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Expression of Hop Stunt Viroid from Its cDNA in Transgenic Tobacco Plants: Identification of Tobacco as a Host Plant

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Hop stunt viroid (HSV) cDNAs were introduced into tobacco plants by a Ti plasmid-mediated transformation. The replication of infectious HSV was detected in transgenic tobacco plants, carrying two tandemly repeated HSV cDNA sequences, and in their progeny as well. This is the first report of transgenic plants expressing viroids and offers a novel strategy for the study of viroid-host interactions. Previously, tobacco had been reported to be resistant to HSV infection. Accordingly, we examined the susceptibility of tobacco to HSV by two different methods, agroinfection and mechanical inoculation. Tobacco was readily agroinfected with HSV; however, tobacco was mechanically infected at a greatly reduced level of efficiency. These results identify *Nicotiana tabacum* as a host of HSV.

Viroids are the smallest known plant disease agents (Diener 1979). They are single-stranded circular and linear RNAs of 250–400 nucleotides. They are not encapsidated, instead they are highly intramolecularly base-paired to form rod-shaped structures (Keese and Symons 1987). Viroids do not code for proteins, and their replication is totally dependent on host enzymes (Diener 1982). Viroids have been shown to be associated with the nuclei of the host cells, with the exception of avocado sunblotch viroid (Semancik and Conejero-Tomas 1987).

The replication cycle of the viroids depends on RNA-directed RNA synthesis. In viroid infected cells, longer than unit-length plus- and minus-strand viroid RNAs, which are most likely the replication intermediates, have been found for several viroids. From these observations, rolling circle models have been proposed for viroid replication. The host enzymes responsible for viroid replication are not clearly identified, although involvement of DNA-dependent RNA polymerases II and III has been suggested (Robertson and Branch 1987).

Infectious cDNA clones have been constructed for several viroids (Cress *et al.* 1983; Meshi *et al.* 1984, 1985; Tabler and Sänger 1984; Visvader *et al.* 1985); tandem multimers of viroid cDNA sequences are highly infectious when mechanically inoculated. Recent progress in plant transformation utilizing Ti plasmid vectors, in combination with these infectious viroid cDNA clones, has offered novel strategies for the study of viroid-host interactions. One such system is the agroinfection procedure in which an infectious viroid cDNA is placed in the T-DNA of a virulent strain of *Agrobacterium*. Upon inoculation with such *Agrobacterium* and resultant formation of galls on the plants, the viroid first replicates in the gall tissue and then spreads systemically. Gardner *et al.* (1986) successfully agroinfected tomato plants with potato spindle tuber viroid (PSTV). Using this system, Salazar *et al.* (1988) investigated the replication of PSTV in several nonhost plant species and showed that in all but one species, PSTV did not replicate, not even in the galls. They also showed that one species that had not been susceptible to PSTV infection by mechanical inoculation was infectible with PSTV by the agroinfection procedure.

Another plant transformation system utilizes transgenic plants. However, transgenic plants have not been reported in viroid studies, although transgenic plants expressing tomato golden mosaic virus genomic DNA (Rogers *et al.* 1986) and tobacco mosaic virus genomic RNA (Yamaya *et al.* 1988), as well as the satellite RNAs of cucumber mosaic virus and tobacco ringspot virus (Baulcombe *et al.* 1986; Gerlach *et al.* 1987), have been reported.

Hop stunt viroid (HSV) is the causal agent of hop stunt disease (Sasaki and Shikata 1977), and the complete nucleotide sequence has been determined (Ohno *et al.* 1983). We have shown that the double-stranded cDNA sequences of HSV are infectious when organized into more than two-unit tandem repeats (Meshi *et al.* 1984, 1985). Here, we have introduced HSV cDNA sequences into tobacco plants, *Nicotiana tabacum* L., by a Ti plasmid-mediated plant transformation. Infectious HSV was expressed from tandemly repeated HSV cDNA constructs and replicated in the transgenic tobacco plants.

The successful expression of HSV in transgenic tobacco was a surprising result, because tobacco has been reported to be a nonhost plant of HSV (Sasaki and Shikata 1980; Yaguchi and Takahashi 1984). Accordingly, the susceptibility of tobacco to HSV infection was reexamined by both mechanical and *Agrobacterium*-mediated inoculation. The results of these inoculation experiments showed that tobacco can be infected with HSV by both methods, although the efficiency of mechanical inoculation was very low. The results reported here establish tobacco as a host plant species of HSV.

MATERIALS AND METHODS

Plasmid constructions. Plasmid pHS-P4P, which carries four units of the HSV cDNA sequences in tandem (Meshi *et al.* 1984), was partially digested with *Eco*RI and the resultant fragment consisting of two units of the HSV double-stranded cDNA was isolated. This fragment was

inserted into the unique *Eco*RI site of pLGVneo1103 (Hain *et al.* 1985) and pBI121 (Jefferson *et al.* 1987) to give plasmids pOKHS22 and pBKHS21 or 22, respectively. The structures of these plasmids are shown in Figure 1.

HSV cDNA transgenic tobacco plants. The mobilization of the pOKHS plasmids to *Agrobacterium tumefaciens* C58ClRif^r, carrying a disarmed Ti plasmid, pGV3850 (Zambryski *et al.* 1983), and the transformation of tobacco plants (*N. tabacum* cv. Samsun) were carried out as described previously (Yamaya *et al.* 1988). Throughout the experiments, conditions for handling the transformed plants *in vitro* were at 25° C under 16-hr light per 8-hr dark periods.

Southern blot analysis was carried out essentially as described (Yamaya *et al.* 1988), using a unit-length HSV cDNA fragment as a probe.

Dot and northern blot analyses. Total nucleic acids were extracted from tobacco leaves or galls and treated with DNaseI as described previously for cucumber leaves by Ishikawa *et al.* (1984). Leaf samples used in the dot and northern blot analyses were taken from plants grown *in vitro* in order to avoid possible contamination in greenhouses, except for HSV-infected cucumber plants. Typical yields of nucleic acids from the leaves of tobacco plants grown *in vitro* or cucumber plants grown in greenhouses after the DNase treatment were 150–250 μg/g of fresh weight. Dot and northern blot analyses were carried out as described by Ishikawa *et al.* (1984, 1985).

Agroinfection of HSV onto tobacco plants. Plasmids pBKHS21 and 22 were mobilized into *A. tumefaciens* A208 carrying the nopaline Ti plasmid pTiT37 by the triparental mating procedure (Ditta *et al.* 1980). Resultant transconjugants were selected for their kanamycin resistance and ability to grow on a minimal medium. Tobacco plants (cv. Samsun) were grown *in vitro* to about 10 cm and were decapitated, with a few leaves being left. The *Agrobacterium* transconjugants were inoculated on top of the cut stems of these tobacco plants. Two pairs of plants were inoculated with two *Agrobacterium* strains carrying either pBKHS21 or 22, and grown at 25° C *in vitro*. Six to eight weeks after inoculation, upper parts of the plants, which resulted from the development of the lateral bud just below the inoculation site, were cut off the galls and transferred to a medium containing Claforan to eliminate possible contamination by the agrobacteria. The plants were grown on this medium for another month until we were able to collect enough leaf tissue for the dot blot assay. β-glucuronidase (GUS) activity was assayed as described by Jefferson *et al.* (1987).

Mechanical inoculation of HSV onto tobacco plants. Tobacco plants (cv. Samsun) with three to four true leaves were inoculated with 1 μg/ml of purified HSV by rubbing the viroid solution onto leaves dusted with Carborundum, and they were kept in a greenhouse at 25–32° C. Four weeks after inoculation, the upper leaves were harvested. Four grams of tobacco leaves were homogenized and extracted with phenol, and the crude preparations of total nucleic acids, recovered by ethanol precipitation, were dissolved in 1 ml of 0.1 M Tris-HCl (pH 7.5), 10 mM EDTA, and 0.1% bentonite and inoculated onto cotyledons of cucumber plants (*Cucumis sativus* L. cv. Suyo) in order to test for HSV replication. Symptoms of HSV infection were scored at weekly intervals after inoculation.

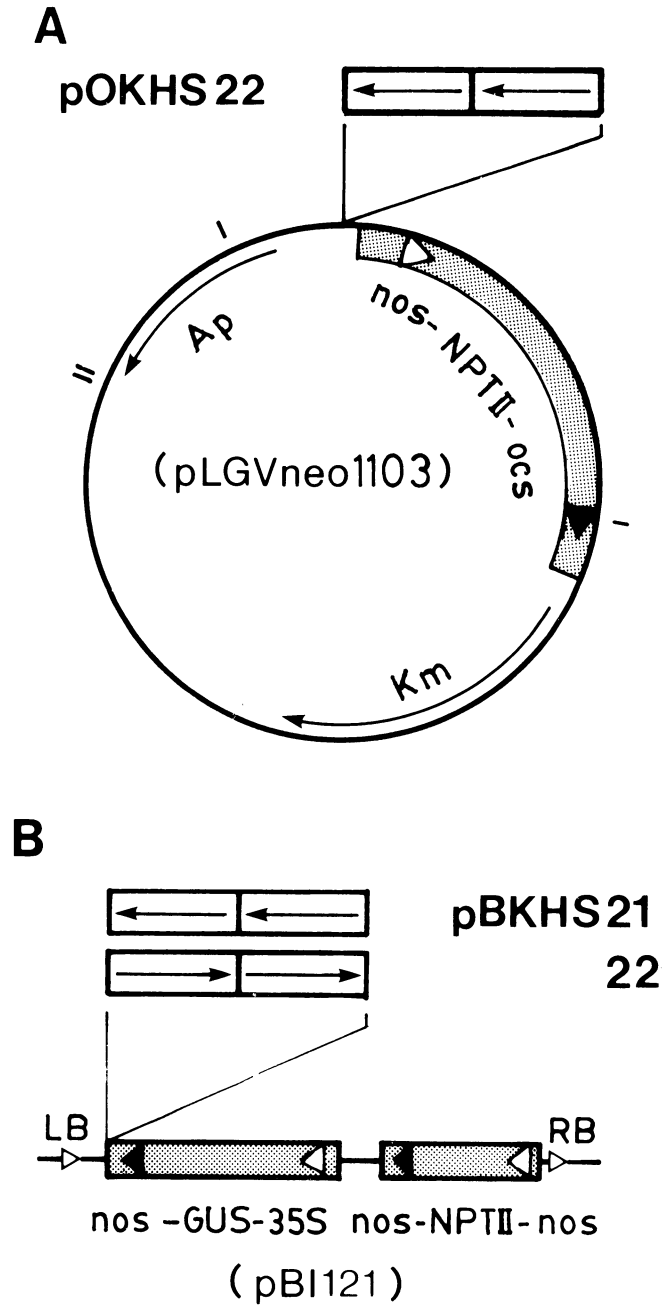

Fig. 1. Structures of vectors carrying the HSV cDNA. Open boxes with arrows indicate the HSV cDNAs; each arrow corresponds to one unit of the cDNA. The direction of the arrows is from 5′ to 3′ of the viroid plus-strand RNA. Shaded boxes indicate marker genes for plant transformation, with open and closed triangles representing promoters and polyadenylation signals, respectively. **A,** Structure of pOKHS22. Ap and Km indicate the bacterial marker genes for ampicillin and kanamycin resistances, respectively. nos-NPTII-ocs: the chimeric gene consisting of nopaline synthase (NOS) promoter, neomycin phosphotransferase II (NPTII) structural gene, and octopine synthase polyadenylation signal. Bars indicate the positions of *Dra*I sites. **B,** Structure of pBKHS21 and 22. Only the T-DNA region is shown. LB and RB indicate the left and right borders of the T-DNA. nos-GUS-35S: chimeric gene consisting of the cauliflower mosaic virus (CaMV) 35S promoter, β-glucuronidase structural gene, and NOS polyadenylation signal. nos-NPTII-nos: chimeric gene consisting of the NPTII structural gene and the NOS promoter and polyadenylation signal.

RESULTS

Transgenic tobacco plants carrying the HSV cDNAs. We have shown that plasmids carrying more than two units of HSV cDNA sequences in tandem are infectious for cucumber plants and give rise to replication of HSV (Meshi *et al.* 1985). These results strongly suggest that the cDNA sequence is somehow transcribed into RNAs containing the HSV sequences in the cucumber cells. Therefore, we presumed that the HSV cDNA introduced into the tobacco genome would also be transcribed without external plant promoter sequences. In the intermediate vector pOKHS22 (Fig. 1A), the HSV cDNA sequences are located about 100 bp upstream of the nopaline promoter of the chimeric kanamycin resistance gene, so they would not be transcribed by this promoter.

Plasmid pOKHS22 was introduced into tobacco (cv. Samsun) by a Ti plasmid-mediated transformation as described in Materials and Methods. Several transformed plants were obtained; they were morphologically indistinguishable from nontransformed tobacco plants when grown *in vitro* continuously at 25° C. We also did the transformation experiments with an intermediate vector carrying the HSV cDNA sequences in the reverse orientation, but no transformant was obtained with this construct. The reason for this failure is not clear.

Three plants, designated HS2203, HS2205, and HS2206, were arbitrarily chosen, and the total DNA was prepared from the leaves of these transformed plants, digested with *Dra*I, and subjected to Southern blot analysis using an HSV cDNA fragment as a probe (Fig. 2). Because the HSV cDNA sequence does not have the *Dra*I site, a band of 2.8 kb encompassing the HSV cDNA sequences is expected (Fig. 1A). A single band of the expected size was detected for all three plants, indicating that the HSV cDNA sequence was integrated into the plant genome without apparent rearrangements. The copy number of the integrated DNA was estimated to be one for HS2203 and three to five for HS2205 and 2206 (Compare lanes 1–3 with lanes 6 and 7 of Fig. 2).

Expression of HSV RNAs in transgenic tobacco plants. Leaf samples from HS2203, 2205, and 2206 tobacco plants, which were grown *in vitro*, were homogenized and their pathogenicity was tested on cucumber plants. The homogenates of the pOKHS22 transgenic tobacco leaves induced typical symptoms of HSV infection on all the inoculated cucumber plants (data not shown). As expected, the homogenate of the tobacco plants carrying only the kanamycin resistance marker gene induced no symptoms on the assay plants.

Total nucleic acids were prepared from the leaves of a

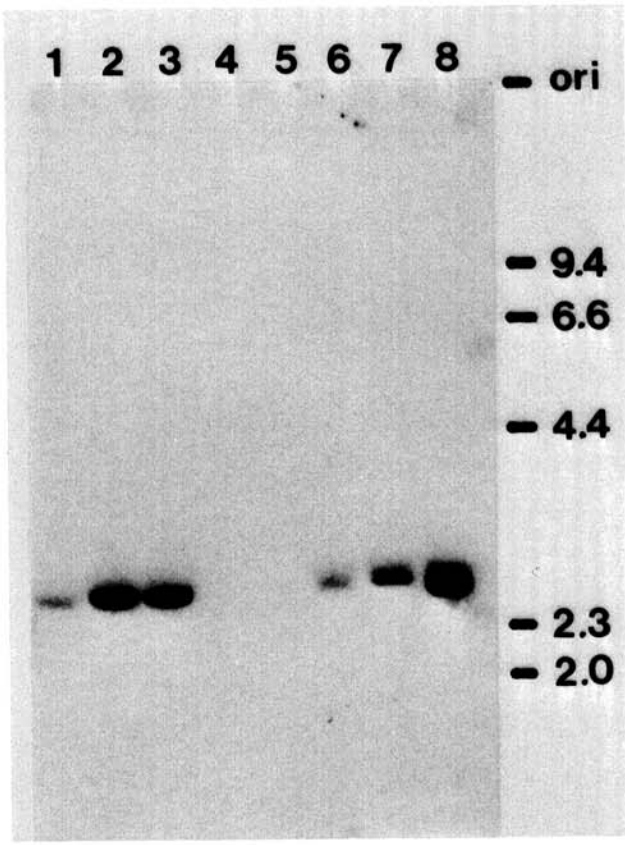

Fig. 2. Southern blot analysis of the total DNAs from transformed tobacco plants. Lanes 1–3, HS2203, HS2205, and HS2206, respectively. Lane 4, pLGVneo1103 transformed tobacco. Lane 5, nontransformed tobacco. Lanes 6–8, 1, 2, and 5 copy number standards, which are the reconstructions of appropriate amounts of *Dra*I-digested pOKHS22 and the total DNA from nontransformed tobacco. Ori: The origin of electrophoresis. Numbers on the right are the sizes of DNA size markers (in kb). Probe = unit-length HSV cDNA.

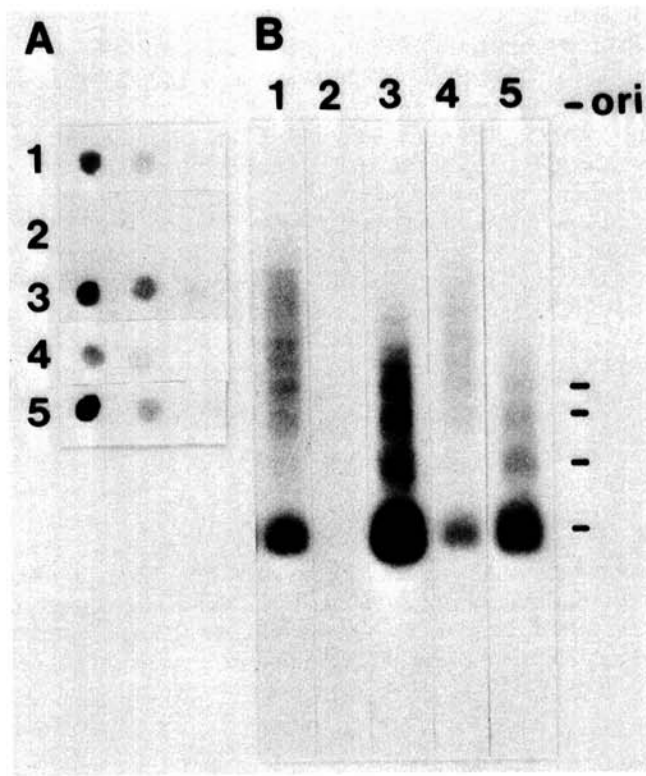

Fig. 3. Evidence for replication of HSV RNAs in transgenic tobacco plants. One plant (HS2203) was arbitrarily chosen and analyzed. **A,** Dot blot hybridization and **B,** Northern blot analysis of the total nucleic acids from transgenic tobacco plants. Each lane in the dot blot contains nucleic acids from 20, 2, and 0.2 mg (left to right) of fresh leaves, and each lane in the northern blot contains those from 20 mg of fresh leaves. Samples in lanes 4 and 5 were treated with DNaseI. Lanes 1 and 4, HS2203. Lane 2, pLGVneo1103-transgenic tobacco. Lanes 3 and 5, HSV-infected cucumber. The bars on the right indicate the positions of one-, two-, three-, and four-unit-length HSV RNAs.

HS2203 tobacco plant and analyzed for the presence of HSV-related RNAs by dot blot hybridization (Fig. 3A). The total nucleic acids from HS2203 gave as strong a signal as those from the HSV-infected cucumber plants, while those from the pLGVneo1103 transgenic plant did not give any detectable signal. DNase treatment did not affect the signal intensity to a significant extent, indicating that the HSV-related nucleic acids detected in the HS2203 tobacco were RNA.

To confirm that the signal was due to replicating HSV-related RNAs, we performed northern blot analysis (Fig. 3B). The electrophoretic pattern of HSV-related RNAs from HS2203 leaves was essentially the same as that from the HSV-infected cucumber leaves, unit-length HSV RNA and longer than unit-length replication intermediates (Ishikawa *et al.* 1984), and was not affected by DNase treatment. When the identical blot was hybridized to the vector pLGVneo1103 (Fig. 1A), no signal was detected, even after prolonged autoradiography exposure (not shown), which indicates that HSV-related RNAs described above do not contain detectable amounts of sequences derived from the vector. The presence of longer than two-unit HSV-related RNAs strongly suggests that HSV RNAs in the transgenic tobacco plants are, in fact, replicating viroid RNAs and do not reflect the processing and accumulation of the primary transcript RNAs.

Progeny of HSV cDNA transgenic plants. When HS2203, 2205, and 2206 plants were selfed, they set seeds normally. The R1 seeds were assayed for their kanamycin resistance. The offspring from HS2203 segregated in a 3:1 ratio, which was consistent with the integration of one HSV cDNA copy per cell in this line (Table 1). Segregation ratios of HS2205 and 2206 R1 seeds deviated greatly from 3:1, indicating the complexity of the organization of integrated sequences in the genome of these two lines.

Eight kanamycin-resistant R1 plants from HS2203 and two each from HS2205 and 2206 were arbitrarily chosen and tested for HSV expression. Dot blot analysis detected HSV-related RNAs in all R1 plants tested (data not shown). These results further suggest that HSV is expressed from its cDNA in the transgenic tobacco plants. HSV is not transmitted through seeds in either of two studied host species, hop and tomato (Yaguchi and Takahashi 1984).

Agroinfection of HSV onto tobacco. Results shown above strongly suggest that HSV replicates and accumulates in the HSV cDNA transgenic tobacco plants, although a previous report described tobacco as a nonhost of HSV (Yaguchi and Takahashi 1984). To clarify this apparent contradiction, we investigated the systemic infection of HSV in tobacco plants by the agroinfection procedure.

To construct a virulent *Agrobacterium* strain capable of transferring HSV cDNA sequences to the gall cells, we constructed binary vectors pBKHS21 and 22, which carry two units of HSV cDNA sequences on the T-region (Fig. 1B). In contrast to the pOKHS plasmids, the HSV cDNA sequences in the pBKHS plasmids are located just downstream of the polyadenylation signal of the chimeric GUS gene, so that the transcription of the GUS gene might proceed past the HSV cDNA.

Decapitated tobacco stems were inoculated with *A. tumefaciens* A208 strains, carrying the nopaline Ti plasmid pTiT37 and the binary vector pBKHS21 or 22. The inoculation and subsequent handling of the inoculated plants were carried out *in vitro* at 25° C, the same conditions as the transformation and subsequent handling of the transgenic plants, in order to allow direct comparison of two experiments.

Galls began to proliferate 2 wk after the inoculation while the lateral bud below the site of each inoculation developed into a mature plant. The galls remained on the tobacco plants for 6–8 wk, and then the upper part of the plants were cut off above the galls. Significant GUS activity was detected from the galls but not from the upper leaves (data not shown).

The presence of HSV RNA in the the upper leaves of the agroinfected tobacco plants was investigated by dot blot hybridization (Fig. 4). HSV RNA was detected at a high concentration, almost the same as that in the HSV-infected cucumber plants, in one of each pair of plants that had been agroinfected with either pBKHS21 or 22, and at a lower concentration in the other pBKHS22 agroinfected plant. We could not detect HSV RNAs in the other pBKHS21 agroinfected plant, although the galls formed on this plant contained HSV RNAs (not shown). These results indicate

Table 1. Segregation of kanamycin resistance in HSV cDNA transgenic plants

	Copy Number[a]	Number of R1 plants[b]		
		Km^r	Km^s	χ^2
HS2203	1	159	48	0.272
HS2205	3–5	18	130	308
HS2206	3–5	123	0	39.7

[a] Estimated from the data in Figure 2.
[b] Km^r = kanamycin resistant, Km^s = kanamycin sensitive.

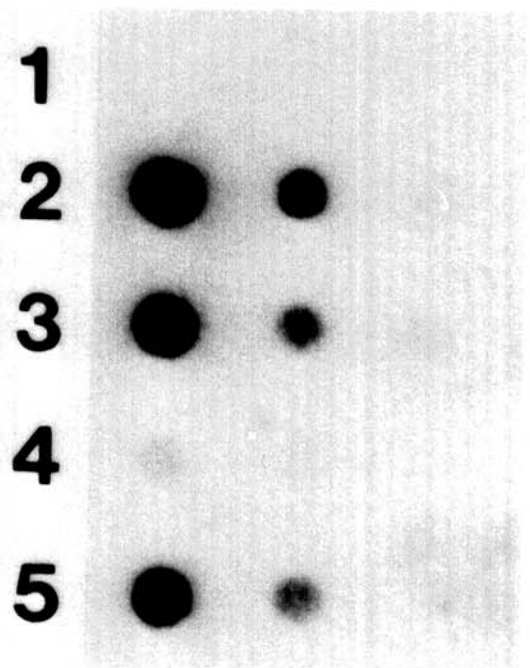

Fig. 4. Agroinfection of tobacco plants with HSV. Total nucleic acids from upper leaves of the agroinfected tobacco plants were treated with DNaseI and analyzed by dot blot hybridization. Each lane contains nucleic acids from 20, 2, and 0.2 mg of leaves (left to right). Lanes 1 to 4, tobacco plants inoculated with *Agrobacterium* carrying either pBKHS21 (lanes 1 and 2) or pBKHS22 (lanes 3 and 4). Lane 5, HSV-infected cucumber.

that HSV readily replicates and spreads systemically in tobacco plants when agroinfected.

Mechanical inoculation of HSV onto tobacco. Yaguchi and Takahashi (1984) reported a failure to detect HSV replication in mechanically inoculated tobacco plants by back inoculation to cucumber plants. However, it is possible that their results might have been a reflection of a low concentration of HSV RNAs in the inoculum used in their study. Therefore, we reexamined the susceptibility of tobacco to mechanical inoculation with HSV by using a high concentration (1 μg/ml) of purified HSV as the inoculum. This inoculum induces typical HSV symptoms on cucumbers within 18 days.

As shown in Table 2, of five inoculated tobacco plants, three were shown to contain a low amount of HSV by back inoculation onto cucumber plants. HSV concentration in the infected tobacco plants is estimated to be 250 pg/g of fresh leaf or less, which is about 0.5% or less of the HSV concentration in infected cucumber (T. Sano, unpublished observation). Dot blot analysis of total nucleic acids from the HSV-inoculated tobacco plants barely detected the presence of HSV-related RNAs (not shown). Thus, tobacco can also be infected with HSV by mechanical inoculation, but the inoculation efficiency is very low.

DISCUSSION

The results clearly indicate that HSV RNA is expressed from its cDNA sequence in the transgenic tobacco plants carrying tandemly repeated dimeric cDNAs. No external plant promoter was necessary for the expression of HSV RNA from the dimeric cDNA, as expected from the results of cDNA inoculation experiments (Meshi *et al.* 1985). Recently, two different systems for the introduction of viroids into plant materials have been described as alternatives to mechanical inoculation. These are the protoplast infection system (Faustmann *et al.* 1986) and the agroinfection system (Gardner *et al.* 1986). The use of transgenic plants expressing viroids is the third alternative.

The advantage of transgenic plants over agroinfection or protoplast infection in the studies of viroid-host interaction is that the host response can be studied under a physiologically normal cellular environment. The control of viroid replication may be different in normal differentiated plant cells and in dedifferentiated gall cells or isolated protoplasts. Galls or protoplasts may be more susceptible to viroid infection as observed for many viruses (Beier *et al.* 1977; Ponz and Bruening 1986). On the other hand, if the replication of a viroid is restricted at the level of systemic movement, a combined study employing transgenic plants and agroinfection would clearly distinguish such a case from the restriction at the cell level. A protoplast infection experiment cannot rule out the possible involvement of increased susceptibility in the protoplast.

Previous studies have revealed that HSV infects hop, *Humulus lupulus* L., its related weed, *H. japonicus* Sieb. et Zucc., and several *Cucurbitaceae* species. HSV also infects tomato, *Lycopersicon esculentum* Mill., but is symptomless in this host (Sasaki and Shikata 1980; Yaguchi and Takahashi 1984). On the other hand, tobacco, *N. tabacum*, was reported to be resistant to HSV infection; it did not develop any symptoms when inoculated with HSV, nor was HSV detected by back inoculation onto cucumber plants (Yaguchi and Takahashi 1984). Here we reexamined the susceptibility of tobacco to HSV and found that tobacco is readily infected with HSV by *Agrobacterium*-mediated inoculation. In addition, we were also able to mechanically infect tobacco plants with HSV, but the inoculation efficiency was greatly reduced when compared to agroinfection. These results suggest that the previous failure to infect tobacco with HSV was most likely due to the low infectivity of the inoculum used in that study. Salazar *et al.* (1988) also found that previously reported resistance of a wild potato species, *Solanum acaule* accession OCH11603, to PSTV infection was actually resistance to mechanical inoculation, which was shown by the successful agroinfection of this plant with PSTV.

The inefficiency of mechanically inoculating tobacco with HSV is interesting because tobacco is readily infected by the same procedure with various plant viruses and naked viral RNAs. Considering the small size and rod-shaped structure of viroids (Keese and Symons 1987), HSV must be much more resistant to cellular nucleases than viral RNA molecules. HSV, as well as other viroids, is enriched in the nuclear fraction of the infected host cells, which suggests that its replication occurs in the nucleus (Takahashi *et al.* 1982; Schumacher *et al.* 1983; Semancik and Conejero-Tomas 1987). On the other hand, the first step of the infection established by naked viral RNAs is, in most cases, the translation of the viral RNA, which occurs in the cytoplasm. Thus, one possible explanation for the inefficient mechanical inoculation of tobacco with HSV is the inefficient transport of the viroid RNA to the nuclei in the tobacco cell.

Our results with HSV on tobacco and those of Salazar *et al.* (1988) with PSTV on *S. acaule* indicate that mechanical inoculation does not work efficiently for some viroid-host combinations. Therefore, one must be very careful to define the host range of viroids with conventional inoculation technology.

Table 2. Infectivity on cucumber plants of crude total nucleic acids from tobacco plants inoculated with HSV[a]

	Days after inoculation on cucumber					
Inoculum	**7**	**14**	**21**	**28**	**35**	**42**
Tobacco Plant 1	0/6[b]	0/6	1/6	1/6	1/6	1/6
Tobacco Plant 2	0/6	0/6	2/6	2/6	2/6	2/6
Tobacco Plant 3	0/6	0/6	1/6	1/6	1/6	1/6
Tobacco Plant 4	0/6	0/6	0/6	0/6	0/6	0/6
Tobacco Plant 5	0/6	0/6	0/6	0/6	0/6	0/6
Purified HSV						
100 ng/ml	0/4	1/4	4/4	4/4	4/4	4/4
10 ng/ml	0/4	0/4	2/4	3/4	4/4	4/4
1 ng/ml	0/4	0/4	1/4	1/4	1/4	3/4

[a] Crude total nucleic acids were prepared from tobacco plants 4 wk after mechanical inoculation with HSV, dissolved in a Tris buffer at 4 g of leaf equivalent per milliliter, and their infectivity was assayed on cucumber plants.

[b] Cucumber plants showing symptoms per total plants inoculated.

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LITERATURE CITED

Baulcombe, D. C., Saunders, G. R., Bevan, M. W., Mayo, M. A., and Harrison, B. D. 1986. Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature 321:446-449.

Beier, H., Siler, D. J., Russell, M. L., and Bruening, G. 1977. Survey of susceptibility to cowpea mosaic virus among protoplasts and intact plants from *Vigna sinensis* lines. Phytopathology 67:917-921.

Cress, D. E., Kiefer, M. C., and Owens, R. A. 1983. Construction of infectious potato spindle tuber viroid cDNA clones. Nucleic Acids Res. 19:6821-685.

Diener, T. O. 1979. Viroids and Viroid Diseases. John Wiley & Sons Inc., New York.

Diener, T. O. 1982. Viroids and their interactions with host cells. Annu. Rev. Microbiol. 36:239-258.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Faustmann, O., Kern, R., Sänger, H. L., and Mühlbach, H.-P. 1986. Potato spindle tuber viroid (PSTV) RNA oligomers of (+) and (−) polarity are synthesized in potato protoplasts after liposome-mediated infection with PSTV. Virus Res. 4:213-227.

Gardner, R. C., Chonoles, K. R., and Owens, R. A. 1986. Potato spindle tuber viroid infections mediated by the Ti plasmid of *Agrobacterium tumefaciens*. Plant Mol. Biol. 6:221-228.

Gerlach, W. L., Llewellyn, D., and Haseloff, J. 1987. Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus. Nature 328:802-805.

Hain, R., Stabel, P., Czernilofsky, A. P., Steinbiss, H. H., Herrera-Estrella, L., and Schell, J. 1985. Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts. Mol. Gen. Genet. 199:161-168.

Ishikawa, M., Meshi, T., Ohno, T., Okada, Y., Sano, T., Ueda, I., and Shikata, E. 1984. A revised replication cycle for viroids: The role of longer than unit length RNA in viroid replication. Mol. Gen. Genet. 196:421-428.

Ishikawa, M., Meshi, T., Okada, Y., Sano, T., and Shikata, E. 1985. *In vitro* mutagenesis of infectious viroid cDNA clone. J. Biochem. 98:1615-1620.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Keese, P., and Symons, R. H. 1987. The structure of viroids and virusoids. Pages 1-47 in: Viroids and Viroid-like Pathogens. J. S. Semancik, ed. CRC Press, Boca Raton, FL.

Meshi, T., Ishikawa, M., Ohno, T., Okada, Y., Sano, T., Ueda, I., and Shikata, E. 1984. Double-stranded cDNAs of hop stunt viroid are infectious. J. Biochem. 95:1521-1524.

Meshi, T., Ishikawa, M., Watanabe, Y., Yamaya, J., Okada, Y., Sano, T., and Shikata, E. 1985. The sequence necessary for the infectivity of hop stunt viroid cDNA clones. Mol. Gen. Genet. 200:199-206.

Ohno, T., Takamatsu, N., Meshi, T., and Okada, Y. 1983. Hop stunt viroid: Molecular cloning and nucleotide sequence of the complete cDNA copy. Nucleic Acids Res. 11:6185-6197.

Ponz, F., and Bruening, G. 1986. Mechanisms of resistance to plant viruses. Annu. Rev. Phytopathol. 24:335-381.

Robertson, H. D., and Branch, A. D. 1987. The viroid replication process. Pages 49-69 in: Viroids and Viroid-like Pathogens. J. S. Semancik, ed. CRC Press, Boca Raton, FL.

Rogers, S. G., Bisaro, D. M., Horsch, R. B., Fraley, R. T., Hoffmann, N. L., Brand, L., Elmer, J. S., and Lloyd, A. M. 1986. Tomato golden mosaic virus A component DNA replicates autonomously in transgenic plants. Cell 45:593-600.

Salazar, L. F., Hammond, R. W., Diener, T. 0., and Owens, R. A. 1988. Analysis of viroid replication following *Agrobacterium*-mediated inoculation of non-host species with potato spindle tuber viroid cDNA. J. Gen. Virol. 69:879-890.

Sasaki, M., and Shikata, E. 1977. On some properties of hop stunt disease agent, a viroid. Proc. Jpn. Acad. 53B:109-112.

Sasaki, M., and Shikata, E. 1980. Hop stunt disease, a new disease occurring in Japan. Rev. Plant Prot. Res. 13:97-113.

Schumacher, J., Sänger, H. L., and Riesner, D. 1983. Subcellular localization of viroids in highly purified nuclei from tomato leaf tissue. EMBO J. 2:1549-1555.

Semancik, J. S., and Conejero-Tomas, V. 1987. Viroid pathogenesis and expression of biological activity. Pages 71-126 in: Viroids and Viroid-like Pathogens. J. S. Semancik, ed. CRC Press, Boca Raton, FL.

Tabler, M., and Sänger, H. L. 1984. Cloned single- and double-stranded DNA copies of potato spindle tuber viroid (PSTV) RNA and co-inoculated subgenomic DNA fragments are infectious. EMBO J. 3:3055-3062.

Takahashi, T., Yaguchi, S., Oikawa, S., and Kamita, N. 1982. Subcellular location of hop stunt viroid. Phytopathol. Z. 103:285-293.

Visvader, J. E., Forster, A. C., and Symons, R. H. 1985. Infectivity and *in vitro* mutagenesis of monomeric cDNA clones of citrus exocortis viroid indicates the site of processing of viroid precursors. Nucleic Acids Res. 13:5843-5856.

Yaguchi, S., and Takahashi, T. 1984. Survival of hop stunt viroid in the hop garden. Phytopathol. Z. 109:32-44.

Yamaya, J., Yoshioka, M., Meshi, T., Okada, Y., and Ohno, T. 1988. Expression of tobacco mosaic virus RNA in transgenic plants. Mol. Gen. Genet. 211:520-525.

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., and Schell, J. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. 2:2143-2150.