16. G. Gaspari, J. Rudnick, A. Beldjenna, *J. Phys. A*, in press.
17. K. Solc and H. Stockmeyer, *J. Chem. Phys.* **54**, 2756 (1971); K. Solc, *ibid.* **55**, 335 (1971).
18. K. Solc, *Macromolecules* **6**, 378 (1973).
19. P. Debye, *J. Chem. Phys.* **14**, 636 (1946).
20. H. A. Kramers, *ibid.*, p. 415 (1946).
21. B. Zimm and W. H. Stockmeyer, *ibid.* **17**, 1301 (1949).
22. D. N. Theodorou and U. W. Suter, *Macromolecules* **18**, 1206 (1985).
23. J. Rudnick and G. Gaspari, *J. Phys. A* **19**, L191 (1986).
24. J. Aronovitz and D. R. Nelson, *J. Phys. (Paris)* **47**, 1445 (1986).
25. V. Ginzburg, *Sov. Phys.-Solid State* **2**, 1824 (1960); E. Helfand and J. S. Langer, *Phys. Rev.* **160**, 427 (1967).
26. J. Rudnick, A. Beldjenna, G. Gaspari, *J. Phys. A* **20**, 971 (1987).
27. M. Bishop and C. Saltiel, *J. Chem. Phys.* **85**, 6728 (1986).
28. M. Bishop and J. P. J. Michels, *ibid.*, p. 1074.
29. A. Messiah, *Quantum Mechanics* (Wiley, New York, 1968), vol. 1.
30. M. Fixman, *J. Chem. Phys.* **36**, 306 (1962).
31. The theoretical predictions based on Eq. 15 overestimate the number of walks in the region near the origin. As $R_1^2 \rightarrow 0$, the walks become extremely compact and the probability of finding such walks drops off abruptly. We are grateful to M. Nauenberg for helpful discussion on this point and for providing us with the results of his numerical simulations.
32. J. Straley and M. J. Stephen, *J. Phys. A*, in press.
33. S. Quandt and A. P. Young, *ibid.*, in press.
34. M. Bishop and C. Saltiel, in preparation.
35. We acknowledge useful discussions with A. Beldjenna, M. Bishop, M. Nauenberg, V. Privman, and A. P. Young. We thank J. Aronovitz, D. R. Nelson, M. J. Stephen, and J. Straley for sending reports of their work prior to publication.

Research Articles

The 30-Kilodalton Gene Product of Tobacco Mosaic Virus Potentiates Virus Movement

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The proposed role of the 30-kilodalton (kD) protein of tobacco mosaic virus is to facilitate cell-to-cell spread of the virus during infection. To directly define the function of the protein, a chimeric gene containing a cloned complementary DNA of the 30-kD protein gene was introduced into tobacco cells via a Ti plasmid-mediated transformation system of *Agrobacterium tumefaciens*. Transgenic plants regenerated from transformed tobacco cells expressed the 30-kD protein messenger RNA and accumulated 30-kD protein. Seedlings expressing the 30-kD protein gene complemented the Ls1 mutant of TMV, a mutant that is temperature-sensitive in cell-to-cell movement. In addition, enhanced movement of the Ls1 virus at the permissive temperature was detected in seedlings that express the 30-kD protein gene. These results conclusively demonstrate that the 30-kD protein of tobacco mosaic virus potentiates the movement of the virus from cell to cell.

THE INITIAL ENTRY AND REPLICATION OF A PLANT VIRUS IN a susceptible host is followed by movement of progeny virus into adjacent healthy cells, a process that is necessary for spread of the infection. In many virus-host interactions, movement (transport) also includes the systemic spread of the virus (that is, into other leaves) via the conductive tissues. In contrast, in nonhost plants the virus either fails to replicate or replicates in initially infected cells but fails to move to neighboring cells. If the virus replicates but fails to spread the plant remains healthy with only the few initially infected cells containing viral progeny (*1*). Effectively, the infection is aborted, and the plant reacts as if resistant to the virus.

Little is known about how plant viruses move in their hosts, either cell-to-cell or systemically. However, in recent years evidence has accumulated which suggests that a specific virus-encoded product is involved in the movement process. Such a virus-encoded function could be instrumental in determining the host range of the virus (*2, 3*), and within a given virus-host interaction it may be an important factor in determining virulence. In the case of tobacco mosaic virus (TMV) it has been suggested that the virus-encoded 30-kD protein is responsible for the movement function.

The genome of TMV is a single-stranded RNA of positive polarity encapsidated by a single type of capsid protein. The complete nucleotide sequence of the U_1 (common) strain has been determined (*4*), and shown to encode at least four proteins. Translation of the genomic RNA in vitro directs the synthesis of the 126-kD and 183-kD proteins. The 183-kD protein is a readthrough product of the amber termination codon of the 126-kD protein. Both proteins are postulated to be subunits of the TMV replicase (*5, 6*), and have been identified in virus-infected cells (*7*). Translation of two other open reading frames to produce the 30-kD and coat proteins requires the formation of two subgenomic RNA's, the 30-kD protein messenger RNA (mRNA), designated I_2 RNA (*8*), and the coat protein mRNA (*6, 9*).

Evidence implicating the 30-kD protein of TMV in movement comes from studies with temperature-sensitive (ts) mutants of TMV that are defective in cell-to-cell movement (*10, 11*). One well-characterized ts mutant is the Ls1 strain of TMV, a spontaneous mutant of the tomato strain L. At nonpermissive temperatures, the Ls1 strain replicates and assembles normally in inoculated leaves and in leaf protoplasts, but is not capable of moving from cell to cell in inoculated leaves (*10, 12*). When two-dimensional peptide mapping analysis was used to compare the 30-kD proteins encoded by I_2 RNA's obtained from Ls1 and L virus preparations, only a minor difference was detected (*13*). A comparison of the nucleotide sequences of the L and the Ls1 strains revealed that the the Ls1 virus had a single base change in the 30-kD protein gene which substituted a serine for a proline residue (*14*). Using the Ls1 mutant,

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Taliansky *et al.* (*15*) demonstrated that the ts defect in virus movement could be complemented in plants infected by related TMV strains, suggesting that the movement function is essential for spread of the virus. However, proof that the 30-kD protein is directly involved, or how it may be involved, remains to be determined.

To gain further insight into the specific biological function of the TMV 30-kD protein, we introduced a chimeric gene encoding the 30-kD protein into tobacco plants via a modified Ti plasmid in *Agrobacterium tumefaciens*. The expression of the gene in transgenic plants allows its function to be studied in the absence of the expression of other viral genes. We now show that expression of the 30-kD protein gene in transgenic plants complements the Ls1 strain of TMV. Transgenic plants infected with Ls1 and maintained at the nonpermissive temperature, potentiate cell-to-cell movement of the Ls1 virus in both inoculated and upper systemic leaves. Moreover, at the permissive temperature, movement of the Ls1 mutant is enhanced in the transgenic plants. These findings provide direct evidence that the 30-kD protein of TMV is necessary for virus movement.

Construction of and plant transformation with a chimeric TMV 30-kD protein gene. We previously described the isolation and the characterization of cloned complementary DNA's (cDNA's) containing the 30-kD protein coding sequence from the U_1 strain of TMV (*16*). When capped transcripts synthesized in vitro from cloned cDNA's containing the intact 30-kD protein-coding sequence were translated in a wheat germ cell-free system, a 30-kD protein was produced. This protein could be immunoprecipitated with antibodies to a synthetic peptide representing the predicted 11 COOH-terminal amino acids of the 30-kD protein (*17*). Moreover, the protein was indistinguishable, on the basis of apparent molecular size, from the 30-kD protein synthesized from the in vitro translation of I_2 RNA.

The TMV sequence in plasmid pTM 934 (TMV nucleotides 4855 to 5868) (*4, 16*) includes the 30-kD protein gene flanked on the 5′ end by 48 base pairs (bp) of the 183-kD protein gene sequence and on the 3′ end by 159 bp of the coat protein sequence. The TMV sequence from pTM 934 was excised and inserted into the expression vector pMON 316 (*18*) to create a chimeric gene containing the 35*S* promoter of cauliflower mosaic virus (CaMV) and the polyadenylation signal of the nopaline synthase gene (NOS) (Fig. 1). The chimeric gene was introduced into cells of *Nicotiana tabacum* cv. Xanthi. Transformed cells were selected for kanamycin resistance and regenerated into plants (*19*).

Table 1. Segregation of the TMV 30-kD protein gene in progeny of transgenic plants and chi square (χ^2) analysis. A crude cell wall fraction (Fig. 3) was obtained from 0.5 g of leaf tissue from 4-week-old seedlings. Crude cell wall extract (12 μl) was combined with 3× Laemmli sample buffer (*38*) in a 2:1 ratio and placed in a boiling water bath for 3 minutes. Samples were analyzed for 30-kD protein by SDS-PAGE and immunoblotting reactions as described in Fig. 3.

Transformant	Number of seedlings		χ^2 (3:1)*	χ^2 (15:1)*
	Expressing 30-kD protein	Not expressing 30-kD protein		
274	29	11	0.13	38.83
277	93	7	17.28	0.18

*A χ^2 of 3.84 is significant at the 0.05 probability level.

Copy number and expression of the chimeric gene in transgenic plants. Genomic DNA was isolated from fresh leaf tissue (*20*) of seven different transgenic plants, restricted with endonucleases, and analyzed by Southern blot hybridization reactions (*21*). Each DNA sample showed a single band of hybridization which corresponded to the expected size of the fragment containing the 30-kD protein gene. Comparison of the degree of hybridization with that of reconstructions containing one, five, and ten copies of the 30-kD protein gene from plasmid pTM 934:316 (Fig. 1) indicates that the copy number per diploid genome was one to three copies for six of the transgenic plants and more than five copies for one other plant. The transgenic plants 274 and 277 used in our study contained one and two gene copies, respectively. DNA blot analysis from a control

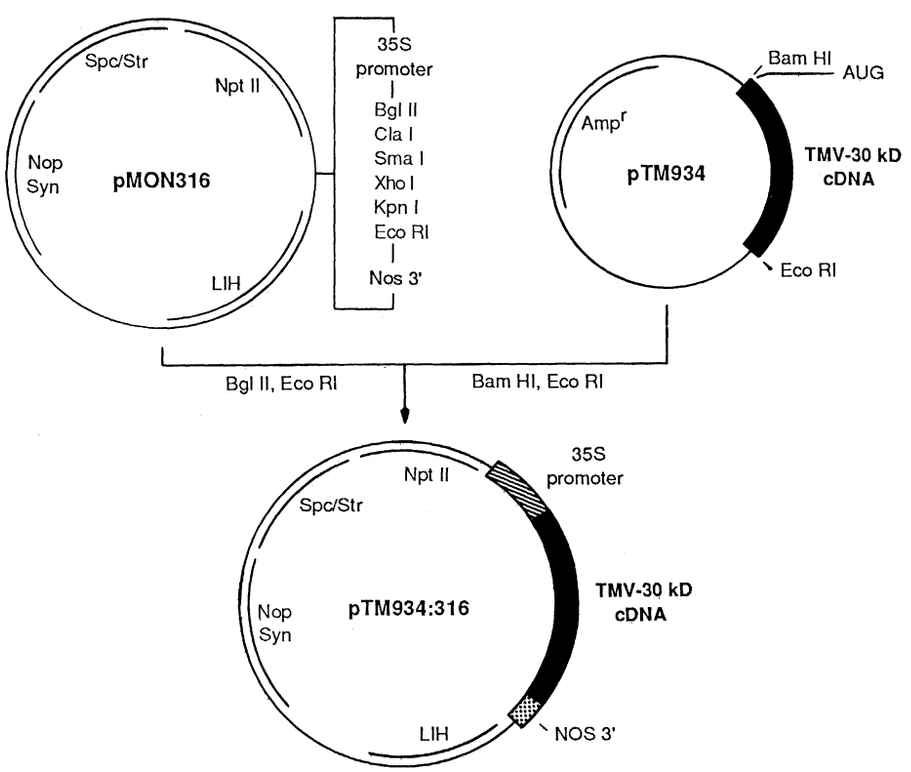

Fig. 1. Construction of the chimeric 30-kD protein gene. The TMV cDNA present in pTM 934 (*16*) was excised with Bam HI and Eco RI restriction enzymes. This fragment was ligated into the vector pMON 316 (*18*) at the Bgl II and Eco RI sites in the polylinker region between the CaMV 35*S* promoter and the NOS 3′ untranslated region. The resultant plasmid pTM 934:316 was transformed into *Escherichia coli* and subsequently mobilized into *A. tumefaciens* strain GV 3111SE carrying a "disarmed Ti" plasmid (pTiB6S3-SE) (*40*). Leaf disks from *N. tabacum* cv. Xanthi were transformed with *A. tumefaciens* carrying the pTM 934:316::pTiB6S3-SE cointegrated plasmid (*19*). Spc/Str represents the bacterial resistance genes for spectinomycin/streptomycin and Npt II represents a chimeric gene that confers resistance to transformed plant cells to neomycin phosphotransferase (type II). Nop Syn is the nopaline synthase gene and LIH represents a region of homology for recombination with a resident Ti plasmid in *A. tumefaciens* (*18*).

plant (transgenic line 306), which was transformed with the intermediate plasmid (pMON 316) alone, showed no fragments complementary to the 30-kD protein gene probe.

Total RNA was isolated from the transgenic plants and analyzed for the presence of RNA that encodes the 30-kD protein (Fig. 2). The expected size of the chimeric gene transcript is equal to 60 nucleotides of 5′ untranslated sequence, 807 nucleotides of the 30-kD protein gene, 159 nucleotides of TMV sequence located 3′ to the 30-kD protein coding region, and 160 nucleotides of the NOS 3′ end, plus the polyadenylation residues resulting in a transcript of 1.2 to 1.4 kilobases (kb) in length. The RNA blot analysis revealed two strongly hybridizing bands of 1.4 kb and 1.3 kb and a minor band of 1.1 kb. The difference in the amount of viral RNA between transgenic plants 274 and 277 is two- to threefold. In the RNA from control plant 306, there were no TMV-related sequences. Each of the RNA species became bound to oligo(dT)-cellulose (Fig. 2, lane 5), indicating that they are each polyadenylated. In addition, when RNA was extracted from polysomes that were isolated from leaf tissue of transgenic plant 277 and was similarly analyzed, most of each of the three transcripts was associated with heavy polysomes (≥5 ribosomes), an indication of their role as mRNA (Fig. 2, lane 6). The basis of the differences between the three transcripts is unknown but could be due to multiple sites of transcription initiation, multiple sites of polyadenylation, or to internal splicing of the transcript.

The presence of the 30-kD protein in transgenic plants was determined as described (Fig. 3). Subcellular fractions of leaf tissue were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antibodies to the 30-kD protein. The antiserum to the 30-kD protein used in these experiments was generated against a synthetic peptide corresponding to the 19 amino acids predicted by nucleotides 5515 to 5571 (*4*) of the 30-kD protein gene from the U_1 strain of TMV (*22*). This sequence was chosen because of its predicted strong degree of hydrophilicity. Immunoblot analysis indicates that the 30-kD protein of TMV accumulates in transgenic plant 277 and is present in both the crude cell wall and soluble fractions (Fig. 3, lanes 2 and 7) of leaf extract. Identical results were obtained with tissue from transgenic plant 274 (*23*). The 30-kD protein synthesized in plant 277 comigrates with the 30-kD protein produced in virus-infected leaf tissue (Fig. 3, lanes 1, 2, and 7). In TMV-infected tissue the virus-encoded 30-kD protein was detected in the crude cell wall fraction but not in the soluble fraction (Fig. 3, lanes 1 and 6). The 30-kD protein is not present in leaf tissue from control plants, nontransformed tobacco, transgenic plant 306, or transgenic line 3404, which expresses the TMV coat protein (Fig. 3, lanes 3 to 5 and 8 to 10). Because of the highly denaturing reagents used to extract the protein from the crude cell wall fraction, the relative amounts of 30-kD protein in transgenic compared to TMV-infected plants could not be determined. The specificity of the immunoblot reactions was illustrated by competitively inhibiting the antibody: antigen reaction with added oligopeptide to which the antibody was

Table 2. Development of chlorotic lesions on inoculated leaves. The three youngest expanded leaves from seedlings of plant 277 (with 30-kD protein) and of transgenic control line 306 (without 30-kD protein) were inoculated with a suspension (20 μg/ml) of purified Ls1 or L virus. Inoculated leaves were rinsed with water, and the plants were placed in growth chambers with 14-hour-light and 10-hour-dark periods. Light flux density was 300 to 350 $\mu E\ m^{-2}\ sec^{-1}$ and relative humidity was approximately 50 percent. Seedlings were maintained at 22°C, or at 22°C for 1 day followed by a temperature shift to 32°C. Lesion size was determined on day 7 after inoculation. Accumulation of virus in the lesions was determined by quantitating coat protein by immunoblot analysis (*39*) with ^{125}I-labeled secondary antibody. Immunoreactions were quantitated in a gamma counter.

Transformant	Genotype 30-kD protein	Virus	Temperature (C°)	Size of lesions* (mm)	Coat protein†
277	Present	Ls1	22	4–5	ND‡
			32	4–5	3.0
		L	22	4–5	ND
			32	5–6	4.0
306	Not present	Ls1	22	3–4	ND
			32	0.5–1.5	1.0
		L	22	4–5	ND
			32	5–6	3.5

*Sizes were determined from lesions in isolated areas on the top inoculated leaves. †Numbers represent the amount of coat protein detected in chlorotic lesions relative to the amount of coat protein in lesions from seedlings of plant 306 (standardized to equal 1) inoculated with Ls1 and maintained at 32°C. ‡ND, Not determined.

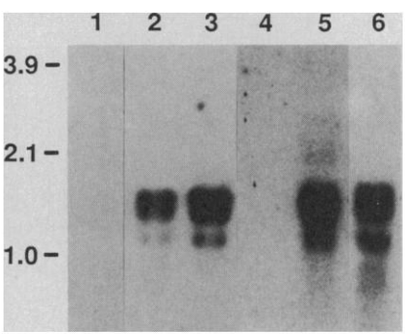

Fig. 2. Detection of RNA that encodes the TMV 30-kD protein in transgenic plants. Total RNA was isolated from leaf tissue as described by Abel *et al.* (*20*). Polyadenylated RNA was isolated from total leaf RNA by oligo(dT)-cellulose chromatography (*41*). Polyribosome-associated RNA was extracted from polyribosomes that were isolated and subsequently fractionated into size classes (*42*). Total, oligo(dT)-selected, and polyribosome-associated RNA's were separated on 1.0 percent agarose gels containing formaldehyde and transferred to nitrocellulose (*41*). The blotted RNA's were hybridized with nick-translated cDNA (*41*) encoding the 30-kD protein gene of TMV. Lanes 1, 2, and 3 contain total RNA (25 μg per lane) from transgenic control plant 306 and transgenic plants 274 and 277, respectively. Lanes 4 and 5 contain poly(A)$^+$ RNA (5 μg per lane) from control plant 306 and transgenic plant 277, respectively. Lane 6 contains polyribosome-associated RNA (30 μg) from polyribosomes containing ≥5 ribosomes per mRNA. The positions of size markers (in kilobases) are indicated at the left; 3.9 and 2.1 kb are the sizes of the 25*S* and 18*S* ribosomal RNA's of tobacco leaves (*8*). The position of the TMV-coat protein mRNA, 1.0 kb, from transgenic plants (*20*) is also indicated.

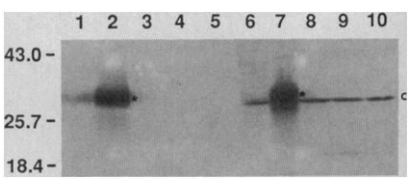

Fig. 3. Detection of TMV 30-kD protein in leaves of transgenic plants. A crude cell wall fraction and a soluble fraction were extracted from leaf tissue (*32*). The protein concentrations of the soluble fractions were determined (*43*). The protein concentration of the crude cell wall fractions could not be determined because of the presence of the denaturing reagents (SDS, urea, and 2-mercaptoethanol) used in extracting this fraction. Samples of the crude cell wall (25 μl) and the soluble fractions (200 μg of protein) were separated on a 10 percent SDS-polyacrylamide gel and transferred to nitrocellulose (*44*). The 30-kD protein was detected by reacting the nitrocellulose with rabbit antibody to TMV 30-kD protein (*22*) and then with a second antibody to rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Promega Biotec). Crude cell wall fractions (lanes 1 to 5) and soluble fractions (lanes 6 to 10) of leaf tissue from transgenic control plant 306 at 3 days after infection with the U_1 strain of TMV (lanes 1 and 6), from transgenic plant 277 (lanes 2 and 7), from transgenic control plant 306 (uninfected) (lanes 3 and 8), from normal tobacco (Xanthi) (lanes 4 and 9), and from transgenic plant 3404 (lanes 5 and 10). The stars indicate the 30-kD protein. The circle indicates the cross-reactive host protein detected only in the soluble fraction. The positions of molecular size markers are indicated at the left in kilodaltons.

produced and by the absence of a reaction when preimmune serum was used (*23*). A cross-reactive host protein that migrates slightly faster than the 30-kD protein was detected in the soluble fraction, but was not further examined.

Spread of Ls1 and L viruses in transgenic plants. Experiments were undertaken to determine whether transgenic plants expressing the 30-kD protein gene would complement a ts mutant of TMV that was defective in cell-to-cell movement. In order to obtain large numbers of uniform plants for statistically valid experiments, we used seedling progeny from self-fertilized transgenic lines 274 and 277 and transgenic control plant 306. Seedlings were analyzed for the presence of nopaline (*24*) and subsequently by immunoblot analysis for the presence of 30-kD protein (Table 1). Progeny from plant 274 segregated 3:1 (with 30-kD protein:without 30-kD protein), suggesting that the 30-kD protein gene was expressed from a single genetic locus. The segregation ratio of seedlings from plant 277 was approximately 15:1, an indication that the 30-kD protein gene was expressed at two genetic loci. Nopaline production co-segregated with expression of the 30-kD protein gene in the progeny of plants 274 and 277.

Transgenic seedlings of plants 274 and 277 were inoculated with a suspension (20 μg/ml) of either the Ls1 or L strain of TMV. Inoculated seedlings of control plant 306 and of nontransformed plants, as well as seedlings of plant 277 which did not express the 30-kD protein gene, served as controls. After inoculation, seedlings were maintained in growth chambers at either 22°C, the permissive temperature for the Ls1 strain, for 11 days, or at 22°C for 1 day and then the temperature was shifted to 32°C, which is the nonpermissive temperature, for 10 days.

The first indication that transgenic seedlings expressing the 30-kD protein gene could complement the ts defect of the Ls1 virus was indicated by the size of chlorotic lesions on inoculated leaves (Table 2). Inoculated leaves of seedlings of transgenic line 277 held at 32°C developed lesions that continued to increase in size to a diameter of 4 to 5 mm. In contrast, the size of lesions on inoculated leaves of seedlings not expressing the 30-kD protein gene, and maintained at 32°C, was 0.5 to 1.5 mm. These results suggest that virus movement, as defined by the increase in the size of chlorotic lesions on inoculated leaves and the relative accumulation of virus in these lesions (Table 2), correlates with the expression of the 30-kD protein gene. However, the size of chlorotic lesions induced by the L virus was independent of the expression of the 30-kD protein gene in the host (Table 2). When seedlings expressing the 30-kD protein gene were inoculated with the Ls1 or L strain and maintained at 22°C, chlorotic lesions, approximately 2 mm in diameter, developed by 3 days after inoculation and continued to increase in size at similar rates. This was also the case for nonexpressing seedlings inoculated with the L strain. But lesions produced on nonexpressing seedlings held at 22°C and inoculated with the Ls1 strain were smaller (~1 mm) at 3 days after inoculation and continued to enlarge more slowly than on seedlings expressing the 30-kD protein gene.

The transgenic seedlings (Table 2) were also observed for development of systemic disease symptoms caused by the Ls1 and L strains (Fig. 4). At the nonpermissive temperature, 90 percent of the seedlings expressing the 30-kD protein that were inoculated with the Ls1 virus showed systemic disease symptoms between 4 and 8 days after inoculation. The single seedling that did not show systemic symptoms did produce chlorotic lesions of 4 to 5 mm on the inoculated leaves (see above). In contrast, control seedlings that did not express the 30-kD protein gene and that failed to complement the Ls1 virus in inoculated leaves also failed to complement the virus in systemic movement at the nonpermissive temperature (*25*). To confirm that these symptoms reflected virus movement and infection, we analyzed tissue from inoculated and upper leaves of each seedling for the presence of virus by an immunological dot blot assay. Accumulation of virus in upper leaf tissue was positively correlated with visual symptoms of systemic movement (Figs. 4 and 5). In addition, virus in the upper leaves of each seedling was phenotypically identified as Ls1 (*10*), an indication that the virus moving into the upper leaves was not virus that had reverted to the L phenotype. Therefore, as with the movement of the Ls1 virus in

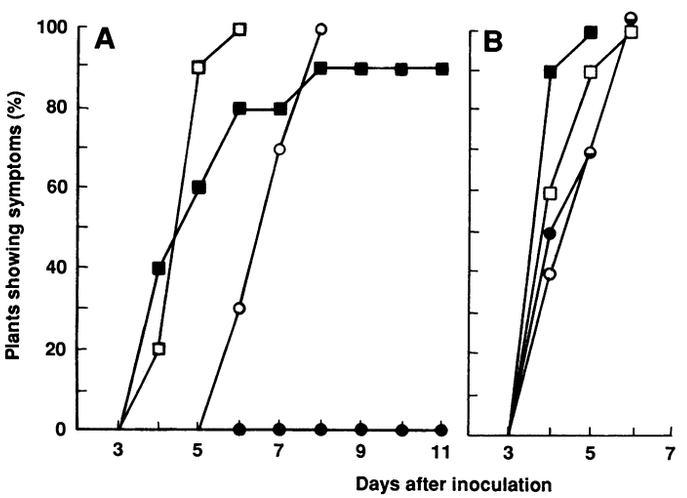

Fig. 4. The percentage of seedlings showing systemic disease symptoms at successive days after inoculation with the Ls1 or L virus. The three youngest expanded leaves of seedlings from transgenic line 277 (with 30-kD protein) and from transgenic control line 306 (without 30-kD protein) were inoculated with Ls1 or L virus and placed in growth chambers (Table 2). Plants were maintained at 22°C (□, ○), or at 22°C for 1 day followed by a temperature shift to 32°C (■, ●). Ten seedlings were used for each treatment. (**A**) Systemic spread of the Ls1 virus in seedlings of transgenic line 277 at 22°C (□) and 32°C (■) and in seedlings of transgenic control line 306 at 22°C (○) and 32°C (●). (**B**) The same as in (A) except that seedlings were inoculated with the L strain of TMV.

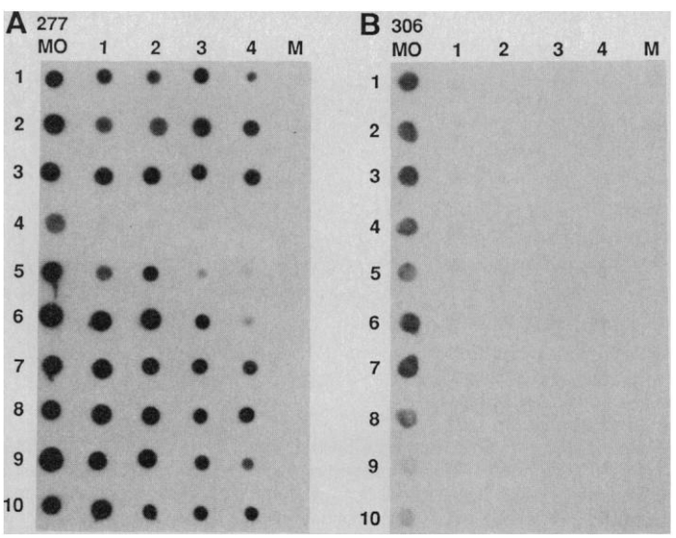

Fig. 5. Accumulation of the Ls1 virus in upper leaves from seedlings expressing the 30-kD protein as determined by immunoblot analysis (Table 2). Half leaves were sampled from the middle inoculated leaf and the first, second, third, and fourth upper leaves from the seedlings of plants 277 and 306. Extracted protein (10 μg) from each sample was dotted onto nitrocellulose. Seedlings of plants 277 and 306 are numbered vertically. MO, middle inoculated leaf; 1, 2, 3, and 4 represent the first, second, third, and fourth upper leaves, respectively; M, mock-inoculated tissue.

inoculated leaves, movement of the virus at 32°C in upper leaves correlates with accumulation of the 30-kD protein. The only seedling of transgenic line 277 that did not show systemic symptoms (seedling number 4) also did not contain detectable virus in upper leaf tissue. As would be expected, virus was also not detected in upper leaves of the control seedlings. When seedlings inoculated with the Ls1 virus were maintained at 22°C, plants expressing the 30-kD protein gene showed systemic disease symptoms 2 days before seedlings not expressing the gene, an indication that the 30-kD protein in transgenic seedlings enhanced movement of the Ls1 virus at the permissive temperature (*26*). This result correlates with the increased rate in development of chlorotic lesions on inoculated leaves at 22°C of seedlings expressing the 30-kD protein gene compared to seedlings not expressing the gene (as described). No statistically significant difference in systemic disease symptoms caused by the L virus was detected in transgenic seedlings that expressed or that did not express the 30-kD protein gene at 22°C or 32°C (Fig. 4B).

In other experiments (*23*) involving 22 seedlings of plant 277 and 10 seedlings of plant 274, all of which expressed the 30-kD protein gene, only one seedling from each transgenic line failed to show systemic disease symptoms at 32°C when inoculated with the Ls1 virus. However, both seedlings did show large chlorotic lesions on the inoculated leaves. In addition, 22 seedlings of control plant 306 and 8 seedlings of plant 277 that did not express the 30-kD protein failed to complement the Ls1 virus at 32°C in inoculated or upper leaves. At 22°C the enhanced local and systemic movement of Ls1 in seedlings expressing the 30-kD protein gene was again observed.

The TMV 30-kD protein gene and virus movement. It has been proposed that the 30-kD protein of TMV facilitates the cell-to-cell movement of the virus (*13, 14*). We now provide direct evidence for the role of the 30-kD protein in virus movement by demonstrating that expression of the 30-kD coding sequence in transgenic plants complements the ts defect in cell-to-cell movement of the Ls1 strain of TMV. Complementation occurs both locally, in inoculated leaves, and systemically, in upper leaves. In addition, we found enhanced movement of the Ls1 virus in seedlings expressing the 30-kD protein gene at the permissive temperature. This suggests that the 30-kD protein encoded by the Ls1 virus does not function as efficiently at the permissive temperature as the 30-kD protein encoded by the L virus and indicates that the 30-kD protein is an important factor in determining virulence. From these results, we conclude that expression of the 30-kD protein gene of TMV is responsible for the cell-to-cell movement of the virus and required for the establishment of a productive virus infection.

The designation "30-kD protein" was initially adopted because of the apparent molecular size of the protein. However, since evidence presented in this article establishes the role of the viral protein, we propose to designate it the M (movement) protein in order to reflect its function. This term has been used previously in postulating the role of the 30-kD protein (*27, 28*). The redesignation of the 30-kD protein is not meant to imply that other viral genes do not affect virus movement. For example, TMV mutants that produce defective coat proteins (*29*) do not move systemically and spread slowly from cell to cell. Also, mutations in the genes encoding the 126- and 183-kD proteins result in a decrease in virus yields and the masking of symptom development (*30*). However, we feel that involvement of the coat protein, or 126- and 183-kD proteins in virus spread are secondary effects that result in aberrant movement of the infectious form of the virus.

How the M protein of TMV functions to potentiate virus movement is unknown. Two general mechanisms by which a virus-encoded function might mediate the cell-to-cell spread of a virus have been proposed (*2*). The first states that plasmodesmata, cytoplasmic tubules that connect plant cells and through which viral genomes are believed to move (*31*), are modified by the virus encoded movement function. In light of this proposed mechanism it is interesting that the P3 protein of alfalfa mosaic virus (AlMV), a 32-kD protein believed to be analogous to the M protein of TMV, has been localized to the cell wall of infected tobacco cells (*32*), specifically to the middle lamellar region (*33*). The second mechanism proposes that the virus product necessary for movement functions by suppressing plant defense responses (*2*).Virus infection can induce host defense responses, and certain antiviral factors may spread from infected cells into adjacent healthy cells and provide resistance (*34*). The virus-encoded movement function may interfere with host defense mechanisms thereby making surrounding healthy cells susceptible to virus infection. This mechanism, operating at the level of transcription, has been invoked in part to explain the nuclear location of the M protein in tobacco protoplasts inoculated with TMV (*35*). The M protein would function by selectively suppressing host cell transcription and subsequently shutting off the defense response. However, since protoplasts lack cell walls and effectively are in a dedifferentiated state (*3, 36*), the possibility cannot be excluded that in infected plant tissue the target of the TMV-M protein is the cell wall. A more precise localization of the protein in intact infected tissue by immunocytochemical methods may help to define the mechanism by which the TMV-M protein functions.

Plant transformation experiments have been used to study both the regulational elements and the expression of various genes (*18, 37*). To our knowledge the complementation experiments described in this article are the first utilizing plant transformation technology to directly define the function of a gene. This system should prove convenient for analyzing in vivo the effect of mutations, induced by ordinary recombinant DNA techniques, that modify the amino acid composition of the TMV-M protein on its structure and function.

REFERENCES AND NOTES

1. M. A. Sulzinski and M. Zaitlin, *Virology* **121**, 12 (1982).
2. J. G. Atabekov and Y. L. Dorokhov, *Adv. Virus Res.* **29**, 313 (1984).
3. F. Ponz and G. Bruening, *Annu. Rev. Phytopathol.* **24**, 355 (1986).
4. P. Goelet *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5818 (1982).
5. M. Zaitlin, C. T. Duda, M. A. Petti, *Virology* **53**, 300 (1973); R. Scalla, P. Romaine, A. Asselin, J. Rigaud, M. Zaitlin, *ibid.* **91**, 182 (1978).
6. T. R. Hunter, T. Hunt, J. Knowland, D. Zimmern, *Nature (London)* **260**, 759 (1976).
7. F. Sakai and I. Takebe, *Virology* **62**, 426 (1974).
8. G. Bruening, R. N. Beachy, R. Scalla, M. Zaitlin, *ibid.* **71**, 498 (1976); R. N. Beachy and M. Zaitlin *ibid.* **81**, 160 (1977).
9. R. N. Beachy, M. Zaitlin, G. Bruening, H. W. Israel, *ibid.* **73**, 498 (1976).
10. M. Nishiguchi, F. Motoyoshi, N. Oshima, *J. Gen. Virol.* **39**, 53 (1978).
11. H. Jockusch, *Virology* **35**, 94 (1968).
12. M. Nishiguchi, F. Motoyoshi, N. Oshima, *J. Gen. Virol.* **46**, 497 (1980).
13. D. A. Leonard and M. Zaitlin, *Virology* **117**, 416 (1982).
14. T. Ohno *et al.*, *ibid.* **131**, 255 (1983).
15. M. E. Taliansky *et al.*, *ibid.* **122**, 318 (1982).
16. M. J. Oliver, C. M. Deom, B. K. De, R. N. Beachy, *ibid.* **155**, 277 (1986).
17. P. A. Kiberstis *et al.*, *FEBS (Fed. Eur. Biochem. Soc.) Proc. Meet.* **164**, 355 (1983).
18. S. G. Rogers *et al.*, in *Biotechnology in Plant Science: Relevance to Agriculture in the Nineteen Eighties*, M. Zaitlin, P. Day, A. Hollaender, Eds. (Academic Press, New York, 1986), p. 219.
19. R. B. Horsch *et al.*, *Science* **227**, 1229 (1985).
20. P. Powell Abel *et al.*, *ibid.* **232**, 738 (1986).
21. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
22. New Zealand White rabbits were immunized by intradermal injections with 300 μg of bovine thyroglobulin–conjugated peptide in complete Freund's adjuvant. Three injections were given at 4-week intervals. Two weeks after the third intradermal injection, the rabbits were given 3 mg of conjugated peptide intramuscularly in incomplete Freund's adjuvant and then 4 weeks later they were given 1 mg of conjugated peptide intravenously in saline. Antiserum was drawn 10 days later and was purified by passage over a peptide affinity column.
23. C. M. Deom and R. N. Beachy, unpublished results.
24. L. A. B. M. Otten and R. A. Schilperoort, *Biochim. Biophys. Acta* **527**, 497 (1978).
25. These results were determined to be valid by the Mann-Whitney U test ($\alpha = 0.01$).
26. This result was determined to be valid by the Mann-Whitney U test ($\alpha = 0.001$).
27. P. Palukaitis and M. Zaitlin, in *The Plant Viruses 2*, M. H. V. Van Regenmortel and H. Fraenkel-Conrat, Eds. (Plenum Press, New York, 1986), p. 105.
28. M. Zaitlin and R. Hull, *Annu. Rev. Plant Physiol.* **38**, 291 (1987).
29. A. Siegel, M. Zaitlin, O. P. Sehgal, *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1845 (1962).
30. M. Nishiguchi, Y. Nozu, N. Oshima, *Phytopathol. Z.* **109**, 104 (1984); M. Nishiguchi *et al.*, *Nucleic Acids Res.* **13**, 5585 (1985).
31. M. Weintraub, H. W. J. Ragetli, E. Leung, *J. Ultrastruct. Res.* **56**, 351 (1976).

32. T. Godefroy-Colburn, M. J. Gagey, A. Berna, C. Stussi-Garaud, *J. Gen. Virol.* **67**, 2233 (1986).
33. C. Stussi-Garaud, J. C. Garaud, A. Berna, T. Godefroy-Colburn, *ibid.* **68**, 1779 (1987).
34. L. Sequeira, *Trends Biotechnol.* **2**, 25 (1984).
35. Y. Watanabe, I. Ooshika, T. Meshi, Y. Okada, *Virology* **152**, 414 (1986).
36. C. Gigot, M. Kopp, C. Schmitt, R.G. Milne, *Protoplasma* **84**, 31 (1975).
37. R. N. Beachy *et al.*, *EMBO J.* **4**, 3047 (1985); Z. L. Chen, M. A. Schuler, R. N. Beachy, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8560 (1986); D. E. Rochester, J. A. Winter, D. M. Shah, *EMBO J.* **5**, 451 (1986).
38. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
39. R. S. Nelson, P. Powell-Abel, R. N. Beachy, *Virology* **158**, 126 (1987).
40. R. T. Fraley *et al.*, *Biotechnology* **3**, 629 (1985).
41. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
42. A. O. Jackson and B. A. Larkins, *Plant Physiol.* **57**, 5 (1976).
43. M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
44. H. Towbin, T. Staelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979); J. Symington, M. Green, K. Brackmann, *ibid.* **78**, 177 (1981).
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Engineering Enzyme Specificity by "Substrate-Assisted Catalysis"

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A novel approach to engineering enzyme specificity is presented in which a catalytic group from an enzyme is first removed by site-directed mutagenesis causing inactivation. Activity is then partially restored by substrates containing the missing catalytic functional group. Replacement of the catalytic His^{64} with Ala in the *Bacillus amyloliquefaciens* subtilisin gene (the mutant is designated His64Ala) by site-directed mutagenesis reduces the catalytic efficiency (k_{cat}/K_m) by a factor of a million when assayed with *N*-succinyl-L-Phe-L-Ala-L-Ala-L-Phe-*p*-nitroanilide (sFAAF-pNA). Model building studies showed that a His side chain at the P2 position of a substrate bound at the active site of subtilisin could be virtually superimposed on the catalytic His side chain of this serine protease. Accordingly, the His64Ala mutant hydrolyzes a His P2 substrate (sFAHF-pNA) up to 400 times faster than a homologous Ala P2 or Gln P2 substrate (sFAAF-pNA or sFAQF-pNA) at *p*H 8.0. In contrast, the wild-type enzyme hydrolyzes these three substrates with similar catalytic efficiencies. Additional data from substrate-dependent *p*H profiles and hydrolysis of large polypeptides indicate that the His64Ala mutant enzyme can recover partially the function of the lost catalytic histidine from a His P2 side chain on the substrate. Such "substrate-assisted catalysis" provides a new basis for engineering enzymes with very narrow and potentially useful substrate specificities. These studies also suggest a possible functional intermediate in the evolution of the catalytic triad of serine proteases.

RATIONAL DESIGN OF ENZYME SPECIFICITY BY PROTEIN engineering should expand the range and utility of biological catalysts. Site-directed mutagenesis techniques (*1*) have already been applied to modify the substrate specificity of subtilisin (*2–4*), trypsin (*5*), tyrosyl-tRNA (transfer RNA) synthetase (*6*), carboxypeptidase (*7*), alcohol dehydrogenase (*8*), and aspartate aminotransferase (*9*). These studies have focused on altering specificity by changing residues that make direct contact with the substrate. Here we present an alternative approach, termed "substrate-assisted catalysis," in which part of the catalytic machinery of the enzyme is removed and appropriately supplied by a similar functionality from a bound substrate. In this way substrates are distinguished primarily by their ability to actively participate in the catalytic mechanism permitting the design of extremely specific enzymes.

We have chosen subtilisin, a serine class endopeptidase, as a model to test the concept of substrate-assisted catalysis. In the hydrolysis of peptide bonds by subtilisin, His^{64} acts both as a catalytic base and acid in the formation of the acyl-enzyme intermediate and in a similar fashion in the subsequent deacylation step (*10*). A model of a substrate containing a histidine residue at the P2 position (*11*) bound to subtilisin (Fig. 1) indicated that the Nδ1 and Nε2 nitrogens can almost be superimposed (to about an angstrom unit) on the corresponding nitrogens of the catalytic histidine (His^{64}). This suggested that if the histidine in the catalytic triad of subtilisin was replaced by an alanine by means of site-directed mutagenesis, a histidine from the substrate might supply the missing catalytic functional group.

Construction and expression of His64Ala mutant subtilisin. Maturation of the primary subtilisin gene product (preprosubtilisin) to subtilisin in *Bacillus subtilis* is believed to be mediated by autoproteolysis that involves trace amounts of active subtilisin (*12*). The His64Ala mutation (*13–19*) caused a severe reduction in secretion of mature subtilisin that was presumed to result from a large reduction in catalytic activity (*20*). However, it was possible to process and subsequently purify the weakly active His64Ala enzyme by co-culturing *B. subtilis* cells harboring the His64Ala mutant gene with *B. subtilis* cells carrying an active subtilisin gene (helper) (*21–24*).

Stringent precautions were taken to ensure the purification of His64Ala subtilisin away from helper subtilisin and any other contaminating proteases. First, the mutant subtilisin was expressed in the *B. subtilis* host BG2036 (*19*), which is deficient in chromosomal copies of the genes for alkaline protease (subtilisin) and neutral protease. To minimize helper contamination, the ratio of helper cells to His64Ala cells in the fermentation culture was adjusted to 1:1000. A functionally silent Ser24Cys mutation that is located on the surface of subtilisin (*17*) was introduced into the

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