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Association of Potato Virus Y Gene Products with Chloroplasts in Tobacco

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Symptom expression in potyvirus-infected plants may be due to altered plastid function. Chloroplasts from potato virus Y (PVY)-infected plants were assayed for the presence of PVY coat protein (CP), helper component protein (HC), and genomic RNA. PVY-CP, -HC, or -RNA binding to the exterior of chloroplasts was eliminated by treating plastids with thermolysin or RNase A, respectively, and by including reconstitution experiments as controls. By western and northern blotting analyses, PVY CP, HC, and RNA were found within infected leaf chloroplasts. Furthermore, the majority of CP was associated with the thylakoids rather than the stroma. These data agree with the work of others, implicating an inhibition of photosystem II in PVY pathogenesis, and suggesting an intimate relationship between PVY RNA, PVY gene products, and host cell plastids.

Infection with potyviruses results in a variety of symptoms, depending on host plant, environmental conditions, and virus strain (Bokx and Huttinga 1981). In general, virus symptoms are associated with plant foliage and result in mosaic patterns, vein banding, or vein clearing. Stunting, chlorosis, leaf distortion, and necrotic lesions may also occur. Such symptoms could result from disruption of organelle function as a result of an interaction(s) with virus genes or gene products. Many plant stress conditions significantly affect photosynthesis, and mosaic symptoms associated with virus infection may be due to plastid breakdown (e.g., Cronshaw *et al.* 1966; Esau 1948; Esau and Cronshaw 1967).

Reduced photosynthesis has been observed in plants infected with various viruses such as beet yellows closterovirus (Hall and Loomis 1972), peach rosette (Smith and Neales 1977) and tobacco ringspot nepoviruses (Roberts and Corbett 1965), barley stripe mosaic hordeivirus (Carroll 1970), and tomato aspermy cucumovirus (Hunter and Peat 1973). However, direct effects on photosynthesis arising from interactions between plastids and virus have been documented in only a few cases.

The relationship of turnip yellow mosaic virus (TYMV) with plastids is well known (Matthews 1973). During the course of TYMV infection, distinct invaginations develop in the chloroplast membrane. The chloroplasts may also become rounded and clumped, although this effect is variable and appears to depend on virus strain. The invaginations ultimately enclose to form vesicles that are associated with dsRNA as well as virus-induced RNA-dependent RNA polymerase. Thus, the vesicles may be a major site of viral RNA synthesis (Hatta and Matthews 1976; Mouches *et al.* 1974). Abutilon mosaic geminivirus (AbMV) has been less studied, but recent evidence suggests that it is intimately associated with plastids (Gröning *et al.* 1987a,b). Apparently, its ssDNA can be transferred to plastids, and dsDNA replicative forms have been found within infected chloroplasts (Gröning *et al.* 1990). Interestingly, progeny virions are found only in nuclei. It has also been observed that the AbMV nucleotide sequence contains prokaryotic as well as eukaryotic elements, and this may help account for AbMV-plastid interactions (Frischmuth *et al.* 1990)

The association of viral coat protein (CP) and genomic RNA of tobacco mosaic virus (TMV) with tobacco chloroplasts has been reported (Reinero and Beachy 1986; Schoelz and Zaitlin 1989). Accumulation of viral CP in chloroplasts of tobacco plants infected with a severe strain of TMV was higher than that of plants infected with a mild strain of the virus (Reinero and Beachy 1989). Reduced photosystem II activity of TMV-infected tobacco has also been reported (Reinero and Beachy 1989; Zaitlin and Jagendorf 1960), and it was observed that the majority of CP is associated with thylakoid membranes. Moreover, Schoelz and Zaitlin (1989) reported genomic TMV RNA in plastids, but detected no subgenomic RNA. These observations suggest that symptom expression in plants infected with TMV may result from interactions between viral gene products and chloroplasts.

Limited data suggest that a similar phenomenon may also be occurring in potyvirus-infected plants. Mayhew and Ford (1974) found that chloroplasts from maize dwarf mosaic virus (MDMV)-infected maize contained "ribonuclease-resistant RNA," presumably dsRNA replicative forms of MDMV. This observation suggests that viral RNA synthesis may occur in plastids and that potyviruses can have an effect on photosynthesis. In experiments similar to those performed by Beachy and co-workers (Hodgson *et al.* 1989; Reinero and Beachy 1989), Naidu *et al.* (1984a,b) showed a direct physiological effect of virus infection on photosystem II activity. Based on previous work by others and the work reported below, the potyvirus and tobamovirus systems appear to be analogous in terms of the physiological effects of virus infection on photosynthesis. Gadh and Hari (1986) reported that the full-length (−)RNA of tobacco etch virus (TEV) was associated exclusively with the chloroplast fraction from tobacco. Further tests sug-

gested that this (−)RNA was present in plastids (as dsRNA). Although partially purified plastids were not subjected to RNase treatment to destroy any non-internalized (surface RNA) molecules, their data (along with the MDMV data) suggest that chloroplasts are capable of supporting potyvirus replication. An alternative hypothesis is that single-stranded genomic RNA as well as double-stranded replicative form RNA is imported into chloroplasts.

In this report, we demonstrate that potato virus Y (PVY) CP, helper component (HC), and viral RNA are found within tobacco chloroplasts.

MATERIALS AND METHODS

Virus propagation. *Nicotiana tabacum* L. 'KY 14' was used for virus propagation and chloroplast isolation. Plants at the 4–6 leaf stage were mechanically inoculated with PVY (strain O). Ten to 12 days after inoculation, the upper leaves showing mosaic symptoms were harvested and used for chloroplast isolation.

Isolation of chloroplasts for protein analysis. Chloroplasts were isolated according to Fish and Jagendorf (1982) as modified by Reinero and Beachy (1986). Fifteen grams of deveined infected tissue was ground in a blender at medium speed in grinding buffer A (350 mM sorbitol, 50 mM HEPES-KOH, pH 8.3, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5% bovine serum albumin (BSA), and 4 mM ascorbic acid). The homogenate was strained through eight layers of cheesecloth, and chloroplasts were pelleted by centrifugation at 1,075 × *g* at 4° C for 10 min. Pelleted chloroplasts were resuspended in 2 ml of resuspension buffer A (375 mM sorbitol, 35 mM HEPES-KOH, pH 8.3, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.96 mM dithiothreiotol [DTT]). Resuspended chloroplasts were layered on sucrose density gradients (20, 40, 60, and 80%) made in resuspension buffer A. Sucrose gradients were centrifuged in an AH629 SW (DuPont, Wilmington, DE) rotor for 30 min at 150,000 × *g*. The lower chlorophyll band, which contained intact chloroplasts, was recovered by using a Pasteur pipette and was gently washed once with resuspension buffer A and centrifuged at 1,020 × *g* for 10 min at 4° C. This pellet was resuspended in resuspension buffer A, thermolysin and $CaCl_2$ were added to final concentrations of 200 μg/ml and 0.5 mM, respectively, and it was incubated on ice for 1 hr (Cline *et al.* 1984). Chloroplasts were washed twice in 5 vol of resuspension buffer A and then lysed in 1 ml of lysis buffer A (25 mM Tris-HCl, pH 8.0, 10% glycerol, 2% 2-mercaptoethanol, 0.5% sodium dodecyl sulfate [SDS], and 5.8 mM EDTA). Samples were boiled for 5 min, centrifuged at 10,000 × *g* for 5 min, and the supernatant was collected and used for SDS-PAGE and western blotting.

Separation of stroma and thylakoid fractions. Intact chloroplasts were prepared as described above, and thylakoid and stromal fractions were obtained by using the method of Roscoe and Ellis (1982). Chloroplasts recovered from sucrose gradients as above were treated with thermolysin, washed twice in resuspension buffer A, and lysed in lysis buffer B (25 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$). The suspension was centrifuged at 2,500 × *g* for 5 min at 4° C, and the supernatant (stromal fraction) was carefully removed by using a Pasteur pipette, lyophilized, and dissolved in lysis buffer A. The pellet, containing the thylakoid fraction, was lysed in lysis buffer A. Proteins from both fractions were precipitated with 9 vol of acetone, centrifuged, dried, dissolved in lysis buffer A, boiled, equalized on the basis of OD_{280}, and stored at −20° C for use in SDS-PAGE and western blotting.

RNA analysis. Percoll gradients were used rather than sucrose for isolating chloroplasts for RNA isolation. Fifteen grams of tobacco leaf tissues was ground in 50 ml of buffer by using a blender for three medium speed cycles of 3 s each in grinding buffer B (350 mM sorbitol, 50 mM HEPES-KOH, pH 7.5, 2 mM EGTA, 0.5 mM $MgCl_2$, 1 mM DTT, and 10 mg/ml of BSA). The homogenate was treated as described (for protein analysis) except that the resuspension buffer (B) was 350 mM sorbitol, 35 mM HEPES-KOH, pH 8.3, 10 mM K_2HPO_4, 0.5 mM $MgCl_2$, and 1 mM DTT. Resuspended chloroplasts were layered on Percoll gradients (80, 65, 45, 25, and 10%) in buffer containing 40 mM HEPES-KOH, pH 7.5, 0.5 mM $MgCl_2$, 350 mM sorbitol, and 1 mM DTT. Percoll gradients were centrifuged at 20,000 × *g* for 15 min at 4° C in an AH629 (DuPont) rotor. After centrifugation, intact chloroplasts were collected, washed twice with resuspension buffer B, resuspended in 1 ml of the same buffer, and incubated for 1 hr on ice with RNase A at 10 μg/ml. Chloroplasts were then washed, resuspended, and treated with thermolysin as described. Chloroplasts were washed twice with resuspension buffer B, and total RNA was isolated by lysis of chloroplasts in lysis buffer C (50 mM Tris-HCl, pH 7.6, 2% SDS, and 2 mM EDTA). After extraction with phenol and chloroform, RNA was precipitated with sodium acetate and ethanol.

Extraction of total RNA from tobacco plants. Leaf tissue, 0.5 g, was ground in liquid nitrogen and thawed in lysis buffer C used for chloroplast RNA extraction. Samples were then extracted with an equal volume of phenol/chloroform (1:1, v/v) and, after centrifugation, the aqueous phase was collected and extracted twice with chloroform. Nucleic acids were precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.5) and 2 vol of ethanol at −20° C. Nucleic acids were collected by centrifugation at 10,000 × *g* and resuspended in DEPC-treated water.

SDS-PAGE and western blot analysis of chloroplast proteins. Five micrograms of total plastid proteins from each sample was analyzed by 12% (w/v) SDS-PAGE, (Laemmli 1970). Gels were run at 6 W constant power until the bromophenol blue dye passed through the bottom of the gel. Gels were stained with silver by the method of Wray *et al.* (1981) or with Coomassie Brilliant Blue R-250.

For western blot analysis, proteins were electrophoretically transferred to nitrocellulose. Blotting was performed at 4° C at 100 V for 1 hr followed by 20 min at 200 V. After transfer, nitrocellulose was incubated in blocking solution (5% [w/v] dried milk in TBS [0.05 M Tris-HCl, pH 7.4, containing 200 mM NaCl]) for 0.5–1 hr and then overnight with the primary antibody at 5 μg/ml in blocking solution. Blots were washed five times for 10 min each in TBS, reblocked as described, and incubated for 3 hr with goat anti-rabbit IgG-alkaline phosphatase conjugate.

Blots were washed again, as described, and substrate solution was added (Leary *et al.* 1983). After color development the reaction was terminated by washing several times with water. Antibodies used were anti-PVY HC or anti-PVY, both provided by T. P. Pirone and D. W. Thornbury (University of Kentucky).

Agarose gel electrophoresis of chloroplast RNA and northern blot analyses. Isolated nucleic acids from chloroplasts and plant tissues were denatured by heating at 65° C for 10 min in MOPS-EDTA buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 50% deionized formamide, 6% formaldehyde, and 0.1% bromophenol blue) (Fourney *et al.* 1988). Samples were then loaded onto 1.4% agarose gels made in MOPS-EDTA buffer with 5% formaldehyde (Fourney *et al.* 1988; Sambrook *et al.* 1989). Electrophoresis was performed in the same buffer without formaldehyde for 2 hr at 60 V. After electrophoresis, nucleic acids were transferred to nitrocellulose membranes by overnight capillary transfer in 10× SSC, (1× = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7) baked at 80° C, prehybridized and hybridized as described (Sambrook *et al.* 1989), except formamide was omitted from the hybridization solution, and hybridization was performed at 65° C. After hybridization, filters were washed and exposed to Kodak XAR-5 film overnight at −80° C.

Preparation of cDNA probes. Hybridization probes were derived from a cloned 2.2 kb 3′-cDNA of PVY (provided by D. W. Thornbury, University of Kentucky). The gel-purified fragment was labeled with a random primed DNA labeling kit (US Biochemicals, Cleveland, OH) and used in hybridization reactions.

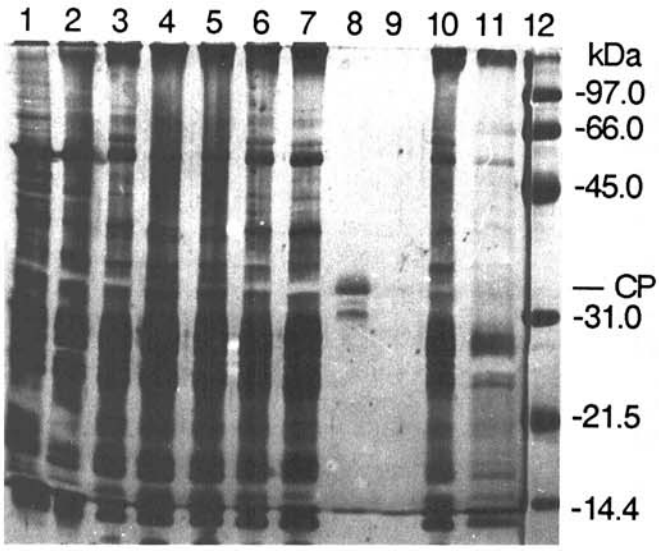

Fig 1. SDS-PAGE of total protein extracted from leaf tissue or thermolysin-treated purified chloroplasts. Lanes 1 and 2, total proteins extracted from infected or healthy tobacco, respectively; lane 3, chloroplasts from potato virus Y (PVY)-infected tobacco; lane 4, chloroplasts from healthy tobacco; lane 5, chloroplasts from mock-inoculated tobacco; lane 6, chloroplasts from healthy tobacco + purified PVY; lane 7, the same as lane 6 but without added thermolysin. Lanes 8 and 9 contain protein from 1 μg of purified PVY without or with thermolysin, respectively. Lanes 10 and 11 contain total protein extracts from thylakoid and stromal fractions, respectively. Lane 12 includes molecular weight markers. CP denotes the position of viral coat protein.

Reconstitution experiments. For both RNA and protein analyses, reconstitution experiments were performed to address the possibility of false signals due to protein or RNA bound to the exterior of isolated chloroplasts. Two types of reconstitution experiments were performed. Chloroplasts were isolated from healthy plants and, before loading onto sucrose or Percoll gradients (after the first low speed centrifugation), mixed with purified virus, or HC, at amounts approximating that estimated to be present in infected tissue; or chloroplasts isolated from healthy plants were mixed with the supernatant of a chloroplast preparation from the same amount of diseased plant tissue. The mixture was centrifuged at 1,020 × *g*, and the chloroplast pellet was washed twice in resuspension buffer and loaded onto Percoll or sucrose gradients. In both cases, after chloroplasts were collected from gradients, they were treated as described, and RNA or proteins were extracted as described. Purified HC (for reconstitution experiments and SDS-PAGE) was provided by T. P. Pirone, University of Kentucky.

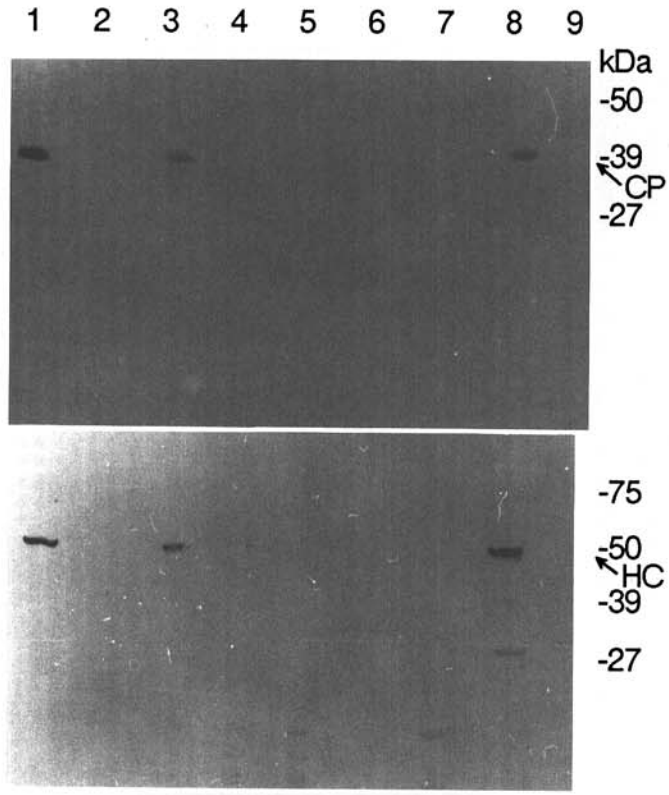

Fig. 2. SDS-PAGE fractionated protein extracts electroblotted onto nitrocellulose and probed with anti-potato virus Y (PVY) serum (**A**) or anti-PVY-helper component protein (HC) serum (**B**). **A,** lanes 1 and 2, total protein extracted from PVY-infected and healthy tobacco plants, respectively; lanes 3, 4, and 5, chloroplast extracts from PVY-infected, healthy, and mock-inoculated plants, respectively. Lanes 6 and 7 include extracts from reconstitution experiments (see Materials and Methods), with and without thermolysin added, respectively. Lanes 8 and 9 include protein from purified virus and molecular weight markers, respectively. Arrow shows position of PVY coat protein (CP). **B,** SDS-PAGE as in A, except the nitrocellulose blot was probed with anti-PVY-HC serum. Lane 8, partially purified PVY-HC. Arrow shows position of PVY-HC protein.

RESULTS

Chloroplast protein analysis. By using western blot analysis, our results indicate that both PVY CP and HC occur within chloroplasts from infected tissue. Chloroplasts from PVY-infected leaves were isolated as described, and chloroplasts from mock-inoculated or healthy tobacco were used as controls. Except where specified, these were treated with thermolysin. Lanes 8 and 9 of Figure 1 show that this proteinase effectively degrades PVY. Note that thermolysin fails to stain with silver but does take up Coomassie Brilliant Blue (not shown). SDS-PAGE of chloroplast proteins from infected or healthy plants revealed similar protein profiles in silver-stained gels and closely resembled those reported by Reinero and Beachy (1986) (Fig. 1). However, western blot analysis indicated a specific band reacting to anti-CP antibody in lanes loaded with proteins extracted from infected plants (Fig. 2A, lane 1) or infected chloroplasts (Fig. 2A, lane 3) but not in the lanes loaded with protein extracted from healthy plants or from reconstitution experiments (Fig. 2A, lanes 2, 4–7).

A similar experiment was done to determine if HC could also be detected in the same chloroplast extracts. Western blot analysis with anti-HC antiserum revealed a band comigrating with authentic HC, which was present in lanes loaded with protein extracted from diseased plants and from chloroplasts from diseased plants (Fig. 2B, lanes 1 and 3). No signal was detected in lanes loaded with aliquots of protein from reconstitution experiments or in lanes loaded with protein from healthy plants (Fig. 2B, lanes 2, 4–7).

To compare a possible relationship between PVY and photosystem II, as reported by Naidu *et al.* (1984a,b) and Reinero and Beachy (1986) for a potyvirus and TMV, respectively, chloroplast extracts were separated into stromal and thylakoid fractions. Protein profiles of stained gels of stromal or thylakoid proteins indicated that these preparations were reasonably free from contaminants (Fig. 1, lanes 10 and 11). In these experiments, it was observed that most of the PVY CP was present in the thylakoid fraction, and little signal was observed in the lanes loaded with stromal fractions (Fig. 3). A similar test with anti-HC was not done.

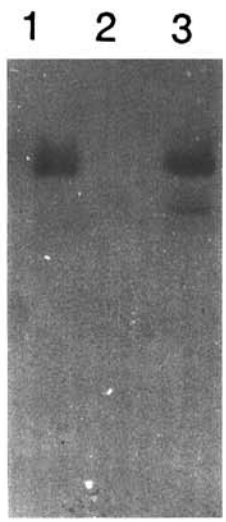

Fig 3. SDS-PAGE fractionated proteins from stroma and from thylakoid membranes prepared from potato virus Y (PVY)-infected chloroplasts, electroblotted onto nitrocellulose and probed with anti-PVY serum. Lane 1, purified PVY; lane 2, stromal fraction; lane 3, thylakoid fraction.

Northern blot analysis. Northern blot analysis of chloroplast RNA, using a 2.2 kb 3′-end PVY genomic clone, revealed a virus-specific band that comigrated with RNA isolated from purified virus and was observed only with chloroplast RNA isolated from diseased plants. In the lanes loaded with RNA isolated from healthy chloroplasts following the reconstitution experiments, a band corresponding to viral RNA was not observed. The gel shown in Figure 4 was also stained with ethidium bromide to reveal total RNA per lane (data not shown). The amount of RNA loaded in lane 4 (infected chloroplast RNA) was less than in lane 2, which was loaded with total RNA extracted from diseased tissue. Even with this lower amount of total chloroplast RNA, a band corresponding to authentic viral RNA was observed with the specific probe.

DISCUSSION

In this report, we demonstrate that two PVY gene products, CP and HC, are present within chloroplasts of infected tobacco plants. Two different reconstitution experiments were performed each of which supports these conclusions.

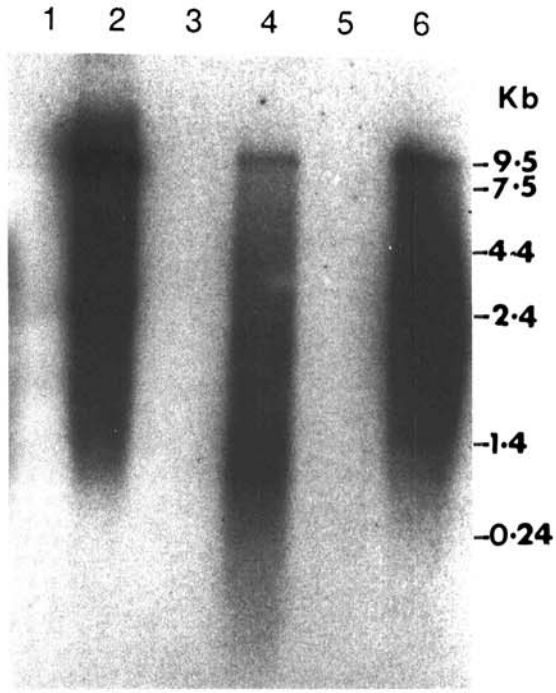

Fig. 4. Formaldehyde and 1.4% agarose gel-fractionated RNA, blotted onto nitrocellulose and probed with a 2.2 kb 3′-potato virus Y (PVY) cDNA clone. Lanes 1 and 2, total RNA isolated from healthy or PVY-infected tobacco respectively; lanes 3 and 4, RNA from chloroplasts isolated following RNase A and thermolysin treatment, from healthy or infected tobacco, respectively. Lane 5, RNA isolated from healthy chloroplasts incubated with PVY and PVY RNA; and lane 6, supernatant from purification of chloroplasts from infected tissue. Numbers on the right side indicate the position of molecular weight markers in kilobases (full-length PVY RNA ~9.5 kb).

In reconstitution experiments in which aliquots of healthy chloroplast samples had PVY added, no antigenic signals reacting against CP or HC antisera were detected, regardless of whether or not they were treated with thermolysin. These controls appear to rule out the possibility of a signal due to viral protein adhering to the outer membrane of chloroplasts (Fig. 2). This further supports the hypothesis that in infected leaves these gene products are indeed inside the chloroplast membranes. Similar experiments have demonstrated the presence of TMV CP and RNA inside the chloroplasts of infected tobacco, and our observations appear to be analogous (Reinero and Beachy 1986; Schoelz and Zaitlin 1989).

To our knowledge, this is the first report of a direct association of potyviral gene products with plastids. Naidu *et al.* (1984a,b) reported reduced photosystem II activity and alteration of plastid protein patterns with a potyvirus (peanut green mosaic virus) in peanut. Their results and those of Beachy and co-workers (Reinero and Beachy 1986, 1989; Hodgson *et al.* 1989) also implicate an interaction of viral proteins with chloroplast membranes, particularly the thylakoid membrane system (Fig. 3). In the experiments reported here, we observed the majority of the CP detected by western blotting to be associated with the thylakoid fraction and not with the stromal fraction.

Investigation of the association of these gene products with chloroplasts in plants infected with PVY strains that produce different symptoms becomes important because symptom expression can be related to the degree of association of viral gene products with chloroplasts. With TMV-infected tobacco plants, Reinero and Beachy (1989) demonstrated that a strain that produces mild symptoms had significantly less CP associated with chloroplasts and especially the thylakoids.

Gadh and Hari (1986) reported that (−)RNA of the potyvirus TEV was present as dsRNA in plastids. Unfortunately, isolated chloroplast fractions were not subjected to RNase A treatment, and no reconstitution experiments were reported in this study. Our results demonstrate that viral RNA was present in chloroplast fractions treated with RNase A. In reconstitution experiments in which virus was added to plant extracts, no viral RNA was detected, even in the absence of RNase A treatment.

Using a temperature-sensitive mutant of TMV, Schoelz and Zaitlin (1989) demonstrated that authentic TMV RNA entered chloroplasts. How this occurs is unknown. A temperature-sensitive PVY mutant is unavailable, and a different experimental approach needs to be adopted. The mechanism(s) of viral RNA transport into plastids and evidence for translation within chloroplasts remain to be resolved. We are attempting to test the infectivity of PVY RNA found in chloroplasts. Finding replicative forms of MDMV or TEV within chloroplasts suggests that they have the ability to replicate in chloroplasts. Whether potyviral gene products are transported into the chloroplasts or are translated exclusively in the organelles also needs to be resolved. Work is underway to investigate this. We have obtained evidence suggesting that PVY is translated *in organello*. Should this be confirmed, then it may be a significant divergence from observations on TMV, because *in organello* synthesis of TMV CP is unclear due to the apparent lack of the CP subgenomic RNA. Even if *in organello* synthesis of PVY CP (and other gene products) is confirmed, the possibility of import still exists and needs to be tested. If viral gene products are transported into chloroplasts, mechanism(s) of transport must be resolved.

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