Selective recovery of foreign gene transcripts as virus-like particles in TMV-infected transgenic tobaccos

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ABSTRACT

A short origin-of-assembly sequence (OAS) located in the 30kDa movement protein gene, about 1.0kb from the 3'-end of the common strain of tobacco mosaic virus (TMV) RNA, nucleates encapsidation of the 6395-nucleotide-long genome by TMV coat protein in vitro, and presumably also in vivo. Single-stranded RNAs containing a foreign reporter gene sequence and the TMV OAS at their 5'- and 3'-ends, respectively, can be synthesized in vitro from recombinant SP6-transcription plasmids and will assemble spontaneously in vitro to form TMV-like 'pseudovirus' particles. In this paper, we show that foreign gene transcripts derived from the nuclear DNA of plants transformed by *Agrobacterium tumefaciens*, and which contain the TMV OAS, can be assembled into stable 'pseudovirus' particles in vivo during a systemic infection by TMV (helper). This is the first report of structural complementation between a heritable function bestowed on a transgenic plant and an infecting virus. As a route to protect, accumulate and recover a specific mRNA in vivo, in transgenic plant cells, this novel approach may find wider applications in developmental plant molecular biology.

INTRODUCTION

Despite extensive studies on the self-assembly mechanism of TMV in vitro (reviewed in [1,2]), little is known about the sub-cellular site or equivalent mechanism of virus assembly in vivo. We have shown [3,4] that a short (0.44kb), 3'-proximal OAS derived from TMV RNA [5,6] will initiate complete encapsidation of foreign single-stranded RNA molecules (but not ssDNA [7]) into TMV-like 'pseudovirus' particles in vitro. Included in these studies were SP6 transcripts [8] of reporter genes encoding chloramphenicol acetyltransferase (CAT [4]), lysozyme [3] and neomycin phosphotransferase [9]. The resulting 'pseudovirus' particles have been shown to disassemble and express their 'pseudo-genomes', transiently, in a wide variety of plant and

animal cell types [4,10].

To extend this work and examine the accessibility of the (presumed) cytoplasmic site of TMV assembly in vivo to foreign reporter gene transcripts, we introduced CAT gene constructs, with or without a 3'-OAS cDNA region, into tobacco DNA via the A. tumefaciens binary vector pROK2 (Fig. 1). We also constructed transgenic plants expressing a chimaeric gene encoding the 30kDa movement protein (MP) of TMV. In TMV common strain, the coding sequence of MP contains the OAS. Control transgenic plants were constructed which lacked the OAS. The protein component of the TMV assembly machinery was supplied by establishing systemic (helper) virus infections with TMV (common strain). If the mechanism of TMV assembly in vivo is analogous to that in vitro, and if the site of viral RNA encapsidation is accessible to RNAs derived from nuclear genes, then we anticipated that among the TMV progeny should be significant numbers of 'pseudo-virus' particles containing reporter sequences engineered into the host plant chromosome. Apart from its obvious implications for plant molecular virology, the ability to protect, accumulate and recover a host mRNA selectively in vivo, by exploiting the self-assembly machinery of TMV (or any other virus), could be a useful tool for those engaged in studies on the homeostatic or developmental aspects of plant molecular biology.

MATERIALS AND METHODS

Plasmids, enzymes and media

The A. tumefaciens binary vector pROK2, containing the strong constitutive 35S promoter from cauliflower mosaic virus (CaMV; Fig. 1), was a generous gift from M.W. Bevan (AFRC IPSR, Cambridge). pROK2 is a derivative of the binary vector pBIN19 [11]. Plasmids pJII102 and pJII2 have been described [12], and were used to construct pROK2 derivatives containing the CAT gene, with or without a 5'-proximal derivative (Ω') of the TMV RNA leader sequence (Ω) [12], respectively. A third class of pROK2 construct contained the Ω' -CAT-OAS sequence. The coordinates of the 0.44kb OAS region have been published elsewhere [3]. The intermediate plasmid pMON316 and the chimaeric TMV-MP gene have been described [13]. The chimaeric gene included the

Fig. 1. Schematic diagram of the binary vector pROK2 containing the Ω' -CAT-OAS construct. NT is the termination/polyadenylation sequence from the nopaline synthase gene. The nucleotide sequence from the CaMV 35S promoter to the start of Ω' reflects the origin of pROK2 (M.W. Bevan, personal communication), and insertion of a (Klenow) blunt-ended HindIII/BglII fragment of pJII102 into the infilled BamHI site of pROK2. Restriction sites shown in brackets are no longer functional.

CaMV 35S promoter, cDNA to nucleotides 4855-5868 of TMV RNA and the termination/polyadenylation sequence of the nopaline synthase gene (NT). All enzymes and media used were as before [12, 14].

Plant transformation and virus infection

Transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) which contained either the pROK2 CAT, Ω' -CAT or Ω' -CAT-OAS construct, or the TMV-MP gene (tobacco line 277 [13]), and the transgenic control line 306 (which does not express the MP gene [13]) were prepared by the leaf-disc method [14]. All the CAT-containing plants were vegetatively propagated for these experiments.

After preliminary screening of transgenic and control plants by Southern [15] and Northern [16] blotting, and CAT assays (see below), leaves were dusted lightly with an abrasive (CarborundumTM) and mechanically inoculated with a suspension of TMV (common or U1 strain). Transgenic plant lines 277 and 306 (control) have been screened and described elsewhere [13]. On these plants, only the two youngest expanded leaves were in-

oculated with a 1µg/ml suspension of TMV. Plants in each of the CAT-transgenic lines were heavily inoculated with TMV at 100µg/ml on all leaves except the youngest crown. In all cases, systemic infection by TMV was established well within the 2-3 weeks before progeny particles were harvested.

Nucleic acid hybridization-blotting procedures

Southern hybridization analyses [15] were performed on 10µg total genomic DNA from each CAT-transgenic line of tobacco. DNAs were first digested with SalI to produce a 0.78kbp fragment containing the CAT gene sequence. A ³²P-labelled probe was prepared from the cloned CAT gene fragment and used for hybridization as described [15,17]. Total genomic DNA was isolated by a modified version of a published procedure [18]. Five grams of frozen leaf tissue were homogenized in 8ml 5M guanidinium thiocyanate, 50mM Tris-HCl, pH 7.5, containing 50mM EDTA (sodium salt). After adding 80µl 20% (w/v) sodium dodecyl sulphate and 80µl 10% N-laur-oyl sarcosine, the homogenate was heated to 50°C for 5 min and centrifuged for 15 min at 15,000 rpm in the SS34 rotor of a Sorvall RC5 refrigerated centrifuge at 4°C. The supernatant was loaded onto 8ml 5.7M caesium chloride in 50mM sodium EDTA, pH 7.0, and centrifuged for 18hr at 35,000 rpm in a Beckman SW40 rotor at 4°C. DNA was extracted from the guanidinium thiocyanate-caesium chloride interface and RNA for Northern blotting [16] was extracted from the pellet. Alternatively, total leaf RNA was isolated from the TMV-MP and control transgenic plants (lines 277 and 306) as published previously [19]. RNAs were separated by electrophoresis in 1.0-1.5% (w/v) agarose gels in the presence of formamide/formaldehyde and transferred to nitro-cellulose or Zeta-Probe membrane (Bio-Rad Laboratories) as described [20,16]. The ³²P-labelled hybridization probes used [15,17] were the nick-translated CAT gene fragment or MP cDNA.

Recovery of progeny virus/pseudovirus particles and purification of encapsidated RNAs

Two weeks (CAT transgenic lines) or 3 weeks (MP line 277 and control line 306) after inoculation with TMV, progeny virus and virus-like particles were purified from infected leaf tissue. For individual plants of each CAT-transgenic class, total "virus" was prepared by polyethylene glycol co-acervation

[21] and one cycle of low/high-speed (35,000 rpm for 18hr) centrifugation. This separated the majority of full-length (300nm) TMV rods (the pellet) from the shorter-than-full-length rodlets (the "pellicle", or overlayer). The "pellicle" was separated by gentle washing with water. Total encapsidated RNA was then extracted from each fraction using phenol-SDS and precipitated with ethanol at -20°C [22]. Similarly, for virus-infected transgenic plant lines 277 and 306, fractions enriched in short rodlets (e.g. I₂-rodlets [22]) were obtained and the RNAs extracted. From each sample of short rodlet-enriched RNAs, polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography [20].

In vitro translation and Western blotting

Total RNAs extracted from the virus pellet and "pellicle" fractions, prepared from each class of CAT-transgenic tobacco, were translated in vitro at 30°C for 90 min in 100µl reactions containing mRNA-dependent rabbit reticulocyte lysate prepared and used according to Pelham and Jackson [23], except that all 20 amino acids were unlabelled. Final concentrations of pellet and "pellicle" fraction RNAs were 150µg/ml and 50µg/ml, respectively. Twenty microlitres of each incubation were taken directly to assay for CAT activity among the polypeptide products. A 50µl sample of each incubation was also added to 50µl gel-loading buffer [24] containing SDS and boiled for 5 min. These samples were subjected to SDS-PAGE [24], transferred to nitrocellulose [25] and probed sequentially with rabbit anti-CAT antiserum (a generous gift from W.V. Shaw, University of Leicester) and peroxidase-conjugated goat anti-rabbit-IgG antiserum as described [26].

CAT assay

For our preliminary plant screening, small leaf-discs (15mg) were taken from several random sites on each 20-40cm-high plant, pooled and homogenized in an Eppendorf tube containing 0.25M Tris-HCl, pH 7.4 and 10mM dithiothreitol. Each extract was clarified by centrifugation for a few mins and the supernatant assayed for CAT activity as described [4,12,27]. Samples from in vitro translation reactions were also assayed for CAT activity by this procedure.

RESULTS

Characterization of CAT-transgenic tobacco lines

Fresh leaf tissue from selected tobacco plants transformed with pROK2 constructs containing the CAT, Ω' -CAT or Ω' -CAT-OAS sequences, was assayed for CAT activity as described. Typical results are shown in Fig. 2a, together with the low endogenous CAT activity (% conversion of ^{14}C -chloramphenicol to acetylated products) found in non-transformed Xanthi tobacco leaf extracts (Fig. 2a, lane 1). Southern and Northern hybridization analyses of total DNA and RNA from these same transgenic plants confirmed that the CAT gene sequence was present in the chromosomal DNA (Fig. 2b) and was transcriptionally active (Fig. 2c). In Fig. 2c, the relative sizes of in vivo transcripts in the Ω' -CAT-OAS (lanes 2,3) and Ω' -CAT (lane 4) transgenic plants and the SP6 polymerase-produced [8] Ω' -CAT-OAS RNA standard (lane 5) confirm that the former approximate to the size predicted for termination at the NT site (Fig. 1). Similar experiments with tissue from transformed plants containing the TMV-MP gene (line 277) have demonstrated that the appropriate mRNA and 30kDa protein accumulate in these plants [13].

In vivo assembly of foreign nuclear gene transcripts

CAT transcripts Four transgenic Xanthi tobacco plants were chosen, two independently transformed with the Ω' -CAT-OAS construct, one with the Ω' -CAT construct (the same individuals as screened in Fig. 2a-c), and one additional control containing the CAT region alone, devoid of any TMV-derived sequence. Two weeks after inoculation with high levels of TMV, four separate "virus" preparations were made from the leaves of these plants. In the electron microscope we could detect no significant variation in the abundance of shorter-than-full-length rodlets in either the sap or purified "virus" preparations from these four TMV-infected transgenic plants. Large numbers of short rods are a natural feature of TMV preparations [22]. In part, they arise by breakage of full-length (300nm) particles, but more significantly by encapsidation of 3'-coterminial subgenomic mRNAs, in particular the 1.5kb I_2 -RNA [22] of the common strain of TMV.

Fig. 2. Analyses of CAT-transgenic tobacco plants before TMV-infection.

(a) CAT activities in leaf-disc extracts from: lane 1, non-transformed Xanthi tobacco; lane 2, as lane 1 but with 0.1 unit CAT enzyme added; lane 3, Ω' -CAT-OAS plant 1; lane 4, Ω' -CAT-OAS plant 2; and lane 5, Ω' -CAT plant. Positions of the 1- or 3-monoacetylated, or 1,3-diacetylated, derivatives of [^{14}C]-chloramphenicol are shown on the left. Conversion (%) of substrate into monoacetylated products is shown above each lane. Numbers with asterisks (*) represent conversion (%) to the diacetylated form. The tlc plate was exposed to X-ray film for 18hr.

(b) Southern blot of 10 μg *Sal*I-digested DNA from each class of transgenic tobacco. Lane 1, non-transformed (control) Xanthi tobacco; lane 2, Ω' -CAT-OAS plant 1; lane 3, Ω' -CAT-OAS plant 2; and lane 4, Ω' -CAT plant. Standard reconstructions representing one (lane 5) or 10 copies (lane 6) of the CAT gene per tobacco genome-equivalent were based on size-estimates of 1 x 10⁶ kbp and 0.8 kbp for the tobacco genome and the *Sal*I CAT DNA fragment, respectively. The blot was probed with a ^{32}P -labelled CAT gene clone as described.

(c) Northern blot of 20 μg total RNA isolated from non-transformed Xanthi tobacco leaves (lane 1) or CAT-transgenic lines, as described. Lane 2, Ω' -CAT-OAS plant 1; lane 3, Ω' -CAT-OAS plant 2; lane 4, Ω' -CAT plant. Lane 5 received 10pg pure Ω' -CAT-OAS RNA produced *in vitro* by SP6 RNA polymerase [8]. The radioactive CAT-sequence probe was as above.

Panels **(b)** and **(c)** were exposed to X-ray film for 3 days with intensifying screens.

Fig. 3. (a) CAT activity among the products of in vitro translation reactions programmed with total RNA extracted from the "virus" pellet and "pellicle" fractions from TMV-infected transgenic tobaccos. Control incubations contained: lane 1, no added RNA; lane 2, no RNA but 0.1 unit CAT enzyme added; lane 3, 10 μ g/ml Ω' -CAT-OAS mRNA produced in vitro [8]; and lane 4, 50 μ g/ml TMV RNA. Incubations assayed in lanes 5-8 and 9-12 contained RNAs from the "virus" pellet and "pellicle" fractions, respectively at 150 μ g/ml and 50 μ g/ml, from the following TMV-infected transgenic tobaccos: lanes 5 & 9, Ω' -CAT-OAS plant 1; lanes 6 & 10, Ω' -CAT-OAS plant 2; lanes 7 & 11, CAT only; and lanes 8 & 12, Ω' -CAT. Spot identities are as in Fig. 2a. The tlc plate was exposed to X-ray film for 18hr.

(b) Western blot of 50 μ l aliquots of the translation reactions described in (a), probed with anti-CAT antiserum. Lane designations as described above.

In vivo assembly of polyadenylated Ω' -CAT-OAS transcripts (approx. 1.45kb) would create rodlets of the same length (75nm) as the natural I₂-rodlets.

To detect any encapsidated CAT mRNA we extracted total RNA from the pellet and "pellicle" fractions (see Materials and Methods) of each "virus" preparation, translated these RNAs in vitro and assayed the polypeptide products directly for CAT

Fig. 4. Northern blot of 15 μ g or 5 μ g samples, respectively, of total RNA extracted from the "virus" pellet and "pellicle" fractions from TMV-infected transgenic tobaccos. Lane 1, TMV RNA control. Lanes 2-5 and 6-9 represent "virus" pellet or "pellicle" fraction RNAs from the following transgenics: lanes 2 & 6, Ω' -CAT-OAS plant 1; lanes 3 & 7, Ω' -CAT-OAS plant 2; lanes 4 & 8, CAT only; and lanes 5 & 9, Ω' -CAT. Lane 10 received 10ng Ω' -CAT-OAS RNA (1.2kb) made *in vitro* [8] from BglIII-linearized pJII102 [12]. Lanes 1-10 were exposed to X-ray film for 18hr. Lanes 11 & 12 are a longer (4 day) exposure of lanes 8 & 9, respectively. *In vivo* transcripts containing Ω' -CAT-OAS are about 1.45 kb in length, whereas those from the CAT or Ω' -CAT constructs are about 0.9kb and 1.0kb, respectively (lanes 11,12).

activity as described. The results are shown in Fig. 3a. The pellet and, more especially, the "pellicle" fraction RNAs isolated from the two Ω' -CAT-OAS transgenic plants alone produced substantial CAT activity when assayed in this way (Fig. 3a, lanes 5,6,9 and 10). Transgenic plant constructs which lacked the TMV OAS (i.e. CAT or Ω' -CAT) gave virus preparations which, by *in vitro* translation of extracted RNA, produced vanishingly low levels of CAT activity (Fig. 3a, lanes 7,8,11 and 12) and so must have contained little or no encapsidated CAT mRNA (c.f. Fig. 3a, control lanes 1,4). Prior to TMV-infection, near-equivalent levels of CAT enzyme activity and CAT mRNA were detected in leaf tissue from all these plants (e.g. see Fig. 2a,c).

As predicted, on a size-basis, the "pellicle" fraction from both TMV-infected Ω' -CAT-OAS transgenic plants contained the majority of the encapsidated reporter gene transcripts. Hence translation of these "pellicle" fraction RNAs at 50 μ g/ml resulted in higher levels of CAT activity (Fig. 3a, lanes 9,10) than

Fig. 5. Northern blot of 1 μ g samples of total (lanes 1 & 2) or poly(A)⁺ (lanes 3 & 4) RNAs extracted from virus preparations enriched for short rodlets, purified from TMV-infected transgenic plants that express the TMV-MP (line 277, lanes 1 & 3) or transgenic control plants (line 306, lanes 2 & 4). Lanes 5 & 6 contained 15 μ g poly(A)⁺ enriched RNA from uninfected leaf tissue of plant line 277 and control line 306, respectively. The position of TMV-genomic, -I₁ and -I₂ RNAs are indicated on the right. MP_t shows the positions of the TMV-MP related transcripts (1.4, 1.3 and 1.1 kb [13]).

were detected with 150 μ g/ml virus pellet fraction RNAs (lanes 5, 6). A similar distribution of CAT enzyme production could be seen indirectly on Western immunoblots, when the products of *in vitro* translation were separated by SDS-PAGE and probed with polyclonal rabbit anti-CAT antiserum (Fig. 3b).

Alternatively, total RNAs extracted from both fractions of the "virus" preparation from each class of transgenic plant were subjected to Northern hybridization analysis. This confirmed, directly, that only in the two Ω '-CAT-OAS plants were nuclear transcripts encapsidated and recovered to a significant extent in the respective TMV pellet (Fig. 4, lanes 2,3) and "pellicle" (Fig. 4, lanes 6,7) fraction RNAs. Blots were probed for the presence of the CAT mRNA sequence.

30kDa protein gene transcripts Northern hybridization analysis was also performed on total RNA and poly(A)⁺ RNA extracted from "virus" isolated from TMV-infected transgenic plant line 277 and from transgenic control line 306 (which contains the intermediate plasmid pMON316 without the TMV-MP gene [13]). Total "viral"

RNA prepared from the TMV-MP transformant line 277 appeared heterogeneous and equivalent to that from control line 306 (Fig. 5, lanes 1,2). However, encapsidated RNAs purified from TMV-infected 277 plants contained poly(A)⁺ MP-RNAs (MP_t in Fig. 5) not present in equivalent RNAs from infected 306 plants (Fig. 5, lanes 3,4). These poly(A)⁺ RNA species represent the MP mRNA encoded by the chimaeric gene construct (Fig. 5, lane 5) in uninfected plants.

DISCUSSION

We believe that these experiments, performed independently in two laboratories, with transgenic tobacco plants containing a reporter gene sequence (CAT or MP) and contiguous TMV OAS region, provide the first direct evidence for an efficient interaction (complementation) between functions encoded by the host genome and the infecting virus.

The occurrence of packaged subgenomic mRNAs and studies on TMV mutants temperature-sensitive for RNA packaging in vivo [28], suggest that the TMV OAS mapped by in vitro experiments [29,30] also functions in vivo during the self-assembly of progeny virus. However the work reported here provides direct support for this conclusion. A contiguous TMV OAS is required to sequester foreign gene transcripts efficiently in vivo during a helper virus infection.

Curiously, in Fig. 4 a very small amount of CAT probe-specific RNA appeared to be encapsidated in both the "virus" pellet and "pellicle" fraction RNAs from infected CAT (lanes 4,8) and Ω '-CAT (lanes 5,9) transgenic plants. Although not required for encapsidation, the Ω '-sequence has been shown to act in cis to enhance translation of foreign mRNAs, both in vitro and in vivo [12]. The level of encapsidation was estimated to be about 100-fold less than that in the Ω '-CAT-OAS transgenics and can be seen better by prolonged exposure of the Northern blot (Fig. 4, lanes 11,12). Since neither former construct possessed a true TMV OAS, these CAT mRNAs must have been packaged inefficiently and illegitimately. It is extremely unlikely that any unencapsidated CAT mRNA survived intact or was co-purified with "virus" material during the lengthy virus-

extraction protocol [21]. Thus we conclude that a small proportion of the CAT or Ω -CAT transcripts were fully-encapsidated in vivo, even in the absence of a cis-acting OAS. It is known that endogenous tobacco RNAs, particularly chloroplast mRNAs [31-34], become encapsidated during a normal systemic TMV infection to form a small population (1-2% of total virions) of natural "pseudovirions" [31]. The pseudo- or cryptic OAS region(s) responsible for these cases of 'mistaken identity' during self-assembly appear unrelated to the true OAS of TMV in either primary or secondary structure. Recently, two additional cryptic OAS regions have been mapped on the mRNA encoding the large subunit of ribulose biphosphate carboxylase in Petunia (A. Siegel, personal communication). There may be a similar, fortuitous sequence in CAT mRNA which can act, albeit inefficiently, to nucleate encapsidation by TMV coat protein in vivo. Nevertheless, our results confirm the need for a true OAS to achieve efficient packaging of foreign RNA both in vitro [3,4,9] and in vivo.

The ability to protect, accumulate and recover intact, specific mRNAs in vivo by exploiting components of the self-assembly machinery of TMV, offers a unique experimental technique to those engaged in studies on the homeostatic or developmental aspects of plant molecular biology. For example, we have some preliminary evidence (K.A. Plaskitt, D.E.S. and T.M.A.W., manuscript in preparation) that encapsidation sequesters and thereby 'switches-off' translation/expression of a particular mRNA species in vivo. Eventually it would be more desirable to engineer a non-pathogenic route to achieve this result. For example, the TMV coat protein gene itself could be introduced into the plant chromosome under the control of a strong but inducible promoter.

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