The EMBO Journal vol.9 no.10 pp.3077 – 3084, 1990

Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation

Remko Offringa[1], Marcel J.A.de Groot, Henne J.Haagsman, Mirjam P.Does, Peter J.M.van den Elzen and Paul J.J.Hooykaas

[1]MOGEN International nv, Einsteinweg 97, 2333 CB Leiden and Department of Plant Molecular Biology, Leiden University, Clusius Laboratories, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

Communicated by C.J.Leaver

We determined whether T-DNA molecules introduced into plant cells using *Agrobacterium* are suitable substrates for homologous recombination. For the detection of such recombination events different mutant versions of a NPTII construct were used. In a first set of experiments protoplasts of *Nicotiana tabacum* SR1 were cocultivated with two *Agrobacterium tumefaciens* strains. Each strain contained a different T-DNA, one carrying a 5′ deleted NPTII gene and the other a NPTII gene with a 3′ deletion. A restored NPTII gene was found in 1–4% of the protoplasts that had been cotransformed with both T-DNAs. Restoration of the NPTII gene could only be the consequence of homologous recombination between the two different T-DNAs in the plant cell, since the possibility of recombination in *Agrobacterium* was excluded in control experiments. In subsequent experiments we investigated the potential use of *Agrobacterium* for gene targeting in plants. A transgenic tobacco line with a T-DNA insertion carrying a defective NPTII gene with a 3′ deletion was transformed via *Agrobacterium* with a T-DNA containing a defective NPTII repair gene. Several kanamycin resistant plant lines were obtained with an intact NPTII gene integrated in their genome. In one of these lines the defective NPTII gene at the target locus had been properly restored. Our results show that in plants recombination can occur between a chromosomal locus and a homologous T-DNA introduced via *A.tumefaciens*. This opens the possibility of using the *Agrobacterium* transformation system for site directed mutagenesis of the plant genome.

Key words: cotransformation/NPTII gene/site directed mutagenesis/T-DNA transfer/tobacco protoplasts

Introduction

For genetic engineering of plant cells a number of methods for DNA transfer have been developed in the last few years. Gene transfer to protoplasts is accomplished via Ca/PEG precipitation (Krens *et al.*, 1982; Negrutiu *et al.*, 1987), electroporation (Shillito *et al.*, 1985), liposome mediated introduction (Deshayes *et al.*, 1985; Rosenberg *et al.*, 1988) or microinjection (Crossway *et al.*, 1986) of DNA. It is also possible to introduce DNA into plants cells via particle bombardment of plant tissues (Klein *et al.*, 1987). In addition to these methods of direct gene transfer the most commonly used procedures are based on the natural transformation system of *Agrobacterium* (reviewed by Gasser and Fraley, 1989). The high efficiency with which *Agrobacterium* can transform plant cells is demonstrated by protoplast cocultivation experiments. Twenty to 50 percent of the microcalli which regenerate from tobacco protoplasts after cocultivation with *Agrobacterium* are found to be transformed (Depicker *et al.*, 1985; Van den Elzen *et al.*, 1985). Additionally, *Agrobacterium* can introduce DNA not only into protoplasts but also into cells of explants such as leaf discs (Horsch *et al.*, 1985), tuber discs (Sheerman and Bevan, 1988) or root segments (Valvekens *et al.*, 1988). Using such tissues as targets for transformation via *Agrobacterium* allows the immediate regeneration of transgenic shoots.

Exogenous DNA which is introduced into the plant cell via any of the above mentioned transformation methods is thought to be integrated into the genome via illegitimate recombination. Little or no homology is required for efficient integration of introduced DNA molecules in the plant genome (Shillito *et al.*, 1985; Negrutiu *et al.*, 1987). This implies that integration is a more or less random process. For genetic engineering purposes it would be useful to be able to direct the integration of exogenous DNA to a particular locus in the genome of plant cells. In some lower eukaryotes like the yeast *Saccharomyces cerevisiae*, foreign DNA is predominantly integrated via homologous recombination (Hinnen *et al.*, 1978). Directed integration via homologous recombination, also called gene targeting, has been accomplished successfully in animal cells (reviewed by Capecchi, 1989). Although it occurs at low frequencies relative to normal integration of foreign DNA, gene targeting is now becoming a standard method for the site directed mutagenesis of the animal genome (Baker *et al.*, 1988; Nandi *et al.*, 1988; Zijlstra *et al.*, 1989; Zimmer and Gruss, 1989). Only a few groups have reported homologous recombination in plant cells, i.e. homologous recombination between co-infected viral DNAs of CaMV (Lebeurier *et al.*, 1982), co-introduced plasmids (Wirtz *et al.*, 1987; Baur *et al.*, 1990) or an introduced plasmid and a plasmid residing at a chromosomal locus (Paszkowski *et al.*, 1988). In all cases exogenous DNA was introduced via a method of direct gene transfer. So far, *Agrobacterium* mediated DNA transfer has not been used in experiments concerning homologous recombination in plant cells. Because *Agrobacterium* provides us with a very efficient system for transformation of plants, it would be an asset to use this system for gene targeting in plants.

Our understanding of the transfer of T-DNA molecules from *Agrobacterium* to its host plant cell is limited to the first steps, which occur in the bacterium itself. The virulence genes which reside partly on the Ti plasmid and partly on the chromosome of *Agrobacterium* are involved in the transfer process. Two imperfect 24 bp direct repeats that

flank the T-region are essential for transfer (reviewed by Melchers and Hooykaas, 1987). Several mechanisms have been proposed for the transfer of the T-DNA molecule (reviewed by Koukolikova-Nicola *et al.*, 1987). Based on available evidence it is most likely that the T-DNA is transferred as a single-stranded molecule to the plant cell (Stachel and Zambryski, 1986; Albright *et al.*, 1987). Several groups have observed that VirD2, a protein encoded by one of the virulence genes, is covalently attached to the 5′ terminus of the T-strand (Herrera-Estrella *et al.*, 1988; Ward and Barnes, 1988; Young and Nester, 1988; Howard *et al.*, 1989). Additionally another virulence gene encoded protein, VirE2, shows non-specific single-stranded DNA binding activity (Gietl *et al.*, 1987; Das, 1988; Citovsky *et al.*, 1989). Christie *et al.* (1988) demonstrated the association of VirE2 with T-DNA *in vivo*. Until now nothing has been known about transfer of the T-DNA to the plant cell and about processes involved in integration of the T-DNA. Possibly the T-DNA is protected by bacterial (virulence) proteins during its transfer and/or some of these proteins may be essential for efficient integration of the T-DNA. Protection of the 5′ terminus of T-DNA by the VirD2 protein against exonucleolytic degradation could be demonstrated *in vitro* (Dürrenberger *et al.*, 1989). Proteins attached to the T-strand might prevent homologous recombination of this DNA with homologous sequences in plants.

We performed transformation experiments to find out whether T-DNA (like naked DNA) is a suitable substrate for homologous recombination in plant cells. First homologous recombination was studied between T-DNAs that were co-introduced from different bacterial strains into tobacco protoplasts. Subsequently, a T-DNA copy was integrated into the plant genome and protoplasts containing this artificial target locus were transformed with a 'repair' T-DNA. In this way recombination between target locus and incoming T-DNA was studied.

Results

Cotransformations, experimental design

Tobacco protoplasts were cocultivated with a mixture of two *Agrobacterium tumefaciens* strains, called SDM102 and SDM201. Both strains were derived from the non-oncogenic helper strain GV2260, but harbour different binary vectors called pSDM102 and pSDM201 respectively (Figure 1A). The T-DNA region of vector pSDM102 contains a chimeric gene encoding hygromycin resistance at the left border side and a NPTII gene construct with a 5′ deletion at the right border side. Vector pSDM201 contains a T-DNA region with the 3′ deleted NPTII gene construct between its border sequences. When both T-DNAs are transferred into one plant cell an intact NPTII gene construct can theoretically be formed via homologous recombination between the two T-DNAs. Since both *Agrobacterium* strains SDM102 and SDM201 carry a defective NPTII construct on their T-DNA it was not possible to determine the transformation frequency by selection on kanamycin. For strain SDM102 the transformation frequency was determined by using the hygromycin resistance gene present on the T-DNA of plasmid pSDM102. Strain SDM200 was used to estimate the frequency with which the T-DNA of strain SDM201 is transferred to tobacco cells. The T-DNA of the binary vector of strain SDM200 (pSDM200) is similar to that of pSDM201 except that it contains an intact NPTII gene construct between right and synthetic left T-DNA borders instead of the 3′ deleted gene.

Restoration of kanamycin resistance after cotransformation

(Co-)transformation frequencies were determined in an experiment where 5×10^5 protoplasts were cocultivated with a mixture of *Agrobacterium* strains SDM102 and SDM200. Without selection an average of 7% of the treated protoplasts could be regenerated into microcalli. When primary selection was done with hygromycin or with kanamycin, 22% of these microcalli showed resistance to hygromycin and 14% were resistant to kanamycin. Of the hygromycin resistant calli 38% grew on kanamycin containing medium and 34% of the kanamycin resistant calli grew on hygromycin containing medium. Thus, with direct selection for kanamycin resistance 0.33% ($0.07 \times 0.14 \times 0.34 \times 100\%$) of the initial number of protoplasts regenerated to callus and were cotransformed with both T-DNAs. Similarly, 0.58% ($0.07 \times 0.22 \times 0.38 \times 100\%$) regenerated to callus and were cotransformed with both T-DNAs. Similarly, 0.58% ($0.07 \times 0.22 \times 0.38 \times 100\%$) regenerated to callus and were cotransformed when hygromycin was used in the primary selection. These figures are comparable with cotransformation frequencies found by Depicker *et al.* (1985).

To assess homologous recombination, 1.5×10^6 tobacco protoplasts were cocultivated with both bacterial strains SDM102 and SDM201. Assuming a cotransformation of 0.33% based on pilot experiments described above, the total number of surviving cotransformed cells in this experiment was calculated to be 5.0×10^3. After selection for

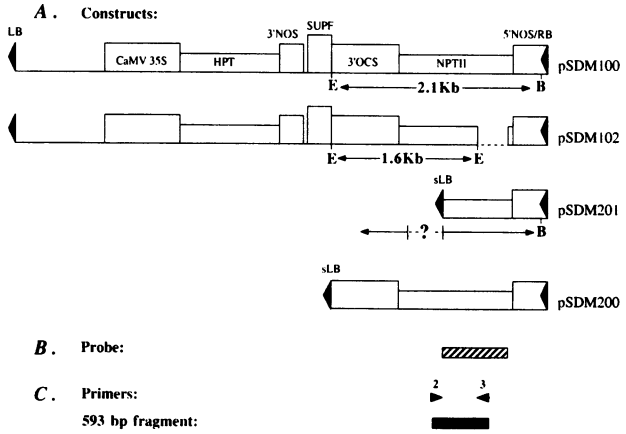

Fig. 1. (**A**) T-DNA constructs used in the cotransformation experiments and detection of a restored NPTII gene in kanamycin resistant cotransformants via molecular analysis. Abbreviations: CaMV 35S: promoter region of the CaMV 35S transcript; HPT: region coding for hygromycin phosphotransferase; 3′NOS: transcription terminator of the nopaline synthase gene; SUPF: *E.coli supF* gene; 3′OCS: transcription terminator of the octopine synthase gene; NPTII: region coding for neomycin phosphotransferase; 5′NOS/RB: promoter region of the nopaline synthase gene including the right T-DNA border repeat; LB: left nopaline T-DNA border repeat; sLB: synthetic octopine left T-DNA border repeat. The fragments that can be expected after digestion of genomic DNA with *Eco*RI (E) and *Bcl*I (B) are presented. ? indicates that the length of the fragment cannot be predicted. (**B**) The *Xma*III−*Rsr*II fragment of the NPTII gene is used as a probe to detect these fragments. (**C**) PCR analysis: primers 2 and 3 are used which anneal within the deleted regions. Only if an intact gene is present in the genome of the plant will a 593 bp fragment be amplified.

kanamycin resistance 51 calli were obtained indicating that restoration of kanamycin resistance occurred in 1% of the cotransformants (Table I). In a control experiment where 1.5×10^6 protoplasts were cocultivated with strain SDM201 alone no kanamycin resistant calli were found. This excluded both the spontaneous restoration of the 3′ deleted gene and contamination of strain SDM201 with a strain containing an intact NPTII gene on the T-region. Strain SDM102 was also tested for this purpose (see below).

Another cotransformation experiment was performed using strains SDM102 and SDM201 but now calli were selected first for hygromycin resistance. Of 2000 preselected hygromycin resistant calli, 33 were also resistant to kanamycin (Table I). From the cotransformation frequency of 38% (760 per 2000 calli) we calculated that restoration of kanamycin resistance occurred in 4% of the cotransformed calli. In the control experiment in which protoplasts were cocultivated with strain SDM102 alone, one of the 2000 hygromycin resistant calli was able to grow on kanamycin (FK-callus).

We tested whether the NPTII gene could have been restored via homologous recombination in the bacterial background. This could only be possible if transfer of the binary vectors between the two bacterial strains occurred. In conjugation experiments with a detection limit of one conjugant in 1×10^9 bacteria (see Materials and methods) transfer of the binary vector between agrobacteria was found not to occur, either in the presence or absence of plant cells. Since the number of bacteria used in the cocultivations with protoplasts did not exceed 1.5×10^8, the likelihood of restoration after recombination between the different binary vectors in *Agrobacterium* is extremely low.

Molecular evidence for the occurrence of homologous recombination

Plants were regenerated from calli having a restored kanamycin resistant phenotype. Total DNA prepared from 12 regenerated plant lines (including the FK-line) was subjected to Southern blot analysis. Figure 2 shows a Southern blot from the plant DNAs digested with *Eco*RI−*Bcl*I and probed with the 609 bp *Xma*III−*Rsr*II fragment of the NPTII coding region. If an intact gene is present a 2.1 kb fragment is expected whereas a 1.6 kb fragment is expected if the NPTII gene construct with the 5′ deletion is present and a fragment of at least 980 bp if the 3′ deleted NPTII gene construct is integrated into the plant genome (Figure 1A and B). The 2.1 kb fragment was found in nine of the plant lines tested. In six of these lines additional hybridizing fragments such as the 1.6 kb fragment could be detected, indicating that these lines contained T-DNA copies which were integrated without restoration of the NPTII gene. Plant lines 9 and 39 each showed one hybridizing fragment of aberrant size. In the FK-line a 1.6 kb and a 2.6 kb fragment were found. At least two T-DNAs were integrated in the genome of the FK-line and one of these T-DNAs carries a 5′ deleted NPTII construct. From this analysis it was concluded that a restored gene is present in at least nine of the 12 lines tested. The cleavage sites that were used to detect the wild-type size fragment of 2.1 kb are located at the edges of the NPTII gene construct. An explanation for the absence of a 2.1 kb NPTII fragment in some kanamycin resistant plant lines could be that by degradation of T-DNA sequences these restriction sites were removed without disturbing the integrity of the resistance gene. Polymerase chain reaction (PCR) amplification was performed to test whether an intact gene was present in the lines 9, 39 and in the FK-line. For this purpose a set of two primers was used, one annealing within the 5′ deletion area and the other annealing within the 3′ deletion area. Using these primers a 593 bp fragment will be amplified only if a restored gene is present (Figure 1C).

In addition to lines 9, 39 and the FK-line the lines 12, 21, 29, 32 and 37 were also analysed in this way. All plant lines tested were PCR positive except for the FK-line (Figure 3A). Two smaller fragments which were also amplified in the SR1 control appeared to be non-specific for the NPTII gene. Southern analysis of the PCR reactions confirmed that only the 593 bp fragment hybridized to the *Xma*III−*Rsr*II NPTII probe (Figure 3B). Thus, molecular analysis demonstrated that the frequency of kanamycin resistant calli in the transformation experiment accurately reflects the

Table I. Restoration of kanamycin resistance after cotransformation of T-DNAs carrying mutant NPTII gene constructs

	T-DNAs[a]	Selection[b]	Resistant calli	% Restoration[e]
No preselection[c]	102 + 200	$Hm^r + Km^r$	5.0×10^3	
	102 + 201	Km^r	51	1
	201	Km^r	0	
Preselection Hm^r	102 + 200	Km^r	7.6×10^2	
(2000 Hm^r calli)[d]	102 + 201	Km^r	33	4
	102	Km^r	1	

[a]The T-DNA constructs are described in Figure 1.
[b]Calli were selected for resistance to hygromycin (Hm^r) and/or resistance to kanamycin (Km^r).
[c]1.5×10^6 tobacco protoplasts were cocultivated with strains SDM102 and SDM201 or with strain SDM201 alone. In the control experiment 5×10^5 protoplasts were cocultivated with strains SDM102 and SDM200 and from this (co)transformation frequencies were determined (see Results). The amount of resistant cotransformed calli given in the table was calculated for a starting population of 1.5×10^6 protoplasts.
[d]Protoplasts were cocultivated with strains SDM102 and SDM201 or with strain SDM102 alone. After preselection for Hm^r 2000 calli were tested for Km^r. The number of cotransformants in a population of 2000 Hm^r calli was calculated from the control experiment (102 + 200).
[e]% restoration = no. of Km^r cotransformants/total no. of cotransformants.

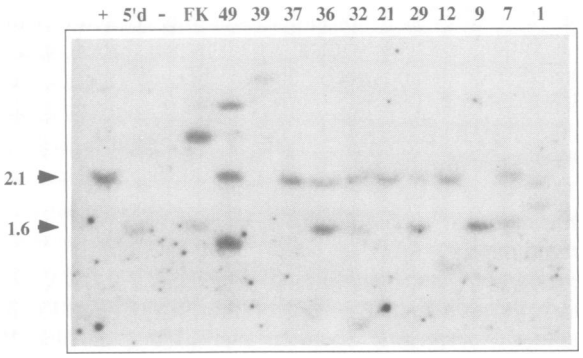

Fig. 2. Southern blot analysis of *Eco*RI−*Bcl*I digested DNA of the kanamycin resistant cotransformants (see also Figure 1). + = a tobacco plant transformed with an intact NPTII gene construct; − = a non-transgenic tobacco; 5′d = a tobacco plant with one copy of T-DNA construct SDM102 stably integrated into the genome. Numbers 1−49 and FK refer to the plant lines.

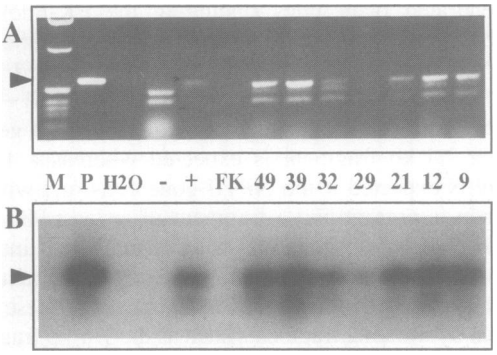

Fig. 3. (**A**) PCR analysis of several kanamycin resistant cotransformants (see also Figure 2B). The reactions were analysed on a gel and (**B**) the DNA was transferred to a nylon filter that was subsequently hybridized with the NPTII probe. P = plasmid control, i.e. PCR reaction which contains 1 pg of plasmid carrying an intact gene; H_2O = water control, i.e. PCR reaction without DNA. The arrows indicate the position of the 593 bp fragment.

recombination frequency. The FK-line obtained from the negative control experiment appeared to be an escape from the selection procedure. T-DNA sequences were found to be integrated in the plant genome of the FK-line but the presence of a restored NPTII gene could not be demonstrated. Such escapes were not found in other experiments.

Gene targeting, experimental design

From the cotransformation experiments described above we concluded that *Agrobacterium* T-DNA is a suitable substrate for homologous recombination in the plant cells. However, in general, recombination between cotransformed DNA molecules (in our case T-DNAs) is thought to occur extrachromosomally before integration in the host genome (Folger *et al.*, 1982; Kucherlapati *et al.*, 1984; Wake *et al.*, 1985; Rauth *et al.*, 1986). Therefore, we subsequently studied whether recombination can also occur between an introduced T-DNA copy and a copy that has already been integrated into the plant genome. This would reveal the potential of the *Agrobacterium* transformation system to be used for gene targeting in plant cells. A T-DNA construct containing a defective NPTII gene next to a hygromycin resistance gene was introduced into *Nicotiana tabacum* SR1 by leaf-disc transformation via *Agrobacterium*. Hygromycin resistant plants were regenerated and analysed by Southern blotting. Thus, an artificial chromosomal locus was created to which an incoming T-DNA with a NPTII repair gene could be targeted. Homologous recombination between the incoming repair T-DNA and the target locus should result in restoration of the NPTII gene so that the recombinants could be distinguished by the kanamycin resistant phenotype.

Introduction of repair T-DNA and detection of recombination

We used transgenic plant line 104 which has a T-DNA insert with a deletion in the 3′ end of the NPTII gene for our targeting experiments. Southern analysis of plant line 104 showed one *Hin*dIII border fragment hybridizing with the NPTII probe (Figures 4A and 5A) whereas two fragments were detected after hybridization with the HPT probe (Figures 4A and 5B). Apparently, two T-DNA copies were present at the same locus in an inverted orientation. Indeed, the sizes of the NPTII hybridizing junction fragments after *Hin*dIII, *Eco*RI or *Nco*I digestion, 2.5, 2.0 and 1.8 kb respectively (Figure 5A and C), were characteristic for the presence of the inverted repeat structure as shown in Figure 4A.

In two independent experiments, protoplasts of plant line 104 were cocultivated with an *Agrobacterium* strain harbouring the binary vector pSDM101. The plasmid pSDM101 contains a NPTII gene with a 5′ deletion, next to the hygromycin resistance marker (Figure 4A). The transformation experiments resulted in 285 and 281 kanamycin resistant calli respectively (Table II). In most of these calli gene targeting had not occurred. Results which are not shown here suggested that the 5′ deleted NPTII gene at the repair T-DNA had been fused to an endogenous plant gene.

In order to screen for kanamycin resistant calli in which an intact NPTII gene had been formed via homologous recombination we used the PCR technique (Figure 4B). A PCR with two primers which anneal within the region deleted in either the target NPTII gene or the repair construct should result in amplification (of a 979 bp size fragment) only if an intact NPTII gene is present. In this way a total number of 213 calli was screened. Three calli appeared to be PCR positive and plants were regenerated from these calli resulting in plants 1, 2 and 3.

Molecular evidence for gene targeting

Southern analysis was performed on plant lines 1, 2 and 3 as illustrated and described in Figures 4A, 4C and 5. The two defective NPTII genes at the target locus of plant line 104 were present on one *Hin*dIII and one *Eco*RI fragment and restoration of one of the defective gene copies at the target locus should result in a 1.2 kb shift of both the *Hin*dIII and the *Eco*RI fragments. In addition, an *Eco*RI−*Bcl*I fragment (2.1 kb) diagnostic of an intact NPTII gene should be present. Digestion of the DNA of line 1 with *Eco*RI, *Hin*dIII and *Eco*RI−*Bcl*I and hybridization with the NPTII probe resulted in these characteristic fragments (Figure 5A). Other sequences of the target locus were left unchanged in plant line 1 as shown after *Hin*dIII digestion and hybridization with the HPT probe. The *Hin*dIII T-DNA−plant DNA junction fragments (J1 and J2, see Figure 4A) that were found in the target line were still present in line 1 (Figure 5B). Two additional fragments were detected, the presence of which could only be explained by the integration of truncated copies of the repair T-DNA containing HPT sequences. Digestion with *Nco*I and hybridization with either the NPTII probe or the HPT probe confirmed the occurrence of gene targeting in line 1. A decrease in intensity of the 1.3 kb band corresponding to the junction fragment between the hygromycin marker and the NPTII construct was detected in plant line 1 with both the NPTII and the HPT probes (Figure 5C). Additionally, a 2.5 kb fragment was found, which could be expected if one of the defective NPTII genes at the target locus is restored. Thus, gene targeting had occurred in plant line 1 resulting in exact restoration of one of the defective NPTII genes at the target locus.

In lines 2 and 3 the target locus was left unchanged, although PCR analysis indicated the presence of a restored NPTII gene. The defective NPTII gene on one of the incoming T-DNAs must have been restored via recombination with the homologous target locus prior to integration of the T-DNA elsewhere in the plant genome. The restoration did not lead to the presence of the 2.1 kb *Eco*RI−*Bcl*I fragment diagnostic of the intact NPTII gene (Figure 5A). The *Bcl*I site, which

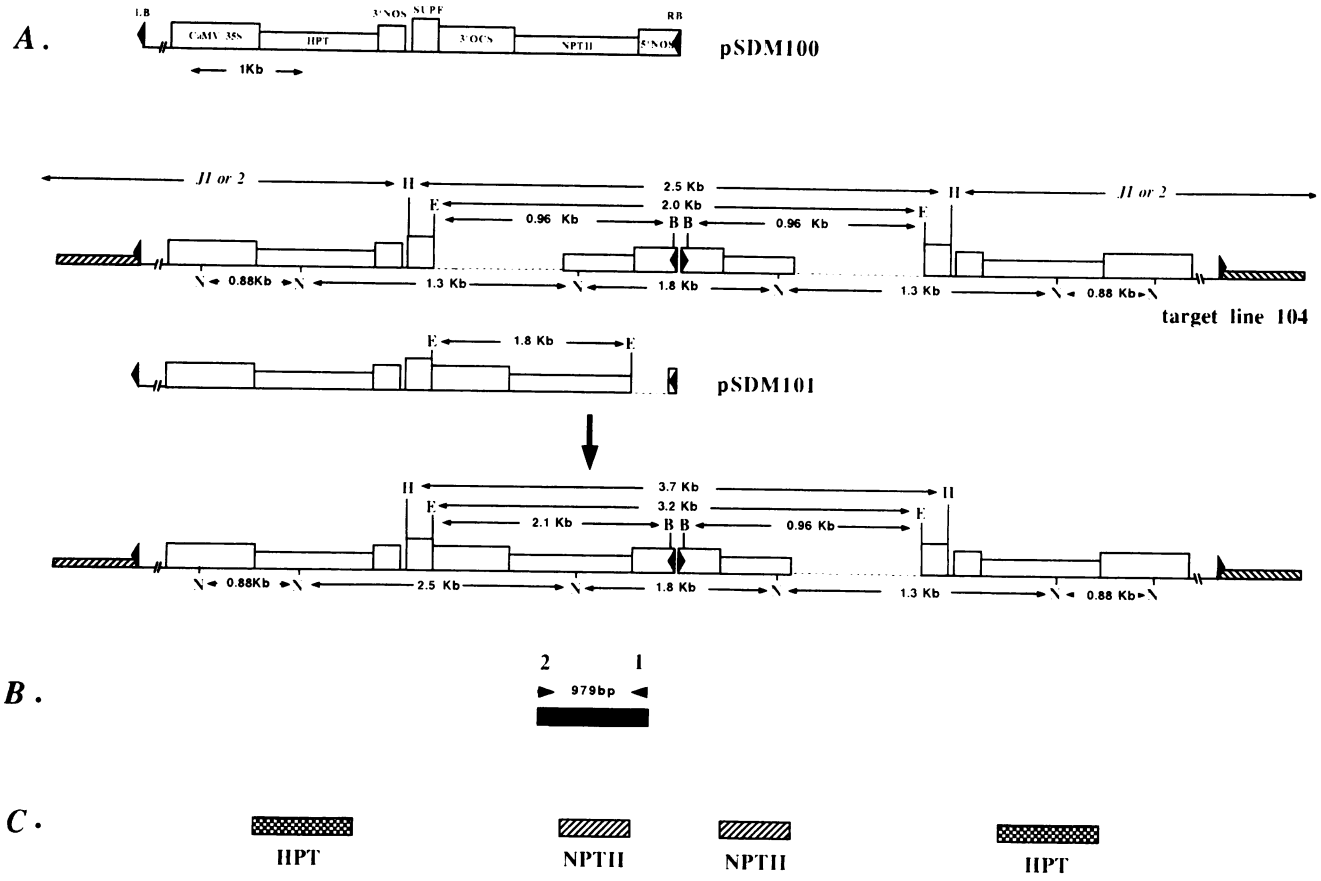

Fig. 4. Restoration of a defective NPTII gene construct present at a chromosomal locus of tobacco cells after homologous recombination with an *Agrobacterium* repair T-DNA. Schematic representation of the artificial target locus in plant line 104 and of the construct that was used in the gene targeting experiments. (**A**) Binary vectors pSDM101 and pSDM104 were both derived from pSDM100 as described in Materials and methods. For explanation of the abbreviations used see Figure 1 legend. Southern analysis was performed by digestion of genomic DNA with *Hin*dIII (H), *Eco*RI (E), *Nco*I (N) or *Eco*RI−*Bcl*I (B). The expected fragments are indicated. (**B**) A PCR with primers 1 and 2 should result in amplification of a 979 bp fragment if an intact NPTII gene is present. (**C**) DNA probes used in the Southern blot analysis. The NPTII probe comprised the 609 bp *Xma*III−*Rsr*II fragment of the NPTII coding region. The HPT probe was an *Eco*RV−*Sst*II fragment of ~900 bp containing the fusion between the 35S promoter and the coding region of the hygromycin resistance gene.

is positioned near the right T-DNA border, was apparently lost in plant lines 2 and 3 due to incomplete repair of the defective NPTII gene on the incoming T-DNA or during integration of the restored T-DNA copy elsewhere in the plant genome.

Discussion

In previous studies it was demonstrated that an enzymatic apparatus which can mediate homologous recombination in mitotically dividing cells exists in cells of higher plants (Wirtz *et al.*, 1987; Paszkowski *et al.*, 1988; Baur *et al.*, 1990). So far, only methods of direct DNA transfer were used to assess homologous recombination in plant cells. In this report we show for the first time that T-DNA molecules are suitable substrates for homologous recombination after *Agrobacterium* mediated introduction into plant cells. The frequencies of extrachromosomal recombination between T-DNAs in our experiments do not differ significantly from recombination frequencies reported for experiments in which a method of direct gene transfer was used (Wirtz *et al.*, 1987; Baur *et al.*, 1990; M.J.A.de Groot and R.Offringa, in preparation). We also show that an introduced T-DNA copy can be targeted to a homologous T-DNA that has already been integrated in the plant genome. Paszkowski *et al.* (1988) reported homologous recombination in plant cells between a chromosomally located defective kanamycin resistance gene and a repair construct introduced via a method of direct DNA transfer at a frequency of 1 in $0.2-2 \times 10^4$. In our experiments homologous recombination occurred between T-DNA and the target locus at a comparable frequency (1 in 3×10^4). It is characteristic of *Agrobacterium* mediated gene transfer that the majority of the T-DNA copies are integrated intact, whereas direct gene transfer gives rise to abundant scrambling of introduced sequences (Hain *et al.*, 1985; Czernilofsky *et al.*, 1986; Deroles and Gardner, 1988). Molecular analysis of the plant lines obtained in our experiments shows simple and easily interpretable integration patterns in contrast to the rather complex patterns found by Wirtz *et al.* (1987) and M.J.A.de Groot and R.Offringa (in preparation) after co-introduction of two defective variants of the NPTII gene into tobacco protoplasts via a direct DNA transfer method. An explanation for this observation is the apparent protection of the *Agrobacterium* T-DNA during its transfer, possibly by the VirD2 protein at the 5′ end (Herrera-Estrella *et al.*, 1988; Ward and Barnes, 1988; Young and Nester, 1988; Dürrenberger *et al.*, 1989) and by single-stranded DNA binding proteins like VirE2 (Gietl *et al.*, 1987; Das, 1988; Citovsky *et al.*, 1989; Christie *et al.*, 1988). Our results suggest that either the conformation of the proposed

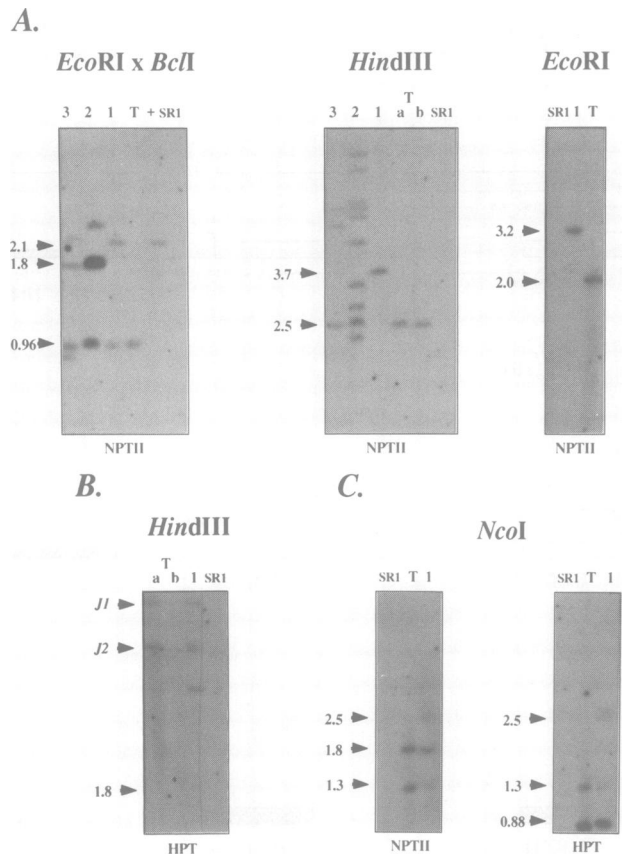

Fig. 5. Molecular evidence for gene targeting to the target locus of plant line 104 via *Agrobacterium*. (**A**) Genomic DNA of PCR-positive plants 1, 2 and 3, the target line 104 (T, Ta or Tb), a non-transgenic tobacco (SR1) and a transgenic tobacco containing an intact NPTII gene (+) was digested with *Eco*RI–*Bcl*I, *Hin*dIII or *Eco*RI. After Southern transfer the DNA was hybridized with the NPTII probe. Sizes of characteristic fragments, which are shown in Figure 4A, are indicated. (**B**) Southern blot of *Hin*dIII digested DNA of two different plants of the target line (Ta and Tb) and plant 1 hybridized with the HPT probe (see Figure 4C). Fragments J1 and J2 indicate the length of the two *Hin*dIII border fragments between the tandem T-DNA insert and the plant chromosomal DNA (see Figure 4A). The arrow indicating 1.8 kb serves as an orientation point. (**C**) Southern blot of *Nco*I digested DNA was first hybridized with the NPTII probe. Subsequently the blot was stripped and rehybridized with the HPT probe.

Table II. Gene targeting: introduction of the NPTII repair construct via *Agrobacterium tumefaciens* into tobacco protoplasts

Exp.	Plant	Plasmid[a]	pps.[b] no.	Number of calli		Ratio[d]
				transf.[c]	Km^r	
1	104	101	2.5×10^7	1.5×10^5	285	1:526
2	104	101	2.0×10^7	1.6×10^5	281	1:577

[a]Protoplasts of plant line 104 were cocultivated with bacterial strain SDM101 harbouring pSDM101 as illustrated in Figure 1A. To estimate the transformation frequency of the T-DNA of pSDM101 plasmid pSDM100 was used in additional cocultivation experiments. This plasmid is identical to pSDM101 but it has an intact NPTII gene construct between the T-DNA borders (Figure 1A).
[b]The initial number of protoplasts.
[c]Total number of transformed cells calculated from the initial number of protoplasts using the survival and transformation frequencies. In general, 5% of the initial protoplasts survived and 25% of the regenerating calli appeared to be transformed after cocultivation.
[d]The ratio of kanamycin resistant calli per number of calli transformed with the repair T-DNA.

T-DNA–protein complex is open enough to permit recombination with homologous sequences in the plant nucleus, or the protein complex is lost after the T-DNA has entered the nucleus of the plant cell.

According to the conjugal transfer model, the T-DNA is introduced into the plant cell as a single-stranded molecule. This so-called T-strand is a copy of the bottom strand of the T-region (Stachel and Zambryski, 1986). In animal cells single-stranded DNA was shown to be an excellent substrate for homologous recombination (Rauth *et al.*, 1986; Lin *et al.*, 1987). The two general hypotheses or models of homologous recombination involve the formation of single-stranded exchanges between homologous double-stranded DNA sequences which includes basepairing between complementary DNA strands (reviewed by Fincham and Oliver, 1989). Since the two homologous T-DNAs in our first experiments are thought to be co-introduced into the tobacco protoplasts as non-complementary DNA strands, at least one of these must be converted to double-stranded DNA before recombination can occur. Recombination between co-transformed T-DNAs is thought to occur extrachromosomally before integration. This is confirmed by the fact that in our experiments homologous recombination between two co-introduced T-DNA molecules in the plant cell occurred at a much higher frequency than recombination between an introduced T-DNA copy and a chromosomally located copy. Therefore, at least some of the T-DNAs have to be present in the plant nucleus as double-stranded DNA molecules before integration into the plant genome. The detection of transient expression of GUS in leaf discs soon after cocultivation with *Agrobacterium* already indicated that T-DNA must be in a transcriptionally active double-stranded form before integration into the plant genome (Janssen and Gardner, 1989).

In this report we have shown the potential of the *Agrobacterium* vector system to achieve gene targeting in plant cells. The major advantage of the *Agrobacterium* system over direct DNA transfer methods is that transformed plants can be obtained with high efficiency from both protoplasts and intact plant tissues. Therefore, we feel that our finding will have implications for the use of reverse genetics in plant research as well as for the advances of genetic engineering technology of crop plants.

Materials and methods

Plasmid constructions

For bacterial cloning *Escherichia coli* K12 strain DH5α [F$^-$, 80d*lac*Z ΔM15, Δ(*lac*ZYA-*arg*F)U169, *rec*AI, end AI *hsd*R17(r_k^-, m_k^+), *sup*E44, λ$^-$, *thi*-1, *gyr*A, *rel*AI] was used. All constructs described in this paper were derived from the binary vector pSDM100 (Figures 1 and 4A) using standard DNA techniques (Sambrook *et al.*, 1989). This plasmid contains two plant selection marker genes between the right and left T-DNA borders which code for hygromycin and kanamycin resistance respectively. A bacterial kanamycin resistance gene that was present outside the T-region could be used to select for bacteria (*E.coli* or *Agrobacterium*) harbouring pSDM100. The plasmid was obtained by modifying the T-DNA part of pBIN19 (Bevan, 1984). A hygromycin resistance gene for selection in plant cells was introduced next to the NPTII gene construct. The nopaline synthase terminator for the chimeric NPTII gene was replaced by the terminator of the octopine synthase gene. An *Eco*RI fragment carrying the *E.coli supF* gene (Seed, 1983) was inserted between the two plant resistance genes.

Plasmids pSDM101 (Figure 4A) and pSDM102 (Figure 1) contained defective NPTII genes that were obtained by replacing a part 5′ of the NPTII gene by a 10 bp *Eco*RI linker. In pSDM101 the *Bcl*I–*Xma*III fragment was deleted and in pSDM102 the deletion comprised the *Xma*III–*Tth*III.1 fragment. For the construction of the 3′ deletion mutation part of the chimeric NPTII gene was eliminated up to the *Rsr*II site in the NPTII coding region (pSDM104,

see Figure 4A). A synthetic 75 bp *Hin*dIII–*Bam*HI fragment carrying the octopine type left T-DNA border was cloned next to the 3′ deleted or the intact NPTII gene construct for the construction of pSDM201 and pSDM200 (Figure 1). A more detailed description of the constructs can be obtained on request.

Plant cell transformation

Protoplasts were prepared from leaves of 5–8 week old axenically grown tobacco plants (*N.tabacum* cv Petit Havana line SR1) by overnight incubation at 26°C in K3 0.4 M sucrose medium (Nagy and Maliga, 1976), 1% cellulase R10, 0.1% Macerozyme R10 and 0.1% MES. The protoplasts were washed once in K3 sucrose medium, diluted to 1×10^5 cells/ml in K3 medium containing 0.4 M glucose (K_3G) and distributed in batches of 7 ml in 9 cm Petri dishes. They were incubated overnight in the dark prior to cocultivation with the bacteria. *Agrobacterium* strains were grown at 29°C in LB medium containing 20 mg/l rifampicin and 50 mg/l kanamycin. End log phase cultures were diluted in K_3G medium and the bacteria were added to the protoplasts at a ratio of ~100 bacteria per protoplast. After 3 days of cocultivation the protoplasts were embedded in agarose discs by mixing 5 ml protoplasts with 5 ml 0.8% low-melting-type agarose (Sigma) in SII medium (Muller *et al.*, 1983) containing 0.1 M sucrose and 0.2 M mannitol. The bacterial growth was stopped by the addition of cefotaxim and vancomycin to final concentrations of 200 mg/l and 100 mg/l respectively. After 10 days 15 ml SII medium containing either 50 mg/l kanamycin or 10 mg/l hygromycin was added to the discs. Seven days later 15 ml SII medium with kanamycin at 100 mg/l and hygromycin at 20 mg/l was added. From this moment on the medium was refreshed weekly by replacing 15 ml old medium with 15 ml fresh SIII medium (100–150 mg/l kanamycin, 20–30 mg/l hygromycin). This medium is identical to the SII medium except for the mannitol concentration which is 0.1 M instead of 0.2 M. The plating efficiency was determined by incubation of 1/8 part of an agarose disc on liquid medium without selection. The hormone regime in the K3 and SII media was 1 mg/l NAA, 0.2 mg/l BAP and 0.1 mg/l 2,4D. In the SIII medium, 2,4D was omitted. Cefotaxim and vancomycin were added to final concentrations of 200 mg/l and 100 mg/l respectively.

Microcalli were harvested from selective or non-selective medium 4–5 weeks after embedding the protoplasts and were transferred to MS30 medium (Murashige and Skoog, 1962) containing 3% sucrose, 1.0 mg/l NAA and 0.2 mg/l BAP and solidified with 0.6% agar (Daichin). Shoots were induced on solid MS15 medium containing 1.5% sucrose, 1.0 mg/l BAP and 0.1 mg/l NAA. Solid medium also contained 100 mg/l cefotaxim and 50 mg/l vancomycin and for selection 100 mg/l kanamycin or 20 mg/l hygromycin was added.

The target line 104 was obtained by cocultivation of tobacco leaf discs with *Agrobacterium* strain SDM104 containing the binary vector pSDM104 (Horsch *et al.*, 1985). Selection for hygromycin resistant calli (20 mg/l hygromycin) was started 7 days after cocultivation. Resistant calli were transferred to shoot inducing medium.

Bacterial conjugations

The binary plasmids pSDM101, 102, 104, 200, 201, 210 and 211 were mobilized by a triparental mating (Ditta *et al.*, 1980) to a rifampicin resistant (rif^r) *A.tumefaciens* strain C58C1 harbouring the disarmed Ti plasmid pGV2260 (Deblaere *et al.*, 1985). Conjugants were selected on LB agar medium (Maniatis *et al.*, 1989) containing 20 mg/l rifampicin and 50 mg/l kanamycin.

To test whether transfer of binary vectors occurred between *Agrobacterium* strains, a donor and a recipient strain were co-incubated for 3 days at 28°C. A total of 10^9 bacteria of each strain was mixed and spotted on a nitrocellulose filter lying on either solid LB medium or on a layer of tobacco suspension cells that had been plated on solid MS30 medium containing 0.5 mg/l of the plant hormone 2,4D. In addition similar co-incubations were performed in the presence of *E.coli* helper strain RK2013 which is used in triparental matings (Ditta *et al.*, 1980). The donor strain SDM201 is rif^r and contains the binary vector pSDM201 that carries a bacterial gene for kanamycin resistance (Km^r). The recipient strain LBA285 is a spontaneous spectinomycin resistant (spc^r) derivative of strain LBA202 and does not contain any plasmid. LBA285 behaves like a wild-type recipient for Ti plasmids in conjugation experiments (Hooykaas *et al.*, 1980). If transfer of the binary vector pSDM201 should occur from SDM201 to LBA285, spc^rKm^r colonies would be found on selective plates. The bacteria were plated on LB medium containing 250 mg/l spectinomycin and 50 mg/l kanamycin after co-incubation. Resistant colonies were found at a low frequency (0.8×10^{-8}). These were not genuine transconjugants, because they were all rif^r. Indeed, incubation of strain SDM201 alone gave rise to $spc^rrif^rKm^r$ colonies at a comparable frequency. From this we concluded that these colonies represent spontaneous spc^r derivatives of strain SDM201. Transfer of the binary vector did occur when the donor and recipient strain were co-incubated together with *E.coli* helper strain RK2013 (Ditta *et al.*, 1980). This confirmed that genes essential for efficient transfer of binary vectors are not present in the strains used in our transformation experiments but have to be provided in *trans* to obtain conjugation. When *E.coli* strain RK2013 was provided as helper, the frequency of transfer after co-incubation on MS medium in the presence of plant cells (8×10^{-7}/recipient) was even lower than when co-incubation was performed on bacterial (LB) medium (1×10^{-3}/recipient).

DNA isolation and Southern analysis

Plant DNA was isolated from not fully expanded leaves of plants in the growth room as described (Mettler, 1987) and purified on a CsCl gradient. The concentration of the obtained DNA suspension was determined by measuring the OD_{260}. Approximately 10 μg of genomic DNA was used for digestion with restriction enzymes. Following separation on a 0.7% agarose TBE gel (Sambrook *et al.*, 1989) the DNA was transferred to a Hybond N membrane (Amersham; Cat. No. RPN.303N) by capillary blotting and the membrane was (pre-)hybridized according to the Hybond N protocol. Final washing was performed in 0.3 × SSC, 0.1% SDS at 65°C. DNA probes labelled with [α-^{32}P]dCTP (specific activity: $0.5-1 \times 10^9$ d.p.m./μg DNA) were obtained using the mixed primer method (Boehringer Mannheim kit; Cat. No. 10044 760).

PCR analysis

The PCRs were performed in a Sensa 949 E DNA Processor using 1 μg genomic DNA in a total volume of 100 μl. The reaction mixture contained 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% (v/v) Triton X-100, 200 μM of each nucleotide (Sigma) and 1 μM of each primer. Reactions were overlaid with 50 μl mineral oil. The DNA was denatured at 95°C for 10 min, followed by 1 min annealing of the primers at 55°C. Subsequently the temperature of the mixture was brought to 72°C and 3 U of *Taq* polymerase (Promega) were added. The actual amplification occurred during 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C and 2 min elongation at 72°C. The final elongation step proceeded for 10 min. One fifth of the reaction mixture was analysed on a 1% agarose TBE gel (Maniatis *et al.*, 1989). The sequences of the primers that were used are: PCR1: 5′-GAACTGACAGAACCGCAACG-3′; PCR2: 5′-ACCGTAAAGCACGAGGAAGC-3′; PCR3: 5′-TTGTCAAG ACCGACCTGTCC-3′.

Acknowledgements

We wish to thank Professor M.van Montagu for providing strain GV2260, Ben Dekker for advice and assistance and Monique Duyndam for her general technical assistance. In addition we thank Leo Melchers, Charles Woloshuk, Ben Cornelissen and André Hoekema for discussion and Johan Memelink for critical reading of the manuscript. This work was supported by the Netherlands Organization for the Advancement of Scientific Research (NWO).

References

Albright,L.M., Yanofsky,M.F., Leroux,B., Ma,D. and Nester,E.W. (1987) *J. Bacteriol.*, **169**, 1046–1055.

Baker,M.D., Pennell,N., Bosnoyan,L. and Shulman,M.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6432–6436.

Baur,M., Potrykus,I. and Paszkowski,J. (1990) *Mol. Cell. Biol.*, **10**, 492–500.

Bevan,M. (1984) *Nucleic Acids Res.*, **22**, 8711–8721.

Capecchi,M.R. (1989) *Science*, **244**, 1288–1292.

Christie,P.J., Ward,J.E., Winans,S.C. and Nester,E.W. (1988) *J. Bacteriol.*, **170**, 2659–2667.

Citovsky,V., Wong,M.L. and Zambryski,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1193–1197.

Crossway,A., Oakes,J.V., Irvine,J.M., Ward,B., Knauf,V.C. and Shewmaker,C.K. (1986) *Mol. Gen. Genet.*, **202**, 179–185.

Czernilofsky,A.P., Hain,R., Herrera-Estrella,L., Lörz,H., Goyvaerts,E., Baker,B.J. and Schell,J. (1986) *DNA*, **5**, 101–113.

Das,A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2909–2913.

Deblaere,R., Bytebier,B., de Greve,H., Deboeck,F., Schell,J., Van Montagu,M. and Leemans,J. (1985) *Nucleic Acids Res.*, **13**, 4777–4788.

Depicker,A., Herman,L., Jacobs,A., Schell,J. and Van Montagu,M. (1985) *Mol. Gen. Genet.*, **201**, 477–484.

Deroles,S.C. and Gardner,R.C. (1988) *Plant Mol. Biol.*, **11**, 365–377.

Deshayes,A., Herrera-Estrella,L. and Caboche,M. (1985) *EMBO J.*, **4**, 2731–2737.

Ditta,G., Stanfield,S., Corbin,D. and Helinski,D.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7347–7351.

Dürrenberger,F., Crameri,A., Hohn,B. and Koukolikova-Nicola,Z. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9154–9158.
Fincham,J.R.S. and Oliver,P. (1989) *Nature*, **338**, 14–15.
Folger,K.R., Wong,E.A., Wahl,G. and Capecchi,M.R. (1982) *Mol. Cell. Biol.*, **2**, 1372–1387.
Gasser,C.S. and Fraley,R.T. (1989) *Science*, **244**, 1293–1299.
Gietl,C., Koukolikova-Nicola,Z. and Hohn,B. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 9006–9010.
Hain,R., Stabel,P., Czernilofsky,A.P., Steinbiß,H.H., Herrera-Estrella,L. and Schell,J. (1985) *Mol. Gen. Genet.*, **199**, 161–168.
Herrera-Estrella,L., Chen,Z.-M., Van Montagu,M. and Wang,K. (1988) *EMBO J.*, **7**, 4055–4062.
Hinnen,A., Hicks,J.B. and Fink,G.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1929–1933.
Hooykaas,P.J.J., Den Dulk-Ras,H., Ooms,G. and Schilperoort,R.A. (1980) *J. Bacteriol.*, **143**, 1295–1306.
Horsch,R.B., Fry,J.E., Hoffmann,N.L., Eichholtz,D., Rogers,S.G. and Fraley,R.T. (1985) *Science*, **227**, 1229–1231.
Howard,E.A., Winsor,B.A., De Vos,G. and Zambryski,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4017–4021.
Janssen,B.-J. and Gardner,R.C. (1989) *Plant Mol. Biol.*, **14**, 61–72.
Klein,T.M., Wolf,E.D., Wu,R. and Samford,J.C. (1987) *Nature*, **327**, 70–73.
Koukolikova-Nicola,Z., Albright,L. and Hohn,B. (1987) In Hohn,T. and Schell,J. (eds), *Plant Gene Research, Plant DNA Infectious Agents*. Springer-Verlag, New York, pp. 109–148.
Krens,F.A., Molendijk,L., Wullems,G.J. and Schilperoort,R.A. (1982) *Nature*, **296**, 72–74.
Kucherlapati,R.S., Eves,E.M., Song,K.-Y., Morse,B.S. and Smithies,O. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3153–3157.
Lebeurier,G., Hirth,L., Hohn,B. and Hohn,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2932–2936.
Lin,F.L.M., Sperle,K,M. and Sternberg,N.L. (1987) *Mol. Cell. Biol.*, **7**, 129–140.
Melchers,L.S. and Hooykaas,P.J.J. (1987) *Oxford Surveys Plant Mol. Cell Biol.*, **4**, 167–220.
Mettler,I.J. (1987) *Plant Mol. Biol. Reporter*, **5**, 346–349.
Muller,J.F., Missianier,C. and Caboche,M. (1983) *Physiol. Plant*, **57**, 37–41.
Murashige,T. and Skoog,F. (1962) *Physiol. Plant*, **15**, 473–497.
Nagy,J.I. and Maliga,P. (1976) *Pflanzerphysiol.*, **78**, 453–455.
Nandi,A.K., Roginski,R.S., Gregg,R.G., Smithies,O. and Skoultchi,A.I. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3845–3849.
Negrutiu,I., Shillito,R., Potrykus,I., Biasini,G. and Sala,F. (1987) *Plant Mol. Biol.*, **8**, 363–373.
Paszkowski,J., Baur,M., Bogucki,A. and Potrykus,I. (1988) *EMBO J.*, **7**, 4021–4026.
Rauth,R., Song,K.-J., Ayares,D., Wallace,L., Moore,P.D. and Kucherlapati,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5587–5591.
Rosenberg,N., Gad,A.E., Altman,A., Navot,N. and Czosnek,H. (1988) *Plant Mol. Biol.*, **10**, 185–191.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. (2nd edn), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Seed,B. (1983) *Nucleic Acids Res.*, **8**, 2427–2445.
Sheerman,S.A. and Bevan,M.W. (1988) *Plant Cell Rep.*, **7**, 13–16.
Shillito,R.D., Saul,M.W., Paszkowski,J., Muller,M. and Potrykus,I. (1985) *Bio/Technology*, **3**, 1099–1102.
Stachel,S.E. and Zambryski,P.C. (1986) *Cell*, **47**, 155–157.
Valvekens,D., Van Montagu,M. and Van Lijsebettens,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5536–5540.
Van den Elzen,P.J.M., Lee,K.Y., Townsend,J. and Bedbrook,J.R. (1985) *Plant Mol. Biol.*, **5**, 149–154.
Wake,C.T., Vernaleone,F. and Wilson,J.H. (1985) *Mol. Cell. Biol.*, **5**, 2080–2089.
Ward,E.R. and Barnes,W.M. (1988) *Science*, **242**, 927–930.
Wirtz,U., Schell,J. and Czernilofsky,A. (1987) *DNA*, **6**, 245–253.
Young,C. and Nester,E.W. (1988) *J. Bacteriol.*, **170**, 3367–3374.
Zijlstra,M., Li,E., Sajjadi,F., Subramani,S. and Jaenisch,R. (1989) *Nature*, **342**, 435–438.
Zimmer,A. and Gruss,P. (1989) *Nature*, **338**, 150–156.

Received on February 13, 1990; revised on June 21, 1990

Note added in proof

Recently a publication by Lee *et al.* (1990, *Plant Cell*, **2**, 415–425) confirmed that homologous recombination can occur in the plant cell between an endogenous gene and a homologous gene introduced via *A.tumefaciens*. However, it could not be demonstrated unequivocally that the endogenous gene copy was changed after recombination with the homologous T-DNA, i.e. that gene targeting had occurred.