Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7752–7756, October 1990
Genetics

Wheat dwarf virus *Ac/Ds* vectors: Expression and excision of transposable elements introduced into various cereals by a viral replicon

(geminivirus/viral vector/DNA replication/monocotyledonous plants/plant genetic engineering)

JÜRGEN LAUFS*, UWE WIRTZ*†, MATTHIAS KAMMANN*, VOLKER MATZEIT*, SABINE SCHAEFER*, JEFF SCHELL*, ARMIN PETER CZERNILOFSKY‡, BARBARA BAKER†, AND BRUNO GRONENBORN*§¶

*Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, Federal Republic of Germany; †Plant Gene Expression Center, U.S. Department of Agriculture, Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710; §Institut des Sciences Végétales, Centre National de la Recherche Scientifique, 91198 Gif sur Yvette Cedex, France; and ‡Rheinisch Westfälische Technische Hochschule, 5100 Aachen, Federal Republic of Germany

Contributed by Jeff Schell, June 15, 1990

ABSTRACT **The maize transposon Activator (*Ac*) and *in vitro*-generated nonautonomous derivatives thereof [*Ac*Δ or Dissociation (*Ds*) elements] were inserted into the genome of a geminivirus of graminaceous plants, wheat dwarf virus, at a site that does not interfere with viral replication. These recombinant viral genomes were introduced into wheat, maize, and rice protoplasts, where rapid and efficient excision of *Ac* was observed. Excision was detected only in vectors in which, after transfection, the virus could replicate. This result is not restricted to the autonomous *Ac*; excision of *Ds* elements was also induced by transposase activity provided in trans by plasmids expressing the cDNA of *Ac*. The potential of this combination of a transposon with a viral replicon for plant molecular genetic engineering is discussed.**

The Activator (*Ac*) and Dissociation (*Ds*) transposons (1) isolated from *Zea mays* are genetically and molecularly well characterized (for reviews, see refs. 2–4). Genetic and molecular studies have shown that these elements transpose in a nonreplicative manner, but transposition is associated with DNA replication (5, 6).

Ac and *Ds* have been introduced in the dicotyledonous plant species tobacco, tomato, potato, carrot, and *Arabidopsis* and shown to transpose (7–10). The rapidly increasing knowledge concerning the molecular requirements of *Ac* and *Ds* to transpose emanates largely from *in vitro* systems or reverse genetics using nonhost plants. However, neither *Ac* nor *Ds* has been reintroduced into maize or any other monocotyledonous species.

We have shown (11, 12) that vectors based on the genome of the geminivirus wheat dwarf virus (WDV) replicate in monocotyledonous suspension culture cells, wheat embryos, and cells of intact plants (refs. 13–15 and B.G., unpublished work). In addition, these experiments demonstrated that foreign genes are stably maintained in the viral genome and are expressed over a period of ≈2 weeks (13).

Vectors comprising a virus replicon and the *Ac/Ds* transposons may prove useful for investigation of the process of transposition itself as well as provide a means for the delivery of these transposons along with other genes into the genome of monocotyledonous species.

Here we describe the excision of *Ac* and *Ds* from the WDV replicon.

MATERIALS AND METHODS

Standard molecular biological techniques were used as described (16).

Construction of Recombinant WDV Genomes. (*i*) pWDV::*Ac* and pWDV::*Ac*Δ. The vector molecule for the *Ac* and *Ac*Δ elements was the plasmid pWDVΔ4X6 (Fig. 1). This is a deletion derivative of a Swedish isolate of WDV (15), in which 918 base pairs (bp) (nucleotides 253–1170, according to ref. 15) of the WDV(+) strand open reading frames (ORFs) are replaced by a unique *Xho* I site. This deletion mutant of WDV is cloned in the *Sst* I site of pUC18. Unique *Sal* I restriction sites were introduced into the sequences flanking the 11-bp inverted terminal repeats of *Ac*(*Ac wx-m9*, refs. 18–20) by site-directed mutagenesis (21) with the two oligonucleotides 5′-CCTACTTTCATCCCTGCGTCGACTTGGTGGAGGACGTGC and 5′-CCGTTTTCATCCCTATGTCGACGAGCTGCGGGATGGC for the 5′ and 3′ ends of *Ac*, respectively. Only one nucleotide of the waxy locus is left between the inverted repeats and the *Sal* I recognition sites (see Fig. 1). The resulting 4579-bp *Sal* I fragment containing *Ac* was inserted into the *Xho* I site of pWDVΔ4X6. *Ac*Δ*1* is deleted for 1676 bp between a *Nae* I site in the 5′ leader sequence (position 429, coordinates of the *Ac* sequence according to ref. 22) and the *Nar* I site (position 2105) in exon II of the transposase gene. In this deletion mutant, 337 amino acids of the N terminus of the transposase are missing. *Ac*Δ*2* is a 1855-bp deletion between the *Bal* I sites at positions 268 and 2123 of *Ac*. In *Ac*Δ*3* the 5′ end of the deletion is at position 245 of *Ac* (endpoint of a Bal-31 deletion in plasmid pKU33; ref. 22) and extends to the *Acc* I site at position 4194. *Ac*Δ*3* has a size of only 627 bp and shares no homology with the cDNA clone of the transposase, except for 210 bp in the 3′-untranslated region.

(*ii*) pWDV rep$^-$::*Ac*. pWDVΔ4X6 was cut with *Sty* I (bp 2424 and 2514); the recessed 3′ ends were filled in with Klenow polymerase and ligated. This deletion removes 30 amino acids from the 5′ end of WDV ORF III, which is essential for replication (15). Subsequently the *Ac–Sal* I fragment was inserted into the *Xho* I site of the pWDVrep$^-$ plasmid.

(*iii*) p35ScAc. p35ScAc contains the *Ac* cDNA (22) inserted between the cauliflower mosaic virus 35S RNA promoter and the polyadenylation signal of the nopaline synthase gene (23). The cDNA (22) does not contain the entire untranslated leader (17), and, therefore, it does not overlap with the 5′ end of *Ac*Δ*1* (Fig. 1).

Maintenance of Suspension Cell Lines and Transfection with DNA. The suspension culture of *Z. mays* 'Black Mexican Sweet' was cultured, and protoplasts were prepared and transfected as specified (24); *Triticum monococcum* and *Oryza sativa* cells were maintained, and protoplasts were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: *Ac*, Activator; *Ds*, Dissociation; ORF, open reading frame; PCR, polymerase chain reaction; WDV, wheat dwarf virus.
¶To whom reprint requests should be addressed at * address.

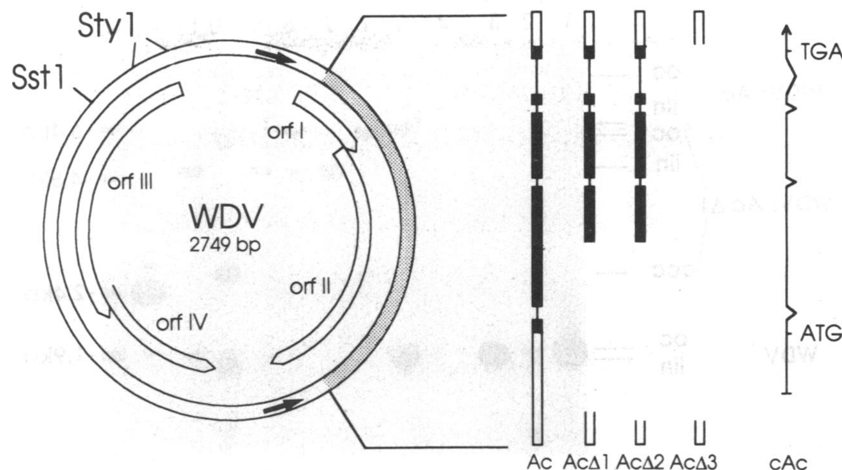

FIG. 1. Insertion of *Ac* and *Ac*Δ elements into the WDV genome. The vector molecule for the *Ac* and *Ac*Δ elements was plasmid pWDVΔ4X6, in which 910 bp of the WDV(+) strand ORFs (grey segment) are replaced by a unique *Xho* I site. The deleted WDV genome is inserted at the *Sst* I site of pUC18. Arrows mark positions of the primers used in the amplification reactions (see Fig. 4). The transcribed region of the cDNA of *Ac* is aligned with the *Ac* element. The translated region of *Ac* (the putative transposase gene) is displayed as black bars (exons) interrupted by four lines representing the introns (17). The nontranslated sequences are represented by open boxes. The deleted regions of *Ac*Δ*1*, *Ac*Δ*2*, and *Ac*Δ*3* are indicated.

prepared and transfected, as described (13, 25, 26). All transfections were done after releasing the bacterial replicon (pUC18) from the WDV vector by *Sst* I.

Protoplasts were treated with 15 μg of linear DNA, except that *O. sativa* protoplasts were transfected with 100 ng of pWDV::*Ac*. Fifty micrograms of supercoiled p35ScAc DNA was applied in cotransfection experiments.

Analysis of Viral Forms Isolated from Transfected Protoplasts. For Southern analysis total cellular DNA was isolated (13) and fractionated either undigested or *Sst* I-digested on 1% agarose gels. The DNA was transferred to nylon membranes (Hybond-N; Amersham) and hybridized to WDV4X6 probes labeled with [α-^{32}P]dCTP by nick-translation.

For the polymerase chain reaction (PCR) (27), 1–2 μg of total DNA was amplified in a volume of 100 μl (10 mM Tris, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/200 μM each of dNTP/0.01% gelatin) by 2 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin–Elmer) and ≈1 μg of the two WDV-specific primers (*i*) 5′-GACGGGGAAATCTGTGCCATGCC and (*ii*) 5′-CTCAGGGTGTTGTATTTCGTTT). Twenty-five cycles of 1 min/94°C, 2 min/60°C, and 3 min/72°C were performed, and one-tenth of the total amplification reaction was run on 2% agarose gel and stained with ethidium bromide.

Analysis of the Empty Donor Site. At days 6 and 13 after transfection total DNA was isolated from the *T. monococcum* cells and enriched for supercoiled viral DNA forms by adding SDS to 0.75% and NaOH to 0.15M. Then the cell debris and the chromosomal DNA were precipitated in a final concentration of 1 M potassium acetate. The resulting DNA was digested by *Sst* I and *Hin*dIII and cloned in plasmid pUC118 (p118 WDV *Sst* I/*Hin*dIII). The DNA sequence was determined on one strand by using a WDV-specific primer (5′-GTCCTAGCGCGGACGGCG). The complementary strand was sequenced with the standard *lac*α sequencing primer after subcloning a suitable WDV *Bcl* I–*Hin*dIII fragment in pUC119 cut by *Bam*HI and *Hin*dIII. Both single-stranded and double-stranded DNAs were used as templates (28). When the *Z. mays* line was used as a host for transfection, *Ac* excision sites of WDV were amplified in a PCR reaction with the two WDV-specific primers (*i* and *ii*). The products were cloned into the *Sma* I site of M13mp18 and sequenced.

RESULTS AND DISCUSSION

AcΔ Is Stably Replicated on WDV, But the Autonomous Ac Is Not. Much is known about the behavior of *Ac* and *Ds* elements residing on plant chromosomes, but there have been no reports of the behavior of these elements as a part of an autonomous—e.g., a viral replicon. Hence the combination of *Ac* with a genome of a virus of monocotyledonous plants may provide a versatile and rapid tool to study transposition.

To facilitate the introduction of the *Ac* element into the WDV genome, unique *Sal* I restriction sites were introduced into the sequences flanking the 11-bp inverted terminal repeats of *Ac*(*Ac wx-m9*; refs. 18–20) (Fig. 2). The resulting 4579-bp *Sal* I fragment containing the *Ac* element was inserted into a WDV vector (pWDVΔ4X6) deleted for most of the plus-strand transcription unit (ORF I and ORF II, Fig. 1). In addition to the wild-type *Ac* element, three different nonautonomous deletion mutants of *Ac*(*Ac*Δ*1*,-Δ*2*, and -Δ*3*) were constructed and inserted into the WDV vector (Fig. 1). These nonautonomous elements lack between 337 amino acids (*Ac*Δ*1*) and the entire reading frame (*Ac*Δ*3*) of the transposase. *Ac*Δ*1* and *Ac*Δ*2* have nearly the same 3′ endpoint of the deletion (bp 2105 vs. 2132) but differ therein, in that the 5′ end of the deletion in *Ac*Δ*2* proceeds beyond the

FIG. 2. DNA sequence of the WDV::*Ac* donor site. DNA of the plasmid pAc 21-5 (*Ac wx m-9*) (19) served as a substrate for the introduction of *Sal* I recognition sites (underlined uppercase letters) flanking the 11-bp inverted terminal repeats of *Ac* (lowercase letters). The resulting *Sal* I fragment (4579 bp) was cloned into the unique *Xho* I site of WDVΔ4X6 (Fig. 1); the flanking WDV sequences are shown in italics (pW::*Ac*, *Ac* in pWDVΔ4X6). Bold uppercase letters represent the transversion obtained upon *Ac* excision. Revertants of type 1 were rescued from both *Z. mays* ('Black Mexican Sweet') and *T. monococcum*, revertants of type 2 were rescued from *T. monococcum*, and revertants of type 3 were from *Z. mays* and twice independently from *T. monococcum*. The orientation of *Ac* fragment corresponds to Fig. 1.

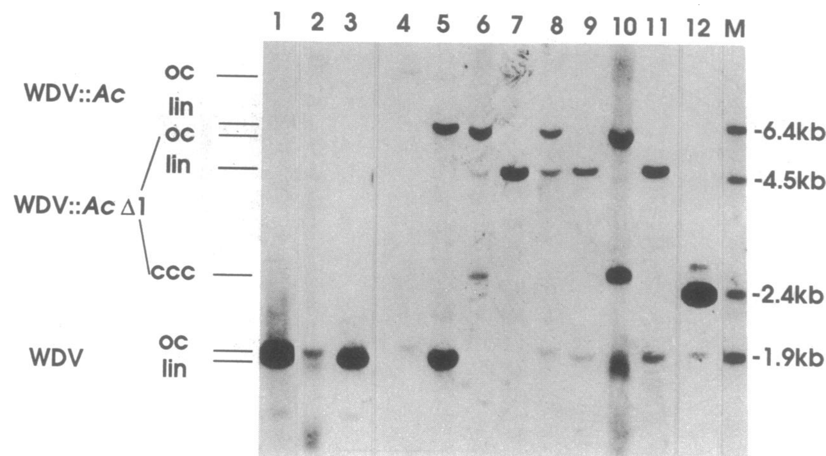

FIG. 3. Replication and excision behavior of WDV::*Ac* and WDV::Δ*Ac* as determined by Southern analysis. After transfection of rice protoplasts with pWDV::*Ac* (lanes 2 and 3) and *T. monococcum* (residual lanes), total DNA was isolated at day 7 or as specified below. DNA isolated from *T. monococcum* protoplasts transfected with pWDVΔ4X6 (lane 1), pWDV::*Ac* (lanes 4 and 5), pWDV::*Ac*Δ*1* (lanes 6–11), or pWDV::*Ac*Δ*3* (lane 12). DNA in lanes 8–12 was isolated from cells additionally transfected with p35ScAc containing the *Ac* cDNA. DNA in lanes 10 and 12 was isolated 13 days after transfection; all other samples were prepared 6 days after transfection. DNA of lanes 1, 3, 5, 7, 9, 11, and 12 was digested by *Sst* I before fractionation. Lane M contains ≈30 pg each of linearized plasmid to mark positions of the linear WDV::*Ac* (6.4 kb), WDV::*Ac*Δ*2* [4.5 kilobases (kb)], WDV::*Ac*Δ*3* (2.4 kb), and WDVΔ4X6 (1.9 kb). The linear *Sst* I fragment of WDV::*Ac*Δ*1* is 4.7 kb and is not part of the marker DNA. oc, open circular; lin, linear; ccc, closed covalent circular.

predominant transcription start (17) in the putative promotor region.

The behavior of the recombinant WDV::*Ac* molecules was analyzed after the transfection of protoplasts derived from cells of suspension cultures of the primitive wheat (*T. monococcum*), rice (*O. sativa* cv. Thaipei), and maize (*Z. mays* cv. Black Mexican Sweet) (23, 25, 29).

Transfection of *T. monococcum* protoplasts with linear WDV::*Ac*Δ*1* leads to two additional bands hybridizing to a WDV-specific probe in Southern blot analysis, the open circular and closed covalent circular form of WDV::*Ac*Δ*1* (Fig. 3, lanes 6 and 7). This result demonstrates that the defective transposons (*Ac*Δ*1* and *Ac*Δ*2*, shown in Fig. 4 lanes of group 7) were stably replicated as a part of the WDV vector. This behavior is the same as was seen for a variety of foreign genes that had been previously inserted into the WDV genome (13).

In contrast, when the authentic *Ac* element was carried by the WDV vector, newly appearing bands of 1.9 kb in size were detected as early as 3–6 days after transfection (Fig. 3, lanes 2–5). These bands correspond to the respective open circular and linear forms of the "empty" WDV replicon after excision of *Ac* and were never seen after transfection with WDV::*Ac*Δ DNA.

To determine whether the disappearance of the *Ac* element from the WDV genome was due to general recombination or to the *Ac*-specific excision mechanism, replicative forms of the WDV chromosome from which *Ac* had apparently been excised were cloned and sequenced. The sequence of a total of nine "empty sites" was determined, six of which were of independent origin. The empty donor sites fall into three groups (Rev1–3, Fig. 2), each characterized by a typical sequence alteration. All three "empty sites" are completely devoid of *Ac* sequences, and the innermost nucleotides

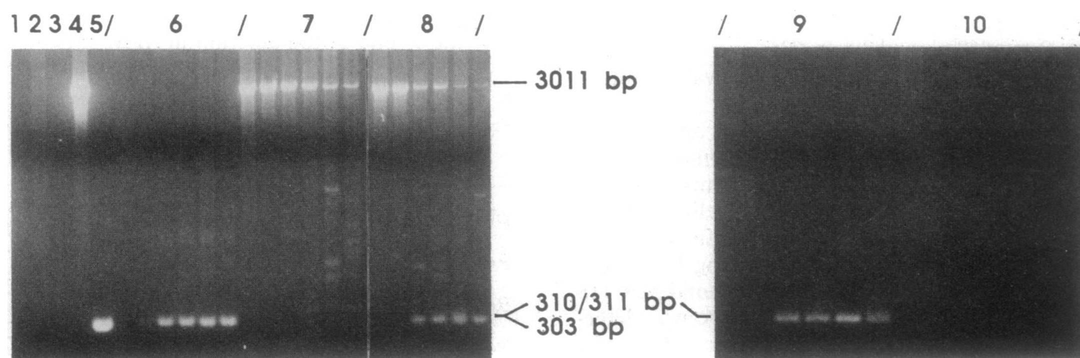

FIG. 4. Analysis of *Ac*/*Ds*-excision in *Z. mays* ('Black Mexican Sweet') cells by PCR. WDV-specific primers *i* and *ii* flanking the position of the inserted *Ac*/*Ds* elements (see Fig. 1) were used to amplify the respective DNA region of plasmid pWDV4X6 before insertion of *Ac* (lane 5), yielding a fragment of 303 bp, or after excision of *Ac* (the six lanes of group 6), leading to 310/311-bp fragments, as identified by cloning and sequencing of two amplification products. PCR of the respective sequence of WDV::*Ac*Δ*2* with the unexcised element leads to the indicated 3-kb band, whereas no PCR product of 4.8 kb corresponding to the entire *Ac* element of pWDV::*Ac* could be obtained, probably due to its G+C-rich promoter region (lane 3). Lanes 1–4, control amplification reactions containing the two WDV-specific primers alone (lane 1), total DNA of the *Z. mays* cells and the WDV primers (lane 2), ≈50 pmol of pWDV::*Ac* (lane 3), pWDV::*Ac*Δ*2* (lane 4), and pWDVΔ4X6 (lane 5). Total DNA of *Z. mays* cells was harvested at 3 hr and 2, 5, 7, 14, and 21 days after transfection. The PCR reactions with DNA of each of these time points are summarized in the 6 lanes (left to right) of groups 6–10. Groups: 6, transfection with pWDV::*Ac*; 7, pWDV::*Ac*Δ*2*; 8, pWDV::*Ac*Δ*2* cotransfected with p35ScAc; 9, pWDV::*Ac*; 10, pWDV rep⁻::*Ac*. The sizes of the products obtained in the different amplification reactions are indicated. Only the reactions of groups 6 and 9 (pWDV::*Ac*) and group B (pWDV::*Ac*Δ*2* plus p35ScAc) lead to the characteristic empty-donor fragments produced by excision of *Ac* or *Ac*Δ*2*, respectively. No empty-donor fragment was seen when the WDV vector carried the nonautonomous transposon *Ac*Δ*2* (group 7) or when a nonreplicating WDV vector was used (group 10).

adjacent to the former insertion site of *Ac* are either transversed or deleted. These alterations fit well into the proposed model of the excision (30) and prove that the *Ac*-encoded function of excision is also operative on a replicating virus genome. So far we have no evidence for any unspecific loss of the transposon from recombination.

Excision from Replication-Deficient WDV Was Not Found. Genetic data indicate that transposition occurs during or after chromosome replication (5, 6, 31–33). The role of replication in transposition is not yet understood in detail, but it is strongly influenced by methylation; recent biochemical analysis revealed a correlation between methylation of *Ac* and its transpositional activity visualized phenotypically (34–36). In addition, transcription (37) and binding of the transposon-encoded protein to subterminal DNA motifs (38) are affected by methylation. Although these results suggest that replication can control transposition through the change in DNA methylation, current data are not sufficient to exclude an additional influence of the chromosome replication process on transposition.

Hence, we were interested in determining whether transposition is linked to replication. Therefore, *Ac* was combined with a WDV molecule deficient in replication resulting from a deletion of 30 amino acids in ORF III (pWDVrep$^-$, Fig. 1). After transfection of *Z. mays* protoplasts, excision of *Ac* from pWDVrep$^-$::*Ac* was assayed by PCR (Fig. 4), but no excision product could be amplified. Nevertheless it is difficult to state that excision does not occur from a replication-deficient WDV molecule because the frequency of excision might remain below the detection level.

Transactivation of *Ac*Δ. Excision of the defective *Ac* elements from the WDV genome by an active transposase provided in trans was investigated by cotransfection of the deletion mutants pWDV::*Ac*Δ*1*, -Δ*2*, and -Δ*3* and a cDNA clone expressing the transposase under control of the strong constitutive 35S RNA promoter of cauliflower mosaic virus (23). As shown in Fig. 3, lanes 8–12, two replication products are detected by the virus-specific probe—the hybrid WDV::*Ac*Δ*1* (or WDV::*Ac*Δ*3* in lane 12) and again an "empty" WDV genome of 1.9 kb, corresponding to the excision product. In contrast to the very efficient excision of *Ac* by itself, its movement by transactivation occurs at a lower level. Whether this is from an escape of the replicating WDV::*Ac*Δ molecules from a limited supply of transposase by the nonreplicative plasmid p35ScAc or whether it reflects an intrinsically lower efficiency in trans complementation remains open. However, the similar results were observed when a cDNA was used to mobilize a nonautonomous *Ac*Δ element in tobacco (22).

Conclusion and Perspective. The combination of the transposable element *Ac* with the autonomously replicating genome of WDV leads to the rapid and genuine excision of *Ac*. Typical footprints, obtained upon excision were of the same type as described for maize, tobacco, or *Arabidopsis* (7, 10, 19). They provide evidence that the mechanism of excision is truly operating to move *Ac* from the WDV molecule. The results also show that the combination of the WDV replicon with the autonomous transposable element *Ac* provides a rapid and simple tool to study the process of excision at the molecular level. Furthermore, the demonstration that the nonautonomous *Ac* elements (*Ac*Δ*1*, -Δ*2*, and -Δ*3*) are stably replicated and that these *Ac*Δ elements can be excised when active transposase is provided in trans suggests the use of a geminivirus replicon, combined with an engineered transposon, for gene delivery in monocotyledonous plants.

Several ways to deliver DNA to the genome of gramineaceous monocots have been described recently; examples include the direct injection of DNA into immature inflorescences of rye (39), the "biolistic" shooting of tungsten or gold particles coated by DNA into plant tissue (40), or the imbibition of a DNA solution by dry embryos (41). Using the latter technique, we have successfully introduced replicative recombinant WDV vectors into embryos of wheat, rye, oats, and other cereals (14).

Replicative gene expression vectors were also developed based on genomes of geminiviruses infecting dicotyledonous plants (42, 43). In conjunction with the aforementioned techniques to introduce DNA or with the method of "agroinfection" (44), the use of geminivirus replicons carrying suitably tailored transposons might pave promising avenues for the genetic engineering of cereals.

We are indebted to G. Coupland for providing plasmids and H. Lörz for providing the rice suspension culture. We thank G. Coupland and D. Furtek for discussion and R. Walden for comments on the manuscript. This work was supported by the Bundesminister für Forschung und Technologie through a grant to B.G. J.L. is a recipient of a German–Israeli Foundation fellowship (I-57-259 12/87) and U.W. acknowledges the receipt of a fellowship from Deutscher Akademischer Austausch Dienst.

1. McClintock, B. (1951) *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13–47.
2. Döring, H. P. & Starlinger, P. (1986) *Annu. Rev. Genet.* **20**, 175–200.
3. Peterson, P. A. (1987) *CRC Crit. Rev. Plant Sci.* **6**, 105–208.
4. Fedoroff, N. V. (1989) *Cell* **56**, 181–191.
5. Van Schaik, N. W. & Brink, R. A. (1959) *Genetics* **44**, 725–738.
6. Chen, J., Grenblatt, I. M. & Dellaporta, S. L. (1987) *Genetics* **117**, 109–116.
7. Baker, B., Schell, J., Lörz, H. & Fedoroff, N. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4844–4848.
8. Yoder, J. I., Palys, L., Alpert, K. & Lassner, M. (1988) *Mol. Gen. Genet.* **213**, 291–296.
9. Knapp, S., Coupland, G., Uhrig, U., Starlinger, P. & Salamini, F. (1988) *Mol. Gen. Genet.* **213**, 285–290.
10. Van Sluys, M. A., Tempe, J. & Fedoroff, N. (1987) *EMBO J.* **6**, 3881–3889.
11. Lindsten, K. (1980) *Vaextskyddsnodiser* **44**, 54–60.
12. Gronenborn, B. & Matzeit, V. (1989) *Cell Cult. Somatic Cell Genet. Plants* **6**, 69–100.
13. Matzeit, V., Kammann, M., Schaefer, S., Schalk, J., Schell, J. & Gronenborn, B. (1989) *Plant Cell*, in press.
14. Töpfer, R., Gronenborn, B., Schell, J. & Steinbiss, H.-H. (1989) *Plant Cell* **1**, 133–139.
15. Schalk, H.-J., Matzeit, V., Schiller, B., Schell, J. & Gronenborn, B. (1989) *EMBO J.* **8**, 359–364.
16. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Kunze, R., Stochaj, U., Laufs, J. & Starlinger, P. (1987) *EMBO J.* **6**, 1555–1563.
18. Fedoroff, N., Wessler, S. & Shure, M. (1983) *Cell* **35**, 235–242.
19. Pohlmann, R. F., Fedoroff, N. & Messing, J. (1984) *Cell* **37**, 635–643.
20. Pohlmann, R. F., Fedoroff, N. & Messing, J. (1984) *Cell* **39**, 417.
21. Kramer, W., Drutsa, V., Jansen, H.-W., Pflugfelder, M. & Fritz, H.-J. (1984) *Nucleic Acids Res.* **12**, 9441–9456.
22. Coupland, G., Baker, B., Schell, J. & Starlinger, P. (1988) *EMBO J.* **7**, 3653–3659.
23. Fromm, M., Taylor, L. P. & Walbot, V. (1986) *Nature (London)* **319**, 791–793.
24. Fromm, M., Callis, J., Taylor, L. P. & Walbot, V. (1987) *Methods Enzymol.* **153**, 351–366.
25. Zimny, J. & Lörz, H. (1986) *Plant Cell Rep.* **5**, 89–92.
26. Maas, C. & Werr, W. (1989) *Plant Cell Rep.* **8**, 148–151.
27. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Ehrlich, H. A. & Arnheim, N. (1985) *Science* **230**, 1350–1354.
28. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153**, 3–11.
29. Lörz, H., Baker, B. & Schell, J. (1985) *Mol. Gen. Genet.* **199**, 178–182.
30. Saedler, H. & Nevers, P. (1985) *EMBO J.* **4**, 585–590.

31. Greenblatt, I. M. & Brink, R. A. (1962) *Genetics* **47**, 489–501.
32. Greenblatt, I. M. & Brink, R. A. (1963) *Nature (London)* **197**, 44–413.
33. Greenblatt, I. M. (1984) *Genetics* **108**, 471–485.
34. Schwarz, D. & Dennis, E. (1986) *Mol. Gen. Genet.* **205**, 476–482.
35. Chomet, P. S., Wessler, S. & Dellaporta, S. L. (1987) *EMBO J.* **6**, 295–302.
36. Schwartz, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2789–2793.
37. Kunze, R., Starlinger, P. & Schwartz, D. (1988) *Mol. Gen. Genet.* **27**, 323–327.
38. Kunze, R. & Starlinger, P. (1989) *EMBO J.* **8**, 3177–3185.
39. de la Pena, A., Lörz, H. & Schell, J. (1987) *Nature (London)* **325**, 274–276.
40. Klein, T. M., Wolf, E. D., Wu, R. & Sanford, J. C. (1987) *Nature (London)* **327**, 70–73.
41. McCabe, D. E., Martinell, B. J. & Christou, P. (1988) *Bio/Technology* **87**, 923–926.
42. Hayes, R. J., Petty, I. T. D., Coutts, R. H. A. & Buck, K. W. (1988) *Nature (London)* **334**, 179–182.
43. Ward, A., Etessami, P. & Stanley, J. (1988) *EMBO J.* **7**, 1583–1587.
44. Grimsley, N., Hohn, T., Davies, J. W. & Hohn, B. (1987) *Nature (London)* **325**, 177–179.