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CHARACTERIZATION OF A cDNA CLONE FOR BARLEY LEAF GLUTAMINE SYNTHETASE

by

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A barley cDNA clone (1182 bp) encoding chloroplastic glutamine synthetase was isolated with a heterologous cDNA probe of the gene specifying the enzyme from alfalfa. The clone, named pGS8, was found in a λgt11 cDNA library prepared from dark grown barley leaves even though the chloroplastic glutamine synthetase is absent from such leaves. In agreement therewith the clone hybridized in Northern blot analyses with a 1.7 kb mRNA species present the in poly A$^+$ mRNA fraction of both dark grown and greened primary leaves of barley.

The nucleotide sequence of the barley clone reveals 75% identity to the Phaseolus vulgaris and Pisum sativum clones encoding chloroplastic glutamine synthetase, while only 69% identity is observed in comparisons with the clones specifying the cytosolic isozymes. At the amino acid level 85% identity is found between the deduced barley glutamine synthetase sequence and that of the corresponding chloroplastic isoenzymes from bean and pea. The chloroplastic glutamine synthetases contain conserved cysteins in the putative ATP and substrate binding sites. In the cytosolic forms these positions are occupied by alanine residues.

1. INTRODUCTION

In the nitrogen metabolism of higher plants, glutamine synthetase (EC 6.3.1.2) effects the primary assimilation of ammonia produced by nitrate reduction, by amino acid catabolism and by photorespiration. In 1979 MANN et al. (13), working with barley, discovered the presence of two glutamine synthetase isoenzymes in the leaves. The two isoforms, named glutamine synthetases 1 and 2, were found in different compartments of the cell, glutamine synthetase 1 being in the cytosol and glutamine synthetase 2 in the chloroplast. Testing both etiolated and illuminated barley seedlings, MANN et al. (13)

Abbreviations: bp = basepairs; kb = kilobasepairs; kDa = kilodalton; SDS = sodium dodecyl sulphate.

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also found that no glutamine synthetase 2 activity could be detected in dark grown plants.

Subsequently, many papers reported the presence of two glutamine synthetase isoforms in higher plant leaves. Clones encoding glutamine synthetase 1 and clones specifying glutamine synthetase 2 have recently been obtained and characterized (6, 10, 21, 22, 23, 24).

So far, no amino acid sequence for a monocotyledonous glutamine synthetase has been reported. The present paper presents the isolation and characterization of a cDNA fragment containing the coding region for the mature form of a barley glutamine synthetase.

2. MATERIALS AND METHODS

2.1. Plant material

Kernels of barley (Hordeum vulgare L.) were germinated in water-moistened vermiculite in the dark. Six-day-old seedlings were used for RNA extraction. Some plantlets were sampled without further treatment (etiolated) whereas other plantlets were illuminated for 12 h before the actual sampling (green).

2.2. RNA isolation and Northern blot analysis

Total RNA was extracted from barley leaves, both from etiolated (dark RNA) and green (light RNA) seedlings, using extraction by the guanidinium chloride method (11). Poly A^+ enriched RNA was isolated by Hybond™-mAP (Amersham) according to the manufacturer's specifications. For Northern blot analysis RNA was denatured at 60 °C for 10 min in gel running buffer (0.04 M morpholinopropane sulfonic acid pH 7.0, 10 mM sodium acetate, 1 mM EDTA pH 8.0) with formamide and formaldehyde (12), electrophoretically separated in either 1.2% or 1.4% agarose gels containing formaldehyde and transferred to nylon filters (Amersham). Filters were hybridized at 42 °C for 36 h in 50% formamide, 1x Denhardt's solution (0.2 g/l each of Ficoll, polyvinylpyrrolidone 4000 and bovine serum albumin), 5x SSC (0.75 M NaCl, 0.075 M sodium citrate pH 7.0), 20 mM $NaPO_4$ pH 6.5, 10% dextran sulfate, 100 µg/ml denatured salmon sperm DNA, to a 1.2 kb alfalfa DNA probe labelled with α-^{32}P (specific activity 10^8 cpm/µg). Filters were washed in 2x SSC, 0.1% SDS at room temperature, then 30′ in 0.1x SSC, 0.1% SDS at 42 °C (heterologous probe) or 60′ in 0.1x SSC, 0.1% SDS at 48 °C (homologous probe), and exposed to X-ray film at -70 °C with intensifying screen. The molecular weight markers were rRNAs according to POULSEN (16).

2.3. Plasmids, cDNA libraries and isolation of GS cDNA

All our clonings were done in pUC18 plasmids (25). The plasmid pGS100, containing a 1.6 kb glutamine synthetase cDNA from alfalfa (3), was a gift from H.M. GOODMAN (Dept. of Molecular Biology and Dept. of Genetics, Harvard Medical School, Massachusetts, USA). The two λgt11 barley cDNA libraries, kindly donated by K. APEL (Botanisches Institut, Christian-Albrechts Universität, Kiel, FRG), had been constructed from poly A' enriched RNA obtained from 5-day-old seedlings, grown, respectively, in complete darkness and in darkness followed by 18 h of light. The insert DNA was in the EcoRI site of the λgt11 vectors.

2.4. DNA preparations

Large scale plasmid DNA preparations were made using the alkaline lysis method (12) followed by equilibrium density gradient centrifugation with CsCl (12). Small scale plasmid DNA preparations were performed according to HATTORI and SAKAKI (7). Preparation of lambda DNA was done on a small scale according to KASLOW (8).

2.5. Labelling of probes

DNA fragments to be used as probes were cut out of the plasmids, electrophoresed on agarose gels, electroeluted and subsequently labelled with α-[^{32}P]-deoxynucleotides according to the random primed DNA labelling technique (5).

2.6. Screening of the cDNA libraries

The cDNA libraries were screened using the labelled 1.2 kb Bgl II-Stu I fragment of the pGS100 plasmid (3). Plaque lifts were made

according to BENTON and DAVIS (1) and heterologous hybridization was performed at 60 °C for 18 h in 10x Denhardt's solution, 4x TES (0.6 M NaCl, 8 mM EDTA, 120 mM Tris-HCl pH 8.0), 0.1% SDS, 100 µg/ml of denatured DNA and salmon sperm to the α-[^{32}P]-labelled Bgl II-Stu I fragment from pGS100 (specific activity of 10^8 cpm/µg). Filters were washed at 48 °C for 90′ in 1x SSC and exposed to X-ray film with an intensifying screen at -70 °C. The inserts of the positive clones were excised from lambda and subcloned into pUC18.

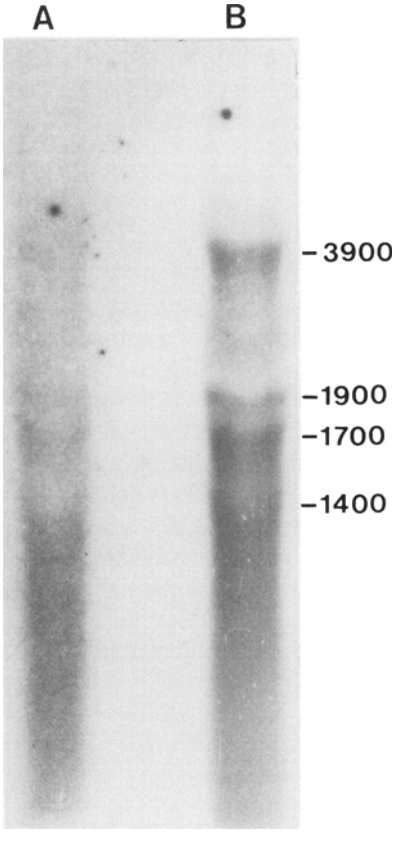

Figure 1. Northern analysis of RNA from etiolated (A) and green (B) barley leaves. 75 µg/lane of total RNA were run in a 1.2% agarose/formaldehyde gel. The probe was the 1.2 kb Bgl II-Stu I fragment from alfalfa glutamine synthetase cDNA. RNA size is expressed in basepairs (bp). The 3900 and 1900 bp bands represent rRNA (16); the 1700 and 1400 bp bands constitute barley glutamine synthetase mRNAs.

2.7. Nucleotide sequence analysis

One of the positive clones, selected from the "dark" λgt11 library, contained an insert of approximately 1200 bp which was subcloned into pUC18 and the resulting plasmid named pGS8. The insert of pGS8 was sequenced by the dideoxy chain termination method with sequence of both strands determined for more than 55% of their lengths. The sequencing was done on double stranded plasmid DNA (2, 7) using chemically modified T7 DNA polymerase (20). We introduced a modification to the above method by using 3.5 µg/µg template DNA of E. coli single strand binding protein (19) in the labelling mix in order to overcome band compression in sequencing gels. Analysis of sequencing data was done with the Microgenie program developed by QUEEN and KORN (17).

3. RESULTS

Northern blot analysis was performed using total RNA, from both etiolated and green leaves, and a probe consisting of the 1.2 kb Bgl II-Stu I fragment from the pGS100 plasmid which contains a cDNA sequence coding for a cytosolic alfalfa glutamine synthetase (3). The result is shown in Figure 1, where 4 bands are visible in each lane. The two upper bands (3900 and 1900 bp) correspond to rRNA (16) whereas the 1700 and 1400 bp bands represent the glutamine synthetase 2 and glutamine synthetase 1 mRNA, respectively. Since our probe was heterologous we used a low stringency in this experiment; this might explain the fact that our probe (i.e. alfalfa GS1) can recognize both glutamine synthetase 1 and 2 mRNA.

By screening the λgt11 cDNA libraries, we were able to isolate 5 positive clones from the "dark" library and 7 from the "light" library, the latter containing only small sized inserts.

One of the "dark" clones (1200 bp) was chosen for further characterization. It was subcloned into pUC18 and named pGS8. On the basis of our Northern blot analysis and of the sizes of glutamine synthetase mRNAs from dicotyledonous plants, it was not expected to be of full-length. The insert of pGS8 was used, under high stringency conditions, as a probe in a Northern blot analysis with poly A$^+$ enriched

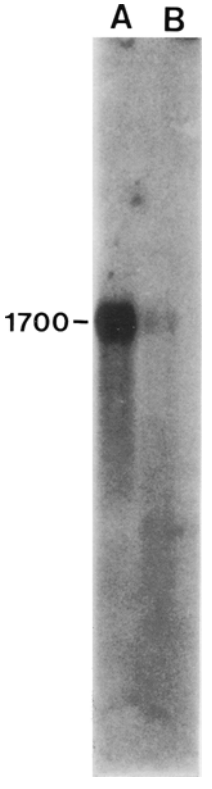

Figure 2. Northern analysis of poly A⁺ enriched RNA from green (A) and etiolated (B) barley leaves. 15 µg/lane were fractionated by size in a 1.4% agarose/formaldehyde gel. The probe was the EcoRI insert from pGS8. Molecular weights were calculated according to POULSEN (16).

RNA from both light and dark grown barley leaves. A strong band of about 1.7 kb is visible in the lane of the "light" poly A⁺ RNA, and a similar but fainter band is visible in the lane of the "dark" poly A⁺ RNA (Fig. 2).

In order to obtain fragments suitable for sequencing, the cloned insert of pGS8 was digested with different restriction enzymes and the selected fragments were re-cloned into pUC18. A restriction map of pGS8 along with the subclones and the sequencing strategy is shown in Figure 3.

Using both the direct and the reverse M13 universal primers it was possible to determine the complete sequence of both strands of the α, β and γ Sau 3A fragments. The remaining sequence was obtained from the δ and ε subclones and directly from sequencing the whole insert of pGS8.

The complete sequence of the pGS8 insert is 1182 bp long and shown in Figure 4 together with the deduced amino acid sequence. Starting with the first base in the sequence the insert contains an open reading frame of 1161 bp which codes for 387 amino acids and ends with an opal stop codon. This is followed by a second opal stop codon separated from the first by two amino acid codons and 9 bp of the 3′ non-coding region. The sequence has a GC content of 56.2% which is higher than the average 45.6% found in barley genomic DNA (15).

The polypeptide deduced from the nucleotide sequence (Fig. 4) has a predicted molecular weight of about 42.5 kDa and is dominatingly composed of acidic (12.66% of Asp+Glu; 9.3% of Lys+Arg; estimated pI 5.76) and hydrophilic amino acids (only 26.6% of hydrophobic residues).

4. DISCUSSION

In 1979 MANN et al. (13) demonstrated that barley leaves contain two glutamine synthetase isoenzymes (GS1 and GS2) with molecular weights of 349 and 363 kDa, respectively. They also showed that no glutamine synthetase 2 activity could be detected in etiolated seedlings.

Our Northern blot analyses with both homologous (pGS8) and heterologous (pGS100) probes demonstrate that the presence of two barley glutamine synthetase isoforms is reflected by the presence of two different mRNAs (1.4 kb

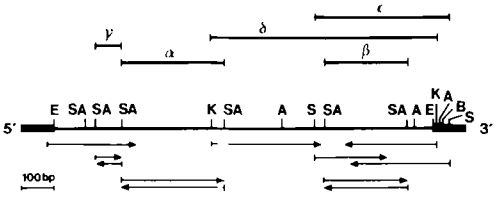

Figure 3. Restriction map and sequencing strategy for the 1.2 kb EcoRI - EcoRI fragment of pGS8. Arrows indicate the positions and directions of sequencing and the greek symbols mark individual DNA fragments subcloned in pUC18. E = EcoRI; SA = Sau 3A; K = KpnI; A = AvaI; B = BamHI; S = SphI.

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                                        30                                  60
TCC GGG TTC AAG GTG CTG GCG CTC GGC CCG GAG ACC ACC GGG GTC ATC CAG AGG ATG CAG
Ser Gly Phe Lys Val Leu Ala Leu Gly Pro Glu Thr Thr Gly Val Ile Gln Arg Met Gln  20

                                        90                                 120
CAG CTG CTC GAC ATG GAC ACC ACG CCC TTC ACC GAC AAG ATC ATC GCC GAG TAC ATC TGG
Gln Leu Leu Asp Met Asp Thr Thr Pro Phe Thr Asp Lys Ile Ile Ala Glu Tyr Ile Trp  40

                                       150                                 180
GTT GGA GGA TCT GGA ATT GAC CTC AGA AGC AAA TCA AGG ACG ATT TCG AAG CCA GTG GAG
Val Gly Gly Ser Gly Ile Asp Leu Arg Ser Lys Ser Arg Thr Ile Ser Lys Pro Val Glu  60

                                       210                                 240
GAC CCG TCA GAG CTG CCG AAA TGG AAC TAC GAC GGA TCG AGC ACG GGG CAG GCT CCT GGG
Asp Pro Ser Glu Leu Pro Lys Trp Asn Tyr Asp Gly Ser Ser Thr Gly Gln Ala Pro Gly  80

                                       270                                 300
GAA GAC AGT GAA GTC ATC CTA TAC CCA CAG GCC ATA TTC AAG GAC CCA TTC CGA GGA GGC
Glu Asp Ser Glu Val Ile Leu Tyr Pro Gln Ala Ile Phe Lys Asp Pro Phe Arg Gly Gly 100

                                       330                                 360
AAC AAC ATA CTG GTT ATC TGT GAC ACC TAC ACA CCA CAG GGG GAA CCC ATC CCT ACT AAC
Asn Asn Ile Leu Val Ile Cys Asp Thr Tyr Thr Pro Gln Gly Glu Pro Ile Pro Thr Asn 120

                                       390                                 420
AAA CGC CAC ATG GCT GCA CAA ATC TTC AGT GAC CCC AAG GTC ACT TCA CAA GTG CAA TGG
Lys Arg His Met Ala Ala Gln Ile Phe Ser Asp Pro Lys Val Thr Ser Gln Val Gln Trp 140

                                       450                                 480
TTC GGA ATC GAA CAG GAG TAC ACT CTG ATG CAG AGG GAT GTG AAC TGG CCT CTT GGC TGG
Phe Gly Ile Glu Gln Glu Tyr Thr Leu Met Gln Arg Asp Val Asn Trp Pro Leu Gly Trp 160

                                       510                                 540
CCT GTT GGA GGG TAC CCT GGC CCC CAG GGT CCA TAC TAC TGC GCC GTA GGA TCA GAC AAG
Pro Val Gly Gly Tyr Pro Gly Pro Gln Gly Pro Tyr Tyr Cys Ala Val Gly Ser Asp Lys 180

                                       570                                 600
TCA TTT GGC CGT GAC ATA TCA GAT GCT CAC TAC AAG GCG TGC CTT TAC GCT GGA ATT GAA
Ser Phe Gly Arg Asp Ile Ser Asp Ala His Tyr Lys Ala Cys Leu Tyr Ala Gly Ile Glu 200

                                       630                                 660
ATC AGT GGA ACA AAC GGG GAG GTC ATG CCT GGT CAG TGG GAG TAC CAG GTT GGA CCC AGC
Ile Ser Gly Thr Asn Gly Glu Val Met Pro Gly Gln Trp Glu Tyr Gln Val Gly Pro Ser 220

                                       690                                 720
GTT GGT ATT GAT GCA GGA GAC CAC ATA TGG GCT TCC AGA TAC ATT CTC GAG AGA ATC ACG
Val Gly Ile Asp Ala Gly Asp His Ile Trp Ala Ser Arg Tyr Ile Leu Glu Arg Ile Thr 240

                                       750                                 780
GAG CAA GCT GGT GTG GTG CTC ACC CTT GAC CCA AAA CCA ATC CAG GGT GAC TGG AAC GGA
Glu Gln Ala Gly Val Val Leu Thr Leu Asp Pro Lys Pro Ile Gln Gly Asp Trp Asn Gly 260

                                       810                                 840
GCT GGC TGC CAC ACA AAC TAC AGC ACA TTG AGC ATG CGC GAG GAT GGA GGT TTC GAC GTG
Ala Gly Cys His Thr Asn Tyr Ser Thr Leu Ser Met Arg Glu Asp Gly Gly Phe Asp Val 280

                                       870                                 900
ATC AAG AAG GCA ATC CTG AAC CTT TCA CTT CGC CAT GAC TTG CAC ATA GCC GCA TAT GGT
Ile Lys Lys Ala Ile Leu Asn Leu Ser Leu Arg His Asp Leu His Ile Ala Ala Tyr Gly 300

                                       930                                 960
GAA GGA AAC GAG CGG AGG TTG ACA GGG CTA CAC GAG ACA GCT AGC ATA TCA GAC TTC TCA
Glu Gly Asn Glu Arg Arg Leu Thr Gly Leu His Glu Thr Ala Ser Ile Ser Asp Phe Ser 320

                                       990                                1020
TGG GGT GTG GCG AAC CGT GGC TGC TCT ATT CGT GTG GGG CGA GAC ACC GAG GCG AAG GGC
Trp Gly Val Ala Asn Arg Gly Cys Ser Ile Arg Val Gly Arg Asp Thr Glu Ala Lys Gly 340

                                      1050                                1080
AAA GGA TAC CTG GAG GAC CGT CGC CCG GCC TCC AAC ATG GAC CCG TAC ACC GTG ACG GCG
Lys Gly Tyr Leu Glu Asp Arg Arg Pro Ala Ser Asn Met Asp Pro Tyr Thr Val Thr Ala 360

                                      1110                                1140
CTG CTG GCC GAG ACC ACG ATC CTG TGG GAG CCG ACC CTC GAG GCG GAG GCC CTC GCT GCC
Leu Leu Ala Glu Thr Thr Ile Leu Trp Glu Pro Thr Leu Glu Ala Glu Ala Leu Ala Ala 380

                                      1170
AAG AAG CTG GCG CTG AAG GTA TGA AGG ACC TGA AAA AAG GAC
Lys Lys Leu Ala Leu Lys Val End                                              387
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Figure 4. Nucleotide and deduced amino acid sequence of the barley pGS8 insert.

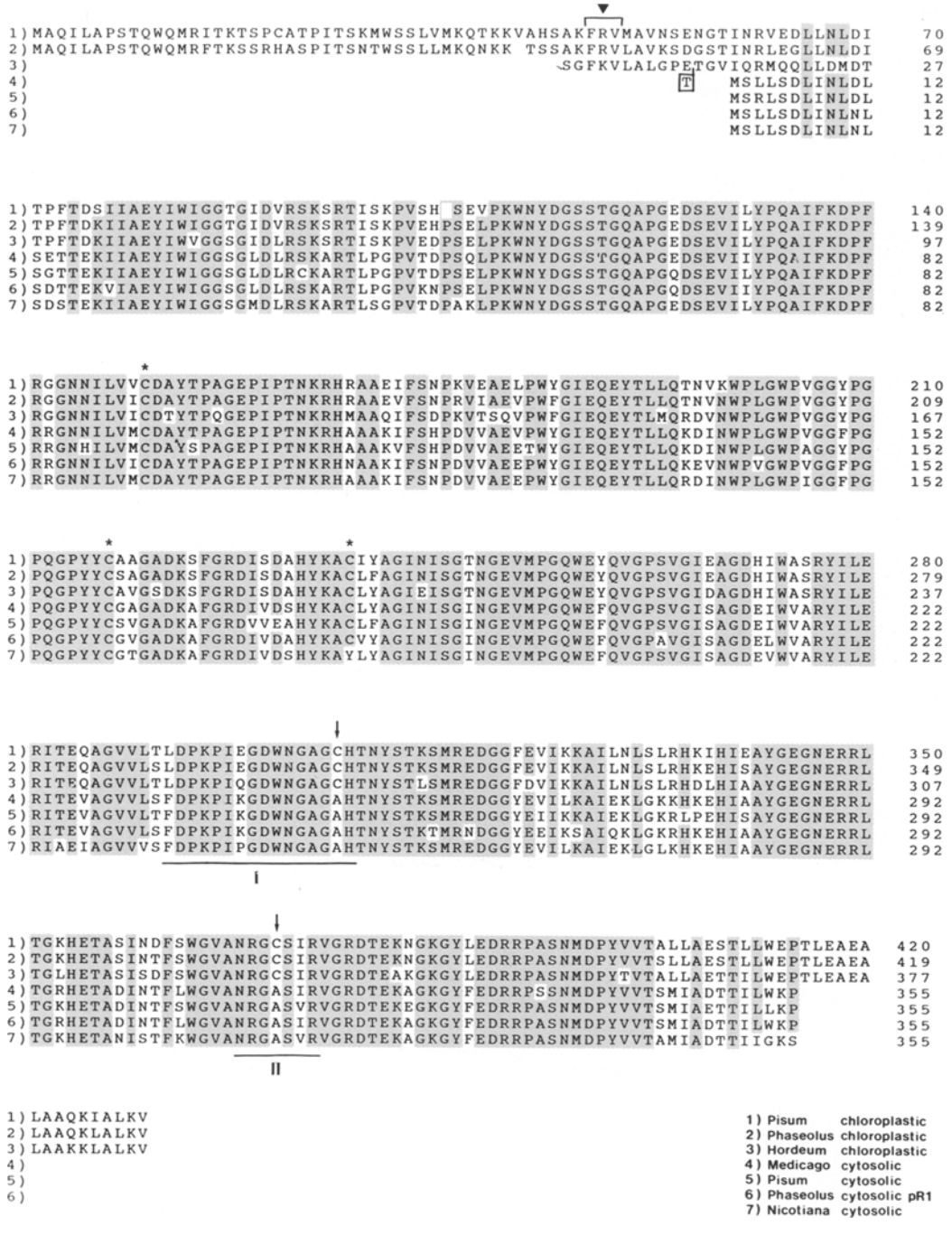

Figure 5. Comparison of the deduced primary structure of higher plant glutamine synthetases from pea (22, 23), bean (6, 10), barley, alfalfa (24) and tobacco (21). Asterisks indicate cysteins common to all glutamine synthetases; arrows indicate cysteins unique for chloroplastic glutamine synthetases. I and II mark regions highly conserved in both prokaryotic and eukaryotic glutamine synthetases (24).

and 1.7 kb). Hybridization under high stringency conditions of poly A$^+$ enriched RNA with the pGS8 probe from barley was exclusively with transcripts of 1.7 kb, suggesting that the pGS8 insert corresponds to a cDNA coding for the higher molecular weight glutamine synthetase 2 located in the chloroplasts. This is substantiated by the closer similarity of the primary structure of this enzyme with the chloroplastic glutamine synthetase from pea and bean (Fig. 5) than with that of the cytosolic isoenzymes in these species.

The presence of a 1.7 kb transcript in the "dark" poly A$^+$ RNA and the fact that the pGS8 insert was actually isolated from the "dark" library reveals that some glutamine synthetase 2 mRNA must be present in etiolated leaves even though MANN et al. (13) could not detect any glutamine synthetase 2 activity. The increase in the steady state level of the 1.7 kb mRNA upon illumination of etiolated leaves might reflect an increase in transcription and/or mRNA stability.

The deduced amino acid sequence of the open reading frame of clone pGS8 from barley has 85% identity with the chloroplastic glutamine synthetases of Phaseolus vulgaris and Pisum sativum, similarity at the nucleotide level being 75% and 74%, respectively (Fig. 5). A corresponding comparison of pGS8 with the deduced amino acid sequences for cytosolic glutamine synthetases of Phaseolus vulgaris, Pisum sativum, Medicago sativa and Nicotiana plumbaginifolia yields a lower degree of identity ranging from 74% to 76% and at the nucleotide level the similarity is only 69% to 70%.

It is apparent from Figure 5 that the primary structures of chloroplastic and cytosolic glutamine synthetases of the dicotyledonous species have large conserved domains also present in the barley enzyme. The chloroplastic glutamine synthetases of pea and bean have an N-terminal extension of 58 and 57 amino acids when aligned with their cytosolic counterparts. The deduced amino acid sequence for the barley glutamine synthetase has the extension beyond the amino terminal methionine of the mature cytosolic glutamine synthetases. It has been suggested that the N-terminal extension of the leguminous chloroplastic glutamine synthetases constitutes the transit peptide for import into the chloroplast (10, 23) and that the cleavage site according to the consensus sequence of KARLIN-NEUMANN and TOBIN (9) is located in the tripeptide FRV (cf. Fig. 5). The barley sequence extends beyond this point and contains at this position the tripeptide FKV. The replacement of the arginine residue with a lysine residue is considered as a conservative amino acid replacement. The barley clone misses the coding region for the major part of the putative transit peptide. If the FKV tripeptide indeed is the cleavage site the 16 amino terminal residues of the mature barley enzyme show little sequence similarity to the chloroplastic glutamine synthetase of pea and bean.

The mature chloroplastic glutamine synthetases are characterized by a 16 amino acid extension of the carboxyterminal end when aligned with the cytosolic isoenzymes (Fig. 5). Fifteen of the 16 amino acids of this carboxyterminal peptide are identical, while a glutamine in the leguminous enzyme is replaced by a lysine in the barley glutamine synthetase at position 7 upstream from the carboxyterminal valine.

According to EVSTIGNEEVA et al. (4) the binding of ammonia and ATP to glutamine synthetase involves cystein SH-groups. Complete inhibition of the enzyme occurred upon modification of two SH-groups in the chloroplastic glutamine synthetase monomer. In Lemna minor, which only has glutamine synthetase 2 activity (14), and in barley the chloroplastic glutamine synthetases are inactivated by thiol reactive agents, while the cytosolic isoenzyme is not affected by these treatments (13, 18). This specificity of thiol reagents may be related to the extra cystein residues consistently found in the chloroplastic enzyme (positions 263 and 328 in barley glutamine synthetase), whereas the four cytosolic enzymes studied contain an alanine residue in these two positions.

Comparisons of the amino acid sequences of eukaryotic and prokaryotic glutamine synthetases led TISCHER et al. (24) to suggest an ATP-binding domain which is marked I in Figure 5. This domain shows faint similarities to the ATP-binding sites of other proteins. Domain II shows sequence relationships to the substrate binding site of bovine glutamate dehydrogenase and is therefore proposed as part of the substrate

binding site of glutamine synthetase (24). It is interesting to note that the two additional cysteine residues of the chloroplastic glutamine synthetases are located within these two domains.

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