Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 391–395, January 1988
Biochemistry

Relocating a gene for herbicide tolerance: A chloroplast gene is converted into a nuclear gene

(Q_B protein/atrazine tolerance/transit peptide)

ALICE Y. CHEUNG*, LAWRENCE BOGORAD*, MARC VAN MONTAGU†, AND JEFF SCHELL†‡

*Department of Cellular and Developmental Biology, 16 Divinity Avenue, The Biological Laboratories, Harvard University, Cambridge, MA 02138; †Laboratorium voor Genetica, Rijksuniversiteit Ghent, B-9000 Ghent, Belgium; and ‡Max-Planck-Institut fur Zuchtungsforschung, D-500 Cologne 30, Federal Republic of Germany

Contributed by Lawrence Bogorad, September 30, 1987

ABSTRACT **The chloroplast gene *psbA* codes for the photosynthetic quinone-binding membrane protein Q_B, which is the target of the herbicide atrazine. This gene has been converted into a nuclear gene. The *psbA* gene from an atrazine-resistant biotype of *Amaranthus hybridus* has been modified by fusing its coding region to transcription-regulation and transit-peptide-encoding sequences of a bona fide nuclear gene. The constructs were introduced into the nuclear genome of tobacco by using the *Agrobacterium* tumor-inducing (Ti) plasmid system, and the protein product of nuclear *psbA* has been identified in the photosynthetic membranes of chloroplasts. Recovery of atrazine-tolerant transgenic plants shows that the product of the transplanted gene functions in photosynthesis. These experiments show that it is possible to modify chloroplast-gene-specified functions via nuclear-genome transformation and also raise evolutionary questions.**

A major puzzle of eukaryotic cell biology is: Why are genes for some organelle proteins located in the nucleus, while genes for others are in the organelles (1)? One commonly entertained view is that proteins coded for by chloroplast and mitochondrial genes (e.g., photosynthetic membrane proteins) have properties that would block their passage through the organelle's outer membrane system. We have tested this proposition as well as the possibility of genetically altering functions normally encoded by chloroplast genes by determining whether a modified chloroplast gene for a photosynthetic membrane protein introduced into the nuclei of tobacco plants can serve as the source of a protein that functions in photosynthetic electron transport.

In photosynthesis, plastoquinone molecules from the large pool present in the thylakoid membrane become associated with the quinone-binding protein, Q_B, of photosystem II, where they are reduced. Once reduced they leave the binding site to be replaced by an oxidized quinone molecule (2). Triazine herbicides compete with the plastoquinone for binding and, thus, interrupt photosynthetic electron flow (3). *In situ* in thylakoid membranes, the Q_B protein of atrazine-sensitive plants, including *Amaranthus hybridus*, binds azidoatrazine; Q_B protein from an atrazine-resistant biotype does not bind the herbicide because of a single amino acid residue substitution (4). Thus, if this mutation in the Q_B protein, encoded by the chloroplast *psbA* gene, is the basis of atrazine resistance, the functional integration of this protein into the photosynthetic apparatus of a plant that is normally sensitive would confer atrazine tolerance.

Plants transformed with the bacterial neomycin phosphotransferase gene spliced behind the promoter and transit peptide-encoding sequences of the small subunit (SSU) of the gene for ribulose bisphosphate carboxylase/oxygenase can transport the protein product into chloroplasts (5). We have spliced the coding region of the *psbA* gene isolated from the chloroplast DNA of the atrazine-resistant biotype of *Amaranthus* to the transcriptional-control and transit-peptide-encoding regions of a nuclear gene, *ss3.6*, for the SSU of ribulose bisphosphate carboxylase/oxygenase of pea (6). The fusion-gene constructions (designated SSU-ATR) were introduced into tobacco plants via the *Agrobacterium* tumor-inducing (Ti) plasmid transformation system using the disarmed Ti plasmid vector pGV3850 (7). Some of the transformed plants tolerated atrazine more than control plants did. This indicated that the modified mutant Q_B protein produced in the cytoplasm had been taken up by the chloroplasts and was functional there. Immunochemical analyses of photosynthetic membrane proteins from transformed atrazine-tolerant plants showed that they contain the imported modified Q_B protein.

METHODS AND MATERIALS

***Agrobacterium* Ti Plasmid Transformation of Plants.** Standard recombinant DNA procedures (8) were used to construct plasmids. Plasmids pSSU-S-ATR and pSSU-L-ATR (Fig. 1*A*) were introduced into *Agrobacterium* GV3850 (7) to generate strains GV3850:SSU-S-ATR and GV3850:SSU-L-ATR. These strains were used to inoculate wounded *Nicotiana tobaccum* Wisconsin 38 or to infect leaf discs of SR1 tobacco (9). Infected plant tissues were propagated, and plantlets were regenerated as described (7).

Plant DNA Analysis. DNA was isolated from leaf tissues as described (10). Total restriction enzyme-digested DNA (10 μg) was separated electrophoretically in 0.8% agarose gels. The DNA was transferred to nitrocellulose, and the blot was hybridized with *psbA* sequences labeled with ^{32}P by nick-translation (8).

Protein Blot Analysis of Tobacco Thylakoid Proteins. Thylakoid membranes were isolated from tobacco plants (11). The membrane proteins were electrophoresed at 4°C in a $NaDodSO_4$/polyacrylamide gel (12.5%) and electrotransferred to nitrocellulose paper (12). The protein blot was treated with an antibody [Q_B-3 (13)] against a synthetic oligopeptide corresponding to an internal region of the Q_B protein. Horseradish peroxidase conjugated to second antibodies was used to locate the antibody.

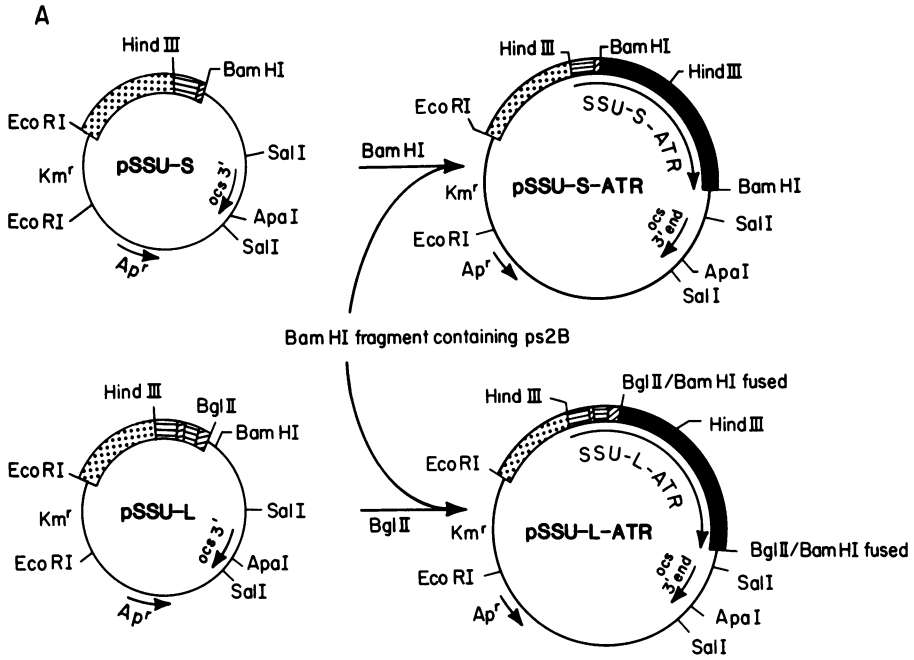
RESULTS

Construction of Chimeric SSU-S-ATR and SSU-L-ATR Genes. All known nuclear-coded, cytoplasmically synthesized proteins destined for chloroplasts contain amino-terminal transit peptides that are removed some time during

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations; SSU, small subunit; Ti, tumor-inducing.

or after import of the proteins into the organelle (11). Furthermore, chloroplast genes from maize and *A. hybridus* are not transcribed from their own promoters when placed in the nuclei of transgenic tobacco plants (A.Y.C. and L.B. unpublished data). We have taken both of these facts into account in constructing two plasmid vectors, pSSU-L and pSSU-S (Fig. 1*A*), into which the coding region of a plastid gene can be cloned to form translational fusion genes that are expressed in the nucleus. This allows for testing the transportability of proteins into chloroplasts by the SSU transit peptide because each plasmid carries the transcriptional-regulatory sequences and the transit-peptide-encoding sequence of a nuclear *rbcS* gene (for the SSU of ribulose bisphosphate carboxylase/oxygenase), *ss3.6*, from pea (6). The first exon of *ss3.6* encodes the entire transit peptide plus the first two amino acids of the mature SSU polypeptide. Plasmid pSSU-S contains only this exon and has a unique *Bam*HI cloning site two amino acid codons behind the transit-peptide-processing site. The second of these two amino acid codons resulted from the construction of the cloning site. Plasmid pSSU-L carries the entire first exon, the first intron, and part of the second exon. A unique *Bgl* II



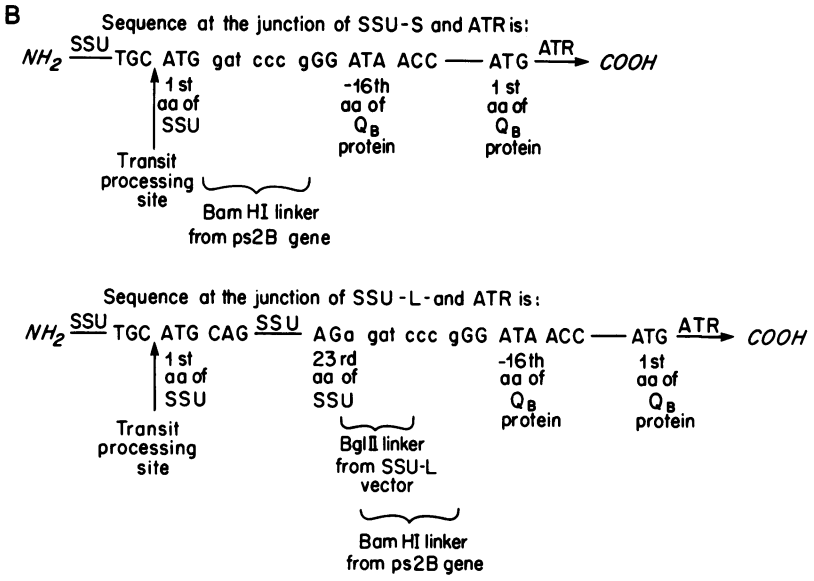


FIG. 1. General cloning vectors pSSU-S and pSSU-L and chimeric gene vectors pSSU-S-ATR and pSSU-L-ATR. (*A*) pSSU-S was constructed by placing a unique *Bam*HI cloning site behind a 900-base-pair (bp) fragment from *ss3.6*, which included the regulatory region (▩) and the coding region for the transit peptide (▤) and two amino acids of the mature SSU (▨) (see also ref. 5). pSSU-L was similar to pSSU-S except that the portion of *ss3.6* in this plasmid extends into amino acid 23 of the SSU peptide, and a unique *Bgl* II cloning site has been engineered behind the *ss3.6* DNA. Both pSSU-S and pSSU-L have a bacterial neomycin phosphotransferase gene (15) and a fragment (*ocs* 3') from the 3' end of the *ocs* gene (14), which includes a mRNA polyadenylylation site. Plasmids pSSU-S-ATR and pSSU-L-ATR were constructed by introducing a *Bam*HI fragment that contained a BAL-31-modified *psbA* gene [from pAh484 (■) (4)] into the *Bam*HI site of vector pSSU-S and the *Bgl* II site of vector pSSU-L, respectively. (*ps2B* is another designation for the gene *psbA*.) (*B*) The nucleotide sequence and derived amino acid positions of the fusion regions of SSU-S-ATR and SSU-L-ATR genes are shown. Km^r, kanamycin resistance; Ap^r, ampicillin resistance; aa, amino acid.

cloning site is located at the 23rd amino acid codon of the SSU in pSSU-L. Plasmids pSSU-S and pSSU-L both have the *ocs* 3′ end (14) to provide a mRNA polyadenylylation signal for transcripts from any inserted genes and a bacterial neomycin phosphotransferase gene (15), which confers kanamycin resistance (Km^r) on both *Escherichia coli* and *Agrobacterium*.

The chimeric genes SSU-S-ATR and SSU-L-ATR were constructed by inserting the coding sequence of the *psbA* gene [modified by BAL-31 nuclease treatment of the gene from plasmid pAh484 (4)] from the atrazine-resistant biotype of *A. hybridus* behind the SSU-encoding region in vectors pSSU-S and pSSU-L (Fig. 1*A*). The inserted fragment contained the entire Q_B protein-encoding region and sequences coding for 16 amino acids beyond the most probable initiation codon ATG of the Q_B gene (Fig. 1*B*). The nucleotide sequences across the fusion junctions of the two chimeric genes were verified by sequencing (16) the DNA (Fig. 1*B*). The construction resulted in the creation of three amino acid codons between the *ss3.6* and *psbA* fusion junctions. The chimeric SSU-S-ATR and SSU-L-ATR genes would code for the fusion proteins SSU-S-Q_B and SSU-L-Q_B, respectively. If these two fusion proteins were processed at the original SSU transit-peptide-processing site, the mature proteins SSU-S-Q_B and SSU-L-Q_B should be 20 and 42 amino acids longer, respectively, than the endogenous Q_B protein, assuming that the designated ATG is the actual initiation codon used *in vivo*. The extra amino acids in SSU-L-Q_B have proved to be useful for distinguishing it from the endogenous tobacco Q_B protein in thylakoid membranes.

Atrazine Tolerance Properties of Transformed Tobacco Plants. In the growth conditions used [sugar-free Murashige–Skoog basal-salt (17) agar medium cultured at 25–26°C, 1500 lux, 16-hr day/8-hr night], normal tobacco plantlets are totally bleached by 100 μM atrazine in 10-14 days (Fig. 2*A*).

The portion of the transforming vector pGV3850 that is transferred (T-DNA) carries the gene for nopaline synthetase (7); thus, tobacco plants transformed by constructs SSU-S-ATR and SSU-L-ATR could be identified by the presence of nopaline in the regenerated plants. To test the response to atrazine of tobacco plantlets grown on agar under "tissue culture conditions," six nopaline-positive regenerated plantlets, designated SSU-S-ATR and SSU-L-ATR, and untransformed regenerated plants from callus tissue were grown up in sterile culture. Cuttings, including the top two to three leaves of plants grown under identical conditions, were transferred to atrazine-containing or control agar medium. The untransformed plant cuttings began to bleach after ≈10 days on 100 μM atrazine and were consistently totally bleached within 2 weeks (e.g., see Fig. 2*A* and Fig. 2*B Right*). Some transformed plants remained green after 14 days in culture under identical conditions (e.g., SSU-S-ATR 1 in Fig. 2*B Left*, SSU-L-ATR 12 in Fig. 2*C*, and SSU-L-ATR 6 in Fig. 2*D*), but most bleached within 3-7 days after the control plants had died. One of the transformed plants, SSU-L-ATR 6, remained green for more than 3 weeks; it developed roots in the atrazine-containing agar

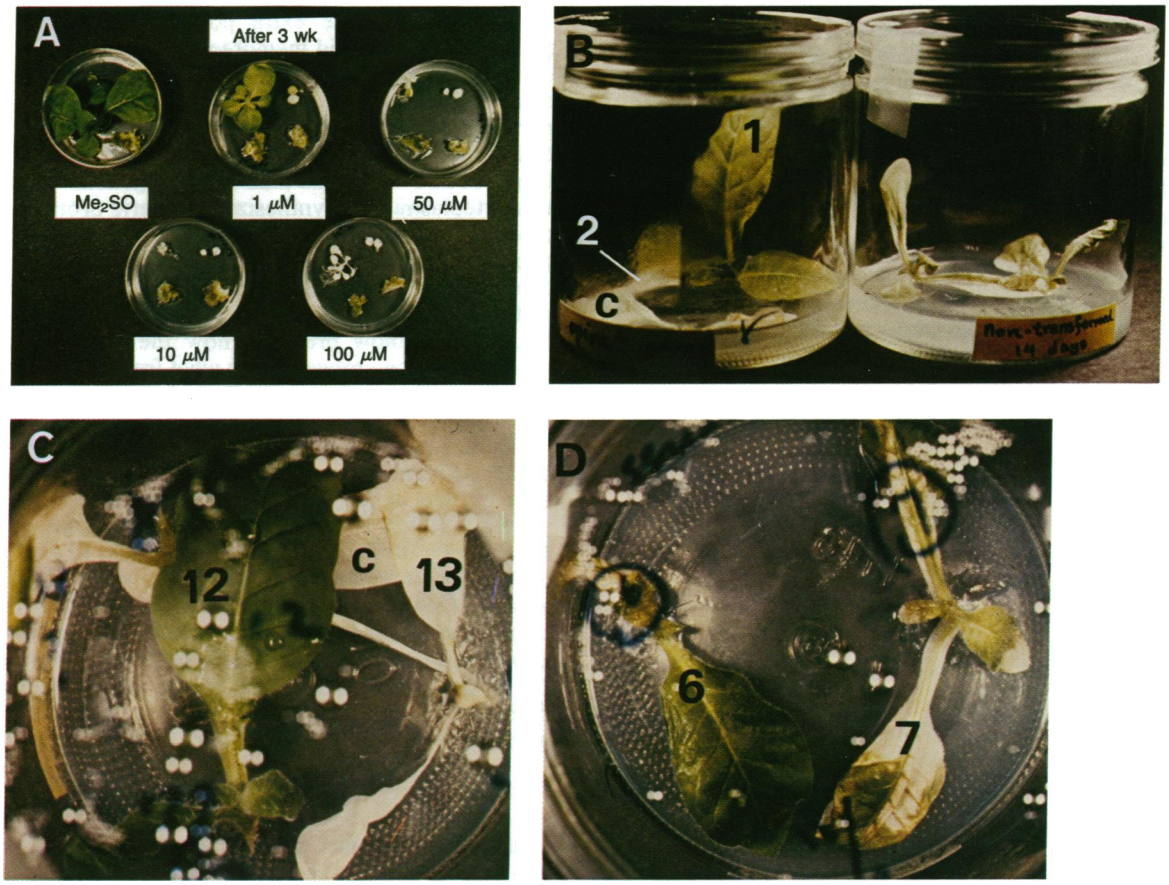


FIG. 2. Atrazine tolerance tests for tobacco. (*A*) Control experiments to establish sensitivity to a range of atrazine concentrations of tobacco grown in tissue-culture conditions. Untransformed tobacco plantlets, leaves, and callus tissues were used. The cultures were incubated for 14 days with 0, 1, 10, 50, and 100 μM atrazine. (*B*) Cuttings of normal plants (*Right*) and SSU-S-ATR-transformed tobacco plants (*Left*) after 14 days in 100 μM atrazine-containing medium. (*Left*) Plants SSU-S-ATR 1 (green) and 2 (bleached) in the left jar were both transformed and plant C (bleached) was an untransformed control. (*C*) Cuttings of transformed-plants SSU-L-ATR 12 and 13 and of a control plant C. (*D*) Cuttings of transformed-plants SSU-L-ATR 6 (green) and 7 (bleached) after 14 days in atrazine-containing medium. Me_2SO, dimethyl sulfoxide.

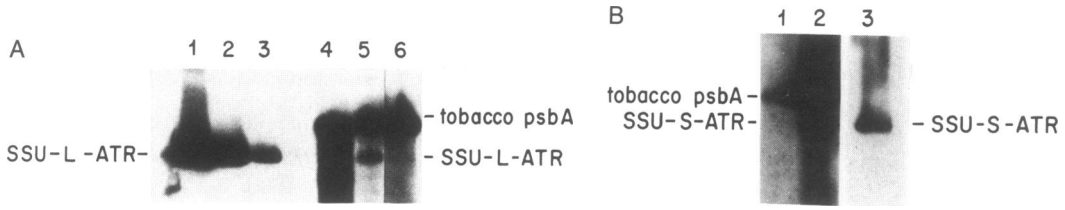


FIG. 3. Analyses of DNA isolated from regenerated SSU-S-ATR- and SSU-L-ATR-transformed plants. (*A*) Lanes: 1–3, 50, 25, and 10 copies of the original *Eco*RI/*Sal* I fragment from the plasmid pSSU-L-ATR containing the SSU-L-ATR gene; 4–6, *Eco*RI/*Sal* I-digested DNA from the control plant and from transformed-plants SSU-L-ATR 6 and 12, respectively. The fragment containing the endogenous tobacco chloroplast *psbA* gene in all of the DNA samples is indicated. (*B*) Lanes: 1 and 2, *Eco*RI/*Sal* I-digested DNA from the control plant and transformed-plant SSU-S-ATR 1; 3, original *Eco*RI/*Sal* I fragment from the plasmid pSSU-S-ATR containing the chimeric gene SSU-S-ATR.

(data not shown). However, this plant also eventually succumbed. Control plants (e.g., SSU-S-ATR C in Fig. 2*B* and SSU-L-ATR C in Fig. 2*C*) and some transformed plants (e.g., SSU-S-ATR 2 in Fig. 2*B*, SSU-L-ATR 13 in Fig. 2*C*, and SSU-L-ATR 7 in Fig. 2*D*) were indistinguishable with regard to their early bleaching behavior. Great variations in the levels of expression of genes introduced into transgenic plants have been observed frequently (18); the unaltered atrazine-sensitive phenotype exhibited by some of the transformed plants might have resulted from poor expression of the nuclear SSU-ATR fusion gene. In contrast to the results described above, all plants transformed with a chimeric *nos* promoter-mutant *psbA* gene (i.e., a nuclear mutant *psbA* gene lacking a transit peptide) were as sensitive to atrazine as were untransformed tobacco plantlets (A.Y.C. and L.B., unpublished data).

***psbA* Gene as a Nuclear Gene in Transgenic SSU-S-ATR and SSU-L-ATR Plants.** Total DNA isolated from transformed-plants SSU-L-ATR 6 and 12 and SSU-S-ATR 1 was digested to completion with *Eco*RI and *Sal* I. DNA blot analyses (10) using the *psbA* gene as a probe showed a fragment of the same size as that of the original 3-kbp SSU-L-ATR or SSU-S-ATR gene in the total DNA from the transformed plants (Fig. 3*A*, lanes 5 and 6; Fig. 3*B*, lane 2). Such fragments were absent from normal control tobacco plant DNA (Fig. 3*A*, lane 4; Fig 3*B*, lane 1). The *psbA* probe also hybridized to the endogenous tobacco chloroplast *psbA* gene fragment. Thus, the chimeric SSU-S-ATR and SSU-L-ATR gene constructs are not rearranged in the regenerated transformed-plants SSU-S-ATR 1 and SSU-L-ATR 6 and 12. When compared to copy-number reconstruction standards (Fig. 3*A*, lanes 1–3), plants 6 and 12 had about five copies and one copy, respectively, of the introduced gene per haploid genome.

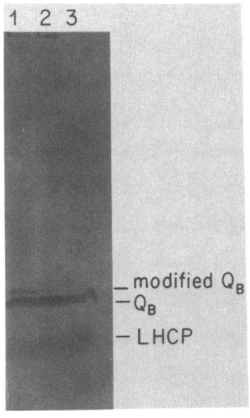


FIG. 4. Immunochemical detection of SSU-Q_B protein among the thylakoid membrane proteins from leaves of SSU-L-ATR- and SSU-S-ATR-transformed plants. Lanes: 1–3, thylakoid membrane proteins (25 μg of chlorophyll) from transformed-plants SSU-L-ATR 6 and SSU-S-ATR 1 and from control tobacco plants, respectively. The location of LHCP (light-harvesting chlorophyll a/b binding proteins; between 25 and 29 kDa) is indicated as a molecular mass reference.

Localization of the Nuclear-Cytoplasmic Synthesized Q_B Protein in Thylakoid Membranes of Transformed Plants. Protein blot analyses (12) using antibodies raised against a synthetic peptide corresponding to an internal region of the Q_B protein (Q_B-3 in ref. 13) were carried out to test for the presence of the cytoplasmically synthesized Q_B protein in the thylakoid membranes of SSU-L-ATR and SSU-S-ATR plants.

Thylakoid membrane proteins prepared from plant SSU-L-ATR 6 (Fig. 4, lane 1) showed two bands that reacted with the antibodies, whereas only one band, namely that containing the endogenous Q_B protein, was detected in the thylakoid membrane proteins of the untransformed control plant shown as well as of plant SSU-S-ATR 1 (Fig. 4, lanes 3 and 2, respectively). The extra polypeptide detected in the SSU-L-ATR thylakoid membranes is about 3–5 kDa longer than the endogenous Q_B protein. This additional size may be contributed by the extended N-terminal region of the imported, modified Q_B protein. The predicted protein product of the chimeric SSU-L-ATR gene would be 42 amino acids longer at its N terminus than the endogenous Q_B protein, if this cytoplasmically synthesized chimeric protein were processed at the site normal for the *ss3.6*-encoded SSU transit peptide. On the other hand, the increased molecular mass could also have resulted from aberrations in the processing of the cytoplasmically synthesized SSU-L-Q_B protein. We also do not know precisely how the primary translation product has been processed or what fraction of the SSU-Q_B protein synthesized in cytoplasm has been imported into chloroplasts.

The failure to detect an additional Q_B protein band in thylakoid membranes isolated from the plant SSU-S-ATR 1 may be due to the smaller molecular mass difference expected between the modified imported and the endogenous Q_B proteins, to lower levels of expression from the SSU-S-ATR gene, and/or to accumulation of less of its gene product. Stability of the protein may also be affected by minor modifications in its structure. Differences in one or all of many processes and features, ranging from transcription to posttranslational stability of the SSU-S-ATR and the SSU-L-ATR gene products, would be consistent with the variable tolerance levels in different transformed plants.

DISCUSSION

Although chloroplast genes, complete with their own promoters, are stable when inserted into the nuclear genome of tobacco via the *Agrobacterium* Ti plasmid system, transcripts are not detectable (A.Y.C. and L.B. unpublished data). In the present work, addition of (*i*) the regulatory region of a pea nuclear gene for the SSU of ribulose bisphosphate carboxylase/oxygenase and (*ii*) the DNA coding for the transit peptide of the SSU to the protein-encoding

DNA of the *A. hybridus* chloroplast gene *psbA* has permitted transcription of the chloroplast gene in the nucleus, translation of the mRNA in the cytoplasm, uptake of this protein by the chloroplast, and insertion of this protein in the photosynthetic membrane. This shows that the transit peptide of the ribulose bisphosphate carboxylase/oxygenase SSU can mediate the transport of this membrane protein across the plastid envelope and that the amino acid sequence of the Q_B protein does not prevent its uptake. These results contradict the reasonable but untested view that genes for some membrane proteins are confined to the organelle genome because an intrinsic property of an amino acid sequence might block passage of the polypeptide across the organelle envelope.

Two sets of observation encouraged us to undertake these experiments. First, the light-harvesting chlorophyll a/b protein of photosystem II is coded for by a family of nuclear genes, although the product is a component of the photosynthetic membrane (see ref. 19). Thus, at least one thylakoid membrane protein is synthesized in the cytoplasm and transported into the chloroplast to function in the photosynthetic membrane. Either the primary amino acid sequence of this thylakoid membrane protein does not interfere with its passage through the outer membranes of the chloroplast or the addition of the transit sequence overcomes such a barrier. Second, the β subunit of the maize chloroplast coupling factor (CF_1) for photosynthetic phosphorylation is about 65% homologous to the β subunit of the mitochondrial complex for phosphorylation (F_1) (20); yet the β subunit of CF_1 is coded for by a chloroplast gene and the β subunit of F_1 of bovine mitochondria is coded for by a nuclear gene. Thus, either the amino acid sequence of the β polypeptide has no bearing on whether the gene is in the nucleus or in the organelle or the addition of a transit sequence circumvents the barrier. That this is the case for the Q_B protein has been demonstrated experimentally here. The notion that the inability of a protein to be transported across membranes dictates its being synthesized within an organelle is weakened further by the observation that the large subunit of ribulose bisphosphate carboxylase/oxygenase, the product of a chloroplast gene, can be imported into yeast mitochondria when linked to a mitochondrial transit peptide (21).

One component of each photosystem II reaction center is a quinone-binding Q_B protein molecule (2). Consequently, in untransformed plants, every photosystem II reaction center contains an atrazine-sensitive Q_B protein; thus, atrazine, particularly at the high concentrations to which the plant cuttings were continuously exposed in these experiments, would block electron transport through it. Transgenic tobacco plants producing the Q_B protein-encoded by the nuclear *psbA* from the atrazine-resistant biotype of *A. hybridus* would be expected to contain a large fraction of photosystem II reaction centers with the endogenous atrazine-sensitive tobacco Q_B protein and a smaller number of reaction centers with the product of the nuclear *psbA* if the mutation in *psbA* were the basis for atrazine resistance. Only the resistant reaction centers would continue to function in the presence of high concentrations of atrazine. However, because of their relatively small number, photosynthesis would be sharply reduced and the plants might starve. Furthermore, the ineffective reaction centers would continue to absorb light and the leaves would bleach. Thus, transformed plants would survive longer than the normal ones but not necessarily indefinitely when atrazine is supplied continuously, as in our experiments. However, we have not determined whether the transgenic plants would survive under field conditions of atrazine application. Fluorescence and oxygen evolution measurements of photosynthetic electron transport by thylakoid membranes isolated from atrazine-tolerant transformed plants showed reproducible but low (between 20% and 30%) resistance to atrazine (data not shown) when compared to thylakoid membranes from untransformed tobacco.

We have converted a chloroplast gene for a membrane protein into a nuclear gene whose protein product is transported into chloroplasts, installed in the photosynthetic membrane, and functions. The experiment has produced plants whose tolerance to atrazine has been increased. This approach should be useful for learning more about the molecular basis for the development of plant cells and organelles as well as about the structural-functional aspects of chloroplast components. Besides addressing the evolutionary question of what accounts for the contemporary distribution of genes for organelle proteins, these experiments also show that it should be possible to alter chloroplast gene functions by converting modified chloroplast genes into nuclear genes and could provide an important method for altering chloroplast phenotypes for crop improvement.

We thank Dr. Anthony Cashmore for the pea SSU gene *ss3.6*, Guido Van den Broeck for sharing plasmids, and Dr. Richard Sayre for the anti-Q_B antibodies. A.Y.C. thanks Sunanda Babu for help in this work and colleagues in Ghent who kindly hosted and helped during her stay there. We also thank Drs. Harald Paulsen, Jean Lukens, and Nestor Carrillo for critical comments on this manuscript. This work was supported by a grant from the Rockefeller Foundation.

1. Bogorad, L. (1975) *Science* **188**, 891–898.
2. Steinback, K. E., Arntzen, C. J. & Bogorad, L. (1985) in *Molecular Biology of the Photosynthetic Apparatus*, eds. Steinback, K. S., Bonitz, S., Arntzen, C. J. & Bogorad, L. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 1–19.
3. Vermaas, W. F. J., Arntzen, C. J., Gu, L.-Q. & Yu, C.A. (1983) *Biochim. Biophys. Acta* **723**, 266–275.
4. Hirschberg, J. & McIntosh, L. (1983) *Science* **222**, 1346–1349.
5. Van den Broeck, G., Timko, M. P., Kausch, A. P., Cashmore, A. R., Van Montagu, M. & Herrera-Estrella, L. (1985) *Nature (London)* **313**, 358–363.
6. Cashmore, A. R. (1983) in *Genetic Engineering of Plants: An Agricultural Perspective*, eds. Kosuge, T., Meredith, C. P. & Hollaender, A. (Plenum, New York), pp. 29–38.
7. Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. & Schell, J. (1983) *EMBO. J.* **2**, 2143–2150.
8. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
9. Horsch, R. B., Fraley, R. T., Rogers, S. G., Sanders, P. R., Lloyd, A. & Hoffman, N. (1984) *Science* **223**, 496–498.
10. Bendich, A. J., Anderson, R. S. & Ward, B. L. (1981) in *Genome Organization and Expression in Plants*, ed. Leaver, C. J. (Plenum, New York), pp. 31–33.
11. Sayre, R. & Cheniae, G. M. (1982) *Plant Physiol.* **69**, 1084–1095.
12. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
13. Sayre, R. T., Andersson, B. & Bogorad, L. (1986) *Cell* **47**, 601–608.
14. DeGreve, H., Dhaese, P., Seurinck, J., Leemans, J., Van Montagu, M. & Schell, J. (1982) *J. Mol. Appl. Genet.* **1**, 499–512.
15. Vieira, J. & Messing, J. (1982) *Gene* **19**, 259–268.
16. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
17. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15**, 473–497.
18. Jones, J. D. G., Dunsmuir, P. & Bedbrook, J. (1985) *EMBO J.* **4**, 2411–2418.
19. Karlin-Neumann, G. A. & Tobin, E. M. (1986) *EMBO J.* **5**, 9–13.
20. Krebbers, E. T., Larrinua, I. M., McIntosh, L. & Bogorad, L. (1982) *Nucleic Acids Res.* **10**, 4985–5002.
21. Hurt, E. C., Goldschmidt-Clermont, M., Pesold-Hurt, B., Rochaix, J. D. & Schatz, G. (1986) *J. Biol. Chem.* **25**, 11440–11443.