Journal of Plant Pathology (1998), **80** (1), 59-62 *Edizioni ETS Pisa, 1998*

SHORT COMMUNICATION

FULL-LENGTH OR TRUNCATED MOVEMENT PROTEINS OF CYMBIDIUM RINGSPOT TOMBUSVIRUS DO NOT CONFER RESISTANCE ON TRANSGENIC *NICOTIANA* PLANTS

P. Ciuffreda, L. Rubino and M. Russo

Dipartimento di Protezione delle Piante, Università degli Studi, and
Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, I-70126 Bari, Italy

SUMMARY

The full-length and truncated versions of the cymbidium ringspot tombusvirus (CymRSV) movement protein gene under the control of the cauliflower mosaic virus 35S promoter and terminator were used to transform *Nicotiana benthamiana* plants. Integration of the viral sequence in the plant genome was confirmed by PCR amplification of DNA extracts from transformed plants and by detection of virus-related transcripts in RNA extracts. Western blot analysis failed to show the presence of the transgene-encoded protein in transgenic plants. None of these plants was protected from infection when challenged with CymRSV, regardless of the type of transgene, whether full-length or truncated. It is suggested that RNA-mediated resistance was not acting in these plants and that failure to obtain resistance was due to lack of accumulation of the transgenic protein.

RIASSUNTO

LA PROTEINA DI MOVIMENTO DEL VIRUS DELLA MACULATURA ANULARE DEL CYMBIDIUM NELLA SUA FORMA INTEGRA O DIFETTIVA NON PROTEGGE LE PIANTE TRANSGENICHE DALL'INFEZIONE. Una versione intera e due difettive della proteina di movimento del virus della maculatura anulare del Cymbidium (CymRSV) sono state poste sotto il controllo del promotore 35S e del segnale di poliadenilazione del virus del mosaico del cavolfiore ed usate per trasformare piante di *Nicotiana benthamiana*. L'integrazione del transgene è stata verificata mediante analisi del DNA e dell'RNA. Al contrario la proteina corrispondente non è stata mai trovata. Nessuna linea delle numerose saggiate è risultata resistente all'infezione. Si suggerisce che questo è dovuto alla mancanza di accumulo della proteina transgenica ed all'assenza di un meccanismo di protezione dovuto all'RNA transgenico.

Corresponding author: M. Russo
Fax: +39.80.5442813
E-mail: csvvmr01@area.ba.cnr.it

Key words: movement protein, transgenic plants, resistance, tombusviruses.

Cymbidium ringspot virus (CymRSV) (genus *Tombusvirus*, family *Tombusviridae*) has icosahedral particles containing single copies of a messenger-sense single-stranded RNA genome of 4733 nucleotides (nt), containing five open reading frames (ORFs) (Grieco *et al.*, 1989) (Fig. 1). The genomic RNA functions as mRNA for translation of a 33-kDa protein (33K) and a readthrough protein of 92-kDa (92K), both of which are essential for replication (Dalmay *et al.*, 1993). The 41-kDa coat protein (CP) gene is internal and is translated from a subgenomic RNA of 2.1 kb. Two nested genes are located near the 3' end of the genome and are translated from a second subgenomic RNA of 0.9 kb into two proteins of 22-kDa (22K) and 19-kDa (19K), respectively. The 22K protein is required for cell-to-cell movement and contains a sequence motif conserved in the «30K superfamily» of movement proteins (MPs) (Mushegian and Koonin, 1993), whereas the 19K protein influences the severity of symptoms in infected *Nicotiana benthamiana* plants (Dalmay *et al.*, 1993).

Expression of active or dysfunctional MPs in transgenic plants has been reported to confer what is currently called pathogen-derived resistance (PDR) (Sanford and Johnston, 1985) to infection by several viruses. With the exception of plants transformed with the full-length MP of cowpea mosaic virus (CPMV), where resistance is suggested to be RNA-mediated (Sijen *et al.*, 1995), MP-mediated resistant plants have been obtained using truncated dysfunctional MPs (Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Beck *et al.*, 1994; Cooper *et al.*, 1995; Seppanen *et al.*, 1997) suggesting that the modified genes operate in transgenic resistant plants as dominant negative mutants (Herskowitz, 1987).

In this study we have transformed *N. benthamiana* plants with full-length or truncated versions of the CymRSV 22K gene and tested them for resistance to the virus.

The sequence encoding the CymRSV 22K protein was excised from a full-length cDNA clone by digestion

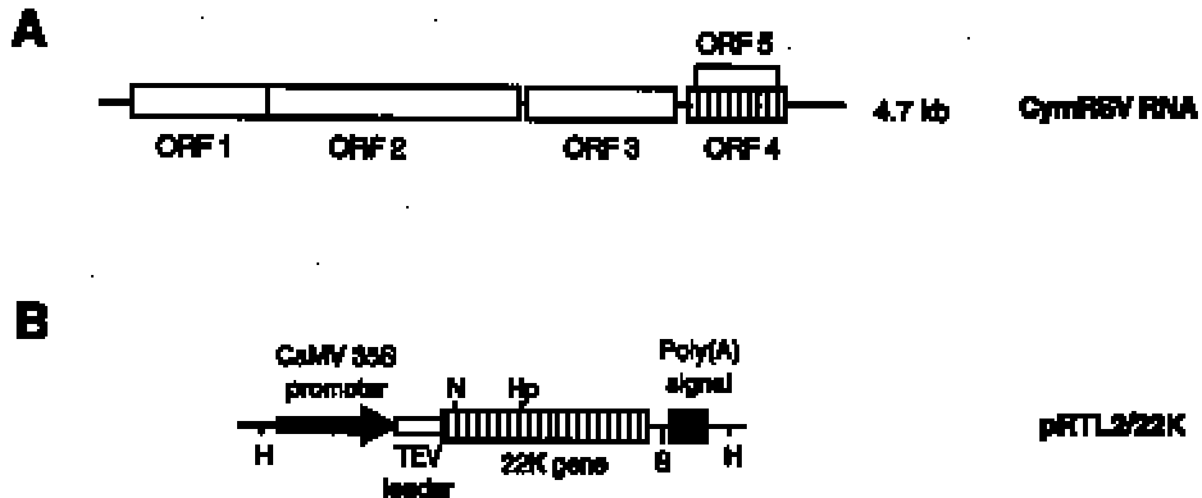

Fig. 1. A. Schematic representation of CymRSV genomic RNA showing the positions of open reading frames (ORFs). ORF 4 encodes the 22K movement protein. **B.** Schematic representation of the expression cassette from vector pRTL2 inserted in the binary vector pGA482 showing the 5' untranslated region of TEV (open box), positioned downstream of the CaMV duplicated 35S promoter, and the CymRSV 22K protein coding sequence, followed by the CaMV poly(A) signal. Relevant restriction sites are indicated: H, *Hind* III; N, *Nco* I; Hp, *Hpa* I; S, *Sma* I.

with *Pfl*M I (position 3806) and *Bst*E II (position 4482). The fragment was made blunt-ended with T4 DNA polymerase, eluted from low-melting-point agarose gel, and cloned into the *Nco* I site (made blunt-ended with Klenow enzyme) of the vector pRTL2, which contains the cauliflower mosaic virus (CaMV) 35S promoter and polyadenylation signal, and the 5' leader sequence of tobacco etch virus (TEV) (Carrington *et al.*, 1990). To obtain transgenic plants expressing truncated versions of the 22K protein, the clone containing the full-length 22K gene in pRTL2 was digested first with *Hpa* I and then either with *Nco* I or *Sma* I, made blunt-ended and religated. In this way, two defective clones were obtained: *i*) clone Hpa-Nco, coding for only the N-terminal 11 amino acids of the 22K protein or the C-terminal 83 residues if internal initiation on ribosomes occurred; (*ii*) clone Hpa-Sma, coding for the N-terminal 105 amino acids. The expression cassette was excised from the recombinant plasmid with *Hind* III and inserted into the binary vector pGA482 (An, 1986), which was mobilized into *Agrobacterium tumefaciens* LBA4404 by the triparental mating procedure (Ditta *et al.*, 1980). Leaf disks of *N. benthamiana* were used for *Agrobacterium*-mediated transformation as described by Horsch *et al.* (1985) and Carrington and Freed (1990).

Twenty lines transformed with the full-length 22K protein gene were selected for analysis by Southern and Northern blotting. DNA was prepared from leaves and amplified by the polymerase chain reaction (PCR) as previously described (Rubino *et al.*, 1992) using as primers two oligonucleotides, homologous and complementary to the sequences in the CaMV transcriptional control regions flanking the cloning site of the 22K gene. A fragment of expected size (*ca* 1000 nt) was amplified, and this hybridized with the CymRSV specific probe (Fig. 2). Northern blot hybridization analysis of RNA extracts showed the presence of transcripts of *ca.* 1 kb, corresponding to the 22K gene sequence plus the plasmid sequences and poly(A) tail. The amount of the 22K-related transcripts was similar in all lines (Fig. 3).

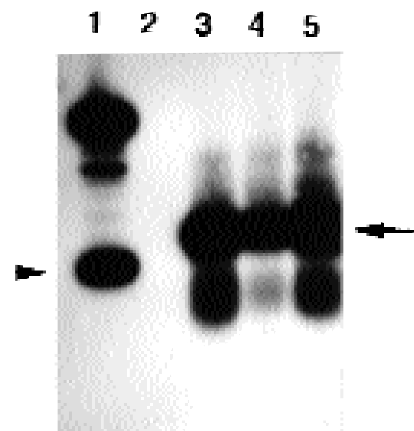

Fig. 2. Southern blot analysis of PCR products amplified from DNA extracts from three lines of *N. benthamiana* plants transformed with the CymRSV 22K protein gene (lanes 3 to 5) and untransformed (lane 2). Lane 1 contains recombinant pRTL2, carrying the 22K gene, digested with *Eco* RI and *Bam*H I. Arrow head points to a DNA fragment of about 800 nt . Arrow indicates the PCR product of the expected size of about 1050 nt. Hybridization was with a nick-translated 22K protein gene clone.

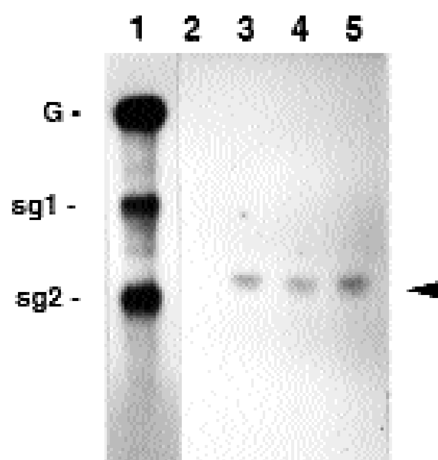

Fig. 3. Northern blot analysis of total RNA extracts from the same transgenic plants as in Fig. 2 (lanes 3 to 5) and untransformed (lane 2). Lane 1 contained RNA from a CymRSV-infected untransformed plant showing the positions of genomic (G) and subgenomic RNAs (sg1 and sg2) (4.7, 2.1, and 0.9 kb, respectively). Arrow head indicates the position of the 22K protein gene transcripts. Hybridization was with a nick-translated clone representing the 3' terminal 1000 nt.

All plants obtained from transformed tissue explants (R_0 generation) were selfed and seeds of the R_1 generation were germinated and selected on MS medium (Murashige and Skoog, 1962) containing 0.1 mg ml^{-1} kanamycin. Kanamycin-resistant seedlings were transplanted to soil and inoculated with *in vitro* synthesized CymRSV RNA as described by Rubino *et al.* (1993). Twelve plants of each line were inoculated along with untransformed control plants and inspected daily for symptom appearance. All inoculated plants reacted with local lesions in 2-3 days after inoculation, followed in 3-4 days by leaf distortion and systemic mosaic, and, after an additional 5 days, by apical necrosis and death. No difference was noticed in symptom development between transformed and untransformed plants.

Several transgenic lines with each mutant clone were obtained, screened and tested for resistance as described above. All lines reacted to inoculation with CymRSV like the controls, showing no difference in severity and time of appearance of symptoms. These plants were not further analyzed.

Proteins were extracted at random from a number of transgenic plants expressing the full-length 22K gene, fractionated, concentrated, and analyzed by Western blotting as described by Lupo *et al.* (1994). An antiserum specific to the tomato bushy stunt virus (TBSV) 22K protein was used as first antibody since it reacts with the homologous protein of CymRSV (K.-B. G. Scholthof, personal communication). The 22K protein was not detected in any transgenic plant. Hindrance to translation of the 22K gene nuclear transcripts is not a likely explanation, since with similar constructs, in which the TEV leader sequence (known to be an efficient enhancer for heterologous genes; Carrington and Freed, 1990) was used, other genes of CymRSV were correctly expressed (Rubino *et al.*, 1992; Lupo *et al.*, 1994). To verify whether the cloning and/or transformation procedures had produced a change in the sequence of the transgene, the PCR product obtained from amplification of a genomic DNA extract was cloned, sequenced, and shown to be identical to the sequence of the intact 22K gene. It was therefore hypothesized that failure to detect the 22K protein was due to its low concentration and/or intrinsic lability and lack of accumulation.

Evidence that the 22K protein did not accumulate in transgenic plants was obtained by inoculating transgenic plants with a CymRSV mutant clone (Δ22) that does not express the 22K protein as its start codon is changed to AGG (Dalmay *et al.*, 1993). It is in fact known that wild type TBSV MP can complement movement defective mutants *in trans* (Scholthof *et al.*, 1995). Clone Δ22 was further manipulated by replacing the coat protein gene with the β-glucuronidase (GUS) gene (clone Δ22-GUS). Preliminary experiments with non-transgenic plants showed that histochemical assays for GUS activity (Jefferson, 1987) allowed visualization of Δ22-GUS RNA replicating essentially in single cells, whereas replication of the clone wt22-GUS, carrying the coat protein gene replaced by the GUS gene and expressing a wild-type 22K gene, resulted in expanded blue lesions. Inoculation of transgenic plants with transcripts from clone Δ22-GUS yielded only single blue cells, thus contrasting with the blue lesions obtained by inoculation with wt22-GUS.

Failure to obtain resistant *N. benthamiana* plants transformed with the MP of CymRSV may be ascribed to the fact that wild-type or mutated transgene-encoded proteins do not accumulate to a level sufficient to interfere with the normal function of the 22K protein synthesized by the replicating viral RNA. On the other hand, in the present instance, an RNA-mediated PDR is unlikely to have been operating in any of the transgenic plants, otherwise at least some of them would have exhibited some protection against the infecting virus.

ACKNOWLEDGEMENTS

The authors thank Dr.K.-B. G. Scholthof, Texas A & M University, College Station, TX, USA for the generous gift of the antiserum to the TBSV 22K protein, Mr. Roberto Lerario for help with photography, and Prof. G. P. Martelli for critical reading of the manuscript.

REFERENCES

An G., 1986. Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cell. *Plant Physiology* **81**: 86-91

Beck D.L., Van Dolleweerd C.J., Lough T.J., Balmori E., Voot D.M., Andersen M.T., O'Brien I.E.W., Forster R.L.S, 1994. Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block. *Proceedings of National Academy of Sciences, USA* **91**: 10310-10314.

Carrington J.C., Freed D.D., 1990. Cap-independent enhancement of translation by a plant virus 5' nontranslatable region. *Journal of Virology* **64**: 1590-1597.

Carrington J.C., Freed D.D., Oh C.S., 1990. Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. *EMBO Journal* **9**: 1347-1353.

Cooper B., Lapidot M., Heick J.A., Dodds J.A., Beachy R.N., 1995. A defective movement protein of TMV in transgenic plants confers resistance to multiple viruses whereas the functional analog increases susceptibility. *Virology* **206**: 307-313.

Dalmay T., Rubino L., Burgyan J., Kollar A., Russo M., 1993. Functional analysis of cymbidium ringspot virus genome. *Virology* **194**: 697-704.

Grieco F., Burgyan J., Russo M., 1989. The nucleotide sequence of cymbidium ringspot virus genome. *Nucleic Acids Research* **17**: 6383.

Herskowitz I., 1987. Functional inactivation of genes by mutant negative mutations. *Nature (London)* **329**: 219-222.

Horsch R.B., Fry J.E., Hoffman N.L., Eichholtz D., Rogers S.G., Fraley R.T., 1985. A simple and general method for transferring genes into plants. *Science* **227**: 1229-1231.

Jefferson R.A., 1987. Assaying chimeric genes in plants: The GUS fusion system. *Plant Molecular Biology Reporter* **5**: 387-405.

Lapidot M., Gafny R., Ding B., Wolf S., Lucas W.J., Beachy R.N., 1993. A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *Plant Journal* **4**: 959-970.

Lupo R., Rubino L., Russo M., 1994. Immunodetection of the 33K/92K polymerase proteins in cymbidium ringspot virus-infected and in transgenic plant tissue extracts. *Archives of Virology* **138**: 135-142.

Malyshenko S.I., Kondakova O.A., Nazarova Ju.V., Kaplan I.B., Taliansky M.E., Atabekov J.G., 1993. Reduction of tobacco mosaic virus accumulation in transgenic plants producing non-functional viral transport proteins. *Journal of General Virology* **74**: 1149-1156.

Murashige T., Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

Mushegian A.R., Koonin E.V., 1993. Cell-to-cell movement of plant viruses. *Archives of Virology* **133**: 239-257.

Rubino L., Carrington J.C., Russo M., 1992. Biologically active cymbidium ringspot virus satellite RNA in transgenic plants suppresses accumulation of DI RNA. *Virology* **188**: 429-437.

Rubino L., Capriotti G., Lupo R., Russo, M., 1993. Resistance to cymbidium ringspot tombusvirus infection in transgenic *Nicotiana benthamiana* plants expressing the virus coat protein gene. *Plant Molecular Biology* **21**: 665-672.

Sanford J.C., Johnston S.A, 1985. The concept of pathogen-derived resistance: Deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113**: 395-405.

Scholthof H.B., Scholthof K.-B. G., Jackson A.O., 1995. Identification of tomato bushy stunt virus host-specific symptom determinants by expression of individual genes from a potato X vector. *The Plant Cell* **7**: 1157-1172.

Seppanen P., Puska R., Honkanen J., Tyulkina L.G., Fedorkin O., Morozov S.Yu., Atabekov J.G., 1997. Movement protein-derived resistance to triple gene block-containing plant viruses. *Journal of General Virology* **78**: 1241-1246.

Sijen T., Wellink J., Hendriks J., Verver J., van Kammen A., 1995. Replication of cowpea mosaic virus RNA1 or RNA2 is specifically blocked in transgenic *Nicotiana benthamiana* plants expressing the full-length replicase or movement protein genes. *Molecular Plant-Microbe Interactions* **8**: 340-347.

Received 20 October 1997
Accepted 22 December 1997