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Nucleotide sequence encoding the precursor of the small subunit of ribulose 1,5-bisphosphate carboxylase from *Lemna gibba* L.G-3

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ABSTRACT

We have sequenced a cDNA clone, pLgSSU, which encodes the small subunit of ribulose 1,5-bisphosphate carboxylase of Lemna gibba L.G-3 a monocot plant. This clone contains a 832 basepair insert which encodes the entire 120 amino acids of the mature small subunit polypeptide (M_r = 14,127). In addition this clone encodes 53 amino acids of the amino terminal transit peptide of the precursor polypeptide and 242 nucleotides of the 3' non-coding region. Comparison of the nucleotide sequence of pLgSSU with Lemna gibba genomic sequences homologous to the 5' end of the cDNA clone suggests that nucleotides encoding four amino-terminal amino acids of the transit peptide are not included in the cDNA clone. The deduced amino acid sequence of the Lemna gibba mature small subunit polypeptide shows 70-75% homology to the reported sequences of other species. The transit peptide amino acid sequence shows less homology to other species. There is 50% homology to the reported soybean sequence and only 25% homology to the transit sequence of another monocot, wheat.

INTRODUCTION

The synthesis of ribulose 1,5-bisphosphate carboxylase (RuBPCase), the enzyme responsible for the first step of the Calvin cycle, requires the coordinate activities of the nuclear as well as the chloroplast genome (1). RuBPCase contains 8 large subunits (LSU) encoded in the chloroplast and 8 small subunits (SSU) coded for by the nuclear genome. The mRNA for the SSU polypeptide is translated in the cytoplasm as a precursor of about 20,000 daltons which is subsequently transported into the chloroplast, processed to its mature size, and combined with LSU to form the mature functional enzyme (2-8). The SSU has been reported to be encoded by a multigene family in a number of species including Lemna (9-13,15).

We have reported the isolation of a cDNA clone encoding an SSU polypeptide of Lemna gibba (14). Using this cDNA clone as a probe we have been able to show in Lemna that light rapidly influences the amount of SSU mRNA and that this effect of light is mediated by phytochrome. Our experiments demonstrated that in Lemna phytochrome action can rapidly influence the expression of the

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gene(s) encoding the SSU polypeptide, probably by increasing transcription. We have now sequenced this cDNA as part of characterizing this gene family in Lemna in order to be able to study the regulation of the expression of these genes.

The complete nucleotide sequence of soybean, wheat, and pea SSU genes have been published (9,13,15). In addition, partial nucleotide sequences from pea, petunia, and tobacco SSU cDNAs and a complete wheat cDNA have been reported (10,11,15-17). The sequence comparison from a number of species should ultimately help elucidate the function of various parts of the polypeptide. For example, the amino acid composition of the transit peptide may be helpful for the analysis of the function of this part of the precursor polypeptide in its transport from the cytoplasm into chloroplasts.

The Lemna gibba SSU cDNA clone encodes 53 amino acids of the transit peptide, 120 amino acids of the mature protein and 241 nucleotides representing the 3' non-coding region of the mRNA. Comparison of the nucleotide sequence of the SSU cDNA clone with partial sequences of two Lemna gibba SSU genomic clones suggests that the SSU cDNA clone may not encode the complete transit peptide but may lack 4 triplets coding for 4 amino terminal amino acids.

MATERIALS AND METHODS

Isolation of nucleic acids

Plasmid DNA was isolated by a standard high-salt isolation procedure using Triton (18) or by the Holmes and Quigley method (19) using boiling to lyse the bacterial spheroplasts. CsCl-ethidium bromide density gradient centrifugation was used to purify the plasmid DNA preparations.

Enzymes

All restriction enzymes and DNA ligase were obtained from Bethesda Research Laboratories (Gaithersburg, MD, USA). DNA polymerase Klenow fragment was obtained from New England Nuclear Corporation (549 Albany St., Boston, MA). All enzymes were used as recommended by the supplier.

Isolation of cDNA clones containing a copy of the SSU mRNA

The construction of cDNA clones containing a copy of the mRNA for the Lemna SSU polypeptide has been described (14). Briefly, a cDNA clone bank derived by reverse transcription of total poly A (+) RNA from Lemna gibba was screened for SSU sequences by filter hybridizations using three different probes. As reported previously, the concentration of SSU mRNA in tissue put into the dark for a number of days is only a fraction of the SSU mRNA amount

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in light-grown plants (4,23). Therefore, those cDNA clones that hybridized with ^{32}P-labelled cDNA made against poly A (+) RNA isolated from light-grown plants and not with ^{32}P-labelled cDNA made against poly A (+) RNA isolated from plants put into the dark were identified as putative SSU cDNA clones. These recombinants were further screened with a ^{32}P-labelled cDNA probe made against poly A (+) RNA enriched for the SSU mRNA by size fractionation. Positive recombinants from this screening were identified as containing copies of the SSU mRNA using the hybrid-release translation method.. One of these cDNA clones, pLgSSU, was shown to hybridize to the *Lemna* SSU polypeptide mRNA (14).

DNA sequence analysis

DNA sequence analysis was performed using the chain termination method (20-22). *E. coli* JM 101 cells were transformed with recombinant bacteriophage M13 RF DNA to obtain single-stranded templates (21). M13mp7 (22), M13mp8 and M13mp9 were used as vectors. Initially we used a M13 DNA sequencing kit containing a 24 bp universal primer (PL Biochemicals, Inc. Milwaukee, WI). The sequencing reactions were performed according to the instructions of this kit. IPTG (isopyropyl β-D-thiogalactoside) and X-Gal (5-bromo-4 chloro-3 indoyl-β-D-galactoside) were purchased from Bethesda Research Laboratories. α-^{32}P-dATP (800 Ci/mmol) was purchased from New England Nuclear Corp. Later in this study a 15 bp universal primer was obtained from Bethesda Research Laboratories. The gel patterns with this primer showed fewer ambiguities than with the 24 bp primer. Thin 6 or 8% polyacrylamide gels (40x20x0.04 cm) were used to separate the DNA fragments. After electrophoresis the gels were dried onto Whatman 3MM paper and the radioactive DNA fragments visualized by autoradiography at room temperature without the use of intensifying screens.

RESULTS AND DISCUSSION

The nucleotide sequence of the Lemna SSU mRNA

Figure 1 shows a restriction map of pLgSSU (see Materials and Methods) and the sequencing strategy used. A first attempt to clone the entire pLgSSU insert into the Pstl-site of M13mp7, M13mp8 or M13mp9 for sequencing of the 5' and 3' border fragments failed because of the GC-tails present at both ends of the sequence. Annealing of the G-tail at one end with the C-tail at the other inhibited the action of the DNA polymerase Klenow fragment. Therefore we used DNA fragments generated by cleavage of the insert with two restriction enzymes for cloning into the M13 vectors and subsequent sequencing. The following DNA fragments of pLgSSU were subcloned into M13mp8 and M13mp9:

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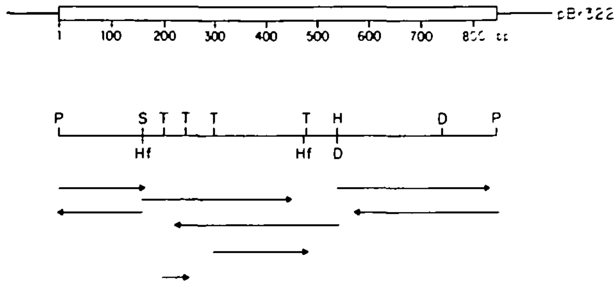

Figure 1. Restriction enzyme cleavage map and DNA sequencing strategy for pLgSSU. The arrows below the cleavage map indicate the direction and extent of the sequencing reactions. P = Pst I; S=Sal I; Hf - Hinf I; T = Taq I; H = Hind III; D = Dde I. The open box shows the insert DNA.

a. The 160 and 700 basepair fragments generated by digestion of pLgSSU with Pst I and Sal I.

b. The 270 and 600 basepair fragments generated by digestion of pLgSSU with Pst I and Hind III.

c. The 35 and 185 basepair fragments generated by digestion of pLgSSU with Taq I.

The fragments were ligated into M13mp8 or M13mp9 cut with AccI. The complete nucleotide sequence of the non-coding strand of the pLgSSU insert is shown along with the deduced amino acid sequence in Figure 2. The insert has a length of 777 basepairs without the GC-tails and the poly (A) tail of 13 residues. Plant mRNAs have been reported to have poly (A) tails averaging about 100-200 residues (e.g., 24). Therefore, we can infer that the average *Lemna* SSU mRNA *in vivo* has a length of about 900 nucleotides, in excellent agreement with the length determined for this mRNA with denaturing formaldehyde-agarose gel electrophoresis (14). The determination of the amino acid sequence of the *Lemna* SSU polypeptide was facilitated by comparison to SSU sequences of other species (9-11,13,15,16,25,26). Nucleotides 38-196 code for 53 amino acids of the transit peptide. However, we think that the first ATG triplet (nucleotides 38-40) may not be the first triplet translated *in vivo*. It is possible that due to artifacts of the reverse transcription of mRNA the 5' end of the mRNA has not been cloned (27-29). Therefore we have compared the 5' sequence of two genomic clones of the SSU gene family in *Lemna* (12) with the cDNA sequence. As shown below (Figure 4) this comparison suggests that the transit peptide contains 4 additional amino acids not included in the cDNA clone. This would extend the length of the transit peptide of

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                                                  M  M  V  S  T  A  A  V
GGGGGGGGGGGGGGGGGGGGGGTCAAAAAACAGATATATGATGGTTTCCACCGCCGCCGT
         10        20        30        40        50        60

  A  R  V  R  P  A  Q  T  N  M  V  G  A  F  N  G  C  R  S  S
GGCCCGCGTCCGCCCTGCCCAGACCAACATGGTGGGCGCCTTCAACGGGTGCCGCTCCTC
         70        80        90       100       110       120

  V  A  F  P  A  T  R  K  A  N  N  D  L  S  T  L  P  S  S  G
CGTCGCCTTCCCCGCCACCCGCAAGGCCAACAACGATTTGTCGACTCTCCCCAGCTCCGG
        130       140       150       160       170       180

  G  R  V  S  C↓ M  Q  V  W  P  P  E  G  L  K  K  F  E  T  L
CGGCAGGGTTAGCTGCATGCAGGTGTGGCCGCCGGAGGGCCTGAAGAAGTTCGAGACCCT
        190       200       210       220       230       240

  S  Y  F  P  L  S  S  V  E  D  L  A  K  E  V  D  Y  L  L  R
CTCCTACTTCCCCCTCTCTTCCGTCGAGGACCTCGCCAAGGAGGTGGACTACCTCCTCCG
        250       260       270       280       290       300

  N  D  W  V  P  C  I  E  F  S  K  E  G  F  V  Y  R  E  N  H
CAACGACTGGGTTCCCTGCATCGAGTTCTCCAAGGAGGGGTTCGTGTACCGTGAGAACAA
        310       320       330       340       350       360

  A  S  P  G  Y  Y  D  G  R  Y  W  T  M  W  K  L  P  M  F  G
CGCGTCGCCCGGGTACTACGACGGGAGGTACTGGACGATGTGGAAGCTGCCCATGTTCGG
        370       380       390       400       410       420

  C  T  D  A  S  Q  V  I  A  E  V  E  E  A  K  K  A  Y  P  E
CTGCACCGACGCCAGCCAGGTGATCGCCGAGGTGGAGGAGGCCAAGAAGGCCTACCCCGA
        430       440       450       460       470       480

  Y  F  V  R  I  I  G  F  D  N  K  R  Q  V  Q  C  I  S  F  I
GTATTTCGTCAGAATCATCGGCTTCGACAACAAGCGCCAAGTCCAGTGCATCAGCTTCAT
        490       500       510       520       530       540

  A  Y  K  P  T  *
CGCCTACAAGCCCACCTAAGCTTGAAATCTAGTTGGGGGCCGCCGTACCCCCTCTCTCAA
        550       560       570       580       590       600

GTTTCTATAAACTTTCCTCGGGCTCTCTGTACTGTTGCCCTTTCATTGCTGGTGACTATC
        610       620       630       640       650       660

TTCAGGTCGGCTTTCGTCTTCGTCGTTAGGTGATTCTAACATGTGAGGAGCGTATTACCG
        670       680       690       700       710       720

TCATATATCTGTTTTTTGATAGTGAGAATTGTCTTAGCTTGGGGTATTATATAAATTAAA
        730       740       750       760       770       780

AAGTATCTGGCAATCTCTAAAAAAAAAAAAACCCCCCCCCCCCCCCCCCC
        790       800       810       820       830
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Figure 2. DNA sequence of pLgSSU encoding the small subunit of RuBPCase from *Lemna gibba* L.G-3. The nucleotide sequence shown is that for the anticodon strand. The deduced amino acid sequence is shown above the codons. Some of the regions discussed in the text are underlined: positions 23-40 and 721-739, inverted repeat; 557-559, stop codon; 774-780, polyadenylation signal. The arrow indicates the position of cleavage of the precursor polypeptide to form the mature protein.

the *Lemna* SSU precursor polypeptide to 57 amino acids, the same length as found in pea (13) but longer than reported for soybean (9), wheat (15) and *Chlamydomonas* (30). The *Lemna* SSU transit peptide is quite basic containing five arginine residues and one lysine, compared with only one acidic residue, aspartic acid. Possibly this basic character reflects the capacity of the SSU

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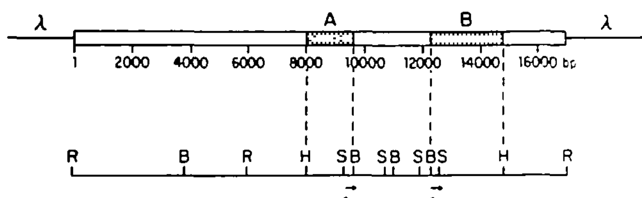

Figure 3. Restriction enzyme cleavage map of the genomic clone λLgSSU5. The open box shows the 17000 basepairs insert of Lemna gibba genomic DNA in Charon 4. The hatched regions, A and B, are that part of the insert DNA hybridizing with pLgSSU. The arrows below the cleavage map indicate the direction and extent of the sequencing reactions. R = EcoRI; B = Bam H1; H = Hind III: S = Sal I.

polypeptide precursor to bind to the negatively charged chloroplast envelope (30).

Nucleotides 197-556 code for the mature SSU polypeptide of 120 amino acids with a molecular weight of 14,127. The mature Lemna peptide is slightly shorter than in wheat (128 amino acids) or pea (123 amino acids). One of the amino acid deletions (position 48) is located in a region where in soybean the coding sequence is interrupted by an intervening sequence (9). Broglie et al. also found a similar deletion in the wheat sequence (15). The other differences are located at the 3' carboxy-terminal end of the SSU polypeptide. The amino acid sequence has high homology to other species around the processing site between a cystine and methionine residue (see below). The coding sequence of the SSU mRNA is flanked at the 3' end by a TAA stop codon and 238 nucleotides. A hexanucleotide sequence "AATTAA" occurs 19 nucleotides upstream from the poly A tail. Such a sequence has been found in many eukaryotic mRNAs, and it is proposed that it has a function as a signal for polyadenylation or termination of transcription (31).

The nucleotide sequence of the 5'-terminal regions of two Lemna gibba SSU genes: the 5'-terminal ATG triplet

We have also contructed a Lemna gibba genomic DNA library (12,14). A number of clones encoding SSU sequences were isolated (14). Figure 3 shows the restriction enzyme map of one of these genomic SSU clones λSSU5 which contains 2 SSU genes, 5A and 5B. The position of the coding sequences was established by Southern hybridization (32). The 5'-terminal ends of the two SSU genes were determined by Southern hybridization using a probe specific for these regions. We used a 160 basepair fragment from the SSU cDNA clone generated by digestion with Pst I and Sal I and purified by electrophoresis

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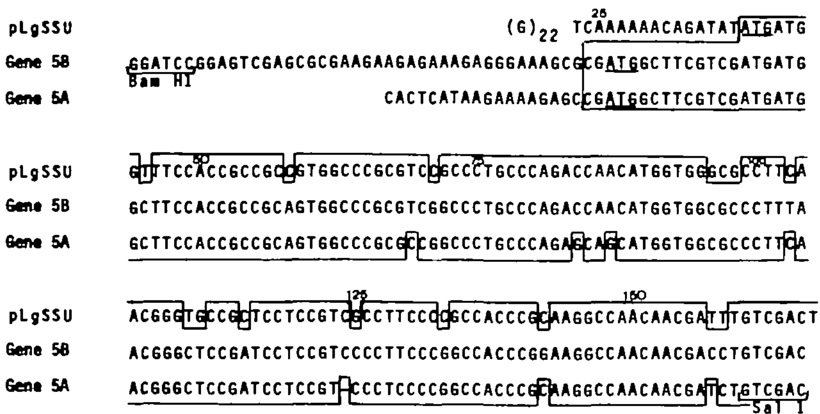

Figure 4. Comparison of cDNA and genomic DNA 5' sequences coding for the amino terminal end of the SSU precursor polypeptide. Genes A and B are those shown in Fig. 3 and include 5' non-coding regions. The homologous sequences are indicated.

as a 5'-terminal specific probe (see Figure 1). We found that the transcription start site of both SSU genes are positioned in the region between the two coding sequences (data not shown). Therefore, the direction of transcription of both genes is in opposite directions.

In order to determine the nucleotide sequence of the region containing the putative translation start sites, λSSU5 Sal I restriction fragments of about 450 and 1500 basepairs were subcloned into the Sal I site of pBR322 (33). The 450 basepair Sal I fragment of SSU 5B contains a Bam HI restriction site dividing this fragment into 183 basepair and 270 basepair fragments (see Figure 3). The 1500 basepair Sal I fragment of SSU 5A also contains a Bam HI restriction site, dividing this fragment into a 180 basepair fragment and a fragment of about 1300 basepairs. To determine the 5'-terminal ends of both Lemna SSU genes the two ∿180 basepair Bam HI-Sal I fragments were force-cloned into M13mp8 and M13mp9. Both strands of the DNA fragments were sequenced using the Sanger dideoxy chain termination method (21). The nucleotide sequence of the 183 basepair Bam HI-Sal I fragment of gene 5B and a part of the nucleotide sequence of the 175 basepair Bam HI-Sal I fragment of gene 5A is shown in Figure 4. The Bam HI-Sal I fragments of both genes 5A and 5B code for 46 amino-terminal amino acids of the SSU precursor polypeptide. Comparison of the nucleotide sequence coding for these 46 amino acids in genes 5A and 5B shows 95% homology. Only 7 base changes are present and of these only three (at nucleotides 69, 84, and 87 of pLgSSU) would result in amino

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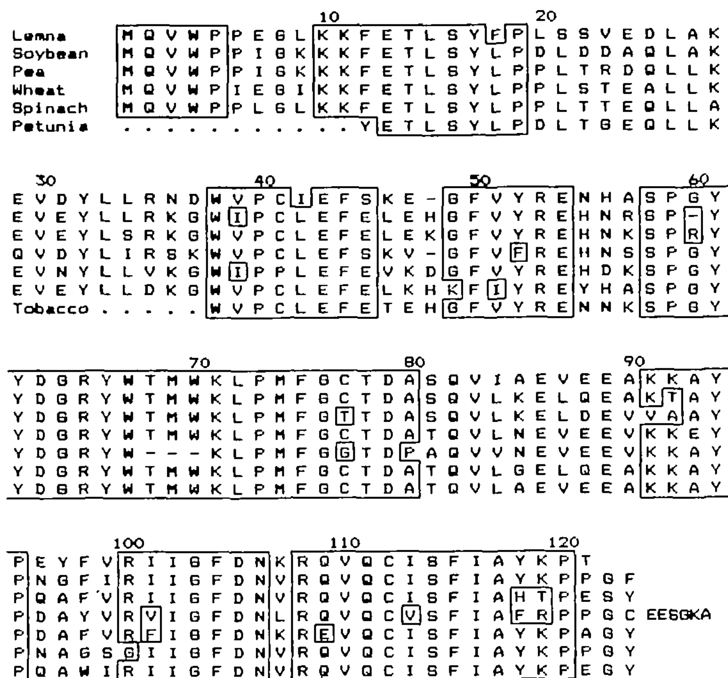

Figure 5. Comparison of amino acid sequences for mature SSU polypeptides. The sequences are from *Lemna*, soybean (9), pea (10), wheat (15), spinach (25), petunia (11), and tobacco (17). Large (5 or more) blocks of conserved (in at least 5 species) amino acids are indicated.

acid substitution. However, there is also an apparent deletion of one nucleotide in gene 5A (at nucleotide position 124 of pLgSSU) which interrupts the reading frame and suggests the possibility that gene 5A is a *Lemna* SSU pseudogene. These genes will be further characterized to answer such questions.

Comparison of the SSU mRNA nucleotide sequence derived from pLgSSU with these two different SSU genes shows that the cloned cDNA is derived from neither. However there is 84% homology between these sequences when the first ATG triplet of the cDNA sequence is aligned with the second ATG in genes 5A and 5B (see Figure 4). In both SSU genes the first and second ATG triplets are separated by three other triplets, which are homologous in both genes. Upstream from the genomic 5' terminal ATG triplet there is a considerable lack of homology over 18 bases between the nucleotide sequences. Therefore it is very likely that the first ATG triplet in the genomic DNA sequences represents the first translated triplet, and we infer that the nucleotide sequence up-

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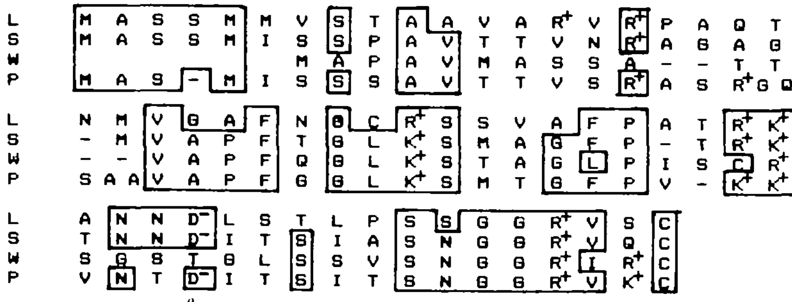

Figure 6. Comparison of amino acid sequences of SSU transit peptides. The sequences were deduced from L: cloned Lemna gibba cDNA (pLgSSU1) and the 5' region of 2 genomic clones (genes 5A and 5B); S: a soybean genomic clone (SRS1) (9); W: cloned wheat cDNA (pW9) (15); and P: a pea genomic clone (pSS36) (13). Conserved regions are indicated.

stream from the first ATG triplet in the cDNA clone may not reflect the actual 5' terminal sequence of the SSU mRNA. It has been reported that reverse transcription of mRNA can be inaccurate at the 5' end (27-29). One type of error that can be generated is an inverted repeat sequence, and in fact such an inverted sequence can be seen in Fig. 2 in nucleotides 23-40 and 721-739. Therefore the discrepancy between the cDNA clone nucleotide sequence and genomic DNA nucleotide sequences might be explained by an artifact created during the cDNA cloning procedure. We infer from these data that the transit peptide from the Lemna SSU precursor consists of 57 amino acids with an amino-terminus of M A S S...and a molecular weight of 5941. The predicted M_r of the entire precursor protein is 20,068.

Comparisons of the Lemna sequence with other species. Surprisingly, the greatest homology between the SSU of Lemna and other species in which the SSU sequence has been determined is found in soybean, a dicot, rather than in wheat, the only other monocot SSU sequence so far reported. There is a 70% homology at the nucleotide level between the Lemna and soybean SSU coding sequences. The currently known amino acid sequences are compared for the mature SSU in Fig. 5, and for the transit peptide in Fig. 6. The amino acid sequence homologies for the mature protein between Lemna and each of the other species range from 70% (for pea and petunia) to 75% (soybean). Only 12 of the 120 amino acids (10%) are unique to the Lemna sequence. Of these, several are conservative substitutions (e.g., I for L). Thus, the amino acid sequence of the mature SSU polypeptides is strongly conserved among diverse species of higher plants. As noted by others (e.g., 15), certain blocks of amino acids

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are essentially invariant; these are marked in Fig. 5. In addition, there is charge conservation in areas outside the large marked blocks (amino acid positions 24, 28-31, 35-36, 86-89).

The transit peptide comparisons show larger differences between species. The largest areas of homology in these sequences occur at the N-terminus and near the processing site. The charged residues also tend to be conserved. There are additional areas of homology indicated in Fig. 6. It is interesting to note that the initial 4 amino acids of Lemna and soybean occur internally as the 6th-10th residues in the wheat sequence. In addition, the N-terminal amino acid sequence of a Lemna light-harvesting chlorophyll a/b-protein clone (14) encoding an entire precursor polypeptide, is also M A A S (G.A. Neumann, C.F. Wimpee, and E.M. Tobin, unpublished work). Perhaps this sequence is a part of the recognition process between chloroplasts and cytoplasmically synthesized chloroplast proteins.

The Lemna transit sequence shows about 50% homology to soybean, 40% to pea, and only 25% to wheat. Evolutionarily Lemna is more closely related to wheat than to soybean. Berry-Lowe et al. (9) have predicted two types of SSU gene in soybean, one strongly related to pea and one which is less homologous. It would be interesting if another Lemna gene with a transit peptide more similar to the wheat sequence were found. If this were the case, it would imply that the subfamilies of SSU genes arose before the divergence of monocots and dicots.

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