Effects of inactivation of the coat protein and movement genes of *Tomato bushy stunt virus* on early accumulation of genomic and subgenomic RNAs

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Accumulation of RNA of *Tomato bushy stunt virus* (TBSV) was examined within the first few hours after infection of *Nicotiana benthamiana* protoplasts to determine the influence of the coat protein (CP), the movement-associated proteins P22 and P19 and RNA sequences at very early stages of replication. The results showed that P19 had no effect on early RNA replication, whereas the absence of CP and/or P22 expression delayed RNA accumulation only marginally. Removal of CP-coding sequences had no added negative effects, but when the deletion extended into the downstream p22 gene, it not only eliminated synthesis of subgenomic RNA2 but also delayed accumulation of genomic RNA by 10 h. At times beyond 20 h post-transfection, RNA accumulated to normal high levels for all mutants. This illustrates that TBSV RNA sequences that have negligible impact on overall RNA levels observed late in infection can actually have pronounced effects at very early stages.

Introduction

The precise biochemical mechanisms of replication and transcription of plus-sense RNA plant viruses remain to be defined, but much has been learned in recent years (Buck, 1996; Miller & Koev, 2000). In particular, our knowledge concerning viral RNA elements and proteins involved in replication is becoming increasingly detailed (Duggal *et al*., 1994; Russo *et al*., 1994; Buck, 1996, 1999; Sit *et al*., 1998; Kim & Hemenway, 1999; Nagy *et al*., 1999; Neeleman & Bol, 1999; Zhang *et al*., 1999; Koev & Miller, 2000; Lewandowski & Dawson, 2000; Qiu & Scholthof, 2000; Qiu *et al*., 2001). For example, for *Tomato bushy stunt virus* (TBSV), it is known that the RNA genome encodes two 5'–proximal replicase-associated proteins (P33 and P92) that are translated directly from the positive-sense genomic RNA (gRNA). These two proteins are required for replication and transcription (Scholthof *et al*., 1995). Cis-acting RNA elements on the TBSV genome are involved in these processes via effects on translation of replicase proteins (Scholthof & Jackson, 1997; Wu & White, 1999), replication (Chang *et al*., 1995; Wu & White, 1998), transcription (Zhang *et al*., 1999) or stimulation of cis-preferential replication (Qiu *et al*., 2001). Tombusvirus RNA replication is associated with multivesicular bodies (Rubino & Russo, 1998) and occurs independently of the coat protein (CP), which is translated from subgenomic RNA1 (sgRNA1). Furthermore, RNA accumulation does not require expression of the nested 3'–proximal p22 and p19 genes, which are translated from sgRNA2 (Scholthof *et al*., 1995) (Fig. 1). Instead, the products of those tombusvirus genes contribute independently to pathogenicity (Russo *et al*., 1994). The TBSV p22 and p19 gene products (P22 and P19), or their equivalents in other *Tombusvirus* species, are involved in cell-to-cell movement and host-specific systemic spread, respectively, and P19 also serves as a major symptom determinant (Rochon, 1991; Dalmay *et al*., 1993; Scholthof *et al*., 1993a, c). Although the CP is not essential in some hosts, it presumably augments long-distance vascular transport of tombusviruses and symptom development (Dalmay *et al*., 1992; McLean *et al*., 1993; Scholthof *et al*., 1993). As exemplified by recent reports (Herzog *et al*., 1998; Olsthoorn *et al*., 1999; Tsai *et al*., 1999; Chu *et al*., 2000; Scheets, 2000; Skaf *et al*., 2000; Turina *et al*., 2000; Yeh *et al*., 2000), the requirements for replication of plant RNA viruses are generally evaluated by using protoplasts to analyse RNA accumulation at 12–48 h post-transfection (p.t.) or beyond. However, some...
viruses, such as members of the genera *Potexvirus* and *Tobamovirus*, replicate with such efficiency that RNA accumulation can be detected at or before 8 h.p.t. (Ishikawa et al., 1991; Kim & Hemenway, 1997; Lewandowski & Dawson, 2000) and tombusvirus RNA accumulates to readily detectable levels at 4–8 h.p.t. (Tavazza et al., 1994; Johnston & Rochon, 1995; Zhang et al., 1999). In fact, our preliminary data (Qiu et al., 1999) illustrated that newly synthesized TBSV RNA was detectable as early as 2.5 h.p.t. These developments imply that, perhaps for many viruses and in particular for TBSV, critical replication- and transcription-associated events may occur considerably earlier than 12 h.p.t. Therefore, contributions of viral proteins or cis-acting RNA sequences to early RNA accumulation may have gone unnoticed in previous evaluations that did not include very early time-points. This consideration alone justified a re-examination of the requirements for TBSV replication, but such a study seemed particularly worthwhile in view of the following aspects: (i) the implication that P19 might somehow be involved in replication (Rochon, 1991); (ii) the possible effects of P19 on RNA accumulation via suppression of post-transcriptional gene silencing (PTGS) (Voinnet et al., 1999; Qiu & Scholthof, 2000a); (iii) the ongoing identification of multiple cis-acting elements on the TBSV genome with previously unknown activities (Scholthof & Jackson, 1997; Wu & White, 1999; Zhang et al., 1999) and (iv) the RNA-binding properties of CP and P22 (B. Desvoyes and H. B. Scholthof, unpublished) that could protect RNA at early stages.

The objective of the present study was to examine and distinguish the effects of TBSV CP, P19 and P22 and the corresponding RNA sequences on viral RNA accumulation at very early stages in the infection cycle. The results showed that TBSV RNA accumulation was detectable at 2–2.5 h.p.t., and this early accumulation was not affected by P19. RNA accumulation was delayed only marginally (1–2 h) by the absence of CP and/or P22. On the other hand, genomic RNA accumulation was delayed substantially by a deletion that removed an RNA segment bridging these two genes. This deletion also abolished transcription of sgRNA2 and affected the relative timing of sgRNA1 accumulation.

### Methods

Standard molecular biology protocols were followed for the isolation and manipulation of plasmid DNA (Sambrook et al., 1989). Bioassays on plants and protoplasts, immuno-detection assays, RNA hybridization and *in vitro* translation assays were performed as described previously (Scholthof et al., 1993, 1995a, b, c, 1999). For the *in vivo* RNA accumulation tests, about 4 × 10⁶ *Nicotiana benthamiana* protoplasts were used per transfection. Methods described previously (Scholthof et al., 1995c) were used to monitor RNA accumulation using hybridization probes that were generated by using random primers with plasmid pBW1. This plasmid contains an insert spanning the 890 nt 3′-terminal portion that is present in equimolar amounts on TBSV gRNA and each of its two sgRNAs, which ensures that the observed hybridization signal intensity is a direct reflection of the relative amount of each viral RNA species.

A schematic representation of the infectious cDNA clone of TBSV and its derived mutants is provided in Fig. 1. Some of the mutants have been described previously (Scholthof et al., 1993, 1995c), but the salient points will be discussed briefly. Transcripts from pHS7 do not express CP due to a small deletion from the *Ncol* site (nt 2724) to the *BalI* site (nt 2773) in the 5′-proximal region, resulting in a frame-shift. The translation of p22 is terminated prematurely in pHS136 by replacement of the codon for amino acid 67 with a stop codon, whereas, in pHS157, a stop codon is introduced at amino acid position 52 to prevent expression of p19. Immuno-blot assays and infectivity studies in protoplasts and plants confirmed that each of the aforementioned mutations abolished expression of the target protein(s) without impeding expression of other non-target proteins, and these mutations are maintained stably during infection (Scholthof et al., 1995a, c). Removal of the *Ncol*–*SacI* fragment (nt 2724–3379 of the TBSV sequence) from pHS45 (Scholthof et al., 1993) followed by incubation with DNA polymerase I Klenow fragment and self-ligation removed most of the CP ORF, to yield pBD-1. The deletion from nt 2724 to 3886 in pTD (Scholthof et al., 1995c) covers most of the CP gene, the core promoter for transcription of sgRNA2 (Johnston & Rochon, 1995) and the extreme 5′-terminal portion of the p22 gene (Fig. 1).
Results

Early detection of TBSV RNA accumulation

Transcripts of the mutants were produced in similar amounts to wild-type RNA (not shown) and all served equally well as templates for in vitro translation of P33 (Fig. 2). These analyses confirmed that the amounts of translationally competent transcripts were comparable in subsequent RNA accumulation assays. The in vitro-generated transcripts were used for transfection of N. benthamiana protoplasts and total RNAs were extracted at different times p.t. Agarose-gel electrophoresis of these RNAs was performed under non-denaturing conditions. Consequently, the hybridization signals (Figs 3 and 4) reflect predominantly the levels of plus-sense viral RNAs (Russo et al., 1994). In our experience, minus-sense gRNA is only readily detectable through its association with plus-sense RNA as double-stranded RNA (Scholthof et al., 1993). Furthermore, the patterns of accumulation of tombus-virus plus-sense and minus-sense RNA are essentially identical over time (Tavazza et al., 1994; Molinari et al., 1998) and, in fact, plus-strand RNA could be detected a few hours earlier than minus-strand. This suggested that detection of plus-strand RNA is the most informative approach to monitor very early events. Furthermore, our experiments with minus-strand-specific probes have not provided usable results reliably (not shown), which may be related to a low non-specific background hybridization of minus-strand-specific probes with plus-sense RNA (Tavazza et al., 1994). This was anticipated to be especially problematic for specific detection of small amounts of minus-strand RNA early in infection. In view of these considerations, we deliberately designed our experiments to monitor the increase in total gRNA, while using the detection of sgRNA accumulation as a second reliable indicator of RNA accumulation.

Accumulation of wild-type TBSV RNA was detected as early as 2 h to 2 h 20 min p.t. of N. benthamiana protoplasts (Fig. 3A). The positive signals were not due to input RNAs, which were generally degraded rapidly. Furthermore, the appearance of sgRNA2 occurred concurrently with gRNA (Fig. 3A), confirming that RNA synthesis had occurred. Subsequent sampling at 4–6 h p.t. at intervals of 40 min showed a further increase in RNA levels (Fig. 3A). These results were reproducible and illustrate that readily detectable TBSV RNA replication occurred in protoplasts between 2 and 6 h p.t. Therefore, this period constituted a good time-frame for analysis of other mutants.

Effects of CP, P22 and P19

The potential contribution of CP, P22 and P19 to early RNA accumulation was investigated in protoplasts transfected with transcripts from pHS7(ACP), pHS136(AP22) or pHS157(AP19) (Fig. 1). The results in Fig. 3(B-D) confirm previous reports (Scholthof et al., 1993, 1995c) that, at later stages of infection (21–22 h p.t.), elimination of CP, P22 or P19 expression had no pronounced negative effect on the overall high level of RNA accumulation. The levels of RNA accumulation for wild-type and mutants were also judged to be similar, based on the intensity of the gRNA bands on ethidium bromide-stained gels (not shown).

Within the first 2–4 h p.t., RNA of pHS157(AP19) accumulated reproducibly at least as efficiently as wild-type RNA (Fig. 3B; and data not shown). On the other hand, RNA accumulation for pHS136(AP22) and pHS7(ACP) was very low at the early time-points (Figs 3C, D; left panels). RNA accumulation for these two mutants was therefore also monitored between 4 and 6 h p.t. (Fig. 3C, D; right panels). These comparisons showed that the accumulation of pHS136(ΔP22) RNA was delayed by about 1–2 h compared with wild-type or pHS157(AP19).

As observed for pHS136(ΔP22), the accumulation of detectable levels of pHS7(ΔCP) gRNA and sgRNA2 was somewhat delayed (Fig. 3D). However, we have found recently that RNA from pHS7(ΔCP) is rearranged in N. benthamiana plants to generate a recombinant genome that has repaired the CP ORF to produce a truncated CP (Desvoyes et al., 2000). In order to eliminate any potential effects associated with a truncated CP, experiments were performed with pBD-1, in which most of the CP ORF is deleted (Fig. 1). The results shown in Fig. 4(A) show that pBD-1 RNA accumulation was detectable between 2 h 40 min and 3 h 20 min p.t., but the accumulation of sgRNA2 was not detectable until 4 h p.t. In
Fig. 3. TBSV RNA accumulation at early stages in *N. benthamiana* protoplasts. Total RNAs were extracted from protoplasts starting at 1 h 40 min (1:40) after transfection with wild-type TBSV RNA, followed by sampling at 20 (left) or 40 (right) min intervals (A). Accumulation of the mutants was evaluated in protoplasts transfected with RNA from pHS157(ΔP19) (B), pHS136(ΔP22) (C) or pHS7(ΔCP) (D). The positions of TBSV gRNA, sgRNA1 and sgRNA2 are indicated to the right of the autoradiograph in (A) only, but their relative positions are similar in (B)–(D).

In order to eliminate the possibility that CP and P22 could substitute for one another (e.g. for RNA protection) and therefore contribute indirectly to efficient RNA accumulation during early stages even if one of the two proteins was absent, a set of additional double or triple mutants was generated. These mutants were defective for expression of both CP and P22 or CP and P19 or all three proteins, and replication assays with these mutants yielded results (not shown) that were essentially similar to those shown in Fig. 3 for the individual P22 and CP mutants.

Collectively, the results showed that measurable early TBSV RNA accumulation still occurs in the absence of CP, P22 or P19. Although the elimination of P19 expression had no significant effect at any stage, the detectable accumulation of mutants inactive for expression of CP and/or P22 was delayed slightly (1–2 h).
Early TBSV RNA accumulation

**Fig. 4.** Effects of deletions in pBD-1 (A) and pTD (B) on early RNA accumulation. As expected, pBD-1 sgRNA1 migrated in close proximity to sgRNA2 due to the deletion. The origin of the RNA species indicated by the asterisk is unknown, but may reflect the appearance of a cryptic sgRNA or defective RNA. Symbols and markings are the same as in previous figures.

**Effect of deletions that bridge RNA sequences of the CP and p22 genes**

In a separate study, the entire p19 gene has been subjected to an extensive deletion mutagenesis analysis to determine the effect of internal *cis*-acting regions on RNA accumulation (Park et al., 2000; J.-W. Park and H. B. Scholthof, unpublished results). The present study is focused on RNA regions within the CP gene and p22, upstream of p19 (Fig. 1). The results shown in Fig. 4 confirm that CP RNA and the 5′ end of p22 are dispensable for overall high levels of RNA accumulation (Scholthof et al., 1995c). Compared with results obtained with pHS7(ΔCP) (Fig. 3D), which retained most of the CP RNA sequences, removal of virtually the entire CP ORF in pBD-1 failed to exert a pronounced additional effect on early RNA accumulation (Fig. 4A). These findings illustrate that RNA sequences within the CP ORF upstream of nt 3379 (the 3′ site of the deletion in pBD-1; Fig. 1) do not contribute measurably to early processes governing the rate of TBSV RNA accumulation.

The same CP sequences that were deleted in pBD-1 were also removed from pTD but, in the latter mutant, the deletion extends into the 5′ end of the p22 gene, encompassing the core promoter for sgRNA2 (Fig. 1). The high level of RNA accumulation obtained with this mutant at 20 h p.t. (Fig. 4A) confirmed an earlier report (Scholthof et al., 1995c) that the aforementioned deletion does not noticeably affect the overall levels of RNA that are ultimately produced. However, a pronounced, unexpected difference became apparent at earlier times when RNA accumulation was compared between pBD-1 and pTD (Fig. 4). Not only did the deletion in pTD result in the predicted elimination of sgRNA2 synthesis, but the accumulation of pTD gRNA was not detectable until approximately 13 h p.t. (Fig. 4B). This effect was consistent in multiple experiments. Therefore, accumulation of pTD RNA is delayed by about 10 h compared with wild-type RNA, but at times beyond 20 h p.t. the levels are back to normal.

In addition to the negative effect produced by the deletion in pTD on early gRNA accumulation (and the abolition of sgRNA2 synthesis), an effect was also noticed on the relative timing of sgRNA1 accumulation. In the experiments with wild-type RNA, the specific ORF-inactivation mutants and pBD-1, sgRNA1 was not detectable until at least several hours after the appearance of gRNA (Figs 3 and 4A). However, instead of this normally delayed appearance of sgRNA1 compared with gRNA, the truncated sgRNA1 produced by pTD RNA appeared concurrently with its gRNA (Fig. 4B). Combined with the results described in the previous section, it can be concluded that the effects provoked by the deletion from nt 3379 to 3886 in pTD are not protein-mediated but instead are caused by the removal of RNA sequences. This deletion has a negative impact on the early rate of RNA accumulation while shifting the relative timing of sgRNA1 to occur concurrently with gRNA accumulation.

**Discussion**

This study showed that TBSV RNA accumulation occurs very rapidly (2–2.5 h p.t.) in *N. benthamiana* protoplasts. RNA accumulation of TBSV or other members of the genus *Tombusvirus* could be detected at 4–8 h p.t. of cucumber or...
artichoke protoplasts (Tavazza et al., 1994; Johnston & Rochon, 1995; Zhang et al., 1999). While this suggests that TBSV RNA accumulation is most rapid in *N. benthamiana*, viral RNA ultimately reaches high levels in other hosts as well (Scholthof et al., 1993, 1995c). Furthermore, the levels and ratios of gRNA, sgRNA1 and sgRNA2 observed in this study at late stages in protoplasts are similar to those observed during daily monitoring of RNA accumulation in inoculated pepper and *N. benthamiana* leaves (unpublished results).

Previously, it was shown that the absence of P19 did not affect the level of TBSV accumulation in *N. benthamiana* protoplasts at about 16–20 h p.t. (Scholthof et al., 1995c; Chu et al., 2000). The present study reveals that elimination of P19 expression has no effect on very early events during RNA amplification in *N. benthamiana* protoplasts. Furthermore, time-course studies extending to 24 h p.t. of cucumber or *N. benthamiana* protoplasts also failed to discern any effect of P19 on RNA accumulation (Zhang et al., 1999; J.-W. Park and H. B. Scholthof, unpublished results). Therefore, these results show collectively that P19 does not affect the levels of RNA accumulation in cucumber or *N. benthamiana* protoplasts, a result that is also in agreement with time-course studies on inoculated spinach leaves (Scholthof et al., 1999; Chu et al., 2000).

The results discussed above have at least two important implications. Firstly, they suggest that the reported effect of P19 inactivation on the accumulation of defective interfering RNAs in *N. benthamiana* plants (Rochon, 1991; Rezende et al., 1998) is probably not caused by direct effects of P19 on replication of gRNA. Nevertheless, the appearance of a relatively abundant RNA species, migrating between sgRNA1 and sgRNA2 for pHS157 (AP19) (Fig. 3B), could support the notion that, in the absence of P19, the accumulation of defective RNAs is accelerated for unknown reasons (Rochon, 1991). Secondly, the results also illustrate that the observed P19-mediated suppression of PTGS in *N. benthamiana* (Voinnet et al., 1999; Qiu & Scholthof, 2000a) is not related to a direct effect of P19 on viral RNA accumulation in protoplasts. Therefore, the suppressor activity associated with P19 is probably different from that of HC-Pro of potyviruses (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau & Carrington, 1998; Voinnet et al., 1999; Qiu & Scholthof, 2000a), which is reported to affect amplification of viral RNA in protoplasts (Kasschau et al., 1997).

The results confirm the conclusions of previous qualitative studies that TBSV CP and P22 (or their analogues for other members of the genus *Tombusvirus*) are dispensable in order to obtain high final levels of RNA accumulation (Dalmay et al., 1993; McLean et al., 1993; Scholthof et al., 1993). However, at early stages, CP and P22 may each contribute slightly to enhance RNA accumulation within the first 6 h p.t. Whether this effect is biologically significant or an indirect consequence of formation of a protective ribonucleoprotein complex is not known.

The RNA accumulation results obtained with mutant pTD (Figs 1 and 4B) revealed a negative effect when the deletion encompassed nt 3379 to 3886, a region that spans most of the CP ORF and the 5’-proximal portion of the p22 gene. However, after sufficient time had passed, overall RNA levels were as high as those produced by wild-type TBSV. We did not determine the precise nucleotides that were responsible for the effects observed with pTD. Based on published results, the active sequence component may consist of several elements, i.e. a combination of the 3’ end of the CP gene, the core promoter or the 5’ end of p22 (Scholthof et al., 1993, 1995c; Johnston & Rochon, 1995; Zhang et al., 1999). The combined deletion of these elements in pTD perhaps exerts a negative impact early in infection by disturbing the formation of essential RNA–RNA or RNA–protein interactions or by generally inducing the formation of RNA structures that are unfavourable for replication. Whether any of these effects observed in protoplasts relates to the proposed avirulence properties of certain tombusvirus CP RNA sequences (Szittya & Burgyan, 2001) remains to be determined.

The RNA accumulation results obtained with the wild-type and mutant transcripts show that sgRNA2 can generally be detected prior to sgRNA1 (Figs 3 and 4A). This agrees with previous observations, that sgRNA2 accumulates to higher levels than sgRNA1 in *N. benthamiana* protoplasts and plants (Scholthof et al., 1995c, 1999; Chu et al., 2000). This was also shown for *Artichoke mottle virus* in artichoke and *N. benthamiana* protoplasts (Tavazza et al., 1994; Molinari et al., 1998) and two other tombusviruses in *N. benthamiana* and *Datura stramonium* plants (Szittya & Burgyan, 2001). The same general trend occurs for TBSV in spinach, *Nicotiana clevelandii*, *Chenopodium quinoa* and *Nicotiana tabacum* (Scholthof et al., 1995c, 1999; Scholthof & Jackson, 1997; Chu et al., 2000; J. W. Park and H. B. Scholthof, unpublished results). In cucumber protoplasts, sgRNA2 can be detected earlier than sgRNA1 but, eventually, both appear to accumulate to similar levels (Scholthof et al., 1993; Johnston & Rochon, 1995; Zhang et al., 1999). Thus, the combined data on sgRNA1 and sgRNA2 accumulation suggest that transcription is regulated temporally and co-ordinately. The observation that sgRNA2 accumulates earlier than sgRNA1 in most host species is consistent with the hypothesis that the two movement genes expressed from sgRNA2 (p22 and p19) represent ‘early’ genes, whereas the CP, translated from sgRNA1, is a ‘late’ gene. In this study, sgRNA2 normally appeared concurrently with gRNA and, as discussed above, sgRNA1 appeared a few hours later. However, in addition to the overall slow rate of accumulation of pTD RNA, its sgRNA1 accumulated simultaneously with gRNA (Fig. 4B). This implies that, in the absence of sgRNA2 transcription, sgRNA1 production mimics the behaviour otherwise associated with sgRNA2. It could be that the deletion in pTD reveals positional effects on synthesis of the 3’-co-terminal sgRNAs, which were previously proposed to contribute to transcriptional control in other
positive-sense RNA viruses (French & Ahlquist, 1988; Boccard & Baulcombe, 1993; Culver et al., 1993; Wang & Simon, 1997). However, the mutant pBD-1, in which the core promoter for sgRNA1 is also positioned substantially closer to the 3’ end, shows a profile of sgRNA accumulation that is comparable to that of the wild-type (Fig. 4A). The removal of the sgRNA2 promoter from pTD can perhaps enhance the binding of transcription complex to the sgRNA1 promoter. In this context, it is possible that the deletion caused a shift in intermolecular RNA interactions involving distal elements on the viral RNA that are required for transcription (Zhang et al., 1999). However, other factors probably also play a role, based on the unpredictable effects of CP deletions on the ratio of sgRNA1 to sgRNA2 (Szittya & Burgyan, 2001).

From a general viewpoint, our findings illustrate the resilience of the TBSV genome. Even though one can argue that TBSV replication is a very streamlined process, to which every nucleotide contributes, the virus appears to possess an inherent structural elasticity that allows it to adapt to changes in genomic composition. This feature provides a capacity to maintain high levels of overall RNA accumulation even when initial replication rates are diminished substantially. Our results also illustrate the importance of incorporating studies on early RNA accumulation events when investigating virus replication and transcription. Such an approach might reveal hidden influences of proteins or RNA elements that remain unnoticed when amplification has passed the exponential phase.

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