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A Test for Transvection in Plants: DNA Pairing May Lead to *trans*-Activation or Silencing of Complex Heteroalleles in Tobacco

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ABSTRACT

To study whether DNA pairing that influences gene expression can take place in somatic plant cells, a system designed to mimic transvection was established in transgenic tobacco. Pairing was evaluated by testing whether an enhancerless *GUS* gene on one allele could be activated *in trans* by an enhancer on the second allele. The required heteroalleles were obtained at four genomic locations using Cre-*lox*-mediated recombination. In one transgenic line, elevated GUS activity was observed with the heteroallelic combination, suggesting that *trans*-activation occurred. Conversely, when the unaltered allele was homozygous, GUS activity dropped to hemizygous levels in a silencing phenomenon resembling dosage compensation. Double-stranded *GUS* RNAs or small *GUS* RNAs indicative of RNA-based silencing mechanisms were not detected in plants displaying reduced GUS activity. These results suggested that a transgene locus capable of pairing, as revealed by *trans*-activation, could also become silenced in an RNA-independent manner, thus linking DNA pairing and gene silencing. The transgene locus was complex and comprised an inverted repeat, which possibly potentiated allelic interactions. The locus was unable to *trans*-activate transgenes at ectopic sites, further implicating allelic pairing in the transvection effects.

HOMOLOGY-DEPENDENT gene silencing (HDGS) refers to a type of epigenetic inactivation that is based on recognition of nucleic acid sequence identity at either the DNA or RNA level. HDGS phenomena have been described in diverse organisms and are probably common to most eukaryotes (Garrick *et al.* 1998; Birchler *et al.* 2000; Fagard and Vaucheret 2000; Faugeron 2000; Wianny and Zernicka-Goetz 2000). Cosuppression, quelling, and RNAi are all terms that have been used to refer to a post-transcriptional gene silencing (PTGS) process in which double-stranded (ds) RNA induces the degradation of homologous RNAs in the cytoplasm (Cogoni and Macino 2000; Meins 2000; Plasterk and Ketting 2000). Other HDGS effects act at the genome level to trigger transcriptional gene silencing and DNA modifications. There is evidence from various organisms suggesting that sequence-specific DNA methylation can be triggered by DNA-DNA or RNA-DNA interactions. RNA-directed DNA methylation has been documented so far only in plants (Wassenegger 2000; Matzke *et al.* 2001) but could conceivably be involved in mammalian X-chromosome inactivation and some cases of parental imprinting that involve overlapping sense/antisense RNAs and DNA methylation (Wolffe and Matzke 1999).

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Evidence that DNA-DNA interactions can trigger the modification of linked and unlinked sequence duplications is provided most convincingly by the repeat-induced point mutation (RIP) and methylation induced premeiotically (MIP) phenomena observed, respectively, in the filamentous fungi *Neurospora crassa* (Selker 1997) and *Ascobolus immersus* (Faugeron 2000). RIP and MIP take place at a specific stage in the sexual cycle of these fungi and probably do not have strict counterparts in other organisms. Nevertheless, these specialized processes have provided precedents for the involvement of DNA sequence interactions in triggering epigenetic silencing and genome modifications in higher eukaryotes.

Pairing-dependent genetic phenomena on the chromosomal level have long been known to occur in Drosophila. The general terms transvection or *trans*-sensing effects are used to refer to cases in which homolog pairing influences gene expression (Henikoff and Comai 1998; Pirrotta 1999; Wu and Morris 1999). Different types of transvection effects involving either gene activation or silencing *in trans* have been reported in Drosophila (Henikoff and Dreesen 1989; Goldsborough and Kornberg 1996; Morris *et al.* 1999). Such pairing-dependent phenomena are not surprising in this organism, where stable associations of homologous chromosomes are observed in somatic cells. Cytologically visible pairing does not appear to be the rule in most mammalian and plant cells. However, persistent pairing might not be necessary for allelic cross talk (Tartof and Henikoff 1991) or for transmission of epigenetic states between homologous sequences (Colot *et*

al. 1997). Pairing thus remains an attractive hypothesis to explain certain epigenetic silencing effects in mammals and plants. Several cases of parental imprinting in mammals have been associated with pairing of chromosomal domains (Lasalle and Lalande 1996; Duvillie *et al.* 1998) and chromosome pairing has been proposed to play a role in mammalian X-chromosome inactivation (Marahrens 1999).

In plants, DNA-DNA pairing has long been postulated to occur in various cases of HDGS that involve inactivation and/or methylation of repeated sequences on different chromosomes (Jorgensen 1992; Matzke and Matzke 1995; Baulcombe and English 1996; Hollick *et al.* 1997; Bender 1998). Pairing is suggested by reports of coextensive methylation of unlinked homologous sequences, which indicates that sequence interactions trigger DNA modification (Stam *et al.* 1998; Luff *et al.* 1999). However, because the involvement of RNA-directed methylation has not been completely ruled out in these cases (Selker 1999), it is still uncertain whether pairing of homologous (trans)gene sequences actually occurs and leads to alterations of gene expression in somatic plant cells.

DNA-DNA pairing, particularly if it occurs transiently, might be difficult to detect in plant somatic nuclei by standard techniques such as fluorescence *in situ* hybridization (FISH). Moreover, FISH alone cannot establish whether any pairing interactions observed are functionally significant. To obtain a positive measure of pairing that leads to altered gene activity, we have set up a system to mimic transvection in transgenic tobacco. Heteroalleles of a 35S*GUS* reporter gene that either contain or lack a 35S enhancer were generated using Cre-*lox*-mediated site-specific recombination. The ability of the 35S enhancer on one allele to activate *GUS* expression *in trans* was then tested. In one tobacco line containing a large complex transgene locus, augmentation of *GUS* expression was observed with the heteroallelic combination, suggesting that pairing of transgene alleles occurred at this locus. As a possible consequence of pairing, plants homozygous for the 35S*GUS* unaltered allele exhibited gene silencing that did not appear to be mediated by dsRNA. These results suggest that allelic interactions capable of influencing gene expression in both positive and negative ways can take place in somatic cells.

MATERIALS AND METHODS

Vector construction: To create a 35S*GUS* chimeric gene that was manipulatable by site-specific recombination, the plasmid pEMBL-GUS-46 (Benfey *et al.* 1989) was cut with *Bgl*II and a 35S enhancer (Pietrzak *et al.* 1986) fragment flanked by synthetic *lox* sites (Dale and Ow 1991) in direct orientation, and an *FRT* site (Odell and Russell 1994) was inserted. The orientation of the first *FRT* site was the same as for a second *FRT* site that was inserted at the end of the *GUS*-coding region-3C transcription terminator in pEMBL-GUS-46. This modified plasmid was linearized with *Bam*HI and inserted into the *Bgl*II site of the BV4 binary vector (Schernthaner *et al.* 1988). Further steps were carried out as described by Matzke and Matzke (1986).

Plant transformation and reporter gene assays: Leaf disk transformation and seed germination assays on kanamycin-containing medium to determine the number of segregating transgene loci were performed as described previously (Matzke *et al.* 1989). Nopaline was detected using high-voltage paper electrophoresis and phenanthrenequinone staining (Matzke *et al.* 1989). Fluorometric and histochemical GUS assays were performed on whole individual seedlings ~6 weeks after germination as described previously (Jefferson 1987). The protein concentration in seedling extracts was determined using the Bradford assay (Bradford 1976). Assays were carried out for 1 hr at 37°. Statistical analyses were performed with the *t*-test using Microsoft Excel.

DNA blot analysis: Plant DNA isolation and DNA blot analysis were carried out as described previously (Matzke *et al.* 1989).

RNA analyses: Total RNA was extracted from young expanding tobacco leaves using the Hybaid-AGS RNAClean system (Chemomedica, Vienna) including the RNAClean extension protocol according to the manufacturer's instructions. Northern blots were performed following standard protocols (Mette *et al.* 1999). Protocols for detection of double-stranded RNAs and small RNAs have been detailed by Mette *et al.* (2000).

λ-cloning, rescue cloning, and nucleotide sequence analysis: The plasmid pEMBL was included in the transgene construct to allow the transgene inserts to be recovered from the genomes of the tranformed tobacco lines by rescue cloning (Papp *et al.* 1996). This procedure was successful with lines 17 and 19, which contained a complete T-DNA region. For lines 13 and 14, rescue cloning was unsuccessful and the respective transgene inserts were isolated by λ-cloning and sequenced as described previously (Mette *et al.* 1999).

RESULTS

The construct used in these experiments contained a 35S*GUS* reporter gene positioned between selection and screening marker genes encoding kanamycin resistance (*KAN*) and nopaline synthase (*NOS*) activity, respectively (Figure 1A). The 35S enhancer was flanked by *lox* sites to allow its removal by the Cre recombinase (Odell and Russell 1994). The *GUS* coding region together with a minimal promoter (P-*GUS*) was flanked by *FRT* sites to permit excision by the FLP recombinase (Kilby *et al.* 1995). Heteroalleles that lacked either the 35S enhancer (Δ35Senh) or the P-*GUS* gene (Δ*GUS*) could thus be created *in planta* by site-specific recombination (Figure 1B). Theoretically, when the two heteroalleles are combined in the same genome, GUS activity should be obtained only when the 35S enhancer on one allele *trans*-activates the enhancerless P-*GUS* gene on the other allele and this would require physical pairing of the alleles [allelic transvection (A-TV); Figure 1B]. Pairing interactions between unlinked loci are also conceivable [ectopic transvection (E-TV)]. The flanking *KAN* and *NOS* genes provide extra sequence homology

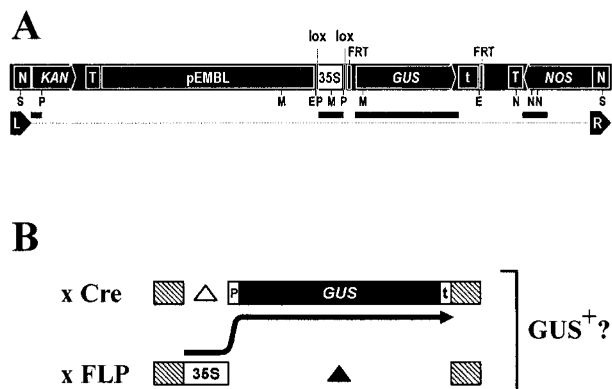

FIGURE 1.—(A) Transgene construct. A minimal promoter-β-*glucuronidase* (*GUS*) gene, flanked by *FRT* sites, was positioned downstream of a 35S enhancer (35S) flanked by *lox* sites. To the right and left were genes encoding nopaline synthase (NOS) and resistance to kanamycin (KAN), respectively, both under the control of the NOS promoter (N). A bacterial origin of replication, pEMBL, was present in the construct and could be used to rescue clone transgene inserts from the tobacco genome. Arrowheads labeled L and R indicate the left and right T-DNA borders, respectively. Enzymes used to digest DNA for the blot analyses are indicated; bars show the probes used. Abbreviations are as follows: S, *Sac*II; P, *Pst*I; M, *Msp*I or *Hpa*II; E, *Eco*RI; N, *Nsi*I; T, NOS transcription terminator; t, transcription terminator from the pea gene encoding ribulose bisphosphate carboxylase 3C gene (accession no. emb/XO4334/PSRBCS3C). (B) Transvection strategy: heteroalleles lacking either the 35S enhancer or the minimal promoter (P)-*GUS* gene were produced by Cre or FLP activity, respectively. Because neither heteroallele can produce GUS activity on its own, a positive GUS result can only be obtained in the heteroallelic combination if the 35SS enhancer on one allele *trans*-activates the minimal promoter on the other allele and this should require pairing of alleles. Diagonally hatched flanking regions represent sequence homology contributed by pEMBL and the *KAN* gene on the left and the *NOS* gene on the right.

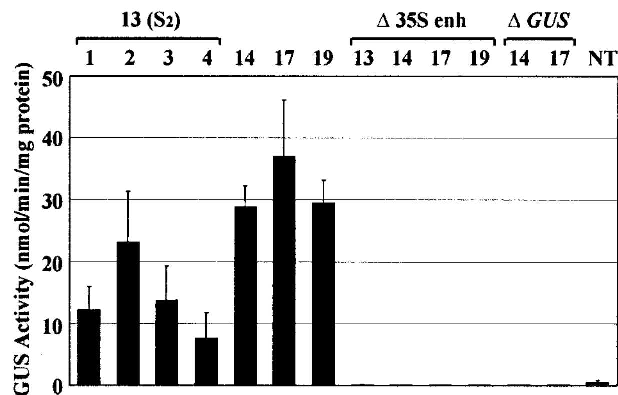

FIGURE 2.—Quantification of GUS activity in transgenic lines and their Cre and FLP derivatives. GUS activity in leaves of adult plants of homozygous transformed lines 14, 17, and 19, as well as line 13 S_2 progeny 1–4 (all are homozygous except 4) is shown. In contrast to the original lines, the Cre-derived Δ35Senh alleles (obtained for all four lines) and the FLP-derived Δ*GUS* alleles (obtained for lines 14 and 17 only) produced no GUS activity. The averages of at least three separate determinations are shown. Data for the parental 13 plant are not available. NT, normal tobacco.

to promote DNA pairing interactions that could potentially affect the 35S*GUS* gene.

The construct was introduced into the tobacco genome by leaf disk transformation. Four individual transformed lines (13, 14, 17, and 19) that expressed all three reporter genes (Figure 2 and data not shown) and that contained single independently segregating transgene loci were recovered. Cre-mediated removal of the 35S enhancer was achieved in all four lines (Figure 3A); successful excision of P-*GUS* by the FLP recombinase was obtained only with lines 14 and 17 (Figure 3B). As discussed below, the transgene locus in line 13 contained multiple *FRT* sites, which probably interfered with P-*GUS* excision. It is not known why P-*GUS* in line 19 was refractory to FLP activity. No GUS activity was detectable in plants containing either the Δ35Senh allele or the Δ*GUS* allele (Figure 2). These plants were backcrossed with untransformed tobacco to remove the *Cre* or *FLP* loci and then selfed until homozygous for the Δ35Senh or Δ*GUS* alleles. Plants homozygous for the respective unaltered 35S*GUS* allele were obtained by selfing each of the four lines.

To test for A-TV in lines 14 and 17, intercrosses were made between plants homozygous for the respective Δ35Senh alleles and Δ*GUS* alleles. Progeny, which all have a genotype of Δ35Senh/Δ*GUS* (Figure 4D), were tested for GUS activity. None was detected in either

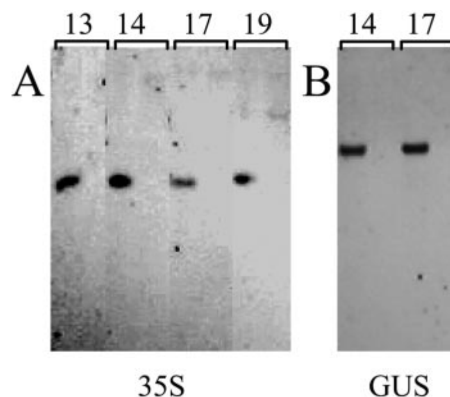

FIGURE 3.—DNA blot analysis of heteroalleles produced by Cre and FLP-mediated site-specific recombination. DNA isolated from representatives of the indicated plant lines was digested with either *Pst*I (A) or *Eco*RI (B). The first lanes show the presence of the 35Senh (A) or *GUS* gene (B) in two or more of the original lines (13, 14, 17, and 19). The second lanes show absence of the sequence following excision by the Cre or FLP recombinase, respectively. Deletion of the 35S enhancer by the Cre recombinase was accomplished for all four transgenic lines. Excision of the P-*GUS* gene by the FLP recombinase was achieved only with lines 14 and 17. The probes used are shown as solid bars under the construct map in Figure 1A and consisted of 35S enhancer sequences (A) and the entire *GUS* coding sequence (B).

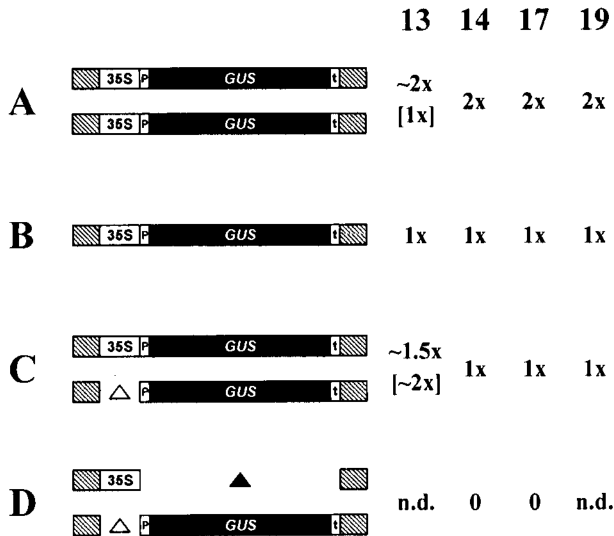

Figure 4.—Possible allelic combinations. (A) Homozygous for unaltered allele—present in 100% of selfed progeny of all lines (exception: line 13 S_2 no. 4, which produced 25% homozygous progeny in selfings); (B) hemizygous for unaltered allele—present in 100% of backcross (BC) progeny from all lines (exception: line 13 S_2 no. 4, where 50% of selfed and 50% of BC progeny were hemizygous); (C) unaltered allele combined with Δ35Senh allele; (D) combination of Δ35Senh and Δ*GUS* alleles. The combination in D did not produce any GUS activity with lines 14 and 17 for which both Δ35Senh and Δ*GUS* alleles were available. Therefore, the allelic transvection (A-TV) and ectopic transvection (E-TV) discussed in the text refer to the combination shown in C. In the case of A-TV, the 35S*GUS* gene and Δ35S variant were from the same line; for E-TV, they were each from a different line. The relative amounts of GUS activity obtained for each of the four lines (13, 14, 17, and 19) with these allelic combinations are shown to the right. The amount measured in hemizygotes (B) was taken as 1×. Line 13 produced variable GUS activity. Average results from the A and C combinations in the line 13 parent, 13 S_2 nos. 2 and 3, approximated unbracketed values; average results from A and C combinations in line 13 S_2 nos. 1 and 4 approximated bracketed values (Figures 5 and 6). n.d., not determined.

histochemical or fluorometric assays carried out on at least 25 seedlings from each cross (data not shown). GUS activity was also not observed in progeny obtained by crossing lines 14 and 17 Δ*GUS* plants with lines 13 and 19 Δ35Senh plants (data not shown), indicating that E-TV did not occur.

To improve the chances of homologous pairing, which might have been disrupted by the deletions in the Δ35Senh and Δ*GUS* alleles, crosses were made to combine the Δ35Senh allele with the respective 35S*GUS* unaltered allele from each of the four lines (Figure 4C). Progeny of these crosses were tested for GUS to see whether the activity exceeded the amount observed in backcross (BC) progeny containing the unaltered allele in the hemizygous condition alone (Figure 4B). Increased GUS activity with the heteroallelic combination would provide evidence for *trans*-activation of the Δ35Senh allele, possibly through the action of 35S enhancer present on the unaltered allele. GUS activity was also measured in selfed progeny of plants homozygous for the unaltered allele (Figure 4A).

For lines 14, 17, and 19, GUS activity was dependent on gene dosage (homozygous selfed progeny contained ∼2 times as much as hemizygous BC progeny) and there was no detectable *trans*-activation (GUS activity in progeny containing the heteroallelic A-TV combination was comparable to that observed in hemizygous BC progeny; Figure 5, lines 14, 17, and 19). In line 13, a different pattern was obtained. Two observations are relevant. First, unlike the relatively uniform GUS values seen with homozygous selfed progeny in the other three lines, selfed progeny of line 13 exhibited a wide range of GUS activity, with some values falling to those observed in hemizygous BC progeny. Second, enhanced GUS activity (on average ∼1.4 times the hemizygous level in BC progeny) was observed with the combination of heteroalleles designed to test for A-TV (Figure 5, line 13). These results suggested that GUS activity was unstable in homozygous progeny of line 13 and that transvection was taking place.

To examine this phenomenon in line 13 further, the homozygous parent was selfed and four progeny (S_2 nos. 1–4) that contained varying amounts of GUS activity (Figure 2) were selected for analysis. Seeds from selfing, backcrossing, and intercrossing to produce the heteroallelic A-TV combination were obtained to test the three allelic pairs shown in Figure 4, A–C, respectively. The variability evident in the original line 13 parental plant was also manifested in the S_2 progeny, where different patterns of GUS activity were observed for each line. In the case of S_2 no. 3, a distribution similar to the parental line was observed, *i.e.*, a wide range of GUS values in homozygous selfed progeny, some reaching hemizygous BC levels or slightly below, and increased GUS activity in the heteroallelic A-TV combination (on average, ∼1.6 times the hemizygous level measured in BC offspring; Figure 6, S_2 no. 3). GUS activity was less variable in selfed progeny of line 13 S_2 no. 2, where a relatively good dosage effect was observed together with a generalized enhancement of GUS activity in the A-TV combination (on average ∼1.3 times the hemizygous level observed in BC offspring; Figure 6, S_2 no. 2).

The most dramatic results were obtained with line 13, S_2 nos. 1 and 4 (Figure 6, S_2 nos. 1 and 4). In both cases, the dosage effect disappeared: homozygous selfed progeny produced amounts of GUS activity similar to hemizygous BC progeny. Moreover, in the heteroallelic A-TV combination, average GUS activity was up to two times that observed in the homozygous and hemizygous offspring. Similar results were obtained in reciprocal crosses, indicating no parental effect. An additional peculiarity of line 13, S_2 no. 4 was that it behaved as a hemizygote, even though it was produced by selfing the homozygous parental line 13 plant. With line 13 S_2 no.

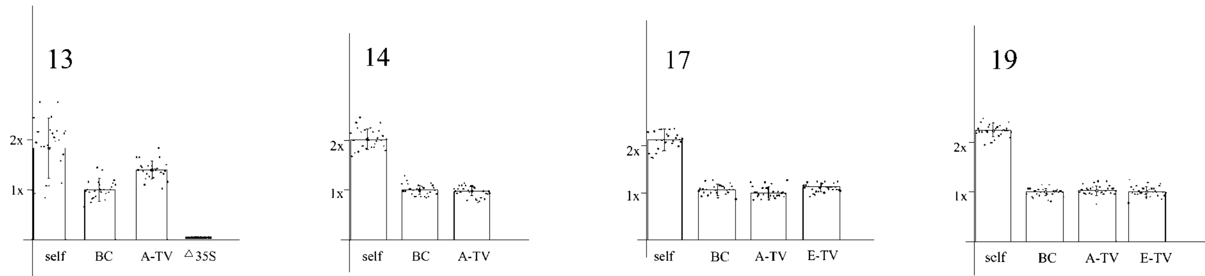

FIGURE 5.—Relative GUS activity with different allelic combinations from the four original transgenic lines. Homozygous seedlings obtained from selfing, hemizygous seedlings from backcrossing (BC), and seedlings containing the heteroallelic combination designed to test for A-TV (Figure 4C) were assayed for GUS activity. Each point represents GUS activity in an extract prepared from an individual seedling. The average hemizygous levels in BC progeny were set at 1×. In line 13, seedlings containing the Δ35Senh allele alone are shown to demonstrate that this allele contributed no GUS activity. Some selfed progeny showed more than 2× GUS activity, which can be considered an additional reflection of the variability of this locus. Tests for ectopic transvection (E-TV) are shown for lines 17 and 19. These seedlings contained the unaltered 35S*GUS* allele from lines 17 or 19 and the Δ35Senh allele from line 13. No E-TV was observed. Statistically significant differences between the BC and A-TV values were only observed with line 13.

4, ~25% of the selfed progeny and 50% of the progeny obtained from the crosses were GUS-negative (Figure 6, S_2 no. 4). DNA blot analysis demonstrated that transgene sequences were missing in GUS-negative progeny (data not shown). This was apparently due to deletion of the transgene sequences, probably during meiosis, and not to chromosome loss because line 13 S_2 no. 4 contained the normal number of chromosomes (data not shown).

In contrast to lines 14, 17, and 19, the transgene locus in line 13 thus appeared to be susceptible to transvection: GUS expression in homozygous progeny could be highly variable and in some cases (S_2 nos. 1 and 4) a gene silencing phenomenon akin to dosage compensation, where two copies of the 35S*GUS* gene were expressed at a level comparable to one copy, was regularly observed. Moreover, in addition to this partial silencing of unaltered 35S*GUS* alleles in the homozygous state, line 13 exhibited *trans*-activation, as revealed by enhanced GUS activity when the unaltered 35S*GUS* allele and the Δ35Senh allele, which contributed no GUS activity, were together in the same genome.

To determine whether structural variation could account for these differences in behavior, the four transgene loci were cloned out of the tobacco genome and the nucleotide sequences were determined. Lines 14, 17, and 19 contained single copies of the transgene construct, although an internal rearrangement was present in line 14 (Figure 7). In contrast, line 13 contained a large scrambled locus comprising reiterated transgene sequences and substantial binary vector DNA. Despite this complexity, line 13 contained only one complete 35S*GUS* gene, which was present in the spacer region between two *NOS* gene sequences arranged as an inverted repeat (IR). The lone 35S enhancer at this locus could thus be present in the loop of a cruciform. Consistent with the simple structures of the transgene loci in lines 14, 17, and 19, the *KAN* and *NOS* genes were expressed strongly in progeny. In contrast, progeny of line 13 displayed only weak kanamycin resistance and

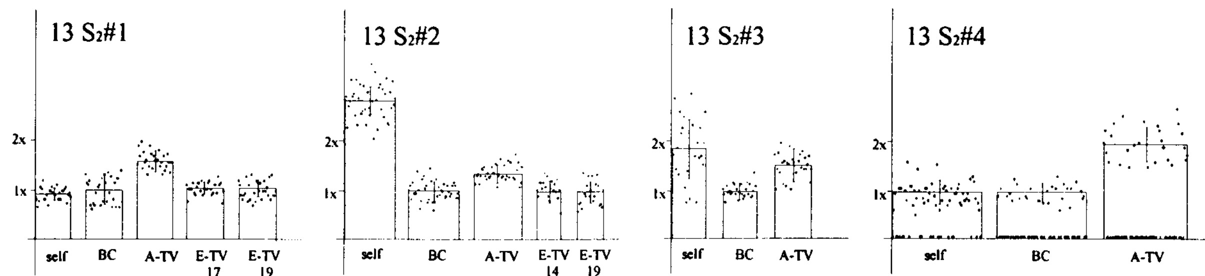

FIGURE 6.—Relative GUS activity with different allelic combinations in S_2 progeny of line 13. Abbreviations and explanations are as in Figure 5. E-TV crosses are shown for S_2 nos. 1 and 2. These seedlings contained the unaltered 35S*GUS* allele from lines 13 S_2 nos. 1 and 2, respectively, and the Δ35Senh alleles from lines 14, 17, or 19 as indicated. In all cases, the differences between the A-TV values and BC values were statistically significant, whereas differences between BC and E-TV values were not statistically significant. In S_2 nos. 1 and 4, differences between the homozygous selfed and hemizygous BC seedlings were not statistically significant. Similar profiles were obtained with populations of seedlings from at least two different pods of each cross. Although derived from selfing a homozygous parent, S_2 no.4 behaved as a hemizygote, producing ~25 and 50% GUS-negative progeny in the selfing and crosses, respectively.

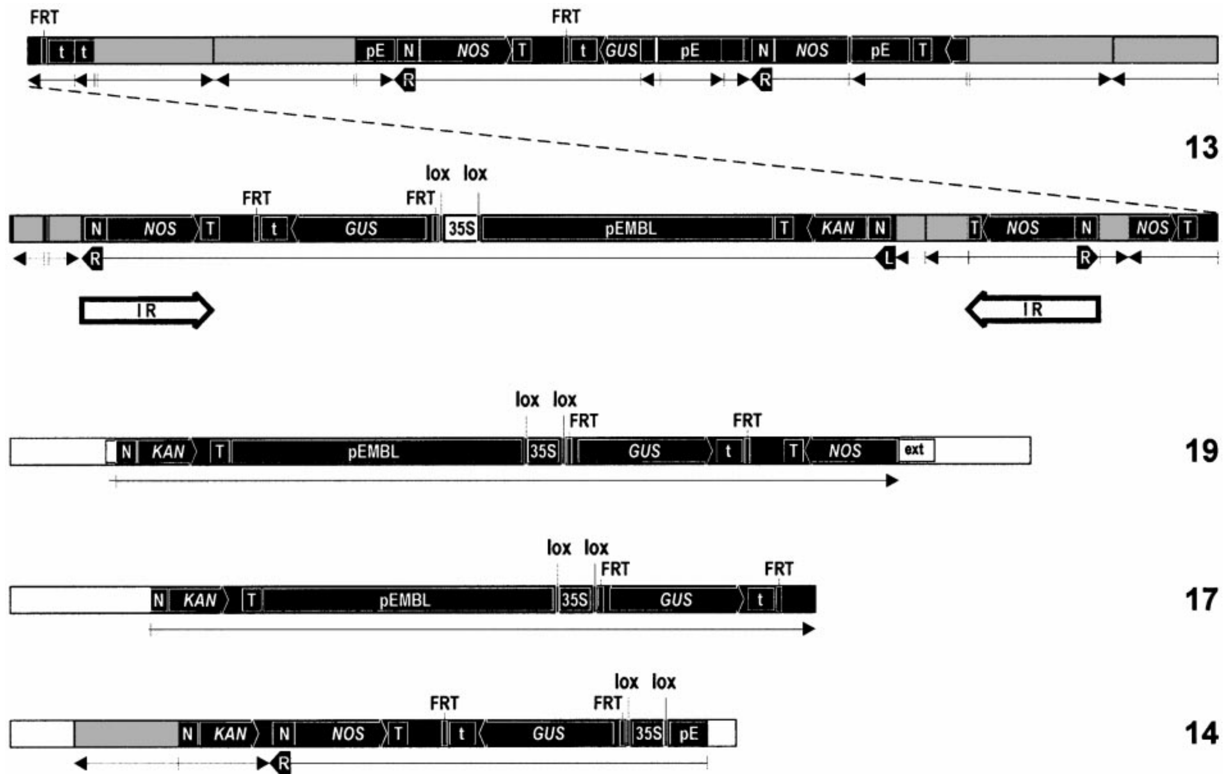

Figure 7.—Structures of the transgene loci in lines 13, 14, 17, and 19. Genomic λ-clones (lines 13 and 14) or rescue clones (lines 17 and 19) containing the respective transgene inserts were isolated and sequenced. Solid regions indicate transgene sequences (described in Figure 1A); shaded blocks denote binary vector sequences; open flanking regions represent plant DNA. Locus *13* is large and scrambled (arrows below maps indicate the relative orientations of different fragments); two contiguous regions of this locus are connected by the dashed diagonal line. Portions arranged as an inverted repeat (IR) are shown. The single 35S enhancer in the locus is present in the spacer of the IR and is highlighted as a white block. Note the two *FRT* sites (top half) in addition to the two surrounding the intact GUS gene (bottom half). Lines 14, 17, and 19 contain essentially single copies of the transgene construct. Right and left borders are missing in line 19, as is the NOS promoter driving the *NOS* gene. Nevertheless this plant is nopaline positive, indicating that the flanking plant DNA, part of which is homologous to a portion of an extensin gene (ext) promoter (accession no. L38908), has promoter activity. A left T-DNA border is missing in line 17; plant DNA to the right of transgene sequences in line 17 was not recovered in the rescue clone. In line 14, the region containing the *NOS* and 35S*GUS* genes was inverted relative to the *KAN* gene and some transgene sequences were largely deleted (*e.g.*, pEMBL), but otherwise no additional scrambling was observed. Some flanking binary vector DNA was present at the left in locus *14*. Abbreviations are as in Figure 1.

negligible amounts of nopaline (data not shown), presumably owing to progressive silencing of the multiple copies of the *NOS* promoter in advanced generations.

Although structural differences provided an explanation for the distinct behaviors of the four individual transgene loci, it was more difficult to account for variations in the four line 13 S_2 lines. DNA blot analysis using probes throughout the transgene construct revealed no major differences in structure or methylation among line 13 S_2 nos. 1–3 (Figure 8, A–D) and their chromosome numbers were normal (data not shown). The only differences among the line 13 S_2 lines were observed with the hemizygote S_2 no. 4, in which methylation in the NOS promoter of the *KAN* gene (Figure 8B, lane 4) was detected. The significance of this difference is not known.

Because dsRNAs that are processed to small (21–25 nucleotide) RNAs have been implicated in both RNA-mediated TGS and PTGS (Matzke *et al.* 2001), we tested whether the gene silencing phenomenon observed in line 13 might be due to the unintentional production of dsRNAs or small RNAs containing *GUS* or 35Senh sequences. *GUS* mRNA was present in plants from lines 13, 14, 17, and 19 (Figure 9A) at steady-state levels, approximating the relative amounts of GUS activity in these plants (Figure 2). Although it is difficult to predict the length of dsRNAs that might be synthesized from locus *13*, RNase protection experiments failed to reveal dsRNAs derived from either *GUS* or 35Senh sequences (data not shown). Moreover, no detectable amounts of 23-nucleotide RNAs containing *GUS* or 35Senh sequences were observed in plants 13 S_2 nos. 1 and 2 (Figure 9B and data not shown), which differed approximately twofold in GUS activity (Figure 2) and steady-state *GUS* RNA levels (Figure 9A). Plants containing the Δ35Senh allele did not produce GUS mRNA

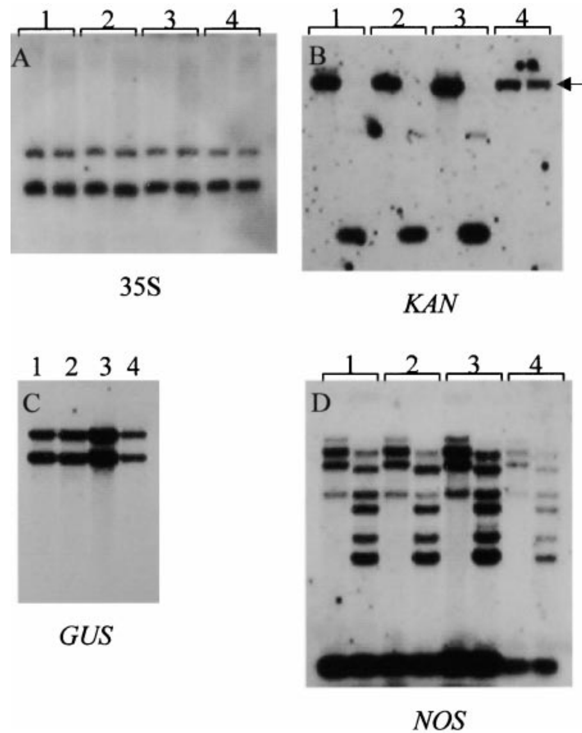

Figure 8.—DNA blot analysis of line 13 S_2 progeny. DNA isolated from line 13 S_2 nos.1–4 was digested with the appropriate restriction enzymes and probed with sequences throughout the transgene construct (Figure 1A) as follows: (A) *Hpa*II or *Msp*I, 35S probe; (B) *Eco*RI/*Pst*I plus or minus *Sac*II, KAN probe; (C) *Eco*RI, *GUS* coding region probe; and (D) *Nsi*I plus or minus *Sac*II, NOS probe (Figure 1). The blots in A, B, and D examine methylation in the four lines. Two lanes representing digests minus and plus a methylation-sensitive restriction enzyme are shown for each line. Digestion with either *Hpa*II (CpG methylation-sensitive) or *Msp*I (CpG methylation-insensitive) produced two 35S bands of the expected size (Figure 1A), indicating no methylation at a *Hpa*II site in this single-copy region (A). For S_2 nos. 1–3, an *Eco*RI/*Pst*I fragment shifted to the smaller fragment following the addition of methylation-sensitive *Sac*II, indicating no methylation of this site in the NOS promoter of the single *KAN* gene (B). In all four plant lines, two GUS bands, corresponding to the intact 35S*GUS* gene and a truncated GUS coding region, were observed (C). Conversion of several *Nsi*I bands to smaller fragments in all four lines after addition of methylation-sensitive *Sac*II revealed little or no methylation in the NOS sequences, which are present in at least five partial or complete copies at locus *13* (D). The only visible difference in structure or methylation was in S_2 no. 4, where a *Sac*II site in the NOS promoter of the *KAN* gene was methylated, as indicated by the maintenance of the large fragment after addition of *Sac*II (arrow in B, lane 4). S_2 no. 4 signals appear fainter because this plant is hemizygous for the transgene locus, whereas other lines are homozygous. The fragments observed with all probes can be accounted for in the locus *13* structure determined by sequencing (Figure 7). All fragments cosegregated in backcross progeny demonstrating that they are linked. The line 13 Δ35Senh allele appeared identical to S_2 nos. 1–3 with these probes (data not shown) except for the absence of the 35S enhancer (Figure 3A).

(Figure 9A), as expected from the absence of GUS activity in these plants (Figure 2), nor did these plants contain dsRNA or small RNAs derived from *GUS* or 35Senh sequences (Figure 9B and data not shown). The absence of small or dsRNAs in plants showing reduced or no GUS activity argues against RNA-mediated silencing of the 35S*GUS* gene in plant 13 S_2 no. 1, supporting the involvement of DNA-DNA pairing in the silencing phenomenon observed.

To determine whether the transgene locus in line 13 was able to *trans*-activate the ectopic loci in lines 14, 17, and 19 (E-TV), crosses were made to combine either the line 13 Δ35Senh allele with the unaltered lines 14, 17, and 19 35S*GUS* alleles or the line 13 unaltered 35S*GUS* allele with the lines 14, 17, and 19 Δ35Senh alleles. No significant enhancement of GUS activity above the hemizygous BC level was observed, indicating no appreciable E-TV (Figure 5, lines –17 and 19; Figure 6, S_2 nos. 1 and 2). These findings further substantiate a role for allelic pairing in the transvection effects observed in line 13.

DISCUSSION

In this study, a system designed to mimic transvection was established to attempt to detect somatic pairing of transgene alleles in tobacco. This system assessed the ability of an enhancer on one allele to *trans*-activate an enhancerless promoter on the second allele. The required heteroalleles were generated at four different locations in the tobacco genome by Cre-*lox*-mediated excision. No evidence for *trans*-activation was obtained for three loci containing simple transgene inserts, indicating that transvection does not normally occur with single-copy, unrearranged transgenes. One complex transgene locus, however, exhibited behavior that can be interpreted in terms of allelic pairing. Depending on which alleles were present, two distinct transvection effects—resulting in either *trans*-activation or *trans*-silencing—were observed. Because *trans*-activation presumably required close physical association of transgene heteroalleles, it can be inferred that the *trans*-silencing phenomenon also involved allelic pairing. Moreover, the frequency and degree of silencing correlated roughly with the strength of *trans*-activation, providing a further connection between the two phenomena. RNA-mediated silencing could be ruled out because the expected hallmarks of RNA silencing—dsRNAs and small RNAs containing *GUS* or 35Senh sequences—were not detected in plants with diminished GUS activity. These results thus provide molecular evidence suggesting that complex alleles can physically associate in somatic plant cells in a manner that influences gene expression.

The large size and repetitiveness of transgene locus *13* possibly induced the formation of heterochromatin capable of forming homologous associations (Henikoff 1997; Marahrens 1999). Despite the complex charac-

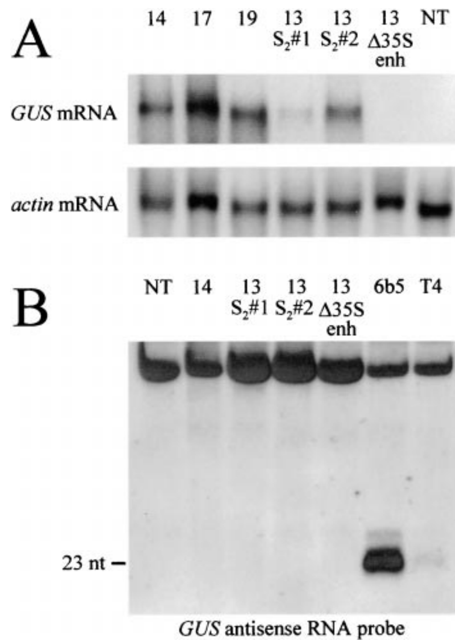

Figure 9.—RNA analyses. (A) Northern blot analysis of GUS mRNA. Total RNA preparations isolated from leaves of adult plants of transgenic lines 14, 17, 19, 13 S_2 no. 1, 13 S_2 no. 2, 13 Δ35Senh, and untransformed tobacco (NT) were electrophoresed, blotted onto nitrocellulose, and probed with the *GUS*-coding sequence (top) or an actin probe (bottom) as a loading control. The relative levels of *GUS* RNA correlate roughly with the average GUS activities measured in plants from these lines (Figure 2): line 17 is the highest; lines 14 and 19 are somewhat less; 13 S_2 no. 2 contains at least twice as much GUS RNA as does 13 S_2 no. 1. Both *GUS* RNA and GUS enzyme activity are absent in line 13 Δ35S. (B) Attempt to detect small RNAs. RNA preparations enriched for small (21–25 nucleotide) RNAs were isolated from the indicated plants, electrophoresed on 15% acrylamide gels, electroblotted onto a nylon membrane, and hybridized to a *GUS* antisense RNA probe. Two tobacco lines, 6b5 and T4, in which a 35S*GUS* gene is silenced by PTGS, were used as positive controls for *GUS* small RNAs (Hamilton and Baulcombe 1999); line 14, in which no *GUS* silencing occurred, and untransformed tobacco (NT) were used as negative controls. Although signals are clearly visible in the 6b5 and T4 lanes, no signals were observed in line 13 plants, including S_2 no. 1, which contains approximately one-half the GUS activity and GUS RNA as S_2 no. 2, or the line 13 Δ35Senh derivative, which augments GUS activity when combined with the unaltered allele in lines 13 S_2 no. 1. Similar results were obtained with a *GUS* sense RNA probe (data not shown). Under identical conditions, no small RNAs derived from the 35Senh were detected (data not shown). The position of a 23-nucleotide marker is indicated to the left.

ter of locus *13*, however, the intact 35S*GUS* gene undergoing transvection was present as a single copy in the original T-DNA sequence context, similar to the 35S*GUS* gene at other three loci. Moreover, the 35S*GUS* gene in line 13 was never fully silenced, as might be expected from wholesale heterochromatinization of the locus. These observations can be reconciled with the two types of transvection effects observed in line 13 by considering the possible importance of an IR that could make up the stem of a cruciform containing the 35S enhancer in the loop. This structural arrangement could facilitate allelic interactions and determine whether *trans*-activation or silencing occurred.

Silencing took place when two unaltered alleles that each possessed a 35S enhancer were present in homozygotes. Conversely, *trans*-activation occurred with the heteroallelic combination in which the 35S enhancer was deleted from one allele (Figure 10). These results suggest that allelic pairing affected the activity of enhancer sequences. Indeed, a close link between transcriptional regulatory elements and homology effects has been suggested by a number of other observations (Morris *et al.* 1999; Wu and Morris 1999). It has also been noted that unpaired loops might be particularly prone to initiating a homology search (Wu and Morris 1999). The presence of the 35S enhancer in the loop of a potential cruciform might thus account for its ability to locate and to interact with allelic partners in a way that modifies gene expression.

While IRs have been repeatedly implicated in *trans*-silencing effects in plants (Muskens *et al.* 2000), this is the first case potentially linking IRs and *trans*-activation. Whether IRs are involved in silencing or activation probably depends on their gene content and length; the size and composition of any intervening spacer; whether the IR is transcribed; and whether any DNA secondary structures, such as hairpins or cruciforms, are produced *in vivo*. In the effects described here, the importance of the IR possibly rested in its potential to form the stem of a cruciform that placed the 35S enhancer in a loop, a position favoring *trans*-interactions. Sequences present in the repeated regions of an IR can lead to different types of HDGS. For example, IRs containing promoter sequences or transcribed regions can trigger *trans*-silencing at the transcriptional (Mette *et al.* 2000) or post-transcriptional (Stam *et al.* 1998) level, respectively. It has been uncertain whether IRs act as *trans*-silencers through DNA-DNA interactions or by means of dsRNA (Selker 1999). While some cases of *trans*-silencing involving IRs do appear to rely on dsRNA (Mette *et al.* 2000; Muskens *et al.* 2000), we were unable to detect dsRNA or 21–25 nucleotide RNAs containing either *GUS* or 35Senh sequences in line 13 plants. Moreover, transvection was restricted to the heteroallelic pair and did not extend to homologous transgenes at ectopic loci as might be expected if a diffusible dsRNA were involved. These data provide support for the idea that IRs have *trans*-sensing ability and can modify gene expression in certain circumstances via DNA-DNA pairing. Given that lines 14, 17, and 19, which did not exhibit transvection, contained only single copies of the transgene construct, it still remains possible that ectopic pairing interactions could occur between two loci that each contain an IR. The information on transgene structural requirements obtained from the tobacco transvection

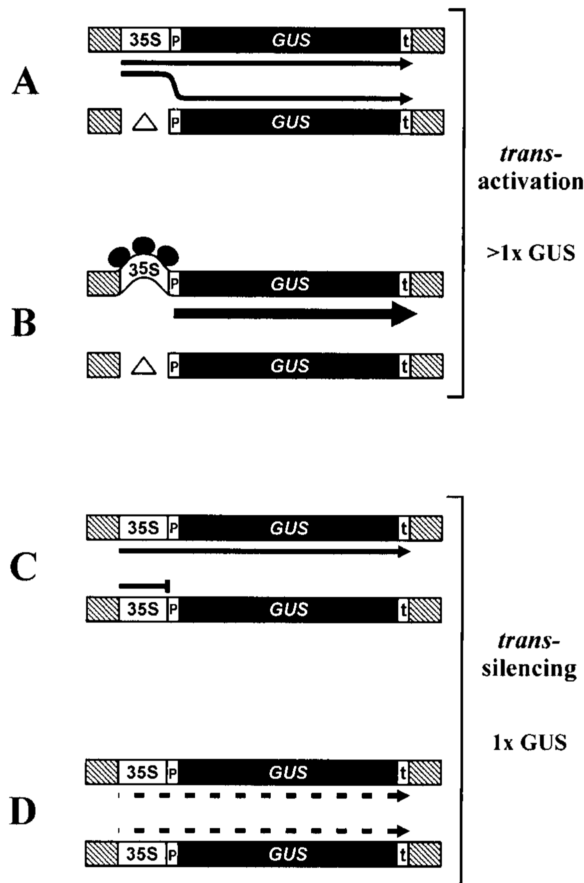

FIGURE 10.—Two types of transvection effects observed with the 35S*GUS* transgene in line 13. *trans*-activation, in which the heteroallelic 35S*GUS*/Δ*GUS* combination produced GUS activity in excess of the 35S*GUS* unaltered allele in the hemizygous condition alone (>1× GUS), provided evidence for allelic pairing. One explanation for this effect is that the 35S enhancer on the unaltered allele acted *in cis* and *in trans* to drive expression from both P-*GUS* genes (A). An alternative possibility is that pairing of two structurally dissimilar alleles deforms one allele, improving the accessibility of the single 35Senh to transcription factors (solid spheres) and increasing expression from one allele (B). When the unaltered 35S*GUS* allele was present in the homozygous state, GUS activity could be reduced to that observed in hemizygotes (1× GUS). This silencing phenomenon, which resembled dosage compensation, was presumably a consequence of allelic pairing. It is not yet known whether one allele was expressed and one was silent (C) or whether expression from both alleles was halved (D).

system is being used to reproduce the effects in Arabidopsis with the hope of obtaining multiple lines that can be used to examine the phenomenon in more detail.

The mechanism of *trans*-activation is not yet known. Conceivably, pairing could permit the enhancer to function both *in cis* and *in trans* to activate expression from two alleles (Figure 10A). Alternatively, pairing of structurally dissimilar heteroalleles could lead to deformation of one allele, causing the 35S enhancer to become more accessible to transcription factors, and boosting expression from one allele (Figure 10B). These possibilities, both of which require physical interactions between alleles, can be tested when similar experiments are performed with marked alleles whose individual expression can be distinguished.

The magnitude of *trans*-activation is notable. In line 13 S_2 no. 4, average GUS activity resulting from the heteroallelic combination was nearly double that observed in hemizygotes, and thus approached a level normally expected in homozygotes. This indicates that certain transgene alleles can locate each other in a large genome containing much repetitive DNA. [The tobacco haploid genome contains 4.4 pg of DNA (Bennett and Leitch 1998), ~45% of which consists of repeated sequences (Zimmerman and Goldberg 1977)]. Moreover, despite the apparent lack of stable pairing of homologous chromosomes in most somatic plant cells, the *trans*-activation we observed suggests that relatively long-lived associations can form between specific regions, in particular those that have one or more special features noted here, such as repetitiveness or the potential to form secondary structures. It is unclear why the strengths of the transvection effects were variable within line 13. If a cruciform is required to promote homology searching and allelic pairing, then perhaps the locus *13* cruciform, with its relatively long spacer (~12 kb) separating IRs that are ~2 kb in length, might not have formed frequently. Many IRs are present in higher eukaryotic genomes and they must be considered sources of not only physical instability but also epigenetic variability. In addition to the transvection effects observed with the transgene locus in line 13, the apparent meiotic loss of one allele in line 13 S_2 no. 4 is consistent with the general lability of IRs.

The silencing phenomenon seen in homozygous line 13 progeny differs from those reported previously in plants by resembling dosage compensation, where two copies of a gene are expressed at a level comparable to one copy. Normally, homozygous transgene loci that undergo HDGS are suppressed considerably more than the 50% observed here (Van Houdt *et al.* 2000). It is not known whether the silencing observed in homozygous line 13 plants resulted from inactivation of just one *GUS* allele (Figure 10C) or whether activity of each allele was reduced by a fractional amount (Figure 10D). We assume that silencing occurred at the transcriptional level because of the apparent involvement of enhancer sequences and the absence of dsRNA or small RNAs containing *GUS* sequences, which would be indicative of PTGS. Dosage compensation suggests some sort of counting mechanism. The IR, which can conceivably pair *in cis*, might already be counted as two copies, the normal diploid number. This could result in the inactivation of one allele in homozygotes where the IR would be perceived as four copies. In mammals, dosage compensation is exemplified by X-chromosome inactivation. Similar to the results reported here, physical interactions between *Xist* alleles have been proposed as

a means to silence and heterochromatize one allele but not the other (Marahrens 1999). Other cases of monoallelic expression in mammals, either with (Lasalle and Lalande 1996; Duvillie *et al.* 1998) or without (Hollander *et al.*, 1998) parental imprints, could also possibly result from homologous pairing, particularly at regions of heterochromatic repeats (Marahrens 1999) or IRs.

None of the three single-copy loci exhibited either *trans*-activation or silencing. This suggests that unlike the examples from filamentous fungi, where simple sequence duplications are sufficient to trigger DNA modifications, single-copy loci do not appreciably pair in somatic plant cells. Alternatively, pairing could occur but be inconsequential for gene expression. The stable activity of the three single-copy loci, all of which expressed the 35S*GUS* gene at levels higher than locus *13*, does not support threshold models for gene silencing. Complex loci, particularly those containing IRs, continue to be most frequently implicated in silencing effects in plants (Stam *et al.* 1998; Luff *et al.* 1999).

In summary, the results presented here provide evidence that alleles of a large and repetitive transgene locus can interact *in trans*—most likely via DNA-DNA pairing—to modify gene expression either positively or negatively in somatic cells. Given the abundance of various types of repeats in most plant and mammalian genomes and the proximity of repeats to many genes, it is possible that pairing interactions involving these repeats affect the activity of endogenous genes more often than currently believed. Transgenes can be useful tools for probing the types of effects that are possible and their sequence and structural requirements.

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