Homologous Recombination in Plant Cells after Agrobacterium-Mediated Transformation

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A single amino-acid change in the acetolactate synthase (ALS) protein of tobacco confers resistance to the herbicide chlorsulfuron. A deleted, nonfunctional fragment from the acetolactate synthase gene, carrying the mutant site specifying chlorsulfuron resistance plus a closely linked novel restriction site marker, was cloned into a binary vector. Tobacco protoplasts transformed with Agrobacterium tumefaciens carrying this vector yielded chlorsulfuron-resistant colonies. DNA gel blot analysis of DNA from these colonies suggested that in three transformants homologous recombination had occurred between the endogenous ALS gene and the deleted ALS gene present in the incoming T-DNA. Plants were regenerated from these chlorsulfuron-resistant colonies, and in two of the transformants, genetic analysis of their progeny showed that the novel gene segregated as a single Mendelian locus. Possible models for the generation of these recombinant plants are discussed.

INTRODUCTION

Foreign DNA introduced into the nuclei of higher eukaryotes generally integrates into nonhomologous sequences, even when the introduced DNA has homology to endogenous sequences. This hampers attempts to direct introduced DNA sequences to homologous locations in the genome. Direct integration, via homologous recombination, of introduced DNA sequences at sites in the genome where homologous sequences exist (gene targeting) would be a powerful technique for the molecular genetic study of higher organisms.

Gene targeting by homologous recombination has been described in several eukaryotic systems, including human cells (Smithies et al., 1985), mouse cells (Thomas et al., 1986), dictyostelium (DeLozanne and Spudich, 1987; Wilke et al., 1987), yeast (Hinnen et al., 1978), and the filamentous fungi (Timberlake and Marshall, 1989). Homologous recombination between newly introduced DNA sequences and the corresponding genomic DNA sequences has been used to modify specific loci in mammalian cell culture (Smithies et al., 1985; Song et al., 1987; Doetschman et al., 1988; Adair et al., 1989). Both endogenous genes (e.g., β-globin, hprt, or aprt) and experimentally introduced genes (defective bacterial or viral genes) have been targeted. In these experiments, the ratio of homologous to nonhomologous (random insertion) recombination events ranged from $10^{-2}$ to $10^{-3}$. Gene targeting via homologous recombination has recently been reported in tobacco cells (Paszkowski et al., 1988). The target was a stably integrated defective NPTII gene that was present in multiple copies at one locus. The exogenous DNA in these experiments was introduced by polyethylene glycol-mediated transformation, and the ratio of homologous events to random insertion events was $10^{-4}$.

When exogenous DNA is introduced into plant cells by infection with Agrobacterium tumefaciens, the site of integration of the T-DNA is apparently random (Chyi et al., 1986). As a first step in investigating the suitability of Agrobacterium as a means of delivering DNA into plant cells for targeting endogenous plant genes, we were interested to see whether homologous recombination could be detected between sequences present on the T-DNA and in the plant genome. We have developed a model system in which such an event would give a selectable phenotype to any plant cells where it had occurred.

The gene we chose for this study is the acetolactate synthase (ALS) gene. ALS performs the first common step in the biosynthesis of branched-chain amino acids in plants; it is the site of action for the sulfonylurea herbicides (LaRossa and Schloss, 1984). Previous reports (Chaleff et al., 1986) have shown that there are two genetically unlinked (probably homeologous) ALS loci (SuRA and SuRB) in Nicotiana tabacum. Molecular analysis of the ALS genes from mutant and wild-type plants has demonstrated that a single amino acid replacement in the ALS protein results in a herbicide-resistant form of the enzyme (Lee et al., 1988); this effect can be mediated by mutation at either the SuRA or SuRB locus.

Here we report the results of experiments in which sulfonylurea herbicide-resistant plants were obtained after the transformation of sensitive cells with T-DNA containing
Figure 1. Construction of Mutant ALS Gene Binary Plasmids for Targeting the Endogenous ALS Genes in Tobacco.

The plasmid pAGS177 contains a modified form of the ALS gene from the SuRB locus in \textit{N. tabacum}; the thick black line corresponds to the ALS protein coding region. The location of the Trp-573-Leu mutation (+) and the introduced Apal restriction site (e) are shown. In pAGS182BV and pAGS180BV, the thick open lines correspond to the genes that encode kanamycin or hygromycin resistance; the pUCl8 plasmid is represented by the thick hatched line; and the T-DNA border regions, which define the sequence of DNA that is transferred to the plant cell, are indicated by black diamonds.

RESULTS

Targeting Strategy

The targeting DNA consists of a mutant ALS gene from the SuRB locus conferring herbicide resistance that has been inactivated by deletion of the 5’-coding sequences and promoter, as illustrated in Figure 1. The resistance mutation is a Trp \textrightarrow{} Leu change at amino acid 573; an Apal restriction site was introduced 25 bp downstream of this mutation to mark the targeting DNA. (This sequence alteration did not change the amino acid sequence of the encoded protein.) When the deleted form of the mutant ALS gene is introduced into plant cells, herbicide-resistant cells could result from (1) spontaneous mutation in either of the two endogenous \textit{SuR} loci, (2) translational fusion of the \textit{ASuRB*} fragment to an active gene, or (3) from homologous recombination between the introduced fragment of the ALS mutant gene and either of the endogenous genes. Spontaneous chlorsulfuron-resistant mutants, which occur at a frequency of \(10^{-6}\) (J. Townsend, unpublished data), can be distinguished from those resulting from homologous recombination by detection of the novel Apal restriction site. [This relies on the assumption that the novel Apal site and amino acid 573 (Trp \textrightarrow{} Leu) are sufficiently closely linked that the majority of reciprocal exchange or gene conversion events between the T-DNA and the endogenous ALS gene will involve both.] Although the coding sequences between the \textit{SuRA} and \textit{SuRB} genes are highly conserved (Lee et al., 1988) and it is possible that recombination could occur at each locus, the products of recombination at either locus can be differentiated by their restriction digest pattern. A translation fusion event or random integration of the fragment,
Homologous Recombination in Plant Cells

Figure 2. A Schematic That Shows the Predicted Outcome of Homologous Recombination between the Introduced Mutant SuRB Gene and the Endogenous ALS Genes.

The relevant restriction sites in the genomic DNA encompassing both the SuRA and SuRB genes are shown. The homologous region within the T-DNA that contains the deleted mutant form of the SuRB gene is shown (ΔSuRB*) together with the products that would result from homologous recombination or gene conversion at either the SuRA or the SuRB locus (SuRA* and SuRB*). The altered restriction fragment patterns that would result from recombination are indicated. * denotes the Trp-573→Leu mutation, Apal denotes the introduced Apal site, and the thick black line represents the ALS coding region.

together with spontaneous mutation, would not result in the introduction of the diagnostic Apal site in the ALS gene. A diagram showing the new restriction fragments that would arise from homologous recombination at either SuRA or SuRB is shown in Figure 2.

Transformation with the Nonfunctional Mutant ALS Gene Yields Chlorsulfuron-Resistant Colonies

Tobacco (cv Wisconsin 38) protoplasts were co-cultivated with A. tumefaciens carrying pAGS180BV (the control plasmid) or pAGS182BV (which carries the deleted fragment from the mutant ALS gene). After co-cultivation, protoplasts were divided and plated on chlorsulfuron (2 ng/mL) or kanamycin (50 μg/mL). The data from this experiment are summarized in Table 1. Transformation with pAGS182BV produced seven chlorsulfuron-resistant clones. Transformed calli and the regenerated plants derived from these calli were maintained on 10 ng/mL chlorsulfuron until transfer to the greenhouse. Each herbicide-resistant transformant was tested for kanamycin resistance by transferring callus pieces to plates containing 200 μg of kanamycin/mL and monitoring survival. Of the seven chlorsulfuron-resistant transformants selected, two (HR11 and HR15) were also kanamycin resistant.

Three Chlorsulfuron-Resistant Clones Result from Homologous Recombination

To determine whether the chlorsulfuron-resistant clones resulted from homologous recombination or spontaneous mutation, we performed DNA gel blot hybridizations on genomic DNA from the seven chlorsulfuron-resistant transformants. As a probe, we used an EcoRI-HindIII fragment from the SuRA gene that spans the introduced mutations and extends 450 bp in the 5' and 750 bp in the 3' direction (Figure 2).

After genomic DNA was digested with Ncol and Apal, the two endogenous ALS genes, SuRA and SuRB, gave rise to hybridizing bands of 4.7 kb and 2.0 kb. Homologous recombination between the pAGS182BV DNA and the ALS genes at SuRA or SuRB should result in the appearance of two new bands of 1.2 kb and 0.8 kb. Random insertion of pAGS182BV will give rise to the same 0.8-kb band and one additional hybridizing fragment resulting from Ncol or Apal sites in the flanking DNA. Random insertion of the control plasmid pAGS180BV (which contains the full-length chlorsulfuron resistance gene and the novel Apal site) will give a hybridization pattern identical to that of homologous pAGS182BV insertion.

Figure 3 shows the hybridization pattern for DNA isolated from three independent chlorsulfuron-resistant trans-
Table 1. Summary of Transformation Experiments

<table>
<thead>
<tr>
<th>Transformation Vector</th>
<th>Plating Efficiency</th>
<th>No. of Colonies</th>
<th>Transformation Frequency</th>
<th>Chlorsulfuron-Resistant Colonies</th>
<th>Homologous/Nonhomologous Insertion Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAGS180BV (5 x 10^5 starting protoplasts)</td>
<td>12.32%</td>
<td>5985</td>
<td>1.19%</td>
<td>1620</td>
<td></td>
</tr>
<tr>
<td>pAGS182BV (7 x 10^6 starting protoplasts)</td>
<td>15.34%</td>
<td>13.1 x 10^4</td>
<td>1.88%</td>
<td>7^a</td>
<td>8.4 x 10^-5b</td>
</tr>
</tbody>
</table>

N. tabacum protoplasts were transformed with pAGS180BV or pAGS182BV, and kanamycin or chlorsulfuron resistance was scored independently.

^a The number of chlorsulfuron-resistant colonies after pAGS180BV transformation was seven; however, four of these colonies showed a hybridization pattern that was indistinguishable from untransformed N. tabacum Wisconsin 38 DNA. Therefore, we consider these four colonies to be spontaneous mutants.

^b The homologous/nonhomologous insertion frequency is the quotient of 3/1620, which is then corrected for the relative transformation rates in the two independent transformation experiments, i.e., 1.19/1.88.

formants (HR11, HR14, and HR15) digested with Ncol and Apal. In each instance the hybridization pattern shows the bands predicted if homologous recombination had occurred. The other four chlorsulfuron-resistant transformants have hybridization patterns that are identical to untransformed tobacco (data not shown); hence, we presume that they have arisen from spontaneous mutation at the SuRA or SuRB loci. The DNA gel blot hybridization patterns for DNA from HR11, HR14, and HR15 (Figure 3A, lanes 4 to 6) show the 1.2-kb and 0.8-kb bands predicted to result from homologous recombination at the SuR loci. In DNA from HR11 and HR14, there appear to be no ALS hybridizing bands other than those attributed to homologous recombination, suggesting that additional T-DNA random insertion events have not occurred; however, in both cases when restriction digest with different enzymes were performed, the DNA gel blot hybridizations indicated that nonhomologous insertions had occurred in these transformants (see below). In the plant HR15 three extra hybridizing ALS bands occurred (3.6 kb, 3.5 kb, and 1.6 kb), suggesting three additional insertions of T-DNA in this transformant.

The DNA isolated from chlorsulfuron-resistant, kanamycin-resistant calli transformed with the positive control pAGS180BV (Figure 3A, lane 2) showed the same hybridization pattern as the putative recombinants (Figure 3A, lanes 4 to 6). DNA extracted from chlorsulfuron-sensitive, kanamycin-resistant calli transformed with pAGS182BV contained randomly inserted T-DNA and only the 0.8 kb fragment hybridized (Figure 3A, lane 3). Because this DNA was isolated from a callus population rather than an individual transformant, a unique band resulting from cleavage at the Apal site in the introduced DNA and the next Ncol or Apal site in flanking genomic DNA was not seen. A unique random insertion band did occur when DNA from a single pAGS182BV transformant was analyzed (see below).

We also carried out DNA blot hybridization after digestion of DNA with Spel, which cuts in the genomic sequences flanking the SuR loci. When tobacco genomic DNA was digested with Spel and Apal, the endogenous ALS genes gave rise to hybridizing bands of 10 kb and 3.4 kb. Homologous recombination between pAGS182BV DNA and SuRA will result in new 5.4-kb and 0.8-kb bands, whereas recombination at SuRB will result in hybridizing fragments of 2.6 kb and 0.8 kb (see Figure 2). Random insertion of pAGS182BV will give rise to the 0.8-kb fragment and an additional fragment(s) resulting from Spel or Apal sites in the flanking DNA. Random insertion of pAGS180BV will give a pattern similar to random insertion of pAGS182BV because the flanking 5' Spel site is not contained in the T-DNA of pAGS180BV.

Figure 3B shows hybridization to the genomic DNA isolated from regenerated plants after digestion with Spel and Apal. The 2.6-kb band predicted after homologous recombination at SuRB appeared only in DNA from the putative recombinants HR11, HR14, and HR15 (Figure 3B, lanes 4 to 6). The other hybridizing fragments in these DNAs represent the Spel-Apal fragments generated from the random insertion of the T-DNA carrying the targeting ALS gene into the genome. As expected, based on the Ncol-Apal hybridization patterns, HR15 had at least two other hybridizing bands in the Spel-Apal digests (3.6 kb and 1.7 kb). An additional band was also seen in HR11 (6.6 kb) DNA, suggesting at least one T-DNA random insertion (Figure 3B, lane 4). These data are consistent with the observation that HR11 and HR15 are also kanamycin resistant, indicating that they contain a functional NPTII gene. The results from Spel-Apal hybridizations showed that the novel Apal restriction site marker in the
targeting DNA was linked to a restriction site outside the SuRB gene.

In addition to characterizing the homologous recombination of sequences in the transformed plants, we have attempted to define the nonhomologous or random insertion events that have also occurred in these plant cells. To do this we have used two probes in addition to the ALS probe already described: the left-border probe is a 1.2-kb fragment that extends 900 bp into the T-DNA region (toward the selectable marker) and 300 bp beyond the 25-bp left border (Zambryski, 1988), and the NPTII probe, containing 1000 bp from the neomycin phosphotransferase gene-coding region.

When genomic DNA was digested with a restriction
enzyme such as EcoRI, which cleaves only once within the T-DNA (once in the ALS genes; Figure 2), then it was possible, by using these probes, to define the structure and number of randomly inserted T-DNAs in the genome. The data from such experiments are summarized in Table 2. In the case of HR11, we observed a single 13.5-kb EcoRI fragment that hybridized with all three probes, indicating a single random insertion of T-DNA. This indication was corroborated by genetic analysis (Table 3). The transformant HR15 appeared to have three intact T-DNA insertions (Table 2), and this was confirmed by genetic analysis (Table 3).

In HR14 an intact T-DNA insertion did not occur; the NPTII gene had apparently been deleted, and the ALS probe and the LB probe hybridized to different-sized EcoRI fragments in DNA gel blot analysis. Because there was no additional EcoRI site in the T-DNA between the EcoRI site in the ALS gene fragment and the left border, this implied that fragments of DNA containing the left border or the ALS gene had inserted separately in the genome, or that the T-DNA had undergone rearrangement, resulting in the deletion of the NPTII gene and introduction of a novel EcoRI site between the ALS fragment and the left border.

Molecular analyses of HR14 progeny plants (see below) showed that the left-border fragment and ALS gene fragment were both linked to the recombinant-resistant form of the ALS gene (data not shown).

We noted that in all of our DNA gel blot hybridizations the bands corresponding to the introduced ALS gene were generally less intense than those corresponding to the endogenous genes. This was the case irrespective of whether the plant being analyzed was the product of a homologous or nonhomologous event (see Figure 3B, lane 3). The reason for this difference in intensities is not known. We have performed reconstruction experiments with pAGSl80BV DNA added to N. tabacum var Wisconsin 38 genomic DNA to determine whether the SuRA and SuRB loci actually encompass many identical or near-identical copies of the respective ALS genes. Our data (not shown) do not support this hypothesis. A second possible explanation for the reduced intensity of the recombinant fragments in DNA blots is that there was methylation of the newly integrated DNA at the Apal restriction site. Although an Apal methylation activity is known (McClelland and Nelson, 1986), it has not been detected in plant cells. A third possibility is that the transformants are actually chi...
meric, resulting from the transformation of only one pro-
toplast in a lump of several cells that together gave rise to
the regenerated plant. However, we would expect, then,
that the band intensities would be normal when the prog-
eny were examined by DNA blotting, which was not the
case (see next section). A similar phenomenon of reduced
band intensity was sometimes observed by Paszkowski
et al. (1988) in their gene-targeting experiments in tobacco.

Chlorsulfuron Resistance Segregates as a Single
Mendelian Gene in the Recombinant Plants HR11 and
HR14

The results of genetic analysis of the transformants HR11,
HR14, and HR15 are shown in Table 2. The segregation
of chlorsulfuron resistance to sensitivity in the progeny
from self-pollinated HR14 was consistent with a 3:1 re-
sistant:sensitive ratio, indicating a single dominant locus
for chlorsulfuron resistance. All seed derived from HR14
were kanamycin sensitive. Progeny from self-pollinated
HR11 gave a ratio of chlorsulfuron resistance to sensitivity
consistent with either a 3:1 or a 2:1 ratio, indicating a
single Mendelian locus with the possibility of partial pe-
etrance or homozygous lethality. The segregation of kan-
amycin resistance in these progeny was consistent with a
single locus for kanamycin resistance. When grown on
media containing both kanamycin and chlorsulfuron, these
progeny segregated 9:7 resistant:sensitive. This sug-
gested that the markers for kanamycin resistance and
chlorsulfuron resistance were not linked (the value of $\chi^2$
for a 3:1 ratio, which would have been predicted if the two
markers were tightly linked, is 23.25, $P<0.01$).

To confirm the lack of linkage, 12 progeny from self-
pollinated HR11 were grown in the absence of selection
and then tested independently for kanamycin and chlo-
sulfuron resistance using a leaf-callusing assay. For each
antibiotic, two sensitive plants were found in the popula-
tion; no plants were found that were sensitive to both
antibiotics, confirming that the two markers were not
linked. Progeny from HR15 were not chlorsulfuron resist-
ant (0/500 seed). On kanamycin the ratio of resistant to
sensitive progeny was consistent with a 63:1 ratio, indi-
cating the presence of three independent loci for kana-
mycin resistance.

If the chlorsulfuron resistance phenotype has indeed
arisen from a homologous recombination event, then the
resistance phenotype and the novel diagnostic fragment
should be completely linked. If the event that occurred
was indeed a gene targeting (homologous recombination
at the $SuRB$ locus), then in one-third of the chlorsulfuron-
resistant progeny, the band corresponding to the parental
allele should disappear because these plants would now
be homozygous for the targeted gene. To test these
hypotheses, DNA gel blot hybridization was carried out on
the DNA from progeny derived from the self-fertilization of
both HR11 and HR14 plants. Some of these progeny were
preselected for chlorsulfuron resistance, others were
grown in the absence of chlorsulfuron and subsequently
tested for resistance in a leaf-callusing assay. Of 39 prog-
eny studied, 35 (of which 12 were preselected as chlo-
sulfuron resistant) contained the predicted Ncol-Apal or
Spel-Apal recombinant fragment. As shown in Figure 4,
lane 7, all of these progeny plants were chlorsulfuron
resistant. Four plants that did not contain the predicted
recombinant band were found to be chlorsulfuron sensitive
(Figure 4, lanes 5 and 6). However, in no case did the

![Figure 4. Hybridization to ALS Genes in Progeny of the HR14
Transformant.](image)

Ten micrograms of genomic DNA digested with Ncol and Apal
was electrophoresed on an agarose gel and transferred to a
nitrocellulose filter that was hybridized with a $^{32}$P-labeled ALS
gene fragment. DNA from *N. tabacum* leaves is shown in lane 1;
DNA was extracted from young leaves of individual transformants
in all other lanes. Lane 2 contains DNA from a chlorsulfuron-
resistant pAGS180BV transformant, and lane 3 contains DNA
from a chlorsulfuron-sensitive/kanamycin-resistant pAGS182BV
transformant. Lane 4 contains DNA from the homologous recom-
binant HR14. Lanes 5, 6, and 7 correspond to DNA isolated from
progeny of self-fertilized HR14. The phenotype with respect to
growth on chlorsulfuron is indicated: R, resistant; S, sensitive.
band corresponding to the parental SuRB gene disappear, as would have been predicted in homozygous progeny of plants in which a single gene had been targeted.

To determine whether the assayed F1 plants were homozygotes or heterozygotes, the F1 plants derived from self-fertilized HR11 and HR14 were themselves self-fertilized and their progeny scored for chlorsulfuron resistance. Seven HR14 F1 plants were tested in this way and one was homozygous; however, no homozygous plants were detected among seven HR11 F1 plants tested.

**DISCUSSION**

In this paper we presented evidence showing that homologous recombination can occur between an endogenous plant gene and a homologous gene introduced via A. tumefaciens infection. This report differs from the previous example of homologous recombination after transformation of plant cells (Paszkowski et al., 1988) in two ways: we used a different method of DNA introduction and the recombination was with an endogenous plant gene rather than one introduced experimentally. We presented several lines of evidence for homologous recombination having occurred.

First, although spontaneous mutations to the herbicide-resistant phenotype occurred at a relatively high frequency (10^{-5}), they could be distinguished from targeted events by the presence of the novel restriction enzyme site on the introduced DNA. Our analysis of the integrated structure in the primary transformants was consistent with the site of integration of the introduced gene being at the SuRB locus. This was shown by the size of the Spel-Apal fragment detected by the ALS gene probe. Homologous recombination at the SuRB locus would yield a 2.6-kb band, as was seen, whereas homologous recombination at the SuRA locus would yield a 5.4-kb band. Second, in analysis of the progeny from the self-fertilization of plants HR11 and HR14, the resistance phenotype segregated with the novel bands, as would be predicted if the chlorsulfuron-resistant phenotype were due to homologous recombination. Finally, the introduced gene was only a small fragment of the complete gene, and it is unlikely that expression of a functional gene product could be obtained even if random insertion of this fragment were to result in an in-frame fusion to a heterologous gene.

In our analysis of the F1 progeny from plants HR11 and HR14, we expected that in approximately one-third of the chlorsulfuron-resistant plants the Ncol-Apal band corresponding to the original wild-type gene (2.0 kb) would be absent because these plants would be homozygous for the targeted locus. However, of 35 chlorsulfuron-resistant F1 plants examined, all contained the bands corresponding to the wild-type genes as well as the mutant gene. There are several possible explanations for this. One is that there is more than one copy of the ALS gene at the SuRB locus in *N. tabacum* and that in our experiments we have successfully targeted only one of them. This explanation would also help explain the discrepancy in the relative intensities of the bands corresponding to the endogenous and introduced genes. We have performed in vitro reconstruction experiments that suggest that there are not a large number of ALS gene copies at the SuRA or SuRB loci; however, this type of DNA blot experiment may not be sufficiently sensitive to discriminate between one and two copies.

A second possibility is that the events we detected result from a recombination event between the incoming T-DNA and the endogenous gene such that the incoming DNA retains the resistant marker and the novel Apal DNA site and receives a functional 5' end (including the promoter) for the ALS gene. This could be followed by random insertion of the T-DNA. For this to have occurred, one end of the recombination event would have to be in a region of nonhomology because the incoming DNA has no homology to the 5' end of the ALS gene. The minimum length of nonhomologous DNA involved in this event would be 1.5 kb, which is the distance from the CiaI site (the start point for homology between the endogenous ALS gene and the ALS gene in pAGS182BV) to the Spel site upstream of the SuRB locus. Moreover, conversion of the incoming DNA, followed by its random insertion elsewhere in the genome, would be expected to yield plants with tight linkage between kanamycin and chlorsulfuron resistance, but this was not seen. In view of these two caveats, this second model seems less likely; however, more extensive mapping of the regions flanking the endogenous gene and the novel resistant gene will be required before formally excluding this possibility.

A third possibility is that the new insertion is lethal when homozygous. This can be ruled out for HR14 because we have shown genetically that one of the progeny from the self-fertilization of the original recombinant plant is homozygous for the chlorsulfuron resistance marker; it remains a possibility for plant HR11. A fourth possibility, that these events arose from trace contamination of the pAGS182BV plasmid with the control plasmid pAGS180BV, can be ruled out by the presence of the 2.6-kb Spel-Apal fragment in DNA from HR11, HR14, and HR15. The Spel site is not present in the plasmid pAGS180BV, and a fragment of 2.6-kb is not seen in Spel-Apal digest of DNA from plants transformed by this plasmid. Thus, although a homologous recombination event has clearly occurred to give rise to the chlorsulfuron resistance phenotype, the determination of the precise nature of the event or events must await more detailed analysis.

T-DNA is believed to enter the plant cell as a single-stranded DNA molecule that is associated with one or more bacterial proteins (Zambryski, 1988). Little is known of the intermediates that must exist in the plant nucleus before integration occurs, although there are some indications that circular molecules may be formed (Bakkeren...
et al., 1989). It is impossible at this stage to determine whether the recombination events described here arose from interaction of endogenous plant sequences with the same T-DNA structures that are normally intermediates for nonhomologous integration, or whether the precursors to homologous integration are different in some way from those for nonhomologous integration. However, as Agrobacterium has evolved a method of delivering its DNA into the plant nucleus and eventually the plant genome, it is not surprising that this DNA also can act as a substrate for recombination enzymes at a measurable frequency. The data presented here are insufficient for us to draw any detailed conclusions about the mechanism by which the observed recombinants arose. We can, however, rule out the possibility in plants HR11 and HR14 that they arose simply by a single cross-over event between the endogenous ALS gene and a circular form of the T-DNA because this would have integrated the NPTII gene at the same locus. The genetic analysis on the progeny of plant HR11 showed that kan^R and cf^R segregate as two independent loci. DNA gel blot hybridization analysis showed that plant HR14 contains no NPTII DNA but does contain left border homology and ALS gene homology. In EcoRI digests of DNA from HR14 progeny, we did not find any examples in which the left-border fragment segregated from the ALS gene fragment and the resistance phenotype; thus, it is possible that one end of the T-DNA was associated with the homologous recombination event from which HR14 arose.

The HR15 primary transformant was chlorsulfuron resistant, being maintained on herbicide until it was transferred to the greenhouse, as were HR11 and HR14. Based on DNA blotting and segregation data, this transformant appeared to have three independent random insertions of T-DNA in addition to the homologous recombination event. Surprisingly, none of the progeny from this plant was chlorsulfuron resistant. Genomic DNA gel blot analysis of the DNA extracted from two independently regenerated HR15 plants showed the 1.2-kb NcoI-Apal band, as expected from a homologous recombination. Leaf discs from these plants placed on callusing medium containing 200 ng/mL chlorsulfuron failed to form callus, whereas discs from HR11 and HR14 did form callus on this medium. It is possible that the HR15 plants were chimeras of tissue arising from both homologous and nonhomologous integration events, with the germinal tissue being derived entirely from tissue where nonhomologous integration had occurred. A second alternative is that the structure arising from homologous recombination may have been mitotically unstable in HR15 and may have been lost from the cells as they divided. A third possibility is that the recombinant Sur^R gene is gradually “switched off” during regeneration of HR15 plants. There are now several examples in which the expression of an introduced gene is suppressed if multiple copies of the gene are present in the cell (Goyon and Faugeron, 1989; Matzke et al., 1989; Napoli et al., 1990). This inactivation phenomenon has been correlated with de novo methylation of the duplicated DNA sequences. We have not examined the methylation status of the Sur^R gene in the HR15 transformant.

The relative frequency of homologous to nonhomologous recombination (8.4 x 10^-5) that we saw in this study is comparable with that obtained in the previous study of gene targeting in tobacco (5 x 10^-5 to 4.2 x 10^-4) (Paszowski et al., 1988). In mammalian systems the ratio of homologous to nonhomologous recombination ranges from 10^-2 to 10^-5 (Thomas and Capecechi, 1987; Doetschman et al., 1988; Adair et al., 1989) in studies that have employed a variety of target genes and methods of DNA introduction. A number of different approaches are now being pursued in mammalian systems aimed either at increasing the relative frequency of homologous to nonhomologous events or at improving the ease with which they can be detected, a factor that is of particular importance when genes with a nonselectable phenotype are being targeted. Many of the strategies being tested in these studies could also be applied to the model system described here. These include selection against nonhomologous events (Mansour et al., 1988), increasing the length of homology between the endogenous and the introduced genes (Thomas and Capecechi, 1987), and the use of the polymerase chain reaction to detect rare recombinants in a large population (Kim and Smithies, 1988).

METHODS
Construction of the Targeting Plasmid

The plasmid pAGS157 contains a mutant ALS gene (isolated from Nicotiana tabaccum var Xanthi) with three nucleotide replacements (586, 587, and 1719) that give rise to the two amino acid replacements (Pro-196 → Ala and Trp-573→Leu). Either one of these amino acid replacements confers herbicide resistance to the encoded ALS enzyme (Lee et al., 1988; C. Falco, personal communication). pAGS167 carries the corresponding wild-type gene. These plasmids were used to construct the targeting plasmid. In vitro mutagenesis (Kunkel, 1985) was used to introduce a diagnostic Apal site (AGAGCACACAGAGGCCCAGAGG) 25 bp downstream from the Trp-573→Leu mutant site in pAGS157 to form the plasmid pAGS175. This new site did not change the amino acid sequence of the protein. Subsequently, a 1.0-kb Ncol-HpaI fragment from pAGS175, containing the Pro-196→Ala mutation, was replaced by the equivalent fragment from pAGS167, containing the wild-type sequence. The resulting plasmid, pAGS177, contains the single Trp-573→Leu mutation and the introduced Apal site (Figure 1). pAGS177 was ligated between the T-DNA borders at the BamH1 site of the binary vector pAGS140 (Dean et al., 1986) to give the plasmid pAGS180BV. This plasmid was used as the positive control in the targeting experiments. Plant cells arising from transformation with this plasmid are resistant to hygromycin, kanamycin, and chlorsulfuron. An inactive form of the herbicide-resistant ALS gene with a
Transformed plants were self-pollinated and the resulting seeds were scored after 10 days to 14 days as either sensitive or resistant to 200 pg/mL kanamycin or 50 ng/mL chlorsulfuron. Seedlings were plated on MS media (Murashige and Skoog, 1962) containing 0.1 pg/mL 6-benzyl-aminopurine, and 10 ng/mL chlorsulfuron.

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