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Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus

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ABSTRACT **Beet western yellows luteovirus, like other luteoviruses, cannot be transmitted to host plants by mechanical inoculation but requires an aphid vector, a feature that has heretofore presented a serious obstacle to the study of such viruses. In this paper we describe use of agroinfection to infect hosts with beet western yellows virus without recourse to aphids. Agroinfection is a procedure for introducing a plant virus into a host via *Agrobacterium tumefaciens* harboring a Ti plasmid, which can efficiently transfer a portion of the plasmid (T-DNA) to plant cells near a wound. The viral genome must be inserted into the T-DNA in such a way that it can escape and begin autonomous replication, a requirement that has, so far, limited agroinfection to pathogens with a circular genome. We have cloned cDNA corresponding to the complete beet western yellows virus RNA genome between the cauliflower mosaic virus 35S promoter and the nopaline synthase transcription termination signal. In one construct, a self-cleaving (ribozyme) sequence was included so as to produce a transcript *in planta* with a 3′ extremity almost identical to natural viral RNA. When inoculated mechanically to host plants, the naked plasmid DNA was not infectious but, when introduced into T-DNA and agroinfected to plants, both the construct with and without the ribozyme produced an infection. This approach should be applicable to virtually any plant virus with a linear plus-strand RNA genome.**

The luteoviruses are a large group of plant viruses with a monopartite plus-strand RNA genome packaged in small isometric virions (for reviews, see refs. 1 and 2). They are transmitted in a persistent circulative fashion by several aphid species and are generally confined to host phloem tissues. Luteovirus RNA and virions cannot be transmitted by mechanical inoculation. Recently, the genomes of several luteoviruses have been characterized (3–6), and *in vitro* transcripts of full-length cDNA clones of barley yellow dwarf virus and beet western yellows virus (BWYV) have been shown to be infectious in protoplasts (7, 8). These transcripts lend themselves to the study of gene function by "reverse genetics," in which specific mutations are introduced into the cloned cDNA and the biological properties of transcripts carrying the mutations are investigated. However, the aforesaid nontransmissibility of luteoviruses to whole plants by mechanical inoculation impedes many interesting applications of this approach.

Agrobacterium tumefaciens harboring a Ti plasmid can efficiently transfer a portion of this plasmid called the T-DNA to plant cells in the vicinity of a wound (9). This property has provided the basis for a technique called "agroinfection" or "agroinoculation" in which a viral genome is inserted into the T-DNA in such a way that it can escape and initiate an infection once within a target cell (10). Up until now, agroinfection has been limited to infectious agents with circular genomes [i.e., cauliflower mosaic virus (CaMV), geminiviruses, and potato spindle tuber viroid (for review see ref. 11)]. In this paper we describe an agroinfection system suitable for a plant virus with a linear single-stranded RNA genome and its use to infect plants with cloned BWYV cDNA.

MATERIALS AND METHODS

Plasmid Construction. The previously described full-length clone of BWYV isolate FL1 cDNA (pBW_0; ref. 8) served as the source for all viral sequences. *Escherichia coli* strain SURE (Stratagene) was used as host for constructions. A CaMV 35S promoter cassette (ref. 12; and further modifications), containing nucleotides (nt) −417 to −1 relative to the CaMV transcription initiation site, was introduced into pBluescript SK(−) (Stratagene) to produce pBK35. pBK35 contains a unique *Stu* I site with the point of cleavage coincident with the transcription initiation site (12). A DNA fragment (P1; Fig. 1*A*) containing nt 1–376 of the BWYV sequence was synthesized by PCR. The upstream primer, 5′-CCG*GTCGACAAAAGAAACCAGGAGGGA*, contained a *Hin*cII site (italicized) overlapping the 5′ terminal viral sequence (underlined). P1 was cut with *Hin*cII and *Xho* I (nt 256), and the resulting fragment was inserted between the *Stu* I and *Xho* I sites of pBK35 to give p35BW51 (Fig. 1*A*). A *Bam*HI–*Xho* I fragment from p35BW51 containing the promoter and cDNA insert was then cloned between the *Bam*HI–*Xho* I sites of pRT103 (14) to give p35BW52. The insert region was excised from p35BW52 as a *Bam*HI–*Hin*dIII fragment and placed between the *Bam*HI–*Hin*dIII sites of pUC18S (a derivative of pUC18 with an additional *Sal* I site preceding the polylinker *Hin*dIII site) to give p35BW53. The foregoing steps were necessary to obtain an appropriate configuration of polylinker sites. The rest of the BWYV sequence (4) was inserted in two steps as an *Xho* I–*Eco*RV fragment (nt 256–1412) between the *Xho* I–*Hin*cII sites of p35BW53 and as a *Spe* I–*Sal* I fragment (nt 1350–5641) between the *Spe* I and polylinker *Sal* I sites to produce $pBW.A^+$ (Fig. 1*A*).

The 3′ poly(A) tail of $pBW.A^+$ was replaced by *Xba* I and *Sst* I sites by oligonucleotide-directed mutagenesis (8) to yield $pBW.A^-$ (Fig. 1*A*), and an *Sst* I–*Sal* I fragment containing the nopaline synthase (NOS) transcription termination signal and polyadenylylation site (15) was inserted between the vector *Sst* I and *Sal* I sites to create pBW.N (Fig. 1*A*). The ribozyme sequence was provided as a 54-mer

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Abbreviations: BWYV, beet western yellows virus; CaMV, cauliflower mosaic virus; NOS, nopaline synthase; nt, nucleotide(s); T-DNA, transferred DNA.

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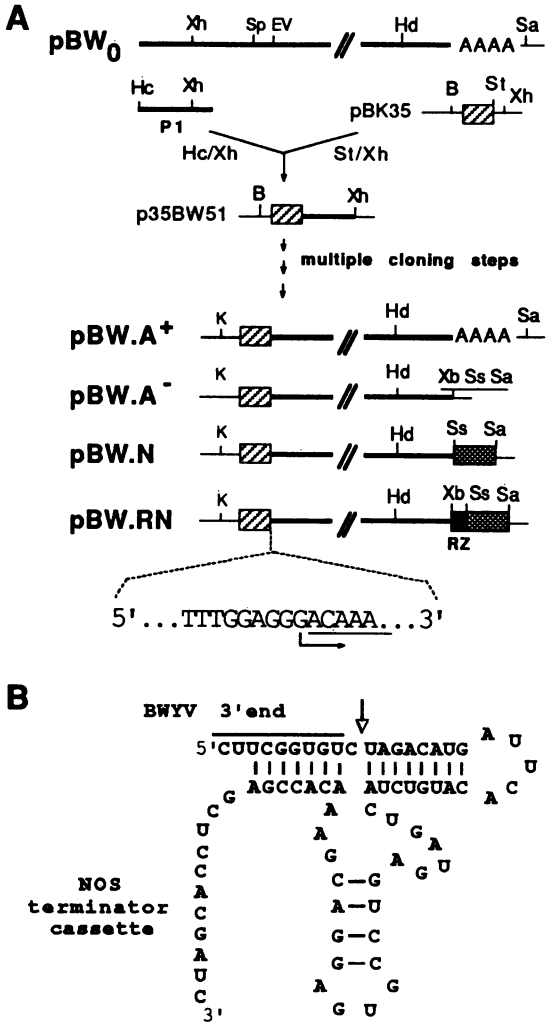

FIG. 1. Structure of clones used in agroinfection. (*A*) A simplified scheme for construction of pBW.N and pBW.RN. Thick lines represent BWYV cDNA sequences (not to scale), and thin lines represent flanking vector sequences. Hatched rectangle, 35S promoter cassette; black rectangle, ribozyme (RZ) cassette; cross-hatched rectangle, NOS terminator cassette. The sequence at the 35S promoter–viral cDNA junction shows the transcription initiation site (arrow) and the beginning of the viral sequence (underlined). Relevant restriction sites are *Bam*HI (B), *Eco*RV (EV), *Hin*cII (Hc), *Hin*dIII (Hd), *Kpn* I (K), *Sal* I (Sa), *Spe* I (Sp), *Stu* I (St), *Sst* I (Ss), *Xba* I (Xb), and *Xho* I (Xh). (*B*) Structure of the ribozyme separating the BWYV 3′ terminus from the NOS terminator cassette. The sequence of the nonviral portion of the ribozyme sequence was based on the consensus hammerhead structure given in ref. 13. The arrow indicates the predicted site of self-cleavage.

(5′-CTAGACATGATTCACATGTCTACTGATGAGTC-CGTGAGGACGAAACACCGAGCT) hybridized with a 46-mer complementary to all but the underlined extremities of the 54-mer. The hybrid was inserted between the *Xba* I and *Sst* I sites of pBW.A$^-$ to produce pBW.R. The NOS cassette was then introduced into pBW.R to yield pBW.RN (Fig. 1*A*).

Transcription vectors p3′BW.N and p3′BW.RN were obtained by placing the *Hin*dIII–*Sal* I fragment from pBW.N and pBW.RN between the *Hin*dIII and *Sal* I sites of BS(−) (Stratagene). DNA fragments containing the insert regions were amplified by PCR using the M13mp19 universal and reverse sequencing primers (Pharmacia). *In vitro* run-off transcription of amplified DNA was as described (8).

Infection of *Chenopodium quinoa* Protoplasts. *Ch. quinoa* protoplasts were prepared and inoculated with circular plasmid DNA (25 μg) by electroporation as described (8) except that a high-voltage pulse of 750 V/cm was applied.

Agroinfection. The BWYV cDNA flanked by the control sequences of pBW.RN and pBW.N was moved into pBin19 (16) on a *Kpn* I–*Sal* I fragment (pBinBW.RN and pBinBW.N, respectively). Recombinant vectors were introduced into *A. tumefaciens* LBA4404 by electroporation (17) or by triparental mating (18). The chromosomal background of the resulting transformants is LBA4404 (pAL4404::pBinBW.N) and LBA4404 (pAL4404::pBinBW.RN). For agroinfection, the *A. tumefaciens* in a 48-hr culture were collected by centrifugation and resuspended in 1/100th volume of 20 mM Tris Cl at pH 8. *Physalis floridana* (six-leaf stage), *Nicotiana benthamiana* (six- to eight-leaf stage), and *Capsicum annuum* (four-leaf stage) were inoculated by making several incisions of 1 mm depth in the stem and applying 20 μl of inoculum (containing ≈10^9 bacteria) to the cuts. Inoculation of *Nicotiana clevelandii* (6- to 8-cm diameter) was performed by using a Hamilton syringe to inject a total dose of 20 μl of inoculum at about 10 sites in the leaf midribs and petioles.

Aphid Transmission Tests. Aphids (*Myzus persicae*) were reared on healthy *Ca. annuum*. Apterous adults and fourth-instar larvae were deposited on excised leaves of agroinfected plants in small plastic boxes. On *N. benthamiana*, aphid survival was greatly improved by first washing the leaves with 2% DDN150 (Franklab, Montigny-le-Bretonneux, France) and then with water. After a 1- to 2-day acquisition period, the aphids were transferred to healthy *Montia perfoliata* (approximately five aphids per plant). The aphids were killed by insecticide treatment after 5 days, and virus detection by ELISA was performed 3 weeks later.

Detection of Virus Coat Protein and RNA. Infection after agroinoculation and aphid-transmission experiments was tested by double-antibody sandwich ELISA (19) using BWYV-specific antisera. In each set of experiments, the threshold for infection was defined as the mean ELISA absorbance value for four healthy plants plus three times the standard deviation of the healthy plant values (19). Total RNA was extracted from protoplasts (8) 72 hr after electroporation or from randomly selected leaves of plants (20) 3–6 weeks postinoculation, and viral RNA was detected by Northern hybridization using a ^{32}P-labeled antisense RNA probe (8). Electron microscopic detection of virus in extracts of agroinfected plants was as described (21).

The 3′ termini of progeny viral RNA were analyzed by RNase mapping (22) using radioactive antisense RNA probes made by transcribing *Hin*dIII-linearized p3′BW.RN or p3′BW.N. Dried RNA samples (5–10 μg of total plant RNA, 2–4 μg of total protoplast RNA, 10–30 ng of transcript) were dissolved in 30 μl of hybridization buffer with 10^6 cpm of probe, allowed to hybridize, and then treated with RNase as described (22). RNase-resistant products were sized by electrophoresis through a 6% polyacrylamide/8 M urea sequencing gel.

RESULTS AND DISCUSSION

Plasmids for Agroinfection. In previous agroinfection experiments it has generally proven necessary to insert tandem copies of the viral genome into the T-DNA (for review, see ref. 11). For the geminiviruses and CaMV, which have DNA genomes, the viral sequence is probably liberated from the T-DNA by homologous recombination between the sequence repeats, but, in the case of CaMV, escape can also involve synthesis of the genome-plus length 35S transcript, an intermediate in DNA replication (23). The mechanism by which a viroid sequence might escape and begin autonomous replication is not known.

In this paper we have devised an agroinfection strategy for delivery of a virus with a linear RNA genome. Evidently, a

plus-strand RNA virus such as BWYV with no DNA intermediate in its replication cycle is not expected to possess transcription signals active at the DNA level. Consequently, we have provided exogenous transcription initiation and termination signals flanking the cloned viral cDNA. The CaMV 35S promoter, which is active in many plant tissues (24), was used to initiate transcription. The promoter cassette was placed so that the resulting transcript would have only one nonviral residue (guanosine) at its 5′ end (Fig. 1*A*).

To provide a transcription termination signal, a 333-nt DNA fragment containing the NOS 3′ noncoding sequence was inserted behind the cDNA to produce pBW.N (Fig. 1*A*). Viral RNA transcripts terminating *in planta* at the predicted site will possess a 3′ nonviral extension of 156 nt derived from the NOS cassette plus a 3′ poly(A) tail. BWYV run-off transcripts with a nonviral 3′ extension of 32 nt are infectious in protoplasts (8). However, experience with other viral RNAs suggested that a very long 3′ nonviral extension might greatly diminish biological activity (25, 26). Therefore, an autocatalytic sequence (27), or "ribozyme," was placed just downstream of the viral RNA 3′ terminus (see *Materials and Methods*) to produce pBW.RN (Fig. 1*A*). The ribozyme (Fig. 1*B*) was modeled after the self-cleaving RNA sequences characteristic of avocado sunblotch viroid, tobacco ringspot virus satellite RNA, and many virusoids (28). These sequences can fold into a "hammerhead" structure, which, in the presence of Mg^{2+}, directs spontaneous autocleavage at a specific site on the RNA (13). Based upon the hammerhead model, the ribozyme in pBW.RN should cleave the primary transcript 1 nt downstream of the viral 3′ end (Fig. 1*B*). A similar approach has been used to eliminate nonviral sequences from the 3′ end of *in vitro* run-off transcripts of cloned brome mosaic virus cDNAs (26).

The activity of the ribozyme was tested by using an *in vitro* run-off transcript of p3′BW.RN, which contains the 3′ terminal portion of the BWYV sequence plus the ribozyme and NOS termination cassette. PAGE revealed that an estimated 50% of the transcript underwent spontaneous cleavage during transcription to yield two fragments of the size expected for ribozyme-induced scission (Fig. 2, lane 2). An antisense transcript of p3′BW.RN or a sense transcript of p3′BW.N (no ribozyme sequence) did not undergo cleavage (Fig. 2, lanes 1 and 3). We conclude that the ribozyme in p3′BW.RN cuts the primary transcript at or near the predicted site.

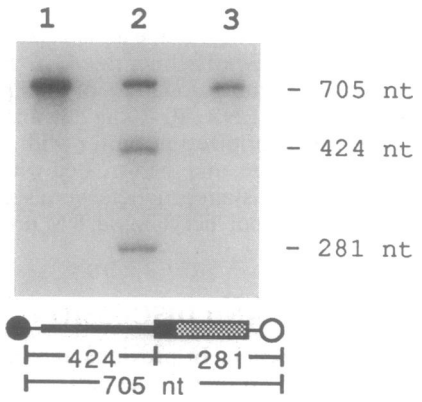

FIG. 2. Self-processing of a transcript containing the ribozyme sequence. ^{32}P-labeled sense RNA was transcribed from PCR-amplified fragments of p3′BW.RN (lane 2) and p3′BW.N (lane 3) with bacteriophage T3 RNA polymerase. ^{32}P-labeled antisense RNA of p3′BW.RN (lane 1) was transcribed with bacteriophage T7 RNA polymerase. After 30 min, the transcription reactions were stopped, and the products were analyzed by electrophoresis through a 6% polyacrylamide/8 M urea gel followed by autoradiography. Sizes of uncleaved transcript and expected cleavage products are indicated on the map (below) and beside the autoradiograph. ●, T3 promoter; ○, T7 promoter. Other symbols are as in Fig. 1.

Protoplast Infection Experiments. We have shown previously that transcripts of cloned BWYV cDNA are infectious to *Ch. quinoa* protoplasts (8). When circular pBW.RN and pBW.N plasmids were introduced into *Ch. quinoa* protoplasts by electroporation, both constructs were infectious as judged by the appearance of viral RNA (Fig. 3, lanes 2 and 3) in extracts of protoplasts harvested 72 hr postinoculation. The ability of pBW.N to initiate an infection suggests that the BWYV replicase can recognize and initiate minus-strand RNA synthesis on the viral 3′ sequence even when it is embedded in a longer transcript. Alternatively, RNA molecules capable of being replicated could be engendered by premature termination of transcription (or posttranscriptional cleavage) near the authentic viral RNA 3′ terminus of the primary transcript. A similar finding has been reported for tobacco transformed with tobacco mosaic virus cDNA under control of the 35S promoter but with no transcription terminator (12).

Agroinfection. Three known hosts for BWYV (30) were inoculated as described in *Materials and Methods* with *A. tumefaciens* carrying pBinBW.RN. With *P. floridana*, symptoms (leaf yellowing, leaf curling, and petiole necrosis) began to appear 10 days postinoculation. Early symptoms were less severe on *N. benthamiana* and *N. clevelandii*, with interveinal leaf yellowing becoming pronounced only 4–6 weeks after infection. Virus could be detected by ELISA 3 weeks postinoculation in segments of randomly selected leaves of 33–100% of the plants inoculated with pBinBW.RN in different experiments (Table 1). ELISA values for all the plants scored as infected in Table 1 were strongly positive, at least 5 times and often 10 times the infection threshold value as defined in *Materials and Methods*. Infection of *N. clevelandii* occurred with greater frequency than for the other two hosts (Table 1). It is not yet known if this difference reflects greater susceptibility of *N. clevelandii* to virus infection or if the inoculation procedure (jabbing with a syringe) was more efficient than that employed with the other two hosts. Agroinfection with *A. tumefaciens* carrying pBinBW.N rather than pBinBW.RN was also successful in *N. clevelandii* (Table 1).

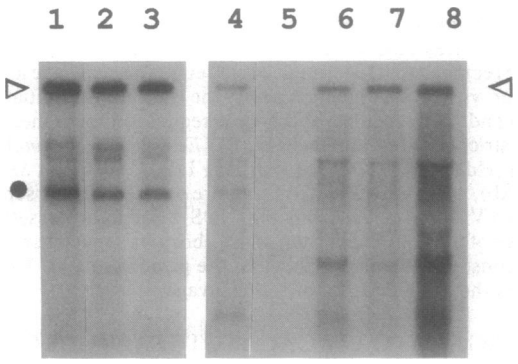

FIG. 3. Detection of BWYV RNA in total RNA from infected protoplasts and agroinfected *N. clevelandii* by Northern hybridization. RNA was extracted from protoplasts electroporated with the full-length transcript of pBW_0 (8) (lane 1), pBW.RN DNA (lane 2), and pBW.N DNA (lane 3). RNA was extracted from aphid-infected *P. floridana* (lane 4), a mock-inoculated *N. clevelandii* plant (lane 5), and three agroinfected *N. clevelandii* plants that tested positive for infection by ELISA (lanes 6–8). Triangles mark the position of BWYV genomic RNA, and the black dot marks the position of the major viral subgenomic RNA (8). The other minor bands in lanes 4–8 have not been characterized, but some may arise from ribosomal RNA shadowing (29). Although it has been detected in other experiments (data not shown), the subgenomic RNA is not readily visible against the background of minor bands in the whole plant extracts in lanes 6–8.

Table 1. Efficiency of transmission of BWYV by agroinfection

Host	Experiment	Inoculum*	Plants infected/ inoculated
P. floridana	1	RN	5/15
	2	RN	7/10
	3	RN	6/14
N. clevelandii	1	RN	8/10
	2	RN	11/12
	3	RN	5/6
	4	RN	4/4
		N	23/25
	5	RN	2/4
		N	18/26
N. benthamiana	1	RN	6/12
	2	RN	5/12
Ca. annuum	1	RN	0/11

*RN, inoculation with LBA4404 (pAL4404::pBinBW.RN); N, inoculation with LBA4404 (pAL4404::pBinBW.N).

Virus-like particles were readily detected by immunosorbent electron microscopy of extracts of agroinfected plants (data not shown). Viral RNA was detected by Northern hybridization of total RNA extracted from ELISA-positive agroinfected plants (Fig. 3, lanes 6–8) but was not detected in a mock-inoculated plant (Fig. 3, lane 5). Indeed, no viral infection was ever detected by ELISA for noninoculated control plants (n = 30) kept in the same growth chamber or for *P. floridana* (n = 24) inoculated with *A. tumefaciens* harboring pBin19 without a cDNA insert. Agroinoculation of *Ca. annuum*, a nonhost for BWYV isolate FL1 (H. Lot, personal communication) but a host for *A. tumefaciens* (31), gave no infection (Table 1). Thus, the failure of viruliferous aphids to transmit BWYV to *Ca. annuum* (a plant on which they feed readily) is apparently due to incompatibility between the virus and the plant and not to a deficiency in vector transmission, as it seems unlikely that a totally different inoculation route such as agroinoculation would suffer from the same deficiency.

To determine if naked BWYV cDNA (plus flanking control sequences) can initiate an infection, 14 *N. clevelandii* were each inoculated with 50 μg of purified pBW.RN plasmid using the syringe jabbing procedure employed for agroinoculation of *N. clevelandii*. This amount of pBW.RN corresponds to about 5000 times the amount of viral cDNA present in the *A. tumefaciens* inoculum used in the experiments shown in Table 1. None of the plants so inoculated became infected. We conclude that efficient delivery of the pBW.RN cassette to susceptible sites on whole plants requires the transfer functions provided by the *A. tumefaciens* Ti plasmid.

The fate of nonviral 3′ sequences after agroinfection was studied by RNase mapping (22). A ^{32}P-labeled RNA probe complementary to the NOS cassette, the ribozyme sequence, and the 3′ terminal portion of BWYV (nt 5367–5641) was produced by run-off transcription of *Hin*dIII-linearized p3′BW.RN. The antisense probe RNA was hybridized to progeny viral RNA from *N. clevelandii* agroinfected with pBinBW.RN and from transcript-infected protoplasts. The material was then treated with RNase under conditions designed to degrade single-stranded but not double-stranded RNA, and the RNase-resistant portion of the probe was sized by PAGE. Transcript of pBW_0 (8), which does not contain the ribozyme and NOS cassette, was carried through the same hybridization and RNase treatment to provide a mobility marker (predicted size, 274 nt) for the portion of the probe complementary to the viral 3′ terminal sequence. The mobility of this marker (Fig. 4, lane 8) was identical to that of the RNase-trimmed probe following hybridization with progeny RNA from infected protoplasts (lane 7) and from naturally infected (lane 6) and agroinfected (lane 5) plants, indicating that successful multiplication of the virus following agroinoculation is accompanied by elimination of the 3′ nonviral sequences. Similar experiments using progeny RNA from a pBinBW.N agroinfection showed that the downstream nonviral sequences predicted for the primary transcript are likewise absent from the progeny RNA (Fig. 4, lane 11). Thus, to achieve agroinfection with BWYV, no special precautions are needed to generate an authentic or nearly authentic viral RNA 3′ terminus *in planta*. This may not, however, be the case for other luteoviruses.

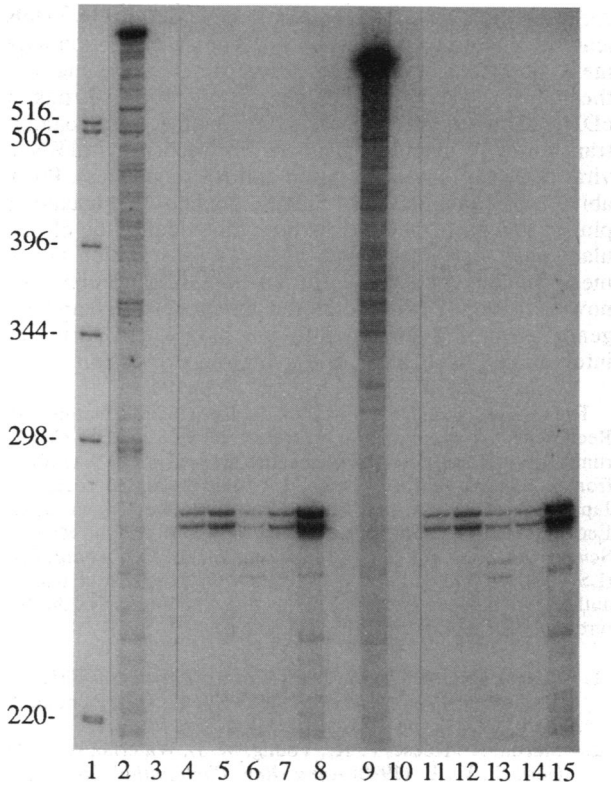

FIG. 4. RNase protection analysis of the 3′ termini of progeny viral RNA from plants agroinfected with pBW.RN and pBW.N. RNA samples were from healthy *N. clevelandii* (lanes 3 and 10), *N. clevelandii* agroinfected with pBW.N (lanes 4 and 11) or with pBW.RN (lanes 5 and 12), naturally BWYV-infected *P. floridana* (lanes 6 and 13), transcript-infected protoplasts (lanes 7 and 14), and pBW_0 transcript (lanes 8 and 15). The RNA was hybridized with ^{32}P-labeled antisense probe from p3′BW.RN (lanes 3–8) or p3′BW.N (lanes 10–15) prior to RNase treatment. Lane 1, single-stranded DNA size markers; lane 2, full-length p3′BW.RN probe; lane 9, full-length p3′BW.N probe. Mobility was determined by electrophoresis through a denaturing 6% polyacrylamide gel and autoradiography. The two heavily labeled bands in lanes 4–8 and 11–15 differ by 4 nt in mobility. The shorter of these two species and most of the other minor bands may arise from overdigestion of the viral RNA–probe hybrid.

To test the transmissibility of virus in agroinfected plants, nonviruliferous *My. persicae* were allowed to feed on such plants and were then transferred to healthy *Mo. perfoliata*. Successful aphid transmission of the virus, as assayed by ELISA, produced typical symptoms and occurred at a high rate for all three types of agroinfected source plants (10 of 10, 12 of 12, and 15 of 24 *Mo. perfoliata* infected for transmission tests from *P. floridana*, *N. benthamiana*, and *N. clevelandii*, respectively). We conclude that the viral sequence transmitted by agroinfection is still fully competent for transmission by aphids and for symptom development.

Concluding Remarks. Cloned cDNA of several mechanically transmitted RNA viruses, when positioned behind the

35S promoter, have been shown to be infectious by mechanical inoculation to plants (32–34). The agroinfection experiments described in this paper differ from these findings in that they offer a means of obtaining an infection starting with cDNA of a virus with a linear RNA genome, which cannot be transmitted by mechanical inoculation of virus, viral RNA, or viral cDNA (plus transcription control sequences). Presumably, such an approach can be applied to virtually any plus-strand RNA plant virus but will evidently be of particular value for viruses such as BWYV for which no other means of delivery is presently available. In particular, we can now envision use of site-directed mutagenesis to map BWYV genes governing viral cell-to-cell movement, virus–aphid interactions, host range, and symptom expression.

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