Venous fragments were incubated at 37°C with collagenase (30 mg in 10 ml of KG solution) for 30 minutes. The collagenase supernatant was discarded and the tissue was exposed to three or four 15-minute incubations in 15 ml of trypsin (1 mg/ml) in KG solution. After each incubation, the supernatant was removed and placed in 10 ml of horse serum on ice. The combined supernatant fractions were then centrifuged for 15 minutes at 200*g*, washed by suspension in CV3M, and centrifuged at 200*g* for 5 minutes. The cell pellets were resuspended in 10 ml of CV3M plus 5 μ*M* 5-bromo-2′-deoxyuridine (BrdU) and sedimented in a 15-ml tissue culture flask. The muscle cells were resuspended in CV3M plus BrdU, diluted to a density of 60,000 to 70,000 cells per milliliter, and plated on poly-L-lysine–coated glass cover slips. Cells were grown in a 5% CO_2 incubator at 95% humidity and 37°C.

10. For voltage clamp analysis of inward currents, cells were placed in a perfusion chamber containing a solution (Ca10s) that contained 10 m*M* $CaCl_2$, 135 m*M* TEACl, 1 m*M* $MgCl_2$, 10 m*M* glucose, 10 m*M* Hepes, *p*H adjusted to 7.4 with TEAOH, or a Na^+-containing solution (Ca20c) which was exactly the same, except that $CaCl_2$ was 20 m*M* and TEACl and NaCl were each 67.5 m*M*. Barium was substituted for Ca^{2+} in some cases; the solutions were Ba20s, which contained 20 m*M* $BaCl_2$, 135 m*M* TEACl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* Hepes (*p*H 7.4), or Ba20c, which contained 20 m*M* $BaCl_2$, 67.5 m*M* TEACl, 67.5 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* Hepes (*p*H 7.4). The pipette solution (PEG 10) consisted of 150 m*M* cesium (Cs) glutamate, 10 m*M* CsEGTA, 5 m*M* $MgCl_2$, and 10 m*M* Hepes (*p*H adjusted to 7.4 with CsOH). After a gigaseal ($\geq 10^9$ ohms) was formed and the cell membrane at the pipette tip ruptured by suction to allow whole cell voltage clamp, ionic currents were recorded. Calcium or Na^+ currents were elicited by 350-msec depolarizations at intervals of 10 to 15 seconds. The currents were amplified by a List EPC-7 amplifier with 0.5-gigaohm feedback resistor and low-pass filtered at 500 Hz. This cutoff, chosen to optimize signal to noise ratio, would have caused an underestimate of Na^+ channel current amplitudes. All data were digitized (the sampling rate was 5000 per second) and stored on floppy disks to permit further analysis. Leak and capacitive currents were subtracted for each record by summation of currents during depolarizing pulses with linearly scaled current elicited in the same cell by 10-mV hyperpolarizing pulses.

11. A. B. Cachelin, J. E. De Peyer, S. Kokubun, H. Reuter, *J. Physiol. (London)* **340**, 389 (1983); C. J. Cohen, B. P. Bean, T. J. Colatsky, R. W. Tsien, *J. Gen. Physiol.* **78**, 383 (1981); R. Horn, J. Patlak, C. F. Stevens, *Nature (London)* **291**, 426 (1981).
12. M. Sturek and K. Hermsmeyer, unpublished observations.
13. K. Hermsmeyer and C. Kuthe, *J. Cardiovasc. Pharmacol.* **6**, 5933 (1984); K. Hermsmeyer and M. Sturek, *ibid.*, in press; C. van Breemen, P. Aaronson, R. Loutzenhiser, *Pharmacol. Rev.* **30**, 167 (1979).
14. B. P. Bean, M. Sturek, A. Puga, K. Hermsmeyer, *Circ. Res.*, in press.
15. B. P. Bean, *J. Gen. Physiol.* **86**, 1 (1985).
16. C. van Breemen, P. Aaronson, R. Loutzenhiser, *Pharmacol. Rev.* **30**, 167 (1979); G. B. Weiss, *Adv. Gen. Cell. Pharmacol.* **2**, 71 (1977); K. Hermsmeyer and M. Sturek, *J. Cardiovasc. Pharmacol.*, in press.
17. H. Reuter, *Nature (London)* **301**, 569 (1983).

18. Supported by grants HL 16328 and HL 32295 from the National Institutes of Health.

14 January 1986; accepted 22 May 1986

Engineering Herbicide Tolerance in Transgenic Plants

DILIP M. SHAH,* ROBERT B. HORSCH, HARRY J. KLEE, GANESH M. KISHORE, JILL A. WINTER, NILGUN E. TUMER, CATHY M. HIRONAKA, PATRICIA R. SANDERS, CHARLES S. GASSER, SERDAR AYKENT, NED R. SIEGEL, STEPHEN G. ROGERS, ROBERT T. FRALEY

The herbicide glyphosate is a potent inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in higher plants. A complementary DNA (cDNA) clone encoding EPSP synthase was isolated from a complementary DNA library of a glyphosate-tolerant *Petunia hybrida* cell line (MP4-G) that overproduces the enzyme. This cell line was shown to overproduce EPSP synthase messenger RNA as a result of a 20-fold amplification of the gene. A chimeric EPSP synthase gene was constructed with the use of the cauliflower mosaic virus 35*S* promoter to attain high level expression of EPSP synthase and introduced into petunia cells. Transformed petunia cells as well as regenerated transgenic plants were tolerant to glyphosate.

GLYPHOSATE IS A POTENT, BROAD-spectrum herbicide that inhibits the growth of both weed and crop species. Glyphosate (*N*-[phosphonomethyl] glycine) interferes with aromatic amino acid biosynthesis by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase of the shikimate pathway (*1–4*). Inhibition of this enzyme prevents the synthesis of chorismate-derived aromatic amino acids and secondary metabolites in plants.

Glyphosate-tolerant plant cell cultures containing elevated levels of EPSP synthase activity have been reported (*4, 5*). We have recently described a glyphosate-tolerant *Petunia hybrida* cell line (MP4-G) which was established after stepwise selection on increasing amounts of glyphosate (*6*). This cell line overproduces EPSP synthase 15- to 20-fold. In this study, we have determined that the molecular basis for glyphosate tolerance in the MP4-G cell line is the amplification of the EPSP synthase gene and demonstrate that a chimeric EPSP synthase gene, designed to overproduce EPSP synthase, confers tolerance to glyphosate in transformed petunia calli and in regenerated transgenic plants.

A homogeneous preparation of EPSP synthase (M_r, 49,000 to 55,800) was obtained from the glyphosate-tolerant MP4-G cell line (*6*). Gas-phase microsequencing (*7*) of the purified enzyme yielded a partial amino acid sequence from its NH_2-terminus. Three families of oligonucleotides (EPSP1, EPSP2, and EPSP3) were synthesized, based on codons specifying amino acid residues 8 to 13 (Fig. 1). To facilitate screening of the MP4-G complement DNA (cDNA) library, we determined which of the three families of oligonucleotides contained the correct EPSP synthase messenger RNA (mRNA) sequence. Northern blots containing polyadenylated [poly(A)$^+$] RNA's isolated from the glyphosate-sensitive MP4 cell line and the glyphosate-tolerant MP4-G cell line were hybridized with the oligonucleotide probes. Of the three oligonucleotide probes, only EPSP1 hybridized strongly to a transcript of 1.9 kb that was present in the MP4-G RNA but not in the MP4 RNA. This result suggested that the MP4-G cell line overproduces EPSP synthase mRNA and that EPSP1 contains the correct sequence of EPSP synthase mRNA.

A λgt10 cDNA library was constructed from poly(A)$^+$ RNA isolated from MP4-G cells and was screened with the EPSP1 probe. After three cycles of screening, six phage containing DNA hybridizing to the oligonucleotide probe were isolated. These phage contained cDNA inserts ranging from 180 to 330 bp—a size significantly shorter than the 1.9-kb EPSP synthase mRNA. The 330-bp fragment from one of these cDNA clones was recloned into pUC9 (pMON9531) and sequenced. The amino acid sequence deduced from the nucleotide sequence was identical to that obtained by protein sequencing (*8*). It also revealed that the enzyme was translated as a precursor polypeptide containing a transit peptide of 72 amino acids at the NH_2-terminal end (Fig. 2). A genomic clone containing a 20-kb Bam HI fragment that hybridizes to pMON9531 was isolated from a genomic library of MP4-G DNA. Restriction mapping and DNA sequencing indicated that a 4.6-kb subfragment of this clone contained sequences 3′ of the original cDNA clone. This fragment was then used as a probe to rescreen the cDNA library. Two clones con-

Plant Molecular Biology, Division of Biological Sciences, Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, MO 63198.

*To whom correspondence should be addressed.

```
          8    9   10   11   12   13
         Gln  Pro  Ile  Lys  Glu  Ile

5' CAA CCC ATT AAA GAA ATT 3'
   G   A   C   G   G   C
       T   A           A
       G

EPSP1  5' AT CTC CTT AAT AGG CTG 3'
          T   T   G   G   T

EPSP2  5' AT CTC CTT AAT CGG CTG 3'
          T   T   G   T   T

EPSP3  5' AT CTC CTT TAT CGG CTG 3'
          T   T       A   T
                      T
                      G
```

Fig. 1. The partial amino acid sequence of the petunia EPSP synthase enzyme and the sequences of the corresponding oligonucleotides. The purified EPSP synthase enzyme was obtained from the herbicide-tolerant MP4-G cell line as described previously (*6*). The protein was purified to homogeneity with a Bio-Gel TSK DEAE-5-PW column (75 by 7.5 mm) equilibrated with 50 m*M* tris-HCl, *p*H 8.0. The adsorbed proteins were eluted with the use of a linear gradient of 0 to 0.4*M* NaCl in the same buffer. Column fractions were monitored for protein and EPSP synthase activity. Automated Edman degradation chemistry was used to determine the amino terminal sequence of the purified enzyme. An Applied Biosystems model 470A protein sequencer was used for the degradations (*7*) with respective phenylthiohydantoin–amino acid derivatives identified by high-performance liquid chromatography analysis (*15*). The sequence of amino acids 8 to 13 is shown. Three families of 17 nucleotide sequences (EPSP1, EPSP2, and EPSP3), each 32-fold degenerate, were synthesized on an automated DNA synthesizer from Applied Biosystems, Inc.

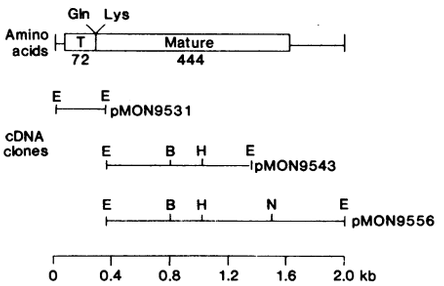


Fig. 2. Restriction maps of the EPSP synthase cDNA clones and the deduced structure of the precursor enzyme. A cDNA library was constructed from MP4-G poly(A)$^+$ RNA in the phage vector λgt10 by procedures described (*16*). This library was screened according to the standard procedures (*17*). A nitrocellulose replica filter of each plate containing the recombinant phage was made and hybridized to ^{32}P-labeled EPSP1 oligonucleotide probe in a solution containing 6× SSC (standard saline citrate), 10× Denhardt's solution, and *Escherichia coli* transfer RNA (200 μg/ml). The hybridization temperature (37°C) was 5°C below the dissociation temperature (T_d) of the oligonucleotide with the lowest GC content. The T_d of the probe was approximated by the formula 2°C × (A + T) + 4°C (G + C) (*18*). After hybridization, the filters were washed in 6× SSC twice for 15 to 20 minutes at room temperature, and then for 5 minutes at 37°C. The positively hybridizing phage were picked and carried through two more rounds of screening under identical conditions. DNA was prepared (*17*) from the cDNA clones that hybridized reproducibly to the probe. Nucleotide sequences of the cDNA inserts were determined (*8*). The lengths of the transit peptide and the mature peptide were determined from the nucleotide sequences of the cDNA clones compared to the mature protein sequence. The cleavage of the precursor polypeptide takes place between Gln^{72} and Lys^{73}. Thus, Lys is the first amino acid of the mature enzyme.

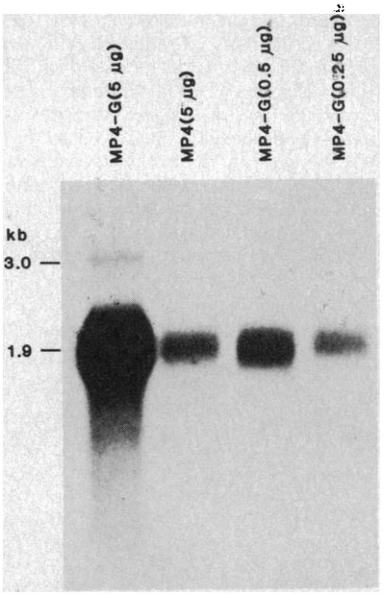


Fig. 3. Northern blot analysis of poly(A)$^+$ RNA's from MP4 and MP4-G cell lines. Poly(A)$^+$ RNA was fractionated on a 1.3% formaldehyde-agarose gel, transferred to nitrocellulose and hybridized with the nick-translated pMON9543 cDNA insert as described in Scheller *et al.* (*19*). The amount of RNA run in each lane is indicated.

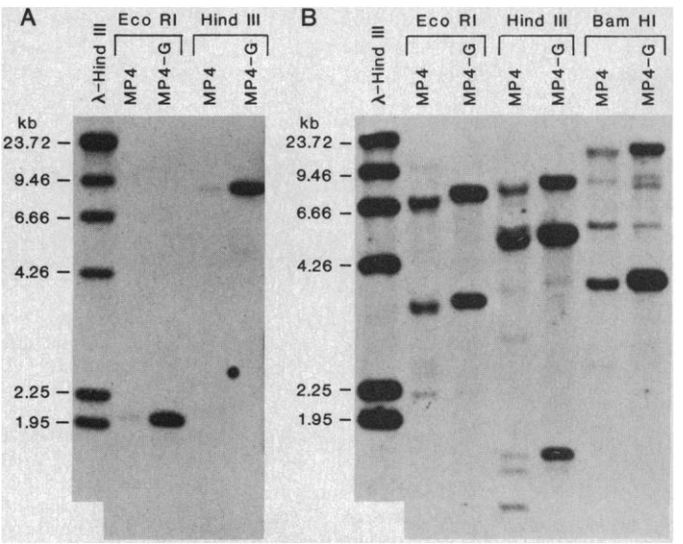
taining the cDNA inserts of 900 and 1600 bp were obtained and were recloned in pUC9 to generate pMON9543 and pMON9556, respectively (Fig. 2). The cDNA inserts in pMON9531 (330 bp) and pMON9556 (1600 bp) may be joined at their common Eco RI site to reconstitute a full-length cDNA clone which spans 27 nucleotides of the 5′ untranslated leader sequence, 72 codons of the transit peptide, 444 codons of the mature enzyme, and the entire 3′ nontranslated sequence of 0.4 kb (*8*).

The steady-state levels of EPSP synthase mRNA in the MP4 and MP4-G cell lines were compared by RNA blot analysis. A 1.9-kb mRNA was detected in the poly(A)$^+$ RNA of these cell lines (Fig. 3). This mRNA was about 20 times more abundant in the MP4-G cells. A minor 3.0-kb transcript of unknown origin was also detected in the RNA from MP4-G cells. The 20-fold increase in the 1.9-kb EPSP synthase mRNA in the tolerant cell line is consistent with 15- to 20-fold increase in the enzyme activity. This increase in EPSP synthase mRNA could result from enhanced transcription of a single gene or from gene amplification. To resolve these two possibilities, Southern blots of genomic DNA's from MP4 and MP4-G cells were hybridized with the cDNA clones, pMON9531 and pMON9556, and the copy number of EPSP synthase genes in each cell line was estimated (Fig. 4). The cDNA clones hybridized to multiple fragments in the restriction digests of both the MP4 and MP4-G genomic DNA's. The cDNA hybridization to multiple fragments in the genomic digests results from the presence of multiple introns in the EPSP synthase gene (*8*). The fragments spanning the gene are present in one copy in the MP4 genome and in approximately 20 copies in the MP4-G genome. The amplification of the EPSP synthase gene in response to glyphosate selection suggests that this enzyme is the primary target of glyphosate. Hybridization to fragments that did not undergo amplification during stepwise selection for glyphosate tolerance indicates that there is an additional EPSP synthase gene (or genes) in petunia. This gene could be a functional EPSP synthase gene or a pseudogene or a gene that is related to EPSP synthase gene.

A binary vector, pMON530, which is a derivative of pMON505, was used for *Agrobacterium*-mediated transformation of the chimeric EPSP synthase gene into petunia cells (*9*). It contains a chimeric NOS5′–NPTII–NOS3′ gene to allow direct selection of transformed plant cells for kanamycin resistance and an expression cassette consisting of the cauliflower mosaic virus (CaMV) 35*S* promoter and a nopaline synthase (NOS) polyadenylation signal. The CaMV 35*S* promoter has been shown to direct high level expression of foreign genes in plants (*10*). The complete open reading frame for petunia EPSP synthase was cloned into pMON530 (Fig. 5). The resulting plasmid, pMON546, was introduced into *Agrobacterium tumefaciens* strain GV3111-SE (*11*) containing the disarmed Ti plasmid, pTiB6S3-SE, and used to transform petunia leaf disks.

Leaf disks transformed with vectors pMON505 and pMON546 both produced large quantities of kanamycin-resistant callus that grew in nearly confluent rings as a result of transformation of many cells at the edges of each disk. This transformed callus was cut into small pieces and transferred to medium containing 0.1, 0.25, or 0.5 m*M* glyphosate.

Fig. 4. Representation of EPSP synthase sequences in the genomic DNA from MP4 and MP4-G cell lines. High molecular weight genomic DNA's were prepared from 10 to 15 g of MP4 and MP4-G cells as described (*20*). DNA's were digested with the indicated restriction enzymes, fractionated on 0.8 percent agarose gels, transferred to nitrocellulose, and hybridized with the appropriate probe (*21*). (A) Eco RI and Hind III digests of 5 μg of MP4 and MP4-G genomic DNA's were hybridized with the nick-translated pMON9531 cDNA insert. (B) Eco RI, Hind III, and Bam HI digests of MP4 and MP4-G DNA hybridized with the nick-translated pMON9556 cDNA insert. Each lane contains 5 μg of DNA; ^{32}P-labeled Hind III fragments of λ DNA were used as size markers.



The callus transformed with pMON546 continued to grow at all levels of glyphosate. In contrast, the callus containing vector pMON505 did not grow on media containing glyphosate. These results have been observed consistently with dozens of leaf disks in five separate experiments.

Glyphosate tolerance was also demonstrated by selecting directly for growth on glyphosate at 0.1, 0.25, and 0.5 m*M*. The callus is able to grow on 0.5 m*M* glyphosate from disks transformed with vector pMON546 but not vector pMON505 (Fig. 6). The clear difference between callus containing vector pMON546 and vector pMON505 confirms that the engineered EPSP synthase gene confers glyphosate tolerance to transformed plant cells. EPSP synthase activity was measured in the extracts of the transformed calli by a phosphate (P_i) release assay at *p*H 7.0 with 1 m*M* phosphoenolpyruvate and 2 m*M* shikimate-3-phosphate as substrates (*4*). The specific activity of the enzyme in crude extracts of pMON546-transformed calli selected for growth on 0.1 m*M* glyphosate was nearly 20-fold (164.8 nmol of P_i released per minute per milligram of protein) higher than that in the pMON505-transformed calli (8.24 nmol of P_i released per minute per milligram of protein). A 40-fold increase (326 nmol of P_i released per minute per milligram of protein) in the enzyme activity was detected in calli selected for growth on 0.5 m*M* glyphosate. Thus, overproduction of EPSP synthase confers glyphosate tolerance in transformed calli.

Four independent transgenic plants containing pMON546 that were selected for resistance to kanamycin were tested for glyphosate tolerance in a greenhouse by spraying them with Roundup herbicide (formulated glyphosate with surfactant) at a dose equal to 0.8 pound per acre. This was two to four times the dose required to kill 100 percent of the wild-type plants. All four independent plants containing pMON546 survived Roundup spraying and grew to maturity while all four wild-type control plants died (Fig. 7). Because the CaMV 35*S* promoter directs constitutive high level expression of foreign genes in plants (*10*), we hypothesize that glyphosate tolerance in the transformed petunia plants results from the overproduction of EPSP synthase. The results of recent subcellular fractionation studies in pea (*12*) and petunia (*13*) have shown that most of the EPSP synthase enzyme activity is localized in chloroplasts which represent a major site of aromatic amino acid biosynthesis. EPSP synthase is

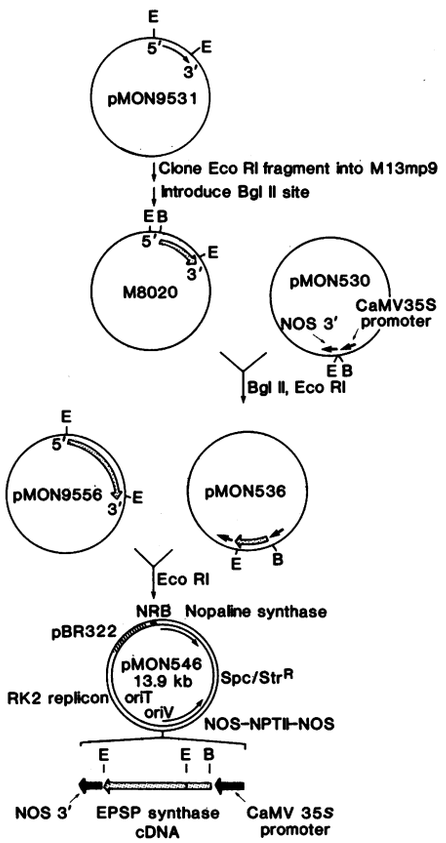


Fig. 5. Construction of pMON546 for expression of EPSP synthase cDNA in plant cells. A 330-bp Eco RI fragment from pMON9531 containing the untranslated leader as well as DNA encoding the first 101 amino acids of the precursor EPSP synthase was cloned into M13mp9 (M8017). A Bgl II site was introduced by site-directed mutagenesis (*22*) immediately upstream of the first ATG codon in the open reading frame. The 310-bp Bgl II–Eco RI fragment was then cloned into the plant expression cassette, pMON530, creating pMON536. An Eco RI fragment from pMON9556 containing the remainder of the EPSP synthase open reading frame was inserted into pMON536, thus reconstructing a full-length coding sequence under the control of the CaMV 35*S* promoter. E, Eco RI; B, Bgl II; NRB, nopaline right border sequence.

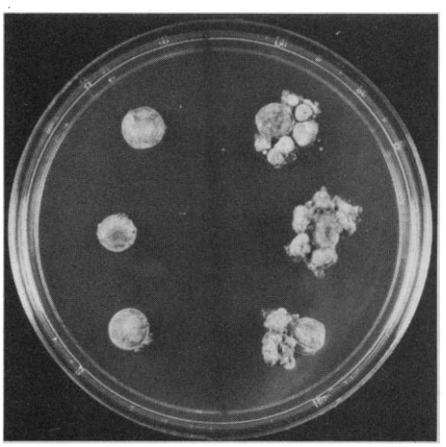


Fig. 6. Selection for glyphosate-tolerant callus from leaf disks infected with *A. tumefaciens* containing pMON546 and pMON505. Leaf disks were infected with *A. tumefaciens* strains carrying vector pMON546, which contains the chimeric EPSP synthase gene or vector pMON505 that lacks the gene as described earlier (*23*) and transferred to medium containing 0.5 m*M* glyphosate. The photograph shows the callus growth on leaf disks transformed with pMON546 (right), but no callus growth on leaf disks transformed with pMON505 (left) 6 weeks after inoculation.

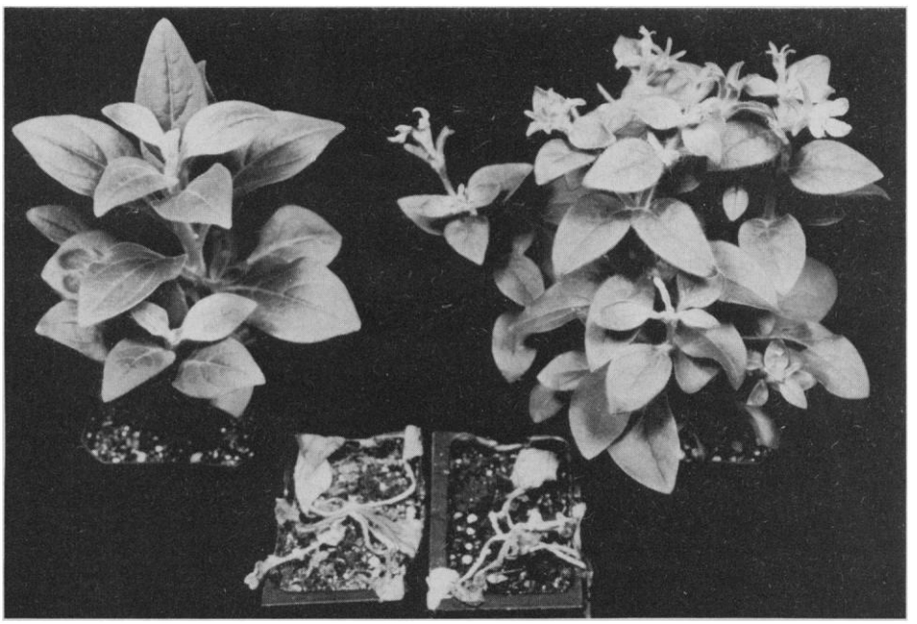


Fig. 7. Glyphosate-tolerant transgenic petunia plants. Transgenic plants were produced by the leaf disk technique (*23*) with selection for kanamycin resistance so that they would be unselected for glyphosate tolerance. These plants and the wild-type control plants were sprayed with Roundup (formulated glyphosate with surfactant) at a dose equal to 0.8 pound per acre. The plants containing pMON546 (upper) survived glyphosate spraying and grew to maturity while the control plants (lower) stopped growing and died. The photograph shows plants 3 weeks after spraying. Transgenic control plants containing pMON505 also died after glyphosate spraying.

synthesized as a precursor polypeptide containing a transit peptide of 72 amino acids. We propose that this transit peptide facilitates the transport of the precursor enzyme into chloroplasts of petunia cells. The precursor EPSP synthase is rapidly imported by isolated chloroplasts from petunia and processed to its mature form. The mature enzyme itself is not imported (*13*). It is therefore expected that in our glyphosate-tolerant plants the overproduced enzyme is localized in the chloroplasts. Comai *et al.* (*14*) have recently reported obtaining glyphosate tolerance in tobacco plants via the expression of an altered bacterial EPSP synthase gene. Given that EPSP synthase is normally localized in the chloroplast, it is surprising that a nontargeted glyphosate-tolerant EPSP synthase is able to confer herbicide tolerance. There may be enough substrate and product exchange across the chloroplast membrane to account for activity from a nontargeted bacterial EPSP synthase. Alternatively, a cytosolic pathway for the aromatic amino acid biosynthesis may exist in plant cells. In light of our observations, we feel that targeting an overproduced unaltered or altered EPSP synthase to the chloroplast via the transit peptide is a reasonable approach to attaining glyphosate tolerance in plants. Our results represent a major step toward establishing selective herbicide tolerance in crop plants.

REFERENCES AND NOTES

1. H. Steinrücken and N. Amrhein, *Biochem. Biophys. Res. Commun.* **94**, 1207 (1980).
2. D. M. Mousdale and J. R. Coggins, *Planta* **160**, 78 (1984).
3. J. L. Rubin, C. Gaines, R. A. Jensen, *Plant Physiol.* **75**, 839 (1984).
4. E. D. Nafziger, J. M. Widholm, H. C. Steinrücken, J. L. Kilmer, *ibid.* **76**, 571 (1984); C. C. Smart, D. Johaänning, G. Müller, N. Amrhein, *J. Biol. Chem.* **30**, 16338 (1985).
5. N. Amrhein, D. Johänning, J. Schab, A. Schulz, *FEBS Lett.* **157**, 191 (1983).
6. H. C. Steinrücken, A. Schulz, N. Amrhein, C. A. Porter, R. T. Fraley, *Arch. Biochem. Biophys.* **244**, 169 (1986).
7. M. Hunkapillar *et al.*, *Methods Enzymol.* **91**, 399 (1983).
8. D. M. Shah, C. S. Gasser, J. A. Winter, R. T. Fraley, in preparation.
9. H. Klee, unpublished data.
10. J. T. Odell, F. Nagy, N.-H. Chua, *Nature (London)* **313**, 810 (1985).
11. R. T. Fraley *et al.*, *Bio/Technology* **3**, 629 (1985).
12. D. M. Mousdale and J. R. Coggins, *Planta* **163**, 241 (1985).
13. G. della-Cioppa and G. M. Kishore, personal communication.
14. L. Comai *et al.*, *Nature (London)* **317**, 741 (1985).
15. M. W. Hunkapillar *et al.*, *Methods Enzymol.* **91**, 227 (1983).
16. T. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning Techniques, A Practical Approach*, D. M. Glover, Ed. (IRL Press, Oxford, 1985), pp. 49–78.
17. T. Maniatis, E. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1983).
18. S. Suggs *et al.*, in *ICN-UCLA Symposium on Developmental Biology Using Purified Genes*, D. Brown and D. Fox, Eds. (Academic Press, New York, 1981), pp. 683–693.
19. R. H. Scheller *et al.*, *Cell* **28**, 707 (1982).
20. M. Shure, S. Wessler, N. Fedoroff, *ibid.* **35**, 225 (1983).
21. N. J. Fedoroff, J. Mauvais, D. Chaleff, *J. Mol. Appl. Genet.* **2**, 11 (1983).
22. M. M. Zoller and M. Smith, *Methods Enzymol.* **100**, 468 (1983).
23. R. B. Horsch *et al.*, *Science* **227**, 1229 (1985).

24. We thank E. Jaworski for his continued support, N. Amrhein for helpful comments and stimulating discussions, and V. Grant and B. Schiermeyer for typing the manuscript.

12 December 1985; accepted 6 May 1986