SHORT COMMUNICATION

NICOTIANA BENTHAMIANA PLANTS TRANSFORMED WITH THE COMPLETE PRE-READTHROUGH DOMAIN OR THE N-PROXIMAL REGION OF THE REPLICASE GENE FROM CYMBIDIUM RINGSPOT VIRUS RNA ARE RESISTANT TO VIRUS INFECTION

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SUMMARY

Nicotiana benthamiana plants were transformed with sequences derived from the 5'-terminal open reading frames (ORF 1 and 2) of the cymbidium ringspot tombusvirus (CymRSV). These sequences included the pre-readthrough region of the replicase or its N-terminal region, the readthrough domain, and the full-length replicase gene in which the stop codon of ORF 1 was abolished. Some lines transformed with the first two sequences showed complete resistance when challenged with CymRSV. Experiments using protoplasts from resistant lines showed that the resistance operates at the single cell level by abolishing viral replication. Lines resistant to CymRSV were susceptible to two other tombusviruses, artichoke mottled crinkle and carnation Italian ringspot. These data and the lack of correlation between transgene expression and resistance would suggest this to be a form of RNA-mediated resistance. Since resistant lines were only found among those transformed with sequences involved in the build-up of the virus replication complex, it is suggested that resistance may be due to the interference of these sequences with those of the infecting virus.

RIASSUNTO

PIANTE DI *NICOTIANA BENTHAMIANA* TRASFORMATE CON IL DOMINIO «PRE-READTHROUGH» O CON LA REGIONE N-TERMINALE DELLA REPLICASI DEL VIRUS DELLA MACULATURA ANULARE DEL CYMBIDIUM SONO RESISTENTI ALL'INFEZIONE. Piante di *Nicotiana benthamiana* sono state trasformate con sequenze derivate dalle griglie di lettura 5'-terminali (ORF 1 e 2) del virus della maculatura anulare del Cymbidium (CymRSV). Queste sequenze sono le regioni "pre-readthrough" e "readthrough" della replicasi, la porzione N-terminale della regione "pre-readthrough" e la replicasi intera in cui è stato soppresso il codone d'arresto dell'ORF 1. Alcune linee trasformate con la sequenza completa dell'ORF 1 o la sua parte N-terminale sono risultate resistenti all'infezione con CymRSV. Poichè la resistenza è risultata operante anche in protoplasti, se ne conclude che essa è dovuta ad un meccanismo di inibizione della replicazione del genoma virale. Le linee resistenti a CymRSV sono risultate suscettibili a due altri tombusvirus, il virus dell'arricciamento maculato del carciofo (AMCV) e il virus italiano della maculatura anulare del garofano (CIRV). Questi dati insieme alla mancanza di correlazione tra espressione del transgene e resistenza suggeriscono che si tratti di una forma di resistenza mediata dall'RNA. Poichè le linee resistenti si trovano solo fra quelle trasformate con le sequenze coinvolte nel complesso replicativo virale, si suggerisce che la resistenza sia dovuta all'interferenza di questa sequenza con quelle del virus infettante.

Key words: replicase, transgenic plants, resistance, tombusviruses.

The genome of cymbidium ringspot virus (CymRSV), a species of the genus *Tombusvirus* in the family *Tombusviridae*, consists of a messenger-sense, single-stranded RNA with five open reading frames (ORFs), encapsidated in 30 nm isometric particles, formed by 180 protein subunits (CP) (Grieco *et al.*, 1989; Russo *et al.*, 1994). The 5'-terminal ORFs 1 and 2 are expressed upon translation of genomic RNA, while the downstream ORFs are expressed via the synthesis of two subgenomic RNAs (Russo *et al.*, 1994). The 33K and 92K proteins encoded by ORFs 1 and 2, respectively, are N-coterminal, the 92K protein being expressed by the readthrough of an *amber* (UAG) termination codon of the 33K protein. Both 33K and 92K are necessary for viral replication (Dalmay *et al.*, 1993) and are localized in the limiting membrane of modified peroxisomes (Bleve-Zacheo *et al.*, 1997). The protein encoded by ORF 3 is the CP; those encoded by ORFs 4 and 5 are involved in virus movement and symptom expression, respectively (Dalmay *et al.*, 1993).

Nicotiana benthamiana plants inoculated with CymRSV react within 3-4 days with local lesions followed

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by systemic leaf distortion and mottling. Apical necrosis develops 10-12 days later, followed by death of the plant.

Transgenic plants transformed with a full-length replicase gene were highly resistant when challenged with CymRSV virions and immune when inoculated with viral RNA (Rubino *et al.*, 1993). The transgene of these plants was shown to be intact and functional which suggested that resistance was not a case of "dominant negative mutant". The resistance was virus-specific and inversely correlated with the level of expression of the transgene, suggesting it to be RNA-mediated (Rubino and Russo, 1995). However, given the occurrence in resistant transgenic plants of both the intact viral replicase and its pre-readthrough domain, it could not be excluded that the resistance was mediated by the protein encoded by the transgene.

The present study was aimed at verifying whether resistant plants could be obtained after transformation with mutated or incomplete forms of the CymRSV replicase gene.

N. benthamiana plants were transformed with defective forms of the CymRSV replicase gene by the same procedure used for the wild type (wt) gene (Rubino *et al.*, 1993). The following modified versions of the replicase gene were used (Fig. 1):

(*i*) the sequence coding for the 33K protein (ORF 1), between restriction sites *Nco* I (*N, Fig. 1, introduced by site-directed mutagenesis so as to include the AUG start codon) and *Eco*R V, located 40 nt downstream of the ORF 1 stop codon;

(*ii*) a truncated frameshift mutant of the 33K protein gene. This was prepared by religating the 33K protein gene clone first digested with *Bss*H II, located at nucleotide (nt) 597, then made blunt-ended with the Klenow enzyme. Lines transformed with this construct were predicted to express a protein of 164 amino acids, 149 being identical to the wt, and the remaining 15 not present in the wt as they result from frameshift, after digestion with *Bss*H II and religation;

(*iii*) the readthrough domain of ORF 2, between restriction sites *Nco* I (**N, Fig. 1, obtained by changing the 33K protein stop codon into a methionine codon) and *Ava* I, located 52 nt downstream of the ORF 2 stop codon;

(*iv*) the full-length replicase gene (ORF 2), identical to wt, except that the stop codon of the 33K protein was mutated to a methionine codon between restriction sites *Nco* I (*N) and *Ava* I.

The sequences were inserted in the pRTL2 vector containing the CaMV 35S promoter and a polyadenylation signal (Carrington *et al.*, 1990), and the expression cassette was excised as a *Hind* III fragment and cloned into the binary vector pGA482 (An, 1986). Recombinant plasmids were conjugated into *Agrobacterium tumefaciens* strain LBA4404 by the triparental mating procedure (Ditta *et al.*, 1980). Leaf disks were transformed as described by Horsch *et al.* (1985) and shoots were regenerated on MS medium (Murashige and Skoog, 1962) containing 0.1 mg ml^{-1} kanamycin. Plants regenerated from kanamycin-resistant shoots (primary transformants, R_0-generation) were further screened by Southern blot analysis of the polymerase chain reaction (PCR) products of DNA extracts.

All plants resistant to kanamycin had an amplified gene fragment of the expected size (not shown). Fourty five lines were obtained which included 12 transformants with the 33K sequence (33K lines), 23 with the readthrough region (RT lines), 10 with the frameshift mutant (Bss lines), and 10 with the readthrough fusion protein (Met lines). All primary transformants were selfed to give R_1-generation plants.

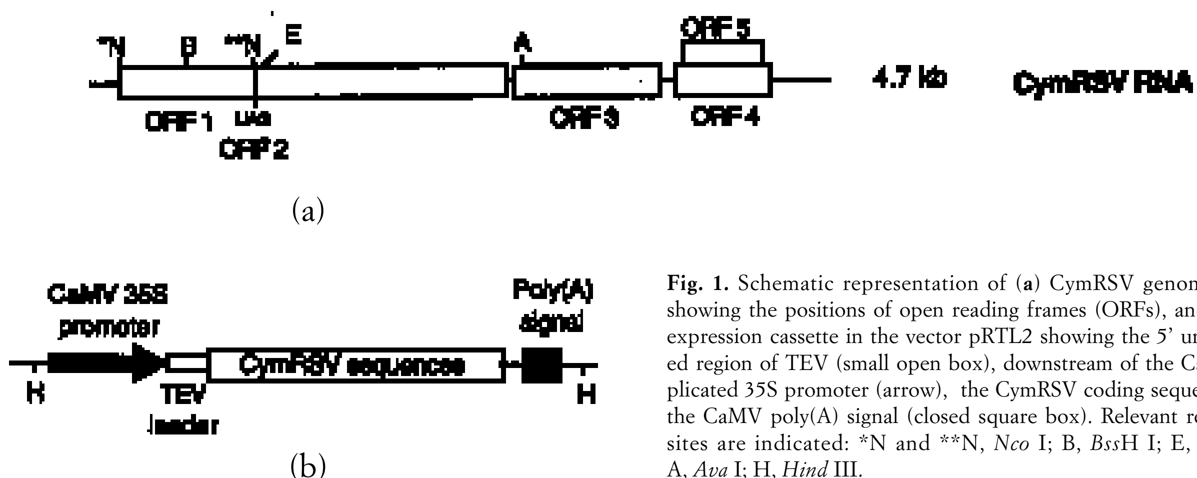

Fig. 1. Schematic representation of (**a**) CymRSV genomic RNA showing the positions of open reading frames (ORFs), and (**b**) the expression cassette in the vector pRTL2 showing the 5' untranslated region of TEV (small open box), downstream of the CaMV duplicated 35S promoter (arrow), the CymRSV coding sequence, and the CaMV poly(A) signal (closed square box). Relevant restriction sites are indicated: *N and **N, *Nco* I; B, *Bss*H I; E, *Eco*R V; A, *Ava* I; H, *Hind* III.

R_1-seeds were spread on kanamycin-containing MS medium and the resistant seedlings were transferred to soil for virus resistance assay.

At least twelve plants of each line and the controls, i.e., untrasformed plants or plants transformed with the vector pGA482 only, were inoculated with CymRSV *in vitro* transcripts as described by Rubino *et al.* (1993). Control plants became infected, as did most of the transgenic plants. However, 70-80% of line 33KA1 plants escaped systemic infection, and all plants of four other lines (33KA33, Bss6, Bss7, and Bss12), proved resistant. About one third of line 33KA1 plants that did not show systemic symptoms had chlorotic lesions on the inoculated leaves.

A number of symptomless plants of the above lines were further analysed by Northern blotting of total RNA extracts from inoculated and noninoculated upper leaves. None of the symptomless plants of lines 33KA33, Bss6, Bss7, and Bss12 contained detectable viral RNA, whereas viral RNA was found in inoculated leaves of line 33KA1 showing local lesions (not shown).

To determine if the partial resistance of line 33KA1 and the apparent immunity of line 33KA33 was at the single cell level and did not depend on inhibition of cell-to-cell movement, a protoplast system was used as described by Rubino *et al.* (1993). Protoplasts were prepared from one leaf each of five plants of line 33KA1, and of two plants of line 33KA33. These plants were then inoculated with *in vitro* synthesized CymRSV RNA and the protoplasts transfected with the same inoculum. Protoplasts were grown for 24 h prior to RNA extraction and examination by Northern blotting. RNA was extracted from the corresponding plants two weeks after inoculation. Two of the five 33KA1 plants proved susceptible to CymRSV infection, as were protoplasts prepared from them. Conversely, three plants and their protoplasts were resistant to infection. Protoplasts from 33KA33 plants did not become infected, and plants remained symptomless. Fig. 2 summarises the results of these experiments.

A number of transgenic lines was analysed for the accumulation of viral nuclear transcripts and virus-specific proteins. These included the resistant lines 33KA33, Bss12, and the susceptible lines 33KA34, RTB1, Bss2, and Met4. Leaf samples were taken at random from R_1 plants before inoculation with CymRSV *in vitro* transcripts and RNA and protein were extracted. Northern blots showed the presence of transgenic RNAs of about 1300 nt in lines 33KA33, 33KA34, Bss2 and Bss12, about 2000 nt in line RTB1 and about 3000 nt in line Met4, corresponding to the size of transgenic CymRSV sequences plus the vector sequences and the poly(A) tail. As shown (Fig. 3), there was no correlation between level of viral transcription and resistance.

Western blots of protein extracts, using a 33K-specific antiserum, were made as described by Lupo *et al.* (1994), except that antibodies to horseradish peroxidase were used in a chemiluminiscent assay (ECL; Amersham). This analysis showed the presence of the virus-encoded 33K protein in line 33KA34 but not in lines 33KA33, Bss2, Bss12 and Met4, even in overloaded gels (Fig. 4).

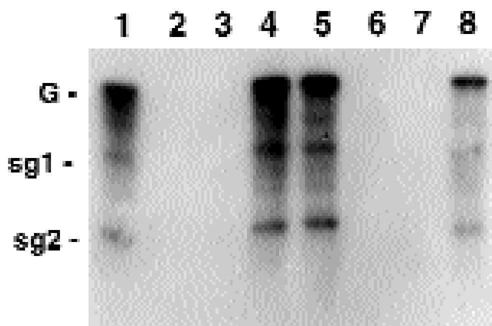

Fig. 2. Northern blots of extracts from protoplasts (lanes 1-4) or plants (lanes 5-8) inoculated with CymRSV. Lanes 1 and 5, and 2 and 6 respectively contain extracts from a susceptible and a resistant plant of line 33KA1; lanes 3 and 7 contain extracts from a plant of line 33KA33; lanes 4 and 8 contain extracts of a non-transgenic plant. G, genomic RNA; sg1 and sg2, subgenomic RNAs 1 and 2, respectively. Hybridization was with a nick-translated full-length clone of CymRSV.

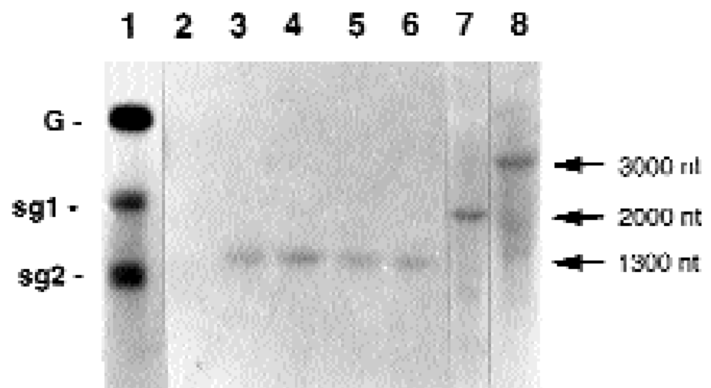

Fig. 3. Northern blots of RNA extracts from uninoculated plants of lines 33KA33 (lane 3), 33KA34 (lane 4), Bss2 (lane 5), Bss12 (lane 6), RTB1 (lane 7) and Met4 (lane 8). Lane 1 contains RNA from a CymRSV-infected untransformed plant and lane 2 from an uninfected untransformed control plant. The autoradiogram was first exposed for a few hours to mark the position of genomic (G) and subgenomic (sg 1 and 2) RNAs (4.7, 2.1, and 0.9 kb, respectively) and reexposed for 3 days. Hybridization was with a nick-translated full-length clone of CymRSV.

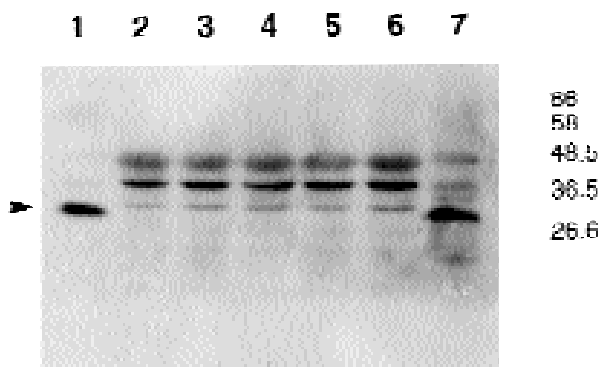

Fig. 4. Western blots of protein extracts from one plant each of lines 33KA34 (lane 1), 33KA33 (lane 2), Bss2 (lane 3), Bss12 (lane 4), Met4 (lane 5), an uninoculated untrasformed plant (lane 6), and from a CymRSV-infected untransformed plant (lane 7). Equivalent amounts of protein were loaded in lanes 1 and 7; the samples in lanes 2 to 6 were three-times more concentrated, since previous runs using the same protein concentrations as the samples in lanes 1 and 7 failed to detect the transgenic protein. Arrow points to the 33K viral product.

Western blots of transgenic lines potentially expressing the readthrough domain of the replicase could not be done because all attempts to express this part of the replicase in bacteria failed, and no antiserum could be prepared (R. Lupo, unpublished results).

As to lines Bss2 and Bss12, it is unclear whether failure to detect virus-encoded proteins was due to instability of the mutated proteins or to absence of antigenic determinants recognized by the antiserum to the entire 33K protein in the 18K predicted product.

Plants of lines 33KA1, 33KA33, 33KA34, RTA6, RTA8, Bss12, Met1, Met2, and Met3 were inoculated with carnation Italian ringspot and artichoke mottled crinkle tombusviruses. All lines were susceptible to infection with both viruses, developing local and systemic symptoms at the same time as the nontransformed controls.

In this study we have shown that transgenic plants transformed with the pre-readthrough region (ORF 1) of the CymRSV replicase gene or its amino-terminal region, are resistant to infection. The resistance was comparable to that of plants transformed with the full-length intact replicase gene (Rubino *et al.*, 1993), in that: (i) it operated at the single-cell level and did not involve inhibition of virus movement, although in one line (33KA1) both mechanisms of resistance seemed to operate; (ii) it was virus-specific; and (iii) it was not correlated with the level of transgene expression. In particular, no transgenic protein was detected in the fully resistant line 33KA33. All these characteristics are hallmarks of RNA-mediated resistance (Baulcombe, 1994).

In plants transformed with the full-length, functional replicase gene (Rubino *et al.*, 1993), both the complete replicase and its pre-readthrough region are synthesized (Lupo *et al.*, 1994). The present data indicate that resistance determinants may be contained in ORF 1 or its N-terminal portion, a sequence important for binding the virus replication complex to cell membranes (Burgyan *et al.*, 1996). It is therefore tempting to speculate that resistance may arise because this sequence interferes with that of the incoming virus in the establishment of RNA replication.

ACKNOWLEDGEMENTS

The authors thank Prof. G.P. Martelli for critical reading of the manuscript.

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Received 6 January 1998
Accepted 2 March 1998