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Teresa Rubio
University of California, Berkeley

Marise Borja
University of California, Berkeley

Herman Scholthof
Texas A&M University, College Station

Paul A. Feldstein
University of California, Davis, California

Thomas Jack Morris
University of Nebraska-Lincoln, jmorris1@unl.edu

See next page for additional authors

Authors
Teresa Rubio, Marise Borja, Herman Scholthof, Paul A. Feldstein, Thomas Jack Morris, and Andrew O. Jackson

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Broad-Spectrum Protection against Tombusviruses Elicited by Defective Interfering RNAs in Transgenic Plants

TERESA RUBIO,[1] MARISÉ BORJA,[1] HERMAN B. SCHOLTHOF,[2] PAUL A. FELDSTEIN,[3] T. JACK MORRIS,[4] AND ANDREW O. JACKSON[1]*

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720[1]; Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843[2]; Center for Engineering Plants for Resistance Against Pathogens, University of California, Davis, California 95616[3]; and School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588[4]

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We have designed a DNA cassette to transcribe defective interfering (DI) RNAs of tomato bushy stunt virus (TBSV) and have investigated their potential to protect transgenic *Nicotiana benthamiana* plants from tombusvirus infections. To produce RNAs with authentic 5′ and 3′ termini identical to those of the native B10 DI RNA, the DI RNA sequences were flanked by ribozymes (RzDI). When RzDI RNAs transcribed in vitro were mixed with parental TBSV transcripts and inoculated into protoplasts or plants, they became amplified, reduced the accumulation of the parental RNA, and mediated attenuation of the lethal syndrome characteristic of TBSV infections. Analysis of F_1 and F_2 RzDI transformants indicated that uninfected plants expressed the DI RNAs in low abundance, but these RNAs were amplified to very high levels during TBSV infection. By two weeks postinoculation with TBSV, all untransformed *N. benthamiana* plants and transformed negative controls died. Although infection of transgenic RzDI plants initially induced moderate to severe symptoms, these plants subsequently recovered, flowered, and set seed. Plants from the same transgenic lines also exhibited broad-spectrum protection against related tombusviruses but remained susceptible to a distantly related tombus-like virus and to unrelated viruses.

Several types of pathogen-derived resistance (PDR) are known to occur in transgenic plants expressing viral genes. The coat protein gene of tobacco mosaic virus (TMV) was the first viral gene used to produce PDR in transgenic plants (37), and subsequently coat protein-mediated protection has been the most frequently attempted approach to obtain PDR (2, 32). In addition to resistance attributed to expression of coat protein genes in transgenic plants, a variety of other viral sequences, including replicase or movement genes, or mutated nonfunctional derivatives of these genes have been shown to provide synthetic resistance (1, 2, 32, 51, 60). Transgenic plants harboring different elements often vary substantially in the level and type of PDR observed, suggesting that fundamentally different cellular mechanisms that affect virus replication may be involved in the protective effects (8, 12–15, 18, 33, 38, 54, 61).

In addition to portions of viral genomes, complete sequences of defective interfering (DI) RNAs (28, 55) and satellite RNAs (17, 20) have also been used to engineer resistance in transgenic plants. DI RNAs are subviral deletion mutants consisting of portions of the parental virus genome that through rearrangements, deletions, or recombination events have evolved to smaller derivatives. These defective molecules lack functions essential for autonomous replication, so they must depend upon the parental helper virus for these functions (24). This dependence reinforces effective competition with the parental virus for *trans*-acting factors required for replication. Consequently, many DI RNAs interfere drastically with virus accumulation and attenuate the disease phenotype elicited by the helper virus. DI RNAs are thought to occur almost universally in animal viruses, and there are increasing numbers of reports of their association with plant viruses (19). DI RNAs associated with tomato bushy stunt virus (TBSV) were the first of this type of symptom-modulating RNA to be definitively characterized in plants (22).

TBSV has a positive-sense RNA genome of ~4.8 kb (Fig. 1A) that encodes five proteins (21). These proteins include replicase proteins p92 and p33 (53), the capsid protein p41 (23), the cell-to-cell movement protein p22, and a nested p19 gene, which functions in systemic invasion in some hosts and which is also involved in eliciting necrosis (49, 50). A sixth gene, pX, contains RNA sequences that have an undefined host-dependent effect on pathogenesis (47), but a direct role for the predicted product has not been identified. TBSV generates DI RNAs reproducibly during high-multiplicity passage experiments (27), and the evolution of tombusvirus DI RNAs likely involves a series of stepwise deletions of various segments of the viral genome (58, 59).

Sequence analyses have revealed that tombusvirus DI RNAs vary in size, but they all contain four conserved regions that contain *cis* elements required for replication (5, 22, 27). The prototypical B10 DI RNA (27) used in this study is 595 nucleotides (nt) long and consists of motifs derived from the 5′ untranslated region (region I), the polymerase gene (region II), and two segments of sequence (regions III and IV) located at the 3′-proximal portion of the viral genome (Fig. 1A). Previous results have demonstrated that coinoculations of TBSV and B10 DI RNAs produce a dramatic attenuation in the development of symptoms in plants (27). The B10 DI RNA has also been shown to inhibit accumulation of viral RNAs in protoplasts (25), as well as contribute to a reduction in viral RNA and protein accumulation in plants (52). A previous study (28) suggested that a DI RNA from a related virus, cymbidium ringspot virus (CymRSV), may have the potential to attenuate disease development. The results described here demonstrate that TBSV DI RNAs protect against the lethal

* Corresponding author. Mailing address: Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720. Phone: (510) 642-3906. Fax: (510) 642-9017. E-mail: andyoj@uclink4.berkeley.edu.

necrosis elicited by TBSV in *Nicotiana benthamiana* and that transgenic plants expressing TBSV DI RNAs have considerable promise for broad-spectrum control of tombusviruses.

MATERIALS AND METHODS

Plasmid constructs. Clones of different tombusviruses suitable for in vitro production of infectious cDNA-derived transcripts have been described previously. These clones include pTBSV-100 (21), pTBSV-B10 (27), pCNV (pK2/M5) (40), pCymRSV (G11) (9), pCIRV (43), and pCLSV (36). In control experiments, we have also used a potato virus X (PVX) cDNA plasmid (6), which contains unique cloning sites engineered downstream of the duplicated coat protein subgenomic promoter, and a PVX construct (pHS-142) containing the p19 gene of TBSV (49).

In order to generate DI transcripts with precise 5′ and 3′ termini, the B10 DI RNA sequence was flanked by ribozymes (RzDI). A modified tobacco ringspot satellite virus (TRSV) ribozyme fusion was generated to provide a precise 3′ end (39), and an avocado sunblotch viroid (ASBVd) ribozyme was engineered for cleavage at the exact 5′ end of the DI RNA (10). To engineer the ribozyme sequences at the ends of the DI RNA, two rounds of PCR were performed with pTBSV B10 DI RNA as a template. The first set of reactions used the oligonucleotide Rz5′-1 (5′ GAGGACGAAA CCCTTTGGGG TCGAAATTCT CCA GGATTTC 3′), which contains the first 17 nt of the 5′ end of the B10 DI RNA and 23 nt of the 3′ end of the ASBVd ribozyme, and the oligonucleotide Rz3′ (5′ GAGCTCACCA GGTAATATAC CACAACGTGT GTTTCTCTGG TAGCC TTCTC TGTCATACGG ACAGGACGGG CTGCATTTTC TGC 3′), which contains the last 16 nt of the DI RNA sequence followed by 67 nt derived from the TRSV ribozyme. A second round of PCR was used to add the remaining 5′ segment of the ASBVd ribozyme by using a 5′ oligonucleotide Rz5′-2 (5′ CCC CCTCGAG AATTTCCTGA TGAGTCCGTG AGGACGAAAC CCTTTG 3′) and the 3′-end Rz3′ oligonucleotide. The PCR product containing the DI RNA sequence flanked by the two ribozymes was cloned into the *Eco*RV site of pBS-SK(−) (Stratagene, La Jolla, Calif.) to place the 5′ end of the DI RNA under the control of the bacteriophage T7 promoter. This resulting transcription plasmid was designated pSK-RzDI. Subsequently, the DI RNA flanked by the two ribozyme sequences was excised with *Xho*I and *Sac*I restriction enzymes and cloned into the *Xho*I and *Sac*I sites of pKYLX71-35S[2], a version of the plant expression vector pKYLX7 (46) with a double 35S promoter and a *nos* terminator. This plasmid was designated pKL-RzDI.

Two other constructs were created for use as negative controls. One plasmid designated pKL-ASDI contained an antisense DI RNA flanked by nonprocessing sequences complementary to the two ribozymes. This plasmid was produced by cloning the *Sma*I/*Xho*I fragment from pSK-RzDI into pKYLX7-35S[2]. For this purpose, pKYLX7-35S[2] was digested with *Hin*dIII and treated with Klenow fragment (New England Biolabs, Beverly, Mass.) to fill in the overhanging sequences and then digested with *Xho*I. The other control plasmid, pKL-ESDI, containing the pTBSV-B10 sequence flanked by extra sequences from pBS-SK+, was produced by first cleaving pTBSV-B10 with *Apa*I and *Hin*dIII and subsequently cloning it into the corresponding sites of pBS-SK+ (Stratagene). Then, the DI RNA and the extra flanking sequences were digested with *Kpn*I, blunt ended with T4 DNA polymerase (New England Biolabs), digested with *Sac*I, and cloned between the *Hin*dIII-digested, Klenow fragment-treated site and the *Sac*I site of pKYLX7-35S[2]. This manipulation produced transcripts with 23 nt from pBS-SK+ abutting the 5′ end of the B10 DI RNA and 73 nt at the 3′ end. The T-DNAs of pKL-RzDI, pKL-AS, and pKL-ES were introduced into *N. benthamiana* by *Agrobacterium tumefaciens* (strain LBA4404) transformation (41), and transformed lines were regenerated from leaf discs in the presence of 50 μg of kanamycin sulfate per ml.

In vitro transcription reactions and plant infections. The pTBSV, pCNV, pCymRSV, pCIRV, and pTBSV-B10 plasmids were linearized with *Sma*I (21), and PVX and pHS-142 were linearized with *Spe*I (49). The linearized plasmids were then treated with T4 DNA polymerase, and in vitro transcripts were produced by bacteriophage T7 RNA polymerase (21) and in the case of the PVX construct in the presence of capping analog. Positive-sense DI-ribozyme transcripts from the plasmid pSK-RzDI were generated by T7 RNA polymerase and the negative-sense DI RNAs were transcribed by T3 RNA polymerase (New England Biolabs). These in vitro transcripts were used to coinoculate plants or protoplasts with TBSV transcripts (25). TMV (11) and belladonna mottle virus (BDMV) (30) were maintained in *N. benthamiana* plants. Sap from these plants was used as inoculum to infect RzDI transgenic plants.

To evaluate ribozyme processing, pSK-RzDI was linearized with *Sma*I, and in vitro transcripts were produced by the T7 RNA polymerase in the presence of [^{32}P]UTP. Then, the reactions were terminated at 0.5, 1, 2, and 4 h by freezing in dry ice and the reaction mixtures were subsequently analyzed in 5% polyacrylamide gels containing 8 M urea.

Nucleic acid and protein analyses. RNA extracted from plants (57) and protoplasts (25) was separated in formaldehyde gels or in 1× TBE (Tris-borate-EDTA) nondenaturing gels. The RNAs were then blotted and hybridized with a ^{32}P-labeled DNA probe corresponding to the 3′-terminal 245 nt of TBSV RNA. Reverse transcription-PCR (RT-PCR) analyses using RNA from noninfected transgenic plants were conducted with 5 μg of total RNA from different transgenic lines. The oligonucleotides 5′TBSV (5′ GAAATTCTCC AGGATTTCTC G 3′) and 3′TBSV (5′ GGGCTGCATT TCTGCAATG 3′) were added to the reaction mixtures to amplify the DI RNA sequences. A second primer pair, 5′Rz2 (5′ CCCCCTCGAG AATTTCCTGA TGAGTCCGTG AGGACGA AAC CCTTTG 3′) and 3′Rz2 (5′ ACGTGTGTTT CTCTGGTAGC C 3′), were used to test whether DI RNA sequences flanked by ribozymes could be amplified. After 30 amplification cycles, the products were electrophoresed on a 1× TBE gel, blotted, and hybridized with a ^{32}P-labeled DNA probe corresponding to the DI RNA sequence. RT-PCR analysis using RNA from infected transgenic plants with the different tombusviruses was performed with 5 μg of total RNA and the four oligonucleotide pairs: 5′TBSV-3′TBSV, 5′Rz2-3′Rz2, 5′TBSV-3′Rz2, and 5′Rz2-3′TBSV. The amplified products were subcloned into the TA cloning vector (Invitrogen, Carlsbad, Calif.) and subjected to sequence analyses by using double-stranded plasmid DNA (45) and Sequenase (U.S. Biochemicals, Cleveland, Ohio). Some sequencing reactions used the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, Foster City, Calif.). In these cases, the analyses were performed on an ABI 377 automated DNA sequencer at the Iowa State University DNA Sequencing Facility. Protein recovered from tissue was analyzed by polyacrylamide gel electrophoresis and immunoblotting (Western blotting) as described previously (50). DNA extractions from transgenic and nontransformed controls were performed with a DNeasy plant minikit (Qiagen, Hilden, Germany). PCR amplification of sequences inserted into the plant genome was performed with primers Rz5′-2 and Rz3′ or TBSV primers 5′TBSV and 3′TBSV. Southern hybridizations were conducted by minor modifications of procedures described previously (45).

RESULTS

Ribozymes flanking DI RNAs produce biologically active molecules. Initial protoplast experiments revealed that the ends of the transgenic DI RNAs needed to match the termini of the native B10 DI RNA closely in order to be recognized and amplified efficiently by TBSV (data not shown). During protoplast cotransfections with TBSV transcripts, replication of DI RNAs with exact 5′ termini but with an additional 73 nt of 3′-terminal sequences from pBS-SK+ (Stratagene) was severely compromised, and sequences corresponding to the DI RNA sequences were difficult to detect by Northern hybridization at 24 h after transfection. Antisense copies of the B10 DI RNA containing 34 extra nt at the 5′ end and an additional 77 nt at the 3′ end also failed to be amplified by the parental virus. Surprisingly, antisense RNAs corresponding exactly in complementary sequence to an equivalent of the B10 DI RNA also failed to accumulate to detectable levels when cotransfected with TBSV (50a). These results thus suggest that efficient DI RNA amplification requires positive-sense DI RNAs with precise or nearly precise termini.

In order to create transgenic plants expressing DI RNAs with sequences identical to those present in the 595-nt precursor B10 DI RNA, we engineered ribozyme sequences at the 5′ and the 3′ termini (Fig. 1B). The resulting transcription products of these constructs were designated RzDIs. Prior to plant transformation, the ribozyme sequences were tested for their ability to process the RzDI to yield synthetic B10 derivatives capable of replication. To evaluate in vitro processing of the ribozymes, a time course experiment (0.5, 1, 2, and 4 h) was performed whereby ^{32}P-radiolabeled transcripts were synthesized in vitro, frozen in dry ice at different times after initiation of the reactions, and subsequently analyzed in polyacrylamide gels (Fig. 1C). These experiments indicated that the RzDI RNA transcripts were processed to liberate two small bands corresponding to the 5′ and 3′ ribozyme sequences (46 and 67 nt, respectively), as well as larger bands corresponding to the unprocessed, intermediate, and cleaved B10 DI RNAs (Fig. 1B and C).

To assess the biological activity of the DI RNAs, protoplasts were cotransfected with TBSV transcripts plus mixtures of ribozyme-flanked transcripts and their reaction products. Although these RzDIs did not reach wild-type B10 DI RNA accumulation levels, they were amplified to high levels in infected protoplasts and they interfered with replication of the

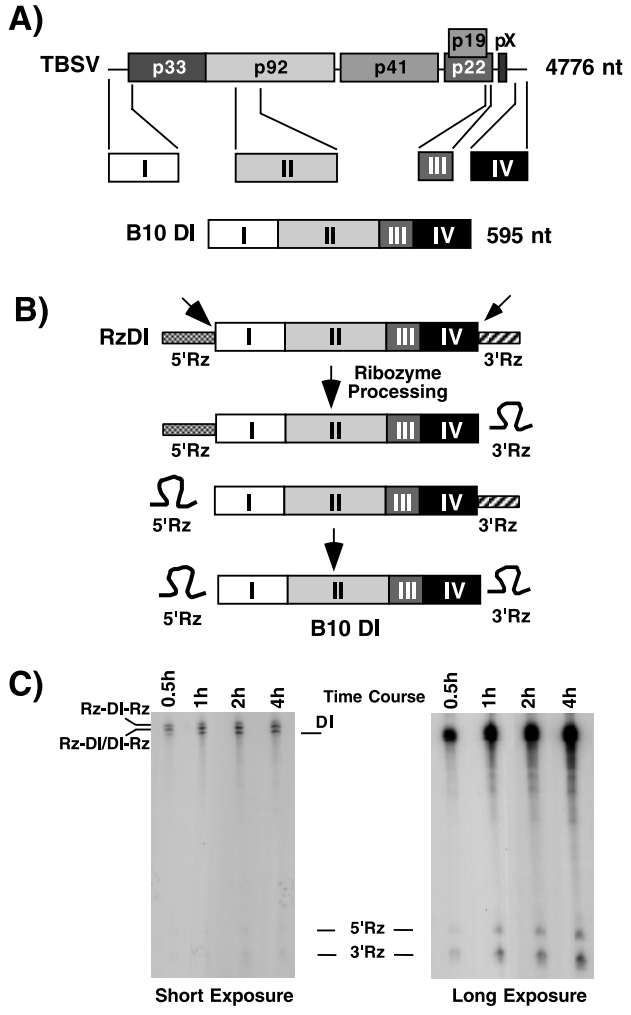

FIG. 1. Processing of TBSV B10 DI RNA flanked by ribozymes. (A) A map of the TBSV genome is illustrated at the top, with the six coding regions (p33, p92, p41, p22, p19, and pX) identified. The four conserved regions of the 595-nt B10 DI RNA are represented below the map by blocks (I, II, III, and IV) to indicate the sequence motifs derived from different regions of the TBSV genome. (B) Schematic illustration of ribozyme processing. The B10 DI RNA was flanked at its 5′ end by the ASBVd ribozyme (5′Rz) and at its 3′ end by the TRSV ribozyme (3′Rz). Sites of ribozyme cleavage are indicated by small arrows flanking the DI RNA. Intermediate and final products (B10 DI, 5′Rz, and 3′Rz) of the in vitro processing reaction are illustrated. (C) Evaluation of ribozyme processing in vitro. Time course experiments showing the in vitro processing of the B10 DI RNA by flanking ribozymes. The positions of the processed and unprocessed DI RNAs, as well as the locations of the liberated ribozymes (5′Rz and 3′Rz), are indicated. The in vitro transcription reactions were performed at 37°C with T7 RNA polymerase in the presence of [^{32}P]UTP, and the samples were separated on 5% acrylamide gels. Short (4-h) and long (20-h) exposures were used to permit visualization of the individual bands.

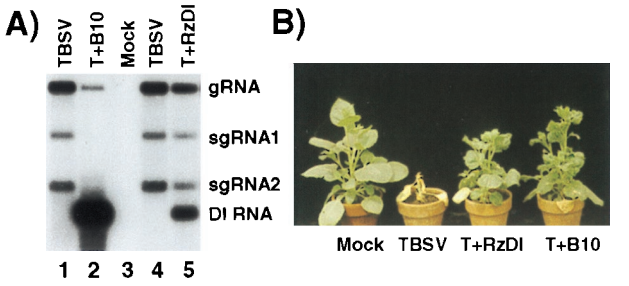

FIG. 2. Amplification of DI RNAs in coinoculations with TBSV in protoplasts and attenuation of symptoms in plants. (A) DI RNA accumulation and inhibition of TBSV replication in transfected *N. benthamiana* protoplasts. Northern blot analyses of total RNA isolated from protoplasts 18 h posttransfection with in vitro TBSV transcripts alone (lanes 1 and 4) or in combination with native B10-DI transcripts (T+B10; lane 2) or pSK-RzDI transcripts (T+RzDI; lane 5) or mock transfections (lane 3). Each lane corresponds to approximately 10^5 protoplasts. The blot was probed with a randomly primed ^{32}P-labeled DNA corresponding to the B10 DI RNA, and the autoradiograph was exposed for 5 h. The positions of the gRNA, sgRNA1, sgRNA2 are indicated. (B) Effects of DI RNAs on symptoms in *N. benthamiana* plants. The plants were mechanically inoculated with TBSV transcripts, TBSV transcripts mixed with transcripts from pTBSV-B10 (T+B10), or TBSV and pKS-RzDI RNA transcript mixtures (T+RzDI), and photographed 2 weeks later. An uninoculated control (Mock) is shown. Note that the presence of DI RNAs (T+RzDI and T+B10) resulted in attenuated systemic symptoms, while TBSV alone was lethal to the plants.

parental TBSV RNAs (Fig. 2A). *N. benthamiana* plants inoculated with wild-type TBSV transcripts alone developed the characteristic lethal necrotic syndrome (Fig. 2B) that is consistently associated with TBSV infections (48). This phenotype was characterized by the appearance of a mild mosaic interspersed with faint yellow flecks within 3 to 4 days postinoculation (dpi) that progressed to necrotic lesions, severe leaf distortion, and apical necrosis. A rapid vascular collapse that culminated in death of the plant between 7 to 10 days dpi subsequently followed (Fig. 2B). However, the symptom severity was greatly diminished by coinoculation of TBSV with transcripts derived from the clones containing the native DI RNA (B10) or the B10 DI RNA flanked by the ribozymes (RzDI). These attenuated symptoms consisted of a mild mosaic and slight stunting, combined with small sporadic chlorotic and necrotic lesions on the systemically infected leaves (Fig. 2B). These plants subsequently flowered and produced seed. Thus, the RzDIs elicited the same protective phenotype in coinfections with TBSV as the native B10 DI RNA.

Transgenic plants accumulate low levels of DI RNAs. The promising results with inoculation of RzDI transcripts suggested that these molecules might provide useful resistance if expressed in transgenic plants. RzDI sequences in positive (RzDI lines) and antisense (AS lines) orientations, as well as a B10 DI RNA flanked by extra sequences (ES lines), 23 nt at the 5′ end and 73 nt at the 3′ end from the plasmid pBS-SK+, were placed under the control of a double 35S promoter, and transgenic *N. benthamiana* plants were obtained via *Agrobacterium*-mediated leaf disc transformation. F_1 and F_2 progeny lines were grown on agar containing kanamycin for selection of transformed lines. Forty of the RzDI lines, seven AS lines, and four ES lines were analyzed for resistance and the relative accumulation of the DI transcripts. In order to determine the nature of the inserted sequences, we performed PCR amplifications using DI (Fig. 3A) and ribozyme (Fig. 3B) primers with DNA from 12 selected RzDI transgenic lines (225, 229, 221, 69, 27′9′, 275, 279, 615, 228, 612, 616, and 611), two AS lines, and one ES line, each of which contained one or two copies of the transgene as deduced by Southern hybridization (data not shown). Control amplifications were also conducted with the plasmid pTBSV-B10 that contained the B10 DI RNA sequence. Amplifications using the pTBSV-B10 and the DI RNA primers produced a band which hybridized to a 595-nt probe encompassing the B10 DI RNA sequence (Fig. 3A and B, lanes 17). Similar-sized bands were amplified from genomic DNA of the 12 RzDI transgenic plants (Fig. 3A, lanes 2 to 16). Slightly larger bands corresponding in size to the DI and flanking ribozyme sequences were amplified by the ribozyme primers (Fig. 3B, lanes 2 to 16), except for the line ES (Fig. 3B, lane 4), for which no product was obtained, as predicted. In contrast,

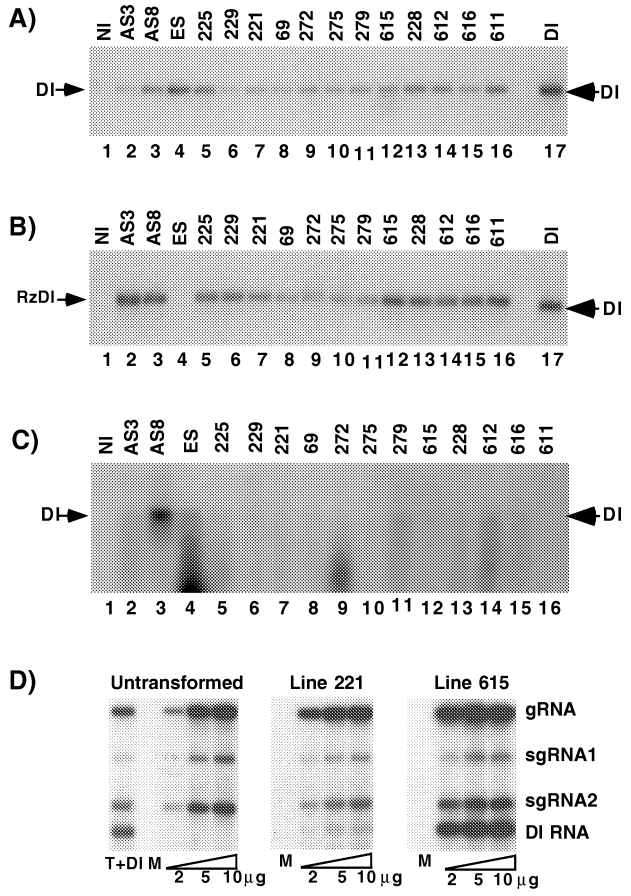

FIG. 3. DI RNA integration and transcript accumulation in transgenic plants. (A) Southern blot analyses of PCR performed with genomic DNA using 5′TBSV- and 3′TBSV-specific primers. Products of 12 RzDI transgenic plant lines are analyzed in lanes 5 through 16. AS3 (lane 2) and AS8 (lane 3) illustrate antisense lanes, and ES (lane 4) represents RNA from plants transformed with the B10 DI RNA flanked by extra plasmid DNA sequences. As a negative control, DNA was extracted from untransformed *N. benthamiana* plants (NI; lane 1), and as a positive control, the PCR amplifications were conducted with the plasmid pSK-RzDI, which contains the RzDI sequence (DI; lane 17). The positions of the DI and RzDI products are indicated by arrows at the sides of the blots to serve as size markers for the amplified plant DNAs. (B) Southern blot as in panel A, except that the PCR amplifications were conducted with the 5′Rz1 and 3′Rz primers. The labeling was done as described above for panel A. (C) Northern blot analysis of RNA from different lines of uninoculated RzDI transgenic plants (noted above each lane). Total RNA (10 μg) extracted from leaves was electrophoresed in a denaturing 1% formaldehyde–agarose gel. The blot was probed with ^{32}P-labeled DNA prepared by random priming of a purified 595-nt fragment encompassing the B10 DI RNA sequence. The arrows indicate the positions of the DI size markers. (D) TBSV amplification of DI RNA from transgenic RzDI protoplasts. Protoplasts were isolated from untransformed (*N. benthamiana*) and transgenic RzDI lines 221 and 615 and transfected with 2, 5, or 10 μg of in vitro-transcribed TBSV RNA. Untransformed protoplasts were also cotransfected with in vitro transcripts from TBSV and the B10 DI (T+DI). Each lane contains approximately 10^5 protoplasts. The blot was probed with a randomly primed ^{32}P-labeled DNA corresponding to the B10 DI RNA. The positions of the gRNA, sgRNA1, sgRNA2, and DI RNAs are indicated. M, mock-inoculated protoplasts.

DNA extracted from control untransformed plants did not contain amplifiable sequences (Fig. 3A and B, lanes 1). These results suggested that each transformed line contained an intact integrated DI RNA sequence plus the flanking ribozyme sequences (Fig. 3A and B).

Accumulation of DI RNA transcripts in uninoculated plants was also estimated by hybridization of total RNA extracted from the same transgenic lines (Fig. 3C, lanes 2 to 16). As expected, no hybridization to the DI probe was observed in untransformed plants (Fig. 3C, lane 1). Except for line AS8 (lane 3) which had a clearly visible band, hybridizing bands with mobility corresponding to that of the B10 DI RNA were also difficult to detect in the other transgenic lines. Lines ES, 279, 612, and 616 appeared to accumulate the highest abundance of sequences with mobilities similar to those of the DI RNAs. Smaller hybridizing entities were also visible in several lines, suggesting that the transcribed RNAs were undergoing some form of degradation (Fig. 3C, lanes 4, 9, 11, 13, and 14).

To further analyze the nature of the DI RNA species expressed in the transgenic plants, we performed RT-PCR analyses on RNA extracted from four transgenic lines using specific primers to amplify sequences containing or lacking the ribozyme sequences and subsequently subjected the RT-PCR products to hybridization with a DI cDNA probe. When DI RNA-specific primers were used (5′TBSV primer and 3′TBSV primer) an amplification product was detected in the RzDI transgenic lines. In contrast, when ribozyme-specific primers were used (5′Rz2 primer and 3′Rz2 primer) products were not amplified from RNA isolated from the RzDI transgenic lines, and only a single band was visible using RNA from the antisense transgenic line AS3 (data not shown). These results suggest that the transgenic RzDI RNAs were efficiently processed in vivo by the flanking ribozymes to generate the native B10 DI RNA.

To assess the amplification of DI RNA in transgenic RzDI plants, protoplasts were isolated from untransformed and transgenic RzDI plants (lines 221 and 615) and transfected with different amounts of TBSV transcripts (2, 5, and 10 μg). We tested two representative transgenic lines (221 and 615) that showed evidence of a DI RNA sequence, each of which had barely detectable levels of constitutive expression of the RzDI RNA. Upon infection with TBSV, high levels of accumulation of replicating DI RNA were present in line 615, lower levels were evident in line 221, and hybridizing bands were not detectable in the untransformed control (Fig. 3D) at 18 h posttransfection. These protoplast results thus demonstrate that the processed RzDI RNAs are biologically active and can be amplified efficiently by TBSV.

Protection against TBSV disease development in transgenic plants expressing DI RNAs. Untransformed *N. benthamiana* plants inoculated with TBSV developed the typical lethal syndrome consistently observed for TBSV infections in this host. However, the transgenic plants from the 12 RzDI lines shown in Fig. 3 exhibited attenuated symptoms that were similar to those illustrated for transgenic line 615 (Fig. 4A and B). These symptoms were also typical of those of untransformed plants following coinoculation with mixtures of the B10 and TBSV transcripts (Fig. 2B). Subsequently, we evaluated the effects of inoculum dosage on the degree of symptom attenuation using in vitro-transcribed TBSV RNA (0.008, 0.04, 0.08, 0.8, 4, and 8 μg/ml), as well as purified virus (0.1 and 1 μg/ml) in transgenic plants. In these experiments, plants infected with various concentrations of in vitro-transcribed RNA had a pronounced mosaic at 2 weeks postinoculation (wpi) (Fig. 4A). By 4 wpi, some plant-to-plant variation was evident, but all plants were in various stages of recovery from the pronounced symptoms that appeared in the early stages of infection (Fig. 4B). In contrast, the antisense (AS3 and AS8) and extra sequence (ES) *N. benthamiana* negative-control lines showed no discernible resistance and died within 2 wpi with TBSV (Fig. 4C). As expected, TBSV infections of control plants progressed rapidly into a severe necrosis, followed by a pronounced vascular collapse and plant death (Fig. 4C).

Minor differences in symptom development were observed

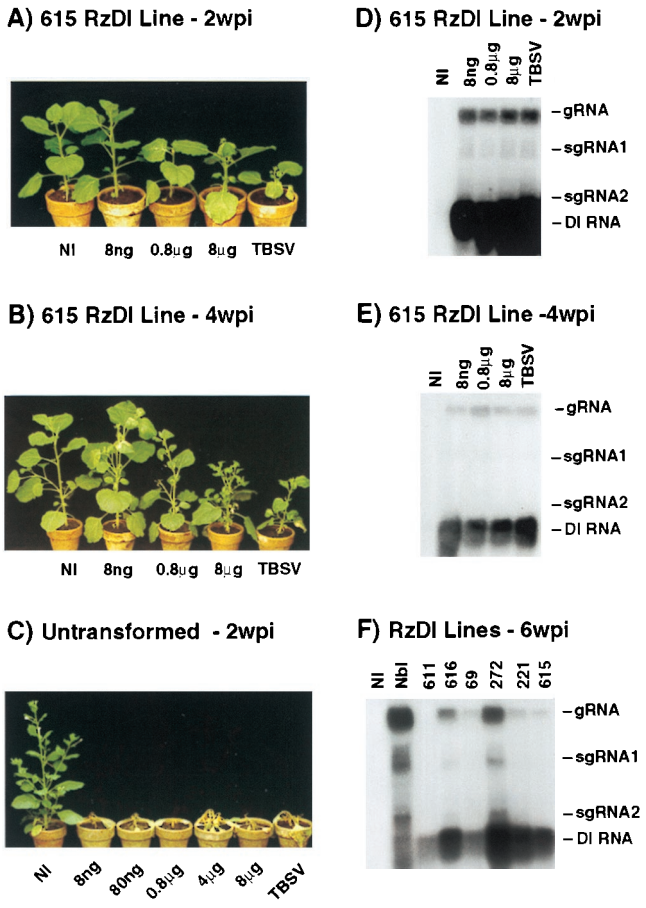

FIG. 4. Resistance to TBSV infection and inhibition of viral replication in RzDI transgenic plants. In the panels on the left (A, B, and C), different amounts of TBSV RNA transcripts and viral particles (TBSV) were used to inoculate each plant. (A) Protected RzDI transgenic plants (line 615) showing a moderate disease phenotype at 2 wpi with TBSV. (B) RzDI transgenic plants illustrating the recovery phenotype at 4 wpi with TBSV. These plants eventually flowered and produced seeds. (C) Nontransformed plants 2 wpi, showing the lethal necrotic syndrome elicited by TBSV. Note that even the plants infected with the lower concentration of in vitro-transcribed TBSV RNA (8 ng) died by 2 wpi. The panels on the right (D, E, and F) show Northern blot analyses performed on RNA extracted from RzDI transgenic lines. (D and E) RNA extracted from line 615 at 2 wpi (D) and at 4 wpi (E) and (F) RNA extracted from different RzDI transgenic lines (611, 616, 69, 279, 221, and 615) at 6 wpi. Blots were probed with a specific probe encompassing the 3′-terminal 245 nt of the TBSV genome. Note that lane NbI contains RNA extracted from dying tissue of untransformed *N. benthamiana* plants at 2 wpi with TBSV transcripts. The NI lanes in all panels correspond to RNA extracted from noninfected plants. The positions of the TBSV gRNA and sgRNAs and the DI RNA are indicated to the right of the blots.

in different infectivity experiments among the 11 remaining RzDI lines. The onset of symptoms was usually delayed slightly in the transgenic plants in comparison to untransformed controls. However, both RzDI and nontransformed plants initially developed the mosaic symptoms characteristic of TBSV infection, and the control plant infections progressed into the lethal syndrome. In contrast, plants expressing the RzDIs displayed moderate disease symptoms between 1 and 2 wpi which included some leaf distortion, curling, small necrotic lesions, and limited apical necrosis in ~15% of the plants. The disease symptoms were also generally more severe under warmer greenhouse conditions exceeding 35°C, but an obvious protective effect was noted in all RzDI transgenic lines (not shown). Irrespective of the temperature, all of the RzDI plants showed signs of recovery from infection by 2 wpi with newly emerging leaves exhibiting a milder mosaic along the apical and lateral stems. By 4 to 6 wpi, the infected RzDI plants were slightly smaller than uninoculated controls and began to develop more lateral branches (Fig. 4B). Subsequently, the DI transgenic plants flowered and produced seeds, although curling, mild chlorosis, and occasional small necrotic spots were still evident in the leaves.

High levels of DI RNAs accumulate during TBSV infection of RzDI plants. Leaves from infected RzDI transgenic plants were collected from 3 dpi to 6 wpi, and RNA analyses were performed. These analyses revealed that the levels of the DI RNAs in inoculated plants exceeded those of uninoculated RzDI transgenic plants as early as 3 dpi, and by 1 wpi, the DI transcripts and the TBSV genomic RNA (gRNA) and subgenomic RNA (sgRNA) accumulated to very high levels (data not shown). Northern blots of RNA isolated from RzDI lines at 2 wpi, which corresponds to the late stages of vascular collapse and necrosis in untransformed plants, showed a high abundance of TBSV-specific RNAs, with even greater amplification of the DI RNA (Fig. 4D). However, by 4 wpi, there was a substantial decrease in the abundance of the TBSV RNAs, with a concomitant reduction in DI RNAs (Fig. 4E), that corresponded to the recovery of the transgenic plants (Fig. 4B). By 6 wpi, the TBSV-specific RNAs were reduced to barely detectable levels in most of the transgenic lines (Fig. 4F). At this stage of infection, the levels of the DI RNAs were also reduced but to a lesser extent than the TBSV RNAs. In addition, the levels of accumulation of the TBSV-encoded p33 and p19 proteins assessed by Western blotting reflected the relative abundance of the TBSV RNAs (data not shown). Thus, these experiments demonstrate that amplification of DI RNAs is highly correlated with the protective effects exhibited in transgenic plants after TBSV infection.

Transgenic DI plants exhibit broad-spectrum protection against tombusviruses. To evaluate resistance of the RzDI transgenic lines to different members of the tombusvirus genus, transgenic and untransformed plants were inoculated with infectious RNA transcripts of the following viruses; TBSV, cucumber necrosis virus (CNV), CymRSV, and carnation Italian ringspot virus (CIRV). Untransformed plants and negative-control lines (AS3, AS8, and ES) infected with TBSV, CNV, and CIRV developed lethal syndromes similar to TBSV (Fig. 5A and B). However, plants infected with CymRSV displayed a less severe disease phenotype, with many plants surviving for 6 wpi. In all cases, the DI transgenic plants exhibited the attenuated symptoms observed in the typical TBSV infections and exhibited strong protective effects against each of the viruses tested. In addition, recovery of the plants was evident by 2 wpi (Fig. 5C). Interestingly, although individual plant lines appeared to be protected to a similar extent, the time course of accumulation of different viral RNAs varied substantially at 2 wpi, and some variation was also observed among different transgenic lines (Fig. 5D, E, and F). For example, the accumulation of gRNA was more pronounced in CNV and CIRV infections (Fig. 5D and E) than in CymRSV infections (Fig. 5F). There also was a reduced level of DI RNA accumulation associated with CIRV infections. For reasons that are not understood, line AS3 (but not line AS8) showed occasional variable accumulation of the DI RNA after infection with CymRSV. The most pronounced accumulation noted is shown in Fig. 5F.

The amplification of the DI transcripts by the related tombusviruses was expected due to the high homology at the nucleotide level among the members of the group. This relatedness is particularly evident within the 5′ and 3′ untranslated

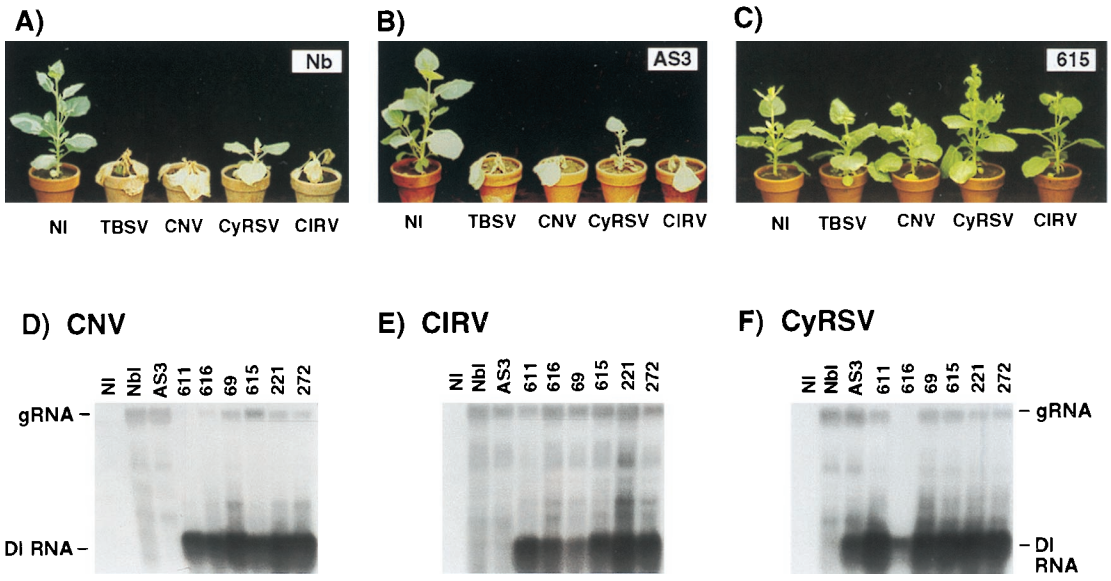

FIG. 5. Broad-spectrum protection of RzDI transgenic plants against different tombusviruses. (A) Untransformed plants (Nb) at 2 wpi with TBSV, CNV, CymRSV (CyRSV), and CIRV. (B) Antisense A3 DI line photographed at 2 wpi. (C) RzDI transgenic line 615 at 2 wpi. The bottom panels show accumulation and amplification of the DI transcript at 2 wpi of different lines with CNV, CIRV, and CymRSV. Northern blots performed with 10 μg of total RNA extracted from the different lines at 2 wpi. The designations of the RzDI lines are as given in the legends to Fig. 3 and 4. The negative controls are noninfected untransformed plants (NbI), untransformed plants infected with each of the different viruses, and AS3, the transgenic line AS3 transformed with an antisense DI RNA. The positions of the gRNAs and sgRNAs of CNV, CIRV, and CymRSV as well as the amplified TBSV DI RNAs, are indicated. Hybridizations were performed using a randomly primed ^{32}P-labeled DNA probe encompassing the 3′-proximal 245 nt of TBSV RNA.

regions which contain *cis*-acting elements necessary for replication. In this regard, infections with cucumber leaf spot virus, a distantly related tombus-like virus, which has a much lower level of sequence homology at the 5′ and 3′ termini than other tombusviruses (36), failed to amplify the TBSV DI RNA after virus inoculation and the transgenic plants developed the same disease phenotype as untransformed *N. benthamiana* controls (data not shown). These results thus show that sequence specificity affects the ability of various tombusviruses to amplify the B10 DI RNA, and provide additional evidence that the protective effect requires efficient amplification of the DI RNA sequences.

To confirm that the DI RNAs amplified by the different tombusviruses originated from the RzDI transgene, we performed RT-PCR analysis of total RNA isolated from transgenic plants infected with the different tombusviruses and then sequenced the amplified products. Total RNA was isolated from transgenic plants infected with TBSV, CNV, CIRV, and CymRSV, and RT-PCR was performed using DI RNA-specific primers (5′TBSV and 3′TBSV), ribozyme-specific primers (5′Rz2 and 3′Rz2), or heterologous combinations of these primers to test for the presence of only one of the ribozymes. Defined amplification products with each tombusvirus were evident only when DI RNA-specific primers were used in the RT-PCRs, and only faint smears were obtained when ribozyme primers alone or heterologous combinations of TBSV and Rz primers were used (data not shown). Sequence analysis of the DI RNA-specific products revealed that in each case the DI RNA amplified in the transgenic plants by the different tombusviruses was derived from the transgene; these results argue against the possibility that the DI RNAs originated from the inoculated virus.

To determine whether the protective effects were restricted to tombusviruses, the transgenic plants were also infected with unrelated viruses including TMV (11), BDMV, PVX, and a PVX clone (pHS-142) containing the sequences of the p19 gene of TBSV whose expression results in the lethal syndrome in *N. benthamiana* (49). In challenges with these viruses, RzDI transgenic plants, the negative controls (A3 and ES plants), and untransformed *N. benthamiana* plants all developed wild-type symptoms characteristic for each virus. As expected, all plants infected with the p19-expressing PVX vector, pHS142, died by 3 wpi following necrosis and vascular collapse elicited by expression of p19 (data not shown). From these experiments, we conclude that the transgenic DI plants were protected against TBSV necrosis and the deleterious effects of related tombusviruses, but protection did not extend to TMV, BDMV, and wild-type PVX, or PVX expressing TBSV p19 sequences.

DISCUSSION

We have analyzed 12 transgenic *N. benthamiana* lines that express DI RNAs associated with TBSV infections. These lines constituted a representative sample of 40 independent resistant lines recovered after transformation with the B10 DI RNA sequence flanked by ribozymes. In vitro and in vivo results demonstrated that accurate 5′ and 3′ processing occurred and that the processed DI transcripts retained biological activity. Negative controls with DI and ribozyme sequences in an antisense orientation (AS lines) or DI sequences flanked by extra nucleotides at each end (ES lines) failed to exhibit biological activity as assessed by the failure of the parental virus to amplify the transcripts or by an inability to protect against death of infected plants. The results also demonstrated that the ribozyme-flanked DI transcripts were amplified rapidly following infection of transgenic protoplasts with the parental TBSV strain and that infected transgenic plants developed an attenuated disease phenotype irrespective of the kind and amount of input viral inoculum. Moreover, resistance to related tombusviruses (CNV, CymRSV, and CIRV), which support amplification of the DI sequences, and the lack of protection with

a distantly related tombus-like virus (cucumber leaf spot virus), and unrelated viruses (BDMV, TMV, PVX, and pHS-142, a PVX vector expressing the p19 protein of TBSV) indicate that the broad-spectrum protective effects require efficient amplification of the transgenic DI RNA. These results suggest that the TBSV B10 DI RNA can provide a useful source of disease resistance against several tombusviruses that cause serious problems in crop plants.

Our study also extends the results of Kollar et al. (28), who previously found a protective effect in plants transformed with DI RNA sequences of CymRSV and noted that antisense sequences failed to provide protection. However, there are several notable differences in the results of the two studies. The most striking difference is that our experiments indicate that the B10 DI sequences require the presence of exact or nearly exact 5′ and 3′ termini in order to replicate efficiently and to provide a consistent protective effect. In contrast, the CymRSV DI transcripts and its flanking sequences appear to be more than a third larger than the DI RNA used for cloning. Based on the cloning description provided by Kollar et al. (28), we estimate that the nonviral sequences consisted of 76 nonviral residues flanking the 5′ terminus of the DI RNA and a large 3′ terminal extension consisting of 19 nt of polylinker sequences plus an undefined number of nopaline synthetase terminator sequences adjacent to a heterologous poly(A) tail. However, the size of the amplified hybridizing component was similar to that of the native DI RNA, suggesting that at some stage during CymRSV infection the extra sequences flanking the transcribed DI RNA were eliminated to give rise to a native protective DI RNA. Alternatively, the DI RNA could have arisen de novo from the inoculum source or via recombination between the transgene and the inoculated CymRSV. In this regard, recombination events that yield defective RNAs have been shown to occur in whole-plant infections without serial passage, but amplification of defective RNAs was not detected in protoplast experiments (29). Our experiments showed that the processed DI RNAs accumulated to high abundance after infection of transgenic protoplasts with TBSV. These results thus provide strong evidence that the amplified DI RNAs were derived directly from the low-abundance transgenic DI RNA sequences, rather than from TBSV. Such experiments, unfortunately were not conducted by Kollar et al. (28); therefore from the data presented, we are unable to directly compare the time required to give rise to actively replicating DI RNA molecules or to assess the rates at which the CymRSV and TBSV DI RNAs increase in abundance following infection of single cells with the respective parental tombusviruses. Thus, although we have no ready explanation for the discrepancies between these two studies, our results with B10 ES derivatives do suggest that nonviral flanking sequences can inhibit DI RNA replication and that these sequences can easily be eliminated by appropriate ribozyme processing.

The requirement for processing of the DI RNA and our demonstration that amplification of the resulting RNA transcripts occurs upon infection of the transgenic plants with TBSV and related viruses provides persuasive evidence that protection requires biological activity of the recombinant DI RNA. The initial high abundance of the DI RNA combined with a subsequent reduction in the DI RNA levels at the later stages of infection is typical of the events occurring during wild-type DI RNA protection in untransformed plants (22, 25, 27, 52). The results are also in agreement with previous findings showing that infections containing DI RNAs exhibit substantial reductions in the accumulation of TBSV gRNA, sgRNA 1, and sgRNA2 and in the levels of the p19- and p22-encoded proteins (25, 52). These results thus are compatible with a model whereby DI RNA-mediated modulation in the symptom phenotype results from competition for available replicase between the helper virus and the DI RNA molecules (25). Milder necrosis due to reduction in accumulation of the p19 protein and reduced spread of the virus as a consequence of interference with synthesis of the p22 movement protein may also contribute to the attenuated disease phenotype (52). Therefore, the broad-spectrum protective effects observed in the transgenic DI plants appear to involve very different mechanisms than those leading to specific resistance that normally is restricted to viral isolates whose sequences are used for coat protein or nontranslatable RNA-mediated resistance (1, 2). Our results also indicate that DI RNA-mediated protection differs fundamentally from the somewhat broader resistance observed in plants transgenic for defective viral movement proteins. In this regard, several studies have shown that plants expressing mutated nonfunctional movement protein genes, analogous to the TMV 30-kDa protein (8, 33) or dysfunctional mutations within the triple gene block movement protein genes (3, 54), exhibit resistance which possibly is mediated via a dominant negative mechanism targeting cell-to-cell movement processes.

The present work demonstrates that DI RNAs have considerable potential for broad-spectrum control of the lethal disease syndrome elicited by TBSV and several other members of the tombusvirus genus. Nevertheless, several questions relevant to the agronomic application of this strategy need to be assessed in direct field tests. (i) Will DI RNA-mediated protection be durable under conditions of widespread distribution of transgenic plants? (ii) Can detrimental effects result from the use of DI RNAs as sources of disease resistance? (iii) What are the prospects for application of the DI strategy to the vast majority of viruses that fail to generate DI RNAs during the normal course of replication? In response to the first question, the DI RNA protective effects appear to rely to a considerable extent on recognition of *cis*-acting 5′- and 3′-terminal sequences by the replicase of the invading virus. However, other less obvious properties of the DI RNAs also affect symptom attenuation, because we have observed that DI isolates from the same source and from different viruses vary substantially in their protective effects. In this regard, our strategy involving analysis of protoplasts and whole plants for efficacy of DI RNA before construction of transgenic plants provides a convenient method to evaluate optimal levels of protection with various derivatives against invasion by several different viruses. We further propose that the protective effects will be quite durable because protection relies on the ability of the DI RNAs to be amplified by the invading virus. Since DI RNA amplification requires recognition of *cis*-acting elements within the DI RNAs, mutations to circumvent protection probably would require fundamental replicase alterations to change the recognition specificity for essential *cis* elements, coupled with simultaneous mutations at the 5′ and 3′ termini of the gRNAs to accommodate the new requirements for the mutated replicase. Therefore, it is likely that the selection of virus mutants able to circumvent protective effects of a particular DI transgene would be negligible, even following widespread release of transgenic varieties. Should such events occur, subsequent DI RNAs generated from the mutant viruses could provide alternative sources of material for construction of second-generation transgenic plants. In addition, we could expect the DI RNAs themselves to self-select to become competitive within protected plants. In terms of environmental safety, the potential detrimental effects of the use of transgenes for crop protection have been discussed in numerous forums (7, 16, 26, 44, 56). We should stress that DI RNAs circumvent most of the

problems envisioned for other sources of PDR, because with only a few rare exceptions (19, 31, 42), DI RNAs attenuate rather than exacerbate the disease phenotype. Moreover, DI RNAs have been identified only in experiments with plant virus infections and have not been shown to exist in nature (27). However, even though transgenic DI RNAs should be able to move to adjacent plants, experimental analyses suggest that introduced DI RNAs do not persist in natural tombusvirus populations (4). In addition, most tombusviruses are soilborne, and inoculum appears to be replenished primarily from decaying plant material killed during infections. Since the protective effects of the RzDI plants are very robust and reduce the levels of virus following infection, a secondary benefit should be a reduction of the available load of inoculum returned to the soil upon death of infected plants.

In conclusion, we anticipate that field studies will demonstrate that transgenic plants relying on DI RNAs for broad-spectrum protection against tombusvirus infections will exhibit predictable disease attenuation, that the protective effects will be durable, and that minimal, if any, adverse environmental consequences will result from the widespread use of such sources of resistance. The possible use of DI RNAs as sources of protection against other viruses will need to be investigated individually. However, the results of in vitro experiments reporting the serendipitous generation of artificial DI RNAs with brome mosaic virus (34, 35) and barley stripe mosaic virus (62) suggest that there is considerable potential for exploitation of DI RNAs as sources of resistance, even with viruses that do not normally generate DI RNAs de novo.

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