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Intermolecular Homologous Recombination in Plants

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To study DNA topological requirements for homologous recombination in plants, we have constructed pairs of plasmids that contain nonoverlapping deletions in the neomycin phosphotransferase gene [APH(3′)II], which, when intact, confers kanamycin resistance to plant cells. Protoplasts isolated from *Nicotiana tabacum* were cotransformed with complementary pairs of plasmids containing these truncated gene constructs. Homologous recombination or gene conversion within the homologous sequences (6 to 405 base pairs) of the protein-coding region of the truncated genes led to the restoration of the functional APH(3′)II gene, rendering these cells resistant to kanamycin. Circular plasmid DNAs recombined very inefficiently, independent of the length of the homologous region. A double-strand break in one molecule only slightly increased the recombination frequency. The most favorable substrates for recombination were linear molecules. In this case, the recombination frequency was positively correlated with the length of the homologous regions. The recombination frequency of plasmids linearized at sites proximal to the deletion-homology junction was significantly higher than when linearization was distal to the homologous region. Vector homology within cotransformed plasmid sequences also increased the recombination frequency.

Homologous recombination has been most thoroughly studied in procaryotes (for reviews, see references 28 and 37) and lower eucaryotes such as yeasts (for a review, see reference 20), for which classical and molecular genetics are most advanced. These studies led to the proposal of several models for the mechanism of homologous recombination (9, 17, 40). Orr-Weaver et al. (21) have shown that the frequency of recombination between genes residing on chromosomes and plasmids can be enhanced as much as 1,000-fold by the introduction of double-strand breaks in the homologous region of the plasmid. This led to the proposal of the double-strand-break repair model of recombination in yeasts (40). In recent years, extensive studies of homologous recombination in higher eucaryotic cells were performed by using cotransformed plasmids or cotransfected viral DNA (1, 10, 11, 14, 18, 31, 36, 39). The DNA plant virus cauliflower mosaic virus (CaMV) was used as a tool to detect homologous DNA recombination in plants. Coinoculation with two noninfectious but complementary viral DNAs resulted in recombined infectious molecules (12). However, the role of viral and plant proteins influencing recombination in this system was not determined.

Recent advances in gene transfer techniques (7, 23, 30, 32) allowed analysis of homologous recombination in plants at the molecular level. Among the various methods, direct gene transfer by either electroporation (35) or polyethylene glycol (PEG) treatment (19) is particularly useful. Direct gene transfer of naked DNA into protoplasts is routinely used for introducing foreign DNA into a variety of plants, resulting in efficient, integrative transformation. The foreign DNA, nonhomologous to the plant genome, was found to be integrated at sites throughout the nuclear genome (19, 24, 26). Thus, the introduced foreign DNA is most probably integrated into the plant genome by nonhomologous, so-called illegitimate, recombination.

In this paper we describe the use of direct gene transfer in the studies of plant-specific processes of homologous recombination. We have constructed pairs of plasmids that contained incomplete but overlapping regions of the hybrid marker gene coding for neomycin phosphotransferase [APH(3′)II], which, when intact, confers kanamycin resistance to plant cells (8, 24). The first set of plasmids contained the beginning of the APH(3′)II gene (the promoter region and 5′ portion of the protein-coding region). In the second corresponding set of constructs, the promoter region and 5′ portion of the protein-coding DNA were deleted. Cotransformation of pairs of the corresponding deletion derivatives into plant protoplasts would be expected to result in the restoration of a functional marker gene by homologous recombination within the common region of the APH(3′)II gene. This region extended from 6 to 405 base pairs (bp), depending on the combination of the deletion derivatives. Using a similar approach, Wirtz et al. (43) demonstrated recombination in tobacco SR1 plant cells, but detected neither correlation between frequencies of recombination and the length of the homologous region required for recombination nor any specific DNA structural characteristics necessary for recombination.

We have examined the influence of circular versus linear forms of the incoming plasmid DNA, the length of the homologous region, and vector homology on recombination frequency in *Nicotiana tabacum*.

MATERIALS AND METHODS

Isolation of protoplasts. Mesophyll protoplasts of *N. tabacum* cv. Petit Havana, line SR1 (15), were enzymatically isolated from leaves of sterile shoot cultures and washed as described by Potrykus and Shillito (27), except that protoplasts were removed from the enzyme solution by sedimentation (5 min at 100 × *g*) in W5 medium (19) (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose [pH 5.8 to 6.0]).

Transformation. Direct gene transfer by the electroporation method developed by Shillito et al. (35) or by the chemical transformation method developed by Negrutiu et

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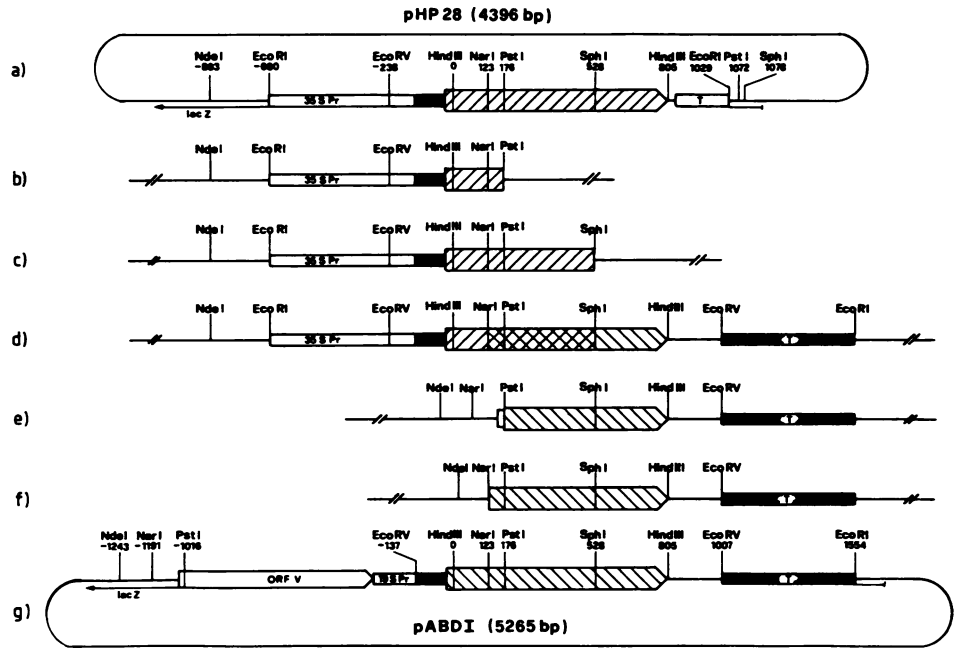

FIG. 1. Scheme of the recombination experiments. Plasmids pHP28 (line a) and pABDI (line g) described previously (22, 24), carrying different constructs of the APH(3')II selectable hybrid marker gene, were used to construct complementing deletion derivatives. The relevant restriction sites are displayed. The 5'-complementing deletion derivatives are shown in lines b and c. Plasmid pHP28ΔPstI (line b) was constructed by deletion of the *Pst*I fragment (positions 176 to 1072) of plasmid pABDI. Plasmid pHP28ΔSphI (line c) was constructed by deletion of the *Sph*I fragment (positions 528 to 1078) of plasmid pHP28. The 3'-complementing deletion derivatives are shown in lines e and f. Plasmid pABDIΔPstI (line e) was constructed by deletion of the *Pst*I fragment (positions −1016 to 176 of plasmid pABDI. Plasmid pABDIΔNarI (line f) was constructed by deletion of the *Nar*I fragment (positions −1191 to 123) of plasmid pABDI. The predicted hybrid gene resulting from a homologous recombination event within the effective homologous region of the protein-coding region of the APH(3')II gene of the two plasmids is shown in line d. An increasing length of homology was provided by the following combinations of plasmids: 6 bp, pHP28ΔPstI and pABDIΔPstI (lines b and e); 53 bp, pHP28ΔPstI and pABDIΔNarI (lines b and f); 352 bp, pHP28ΔSphI and pABDIΔPstI (lines c and e); and 405 bp, pHP28ΔSphI and pABDIΔNarI (lines c and f). Numbers above the restriction sites show their relative position (in base pairs) with respect to the *Hin*dIII site "0" at the 5' end of the APH(3')II protein-coding region. The straight line represents *E. coli* plasmid DNA of pBR322, pUC8 (pABDI), and pUC19 (pHP28). The polarity of the pUC vectors is indicated by the *lacZ* gene. Symbols: ■, promoter and terminator regions of the CaMV 19S RNA transcript; □, end of open reading frame V (ORF V) and the beginning of open reading frame VI of CaMV; □, promoter (Pr) and terminator (T) regions of the CaMV 35S RNA transcript; ▨, APH(3')II protein-coding region of plasmid pHP28; ▧, APH(3')II protein-coding region of plasmid pABDI; ▩, region of homology within which homologous recombination must take place to restore the functional APH(3')II gene.

al. (19) was used for introducing foreign DNA into protoplasts of *N. tabacum*.

For chemical transformation (19), freshly isolated protoplasts were adjusted to a density of 1.6×10^6/ml in 0.5 M mannitol–15 mM $MgCl_2$–0.1% (wt/vol) 2-(*N*-morpholino) ethanesulfonic acid (MES; pH 5.6). Aliquots of 0.75 ml were distributed in 12-ml sterile plastic tubes, heat shocked in a water bath at 45°C for 5 min, and then rapidly cooled room temperature by being placed on ice. During the next 5 to 10 min, DNA dissolved in H_2O (60 μl) was added to the protoplasts. The DNA contained a mixture of plasmid DNA (12 μg) (in cotransformation experiments 12 μg of each plasmid was used) and 60 μg of carrier DNA (calf thymus DNA; Sigma Chemical Co., St. Louis, Mo.) sheared to an average size of 4 kbp. Plasmid and ct-DNA were sterilized by ethanol precipitation (23). Following DNA addition, 0.81 ml of PEG solution [40% (wt/vol) PEG 4000 (E. Merck AG, Darmstadt, Federal Republic of Germany) in 0.4 M mannitol–0.1 M $Ca(NO_3)_2$ adjusted to pH 8.0] was added. After 20 to 30 min of incubation at room temperature, the protoplasts were washed by gradually adding 9 ml of W5 medium over about 10 min, sedimented by centrifugation for 5 min at 100 × *g*, and embedded into agarose-solidified medium (32, 34).

For direct gene transfer by the electroporation method (32, 35), protoplasts were suspended at a density of 1.6×10^6/ml in 0.5 M mannitol solution containing 6 mM $MgCl_2$ and 0.5% (wt/vol) MES (pH 5.6) after being washed twice with W5 medium. The protoplasts were transferred to a 1-ml electroporation chamber (DIALOG GmbH, Düsseldorf, Federal Republic of Germany), and the resistance of the suspension was adjusted to 0.4 kΩ with $MgCl_2$. Protoplasts were distributed in 0.75-ml aliquots and heat shocked at 45°C for 5 min. Plasmid DNA (12 μg) (in cotransformation experiments, 12 μg of each plasmid was added) and calf thymus DNA (60 μg) in a total volume of 60 μl were then added to the chamber, followed by 0.25 ml of PEG solution (24% PEG 6000 [Merck] in 0.4 M mannitol [pH 5.6] with the resistance adjusted to 0.4 kΩ with $MgCl_2$). After 10 min of incubation at room temperature, three pulses (1.4 kV, 30 nF, with a decay time of approximately 12 μs) were applied at 7-s intervals. Protoplasts were then embedded in agarose-solidified medium (32, 34).

Culture and selection of transformed clones. After 1 week of growth of the protoplasts in a thin agarose layer, kanamycin-resistant (Kan^r) clones were selected in the agarose-bead-type culture system (32, 34) containing 50 mg of kanamycin sulfate per liter. The number of Kan^r colonies

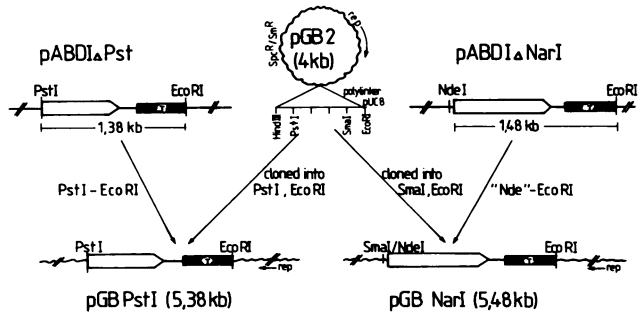

FIG. 2. Construction of 3′ complementing deletion derivatives of the APH(3′)II gene in plasmid pGB2 which show no homology to pUC-type plasmids. Plasmid pGB-PstI was constructed by insertion of the *Pst*I-*Eco*RI fragment of plasmid pABDIΔPstI into the *Pst*I and *Eco*RI sites of plasmid pGB2. After the cohesive ends of *Nde*I sites had been filled in with T4 DNA polymerase, the "*Nde*I"-*Eco*RI fragment of plasmid pABDIΔNarI was inserted into the *Sma*I and *Eco*RI sites of plasmid pGB2 to create plasmid pGB-NarI. Symbols: —, pUC sequences; ∿∿, pGB2 sequences; □, 3′ coding region of the APH(3′)II gene; ■, termination region of the 19S RNA transcript of CaMV. Abbreviation: Spc^r/Sm^r, spectinomycin and streptomycin resistance genes.

was scored after 6 to 9 weeks of culture under selective conditions.

Plant DNA isolation. Total genomic DNA was isolated from Kan^r calli or leaves of transgenic plants by the method described by Paszkowski and Saul (23). Plant tissue (0.5 to 2.0 g of callus or leaf) was homogenized in 5 to 7 ml of ice-cold buffer I (15% [wt/vol] sucrose, 50 mM Tris hydrochloride [pH 8.0], 50 mM disodium EDTA, 250 mM NaCl) with a Dounce glass homogenizer. The homogenate was centrifuged for 7 min at 2,900 × *g*, the pellet was suspended in 10 ml of ice-cold buffer II (15% [wt/vol] sucrose, 50 mM Tris hydrochloride [pH 8.0], 50 mM disodium EDTA), and sodium dodecyl sulfate (SDS) was added to a final concentration of 0.2% (wt/vol). The samples were then incubated at 70°C for 20 min. After they had cooled to room temperature, 1 ml of 5 M potassium acetate was added, and the solution was incubated for 1 h on ice. The samples were then centrifuged for 20 min at 3,600 × *g*, and 2.5 volumes of room-temperature ethanol were added to the supernatant. The formed precipitate was sedimented for 10 min at 3,600 × *g*, and the pellet was dissolved in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM disodium EDTA [pH 8.0]) and purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient. DNA was collected from the gradient and further processed by the method of Maniatis et al. (16).

Southern blot analysis of plant DNA. Approximately 7 μg of total genomic DNA was digested with a fivefold unit excess of the appropriate restriction enzyme. The DNA fragments were separated in a 0.8% agarose gel in TBE buffer (16). After electrophoresis, the gel was subjected to two 15-min treatments with 0.25 N HCl and then rinsed in water. Capillary transfer to Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) was carried out for at least 8 h as described by Reed and Mann (29). The filters were neutralized in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and prehybridized in 1.5× SSPE (20 mM disodium EDTA, 0.12 mM NaOH, 0.2 M $NaH_2PO_4 \cdot H_2O$, and 3.6 M NaCl, adjusted to pH 7.0 with 2 N NaOH)–1% (wt/vol) SDS–0.5% (wt/vol) skim milk powder–preboiled carrier DNA (final concentration, 0.5 mg/ml) for at least 8 h at 62°C. Filters were hybridized with the 0.8-kb *Hin*dIII fragment of the APH(3′)II gene from plasmid pABDI (Fig. 1). The *Hin*dIII fragment was labeled with [^{32}P]ATP by the random primer technique (6). Hybridization was carried out overnight at 68°C in fresh prehybridization buffer. After completion of the hybridization, the membranes were rinsed briefly in 2× SSC and then in 2× SSC–0.1% (wt/vol) SDS, washed for 10 to 15 min at room temperature with agitation in 0.5× SSC–0.1% (wt/vol) SDS and then in 0.1× SSC–0.1% (wt/vol) SDS, and given a final high-stringency wash in 0.1× SSC–0.1% (wt/vol) SDS at 62°C for 15 min. The membrane was exposed to X-ray film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) at −70°C with intensifying screens.

Plasmid construction. The plasmids used in this study are shown schematically in Fig. 1 to 3. Detailed construction of plasmids pABDI and pHP28 is described elsewhere (22, 24). Plasmid pABDI was obtained by insertion of the neomycin

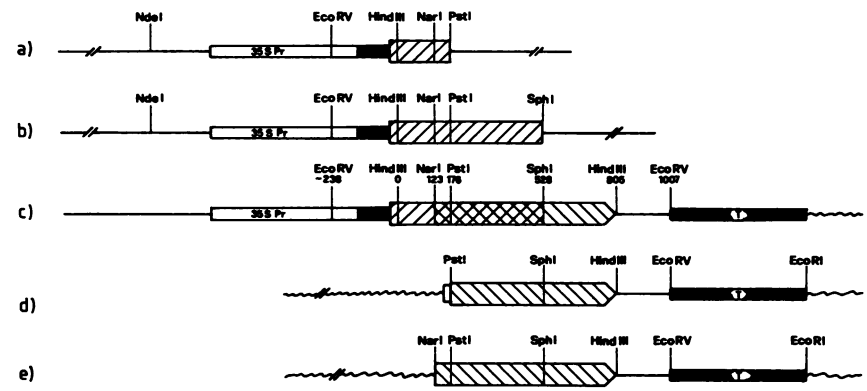

FIG. 3. Scheme of the recombination experiments with plasmids sharing no vector homology. The plasmid constructions are described in Fig. 1 and 2. Lines: a, plasmid pHP28ΔPstI; b, plasmid pHP28ΔSphI; d, plasmid pGB-PstI; e, plasmid pGB-NarI; c, predicted hybrid gene from a homologous recombination process within the homologous region of the APH(3′)II gene between a pair of corresponding plasmids. The length of the homologous region varied from 6 bp (pHP28ΔPstI and pGB-PstI; lines a and d) and 53 bp (pHP28ΔPstI and pGB-NarI; lines a and e) to 352 bp (pHP28ΔSphI and pGB-PstI; lines b and d). Symbols: —, pUC sequences; ∿∿, pGB2 sequences; ■, promoter and terminator (T) regions of the CaMV 19S RNA transcript; □, promoter (Pr) region of the CaMV 35S RNA transcript; ▨ and ▧, APH(3′)II protein-coding regions; ▩, region of homology within which homologous recombination must take place to restore the functional kanamycin gene.

phosphotransferase gene [APH(3')II], from the bacterial transposon Tn*5*, into the linker region of pUC8 (41). The APH(3')II gene is flanked by the promoter region of gene VI and polyadenylation signals of CaMV (Fig. 1, line g). Plasmid pHP28, a derivative of pUC19 (44), contains the hybrid APH(3')II gene consisting of the promoter region of the major transcript (35S) of CaMV fused to the 3' portion of gene VI promoter followed by the protein-coding region of the APH(3')II gene and the polyadenylation signals of CaMV (Fig. 1, line a). The plasmids (pHP28 and pABDI) containing the hybrid APH(3')II gene constructs allowed selection for kanamycin resistance in plant cells and ampicillin resistance in *Escherichia coli*.

Deletion derivatives of the APH(3')II gene deficient in the polyadenylation signals and a portion of the 3' protein-coding region of the APH(3')II gene were derived from plasmid pHP28 (Fig. 1, line a). Plasmids pHP28ΔPstI and pHP28ΔSphI were constructed by a deletion of the *Pst*I and *Sph*I fragment, respectively (Fig. 1, lines b and c). Deletion derivatives of the APH(3')II gene lacking the promoter and some of the 5' protein-coding region of the gene originate from plasmid pABDI (Fig. 1, line g). Plasmid pABDIΔPstI was constructed by deleting the *Pst*I fragment of pABDI (Fig. 1, line e). Plasmid pABDIΔNarI was constructed by deletion of the *Nar*I fragment of pABDI (Fig. 1, line f).

Plasmid pGB2 (5) (Fig. 2) was used to construct complementary deletion plasmids which contain no vector homology to the commonly used vectors, e.g., the pUC type. The *Pst*I-*Eco*RI fragment of plasmid pABDIΔPstI (Fig. 1, line e) was cloned into the *Pst*I and *Eco*RI sites of the polylinker of pGB2 to create pGB-PstI. Plasmid pABDIΔNarI (Fig. 1, line f) was linearized with *Nde*I. After the ends were filled in with T4 DNA polymerase, the "*Nde*I"-*Eco*RI fragment was cloned into the *Sma*I-*Eco*RI site of the polylinker of pGB2 to create pGB-NarI. A scheme of the constructs is shown in Fig. 2.

For the bacterial cloning, *E. coli* K-12 strain JM83 (41) was used. All DNA manipulations were carried out by standard techniques (3, 16). Restriction endonucleases were used under the conditions recommended by the manufacturers (New England BioLabs, Inc., Beverly, Mass.; Boehringer GmbH, Mannheim, Federal Republic of Germany; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). T4 DNA ligase and T4 DNA polymerase were obtained from New England BioLabs, Inc.

RESULTS

Experimental design. To monitor intermolecular recombination between plasmids in *N. tabacum* cells, we used two sets of plasmids containing truncated but overlapping portions of the neomycin phosphotransferase [APH(3')II] gene, which confers kanamycin resistance to plant cells. Two different hybrid gene constructs, the APH(3')II gene under the control of different plant expression signals (plasmids pHP28 and pABDI), were used to construct the nonoverlapping gene deletions by removal of either 3' or 5' regions of the APH(3')II gene (for details, see Materials and Methods and Fig. 1). Control experiments in which up to 10^7 tobacco protoplasts were transformed with both types of truncated genes individually never produced Kan^r cell lines.

Mesophyll protoplasts of *N. tabacum* were cotransformed with a pair of plasmids by the direct gene transfer technique (see Materials and Methods). The use of combinations of plasmids carrying the corresponding 5' and 3' portions of APH(3')II, respectively, allowed us to vary the lengths of homology in the central part of the gene from 6 to 405 bp (Fig. 1). The plasmid pairs pHP28ΔPstI and pABDIΔPstI (Fig. 1, lines b and e), pHP28ΔPstI and pABDIΔNarI (Fig. 1, lines b and f), pHP28ΔSphI and pABDIΔPstI (Fig. 1, lines c and e), and pHP28ΔSphI and pABDIΔNarI (Fig. 1, lines c and f) provided overlaps of 6 (the 6-bp *Pst*I recognition site was common to both plasmids), 53, 352, and 405 bp, respectively.

Homologous recombination within these homologous regions, referred to as effective homologous region (EHR), was expected to restore gene structure and function (Fig. 1, line d), leading to a kanamycin-resistant phenotype. The newly formed selectable marker gene would be distinguishable from the two parental plasmids, pHP28 and pABDI, by the new combination of flanking segments.

The transformation efficiency of tobacco protoplasts was high (around 8%) (19, 35), and cotransformation of plasmid molecules also occurred at high frequency (up to 88%) (33). Therefore, cotransformation of complementary deletion derivatives, when followed by recombination and integration into the plant genome, was expected to result in stable Kan^r cell lines. The relative recombination frequency (RRF) was defined as the ratio of the number of Kan^r clones obtained from these cotransformation experiments to the number of resistant clones from the parallel transformation with the intact APH(3')II gene. No differences in the RRFs were obtained between the transformation methods used.

Factors influencing recombination. The effects of several parameters on the RRF were examined (for a summary of the results, see Tables 1 and 2). These parameters were physical structure of the cotransformed plasmid molecules, length of the EHR, and plasmid vector homology.

(i) Recombination between circular or circular and linear plasmids. Cotransformation with closed-circular plasmids (supercoiled) yielded only one or two Kan^r colonies in some of the experiments with any of the plasmid combinations (Table 1, column A). No Kan^r colonies were obtained in the cotransformation experiments with the shortest EHR (6 bp). The RRFs obtained after linearization of one of the two cotransformed molecules were not significantly different from the low values obtained with two circular plasmids (Table 1, column B). A 352-bp EHR was necessary to obtain a small number of Kan^r clones in all six experiments, with an average of eight resistant clones per experiment. The RRFs between a linear and a supercoiled plasmid were similar, whether the plasmid was cut distal to (*Nde*I) or at the border of (*Pst*I or *Sph*I) the EHR (Fig. 1). Thus, the site of linearization by restriction endonucleases did not appear to affect the RRFs. If a double-strand break, adjacent to the common region, had been the initial site of recombination, more Kan^r clones would have been expected after linearization with the restriction endonucleases *Pst*I or *Sph*I than when the double-strand break was introduced distal to the EHR (*Nde*I).

(ii) Recombination between linear plasmids. Cotransformation of two linearized plasmids containing complementing deletion derivatives of the APH(3')II gene increased the RRFs drastically (15- to 88-fold) in comparison with the RRFs obtained when two circular plasmids or a circular and a linear plasmid were cotransformed.

The recombination capability of linear molecules was influenced by the length of the EHR. Increasing the length of the EHR (53, 352, 405 bp) stimulated the RRFs (Table 1, columns C, D, and E, respectively). The optimal linearization point for efficient recombination of the plasmid molecules appeared to be at the border of the EHR (Table 1,

TABLE 1. Recombination frequency in relation to the topological form of the plasmids and the length of the effective homologous region[a]

EHR	A	B	C	D	E
	structure of two co-transformed plasmids				
6	< 0.18 (5)	< 0.04 (2)	< 0.09 (6)	< 0.09 (1)	≤ 0.18 (8)
53	≤ 0.41 (7)	≤ 0.08 (6)	1.5 ± 0.3 (6)	6.7 ± 2.0 (4)	1.2 ± 0.5 (4)
352	≤ 0.39 (2)	0.37 ± 0.12 (6)	5.2 ± 0.9 (4)	19.0 ± 6.4 (3)	32.4 ± 5.7 (4)
405	≤ 0.18 (4)	n.d.	9.5 ± 1.2 (3)	n.d.	n.d.

[a] The summary of average percentages of RRFs and the standard deviation obtained from different cotransformation experiments are given. The number of experiments is indicated in brackets. The RRF (expressed as percentage) is defined as the ratio between the number of Kan^r clones resulting from the cotransformation experiments and the Kan^r clones obtained with a functional APH(3')II gene on plasmid pHP28. Column A shows the recombination frequencies obtained with two circular plasmids; column B shows the recombination frequencies obtained with a linear and a circular plasmid; columns C to E show the recombination frequencies obtained with two linear plasmids. Plasmids were linearized (columns B to E) at the points indicated by arrows. In column B, plasmids were linearized at either of the sites. The control plasmid pHP28 was used in a circular form in the experiments in column A and linearized with *Nde*I for the experiments summarized in columns B through E. In each experiment 1.2×10^6 protoplasts of *N. tabacum* SR1 were transformed by direct gene transfer as described in Materials and Methods. In experiments in which only a few Kan^r colonies were obtained, the maximal RRF values are given. In cases in which no Kan^r clones were detected, the values are marked with the < sign.

column E). The RRF for plasmids with a 352-bp EHR was as high as 32.4% when the ends of both molecules were close to the EHR. Linearization of the plasmids distal to the EHR decreased the RRF: for a 352-bp EHR the decrease was from 32.4% to 19.0% when one plasmid molecule was linearized in the distal and the other in the proximal position (Table 1, column D) and to 5.2% when both were linearized in distal positions (Table 1, column C).

The correlation between the RRFs and the site of linearization on the plasmids for a 53-bp EHR was less pronounced (Table 1, lines C to E). It should be noted, however, that the restriction endonuclease *Nar*I linearized only 30 to 60% of the plasmid molecules, despite a fivefold excess of units used. This is a common observation (1, 11). Therefore, the reduced RRF observed with the 53-bp EHR in Table 1, column E, is an exception and probably reflects an intermediate value between circular and linear molecules.

The minimal length of a 6-bp EHR (common *Pst*I restriction recognition site) provided by the plasmid pair pHP28ΔPstI and pABDIΔPstI (Fig. 1, lines b and e) was not sufficient for homologous recombination. No Kan^r clones were obtained with two supercoiled plasmids (Table 1, column A), with one supercoiled and one linear plasmid (Table 1, column B), or when both plasmids were linearized with restriction endonucleases other than *Pst*I (Table 1, columns C and D). Only when both molecules were linearized with *Pst*I could Kan^r clones be recovered, but only at a very low frequency and then not in all experiments (Table 1, column E). In these cases, the restoration of the selectable marker was most probably the result of end-to-end ligation.

(iii) Effects of vector homology on RRFs. The plasmids used in the above recombination studies were based on pUC vectors. The polarity of the pUC8 vector of plasmid pABDI

TABLE 2. Recombination frequency of plasmids not sharing any vector homology[a]

EHR	A	B	C	D	E
	structure of two co-transformed plasmids				
6	< 0.04 (1)	n.d.	< 0.04 (1)	< 0.04 (1)	≤ 0.09 (1)
53	≤ 0.04 (3)	≤ 0.04 (3)	0.07 ± 0.03 (2)	0.26 ± 0.03 (3)	0.3 ± 0.02 (2)
352	≤ 0.39 (2)	0.12 ± 0.05 (3)	1.4 ± 0.8 (3)	9.1 ± 0.4 (3)	9.7 ± 2.7 (3)

[a] The summary of results obtained from cotransformation experiments with plasmids which shared no vector homology, analogous to those in Table 1, are shown. pUC-type plasmids are represented by straight lines, and pGB2-type plasmids are represented by wavy lines. The control plasmid pHP28, containing the functional APH(3')II selectable marker gene, was always transformed in a linear form; it was cut with *Nde*I.

and the pUC19 vector of pHP28 was the same. Therefore, each plasmid shared a 2.7-kbp homologous vector sequence. Homologous recombination events within this region should not produce a functional APH(3')II gene, and therefore, no Kan^r colonies would be obtained. To examine the influence of the outside homologous vector sequences on recombination within the EHR of the APH(3')II gene, we constructed a new set of plasmids based on the plasmid pGB2 (5), which has no homology to pUC-type vectors. The plasmid constructions are described in Materials and Methods and illustrated in Fig. 2. The design of the recombination experiments was similar to that described above and is shown in Fig. 3. The results obtained with these plasmids are summarized in Table 2. The RRFs in Table 2 were related to the transformation frequencies obtained with the intact APH(3')II gene on plasmid pHP28, as in Table 1. It should be pointed out that the vector sequences do not influence the transformation frequencies. Comparable transformation frequencies have been obtained with the hybrid marker gene cloned into pUC-type plasmids or into double-stranded M13-type vectors (J. Paszkowski, unpublished data).

Cotransformation with circular plasmids (pGB2 derivatives and pHP28 derivatives) showed the same features as in the above cotransformation experiments based only on pUC vectors. Two circular plasmids recombined very inefficiently (Table 2, column A), and cotransformation with a linear and a circular plasmid also gave low RRFs, with a maximum of 0.39% (Table 2, column B).

Linearization of both plasmids prior to transformation increased the recombination frequencies. As observed in the experiments described above (Table 1), the highest RRF was obtained when both plasmids were linearized at a site adjacent to their EHR. Cotransformation with plasmids sharing a 352-bp EHR resulted in 9.7% resistant clones (Table 2, column E). This is a 3.3-fold reduction from the value obtained with plasmids sharing pUC sequences (Table 1, column E). Surprisingly, equal frequencies were obtained by linearizing both plasmids approximately equidistant from but in the opposite orientation with respect to their EHR (Table 2, column D). The 3′ deletion plasmids were linearized with *Nde*I (1,016 or 1,069 bp upstream of the EHR), and the 5′ deletion plasmids were linearized with *Eco*RI (1,026 and 1,378 bp downstream of the EHR) (Fig. 3; Table 2, column D).

The lowest RRFs were obtained with 3′ deletion deriva-

tives linearized at a site adjacent to their EHR and the 5′ deletion counterparts linearized distal to the EHR with *Eco*RI (Table 2, column C). The RRF of 1.4%, obtained with plasmids sharing a 352-bp EHR was reduced more than 6.5-fold in relation to the combinations in which the plasmids were linearized at a site adjacent to or at a site equidistant but in the opposite orientation to their EHR (Table 2, compare column C with columns D and E).

Structure of the integrated recombined plasmid DNAs. The two cotransformed plasmids containing an inactivated drug resistance gene most probably restored a functional APH(3′)II gene by homologous recombination within the provided common region. By this recombination process, a new hybrid gene structure should be formed which contains the APH(3′)II gene fused to the CaMV 35S promoter, as in plasmid pHP28, and to the CaMV polyadenylation signals, as in plasmid pABDI (Fig. 1, lines a, d, and g). As a result, the 805-bp *Hin*dIII fragment spanning the protein-coding region of the APH(3′)II gene would be restored (Fig. 1, line d). In addition, a new 1,245-bp *Eco*RV fragment would be diagnostic for distinguishing the recombinants from either parental construct. This would not only confirm recombination within the EHR of the APH(3′)II gene but also rule out the possibility of transformation with contaminating parental plasmids. To test the structure of the APH(3′)II gene in the Kan^r clones, we digested total genomic plant DNA with either *Hin*dIII or *Eco*RV. The Southern blot analysis of eight randomly chosen clones, resulting from different recombination experiments with different pairs of plasmids, is shown in Fig. 4.

Seven of eight Kan^r clones (Fig. 4a; clones 1 to 7) showed the restored *Hin*dIII fragment of the intact APH(3′)II gene. As expected, the fragment size corresponded to that of the parental plasmids, pHP28 and pABDI (Fig. 4a, lanes a and b). The same fragment was detected in clones transformed with these parental plasmids (Fig. 4a, lanes A and B). The expected newly formed 1,245-bp *Eco*RV fragment was detectable in five of eight Kan^r clones (Fig. 4b, clones 1, 2, 4, 5, and 6). In one clone the *Hin*dIII as well as the *Eco*RV fragment were missing (Fig. 4, clone 8).

All of the colonies tested were selected on a kanamycin-containing medium and should therefore possess the full-length coding region of the APH(3′)II gene. The 805-bp *Hin*dIII fragment and the 1,245-bp *Eco*RV fragment, however, are expected only when the 5′ and 3′ control regions of the APH(3′)II gene were unaltered during recombination and subsequent integration into the plant genome (Fig. 1, line d, and Fig. 3, line c). Alteration within the flanking regions can be detected by a double digest with *Eco*RV and *Sph*I. This would give rise to a 766-bp *Eco*RV-*Sph*I fragment identical to that of plasmid pHP28 (Fig. 1, line a), spanning parts of the 3′ promoter region and the 5′ protein-coding region of the hybrid APH(3′)II gene. In addition, they should contain the 479-bp *Sph*I-*Eco*RV fragment from derivatives of plasmid pABDI (Fig. 1, lines e to g), which spans the 3′ region of the APH(3′)II gene. To examine which parts of the control region of the APH(3′)II gene were mutated or missing, genomic DNA was cut with *Eco*RV and *Sph*I and analyzed on a Southern blot (Fig. 5). The five Kan^r colonies containing the 1,245-bp *Eco*RV fragment (Fig. 4b; clones 1, 2, 4, 5, and 6) showed the expected pattern of 5′ and 3′ subfragments (Fig. 5, clones 1, 2, 4, 5, and 6). Moreover, the 766-bp *Eco*RV-*Sph*I 5′ fragment was also detectable in all of the Kan^r clones in which the *Eco*RV fragment was missing (Fig. 4b and 5, clones 3, 7, and 8). However, these clones did not show the 479-bp *Sph*I-*Eco*RV fragment, indicative of an altered 3′-flanking region of the APH(3′)II gene.

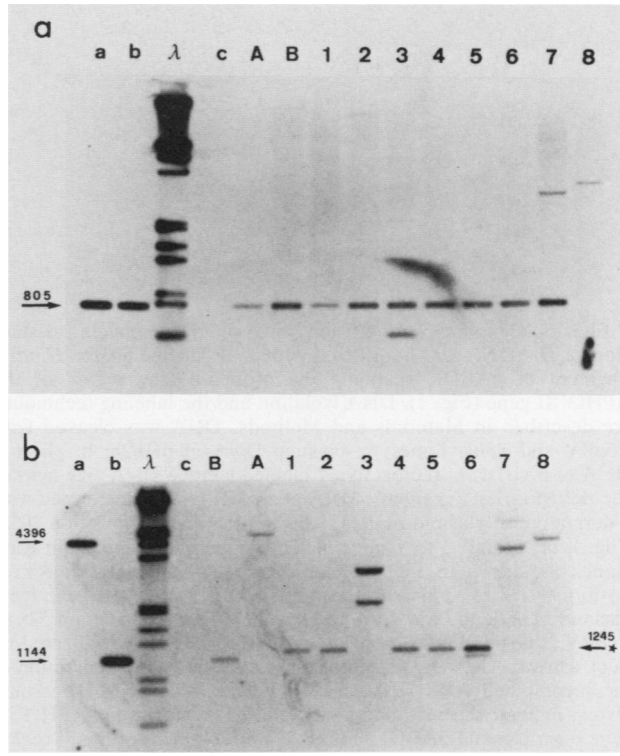

FIG. 4. Southern blot analysis of plant genomic DNA isolated from kanamycin-resistant clones. The DNA was hybridized with a ^{32}P-labeled 805-bp *Hin*dIII fragment of pABDI spanning the protein-coding region of the APH(3′)II gene (Fig. 1). DNA isolation and the labeling techniques are described in Materials and Methods. (a) The DNA was cleaved with *Hin*dIII; (b) the DNA was cleaved with *Eco*RV. Lanes: a, plasmid DNA of pHP28; b, plasmid DNA of pABDI (10 copies reconstruction); λ, radioactively labeled lambda size marker (*Eco*RI/*Hin*dIII); c, genomic DNA of an untransformed SR1 plant; A, genomic DNA of an SR1 plant transformed with plasmid pABDI; B, genomic DNA of an SR1 plant transformed with plasmid pHP28; 1 to 8, genomic DNAs from eight Kan^r clones obtained from cotransformation experiments. The sizes of fragments (in base pairs) are displayed next to the arrows. The expected newly formed *Eco*RV fragment of 1,245 bp (see text) is indicated by an asterisk.

To check the percentage of kanamycin-resistant clones altered in their flanking regions of the APH(3′)II gene, we analyzed 34 clones for enzyme activity and by Southern blotting. All of the colonies tested were positive for neomycin phosphotransferase activity, indicating that the full-length coding region of the selectable marker was present (data not shown). The diagnostic 1,245-bp *Eco*RV fragment could be detected in 53% of the analyzed colonies, and 2 of the 34 Kan^r colonies did not show the 805-bp *Hin*dIII fragment. *Hin*dIII site 805 lies 16 bp 3′ from the stop codon of the APH(3′)II gene. Therefore, a functional protein could still be produced, as indicated by the neomycin phosphotransferase assay. No colonies could be found which were altered in the 5′ region of the APH(3′)II gene, i.e., *Eco*RV (position −238)-*Sph*I (position 528) (Fig. 1). No correlation could be detected between the alteration of the 3′ region and the topological structure of the incoming plasmid DNA or the length of the EHR provided for recombination.

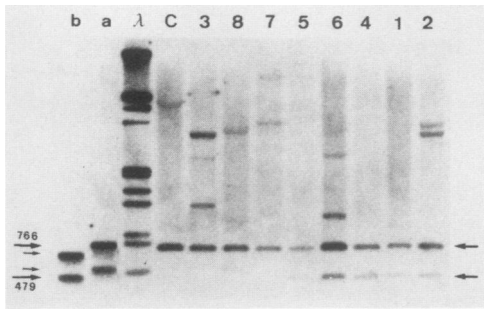

FIG. 5. Genomic Southern blot analysis of kanamycin-resistant clones. The DNA was hybridized with a ^{32}P-labeled 805-bp *Hin*dIII fragment of pABDI, spanning the protein-coding region of the APH(3')II gene (Fig. 1). DNA isolation and the labeling techniques are described in Materials and Methods. DNA was cleaved with *Eco*RV and *Sph*I. Lanes: a, plasmid DNA of pHP28; b, plasmid DNA of pABDI; λ, radioactively labeled lambda DNA size marker (*Eco*RI/*Hin*dIII); c, genomic DNA of an SR1 plant transformed with a derivative of plasmid pHP28, where the *Sph*I site (position 1078) (Fig. 1) is missing; 1 to 8, genomic DNAs from the same eight Kan^r clones as shown in Fig. 4. The 766-bp 5' fragment (*Eco*RV at position −238 to *Sph*I at position 528) of the APH(3')II gene from plasmid pHP28 and the 479-bp 3' fragment (*Sph*I at position 528 to *Eco*RV at position 1007) of plasmid pABDI (Fig. 1) are indicated by thick arrows. These two fragments are expected after integration of the recombined APH(3')II gene with flanking regions. The small arrows indicate the 665-bp fragment of the 5' region of the APH(3')II gene from plasmid pABDI and the 550-bp fragment of the 3' region of the APH(3')II gene from plasmid pHP28 (Fig. 1). The 550- and 665-bp fragments also hybridize with the probe but should not be observed in kanamycin-resistant clones.

DISCUSSION

We have examined homologous recombination, in leaf protoplasts of *N. tabacum*, between two exogenous plasmids carrying nonoverlapping deletions of a marker gene. The mutant plasmids were constructed by deleting parts of the protein-coding region of the APH(3')II gene. Owing to the deletions, the truncated APH(3')II gene was nonfunctional and therefore unable to confer kanamycin resistance to transformed plant cells. Cotransformation with corresponding truncated plasmids yielded Kan^r clones. The ratio between the number of Kan^r clones obtained from these cotransformation experiments and the number of resistant clones obtained from the parallel transformation with the intact APH(3')II gene was used as a measure of the RRF.

The RRFs were influenced by the conformation of the incoming plasmid DNA molecules. A very small number of Kan^r clones were obtained in recombination experiments with circular DNA molecules, as reflected by the low RRFs (Table 1, column A).

Linearization of only one of the two cotransformed plasmids did not markedly stimulate the recombination processes, irrespective of whether the plasmid was linearized adjacent or distal to the EHR. This is in contrast to animal systems (1, 10, 11, 38), in which linearization of one of the molecules increased the recombination frequency at least 10-fold. This difference between tobacco protoplasts and other eucaryotic cells indicates a different substrate specificity or recombination mechanism in plants.

Cotransformation experiments with both plasmids linearized led to the highest RRFs. RRFs up to 32.4% were obtained when a 352-bp EHR was provided (Table 1, column E). A slight (1.7-fold) reduction in the RRF was obtained when one plasmid was linearized distal to the EHR (Table 1, column D). On the other hand, a greater than sixfold reduction in the RRF was obtained when both plasmids were linearized distal to the EHR (Table 1, column C). Thus, not only linearization but also the sites of linearization have a pronounced effect on recombination efficiencies. The most favored substrates for homologous recombination were molecules linearized adjacent to their EHR.

The correlation between the RRFs and the topological structure of the transformed plasmids is most probably related to the underlying molecular mechanism of recombination in plants. This may also reflect differences in the uptake rates or integration efficiencies of linear versus circular DNA molecules. Previous experiments have shown that the transformation frequency of a functional marker is indeed altered by its conformation. A 2-to 10-fold increase in the number of stably transformed colonies is obtained when linear instead of circular DNA is introduced into plant protoplasts (35). The RRFs obtained with two linear plasmids increased 15- to 88-fold in relation to the RRFs obtained with the same plasmids in a circular form (Table 1, compare columns C to E with column A). Therefore, the uptake and integration of two circular DNA molecules cannot entirely account for these reduced RRFs. In addition, previous transformation experiments have shown that more than one copy of a gene is often integrated in the plant genome (26, 35), and cotransformation experiments with two unique marker genes resulted in more than 88% stable integration of at least a portion of both marker genes (33). Thus, the specific structure, and not the uptake, of cotransformed molecules should be taken into account when interpreting these results. This is consistent with the increase in the RRF that is observed when relaxed circular instead of supercoiled plasmid DNA is introduced (data not shown).

To examine whether vector homology influences the RRFs, we cotransformed protoplasts of *N. tabacum* with plasmids containing the same corresponding truncated APH(3')II gene constructs but residing on vectors with no sequence homology (Fig. 3). An effect on the RRF could be detected only between linear plasmids, and it was inversely proportional to the length of the homologous APH(3')II region. A 20- to 26-fold decrease with a 53-bp EHR and only a 2- to 4-fold decrease with a 352-bp EHR were observed in the RRFs relative to the RRFs observed with homologous vectors (compare Table 2, columns C to E, with Table 1, columns C to E). The most likely explanation is that the homologous vector sequences facilitate the aligning of the plasmids and therefore that homologous recombination within the EHR or gene conversion can occur more frequently. Our previous experiments with a plasmid containing both complementary deletion derivatives of the APH(3')II gene have shown that no significant difference in the number of Kan^r clones is obtained as compared with the number obtained by cotransformation with the deletion derivatives on separate plasmids (C. Basse, diploma thesis, FMI and Biozentrum University of Basel, Basel, Switzerland, 1987). Therefore, it is unlikely that an intramolecular recombination event subsequent to intermolecular recombination between homologous vector sequences is responsible for the reduced RRFs with nonhomologous vectors.

In the case of linear substrates, the RRFs increased with the length of the EHR, regardless of whether the plasmids were linearized adjacent or distal to their EHR or whether they shared vector homology. The RRFs were consistently lower with the 53-bp EHR than with a 352-bp homologous portion of the APH(3')II gene. The 6-bp homologous recognition sequence was not sufficient to restore the APH(3')II

gene. In our experimental design, a 53-bp EHR was the minimal length for efficient recombination. Similar minimal lengths of homology (25 and 62 bp) required for intermolecular recombination were also reported for animal cells (2, 4).

The results obtained with molecules containing only protruding ends of four bases and no additional homologous sequences in APH(3')II gene showed that tobacco cells cannot efficiently and precisely end-ligate unlinked DNA molecules (plasmid pairs pHP28ΔPstI and pABDIΔPstI [Table 1, column E] and pHP28ΔPstI and pGB-PstI [Table 2, column E], linearized with *Pst*I). Viral systems used for detection of very efficient ligation steps in plants (13) and animal cells (18) cannot directly be compared with our system. The role of viral proteins during recombination was not determined, and the viruses were self-replicating, whereas our plasmids are nonreplicating in plant cells.

Southern blot analysis showed that the diagnostic 1,245-bp *Eco*RV fragment (Fig. 1 and 3) could be detected in 53% of Kan^r clones analyzed. This new fragment should have been created when recombination occurred within the EHR, and the newly created gene should have integrated with flanking expression signals of CaMV. A particular deletion or mutation of the promoter or polyadenylation region of the hybrid gene would lead to the absence of the predicted *Eco*RV fragment, which was frequently observed. Southern blot analysis by a double digest with *Eco*RV and *Sph*I confirmed that in all Kan^r clones the promoter region was present, but the 3' noncoding region of the APH(3')II gene was altered in some cases (Fig. 5, clones 3, 7, and 8). This can be explained by a tight selection pressure for the maintenance of promoter sequences of the hybrid gene and lack of this pressure for the polyadenylation signals. The two Kan^r clones which did not show the 805-bp *Hin*dIII fragment also did not show the 1,245-bp *Eco*RV fragment. This indicates the occurrence of deletions rather than point mutations. These results suggest that the alterations in the 3' noncoding region of the APH(3')II gene took place at the level of integration rather than during the recombination process.

Although a double-strand break is reported to stimulate recombination in animal cells (1, 10, 11, 38), we observed no such stimulation when providing one plasmid with a double-strand break. This may point to a mechanism other than the double-strand break repair model proposed by Szostak et al. (40). The 15- to 88-fold stimulation of the RRFs that we obtained with plasmids linearized adjacent to (Table 1, column E, and Table 2, column E), nearby (Table 1, column D), or equidistant to (Table 2, column D) the EHR with respect to the RRFs obtained with two supercoiled molecules (Tables 1, column A, and 2, column A) are consistent with the recombination models proposed by Sternberg et al. (39) and Wake et al. (42). Sternberg et al. (39) proposed that DNA ends are the substrate for a specific DNA exonuclease, with either 5'-to-3' or 3'-to-5' activity, which generates complementary single-stranded regions for a strand-annealing reaction. The recombination intermediate generated by that pairing reaction is resolved by an endonuclease activity followed by a gap repair process. On the other hand, Wake et al. (42) proposed a recombination model that involves no single-strand exonuclease activity but does involve an unwinding of the double helix starting at the initial point of the double-strand break. The DNA will be unwound until the homologous region is reached, forming the heteroduplex structure followed by a gap repair process. Both models, the single-strand-annealing and the unwinding model, suggest that the highest RRFs would be obtained when the molecules are linearized at sites adjacent or equidistant to their EHR. The reduced RRFs we have observed either with plasmids linearized distal to the EHR (Table 1, column C) or with one plasmid linearized adjacent and the other 3' distal to the EHR (Table 2, column C) support these models for higher plant cells as well.

The data presented here are the basis for the optimal DNA molecule structures used for gene targeting in plants (22).

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