear genes.

In plants, many enzymes involved in carbohydrate or nitrogen metabolism are located in both the chloroplast and the cytosol (26). It has been shown that the chloroplast and cytosolic forms of the glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase are encoded by divergent nuclear genes which have been proposed to originate from different ancestral lineages (27, 28). We have examined the structure and expression of the genes encoding the chloroplast and cytosolic forms of a nitrogen metabolic enzyme, glutamine synthetase, in the legume *P. sativum*.

Characterization of full length GS cDNA clones has shown that chloroplast and cytosolic GS are encoded by highly homologous but distinct nuclear genes. The cDNA for chloroplast GS2 encodes a polypeptide which is 3–4 kDa larger than the mature chloroplast stromal GSz. This precursor polypeptide is taken up by isolated intact chloroplasts *in vitro*, and the processed product is identical in size and charge to that of mature chloroplast stromal GSz (44 kDa). The 3–4 kDa of peptide cleaved upon import into chloroplasts shares sequence homology with the consensus sequence deduced for the transit peptides of other nuclear encoded chloroplast proteins (20). The processed GS2 polypeptide corresponds to the more basic mature GSz polypeptide in leaves, detected by Western blot analysis on two-dimensional NEpHGE gels. Southern blot analysis (6) and genomic clone analysis3 reveals that there is one gene for GSz in pea. Therefore, the more acidic form of GSz detected on two-dimensional NEpHGE gels may correspond to a GS2 polypeptide which is not fully reduced or to a post-translationally modified version of the pGS185 primary translation product.

The cytosolic forms of GS are encoded by cDNAs which are highly homologous (pGS299 and pGS341) (6). Analysis of *in vitro* generated translation products corresponding to these cDNAs shows that pGS299 encodes the GSz polypeptide (38 kDa) which is present predominantly in roots, whereas pGS341 encodes the most basic GSz polypeptide (37 kDa) which is most abundant in nodules (6). Southern blot analysis (6) and genomic clone analysis3 reveal that there are two nearly identical GSz genes in pea. Therefore, the two other charge variants of the GSz polypeptides seen on two-dimensional NEpHGE gels may be the product of homologous genes and/or may be post-translationally modifications of the pGS341 gene product.

Previous enzyme activity studies showed that the level of chloroplast GSz activity increased during the greening of etiolated rice seedlings (3). We have shown that steady state level of the chloroplast GSz polypeptide is induced upon exposure to light, whereas the levels of cytosolic GS polypeptides are unaffected by the light regimes. Furthermore, we have determined that there is a concomitant increase in the steady state level of GSz mRNA upon greening of etiolated pea seedlings. There are two possible explanations for the increase in GSz mRNA levels in response to light. Phytochrome has been shown to mediate the expression of other nuclear genes encoding chloroplast proteins (i.e. rbcS, Cab) (29, 30). Alternatively, since chloroplast GSz functions to reaminate photoreceptor-ammonia (2), photoreceptor ammonia produced concomitantly with light-induced chloroplast development may be an effector involved in the increased expression of GSz mRNA in the light. Metabolic induction of gene expression has been demonstrated for another nitrogen metabolic gene, nitrate reductase (31). The studies presented here have shown that the light-induced level of GSz mRNA is due at least in part to a phytochrome-mediated response. However, maximal expression of GSz in leaves may also require continuous white light and/or metabolic induction. The light-induced increase of steady state GSz mRNA levels in pea leaves may reflect increased tran-

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3 E. L. Walker and G. M. Coruzzi, unpublished results.
Amino acid homology of chloroplast GS2 to other GS polypeptides

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The high sequence homology for plant cytosolic glyceraldehyde-3-phosphate dehydrogenase (GapA/B) shares higher homology with glyceraldehyde-3-phosphate dehydrogenase, the chloroplast forms of glyceraldehyde-3-phosphate dehydrogenase, and the full length of the GS sequence. In contrast, the overall homology of chloroplast GS2 with GS of the prokaryotes (Anabaena and *E. coli*) is poor (13–6%), the conserved amino acids being limited to a small number of residues in the region proposed to be the active site of *E. coli* GS (36).

In accordance with the endosymbiotic theory, one molecular mechanism to account for the small size of the chloroplast genome is the loss of plastid genes which have been replaced by genes already present in the nucleus (37). This model proposes that genes for plastid isoforms have evolved by duplication of nuclear genes and subsequent specialization of each locus (37). The high sequence homology for plant cytosolic and chloroplast GS suggests that they are derived from a common ancestor, providing molecular evidence which supports the above mechanism of chloroplast evolution (28).

The sequence data for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (27, 28) provides molecular evidence which supports a different mechanism of chloroplast evolution. In the case of glyceraldehyde-3-phosphate dehydrogenase, the chloroplast forms of glyceraldehyde-3-phosphate dehydrogenase (GapA/B) share higher homology with glyceraldehyde-3-phosphate dehydrogenase of thermophilic bacteria than with the higher plant cytosolic form of the enzyme (GapC) (27, 28). These results support a molecular mechanism which predicts that genes of the endosymbiont were transferred to the nucleus during the evolution of the plant cell (37).

The two alternative molecular mechanisms of chloroplast evolution compatible with the endosymbiont hypothesis (i.e. loss of chloroplast genes and functional replacement by nuclear genes, or transfer of chloroplast genes to the nucleus) are not mutually exclusive. In fact, the sequence data for the chloroplast and cytosolic forms of GS and glyceraldehyde-3-phosphate dehydrogenase, indicate that these two different molecular mechanisms have both contributed to chloroplast evolution.

Multigene families in eukaryotes may be categorized in two classes. In one case, multigene families serve to accommodate the need for large amounts of a particular gene product (i.e. ribosomal RNAs) (38). In other cases, multigene families exist when similar but distinct gene products are expressed at different times in development or in response to different factors (i.e. globin) (38). The well studied plant multigene families, rbcS and Cab, fall into the first category. For rbcS and Cab where there is a requirement for large amounts of the gene products, the individual genes encode essentially identical polypeptides and the genes are regulated by identical factors (i.e. light) (39, 40). In contrast, the GS gene family is an example of the second type of gene family, where distinct forms are differentially expressed. The individual GS genes each show distinct patterns of expression during plant development, and each encodes unique gene products which are targeted to separate subcellular locations. We have shown that chloroplast GS2 is expressed predominantly in leaves in a light-dependent fashion, whereas the cytosolic forms of GS (GS1 and GS2) are expressed predominantly in roots and nitrogen-fixing root nodules (6). Our continuing studies are aimed at characterizing the nuclear genes corresponding to the three GS cDNAs that we have identified and to define cis-acting DNA elements responsible for the differential expression of individual members of the GS gene family in vivo.

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