Journal of Plant Biology, March 2000, 43(1) : 1-9

Germinal Virus Vector WDV (Wheat Dwarf Virus)-Mediated Multiple Insertions of a Maize Transposon, *Ds* (Dissociation), in Rice

Hang Gyeong Chin[1,3], Sung Han Park[2,3], Mi Sook Choe[1,3], Su Hyun Park[1,3], Byeong Keun Oh[4], Gi Hwan Lee[4], Hae Choon Choe[4], Moo Je Cho[2,3], Jong Chan Hong[2,3], and Chang-deok Han[1,3]*

[1]Department of Molecular Biology, Gyeongsang National University, Chinju 660-701, Korea
[2]Department of Biochemistry, Gyeongsang National University, Chinju 660-701, Korea
[3]Plant Molecular Biology and Biotechnology Research Center (PMBBRC) Gyeongsang National University, Chinju 660-701, Korea
[4]YeongNam National Agricultural Experiment Station, Milyang 627-130, Korea

Wheat dwarf virus (WDV) is a monocot-infecting geminivirus that replicates in infected tissue as double-stranded DNA. We evaluated whether the WDV vector system bearing *Ds* could be used as an effective insertional mutagen in rice. Molecular data showed that *Ds* was excised from WDV vectors once the WDV-carrying *Ds* (WDV::Ds) and the genomic *Ac* vector were co-introduced into rice calli. Mature T0 and T1 transgenic plants were analyzed for the distribution and inheritance of *Ds* inserts. Southern analysis indicated that the *Ds* elements excised from WDV vectors were stably inserted into genomes. The number of transposed *Ds* ranged from zero to three copies, among independent transformants. Meanwhile, untransposed *Ds* (WDV::Ds) were present in multiple-copies in genomes. Southern analysis of the selfed progeny of T0 plants demonstrated that most WDV::Ds were co-segregated among siblings. This indicated that these elements were integrated into the same single loci. However, a few *Ds* were found to segregate independently from the majority of *Ds*. In this report, we discuss the efficiency of WDV vectors in generating multi-copy *Ds* in rice genomes.

Keywords: *Ac/Ds*, rice, transposon tagging, WDV vectors

Ac (Activator) and *Ds* (Dissociation) in maize (McClintock, 1946) are the most extensively analyzed transposable element family in heterologous plants. A variety of modified forms of *Ac* or *Ds* have been examined to dissect their molecular mechanisms of transposition. The minimal sequence required for the mobility of *Ds* was determined by Coupland et al. (1989). *Ac/Ds* are frequently translocated to linked sites, both in maize and in dicots such as tobacco and *Arabidopsis*. The movement of these elements is coupled with chromosomal replication (Jones et al., 1991; Long et al., 1993; Scofield et al., 1993; Lawson et al., 1994). Most transposition events of *Ac* occur during or shortly after replication of a locus bearing *Ac*. The first genetic evidence was obtained from phenotypic products of *Ac* transposition events from the *P* locus in maize (Greenblatt, 1984). Later, molecular data showed that the transposition of *Ac* was positively correlated with donor DNA replication (Athma et al., 1992; Chen et al., 1992).

In monocotyledonous plants such as maize, wheat, and rice, extra-chromosomal geminivirus (especially wheat dwarf virus) vectors have been utilized to study the relationship between donor DNA replication and *Ac* or *Ds* excision. Wheat dwarf virus (WDV) is a monocot-infecting geminivirus. This genome is monopartite and consists of a circular 2.7 kb molecule of single-stranded DNA (MacDowell et al., 1985). WDV replicates in infected tissue as double-stranded DNA (dsDNA) and encodes four open reading frames (ORF I-IV). ORFs III and IV (or C1 and C2) are required for WDV DNA replication, whereas ORFs I and II (or V1 and V2) are dispensable for WDV DNA replication. Two intergenic regions between the sense ORFs I/II and the complementary sense ORFs III/IV contain promoter sequence for expression of ORFs I-IV, and *cis*-sequences (a stem-loop structure) for WDV DNA replication. Most WDV vectors developed for the study of *Ac/Ds* were deletions of V1 and V2, but carried all the elements for self-replication (Laufs et al., 1990).

Ac transposase-dependent excision of *Ds* from extra-chromosomal replicating WDV-derived vector DNA has been demonstrated in transfected black Mexican sweet (BMS) maize, *Triticum monococcum*, and *Oryza sativa* protoplastids (Hofer et al., 1992; Wirtz et al., 1997). Excision of *Ds1* from the genome of maize streak virus, a geminivirus closely related to

*Corresponding author; fax +82-519-759-9369
e-mail cdhan@nongae.gsnu.ac.kr

WDV, was detected when vector DNA was introduced *via Agrobacterium* infection into *Ac* transposase-expressing maize plants (Shen and Hohn, 1992). Most of these WDV studies have been performed in transient assay with either calli or protoplasts. Therefore, it still remains to examine whether *Ac/Ds* WDV can be used as insertional mutagens in monocotyledonous plants.

Our goal in this study was to evaluate WDV vector systems bearing *Ds* for potential insertional mutagens. After the WDV::Ds vector DNA was introduced into rice calli via particle bombardment, transient assays were used to examine whether *Ds* would be effectively excised from WDV vectors. Mature T0 and T1 transgenic plants were analyzed for the distribution and inheritance of *Ds*. Based on these data, we evaluated the efficiency of WDV vectors as *Ds*-delivering vehicles.

MATERIALS AND METHODS

Construction of Vectors

A 1.3 kb *Ds* element, carrying a BglII site at the 1050th base from the 5′ end of the element, was used for constructing the *Ds* WDV vector. The *Ds* plasmid vector was a kind gift from Sundaresan (Cold Spring Harbor Lab, USA). An expression cassette for HPT (hygromycin phosphotransferase) was installed at the BglII site. The HPT-coding region was expressed by a CaMV 35S promoter and a transcription terminator of the *tml* (tumor morphology large) gene of a Ti plasmid. The 5′ end of *Ac*, including a promoter sequence, was removed with a restriction enzyme NaeI. This truncated *Ac* genomic DNA was fused to a *Ubiquitin* promoter for strong and constitutive expression of *Ac* transposase. Whole *Ac* genomic DNA was donated by Jonathan Jones (John Innes Center, UK). Two prototype pWDV vectors, pWI-11 and pWDV2, were used in this experiment. The pWI-11 vector contained p15A replication origin (Timmermans et al., 1992). *Ds* was cloned into the pWDV2 vector. The backbone plasmid of WDV2 was changed to a low-copy plasmid vector, pMUC. Finally, pWDV::Ds (HPT) was constructed by inserting Ds (HPT) into an EcoRV site of pMUC-WDV2. These plasmids are available upon request.

Particle Bombardment and Tissue Cultures

Surface-sterilized seeds of a Japonica type rice variety (*O. sativa* var. Dong-jin) were placed on NB media for callus induction. After four weeks of culture in the dark at 26°C, embryogenic calli derived from scutella were subcultured two to three times every two weeks.

The gene gun employed in this experiment was the biolistic PDC-100/He system (Bio-Rad). Gold particles (2.1 mg) in 100% EtOH were precipitated to mix with 25 μL of DNA solution (conc. 1 mg/mL). $CaCl_2$ and spermidine were then added to the DNA/gold particle mixtures. After repetitive cycling of precipitation and suspension, DNA/gold particle mixtures were suspended in 36 μL of absolute ethanol. For 4 h prior to bombardment, about 150 embryogenic calli were placed at the center of a Petri dish (9 cm in diameter) that contained an NB medium supplemented with 0.2 M mannitol and sorbitol. Ten μL (around 6 μg DNA and 0.5 mg gold particles) of suspended DNA particles per shot were propelled by 1100 psi of helium gas. After bombardment, calli were maintained on the same medium in the dark at 26°C for 16-20 h.

To regenerate plantlets from bombarded calli, we followed slightly modified culture procedures previously reported (Hajdukiewicz et al., 1994). About 16-20 h after bombardment, those calli were transferred to an NB medium supplemented with 30 mg/L hygromycin. After two weeks of culture, hygromycin-resistant calli were selected and moved to the same medium containing hygromycin (50 mg/L) for the second round of selection.

Two weeks later, calli on the second selective media were transferred to a pre-regeneration medium. After 10 days, hygromycin-resistant calli were placed on regeneration NB media I, which contained 1 mg/L NAA, 5 mg/L kinetin, 30 g/L sorbitol, 5 g/L phytagel, and 30 mg/L hygromycin. Cultures were maintained at 26°C under a photoperiod of 16 h light and 8 h dark. About 10 days later, green-spotted calli were transferred to regeneration media II (NB medium containing 1 mg/L NAA, 5 mg/L kinetin, 30 mg/L hygromycin, and 2.5 g/L phytagel). After plantlets were regenerated from calli, they were transferred to glass bottles containing a half-strength MS medium that had been semi-solidified with 2.5 g/L phytagel and supplemented with 0.5 mg/L NAA and 30 g/L sucrose.

Transient Assay with PCR

PCR was performed with 1 μg genomic DNA. Three primers (primer 1, 2, or 3) were used. The amplification program included an initial step at 94°C

for 3 min, then 30 cycles (1 min at 94°C, 30 s at 65°C, 30 s at 72°C), and a final step at 72°C for 10 min. Primers 1 and 2 recognized the 5′ end of ΔV1 and the 3′ end of C2 of WDV, respectively. The sequence of primer 1 is 5′-cctatacgggactatcaatacca-gaccc-3′; that of primer 2 is 5′-cttcctgggcaaggtctctagg-gacac-3′. Primer 3 recognized the sequence of the 5′ end of genomic *Ac*: 5′-taccggtatatcccgttttcgtttccg-3′. Amplification DNA was size-separated by electrophoresis on a 1.2% agarose gel.

Isolation of Rice Genomic DNA and Southern Blot Analysis

Genomic DNA was prepared from mature leaves or calli, using a urea extraction procedure (K. Cone, Univ. of Missouri, USA; personal communication). Aliquots of 5 µg of purified genomic DNA were digested with appropriate restriction endonucleases, size-fractionated on a 0.8% agarose gel, and transferred to a nylon membrane (GeneScreen, DuPont). The blots were then hybridized to probes in hybridization buffer containing 6× SSC, 5× Denhardts, 0.5% SDS, 50 mM Tris (pH 8.0), 10 mM EDTA, 0.1 mg/mL salmon sperm DNA (heat denatured), and 5% dextran sulfate. When the PCR products were hybridized, blots were incubated in hybridization buffer containing 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA, and 1% BSA. Final washes of the filters were done in 0.2× SSC and 0.1% SDS solution for 30 min at 65°C. Blots were then auto-radiographed at −80°C overnight, using an intensifying screen.

RESULTS

Plasmid Vectors

Schematic diagrams of plasmids used in this study are shown in Figure 1. In the 6.2-kb WDV backbone vector, C1/C2 (or ORF III/IV) were repeated in tandem (Fig. 1A). The repeated sequences facilitate escape from the plasmid construct, via recombination of the viral DNA, in the nuclei of plant cells (Hofer et al., 1992). The consequent WDV replicon not only produces the C1 and C2 protein (presumably replicase), but is also self-replicated. A putative origin of replication is in the region between the 5′ ends of the ΔV1 and C1.

The *Ds* element of 4.1 kb that carried the HPT gene as a selection marker, was inserted into an EcoRV site at the deletion junction of V1. The resultand 10.3 kb vector was called pWDV::Ds (HPT Fig. 1A). Because C1/C2 could act in trans in replicating the viral genome, pWI-11 was co-introduced to maintain a sufficient level of C1/C2 products (Fig. 1B). An *Ac* transposase vector was constructed by fusing a genomic *Ac* element, whose 5′ end had been deleted up to an NaeI site, to a maize *Ubiquitin* promoter. The resulting vector was named pUbi-genomic *Ac* (Fig. 1C).

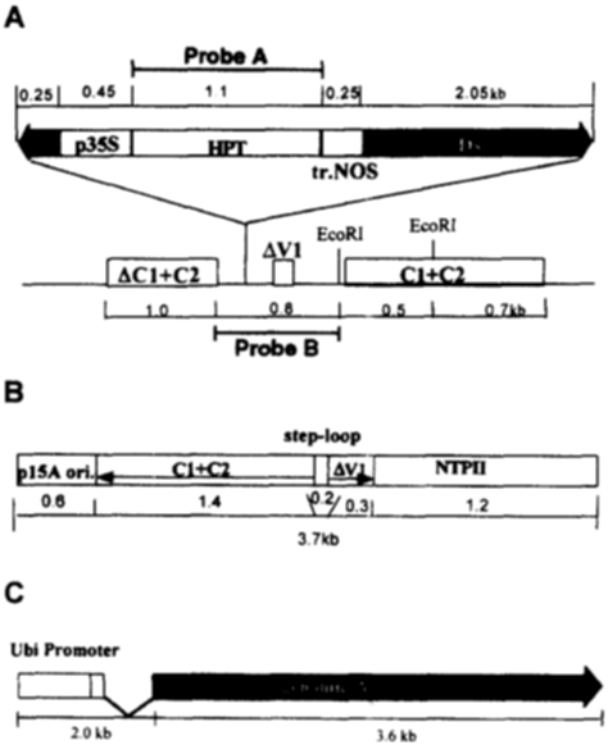

Figure 1. Schematic diagrams of the vectors of pWDV::Ds(HPT) (A), pWDV11 (B), and pUbi-genomic *Ac* (C). **A.** A *Ds* element of 4.1 kb, that carried a HPT (hygromycin phosphotransferase) gene as a selection marker, was inserted into an EcoRV site at the deletion junction of the V2 of the pWDV2 vector of 6.2 kb. In the WDV vector, C1/C2 (or ORF III/IV) were repeated in tandem at both sides of the deleted V1 (ΔV1). **B.** pWI-11 is a vector containing p15A replication origin to supplement ORFIII & IV products (presumably replicase). **C.** Genomic *Ac* whose 5′ end had been deleted up to a NaeI site was fused to a maize *Ubiquitin* promoter.

Ds Excision from WDV Vectors

Eight NBO plates, each containing around 150 calli, were bombarded by gold particles coated with a mixture of three plasmid DNA, pWDV::Ds (HPT), pWI-11, and pUbi-genomic *Ac*. To introduce the same number of molecules of each plasmid DNA

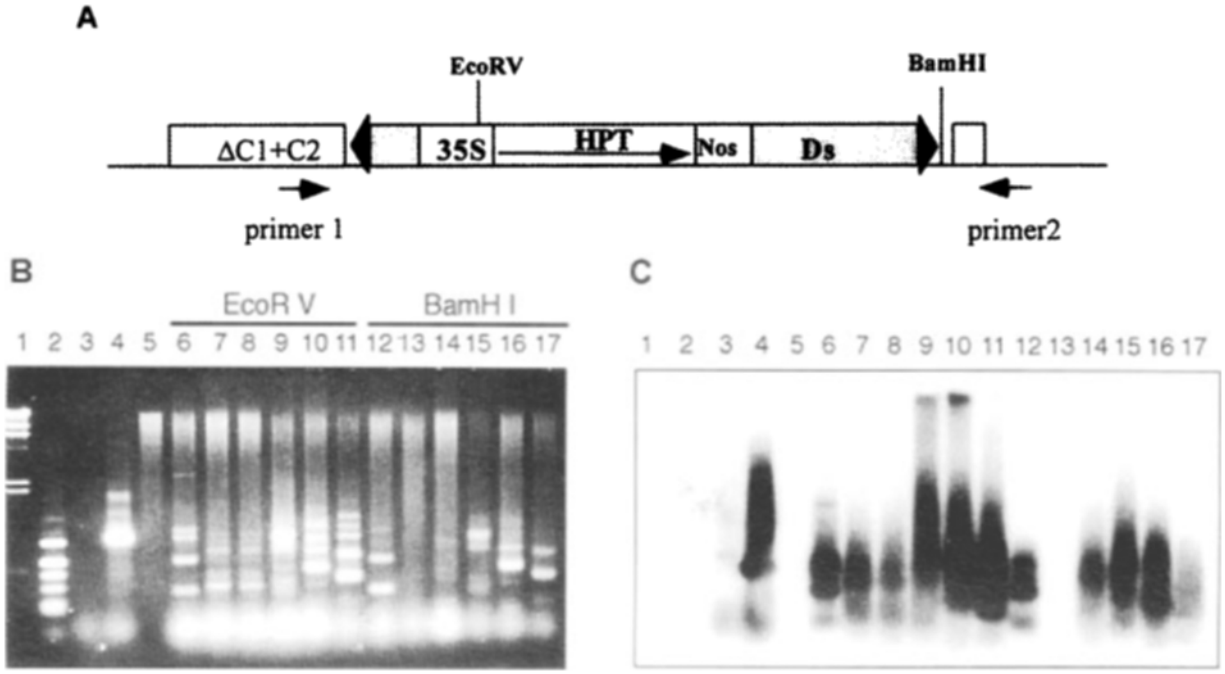

Figure 2. Molecular analysis of *Ds* excision in rice calli by PCR (B) and Southern analysis (C). EcoRV- or BamHI- cut genomic DNA was subjected to PCR amplification by primers 1 and 2, shown in Figure 2 (A). DNA samples were obtained from one-to six-day-old calli after bombardment with WDV11, WDV::Ds, and *Ac* vectors (Lanes 6 to 11 for EcoRV and Lanes 12 to 17 for BamHI of (B) and (C)). The same gel was blotted on a membrane to be hybridized with 900- bp viral DNA (see Lane 4). Lane 1 was HindIII-cut lambda DNA; Lane 2 contained AluI-cut pBR322, 0.9, 0.65, 0.52, 0.42, 0.28-0.22 kb. Lanes 3 and 4 were loaded with PCR products of EcoRV-cut pWDV::Ds (HPT) and linearized pWDV11, respectively. Unamplified EcoRV-cut genomic DNA was loaded in Lane 5 as a negative control.

into cells, three vector DNA of the same molarities were mixed and precipitated with EtOH. Approximately 6 μg of DNA were used per bombardment. After shooting, calli from eight different plates were pooled and equally redistributed on six plates. Total DNA was extracted from calli of all plates, on each of the first 6 days after bombardment.

To detect the excision of *Ds* from pWDV::Ds (HPT), genomic DNA that had been cut with EcoRV was amplified with primers 1 and 2 (Fig. 2A). The pWDV::Ds (HPT) carried only one EcoRV site inside the *Ds* element. These two primers recognized viral sequences next to the ends of *Ds*. Therefore, no DNA should be amplified from the pWDV::Ds (HPT) plasmid DNA that had been digested with EcoRV (see Lane 3 in Fig. 2).

In contrast, the WDV vector from which *Ds* had been excised should produce around 900 bp DNA. The same primers could recognize pWI-11 and result in a 3.0 kb PCR product. However, pWI-11, which contained no EcoRV site, failed to produce any DNA under the PCR condition of this experiment (data not shown). Figure 2B is an ethidium bromide stained gel of PCR products amplified by primers 1 and 2. Lane 2 showed a 900-bp DNA that was an expected product if *Ds* was excised out from the pWDV vector. From Lanes 6 to 11, i.e., the samples from Days 1 to 6 after particle bombardment, respectively, many DNA species of different sizes (including 900-bp DNA) were detected. Such heterogeneous sizes of *Ds*-donor sites could be explained by previous observations that excision of *Ds* frequently accompanied DNA rearrangements, such as by deletions and additions (for details, see Gorbunova and Levy, 1997).

The extent of this DNA rearrangement was estimated by amplifying BamHI-cut genomic DNA with the same primers. Because a BamHI site was between the *Ds* element and the primer 2 binding sequence (Fig. 2A), BamHI-cut genomic DNA should not be amplified by primers 1 and 2. However, as seen in Figure 2B, BamHI-cut genomic DNA produced PCR products. This indicated that the DNA rearrangements related to *Ds* excision were extended into the neighboring sequences. To confirm that these PCR products of different sizes were derived from the pWDV vector, DNA was transferred to a membrane and hybridized

with the 900 bp DNA of the pWDV vector. Afterward, Southern analysis showed that all the amplified DNA were, in deed, from viral DNA (Fig. 2C).

It was still possible, however, that the generation of multiple-sized DNA was related to the instability of WDV-vector DNA inside rice cells, rather than to *Ds* excisions. To test this possibility, DNA from one end of the *Ds* to the flanking viral DNA was amplified from the same samples. If WDV-vector DNA, *per se*, underwent physical rearrangement inside the cells, heterogeneous sizes would also be expected from PCR products of pWDV::Ds (HPT) that still contained *Ds*. However, if excision of *Ds* was responsible for heterogeneity in PCR-product sizes, WDV DNA carrying *Ds* should generate only a single, discrete PCR product. Hence PCR was performed with primers 1 and 3. Primer 1 was the same primer as in Figure 2. Primer 3 recognized the 5′ end of *Ds*. We expected a size of 650 bp for DNA that spanned from the 5′ end of *Ds* to the flanking viral DNA. From all six samples, a single 650 bp DNA fragment was produced from PCR with primers 1 and 3 (Fig. 3). This experiment demonstrated that pWDV::Ds (HPT) was stably maintained in the cells. It was interesting to note that the amount of PCR products decreased in the older samples. In conclusion, these data showed that the WDV::Ds system tended to undergo structural rearrangement after *Ds* was excised out.

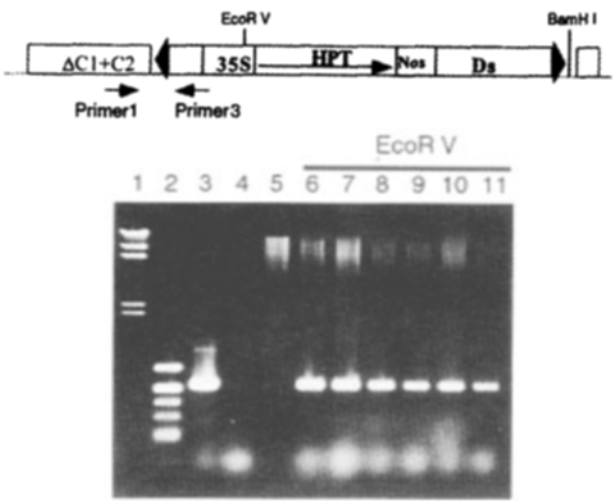

Figure 3. Molecular diagnosis for the stability of pWDV::Ds (HPT) in rice calli by PCR. The same EcoRV-cut genomic DNA samples used in Figure 2 were amplified by primers 1 and 2 (Lanes 6 to 11). Only a 650-bp band of DNA was observed from all the samples. Lane 1 shows HindIII-cut lambda DNA; Lane 2 was AluI-cut pBR322, 0.9, 0.65, 0.52, 0.42, 0.28-0.22 kb. Lanes 3 and 4 were loaded with PCR products of EcoRV-cut pWDV::Ds (HPT) and linearized pWDV11, respectively. Unamplified EcoRV-cut genomic DNA was loaded in Lane 5 as a negative control.

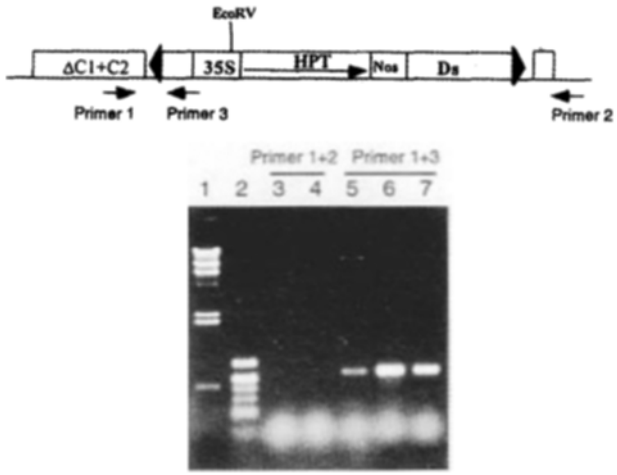

Figure 4. PCR analysis of the mobility of *Ds* without *Ac*. Calli were bombarded only with pWDV::Ds (HPT) and pWI11. After the 1st (Lanes 3 and 6) and 5th days (Lanes 4 and 7) of bombardment, DNA was extracted and digested with EcoRV. With pWDV::Ds (HPT) as control (Lane 5), EcoRV-cut genomic DNA was subjected to PCR with primers shown in Figure 4A.

An additional experiment was performed to examine whether the excision of *Ds* depended solely on the activity of exogenous *Ac* transposase. We wanted to determine if rice cells could somehow provide endogenous cellular factors to mobilize *Ds*. Therefore, calli were transformed with pWDV::Ds (HPT) and pWI-11. Total DNA was extracted from the samples on Days 1 and 5 after bombardment. To detect WDV viral DNA that had lost *Ds* during replication, EcoRV- cut genomic DNA was amplified by primers 1 and 2 that recognized the flanking DNA at either end of *Ds* (top of Fig. 4). The bottom of Figure 4B shows that primers 1 and 2 did not produce any PCR product from EcoRV-digested genomic DNA. In contrast, PCR with primers 1 and 3 produced DNA of the expected size, i.e., 650 bp.

The data indicate that the excision of *Ds* required the expression of exogenous *Ac* transposase. These results also confirmed that heterogeneous sizes of PCR products (shown in Fig. 2) were, in-deed, due to the excision of *Ds*. In summary, the vector systems presented in this study should be suitable for inducing *Ds* insertions during the generation of transgenic rice plants.

Analysis of Transgenic Plants and Inheritance of Transgenes

To evaluate whether the WDV::Ds system could

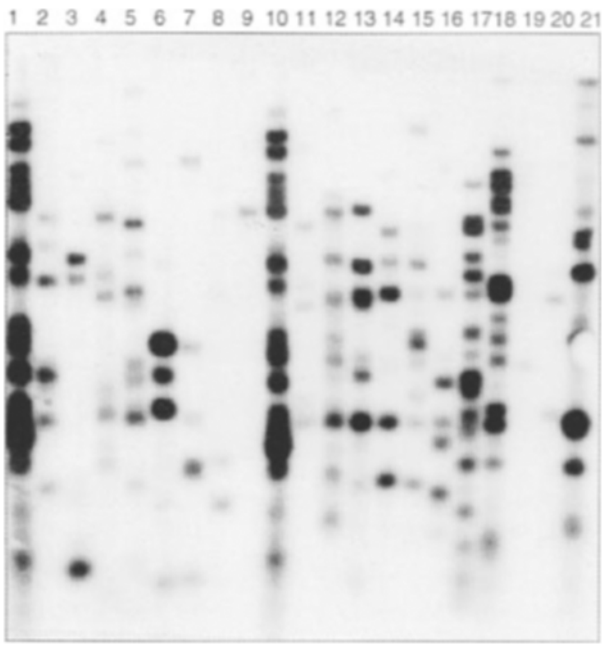

Figure 5. Southern analysis of transgenic rice plants carrying *Ds*. Plants from hygromycin-resistant calli were grown up to maturity. Genomic DNA was cut with EcoRI that did not recognize *Ds*. Each of the DNA hybridized with a hygromycin-coding region DNA (probe A of Fig. 1A) should represent a single *Ds* element..

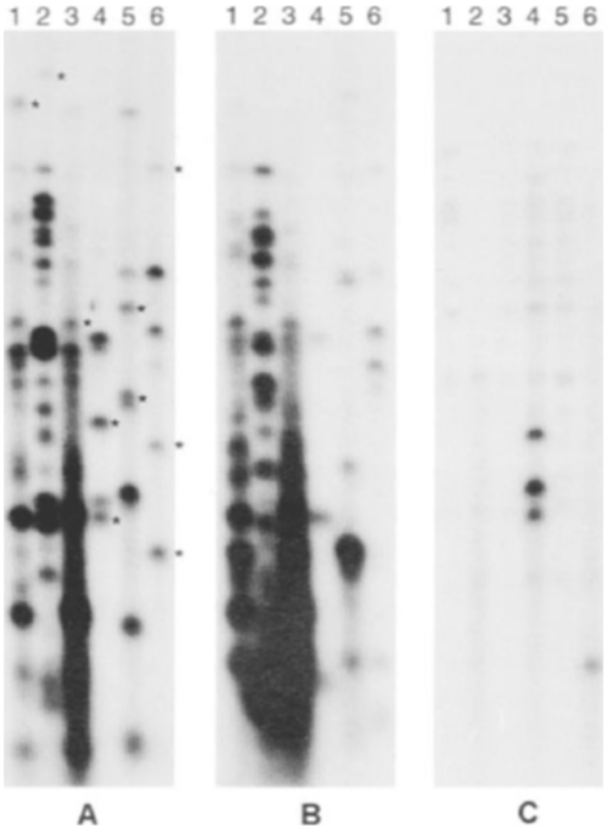

Figure 6. Southern analysis to distinguish transposed *Ds* from one staying with WDV. Independent transgenic lines were examined by Southern hybridization probed with DNA specific to *Ds* (A) and WDV (B). Genomic DNA was cut with EcoRI. As a *Ds*-specific probe, a hygromycin-coding region (probe A in Fig. 1A) was used. The 800-bp viral DNA that spanned a *Ds* (HPT) insertion site was a probe for WDV (Fig. 1B). *Ds* that were not detected with the WDV probe should be ones transposed from WDV vector DNA. To examine whether these *Ds* lines carried *Ac* elements, an *Ac* internal HindIII fragment was used for hybridization (C). DNA hybridized with the *Ds* probe but not with WDV were marked "*". These *Ds* elements were translocated from the pWDV vector.

be utilized for insertional mutagenesis in rice, mature plants regenerated from hygromycin-resistant calli were analyzed for the distribution of *Ds* in genomes. Figure 5 shows the distribution patterns of *Ds* in transgenic mature rice plant. Generally, multi-copy *Ds* were distributed in the genomes. The number of *Ds* was highly variable, from 1 to over 20 copies. Over 100 independent trangenic lines were generated. twenty randomly selected lines are shown in Figure 5. *Ds* was integrated into rice genomes in two different ways. One way involved excising the *Ds* elements from the pWDV vector and integrating them into genomes. The other way was by integrating the WDV::Ds (HPT) DNA molecules, *per se*, into rice genomes.

Both events should endow the transgenic rice plants with hygromycin resistance. To accurately estimate the insertion events of *Ds*, the *Ds* elements transposed from replicating WDV molecules must be distinguishable from those inserted along with WDV viral DNA. As indicated in Figure 1A, two different probes were used: 1) HPT DNA (probe A) to detect *Ds* loci on genomes (Fig. 6A); and 2) a WDV- DNA fragment surrounding the Ds (HPT) element (probe B) to detect WDV loci in transgenic plants (Fig. 6B). Hybridization signals that were detected by probe A but not by probe B, should be *Ds* elements that were excised extra-chromosomally from WDV molecules and inserted into rice genomes.

Southern analysis was performed on six mature T0 plants. DNA fragments that hybridized only with the HPT probe were indicated by dark star markers (Fig. 6A). Hybridization signals seen only by the WDV-DNA probe could be either from WDV carrying 'empty donor sites' of *Ds* or from the pWI-11 that was co-introduced to supply replicase in trans. Southern

analysis showed that mature transgenic plants carried *Ds* elements that were transposed from WDV vectors. The number of transposed *Ds* ranged from zero to three copies in independent transformants.

Figure 6C shows Southern hybridization probed was hybridized with a 1.7 kb *Ac*-internal HindIII fragment that was not present in *Ds*. Plants represented in Lanes 4 and 6 carried *Ac* elements. The weak hybridization signals common to all the lanes were detected even in non-transgenic plants (data not shown). The most noticeable difference between a blot probed with WDV and one with *Ac* (Fig. 6B and C, respectively) was the copy number of DNA integrated into the genome. Because transgenic plants were generated from calli carrying *Ds*, WDV insertions were preferentially selected over *Ac* insertions. However, the copy number of WDV was still very high, compared with one of *Ac* in the same genome. Such a high copy rate for WDV could be due to the replication nature of the molecule.

To determine the organization of the *Ds* loci, the selfed progeny of T0 plants were analyzed by Southern hybridization. Mature plants from 12 independent transgenic lines that were randomly selected for this experiment, were analyzed for the segregation of *Ds* insertion loci (Fig. 7). Because T0 plants carried inserted DNA as a hemizygous state, genetic linkages among insertion loci could be measured in a segregating T1 generation. Figure 7 is Southern hybridization probed with DNA specific to *Ds*. Multiple *Ds* bands were co-segregated among the selfed progeny, which indicated that these *Ds* were integrated into the same single sites. However, we also noticed that a low-copy *Ds* was independently segregated from the majority of *Ds* (marked as "▶" in Fig. 7). This indicated that a few of the *Ds* were located distant from the WDV::Ds loci.

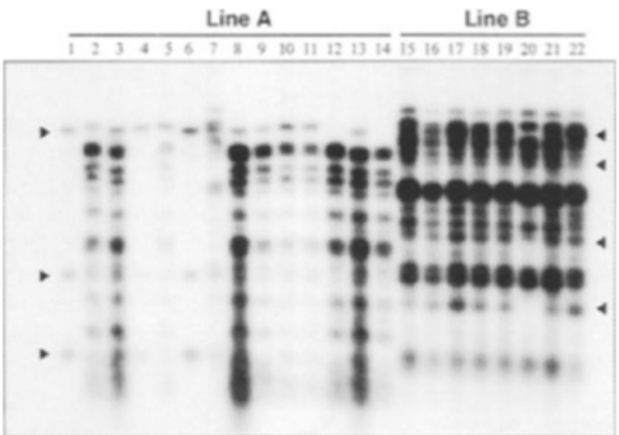

Figure 7. Inheritance of *Ds* insertions. Southern hybridization of EcoRI-cut genomic DNA was performed with a *Ds*-specific probe. Two different lines of 14 and 8 T1 plants are shown. Low-copy *Ds* that were segregated independently from the majority of *Ds* are indicated by filled triangles.

DISCUSSION

International efforts have begun for sequencing the entire rice genome. Along with genes identified by expressed sequence tags (Yamamoto and Sasaki, 1997), vast numbers of rice genes will accumulate from genome-sequencing projects. The emphasis will soon shift toward assessing the functions of sequenced genes. Major tools for dissecting the functional genomes of maize, as well as *Arabidopsis, are* insertional mutagens such as transposable elements or T-DNA. Knocking out a gene and observing the phenotypic effect on plant development and growth are the simplest and most powerful experimental measures by which to assign biological functions to genes.

In rice, efforts have been limited in using insertional mutagens to identify genes. Here, we examined the WDV viral vector as a potential vehicle for delivering the *Ds* element into genomes. Our data showed that not only WDV DNA but also *Ds,* alone, were integrated into rice genomes. WDV vector DNA was integrated in very high copy numbers, compared with plasmid DNA. Viral DNA was mainly integrated into single loci.

This observation was consistent with previous reports that the typical integration pattern of exogenous plasmid DNA, via bombardment, was a multicopy insertion at a single locus (Register et al., 1994; Kohli et al., 1998). Because the *Ds* excised from replicating WDV DNA was present in low copy in rice genomes, the transposition of *Ds* most likely occurred before the WDV vector DNA was integrated into the genomes. This conclusion was based on the following observations. First, transposed *Ds* was detected in plants carrying no *Ac*. Second, if *Ds* was excised from WDV after WDV::Ds was integrated into the genomes, many *Ds* should have been segregated among the T0 siblings regenerated from the same calli. However, no difference was found in the distribution of *Ds* and WDV among T0 siblings of the same transformants (data not shown). In summary, we developed viral vector systems by which *Ds* could be introduced in high copy number into rice genomes. However, only a few *Ds* were generated from transposition from viral DNA.

Future experiments should be directed toward determining whether the activity or mobility of *Ds* can be maintained in subsequent generations. However, it is very unlikely that multi-copy *Ds* in the T1 generation might be re-mobilized by *Ac*. Izawa et al. (1997) and Chin et al. (1999) have shown that *Ds* rapidly becomes inactivated in subsequent generations even though *Ds* is highly active in the first generation of F1 or T0.

In addition, the multimeric arrangement of *Ds* inserts most likely will tend to lower the transposition frequency. When two different *Ds* ends are placed in the same (direct) orientation, chromosomal rearrangement is initiated at the *Ds* ends *via* cut-and-paste processes between sister chromatins or within the same chromatin (English et al., 1993; Weil and Wessler, 1993). Consequently, the frequency of *Ds* transposition is substantially reduced. Therefore, naked *Ds* plasmid DNA introduced through protoplast transfection or particle bombardment is frequently in a physical configuration that is not favorable for active transposition of *Ds* in the presence of *Ac*.

Taken together, the introduction of *Ds* by WDV vectors can accomplish multi-copy insertion of the element. Because *Ds* most likely is not mobilized in the T1 generation, this system is suitable for direct selection of insertional mutants in the T1-segregating generation.

ACKNOWLEGEMENTS

This work was supported by a grant from the Ministry of Education (1996-001-G00056).

Received November 15, 1999; accepted December 17, 1999.

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