Tobacco Mosaic Virus Coat Protein and Reporter Gene Transcripts Containing the TMV Origin-Of-Assembly Sequence Do Not Interact in Double-Transgenic Tobacco Plants: Implications for Coat Protein-Mediated Protection

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The ability of tobacco mosaic virus (TMV) coat protein (CP), expressed in transgenic tobacco plants, to encapsidate RNA was measured by introducing a second reporter gene whose transcripts contained a copy of the TMV origin-of-assembly sequence (OAS). Electrofusion of transgenic mesophyll protoplasts or cross-pollination of singly transformed plants were used to create heterokaryons or plants bearing both transgenes in which complementation between TMV CP and the OAS mRNA could be assessed. Electron microscopy of immune-trapped protoplast or leaf extracts and assays for reporter enzyme activity showed that the endogenous CP was present in insufficient quantities, or was unavailable, to encapsidate significant amounts of OAS mRNA and thereby to affect its expression. Immunogold labeling of fixed, thin cryosections of young leaves from the homozygous CP-transgenic parent suggested that the endogenous CP was located almost exclusively in chloroplast-associated electron-dense bodies. Double-transgenic tobacco plants retained the ability to resist infection by TMV, measured by the delayed appearance of symptoms and the absence of progeny virions in samples of leaf sap. We conclude that substantial reencapsidation of uncoated RNA from challenge virus by endogenous CP in the cytoplasm of TMV CP-transgenic plants is not likely to account for “CP-mediated protection” (Powell Abel et al. 1986; Osbourn et al. 1989).

Recently, we found that transgenic tobacco plants which express tobacco mosaic virus (TMV) coat protein (CP) accumulate stable viruslike rodlets, presumably containing an RNA molecule(s) of host origin but of yet unknown sequence (Wilson 1989). In an attempt to measure the ability of TMV CP expressed in transgenic tobacco plants to encapsidate RNA carrying the true origin-of-assembly sequence (OAS) from TMV, we created mesophyll protoplasts or plants expressing the two complementary transgenes by electrofusion or cross-pollination, respectively.

If complementation occurred in vivo between an OAS mRNA and TMV CP, it is possible that higher numbers of virophage CP might occur due to encapsidation by TMV CP.

In this paper, we describe experiments to investigate these possibilities and attempts to locate the endogenous TMV CP in leaves of singly transgenic tobacco plants. The results are discussed with a view to the possible biological activity of TMV CP during the phenomenon of “CP-mediated protection” (Powell Abel et al. 1986; Osbourn et al. 1989).

MATERIALS AND METHODS

Electrofusion of tobacco mesophyll protoplasts. Mesophyll protoplasts were isolated by the method of Motoyoshi et al. (1973) from leaves of Nicotiana tabacum L. cv. Xanthi. Transgenic tobacco lines 3404 (Powell Abel et al. 1986) and Ω-CAT-OAS (CAT = chloramphenicol acetyltransferase; Ω = a derivative of the 5'-leader sequence of TMV RNA; Sleet et al. 1988a) or nontransformed (control) Xanthi plants were used. Paired samples of protoplasts (3404 + Ω-CAT-OAS or 3404 + Xanthi control) were electrofused following the procedure of Watts and King (1984) and subsequently cultured for 24 hr in 0.7 M mannitol incubation medium (Motoyoshi et al. 1973). Samples of fused protoplasts were also removed immediately, fixed, and embedded for electron microscopy as described previously (Hills et al. 1987). Thus the formation of binucleate cells, some of which would be heterokaryons, was confirmed (Fig. 1, center panel).

After a 24-hr incubation period, fused-protoplast samples were lysed hypotonically and centrifuged at 12,000 × g for 15 min to remove cell debris. Ten-microliter aliquots of each supernatant were placed on dental wax in a petri dish containing moist filter paper to prevent evaporation.

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A 400-mesh rhodium-plated copper grid, precoated with polyclonal anti-TMV serum (Moline and Luisoni 1975), was inverted on each droplet for 2–18 hr to trap any easily identifiable TMV-like structures. Finally, grids were rinsed with “antiserum buffer,” 60 mM sodium phosphate, pH 6.5, and stained with 1% (w/v) uranyl acetate before viewing in a Jeol 1200EX electron microscope.

**Cross-pollination.** Immature flower heads on TMV CP-transgenic tobacco plants (CP+/+) homozygous progeny from “selfed” line 3404; Powell Abel et al. 1986) were emasculated by removing the outer calyx, corolla, and anthers. Pollen from a vegetatively propagated Ω'-CAT-OAS transformant (Sleat et al. 1988a) was applied mechanically to stigmata of the TMV CP transgenic line. The recipient flower heads were bagged to prevent further cross-pollination, and mature seed was later collected from the dried ovaries. Progeny seedlings were then screened for expression of the TMV CP gene (by ELISA or western blotting) and the CAT gene (by enzyme assay, see below). Professor R. N. Beachy (Washington University, St. Louis, MO) kindly provided analogous double-transgenic seed from a cross between a TMV 30-kDa-gene transgenic tobacco plant (line 277; Deom et al. 1987) and TMV CP transgenic line 3404. Expression of the TMV 30-kDa movement protein gene was confirmed by northern blot analysis with a suitable 32P-labeled probe. In the U1 strain of TMV, the OAS resides in the 30-kDa gene (Goel et al. 1982).

For western blots, 30 μl of each leaf extract (= 40 μg total soluble protein per lane) was separated by sodium dodecyl sulfate (SDS)-PAGE on a 15% (w/v) polyacrylamide gel (Laemmli 1970). After electrophoresing on nitrocellulose, bands were probed with rabbit polyclonal antiserum against TMV followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA). All progeny seedlings expressed CP (Fig. 2, lanes 5–10), confirming that the line from “selfed” 3404 was homozogous for the TMV CP gene. Fifty percent of the seedlings grown from the 3404 CP+/Ω'-CAT-OAS cross expressed CAT activity, confirming that the vegetatively propagated Ω'-CAT-OAS parent was heterozygous. The remaining 50% of the seedlings from this cross (CP+/CAT−) provided a valuable reference population of heterozygous CP expressors with which to examine the “protected” phenotype (discussed below).

**Production of a reference heterozygous population of**

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**Fig. 1.** Selected ribonucleoprotein rodlets recovered from electrofused tobacco mesophyll protoplasts by immune-trapping on anti-tobacco mosaic virus coat protein antibody-coated electron microscope grids. Composite panel A = (3404 + Ω'-CAT-OAS) sample, where CAT = chloramphenicol acetyltransferase and OAS = origin-of-assembly sequence. Composite panel B = (3404 + Xanthi control) sample. A low magnification electron micrograph of the (3404 + Ω'-CAT-OAS) sample is shown to confirm that protoplast fusion had occurred (note the double nuclei, Nu). Scale bar = 100 nm.
\(\Omega\)-CAT-OAS transgenic plants. A cutting from the heterozygous \(\Omega\)-CAT-OAS paternal donor plant described above was allowed to self-pollinate. Seed was collected and sown. Thirty-three selfed seedlings were screened. We detected 10 nonexpressors, 15 with intermediate CAT activity and 8 with high CAT activity, a good approximation to the expected ratio of 1:2:1. CAT activities were measured and compared in 6 of these 15 heterozygous \(\Omega\)-CAT-OAS single-transgenic “selfed” progeny and in 6 of the CP\(^{+}\)/CAT\(^{-}\) double-transgenic progeny from the cross-pollination experiment described above.

Screening for TMV-like rodlets in double-transgenic plants. Leaf sap was prepared from several plants of each double-transgenic class (CP\(^{+}\)/CAT\(^{+}\) and CP\(^{-}\)/30-kDa\(^{-}\)) as well as from the CP\(^{-}\)/CAT\(^{+}\) progeny. Leaf tissue was ground in a small volume of 0.1 M Tris-Cl, pH 8.0, containing 10 mM EDTA. Plants selected ranged in size from small seedlings (1-2 cm high) to fully developed, flowering specimens with senescent lower leaves. Leaf extracts were clarified by centrifugation at 10,000 × g for 10 min, and 10-μl aliquots of each supernatant were immune-trapped on electron microscope (EM) grids coated with rabbit polyclonal anti-TMV serum as described above. To confirm that immune-trapping was quantitative (Derrick 1973), five 10-fold serial dilutions of TMV (200–0.02 μg/ml) were made in sap from normal, uninfected Xanthi tobacco and prepared as described above. Five grid squares were scanned for each sample. With each dilution, approximately 10 times fewer TMV rods were bound to the EM grid.

CAT assays. Leaf discs (1 cm in diameter) were removed and ground in 300 μl of CAT assay buffer (0.25 M Tris-Cl, pH 7.4, 10 mM dithiothreitol). Extracts were centrifuged for 10 min at 12,000 × g to remove debris and samples of each supernatant were then assayed for CAT activity (Gorman et al. 1982). The protein content of each supernatant was estimated by the method of Bradford (1976). Equivalent amounts of total protein were assayed from each extract, and the conversion (%) to acetylated products was quantified by cutting out areas corresponding to each [14C]-labeled spot from the thin-layer chromatography plate and counting in a toluene-based scintillator.

Immunogold localization of TMV CP in transgenic tobacco. Narrow (0.5-mm) strips of 1-2-cm-long young leaf tissue from un inoculated plants of CP-transgenic tobacco line 3404 or from nontransformed control Xanthi plants were fixed in 50 mM Pipes, 5 mM MgSO\(_4\), 5 mM EGTA, pH 6.9, (buffer A) containing 2% parafomaldehyde (PF) for 16 hr. After washing briefly in buffer A, an equal volume of 3 M sucrose containing 1% PF was added slowly during a 3-hr period, and the leaf pieces were left to infiltrate for 3 days at 4°C. Samples were then frozen rapidly in liquid ethane cooled with liquid nitrogen and sectioned on a Reichert Ultratrace cryo-ultramicrotome with a dry glass knife at −10°C (Tokuyasu 1986). Sections (100- to 300-nm thick) were placed on carbon-coated grids for immune-staining with rabbit polyclonal anti-TMV serum or mouse monoclonal antibodies specific for TMV CP monomers (“cryptotope”) or helically assembled TMV ribonucleoprotein (“neotope”; Dore et al. 1988). Secondary antibodies used were either goat anti-rabbit IgG or goat anti-mouse IgG (Sera Labs, Crawley Down, Sussex, England), conjugated to 10- or 15-nm colloidal gold, respectively.

RESULTS AND DISCUSSION

The occurrence of ribonucleoprotein structures resembling TMV: Electrophoresed protoplasts. Helical particles of differing lengths, but with the same diameter as TMV (18 nm), were immune-trapped (Fig. 1) in approximately equal numbers in extracts from both classes of electrophoresed protoplasts: (3404 + \(\Omega\)-CAT-OAS) and (3404 + Xanthi), the internal control. The extraordinary lengths of some of the immune-trapped rods (Fig. 1, panels A and B) may be due to the end-to-end aggregation of shorter TMV-like rods, exacerbated perhaps by polarization in the alternating electric field used to aggregate protoplasts or by the single 300 V dc pulse used for fusion (Watts and King 1984). We have not investigated this phenomenon further.

In all cases, it was necessary to scan many EM grid squares to locate a single rodlet. Comparisons with serial dilutions of TMV in leaf sap (described above) suggest that detecting immune-trapped particles at a frequency of less than one per grid square corresponds to a concentration of 20 ng/ml or less.

The presence of TMV-like structures in extracts of both classes of electrophoresed protoplasts could be attributed to the 3404 TMV-CP plant. We have also detected endogenous particles in total leaf extracts from transgenic tobacco plants expressing TMV CP (Wilson 1989).

Failure to detect an increase in rodlet numbers in the (3404 + \(\Omega\)-CAT-OAS) fusion, compared with the (3404 + Xanthi) fusion, may be explained in several ways: 1) there was insufficient time for the two foreign gene products to interact during the 24-hr incubation period, postelectrofusion; 2) the level of TMV CP expressed in the heterokaryons was insufficient to package \(\Omega\)-CAT-OAS mRNA efficiently; or 3) the OAS\(^{+}\) mRNA and TMV CP were segregated within the fused protoplasts.

It is unlikely that a low concentration of OAS\(^{+}\) mRNA is responsible for the absence of a detectable increase in the numbers of rodlets. The steady-state level of \(\Omega\)-CAT-OAS\(^{+}\) mRNA expressed in the parent plant is high (See

![Fig. 2. Western blot of total soluble proteins extracted from transgenic tobacco leaves (40 μg per lane). Lanes 1–3, standards (250, 50, and 10 ng of tobacco mosaic virus [TMV] coat protein [CP], respectively); lane 4, a 3404 homozygous TMV CP\(^{+}\)/CP\(^{-}\) plant; and lanes 5–10, the six heterozygous CP\(^{-}\)/CAT\(^{+}\) double-transgenic plants used for immunoblot analysis and assayed for CAT (chloramphenicol acetyltransferase) activity and resistance to TMV (see text).](image-url)
Cross-Pollinated tobacco plants. As above, immune-trapped leaf extracts from both classes of cross-pollinated, double-transgenic tobacco (CP<sup>+</sup>/CAT<sup>+</sup> and CP<sup>+</sup>/30-kDa<sup>-</sup>), as well as the CP<sup>+</sup>/CAT<sup>-</sup> reference plants, were found to contain very few TMV-like rod structures. The numbers detected were not significantly different from those found in sap of the 3404 CP-homozygous parent. This was the case for plants at all stages of growth and for all periods of trapping (2–18 hr.). Searching many grid squares revealed an occasional TMV-like particle, usually 100–200-nm-long and 18-nm-wide. Again, we estimate that the concentration of endogenous rodetils in undiluted sap from all classes of CP<sup>-</sup>-transgenic plants is approximately 20 ng/ml.

In this line of 3404, 0.1% of the soluble leaf protein is TMV CP (Powell Abel et al. 1986; Fig. 2, lanes 2 and 4). Thus we calculate that sap contains 1.3 µg of TMV CP per milliliter, and hence that approximately 1.5% of the total CP present exists as rodetils. However, we also note that the level of expression of the TMV CP transgene varies significantly between individual progeny plants (Fig. 2, lanes 5–10); a phenomenon reported by many others working with transgenic plants, including Powell Abel and co-workers (1986).

Clearly no substantial complementation had occurred in vivo during the 10 wk or less that the plants were grown. In circumstances in which large amounts of TMV CP were produced (a 3-wk systemic infection by TMV), substantial amounts of Ω-CAT-OAS<sup>+</sup> mRNA became encapsidated, the CAT activity was reduced more than threefold (Sleat et al. 1988b), and sucrose gradient fractionation gave an easily detected peak of shorter than full-length (CAT) particles (T. M. A. Wilson, unpublished data). Therefore, we conclude that in the double-transgenic progeny plants either there was insufficient endogenous CP to package substantial amounts of OAS<sup>+</sup> mRNA, or the endogenous CP and OAS<sup>+</sup> mRNA were located in different subcellular compartments. As stated above, it is unlikely that the level of OAS<sup>+</sup> mRNA limits rodlet accumulation.

Subcellular location of TMV CP in transgenic tobacco.

To determine the subcellular location of TMV CP in transgenic plants, sections of nontransformed Xanthi or homozygous CP<sup>+</sup>/transgenic (3404) tobacco leaves were fixed and cryosectioned as described above. Tissue was also fixed, embedded in Lowi K4M or London Resin (LR White), and sectioned as before (Hills et al. 1987). Extensive probing with rabbit polyclonal antisera against TMV or with mouse monoclonal antibodies specific for free or assembled TMV CP subunits (Dore et al. 1988) showed labeling of CP-transgenic (Fig. 3) but not control tobacco cells. Substantial gold labeling was observed only with PF-fixed and cryosectioned leaf material. This labeling appeared only in vesicles, apparently associated with chloroplasts in the mesophyll cells of very young leaves of transgenic tobacco plants expressing TMV CP (Fig. 3, panels a–c).

Two types of structures were labeled. Polyclonal CP antiserum may have targeted bodies resembling starch grains (Fig. 3, panel a), although these structures do not have the smooth and “opalescent” appearance more typical of a starch grain (for example, see top left corner of Fig. 3, panel c). The substantially more electron-dense structures (Fig. 3, panels b and c) targeted by the “cryptoptoe” monoclonal antibody (Dore et al. 1988) do not bear any resemblance to starch grains and can also be found, unlabeled, in the chloroplasts of very young leaves of nontransformed Xanthi tobacco. Unfortunately, ultrastructural resolution is significantly impared by PF fixation and cryosectioning, and the technique cannot be applied to larger leaves with vacuolated cells (B. Wells, personal communication).

Thus the rare, endogenous TMV-like rodetils found in sap from the parental transgenic tobacco plants expressing TMV CP (Wilson 1989) may be the result of encapsidation of chloroplast DNA transcripts, most likely those from the rbcL gene (Atreya and Siegel 1989). The separate subcellular locations of CP (chloroplasts) and OAS<sup>+</sup> mRNA (cytoplasm) may therefore explain, in part, the lack of interaction in the double-transgenic plants and electrophoresed protoplasts.

During several unsuccessful attempts to purify sufficient quantities of the endogenous rodetils from either single (CP<sup>+</sup>/) or double-transgenic tobacco leaves, to identify the encapsidated RNA by northern blotting with rbcL, CAT, or TMV CP gene-specific probes, it was noted that most particles were present in the low speed pellet fraction that contained chloroplasts.

Other biological effects. Although the CP gene copy number was halved during cross-pollination and the corresponding endogenous level of TMV CP reduced (Fig. 2, lanes 5–10), progeny (CP<sup>+</sup>/CAT<sup>-</sup>) double-transgenic tobacco retained the “protected” phenotype of the homozygous 3404-parent.

Challenge inoculation of the six CP<sup>+</sup>/CAT<sup>-</sup> plants (Fig. 2) with 0.5 µg/ml of TMV (U1 strain) produced no symptoms after 14 days. Although no symptoms could be seen by 16 days, leaf dips revealed a few virions in the systemic leaves of three of these six plants. In this experiment, a small population of homozygous CP<sup>+</sup>/ (3404) and heterozygous CP<sup>+</sup>/CAT<sup>-</sup> plants behaved identically. In a replicate experiment using 10 homozygous CP<sup>+</sup>/ (3404) and 10 CP<sup>+</sup>/CAT<sup>-</sup> tobacco plants inoculated with 1 µg/ml of U1 TMV, no symptoms were apparent in any plant after 13 days, and 50% of both categories of CP expressors showed mosaic symptoms by 16 days. In contrast, all nontransformed Xanthi controls and Ω-CAT-OAS<sup>+</sup> single-transgenic tobaccos inoculated with either concentration of TMV showed severe mosaic symptoms within eight days. At TMV concentrations greater than 1 µg/ml, protection, measured by the delay or absence of symptoms, even in parental homozygous CP<sup>+</sup> plants, is less notable.

We have shown that a 3-week-long systemic infection of Ω-CAT-OAS<sup>+</sup> tobacco seedlings by TMV results in a substantial, measurable decline in CAT activity (Sleat et al. 1988b). Presumably this effect was caused by “mistaken” encapsidation of Ω-CAT-OAS gene transcripts to form recoverable TMV-like particles (Sleat et al. 1988a). In the population of six double-transgenic CP<sup>+</sup>/CAT<sup>-</sup> plants, the average level of CAT activity (85.9% conversion [±SD = 2.8%]) was not significantly different from that in an equivalent number of single-transgenic Ω-CAT-OAS<sup>+</sup>
tobacco plants derived from the paternal parent (85.0% conversion [±SD = 9.6%]). Thus, although the lower level of CP (Fig. 2) expressed in the double-transgenic progeny still gave measurable “protection” against TMV infection (described above), it is clearly insufficient or inaccessible to package and inactivate substantial amounts of Ω'-CAT-OAS mRNA. Some effect might have been expected if the two gene products had coexisted in the cytoplasm throughout the development and growth of the plants. However, our immunocytochemical data (Fig. 3, panels a–c) indicate that most of the immunoreactive CP is localized in the chloroplasts and, hence, would be unavailable to encapsidate OAS mRNA derived from nuclear transgenes.

The immediacy of the “protective” action of TMV CP, expressed in transgenic plants, following challenge virus inoculation poses a perplexing mechanistic problem. Arguably, our results with double-transgenic plants overlook the possibility that the route of virus entry may be significant for CP-mediated “protection” to operate. Virions enter the cytoplasm through wounds in the cell wall and probably also the plasma membrane, or via plasmodesmata in secondarily infected cells (reviewed in Wilson 1988). In contrast, Ω'-CAT-OAS gene transcripts enter the cytoplasm via the nuclear membrane. Whereas the latter are unlikely to enter or interact with the chloroplasts, there are many experimental observations that imply a close interaction between virions, viral CP, and

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Fig. 3. Selected electron micrographs of paraformaldehyde (PF)-fixed and cryosectioned leaf sections of transgenic tobacco plants that express tobacco mosaic virus (TMV) coat protein (CP) (line 3404; Powell Abel et al. 1986) probed with rabbit polyclonal antiserum against TMV (a) or with mouse monoclonal antibodies specific for TMV CP monomers (b and c) or helical nucleocapsids (d) ("neotope"; Dore et al. 1988). Surprisingly, "neotope" gave little or no labeling, even of sections from Xanthi leaves systemically infected with TMV (especially for tissue embedded in London Resin [LR White] or Lowicryl K4M). Controls (healthy leaf sections or Xanthi leaf sections infected with TMV) probed with a mixture of both gold-conjugated second antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) showed no labeling. Colloidal gold in a is indicated by bold arrows. Scale bars = 500 nm.
chloroplasts (reviewed in Schoelz and Zaitlin 1989).

We conclude that the endogenous CP must be more efficient at preventing nucleocapsid disassembly or interfering with a later stage of virus replication (or both) than in repackaging RNA from already uncoated (or partially uncoated) virus particles. Recent results from independent experiments are consistent with this view (Register and Beachy 1988; Osbourn et al. 1989). However, even in mesophyll protoplasts from transgenic tobacco plants that express TMV CP, inhibited virus disassembly alone seems to be unable to account for “CP-mediated protection” (Osbourn et al. 1989).

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LITERATURE CITED


