The Nuclear Shuttle Protein of *Tomato Leaf Curl New Delhi Virus* Is a Pathogenicity Determinant

Mazhar Hussain, Shahid Mansoor,* Shazia Iram, Ayesha Naureen Fatima, and Yusuf Zafar

National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan

Received 29 June 2004/Accepted 21 October 2004

The role of the movement protein (MP) and nuclear shuttle protein (NSP) in the pathogenicity of *Tomato leaf curl New Delhi virus* (ToLCNDV), a bipartite begomovirus, was studied. Both genes were expressed in *Nicotiana benthamiana*, *Nicotiana tabacum*, and *Lycopersicon esculentum* plants with the *Potato virus X* (PVX) expression vector or by stable transformation of gene constructs under the control of the 35S promoter in *N. tabacum*. No phenotypic changes were observed in any of the three species when the MP was expressed from the PVX vector or constitutively expressed in transgenic plants. Expression of the ToLCNDV NSP from the PVX vector in *N. benthamiana* resulted in leaf curling that is typical of the disease symptoms caused by ToLCNDV in this species. Expression of NSP from PVX in *N. tabacum* and *L. esculentum* resulted in a hypersensitive response (HR), demonstrating that the ToLCNDV NSP is a target of host defense responses in these hosts. The NSP, when expressed as a transgene under the control of the 35S promoter, resulted in necrotic lesions in expanded leaves that initiated from a point and then spread across the leaf. The necrotic response was systemic in all the transgenic plants. Deletion of 100 amino acids from the C terminus did not compromise the HR response, suggesting that this region has no role in HR. Deletion of 60 or 100 amino acids from the N terminus of NSP abolished the HR response, suggesting that these sequences are required for the HR response. These findings demonstrate that the ToLCNDV NSP is a pathogenicity determinant as well as a target of host defense responses.

Geminiviruses are single-stranded DNA viruses that infect a broad range of dicotyledonous and monocotyledonous plants. Viruses of the family *Geminiviridae* have been classified into four genera based on host range and genome organization (30). Of these, the whitefly-transmitted geminiviruses (genus *Begomovirus*) are the largest group, with more than 100 virus species identified so far (8). Geminiviruses replicate in the nuclei of host cells by a rolling-circle replication mechanism (reviewed in reference 11). For the propagation of infection in the host, these viruses encode movement proteins that direct the viral genome to the cortical cytoplasm and across the barrier of the cell wall. For bipartite begomoviruses, this process requires two proteins, encoded by the component designated DNA B: the nuclear shuttle protein (NSP) and the cell-to-cell movement protein (MP). These proteins act cooperatively to move the viral DNA from its site of replication in the nucleus to the cytoplasm and into adjacent plant cells (32).

The geminivirus MP and NSP recognize DNA in a form- and size-specific manner (12, 29). The NSP shuttles newly replicated viral DNA between the nucleus and the cytoplasm (42). MP traps these NSP-viral DNA complexes in the cytoplasm and redirects them to and across the plant cell wall (24, 43). In adjacent cells, the NSP-viral DNA complexes are released, and NSP targets the viral DNA to the nucleus to initiate new rounds of infection. This process of movement is highly regulated. Recently, an acetyltransferase has been isolated from *Arabidopsis thaliana* that interacts with the NSP encoded by the bipartite begomovirus *Cabbage leaf curl virus* (22). This protein

regulates the nuclear export of the viral DNA and is highly conserved in plants.

In recent years, geminiviral proteins involved in virulence and pathogenicity have been studied extensively. For bipartite begomoviruses, MP is the major symptom determinant, and the expression of this protein induces disease-like symptoms (2, 6, 7, 15, 17, 26). For monopartite begomoviruses, such as *Tomato leaf curl virus* and *Tomato yellow leaf curl virus*, the C4 protein is involved in symptom development. Expression of their C4 genes in transgenic plants induces disease-like symptoms (28). The transcriptional activator protein (sometimes referred to as AC2 or AL2) of *Tomato golden mosaic virus* and the C2 (sometimes referred to as L2) protein of *Beet curly top virus* are pathogenicity determinants (13, 35). For some geminiviruses, the replication-associated protein (sometimes referred to as AC1 or AL1), transcriptional activator protein, and replication enhancer protein induce a necrotic response in plants (5, 33, 38, 39).

The NSP of *Bean dwarf mosaic virus* is reported to be an avirulence determinant inducing a hypersensitive response (HR) in *Phaseolus vulgaris* (10). The HR is associated with specific recognition and resistance to infection by a range of plant pathogens, which is often accompanied by the collapse of challenged host cells (4, 23). Hypersensitive cell death results in a necrotic lesion delimited from surrounding healthy tissue that is believed to contribute to containment of the pathogen. However, examples have been reported in which the HR and resistance have been uncoupled (19, 27).

Tomato leaf curl New Delhi virus (ToLCNDV) is an Old World begomovirus that induces leaf curling and yellowing in tomato (*Lycopersicon esculentum*) and has also been detected on cucurbits, chilies, and potato on the Indian subcontinent (16, 20, 21, 25, 36). ToLCNDV is a bipartite virus that requires

* Corresponding author. Mailing address: National Institute of Biotechnology and Genetic Engineering, P.O. Box 577, Jhang Road, Faisalabad, Pakistan. Phone: 92 41 651472. Fax: 92 41 651472. E-mail: smansoor@nibge.org.

TABLE 1. Primers used to produce expression constructs of MP and NSP and deletion mutants of NSP

Primer	Sense	Sequence
MP F	Complementary	5'-CACCATGGCAATAGGAAATGATGGTATGGG-3'
MP R	Virion	5'-AAGGATCCTCTTATTTTTTGAATAAATTTGG-3'
NSP F	Virion	5'-GGCATCGATGGTGTTCGGTTGCCACAC-3'
NSP R	Complementary	5'-GGCGTCGACATATCCAATGTAATTAAGAATTAC-3'
N60 NSP	Virion	5'-GGCATCGATGCCATGCCATAGCACGTTCTCCAA-3'
N100 NSP	Virion	5'-GGCATCGATATGTCACGTATCCATCAAAAAC-3'
C30 NSP	Complementary	5'-GGCGTCGACTTATCGGAGGGTGTAGAAATATCG-3'
C100 NSP	Complementary	5'-GGCGTCGACTAATTAACAGATCCAAATAGC-3'
NSP UT	Virion	5'-GGCATCGATTGTGTTCGGTTGCCACAC-3'
NMP	Complementary	5'-CAATCGATATGGCAATAGGAAATGATGGTATGGG-3'
CMP	Virion	5'-AACCCGGGACTCTTATTTTTTGAATAAATTTGGC-3'

DNA B for symptom development, and the coat protein is dispensable for systemic infection and symptom development (25). This established a key role for the NSP and MP gene products in systemic infection, as is the case with other bipartite begomoviruses.

We have analyzed the role of the MP and NSP of ToLCNDV in pathogenicity. We show that the NSP of ToLCNDV is a symptom determinant and an avirulence determinant that is the target of host defense responses in tobacco and tomato leading to an HR.

MATERIALS AND METHODS

Expression constructs. Primers were designed to amplify the MP and NSP based on the published sequence of ToLCNDV DNA B (accession number U15017) (25). The primer sequences used for amplification of MP, NSP, and deletion mutants of NSP are given in Table 1. In deletion analysis of NSP, start and stop codons were added for the N-terminal and C-terminal deletion mutants, respectively. In one of the gene constructs (NSP^{ut}/PVX) a stop codon was introduced just after the start codon. For insertion of PCR fragments into the *Potato virus X* (PVX) vector, ClaI and SalI restriction endonuclease recognition sites were included in the forward (virion-sense) and reverse (complementary-sense) primers, respectively (Table 1). PCR-mediated amplifications (with the cloned DNA B component of ToLCNDV as a template), restriction endonuclease digestion, and cloning were conducted by standard methods with enzymes obtained from Fermentas.

PCR products were cloned into the PVX vector (pgR 107) (3) at ClaI and SalI restriction sites. Features of the gene constructs are summarized in Table 2 and Fig. 1. For plant transformation, the MP and NSP genes were cloned into the expression vector pJIT163 at the NcoI and BamHI restriction endonuclease sites. The expression cassette was then subcloned into the binary vector pBinPlus (37) by using the SacI and SmaI restriction sites. A further NSP construct for the production of double-stranded RNA was produced. The NSP gene was cloned in both the sense orientation (at the XhoI and NcoI sites) and antisense orientation (at the BamHI and XbaI sites) in the vector pFGC5941 (18). This construct was then transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The transgene and agroinfiltrated gene constructs were sequenced to confirm the integrity of the inserts.

Deletion analysis of NSP. Deletion mutants of NSP were produced to determine the protein domains potentially involved in pathogenicity and HR (Fig. 1). Oligonucleotides were designed to delete 60 and 100 amino acids from the N terminus of NSP, yielding mutants NSP^{dmN60} and NSP^{dmN100} respectively. A start codon was introduced in the oligonucleotides for these N-terminal mutations (Table 1). Likewise, 30 and 100 amino acids were deleted from the C terminus with a stop codon introduced in the oligonucleotides, yielding mutants NSP^{dmC30} and NSP^{dmC100} respectively.

Agroinfiltration. *Nicotiana tabacum* (cv. Samsun), *Nicotiana benthamiana*, and *L. esculentum* (tomato; cv. Moneymaker) were grown under controlled conditions at 25°C with a 16-h lighting regimen. The expression constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cultures for inoculation were grown at 28°C for 48 h to an optical density at 600 nm of 0.6 with 50 µg of kanamycin and 50 µg of tetracycline per ml. The bacterial cells were pelleted (5,000 × g for 15 min at 20°C) and resuspended in 10 mM MgCl₂ and 150 µg of acetosyringone per ml. Cells were incubated in this medium for 3 h and then infiltrated into the young and fully expanded leaves of 3- to 4-week-old plants, either directly or following dilution. For coinfiltration of NSP/PVX and NSP/pFGC5941, the *Agrobacterium* cultures transformed with these constructs were activated and then mixed in equal proportions.

Plant transformation. *Nicotiana tabacum* (cv. Samsun) was transformed with two constructs by the *Agrobacterium*-mediated leaf disk method (14). T₀ lines were self-pollinated, and T₁ seeds were germinated on MS medium containing 500 µg of kanamycin per ml before transplantation to soil. Putative transgenic lines were assessed for the presence of transgenes by PCR-mediated amplification.

RESULTS

ToLCNDV NSP is a symptom determinant in *N. benthamiana*. The full-length NSP gene was cloned in the PVX vector pgR107 (NSP/PVX) and inoculated into *N. benthamiana* by agroinfiltration. Infiltrated plants were maintained in a growth chamber. Infiltrated leaves developed downward leaf curling 6 to 8 days postinfiltration (Fig. 2B). The symptoms were systemic and began to appear in the upper, noninfiltrated leaves approximately 15 days postinoculation (Table 3). These symptoms are qualitatively similar to the symptoms induced by

TABLE 2. Gene constructs used in this study

Gene construct	Primer pair used	Insert size (bp)	Cloning sites	Deletion (no. of amino acids)
MP/PVX	NMP + CMP	878	ClaI + SmaI	
MP/pBin	MPF + MP R	878	NcoI + Bam HI	
NSP/PVX	NSP F + NSP R	845	ClaI + SalI	
NSP ^{dmN60} /PVX	N60 NSP + NSP R	665	ClaI + SalI	60, N-terminal
NSP ^{dmN100} /PVX	N100 NSP + NSP R	545	ClaI + SalI	100, N-terminal
NSP ^{dmC30} /PVX	NSP F + C30 NSP	755	ClaI + SalI	30, C-terminal
NSP ^{dmC100} /PVX	NSP F + C100 NSP	545	ClaI + SalI	100, C-terminal
NSP ^{UT} /PVX	NSP UT + NSP R	845	ClaI + SalI	No protein
NSP/pBin	NSP F + NSP R	845	NcoI + BamHI

FIG. 1. *Potato virus X*-based expression cassettes of NSP of ToLCNDV. The deletion mutants of NSP of ToLCNDV are indicated with their amino acid coordinates.

infection of this species with ToLCNDV, which include leaf curling and yellowing. Control plants infiltrated with just the PVX vector or the PVX vector containing a mutated NSP (NSP^{UT}/PVX) did not show any symptoms other than those typical of PVX, ruling out possible RNA-mediated effects. The inoculations were repeated three times, and the results were reproducible. These results suggest that the effects seen with the intact NSP gene are due to protein expression and demonstrate that the NSP of ToLCNDV is a symptom determinant in *N. benthamiana*.

ToLCNDV NSP elicits a hypersensitive response in *N. tabacum* and tomato. We have shown infectivity of the ToLCNDV clones to *N. tabacum* cv. Samsun by biolistic inoculation (results not shown); the infectivity of this species to *N. tabacum* has been demonstrated. Symptoms induced were leaf curling, mild mosaic, and stunting. In order to assess the effect of NSP expression on *N. tabacum* and tomato, plants were agroinfiltrated with NSP/PVX. Necrotic lesions developed on inoculated leaves 4 to 6 days postinoculation (Fig. 2C and H). No necrosis was evident on plants inoculated with the PVX or NSP^{UT}/PVX control (Fig. 2C). Plants inoculated with NSP^{UT}/PVX developed symptoms of systemic PVX infection, whereas the plants infected with NSP/PVX and showing HR did not show such symptoms.

For *N. tabacum* plants stably transformed with NSP under the control of the 35S promoter, all transformed T₀ lines (20 lines examined) showed necrosis on fully expanded leaves. The necrosis was systemic, spreading from a point (usually a secondary vein) across the whole leaf surface (Fig. 2D). All the T₀ plants died before flowering, and it was not possible to recover seeds.

To further confirm the involvement of NSP in eliciting HR in tomato, we attempted to silence NSP expression from the PVX vector by RNA interference. NSP/PVX was coinfiltrated to tomato with NSP/pFGC5941. The HR in this case was con-

siderably delayed and much weaker than in plants inoculated only with NSP/PVX (Fig. 2G). However, systemic symptoms of PVX were not visible on the leaves. These results demonstrate that ToLCNDV NSP is an avirulence determinant in *N. tabacum* cv. Samsun and tomato (cv. Moneymaker) which elicits an HR.

Deletion analysis of NSP. To determine the region(s) of the ToLCNDV NSP potentially involved in eliciting the HR in tomato, four deletion mutants were generated and expressed with the PVX vector. The results of the agroinfiltration of plants with the deletion mutant PVX constructs are summarized in Table 3. Tomato plants inoculated with constructs NSP^{dmN60}/PVX and NSP^{dmN100}/PVX, having 60 and 100 amino acid deletions at the N-terminal end of NSP, respectively, did not exhibit an HR. In contrast, both constructs with deletions of the NSP at the C terminus (NSP^{dmC30}/PVX and NSP^{dmC100}/PVX) remained capable of inducing the HR, although much less efficiently. These results suggest that sequences within the first 60 amino acids of the N terminus of the NSP may play a part in eliciting the HR.

ToLCNDV MP is not a symptom determinant. The MP of ToLCNDV was expressed from the PVX vector and used in agroinfiltration experiments on *N. benthamiana*, *N. tabacum*, and tomato. In this case, agroinfiltrated plants did not develop symptoms over those typical of a systemic PVX infection (results not shown). Similarly, *N. tabacum* plants stably transformed with MP showed no phenotype and were comparable to nontransformed plants. The MP gene was amplified from the transgenic lines and sequenced. The sequence of the transgene was 100% homologous to the wild-type MP sequence, ruling out possible mutations or deletions of MP during plant transformation. There was no problem in generating T₁ lines from the seeds of T₀ lines. These results show that the expression of MP of ToLCNDV is not a pathogenicity determinant in any of the three plant species examined.

DISCUSSION

Geminiviruses replicate within the nuclei of plant cells, and the infection is propagated throughout the plant by systemic movement of the virus. The inter- and intracellular trafficking of viral DNA is mediated by virus-encoded proteins. In the case of bipartite begomoviruses, this is carried out by MP and NSP, encoded on the component designated DNA B (9). The movement protein plays an important role in pathogenicity (6, 15, 26), and both proteins determine viral host range (17). We have analyzed the effects of expressing MP and NSP of the bipartite begomovirus ToLCNDV in *N. benthamiana*, *N. tabacum*, and tomato plants.

The results obtained with both transient expression via a PVX expression vector and constitutive expression from an integrated copy demonstrate that the NSP of ToLCNDV is a

FIG. 2. Symptoms exhibited by plants following agroinfiltration with PVX constructs and phenotype of transgenic plants. *N. benthamiana* plants were inoculated with PVX (A) and NSP/PVX (B). Leaves were agroinfiltrated with NSP/PVX (C; right leaf), NSP^{UT}/PVX (C; middle leaf), and NSP^{dmC30}/PVX (C; left leaf). The leaves of an NSP transgenic *N. tabacum* plant showed systemic spread from the leaf on which HR initiated at the base (D, left leaf) and spread over the vein (D, middle leaf), which then spread to the whole leaf (D, right leaf). Transgenic *N. tabacum* plants show initiation of HR (E) and systemic spread of HR (F). HR in tomato followed agroinfiltration with NSP/PVX (H). The necrosis spread beyond the infiltrated area across the main vein (H). When coinoculated with NSP/PVX and NSP/pFGC5941, the HR in tomato was very limited (G).

TABLE 3. Symptom induction by MP and NSP expressed from the PVX vector following agroinfiltration

Gene construct	No. of plants with symptoms/no. inoculated		
	<i>N. benthamiana</i> (leaf curling)	<i>N. tabacum</i> (necrosis)	<i>L. esculentum</i> (necrosis)
MP/PVX	0/7	0/5	0/5
NSP/PVX	7/7	5/5	5/5
NSP ^{dmN60} /PVX	0/5	0/5	0/5
NSP ^{dmN100} /PVX	0/5	0/5	0/5
NSP ^{dmC30} /PVX	3/5	4/5	3/5
NSP ^{dmC100} /PVX	2/5	3/5	2/5
NSP ^{UT} /PVX	0/3	0/3	0/3

pathogenicity determinant in *N. benthamiana*. When expressed in tomato and *N. tabacum*, NSP induces necrosis. This cell death response is typical of an HR. The necrosis in transgenic tobacco plants is systemic, as it starts from a point on the fully emerged leaf and spreads over the lamina and to other leaves. Thus, ToLCNDV NSP is an avirulence determinant that interacts with the product of a resistance gene encoded by a host defense system, possibly an R gene product, triggering a host defense response involving an HR in *N. tabacum* and tomato. This result is consistent with the findings for the bipartite New World begomovirus bean dwarf mosaic virus (10). The NSP of bean dwarf mosaic virus elicits an HR in some *Phaseolus vulgaris* cultivars. A number of other proteins encoded by geminiviruses have been shown to elicit an HR when overexpressed in plants. These include the C2 proteins of *Tomato leaf curl virus* (33) and *Tomato yellow leaf curl China virus* (39), both monopartite Old World begomoviruses, and the replication-associated protein of *African cassava mosaic virus* (38).

To further investigate the ability of ToLCNDV NSP to induce HR, N- and C-terminal NSP deletion mutants were expressed in the PVX vector. The two C-terminal deletions attenuated but did not abolish the ability of NSP to elicit necrosis in tomato. In contrast, both N-terminal NSP deletion mutants failed to induce an HR, indicating that sequences within the first 60 amino acids of the N terminus are likely important in NSP recognition by tomato in inducing the response. The N-terminal region of the begomovirus NSP contains amino acids that are involved in nuclear targeting of the protein (31). Thus, it is possible that it is the nuclear targeting sequences of the NSP that are involved in eliciting the HR.

In contrast to the NSP, transgenic plants expressing the MP of ToLCNDV showed no abnormal phenotype. This result was confirmed by the transient expression studies with PVX and differs from the findings of similar studies conducted on a number of New World begomoviruses. For *Tomato mottle virus* (6), *Bean dwarf mosaic virus* (15), *Tomato golden mosaic virus* (40), and *Bean golden mosaic virus* (34), the MP was identified as a symptom determinant. Since the MPs of New and Old World begomoviruses show little sequence relatedness (41), it is likely that this protein has evolved different roles in pathogenicity for these geographically and evolutionarily distinct groups of begomoviruses. Since this is the first demonstration of a role of NSP in pathogenicity, the analysis of other Old World bipartite begomoviruses would reveal whether this is a general property of NSP for these viruses.

It is interesting that natural infection of tomato plants by

ToLCNDV does not result in HR. The lack of HR may be due to a lower level of NSP expression during natural infection by ToLCNDV. Alternatively, the virus may have evolved mechanisms, possibly mediated by other virus-encoded proteins, to overcome the HR elicited by NSP. Brigneti et al. (1) postulated that pathogenicity determinants are suppressors of gene silencing. An analysis of the possible suppressor of the gene-silencing activity of NSP and MP and their interaction with host-encoded proteins (for NSP, the products of potential resistance genes) will form the basis of further studies.

ACKNOWLEDGMENTS

We are thankful to David C. Baulcombe for providing the PVX vector and GV strain of *Agrobacterium tumefaciens* and Claude M. Fauquet for providing the ToLCNDV clones.

This work was mainly funded by grants from the International Foundation for Science (grant no. C/3014-1) and the Commonwealth Scientific and Industrial Research Organization (Australia; grant no. CS2/1995/003). The CSIRO project is a collaboration between CSIRO Plant Industry (Adelaide) and National Institute for Biotechnology and Genetic Engineering (NIBGE) (Pakistan). We are also grateful to Rob W. Briddon, Visiting Professor (presently working at NIBGE under the Foreign Faculty Hiring Program of the Higher Education Commission, Pakistan), for assistance with preparation of the manuscript and valuable suggestions.

REFERENCES

- Brigneti, B., O. Voinnet, W.-X. Li, L.-H. Ji, S.-W. Ding, and D. C. Baulcombe. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**:6739–6746.
- Brough, C. L., R. J. Hayes, A. J. Morgan, R. H. A. Coutts, and K. W. Buck. 1988. Effects of mutagenesis *in vitro* on the ability of cloned tomato golden mosaic virus DNA to infect *Nicotiana benthamiana* plants. *J. Gen. Virol.* **69**:503–514.
- Chapman, S., T. Kavanagh, and D. Baulcombe. 1992. Potato virus X as a vector for gene expression in plants. *Plant J.* **2**:549–557.
- Dangl, J. L., R. A. Dietrich, and M. H. Richberg. 1996. Death don't have no mercy: cell death programs in plant-microbe interactions. *Plant Cell* **8**:1793–1807.
- Dong, X., R. van Wezel, J. Stanley, and Y. Hong. 2003. Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. *J. Virol.* **77**:7026–7033.
- Duan, Y. P., C. A. Powell, D. E. Purcifull, P. Broglio, and E. Hiebert. 1997. Phenotypic variation in transgenic tobacco expressing mutated geminivirus movement/pathogenicity (BC1) proteins. *Mol. Plant-Microbe Interact.* **10**:1065–1074.
- Etessami, P., R. Callis, S. Ellwood, and J. Stanley. 1988. Delimitation of essential genes of cassava latent virus DNA 2. *Nucleic Acids Res.* **16**:4811–4829.
- Fauquet, C. M., D. M. Bisaro, R. W. Briddon, J. K. Brown, B. D. Harrison, E. P. Rybicki, D. C. Stenger, and J. Stanley. 2003. Revision of taxonomic criteria for species demarcation in the family *Geminiviridae*, and an updated list of begomovirus species. *Arch. Virol.* **148**:405–421.
- Gafni, Y., and B. L. Epel. 2002. The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. *Physiol. Mol. Plant Pathol.* **60**:231–241.
- Garrido-Ramirez, E. R., M. R. Sudarshana, W. J. Lucas, and R. L. Gilbertson. 2000. Bean dwarf mosaic virus BV1 protein is a determinant of the hypersensitive response and avirulence in *Phaseolus vulgaris*. *Mol. Plant-Microbe Interact.* **13**:1184–1194.
- Gutierrez, C. 2002. Strategies for geminivirus DNA replication and cell cycle interference. *Physiol. Mol. Plant Pathol.* **60**:219–230.
- Hehnle, S., C. Wege, and H. Jeske. 2004. Interaction of DNA with the movement proteins of geminiviruses revisited. *J. Virol.* **78**:7698–7706.
- Hormuzdi, S. G., and D. M. Bisaro. 1995. Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis. *Virology* **206**:1044–1054.
- Horsch, R. B., J. E. Fry, N. L. Hoffmann, D. Eichholtz, S. G. Rogers, and R. T. Fraley. 1985. A simple and general method for transferring genes into plants. *Science* **227**:1229–1231.
- Hou, Y. M., R. Sanders, V. M. Ursin, and R. L. Gilbertson. 2000. Transgenic plants expressing geminivirus movement proteins: abnormal phenotypes and delayed infection by *Tomato mottle virus* in transgenic tomatoes expressing the *Bean dwarf mosaic virus* BV1 or BC1 proteins. *Mol. Plant-Microbe Interact.* **13**:297–308.

16. Hussain, M., S. Mansoor, S. Iram, Y. Zafar, and R. W. Briddon. 2004. First report of *Tomato leaf curl New Delhi virus* affecting chili pepper in Pakistan. New Dis. Rep. vol. 9 [http://www.bspp.org.uk/ndr/].
17. Ingham, D. J., E. Pascal, and S. G. Lazarowitz. 1995. Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity. *Virology* **207**:191–204.
18. Kerschen, A., C. A. Napoli, R. A. Jorgensen, and A. E. Muller. 2004. Effectiveness of RNA interference in transgenic plants. *FEBS Lett.* **566**:223–228.
19. Kim, C. H., and P. Palukaitis. 1997. The plant defense response to cucumber mosaic virus in cowpea is elicited by the viral polymerase gene and affects virus accumulation in single cells. *EMBO J.* **16**:4060–4068.
20. Mansoor, S., S. H. Khan, M. Hussain, N. Mushtaq, Y. Zafar, and K. A. Malik. 2000. Evidence that watermelon leaf curl disease in Pakistan is associated with *Tomato leaf curl virus*-India, a bipartite begomovirus. *Plant Dis.* **84**:102.
21. Mansoor, S., S. H. Khan, and M. Saeed. 1997. Evidence for the association of a bipartite geminivirus with tomato leaf curl disease in Pakistan. *Plant Dis.* **81**:958.
22. McGarry, R. C., Y. D. Barron, M. F. Carvalho, J. E. Hill, D. Gold, E. Cheung, W. L. Kraus, and S. G. Lazarowitz. 2003. A novel Arabidopsis acetyltransferase interacts with the geminivirus movement protein NSP. *Plant Cell* **15**:1605–1618.
23. Morel, J. B., and J. L. Dangl. 1997. The hypersensitive response and the induction of cell death in plants. *Cell Death Differ.* **4**:1318–1328.
24. Noueiry, A. O., W. J. Lucas, and R. L. Gilbertson. 1994. Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell* **76**:925–932.
25. Padidam, M., R. N. Beachy, and C. M. Fauquet. 1995. Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity. *J. Gen. Virol.* **76**:25–35.
26. Pascal, E., P. E. Goodlove, L. C. Wu, and S. G. Lazarowitz. 1993. Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease. *Plant Cell* **5**:795–807.
27. Richael, C., and D. Gilchrist. 1999. The hypersensitive response: a case of hold or fold. *Physiol. Mol. Plant Pathol.* **55**:5–12.
28. Rigden, J. E., L. R. Krake, M. A. Rezaian, and I. B. Dry. 1994. ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity. *Virology* **204**:847–850.
29. Rojas, M. R., A. O. Noueiry, W. J. Lucas, and R. L. Gilbertson. 1998. Bean dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. *Cell* **95**:105–113.
30. Rybicki, E. P., R. W. Briddon, J. E. Brown, C. M. Fauquet, D. P. Maxwell, B. D. Harrison, P. G. Markham, D. M. Bisaro, D. Robinson, and J. Stanley. 2000. Geminiviridae, p. 285–297. In M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wicker (ed.), *Virus taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*. Academic Press, San Diego, Calif.
31. Sanderfoot, A. A., D. J. Ingham, and S. C. Lazarowitz. 1996. A viral movement protein as a nuclear shuttle. *Plant Physiol.* **11**:22–33.
32. Sanderfoot, A. A., and S. G. Lazarowitz. 1995. Cooperation in viral movement: the geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the periphery. *Plant Cell* **7**:1185–1194.
33. Selth, L. A., J. W. Randles, and M. A. Rezaian. 2004. Host responses to transient expression of individual genes encoded by *Tomato leaf curl virus*. *Mol. Plant-Microbe Interact.* **17**:27–33.
34. Smith, D. R., and D. P. Maxwell. 1994. Requirement of the common region of DNA-B and the BL1 open reading frame of bean golden mosaic geminivirus for infection of *Phaseolus vulgaris*. *Phytopathology* **84**:133–138.
35. Sunter, G., J. L. Sunter, and D. M. Bisaro. 2001. Plants expressing tomato golden mosaic virus AL2 or beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. *Virology* **285**:59–70.
36. Usharani, K. S., B. Surendranath, S. M. Paul-Khurana, I. D. Garg, and V. G. Malathi. 2003. Potato leaf curl—a new disease of potato in northern India caused by a strain of *Tomato leaf curl New Delhi virus*. New Dis. Rep. vol. 7 [http://www.bspp.org.uk/ndr/].
37. van Engelen, F. A., J. W. Molthoff, A. J. Conner, J. P. Nap, A. Pereira, and J. Stiekema. 1995. pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Res.* **4**:288–290.
38. van Wezel, R., X. Dong, P. Blake, J. Stanley, and Y. Hong. 2002. Differential roles of geminivirus Rep and AC4 (C4) in the induction of necrosis in *Nicotiana benthamiana*. *Mol. Plant Pathol.* **3**:461–471.
39. van Wezel, R., H. Liu, P. Tien, J. Stanley, and Y. Hong. 2001. Gene C2 of the monopartite geminivirus tomato yellow leaf curl virus-China encodes a pathogenicity determinant that is localized in the nucleus. *Mol. Plant-Microbe Interact.* **14**:1125–1128.
40. von Arnim, A., and J. Stanley. 1992. Determinants of tomato golden mosaic virus symptom development located on DNA B. *Virology* **186**:286–293.
41. von Arnim, A., and J. Stanley. 1992. Inhibition of African cassava mosaic virus systemic infection by a movement protein from the related geminivirus tomato golden mosaic virus. *Virology* **187**:555–564.
42. Ward, B. M., and S. G. Lazarowitz. 1999. Nuclear export in plants: use of geminivirus movement proteins for a cell-based export assay. *Plant Cell* **11**:1267–1276.
43. Ward, B. M., R. Medville, S. G. Lazarowitz, and R. Turgeon. 1997. The geminivirus BL1 movement protein is associated with endoplasmic reticulum-derived tubules in developing phloem cells. *J. Virol.* **71**:3726–3733.