The expression of antisense and ribozyme genes targeting citrus exocortis viroid in transgenic plants

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Four ribozyme and antisense genes targeting citrus exocortis viroid (CEVd) positive- and negative-strand RNA molecules were constructed and used to transform the tomato Lycopersicon lycopersicum cv. UC82B. The tomato is a readily transformable plant and will support replication of CEVd following mechanical inoculation. The ribozyme genes contained three hammerhead catalytic motifs with long hybridizing arms and synthetic RNA transcripts were shown to cleave the target CEVd RNA molecule in vitro. Homozygous transgenic plants were produced from independent transformants expressing either ribozymes or antisense constructs. Inoculation of transgenic seedlings expressing antisense constructs targeting the negative-strand CEVd RNA molecule with CEVd resulted in a moderate reduction in the accumulation of CEVd RNA. In contrast, similarly inoculated transgenic plants expressing constructs targeting the positive-strand CEVd RNA molecule resulted in an increase in the rate of CEVd RNA accumulation. Addition of the ribozyme motifs to the antisense genes did not enhance their efficiency in the suppression of viroid replication and a moderation or elimination of the observed antisense effects was seen in plants expressing the corresponding catalytic RNA-encoding genes.

Introduction

The viroid plant pathogens are unencapsidated, low molecular weight, circular, single-stranded RNA molecules (for review see Diener, 1987). They are the smallest known autonomously replicating agents and are in the size range 246–375 nucleotides. Viroid RNA has been shown to have extensive intramolecular base-pairing, giving viroids a rod-like structure (Sänger et al., 1976). The interaction of viroids with the host plant can result in anything from no apparent effect on host phenotype to an extremely severe pathology. In many cases the infection can lead to significant impairment of plant growth and fruiting, causing economically important losses. Although the pathology of viroid infections has been extensively examined, to date there are no published reports of the identification of natural resistance genes or the design and introduction of synthetic resistance genes into host plants.

A potentially useful approach to the development of viroid-resistant cultivars is the application of pathogen-derived resistance mechanisms (Sanford & Johnston, 1985). Pathogen-derived resistance includes strategies which involve the expression of a component of the pathogen in transgenic hosts resulting in increased tolerance or resistance in the host when infected with that pathogen. Pathogen-derived resistance has been applied to a wide range of plant virus and host combinations. For example, the expression in transgenic plants of virus capsid protein genes (for review see Beachy et al., 1990) or parts of the viral RNA polymerase gene (Golemboski et al., 1990; MacFarlane & Davies, 1992) can result in increased tolerance or resistance to viral infection. Although very effective against viruses, these techniques are unsuitable for application to viroids as the viroid genomes do not encode identifiable ORFs (Sänger, 1987). However, the fact that the viroids are single-stranded RNA molecules makes them potentially sensitive to resistance strategies involving the expression of transgenes encoding sense, antisense or ribozyme RNA molecules. Antisense-mediated control of gene
expression has been demonstrated for a number of endogenous genes (Van der Krol et al., 1988a). In addition, antisense strategies have been successful in conferring a degree of resistance to the appropriate plant hosts of the viral pathogens potato virus X, cucumber mosaic virus and potato leafroll virus (Hemenway et al., 1988; Cuozzo et al., 1988; Kawchuk et al., 1991). Ribozyme-mediated gene inactivation strategies have been successfully employed against the bacterial neomycin phosphotransferase gene in protoplasts of *Nicotiana tabacum* (Steinexce et al., 1992) and tobacco mosaic virus (TMV) replication in protoplasts (Edington & Nelson, 1992). As yet, neither technique has been evaluated against viroids and there have been no published reports of attempts to use ribozyme RNA to regulate gene activity in whole transgenic plants.

This paper describes the testing of strategies to engineer resistance to viroids. Citrus exocortis viroid (CEVd) was used as the target viroid as it is well characterized and will replicate in tomato, a readily transformable greenhouse plant. Tomato plants expressing ribozymes or near full-length antisense genes targeting either the viroid-sense (positive-strand) RNA molecule or the complementary-sense (negative-strand) viroid RNA molecule were tested for inhibition of CEVd replication. Although the full details of the CEVd replication cycle have not been elucidated, it is generally accepted that the replication cycle, completed within the nucleolus (Riesner, 1987) involves the synthesis of a negative-strand template. Copies of positive-strand molecules are formed from the oligomeric negative-strand RNA and are subsequently processed into circular viroid molecules (Symons, 1990). The negative strand of CEVd is much more rare than the positive strand in infected plants and the distribution of RNA, in terms of multimeric units, is different for the two RNA species (Hutchins et al., 1985). The rationale for the selection of two target RNAs was that it is possible that the positive- and negative-strand RNAs may represent quite different targets in terms of abundance, cellular localization and/or structure.

**Methods**

*Construction of antisense and ribozyme genes.* All routine DNA manipulations were as described in Sambrook et al. (1989). A CEVd A cDNA clone (Australian isolate; Visvader et al., 1982) was obtained from Dr P. Keece, (CSIRO Division of Plant Industry, Canberra, Australia). The 371 bp CEVd cDNA was subcloned as a BamHI fragment into *BamHI*-digested pGEM3Z (+) DNA (Promega). Recombinant plasmids were designated pCEV10 or pCEV11 for the sense or antisense orientation to the T7 RNA polymerase promoter, respectively. Three hammerhead ribozyme catalytic domains derived from the sequence of satellite RNA of tobacco ringspot virus (Haseloff & Gerlach, 1988) were introduced sequentially by site-directed mutagenesis (Kunkel et al., 1987) of pCEV10 or pCEV11 (Fig. 1).

Ribozymes were introduced into pCEV11, targeting GUC sequences within the CEVd positive-strand RNA at genomic co-ordinates 116, 145 and 185 and the resultant ribozyme-containing plasmid was designated pRz3+. The construct pCEV10 was mutagenized to include ribozymes targeting ribonucleotide triplets in the CEVd negative-strand RNA at positions 101 (GUU), 130 (GUC) and 173 (GUU) and designated pRz3—. The integrity of all ribozyme constructs was confirmed by DNA sequence analysis (Sanger et al., 1977).

*In vitro ribozyme cleavage reactions.* 32P-labelled target RNA was prepared by in vitro run-off transcription reactions (Melton et al., 1984) using T7 RNA polymerase and *XbaI*-linearized pCEV10 and pCEV11 DNA to produce positive- and negative-strand CEVd RNA, respectively. Unlabelled ribozyme RNAs were prepared using T7 RNA polymerase-directed transcription of *XbaI*-linearized pRz3+ and pRz3—. Yields and the integrity of RNA transcripts were determined spectrophotometrically and by denaturing polyacrylamide gel electrophoresis (PAGE). Cleavage reactions were performed at 37 °C or 50 °C and contained 0·3 μM ribozyme and 0·05 μM target RNA, 50 mM- Tris·HCl pH 7·4 and 10 mM-MgCl2. At each time point, 5 μl was removed from the reaction, added to 95 μl of 0·2 M-NaCl, 20 mM EDTA to stop the reaction and immediately precipitated with ethanol. Samples were glyoxal treated prior to analysis on denaturing PAGE gels (Sambrook et al., 1989). Radiolabelled RNA bands were detected and quantified by phosphorimager analysis (Molecular Dynamics) according to the manufacturer's instructions.

*Tomato transformation.* The antisense genes targeting the CEVd positive-strand (pAs+) and negative-strand (pAs—) were derived from pCEV11 and pCEV10, respectively. The three ribozyme genes were derived from pRz3+ and pRz3—. All the gene fragments were subcloned as Smal–PstI or PstI fragments, after blunt-ending, into the Smal site of pJ35SN (Walker et al., 1987). This cloning method was used to create a 49 bp deletion of the CEVd cDNA from genomic position 41–89. This was to avoid the construction of transgenic plants with full genome-length CEVd cDNA that could potentially produce infectious viroid from an integrated transgene. All constructs were then inserted as blunt-ended *PstI* fragments into the blunt-ended *XhoI* site of the plant transformation vector pGA470 (An et al., 1985). These cloning steps joined the antisense and ribozyme genes to the cauliflower mosaic virus (CaMV) 35S promoter sequence at the 5’ end and the nos gene polyadenylation sequence at the 3’ end and in *in vivo* transcripts are predicted to encode approximately 8 bp and 600 bp of non-viroid sequence at the 5’ end and 3’, respectively. The recombinant constructs were used to transform *Agrobacterium tumefaciens* strain L. The recombinant plasmids were identified by Southern blotting. *Lycopersicon lycopersicum* cv UC82B was transformed with the five constructs by a modification of a procedure described by Fillatti et al. (1987). *L. lycopersicum* was chosen for transformation because CEVd infection of this plant does not cause the devastating symptoms as seen in CEVd infection of *Lycopersicon esculentum*. This was considered desirable as sampling and RNA analysis of infected tissue was expected to be more straightforward with plants showing limited symptoms. Cotyledon explants were cut from 7-day-old tomato seedlings and placed, abaxial side up, onto KCMS medium containing Murashige and Skoog salt mixture (Gibco), myo-inositol (100 mg/l), sucrose (3%), 2,4-D (0·2 mg/l), kinetin (1·0 mg/l), thiamin hydrochloride (1·3 mg/l), KH2PO4 (200 mg/l) and Bactoagar (0·8%) at pH 5·5. Prior to placing explants onto KCMS medium, a sterile piece of filter paper was placed on each plate of KCMS medium. The explants were incubated for 24 h under low-light conditions (20-30 µE) at 23 °C. For co-cultivation, a single colony of *A. tumefaciens* was transferred onto the KCMS plates and diluted to 5 x 106 cells/ml. Fifty cotyledon explants were transferred from the KCMS plates into 5 ml of bacterial suspension. After 30 min, the explants were gently blotted on sterile
Antisense/positive, As(+)

Ribozyme/positive, 3Rz(+)

Antisense/negative, As(−)

Ribozyme/negative, 3Rz(−)

CaMV CEVd nos

Fig. 1. Diagram outlining the structures of the four chimeric genes constructed for plant transformation to produce antisense or long ribozyme complementary to either the CEVd positive or negative RNA strands. The CEVd cDNA fragment (light grey shading) used in all constructs, in the orientation depending on the required sense transcript, was the full-length sequence with a deletion between bases 40 and 90. The positions of the ribozymes (filled boxes) are indicated and the number of the base in the target RNA at which cleavage occurs to the 5′ is shown. The terminology used to describe the genes is as follows: As, antisense gene; 3Rz, long ribozyme containing three catalytic domains; (+), (−) denotes the CEVd positive- and negative-strand RNA, respectively. The CaMV 35S promoter (arrow) and nos 3′ processing sequences are indicated.

Antisense/negative, As(−)

Ribozyme/negative, 3Rz(−)

paper towels and replaced onto the same KCMS plates, adaxial side down. The plates were then incubated for 48 h under low-light conditions. Following co-cultivation, the explants were transferred to 2Z medium containing Murashige and Skoog salt mixture, sucrose (2%), Nitsch vitamins (Thomas & Pratt, 1981), zeatin (2 mg/l), myo-inositol (100 mg/l) and Bactoagar (0.8 %) at pH 6.0. The 2Z medium also contained carbenicillin (500 mg/l) to inhibit growth of A. tumefaciens and kanamycin (100 mg/l) to select for growth of transformed tobacco cells. Explants were transferred onto fresh medium 10 days after co-cultivation and were then transferred every 3 weeks thereafter. After approximately 8 weeks some shoots were large enough (1–2 cm) to transfer to MSSV medium containing Murashige and Skoog salt mixture, sucrose (2%), Nitsch vitamins, carbenicillin (50 mg/l) and kanamycin (50 mg/l) and Bactoagar (0.8 %) at pH 6.0. Roots developed within 10–14 days and plantlets were transferred to glasshouses for seed production.

Transformed plant tissue was selected on the basis of the kanamycin-resistance phenotype conferred by the nptII gene of pGA470. All regenerants of the T1 generation were screened for expression of the CEVd ribozyme or antisense transgenes by Northern blotting and for nptII gene expression by a phosphotransferase dot-blot assay method (McDonnell et al., 1987). Fruits were collected from plants in which expression of both genes was detected and seeds isolated by mild treatment of the fruit with dilute HCl (1/20 dilution of concentrated HCl in deionized water). T1 seedlings were grown from the harvested seeds and were in turn screened for transgene and nptII gene expression as described above. Seeds were collected from those T1 plants that were expressing the transgene and were members of a population where the transgene segregated at approximately 3:1. T2 seedlings were generated from these plants and screened for nptII expression to determine whether they were derived from a homozygous or hemizygous T1 parent. Between 12 and 24 seedlings were screened and shown to be positive for the marker gene before a population was deemed likely to have been derived from a homozygous T1 parent. Once identified, T2 homozygotes were propagated and seed banks prepared for viroid inoculation experiments.

Nucleic acid extraction. Total nucleic acid was prepared from tomato leaves by grinding 100 mg of tissue in a 1:5 ml microcentrifuge tube containing 200 μl of extraction buffer [50:100:1 TE3D (10% w/v NP40, 15% lithium dodecyl sulphate, 10% sodium deoxycholate, 2 mM-EDTA, 20 mM-Tris–HCl pH 8.0):phenol solution:2-mercaptoethanol] with a glass rod. When the material was ground to a paste, 200 μl of 3 M-ammonium acetate and 150 μl of chloroform:isoamyl alcohol (24:1) was added, the tube capped and vortexed for 1 min. Following centrifugation of the leaf extract at 12000 g for 10 min at 4 °C the aqueous phase was recovered and placed in a fresh tube. The required nucleic acid fraction was prepared from this extract by differential precipitation. For the recovery of total RNA for detection of transgene transcripts the recovered solution was adjusted to 2 M-lithium chloride and incubated at 20 °C for 2 h. The insoluble RNA was recovered by centrifugation at 12000 g for 10 min at 4 °C. The pellet was washed in 70% ethanol, dried in vacuo and resuspended in 10 μl of DEPC-treated sterile double-distilled water. For the purposes of detecting viroid and mRNA, total nucleic acid samples were required as the rod-like nature of the viroid RNA ensures it remains soluble in 2 M-lithium chloride. Total nucleic acid was prepared by the addition of 0.1 vol 3 M-sodium acetate (pH 5.2) and 2 vol 100% ethanol followed by incubation and centrifugation as for the total RNA preparation procedure.

Northern blotting. One tenth of the RNA and total nucleic acid samples extracted from 100 mg of leaf tissue were analysed by electrophoresis through 1/2 agarose gels containing formaldehyde as described by Sambrook et al. (1989). The nucleic acid was transferred to Hybond-N membranes (Amersham) by capillary blotting and was cross-linked to the membrane by UV treatment with a Stratalinker (Stratagene) according to the manufacturer’s instructions. All filters were prehybridized in 20 ml of hybridization solution (3 × SSC, 0.5% w/v SDS, 5 × Denhardt’s reagent, 50% v/v de-ionized formamide, 100 μg/ml sheared, denatured herring sperm DNA) in a large hybridization bottle at 45 °C for 3 h. All radiolabelled DNA hybridization probes were prepared by oligo-nucleotide priming of a gel-purified DNA restriction fragment using a Multi-prime kit (Amersham) according to the manufacturer’s instructions. Hybridization was allowed to proceed at 42 °C for 18 h. The filters were washed at 60 °C in a succession of 2 × SSC, 0.1% (w/v) SDS for 15 min and twice in 0.2 × SSC, 0.1% (w/v) SDS for 30 min each. For the detection of the expression of transgenes encoding the CEVd antisense or long ribozyme or viroid RNA, a 371 bp gel-purified BamHI fragment of the CEVd cDNA was used as a probe template. For secondary probing of blots for RNA loading correction, a 9 kb EcoRI fragment representing a portion of the rDNA operon of Triticum aestivum, prepared from the clone pT71 (Gerlach & Bedbrook, 1979), was used for probe preparation. This latter probe hybridized strongly with the tomato 18S and 26S rRNA. Secondary probing was completed by keeping the initially probed blot damp during analysis and then repeating the prehybridizing and hybridizing steps with the second probe. The probe DNA bound to the membranes was visualized by exposure of the membranes to a phosphor storage screen followed by processing of the screen in a phosphorimagery (Molecular Dynamics).

Preparation of infectious CEVd RNA and plant inoculations. Plants used for inoculations were maintained at 30 °C with a 16 h day and 8 h
In vitro cleavage of synthetic full-length CEVd RNA by the CEVd 3Rz(+). (a) Schematic diagram of the cleavage patterns of CEVd (+) RNA by 3Rz(+). The full-length linear RNA molecule is depicted by the thick black line and the positions of cleavage sites are indicated by short vertical lines. The sizes of the cleaved products are indicated on the full-length molecule in nucleotides. The positions and the identity of the intermediate and complete cleavage products detected in the autoradiograph of PAGE analysis of the reaction are indicated. (b) Quantitative analysis of four independent repeats of the reaction shown in (a) to analyse CEV 3Rz(+) activity. The graph shows the proportion of RNA detected in intermediate and complete cleavage products at the time and temperature indicated.

Analysis of viroid RNA replication in CEVd-infected transgenic tomatoes. Populations of wild-type and the various transgenic plants were maintained and inoculated as described above. At specific intervals after inoculation, approximately 100 mg leaf samples were taken from the oldest leaf on each plant using a scalpel blade that had previously been soaked in 1 M-sodium hydroxide and rinsed in sterile double-distilled water. The oldest leaves were sampled as they have previously been shown to be the sites of the lowest level of accumulation of the related viroid, PSTVd (Palukaitis, 1987). This tissue was sampled in the anticipation that the ribozyme and antisense RNA would be in the most favourable ratio with the CEVd RNA. Total nucleic acid was isolated from the leaf tissue as soon as possible after harvesting. The total nucleic acid samples and a standard sample of CEVd-infected plant total nucleic acid were analysed by Northern blotting with the CEVd cDNA probe. Quantitative values were obtained for CEVd signal present using the software supplied with the Molecular Dynamics phosphorimager. The filters were subsequently probed with the rDNA.
probe and the level of 26S RNA in each sample was quantified. The hybridization signals were normalized for variation in loading and in hybridization efficiency from one filter to another by using the values for standard samples on each filter. The final result for each time point post-inoculation was calculated as an average for the population and presented as a histogram. The number of plants at each time point in which CEVd RNA was detectable is indicated above each histogram bar. All data storage and manipulations were completed using the Excel spreadsheet software (Microsoft).

Results

In vitro cleavage activity of CEVd hammerhead ribozymes.

The ribozymes designed to cleave sequences within either the CEVd positive- or negative-strand RNA (Fig. 1) were constructed. In vitro generated RNA transcripts of the ribozyme genes were shown to direct the site-specific cleavage of synthetic CEVd target RNA. Incubation of the CEVd ribozyme RNAs with the appropriate CEVd RNA target results in at least partial cleavage of that target RNA into the expected RNA products. Fig. 2(a) shows the products of cleavage of positive-strand CEVd RNA by Rz3(+) in an analysis of the kinetics of cleavage in experiments with an excess of ribozyme RNA. Four independent time course cleavage reactions were carried out at 37 °C and 50 °C and examples of an autoradiograph of PAGE analysis of cleaved RNA and quantitative analysis of the same experiment are shown in Fig. 2(a) and (b) respectively. At 37 °C the rate of cleavage and maximum total cleavage of approximately 20 % were relatively low whereas the rate of reaction was improved by incubation at 50 °C. At the higher temperature, a plateau of approximately 80 % cleavage was reached after 5 h. In all experiments the cleavage was incomplete and the positions of the intermediate cleavage products are indicated in Fig. 2(a). Similar experiments were completed with Rz3(−) and comparable cleavage rates were obtained (data not shown). Although the ribozyme sequences were inserted within regions of the CEVd RNA with limited predicted self-complementarity, viroids are largely self-complementary and the ability for self-cleavage of the ribozymes was studied. Radiolabelled ribozyme RNA was prepared and incubated at 37 °C and 50 °C under conditions favouring ribozyme activity and no cleavage was detected following analysis of the RNA by denaturing PAGE and autoradiography (data not shown).

Analysis of transgenic plants

Four transformation experiments were completed with the following ribozyme and antisense constructs: As(+), 3Rz(+), As(−) and 3Rz(−). Transgenic plants were designated by the transgenic CEVd ribozyme or antisense gene expressed followed by the primary transformation event the plant was derived from. For example, As(+)#2 refers to plants expressing an antisense to the CEVd positive-strand RNA derived from a primary transformant designated #2. Kanamycin-resistant T₀ seedlings were selected from the initial tomato transformation experiments for each of the four CEVd constructs and were subsequently analysed for the expression of the antisense or ribozyme gene. From these T₀ plants, T₁ seedlings were prepared and analysed for co-segregation of nptII gene and transgene expression. Those T₁ plants identified as having the desired genotype were further characterized to be either homozygous or hemizygous for the transgenes by analysis of the segregation of the nptII gene in the T₂ population. At least two homozygous plants originating from independent primary transformation events were identified for each CEVd transgene. Fig. 3 shows the relative levels of the expression of the transgenes detected by Northern blotting in the 10 identified homozygote plants. The level of transgene expression varied with the highest level in As(+)#2 (lane 6) at approximately 10-fold that of the lowest level expression in 3Rz(−)#2 (lane 11). This wide variation in expression levels of transgenes is a commonly observed phenomenon with transgenic plants and has been suggested to be dependent on the site of integration of the transgene in the plant chromosome (Van der Krol et al., 1988b).

Preparation and titration of CEVd infectious RNA and inoculation of transgenic plants

Viroid RNA was purified from CEVd A-infected L. esculentum Mill cv. Rutgers leaf tissue and resuspended in water at a final concentration of 3 μg/ml. The
Fig. 4. Examples of Northern blot analysis of total nucleic acid extracts from transgenic tomato plants inoculated with CEVd RNA. (a) Results of hybridization with CEVd cDNA. (b) Duplicate series of nucleic acid samples probed with the rDNA probe pTA71 to determine the variation in loading levels of the nucleic acid. Lanes 1 and 2 are RNA samples from uninoculated transgenic plants and lanes 3–12 are samples from 10 CEVd-inoculated transgenic tomato plants.

infectivity of the RNA was assayed by inoculation of *L. lycopersicum* seedlings with dilutions of the viroid RNA to give challenges of 3 ng, 0.3 ng, 0.03 ng or 0.003 ng of CEVd RNA per cotyledon of each seedling. In addition, an *L. lycopersicum* population was mock-inoculated and *L. esculentum* Mill cv. Rutgers seedlings were inoculated with 3 ng of RNA per seedling to identify typical CEVd symptoms. The levels of CEVd RNA were determined by Northern blot analysis of total nucleic acid extracted from each plant at 5 day intervals from 12 to 37 days post-inoculation. All inoculations with CEVd RNA resulted in productive infection by the final time point. The challenge of 0.03 ng per cotyledon was the lowest level that gave a uniform infection in all members of the population. No CEVd RNA was detected in the mock-inoculated plants. Severe epinasty and stunting, symptoms typical of CEVd infection, were detected in the *L. esculentum* Mill cv. Rutgers population at 14 days post-inoculation. Very mild symptoms were detected in about 50 % of the *L. lycopersicum* population infected with 3 ng of CEVd RNA whereas no symptoms were detectable in plants infected with the lower CEVd RNA levels.

The challenge of the transgenic plants was completed once in entirety and included populations of eight plants for each of the 11 homozygous transgenic lines that were challenged with 0.03 ng of CEVd RNA per cotyledon. As no symptoms were detectable following infection with this CEVd RNA level, CEVd replication was monitored by accumulation of CEVd RNA as detected by Northern analysis. The plants were sampled from the oldest leaf at 15, 19, 23, 28, 34 and 46 days post-inoculation and RNA prepared and analysed in batches immediately after tissue harvesting. Fig. 4 shows an example of the Northern blot results for 10 CEVd-infected plants and two mock-inoculated plants. Fig. 4(a) shows the result of hybridization with the CEVd probe and Fig. 4(b) shows duplicate samples hybridized with the rRNA DNA probe.

The average levels of viroid RNA accumulation in plants following inoculation are shown schematically in Fig. 5. The viroid RNA level values were determined only from plants in which viroid RNA was detected. Although this approach was biased against demonstrating immunity or high-level resistance within the plants it also eliminated scoring plants as resistant that were not efficiently inoculated. The numbers of plants within each population in which CEVd RNA was detected is indicated above each histogram bar. CEVd RNA could be detected in only 3/8 of the wild-type plants until 28 days post-inoculation. After this point the proportion of plants with detectable CEVd RNA and the rate of accumulation increased in a linear manner over the remaining time points. Over the early time points there were inconsistencies in levels of CEVd RNA in some plants. This was probably due to the fact that the levels of CEVd RNA were close to the limit of detection and that, although measures were taken to ensure consistency in sampling and hybridization analysis, there were sampling errors. The three independent transgenic families expressing antisense to the CEVd negative-strand RNA [As(−) Fig. 5a] all showed a lower proportion of infected plants and reduced CEVd levels over the whole time course. The only exception to this was for one population [As(−)#3] at one time point (28 days). The plants expressing the ribozymes targeting the CEVd negative strand [3Rz(−) Fig. 5b] showed either a slight reduction [3Rz(−)#1] or wild-type levels [3Rz(−)#2] of CEVd RNA. The only deviation from this pattern was the value for 3Rz(−)#2 at 23 days post-inoculation at which point the viroid level was higher than at 18 and 28 days post-inoculation and the corresponding wild-type levels.

The CEVd accumulation in plants expressing antisense
Fig. 5. Histograms representing the levels of CEVd RNA, detected by Northern blotting, in populations of CEVd-inoculated plants (0.03 ng CEVd RNA/cotyledon) at time intervals post-inoculation. Each bar represents the average value for each time point for a population following correction for hybridization and loading levels between sample batches. The number above each histogram bar is the number of plants in which CEVd RNA was detected. All populations consisted of eight plants except As(+)#1 which was limited to six plants. The Y axis is an arbitrary scale related to the detected intensity of hybridized radiolabelled probe. Each panel shows the time course for families derived from independent transformants for a class of constructs compared to the result from CEVd inoculation of the wild-type population of L. lycopersicum cv UC82B. (a) Plants expressing antisense targeting the negative CEVd RNA strand. (b) Plants expressing ribozymes targeting the negative CEVd RNA. (c) Plants expressing antisense to the positive CEVd RNA. (d) Plants expressing ribozymes targeting the positive CEVd RNA.
to the CEVd positive strand [As(+)#1 and As(+)#2, Fig. 5c] gave an unexpected result. The levels accumulated were significantly greater than for the wild-type population, with the maximum level between three- and eightfold that of wild-type reached at 34 days post-inoculation rather than at the end of the time course. The values for both As(+)#1 and #2 decreased between days 34 and 46, ending at lower levels in the transgenic plants than in the wild-type. Clearly, the latter time points cannot be compared in isolation as the CEVd infection is more rapid in the As(+) plants over the first five time points. Similarly, the results for the populations expressing ribozymes targeting the positive-strand CEVd RNA [3Rα(+), Fig. 5d] shows higher levels of CEVd in the transgenic plants than in the wild-type plants with a peak at 34 days post-inoculation. The CEVd levels that accumulated, although higher than those levels detected in the wild-type population, were not as high as those levels observed in transgenic plants expressing antisense to the CEVd positive-strand RNA.

Discussion

Several independent transgenic homozygous plants were identified that expressed the various antisense or long ribozyme sequences. Homozygous populations expressing transgenes targeting the CEVd negative-strand RNA were generally slightly resistant throughout the time course of infection. This protection is in contrast to the increase in viroid replication rate seen following CEVd inoculation of plants expressing constructs targeting the positive-strand RNA and may reflect the inherent differences of the positive- and negative-strand RNAs. The lower intracellular concentration of the targeted negative-strand molecule may permit the establishment of a more effective ratio of antisense:target. In addition, the detection of the viroid negative-strand RNA in concatemeric forms (Hutchins et al., 1985) may produce a molecule that adopts a RNA secondary structure that is in a conformation more accessible to RNA intermolecular duplex formation. Alternatively, the vulnerability of the negative-strand RNA may be due to its, at least transitory, location in a compartment of the cell such that it becomes accessible to the transgenic RNA transcripts. The possibility also exists that the reduction in CEVd RNA replication may not be the result of a primary interaction between transgenic RNA and CEVd RNA but rather the result of an interaction between the transgenic transcript and the host factors. The effect may be a form of the defective interfering particle phenomenon (Hillman et al., 1987). For example, the transgenic RNA could compete for a plant component normally required for CEVd replication.

All plants expressing transgenes targeting the CEVd positive strand supported viroid replication at a significantly greater level than in the wild-type. Again this result may not only be the result of interactions between the transgenic RNA and the viroid RNA but could be a product of association of the transgenic RNA with host factor(s). Considering candidate host factors, the transgenic RNA could be interacting with host-encoded RNA polymerases or RNA processing reactions involved in viroid replication but could also be modulating a host defence response. The transgenic RNA may have the potential to act as a decoy and reduce the potency of any defence reaction such that authentic CEVd negative-strand RNA is unaffected and replication proceeds unchecked. The enhancement of replication could equally be independent of host plant factors and the transgenic RNA may provide in trans stimulation of the viroid replication by enhancing the proportion of negative-strand replicative intermediate material.

The ribozyme constructs were prepared and demonstrated to be catalytically active in vitro. The CEVd ribozyme cleavage efficiency was enhanced several fold by incubation at a higher temperature. Although an increase in the kinetics of any reaction is expected with increased temperature, cleavage levels were increased by at least fourfold suggesting the possible involvement of RNA secondary structure in the interference of cleavage at the lower temperature. This observation was not unexpected as the viroid RNA structure contains a high proportion of intramolecular base pairing conferring an almost double-stranded rod-like structure. Although the in vitro cleavage rate was low it is possible that even low levels of cleavage of a pathogen at the early stages of an infection cycle may be effective at reducing propagation of the pathogen at later stages in the cycle. However, in both classes of constructs the addition of hammerhead ribozyme sequences to the antisense genes resulted in a decrease of the effects of the expression of the antisense genes. This result could be interpreted as indicating that the inclusion of the hammerhead sequences may reduce the net effect of the interaction of the transgenic RNAs with CEVd by reducing the association of the antisense RNA with either host factors or viroid RNA. This may be occurring via a simple disruption in the duplex-forming potential of the complementary RNA. Equally, the hammerhead ribozyme sequences themselves could be involved in further interactions with plant proteins that reduce the antisense RNA-mediated impact on either inhibition or enhancement of viroid replication.

These results show that expression of antisense genes results in a slightly reduced CEVd RNA accumulation, when targeted to the viroid negative strand. This is the first description of any form of engineered viroid resistance and provides a good starting point from which to develop more efficient resistance genes. Further
understanding of the precise mechanisms of action of the transgenic RNAs will be required to facilitate rational improvement of resistance gene designs. For example, the inclusion of the hammerhead ribozyme sequences failed to have an apparent additive effect on the antisense RNA. To fully interpret this result one would need to be sure RNA:RNA interactions were critical to the observed effects and whether there is any evidence of ribozyme-mediated cleavage of the target RNA. Nevertheless, this is the first report of the use of ribozymes in whole plants and further analysis of the parameters of trans application of such molecules is required. For example, an appreciation of the association of transgenic RNAs with plant proteins and the relationship between the ribozyme transcript structure and in vivo activity will be invaluable. Second generation CVEd resistance-conferring transgenes might target transgenic mRNA to the nucleus, the cell compartment in which viroids are thought to accumulate and replicate (Riesner, 1987). Expression of transgenes using RNAs that are naturally nuclear-localized such as the snRNA U1 may be one way of targeting ribozymes. The apparent enhancement of viroid replication in plants expressing genes targeting the viroid positive-strand RNA was unexpected and requires further investigation. In addition, this latter result is of particular importance with regard to careful design of transgenic experiments and testing of plants in contained facilities.

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