Independent Expression of the First Two Triple Gene Block Proteins of Beet Necrotic Yellow Vein Virus Complements Virus Defective in the Corresponding Gene but Expression of the Third Protein Inhibits Viral Cell-to-Cell Movement

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Cell-to-cell movement of beet necrotic yellow vein furovirus is controlled by three slightly overlapping genes on RNA 2 called the triple gene block (TGB) encoding, in order, P42, P13, and P15. Synthesis of P42 is directed by subgenomic RNA 2sub_a while synthesis of both P13 and P15 is probably directed by a dicistronic subgenomic RNA, 2sub_b. For complementation experiments, each TGB protein gene was inserted into a “replicon” derived from viral RNA 3. In mixed infections, the replicons expressing P42 and P13 complemented RNA 2 mutants defective in the corresponding gene. A P15-containing replicon did not complement a P15-defective RNA 2 but complementation was observed with a dicistronic replicon containing the P15 gene placed behind the P13 gene. In mixed infections with wild-type viral RNAs, the P15-containing replicon did not inhibit viral RNA replication in protoplasts but blocked local lesion formation on leaves. Infection of leaves was also inhibited by an RNA3-derived replicon containing the third TGB gene from another furovirus, peanut clump virus. The results are consistent with a model in which viral cell-to-cell movement requires production of appropriate relative amounts of P13 and P15, and their expression from a dicistronic subgenomic RNA provides a mechanism for coordinating their synthesis.

Additional keyword: plant viruses.

The genome of beet necrotic yellow vein furovirus (BNYVV) consists of five plus-sense RNAs, two of which (RNAs 1 and 2) encode functions essential for infection of all plants while the other three (RNAs 3, 4, and 5) are implicated in vector-mediated infection of sugar beet (*Beta vulgaris* L.) roots (Richards and Tamada 1992). Localized (cell-to-cell) movement of BNYVV, as manifested by formation of local lesions on appropriate hosts, is governed by a set of three successive, slightly overlapping viral genes on RNA 2 known as the triple gene block (TGB) (Gilmer et al. 1992), which en-

code, in order, the viral proteins P42, P13, and P15 (gene products are designated by their calculated molecular mass in kilodaltons; Bouzoubaa et al. 1986). TGB counterparts are present in some other furoviruses (Herzog et al. 1994; Scott et al. 1994), and in potex-, carla- and hordeiviruses (Koonin and Dolja 1993).

BNYVV does not require synthesis of viral coat protein for production of local lesions on leaves of *Chenopodium quinoa* Willd. (Schmitt et al. 1992), indicating that viral RNA or a complex between viral RNA and a protein other than the coat protein can move from cell to cell. The manner in which the TGB components assist in the movement process is not understood although computer-assisted sequence comparisons have detected characteristic conserved sequences that may provide clues to their function. Thus, the 5'-proximal TGB protein (TGB1) invariably contains a series of sequence motifs characteristic of an ATP/GTP-binding helicase while the second protein (TGB2) always has two potentially membrane-spanning hydrophobic domains separated by a hydrophilic sequence that contains a highly conserved peptide motif of unknown significance (Koonin and Dolja 1993). The sequence and size of the third TGB protein (TGB3) is more variable although the N-terminal portion is generally rather hydrophobic. Subgenomic RNAs with 5' termini mapping upstream of the BNYVV TGB1 and TGB2 open reading frames (ORFs) have been detected (Fig. 1A) but no such species has been reported for TGB3 of BNYVV (Gilmer et al. 1992) or of any other of the TGB-containing viruses. In the case of potato virus X (PVX; Morozov et al. 1991) and barley stripe mosaic virus (BSMV; Zhou and Jackson 1996), there is evidence that the TGB2 and TGB3 products are expressed from the same subgenomic RNA.

So far, no example has been reported of a virus in which the three TGB members are arranged differently on the same RNA or are parceled out to different genomic RNAs, suggesting that their association in a particular order is important in regulating their function. In this paper we investigated whether independent expression of the BNYVV TGB proteins during an infection is possible by inserting the ORF of each into a viral replication-dependent “replicon” derived from

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BNYVV RNA 3 (Jupin et al. 1990). We show that, in mixed infections of *C. quinoa* leaves, the BNYVV TGB1 or TGB2 protein so expressed can complement BNYVV RNA 2 containing a mutation disabling the corresponding TGB protein. No complementation was observed with a replicon containing TGB3, however, unless the TGB3 ORF was positioned downstream of the ORF for TGB2 in a dicistronic replicon. When coinoculated with wild-type RNAs 1 and 2, the replicon expressing the TGB3 ORF of BNYVV inhibited infection. The data are consistent with a model for expression of the TGB proteins in which translation of P15 from a dicistronic sub-

genomic RNA regulates P15 expression levels in vivo. Overexpression of P15 interferes with cell-to-cell movement of the viral infection.

RESULTS AND DISCUSSION

Replicons expressing the BNYVV TGB proteins.

Provided that sufficient sequences at the 3' and 5' extremities are retained, a BNYVV RNA 3 transcript from which the central region has been deleted can replicate efficiently on *C. quinoa* leaves when coinoculated with RNAs 1 and 2, and can express a foreign gene inserted in place of the deleted sequence (Jupin et al. 1990; Hehn et al. 1995). In this paper we have used such an RNA 3-based "replicon" to express each of the BNYVV TGB proteins out of their normal context in RNA 2 and tested the capacity of each replicon to complement an RNA 2 mutant defective in the corresponding TGB gene.

The replicons employed in this study are depicted in Figure 1C. In addition to the constructs rep42, rep13, and rep15, each of which contains a TGB gene, a fourth construct (rep1315) was produced containing both the P13 and P15 genes arranged in the same relative configuration as in RNA 2. The ability of each replicon to direct expression of the inserted gene or genes was tested by in vitro translation of the transcript in a wheat germ extract. The rep42, rep13, and rep15 transcripts each directed synthesis of an abundant product (Fig. 2A, lane 2; Fig. 2B, lanes 2 and 3), which was not produced in translations programmed with transcript corresponding to the empty replicon, rep0 (Fig. 2A, lane 1; Fig. 2B, lane 1). P13 migrated slightly more slowly than P15. The anomalous relative mobility of these two products is presumably related to their different amino acid contents. The dicistronic construct rep1315 directed synthesis of both P13 and P15 (Fig. 2B, lane 4) in relative molar amounts of 3:1 (values corrected for the differ-

Fig. 1. Structure of wild-type beet necrotic yellow vein virus (BNYVV) RNA 2 and of replicons expressing triple gene block (TGB) proteins. (A) Genome map of RNA 2. TGB genes are shaded; lines above map indicate approximate extents of subgenomic RNAs 2sub_a, 2sub_b, and 2sub_c. 5'-terminal cap structure denoted by a circle. P21 is the major viral coat protein (CP). RT = readthrough domain (Bouzoubaa et al. 1986). P14 is expressed from subgenomic RNA 2sub_c and is involved in regulation of RNA 2 expression (Hehn et al. 1995). (B) Mutations in RNA 2. Deletions indicated by lines below RNA 2 map; frameshift-inducing insertions shown above. (C) BNYVV RNA 3-derived replicons containing TGB genes of BNYVV (light shading) or TGB3 gene encoding P17 of peanut clump virus (PCV) (dark shading). *Bam*HI site in the empty replicon rep0 used for insertion of polymerase chain reaction-amplified TGB sequences is shown. Positions of frameshift-inducing insertions in various P13 and P15 mutant replicons are indicated.

Fig. 2. In vitro translation of replicons containing triple gene block (TGB) genes in wheat germ extract. (A) ³⁵S-methionine-labeled translation products of empty replicon rep0 (lane 1) and rep42 (lane 2) displayed by autoradiography following polyacrylamide gel electrophoresis (PAGE) (Laemmli 1972). Indicated band identified as P42 by comparing its mobility to that of molecular mass markers (not shown). (B) Translation products directed by rep0 (lane 1), rep13 (lane 2), rep15 (lane 3), and rep1315 (lane 4) displayed by autoradiography following PAGE (Schägger and von Jagow, 1987). Bands tentatively identified as P13 and P15 are indicated to the right. Background band denoted by an asterisk was also synthesized when no transcript was introduced into translation extract (data not shown). Irregularly oriented black "bands" in lanes 1, 2, and 4 are due to wrinkles produced during drying of gel.

ence in methionine contents of the two proteins; if the N-terminal methionine of each protein is removed post-translationally, the molar ratio is 5:1).

The capacity of the replicons to be amplified by the viral replication machinery *in vivo* was tested by coinoculating replicon transcripts to *C. quinoa* protoplasts along with BNYVV RNAs 1 and 2. Northern (RNA) blot analysis of total RNA extracted from the protoplasts 48 h post inoculation (pi) revealed that all the replicons containing the TGB genes were efficiently amplified (Fig. 3A). Western blot (immunoblot) analysis revealed that the P42 level in protoplasts infected with a mixture of rep42 plus transcripts of RNA 1 and the frameshift mutant pB2-14-H, created by filling in an *Spe*I site within the P42 gene of RNA 2 (see Fig. 1B), was about twice that in protoplasts infected with RNA 1 plus wild-type RNA 2 (Fig. 3B, lanes 2 and 4). The presence of rep42 did not block accumulation of two other immuno-detectable RNA 2 gene products, the major viral coat protein and P14 (Fig. 1A). The available antisera were not sufficiently sensitive to immuno-detect P13 and P15 in such experiments.

The BNYVV TGB proteins can be complemented in trans.

The ability of the replicons containing the TGB genes to supply movement functions in whole plants was tested by coinoculating leaves of *C. quinoa* with one of a series of RNA 2 transcripts containing a mutation disabling a TGB gene plus a replicon containing the corresponding wild-type gene. In all experiments, the inoculum also contained transcript of wild-

type RNA 1 as source of viral RNA-dependent RNA replicase, although this fact will not always be stated explicitly below. For the P42 gene, the RNA 2 mutants tested included the frameshift mutant pB2-14-H (Fig. 1B), a series of mutants containing short in-frame deletions at different sites in P42 (mutants pB2-14- Δ S12, - Δ SN, - Δ B1, - Δ B2, - Δ N, and - Δ HPI; Fig. 1B; also see Bleykasten et al. 1996), and a deletion mutant (pB2-14-F; Fig. 1B), where removal of a 935-nucleotide sequence upstream of the P42 ORF has inactivated the promoter for the subgenomic RNA (RNA 2_{sub}) responsible for P42 synthesis (data not shown). Inocula containing RNA 1 transcript plus any one of the above mutant RNA 2 transcripts did not produce local lesions on *C. quinoa* and no progeny viral RNA could be detected in the inoculated leaves 10 days pi (see Figure 4, lanes 3 and 5 for the experiments with pB2-14-F and pB2-14-H and Bleykasten et al. [1996] for similar experiments with the other mutants). When rep42 transcript was included in the inoculum, numerous local lesions (20 to 80 per leaf) appeared on the inoculated leaves in all cases except for the inoculum containing transcript of the RNA 2 deletion mutant pB2-14- Δ HPI, which remained symptomless. The resulting pale green lesions were similar in appearance to those elicited by inoculation with RNA 1 plus wild-type RNA 2 except for the RNA 2 mutant pB2-14-F, where necrotic local lesions were formed. In the latter case, the necrotic lesion phenotype may be related to production of a truncated form of the readthrough protein by this RNA 2 mutant (Schmitt et al. 1992).

Northern hybridization of the inoculated leaves 10 days pi revealed the presence of progeny viral RNAs of the length expected for RNAs 1 and 2 and rep42 for all the RNA 2 mutants (Fig. 4, lanes 2, 4, 6 to 11) except the deletion mutant pB2-14- Δ HPI, where no progeny viral RNAs or replicon could be detected (Fig. 4, lane 12). As will be shown below, the failure of pB2-14- Δ HPI to be complemented by rep42 is probably due to deletion of the promoter for the subgenomic RNA (RNA 2_{sub}), which is believed to direct translation of the downstream TGB proteins.

Similar complementation experiments were carried out with rep13, rep15, and the dicistronic construct rep1315. Both rep13 and rep1315 were able to complement accumulation on leaves (Fig. 4, lanes 14 and 15) of the mutant pB2-14-I, in which the P13 gene had been disabled by insertion of 4 nucleotides (the insertion created an *Xho*I site), although the resulting local lesions were necrotic. Necrotic local lesions were also produced during mixed infections with the aforesaid replicons and wild-type RNA 1 and 2 (see below), indicating that the replicon-related symptom phenotype is dominant over the wild type. The novel symptoms may be related to differences in the time course of synthesis and/or the level of accumulation of P13 when it is expressed from the replicon rather than full-length RNA 2.

In experiments such as those described above, it is important to demonstrate that the mutation originally introduced into the P42 or P13 gene on the RNA 2 transcript was still present in the progeny RNA 2, i.e., the defective copy of the TGB gene on the transcript had not been converted to the wild type by RNA recombination in planta (Simon and Bujarski 1994) with the copy present on the replicon. Therefore, a reverse transcription-polymerase chain reaction (RT-PCR) experiment was carried out on the progeny viral RNA from a

Fig. 3. Amplification of replicons encoding triple gene block (TGB) proteins in protoplasts and expression of P42. (A) Detection by Northern (RNA) hybridization of viral RNAs in *Chenopodium quinoa* protoplasts inoculated with beet necrotic yellow vein virus (BNYVV) RNAs 1 and 2 alone (lane 2) or supplemented with rep0 (lane 3), rep42 (lane 4), rep13 (lane 5), rep1315, (lane 6), and rep15 (lane 7). RNA from mock-inoculated protoplasts analyzed in lane 1. Protoplasts were harvested 48 h post inoculation and viral RNAs detected with 32 P-labeled viral RNA-specific antisense RNA probes. Replicons indicated by arrow heads. (B) Immunodetection of P42 in total protein extracts of *C. quinoa* protoplasts inoculated with BNYVV RNAs 1 and 2 (lane 2), transcript of wild-type RNA 1 plus transcript of the RNA 2 mutant pB2-14-H, which contains a frameshift mutation in the P42 gene (Bleykasten et al. 1996) (lane 3), and RNA 1 and pB2-14-H transcripts plus rep42 (lane 4). Protein extracted from mock-inoculated protoplasts analyzed in lane 1. After polyacrylamide gel electrophoresis (PAGE) (Laemmli 1972) and electrophoretic transfer to nitrocellulose, P42, major viral coat protein (CP), and P14 were immunodetected with a mixture of antisera specific for each protein (Niesbach-Klösgen et al. 1990). The positions of molecular mass standards labeled in kilodaltons to left of the blot.

plant infected with RNA 1, pB2-14-H transcript (P42 gene disrupted by filling in a *SpeI* site) and rep42. The primer pair used in the RT-PCR hybridized to RNA 2 sequences flanking the P42 gene and hence amplifies the copy of the gene present in RNA 2 but not the copy in the replicon, where the flanking sequences are absent. Restriction enzyme analysis revealed that the *SpeI* site was absent in the resulting amplified DNA fragment (data not shown), as expected for the mutated rather than the wild-type form of the TGB gene. Similar analysis of progeny viral RNA from plants infected with RNA 1, pB2-14-I (frameshift mutation in P13 gene creating an *XhoI* site), and either rep13 or rep1315 similarly demonstrated that the mutation disabling the copy of the P13 gene on the RNA 2 transcript was conserved in the progeny RNA 2 (data not shown). We conclude that rep42 and rep13 are indeed complementing P42 and P13 function by supplying the gene product in trans rather than simply serving as a source of the wild-type TGB sequence for recombination.

Unexpectedly, the replicon expressing the wild-type P15 gene (rep15) was unable to complement the P15-defective RNA 2 mutant pB2-14-J in mixed inoculations. No local lesions formed on the inoculated leaves 10 days pi and no viral RNA could be detected in the leaves by Northern blot (Fig. 4, lane 17). On the other hand, when pB2-14-J transcript was coinoculated with rep1315, local lesions (of the necrotic type) appeared and progeny viral RNAs were readily detected (Fig. 4, lane 18). In the latter case, analysis of an RT-PCR product containing the P15 gene in the progeny RNA 2 revealed that the mutation disabling the gene was still present (data not shown). Complementation of pB2-14-J still occurred (data not shown) when the P13 ORF in the dicistronic replicon was interrupted by a frameshift mutation (rep1315-I; Fig. 1C), establishing that expression of full-length P13 from the first

ORF of the dicistronic replicon is not required for complementation by the downstream copy of the P15 gene.

Evidence that P15 is expressed from a dicistronic subgenomic RNA.

An RNA 2-derived subgenomic RNA (RNA 2sub_b) about 1,500 nucleotides long has been detected in BNYVV-infected tissue (Gilmer et al. 1992). The 5' extremity of this species has not been mapped precisely but is predicted to lie near the 5' terminus of the P13 ORF. On the other hand, no subgenomic RNA with a 5' end upstream of the P15 ORF has been detected, suggesting that, as in BSMV (Zhou and Jackson 1996), P13 and P15 are both expressed from RNA 2sub_b.

The aforementioned inability of rep42 to complement the P42-defective RNA 2 mutant pB2-14-ΔHP1 could stem from polar effects of the deletion in RNA 2 on synthesis of downstream TGB proteins if the deletion has disabled the RNA 2sub_b promoter (the right-hand boundary of the deletion in pB2-14-ΔHP1 is only 30 residues upstream of the P13 initiation codon). To test this hypothesis, an experiment was carried out in which the pB2-14-ΔHP1 transcript was complemented with both rep42 and rep1315. Leaves inoculated with this mixture developed local lesions and contained progeny viral RNAs, including both replicons (Fig. 4, lane 20). If, on the other hand, rep13 rather than rep1315 was used along with rep42 to complement pB2-14-ΔHP1, no symptoms appeared and no progeny viral RNAs were detected by Northern blot (Fig. 4, lane 19). These observations are consistent with our hypothesis that the pB2-14-ΔHP1 deletion interferes with expression of the downstream TGB ORFs, presumably by blocking RNA 2sub_b transcription. Furthermore, the fact that complementation was successful with rep1315 but not with rep13 indicates that P15 as well as P13 is translated from RNA 2sub_b.

Fig. 4. Complementation of RNA 2 transcripts containing defects in different triple gene block (TGB) genes by corresponding wild-type gene product supplied from a replicon. Replicon indicated at top of each lane was inoculated to leaves of *Chenopodium quinoa* together with wild-type RNA 1 transcript plus either wild-type RNA 2 transcript (lane 2) or mutant RNA 2 transcript identified above each lane. Designations of various RNA 2 transcripts are abbreviated: 2-F = transcript of pB2-14-F, 2-H = transcript of pB2-14-H, etc. In lanes 19 and 20, inoculum contained rep42 and rep13 (lane 19) or rep42 and rep1315 (lane 20), in addition to RNA 1 and pB2-14-ΔHP1 transcripts. Lane 1 contains RNA from a noninoculated control plant. Inoculated leaves were harvested 10 days post inoculation and tested for viral RNA contents by Northern (RNA) hybridization as described in Figure 3. Positions of replicons are indicated by arrows.

Independent expression of P15 inhibits infection with wild-type viral RNA.

The ability of rep1315 but not rep15 to complement the P15-defective RNA 2 mutant pB2-14-J in leaf infections could indicate that independent expression of P15 from the monocistronic replicon interferes with the viral infection cycle by producing the gene product in excessive quantities relative to P13. To test this hypothesis, an experiment was carried out in which rep15 was inoculated to *C. quinoa* leaves along with wild-type viral RNAs 1 and 2. No lesions appeared on the inoculated leaves, even at late times pi (Fig. 5A), and no viral RNA could be detected by Northern blot (Fig. 5B, lane 6). Under these conditions, leaves inoculated with RNAs 1 and 2 alone were heavily infected (Fig. 5A; Fig. 5B, lane 2). The inhibition of virus infection by rep15 was dose dependent. Addition of 10 times less rep15 to the inoculum mix still resulted in almost complete inhibition of lesion formation but lesser amounts of the replicon were progressively less effective in blocking the infection (data not shown). Rep15 also blocked the appearance of progeny viral RNA in the inoculated leaves and in the roots of *Beta macrocarpa* Guss., a systemic host of BNYVV (Fig. 5C, lanes 5 and 6). The empty replicon, rep0, and replicons expressing the other two TGB proteins (rep42, rep13, and rep1315) did not significantly inhibit BNYVV infection of *C. quinoa* leaves (Fig. 5B, lanes 2 to 5).

Since rep15 did not interfere with amplification of RNAs 1 and 2 in *C. quinoa* protoplasts (see Figure 3), we conclude that the replicon interferes with spread of the viral infection from the initial locus of infection into neighboring cells during formation of visible local lesions. Lesion formation was not inhibited by coinoculation of Stras 12 RNA with the replicons rep15-J or rep15-X (Fig. 5B, lanes 7 and 8), which encode frameshift-truncated forms of P15. This finding confirms that expression of P15 from the replicon, rather than the simple presence of the corresponding RNA sequence, is required for inhibition during the mixed infection experiments. In the pres-

ence of rep15-X, however, the resulting local lesions were about one-third the diameter of the lesions formed by infection with Stras 12 alone or with Stras 12 plus rep15-J, and the content of progeny viral RNA in the inoculated leaves was significantly lower (Fig. 5B, lane 8). This finding suggests that the almost full-length P15 molecule produced by rep15-X, although incapable of substituting for the defective P15 produced by pB2-14-J in a complementation experiment (data not shown), interferes with the cell-to-cell movement activity of the wild-type P15 produced from RNA 2. Presumably, the full-length and truncated forms of P15 compete for binding sites on another component (which could be of either viral or cellular origin) involved in the movement process.

As noted above, sequence comparisons between different viruses possessing a TGB have revealed little sequence similarity between the different TGB3 genes. For example, the 17-kDa TGB3 protein (P17) of peanut clump furovirus (PCV) displays little sequence homology with P15 of BNYVV (Herzog et al. 1994) even though both viruses can infect *C. quinoa*. To determine whether independent expression of the PCV TGB3 protein can interfere with a BNYVV infection in a manner similar to that observed with rep15, a BNYVV RNA 3-derived replicon containing the PCV TGB3 (repPCV-P17; Fig. 1) was constructed. *C. quinoa* leaves inoculated with BNYVV RNAs 1 and 2 plus repPCV-P17 did not develop symptoms and no progeny BNYVV RNAs could be detected by Northern blot (Fig. 5A, lane 9). This observation suggests that the mechanisms by which BNYVV and PCV move locally in *C. quinoa* share at least one common element that interacts with the TGB3 products of both viruses, in spite of their dissimilarity in sequence.

In this paper we have shown that replicons carrying P42 and P13 can complement BNYVV RNA 2 carrying the corresponding defective gene but that a replicon carrying P15 cannot. In the latter case, complementation can occur, however, if the P15 gene is supplied as the second gene on a dicistronic RNA (rep1315) carrying the P13 gene in first position. It

Fig. 5. Effect of replicons on infection with wild-type beet necrotic yellow vein virus (BNYVV) RNAs 1 and 2. (A) Leaves of *Chenopodium quinoa* inoculated with RNAs 1 and 2 (left) or RNAs 1 and 2 plus rep15 (right). Leaves were photographed 20 days post inoculation. (B) Analysis by Northern (RNA) hybridization (as described in Figure 3) of viral RNA contents of *C. quinoa* leaves inoculated with BNYVV RNAs 1 and 2 alone (lane 1) or together with rep0 (lane 2), rep42 (lane 3), rep13 (lane 4), rep1315 (lane 5), rep15 (lane 6), rep15-J (lane 7), rep15-X (lane 8), or repPCV-P17 (lane 9). Positions of replicons indicated by arrows. (C) Analysis by Northern hybridization of viral RNA contents of inoculated leaves (lanes 1, 3, and 5) and roots (lanes 2, 4, and 6) of *Beta macrocarpa* either mock-inoculated (lanes 1 and 2), inoculated with BNYVV RNAs 1, 2, and 3 (lanes 3 and 4) or with RNAs 1, 2, and 3 plus rep15 (lanes 5 and 6). RNA 3 included in inoculum because it is necessary for systemic movement in *B. macrocarpa* (Tamada et al. 1989).

should be noted that the relative disposition of the P13 and P15 genes on rep1315 is identical to their disposition on RNA 2sub_b, the subgenomic RNA that is believed to direct synthesis of both proteins in wild-type infections. We suggest that successful localized movement of BNYVV requires the presence of P13 and P15 in appropriate relative amounts and that production of both proteins from the same subgenomic RNA represents a mechanism for coordinating their synthesis. The inability of rep15 to complement the P15 mutant RNA 2 transcript pB2-14-J and its ability to inhibit infection by wild-type virus would thus both be due to over-production of P15 relative to P13 when the former is translated from the replicon and the latter from RNA 2. When P15 is expressed from the dicistronic replicon rep1315, on the other hand, appropriate relative P13-P15 levels would be produced, allowing cell-to-cell movement to proceed.

The "correct" relative levels of accumulation of P13 and P15 in a wild-type infection are not known. Translation of rep1315 in wheat germ extract produced three to five times more P13 than P15 but such experiments do not necessarily reflect the situation in planta since the turnover rates of the two proteins may differ significantly. TGB-mediated cell-to-cell movement is apparently not sensitive to over-expression of P42 and P13 relative to the levels characteristic of a normal infection, since coinoculation of rep42, rep13, or rep1315 with wild-type virus did not inhibit infection (Fig. 5, lanes 3 to 5) although the lesions produced in the presence of rep13 and rep1315 were necrotic (data not shown). On the other hand, our results indicate that expression of P15 in transgenic plants could provide a mechanism for inducing BNYVV resistance ("pathogen-derived resistance"; Sanford and Johnston 1985) in such plants, if sufficient P15 expression levels can be attained. It is worth noting that expression of a mutant form of the TGB2 protein of white clover mosaic potexvirus in transgenic plants has been shown to confer resistance to systemic infection by other plant viruses with a TGB (Beck et al. 1994).

Gaining a better understanding of how the relative levels of P13 and P15 are regulated during translation will require learning how the P15 cistron is accessed by ribosomes on RNA 2sub_b. Translation initiation at an internal cistron of a eucaryotic messenger RNA may occur by several mechanisms, including (i) leaky scanning, where a fraction of the ribosomal subunits that begin scanning the RNA at the 5' end move past the first (usually non-optimal) upstream AUG without initiating (Kozak 1989) and (ii) internal entry, where ribosomal subunits bind directly to a special sequence on the RNA near the internal initiation codon (Pelletier and Sonnenberg 1988). A third possible mechanism, termination-reinitiation (Kozak 1989), appears unlikely to apply to the TGB-containing viruses because the overlap between the TGB2 and TGB3 cistrons would require ribosomes to scan backwards, sometimes for a considerable distance, after terminating TGB2 to reach the TGB3 initiation codon. It has been suggested that the TGB3 proteins of BSMV and PVX are translated by a leaky scanning mechanism (Morozov et al. 1991; Zhou and Jackson 1996). The BNYVV P15 gene may also be produced by leaky scanning although it should be noted that the context of the BNYVV P13 initiation codon (AUAAUGU) is nearly optimal and that there are two AUGs downstream of the P13 initiation codon that scanning subunits would have to ignore to reach the P15 initiation codon. Addi-

tional experiments are evidently in order to determine the mode of expression of P15 in vivo and the replicon-based system described in this paper will be a useful tool in carrying out such experiments.

MATERIALS AND METHODS

cDNA clones.

The transcription vectors for production of wild-type full-length BNYVV RNAs 1 and 2 were pB15 (Quillet et al. 1989) and pB2-14 (Bleykasten et al. 1996), respectively. Transcription vectors (Fig. 1B) for previously described RNA 2 mutants were pB2-14-F, -H, -I, and -J (Gilmer et al. 1992) and pB2-14-ΔSN, -ΔS12, -ΔB1, -ΔB2, and -ΔN (Bleykasten et al. 1996). The RNA 2 deletion mutant pB2-14-ΔHP1 was produced by elimination of the sequence between nucleotides 3158 and 3258. The empty BNYVV RNA 3-derived replicon, rep0 (Fig. 1C), is identical to the previously described RNA 3 deletion mutant pB35AΔES (Jupin et al. 1990). TGB sequences for insertion into rep0 were amplified by PCR with primers that each contained a nontemplated *Bam*HI site at their 5' extremity. PCR fragments corresponding to the P42 gene (nucleotides 2127 to 3297), the P13 gene (nucleotides 3282 to 3650), the P15 gene (nucleotides 3623 to 4027), and both the P13 and P15 genes (nucleotides 3282 to 4027) were digested with *Bam*HI and inserted into *Bam*HI-cleaved rep0. In all cases, transcription of viral plus-sense RNA 1-, 2-, and 3-based constructs was under control of the bacteriophage T7 RNA polymerase promoter. The structures of the RNA 3-based replicons rep42, rep13, rep15, and rep1315 for independent expression of the TGB genes are shown in Figure 1C. A replicon containing a frameshift mutation in the P15 ORF (Rep15-X) was produced by filling in the overhangs of an insert *Xba*I site (nucleotide 3948). The insert frameshift mutations in rep13-I, rep1315-I, and rep15-J were created as described for the corresponding mutations in full-length RNA 2 (Gilmer et al. 1992). Cloned PCR-amplified sequences were verified to be error-free by sequencing (Sanger et al. 1977).

Synthesis and in vitro translation of transcripts and their inoculation to plants and protoplasts.

Capped transcripts were prepared by bacteriophage T7 polymerase run-off transcription (Quillet et al. 1989) of plasmid DNA linearized by *Hind*III for pB15 and the replicon constructs and by *Sal*I for pB2-14 and related constructs. Transcript concentration and integrity were evaluated by agarose gel electrophoresis. Leaves were mechanically inoculated with 50 µl per leaf of inoculation buffer containing 1 µg of each transcript (Gilmer et al. 1992). In some experiments, the RNA 1 and 2 transcripts were replaced by 0.025 µg of the highly infectious viral RNA purified from BNYVV isolate Stras 12 (Quillet et al. 1989). Preliminary experiments showed that this amount of viral RNA was approximately equivalent in infectivity (as measured by a local lesion assay) to a mixture containing 1 µg each of the RNA 1 and 2 transcripts. For protoplast infections, 0.5 µg of viral RNAs 1 and 2 plus 3 µg of replicon transcript were inoculated to 2×10^5 protoplasts by electroporation (Gilmer et al. 1992).

Transcripts obtained from replicons were translated in a wheat germ extract (Fritsch et al. 1980) and the ³⁵S-labeled translation products were visualized by autoradiography after

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% (Laemmli 1972) or 12.5% (Schägger and von Jagow 1987) polyacrylamide gels. Radioactivity incorporated into translation products was quantified with a Fujix MAS1000 BioAnalyzer and the values were adjusted for methionine content in calculating relative translation levels.

Detection of viral RNA and proteins.

Total RNA was extracted (Gilmer et al. 1992) from inoculated leaves 10 days pi and from protoplasts 48 h pi. Viral RNA was detected by Northern hybridization with ³²P-labeled antisense viral RNA transcripts (Lemaire et al. 1988) as probes. The RNA 1–specific probe was complementary to nucleotides 4740 to 5650, the RNA 2–specific probe to nucleotides 2324 to 3789 and the RNA 3–specific probe to nucleotides 1 to 380. The stability of mutations introduced into RNA 2 was tested by RT-PCR of total RNA extracts from infected plants. Reverse transcripts were produced with an Expand reverse transcription kit (Boehringer, Mannheim, Germany) following the supplier's instructions. PCR was carried out essentially as described (Brault et al. 1995) with 25 cycles of the following regimen: 94°C (30 s), 50°C (30 s), 72°C (3 min). Primer pairs for PCR amplification of different regions of the RNA 2 cDNA corresponded to (or were complementary to, in the case of the second member of each pair of primers) nucleotides 1143 to 1151 and 3393 to 3412 (P42 gene), and nucleotides 3151 to 3169 and 4128 to 4148 (P13 and P15 genes). The primer used to initiate cDNA synthesis prior to the PCR reactions was complementary to nucleotides 4128 to 4148.

P42, P14, and coat protein were detected by Western blot of total protein extracts of infected protoplasts with a rabbit polyclonal antiserum specific for each protein (Niesbach-Klöggen et al. 1990). Immunoreactive bands were visualized by the alkaline phosphatase method as described (Niesbach-Klöggen et al. 1990).

NOTE ADDED IN PROOF

In repPCV-P17, a previously undetected PCR error introduced a point mutation altering the wild-type P17 sequence from isoleucine to valine at amino acid 6.

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