REPLICASE-MEDIATED TRANSGENIC RESISTANCE TO TOBAMOVIRUS INFECTIONS

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Running title: Tobamovirus replicase-mediated resistance.

ABSTRACT.

Plant transformation with tobamovirus replicase gene sequences is one of a number of available strategies for the potential control of tobamovirus disease.

In most cases, a complete, near immunity type of resistance can be induced in plants transformed with fragments or modified forms of the viral replicase gene and the strong resistance is exhibited against high concentrations of both virus and viral RNA inoculum. However, the transgenic plant exhibits a relatively narrow resistance spectrum, manifested only against the virus from which the transgene is derived or against very closely related virus strains.

A more complete understanding of the molecular mechanisms involved in replicase-mediated transgenic resistance against tobamoviruses is needed, in order to increase its efficacy and reduce or eliminate the biosafety concerns related to its use in agriculture as a biotechnological tool for crop protection.

1. INTRODUCTION.

The concept of obtaining resistance to pathogens by transformation with genes derived from the genome of the pathogen, known as pathogen-derived resistance, was first introduced for plant viruses by Hamilton [1] and generalized later to other pathogens by Sanford and Johnston [2]. Since then, this virus-resistance strategy has developed rapidly and has been widely exploited to control certain plant viruses by transforming plants with different viral genes or viral sequences [reviewed in 3,4,5,6,7].

Here, we will focus on transgenic resistance induced by viral replicase gene sequences against the tobamoviruses. Use of replicase sequences to develop resistance against other plant viruses has been considered in previous reviews [8,9,10].

2. TOBAMOVIRUS DISEASES.

Tobamoviruses are disease agents of considerable importance all over the world. This is mainly due to their high infective capability, great particle stability and the large accumulation rate in infected plants. In addition, they are easily transmitted by mechanical means, therefore producing significant economic losses, especially in

protected crops, where they are readily spread by contact with agricultural practices. Different species of the *Tobamovirus* genus cause disease in important crops such as tobacco, tomato, pepper, cucurbits, leguminous, ornamentals and fruit trees [11]. Of special interest are the tobamovirus species which are able to infect pepper cultivars with genetic resistances introduced by classical breeding against other tobamoviruses [12,13].

During past years, there have been considerable advances in our understanding of the molecular nature of tobamovirus genome replication and the pathogenic effects of infection. This knowledge has become crucial to develop and analyze new transgenic resistance strategies to control tobamovirus diseases.

3. TOBAMOVIRUS GENOME.

The tobamovirus genome is a plus-sense (+), single-stranded RNA of about 6.4 kb, that encodes at least four proteins [11,14]. Two proteins, the 126- and the 183-kDa proteins, are involved in virus replication and the 183-kDa protein is an occasional readthrough product of a leaky termination codon in the 126-kDa open reading frame (ORF). The N-terminal portion of the 126-kDa protein shares similarity with methyltransferases whereas the C-terminal portion has similarity with RNA helicases. A third 30-kDa protein, known as movement protein (MP), is involved in the cell-to-cell spread of the virus, and is placed 3' of the replicase gene on the viral genome. The ORF closest to the 3' end of the genome encodes the coat protein (CP) of approximately 17.5-kDa. The MP and CP are synthesized by translation of 3'-coterminal subgenomic RNAs and are considered to be early and late proteins, respectively. A putative third subgenomic RNA contains an ORF for a 54-kDa protein [15,16]. This ORF is in the readthrough portion of the 183-kDa gene and contains the GDD motif characteristic of replicases [14], although no function is ascribed to it, nor has the protein itself been detected in tobamovirus infected tissues [10,17,18, Tenllado *et al.*, unpublished results].

The tobamovirus genome is encapsidated in rigid, elongated virus particles with helical symmetry, about 18 nm in diameter and 300 nm long, that contain approximately 2130 identical CP subunits, each of them interacting with three nucleotides of the viral genome. The type species of the *Tobamovirus* genus is Tobacco mosaic virus (TMV) [19].

4. REPLICASE-MEDIATED RESISTANCE AGAINST TOBAMOVIRUSES.

Replicase-mediated transgenic resistance was first discovered in 1990 by Golemboski *et al.* [20], in experiments looking for the function of the putative 54-kDa protein of TMV. Transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi nn) containing a slightly modified construct derived from this 54-kDa gene sequence yielded plants that were highly resistant to high concentration of both TMV virions and TMV RNA inoculum [20]. The plants did not develop systemic infection and appeared free of virus disease, although a limited virus replication in the inoculated leaves was detected [21]. Protoplasts isolated from the transgenic plants were also highly resistant to TMV RNA inoculum, showing that the inhibition of systemic virus spread was mainly due to a block at the level of virus replication [21]. However, small amount of both (+) and (-) sense TMV RNA accumulated in the inoculated transgenic protoplasts, indicating that the cells were not immune to virus infection [8,21].

In a similar study in our laboratory [22], the sequence of the 54-kDa gene from

Pepper mild mottle virus (PMMoV), a pepper resistant-breaking tobamovirus [23], was expressed in transgenic *N. benthamiana* plants, and the levels of resistance against PMMoV and other tobamoviruses were determined. In contrast to the TMV 54-kDa transgene report of Golemboski *et al.* [20], the transgenic plants exhibited two different types of resistance responses. Some plants exhibited a highly resistant phenotype since no viral symptoms were observed, although a low level of virus replication occurred. The remaining plants showed a delayed, but also highly resistant phenotype since they were early susceptible, but were able to recover from the systemic PMMoV infection (Fig. 1). This was the first time that such a delayed resistance response was reported for plants expressing viral replicase sequences [22].

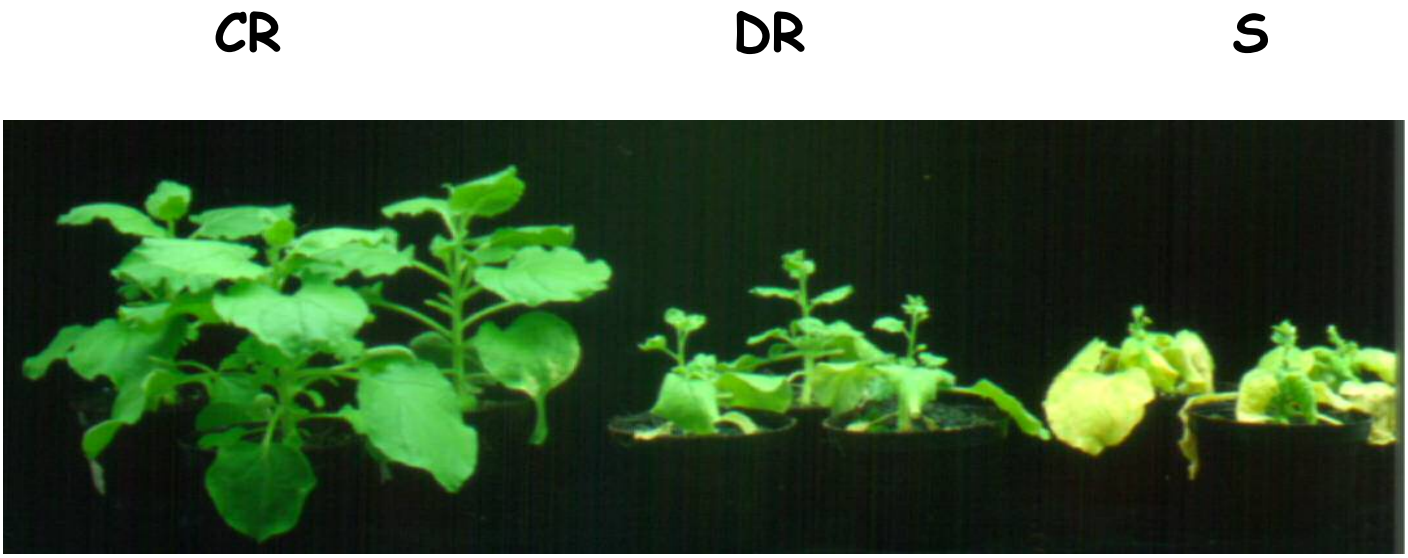

Fig. 1: Comparison of the resistance phenotypes to PMMoV in 54-kDa transgenic plants. Groups of line 12 plants, displaying the complete resistance (CR) or the delayed resistance (DR) phenotypes, and of vector-transformed control plants showing a susceptible (S) phenotype, all photographed after 40 dpi.

The differences found between TMV and PMMoV 54-kDa transgenic resistance could be due to the apparent experimental differences between both studies, that include the transformed host, the transformation vector used and the distinct sequence modifications made to the 54-kDa transgene [20,22]. However, it is not known which of these or other differences account for the contrasting results of these two sets of studies.

In another tobamovirus replicase-mediated resistance study [24], it was found that tobacco plants expressing genes encoding the full length 126-kDa or 183-kDa replicase proteins of TMV did not exhibit resistance to TMV. However, transgenic tobacco plants that contained the gene encoding the 183-kDa protein with an accidental insertion of a bacterial transposon, that resulted in a premature termination of translation at the middle of the 183-kDa ORF, were highly resistant to TMV. The truncated 183-kDa protein maintained the methylase domain and a portion of the helicase domain. The resistant plants

exhibited reduced virus replication in inoculated leaves and reduced systemic spread [24].

More recently [25], it was found that transgenic tobacco plants transformed with cDNA corresponding to the replicase domain 1 sequence of the TMV 126-kDa protein, which comprises the 5'-untranslated and the methyl transferase regions (nucleotides 1-2,149) were completely resistant to high concentration of TMV inoculum, up to as high as 1,000 ug/ml. The presence of the transgene in the plants prevented both local necrotic lesions in *N. tabacum* cv. Xanthi nc and any systemic development of symptoms in *N. tabacum* cv. Xanthi, upon TMV inoculation. Apparently, neither TMV coat protein or viral RNA was detectable in the systemic leaves of the completely resistant transgenic plants by immunochemical tests and Northern blot analysis, whereas they were accumulated in large quantities in the control plants [25].

To date, attempts to detect the 54-kDa protein in the resistant plants transformed with the TMV or the PMMoV 54-kDa gene has not been successful [18,20,22, Tenllado *et al.,* unpublished results], nor the protein has been detected in infections caused by TMV or PMMoV, [17,18, Tenllado *et al.,* unpublished results]. Detection of the truncated 183-kDa protein in the resistant transgenic plants that contain the TMV 183-kDa gene with the frame-shifted insertion [24], or the truncated 126-kDa protein in the resistant plants transformed with the replicase domain 1 sequence of TMV [25], has not been attempted.

SPECIFICITY OF TOBAMOVIRUS REPLICASE-MEDIATED RESISTANCE.

Resistance exhibited by plants expressing the transgene encoding the TMV 54-kDa protein was maintained against TMV and also against a closely related yellow mutant of TMV [20] which differed less than 1% in the CP sequence from the TMV strain from which the transgene was taken [26]. However, resistance was broken by Tomato mosaic virus (ToMV), which differs by 20% at the nucleotide level from TMV. Resistance was also broken by the more distantly related Tobacco mild green mosaic virus (TMGMV). These results suggest that the resistance was effective, only if there was a high level of sequence similarity between the viral RNA and the transgene, therefore indicating a sequence homology-dependant type of resistance.

As in the case of TMV 54-kDa plants, the plants transformed with the transgene encoding the PMMoV 54-kDa protein were resistant not only to the virus from which the 54-kDa gene was taken, but also against another very similar strain of PMMoV whose 54-kDa gene region differs slightly (7%) from the viral strain transgene sequence [27]. However, no resistance was observed against several other more distantly related tobamoviruses, including Paprika mild mottle virus (PaMMoV), which shares a CP sequence identity of 65 % with the CP region of PMMoV [28], nor against ToMV or TMV [22]. These results suggest that replicase-mediated resistance is based on sequence homology with viral RNA, in agreement with the specific spectrum of resistance operating in case of the TMV 54-kDa transgene [20].

Unlike the resistance exhibited by the transgenes encoding the TMV and PMMoV 54-kDa proteins, the resistance exhibited with the gene encoding the frameshifted 126/183-kDa protein showed a broader spectrum of resistance to a wide range of tobamoviruses, including ToMV, TMGMV and Ribgrass mosaic virus (RMV), all of them thought to be quite distinct from TMV [24]. These results indicate that the resistance mechanism is apparently unrelated

to the previous type of resistance based on RNA sequence homology.

The conflicting results described above led several groups to conduct further studies to characterize the mechanisms of resistance in plants that contain transgenes encoding tobamovirus replicase-derived proteins.

MECHANISM OF TOBAMOVIRUS REPLICASE-MEDIATED RESISTANCE.

The mechanisms of pathogen-derived transgenic resistance to viruses in plants have been extensively reviewed [29,30,31,32]. The possible mechanisms responsible for replicase-mediated resistance to tobamoviruses, with fragments or modified forms of the viral replicase gene, have been also reviewed in the last years [7,10,22] and it seems that different mechanisms may apply to each different example of resistance. The mechanism for virus resistance has been more extensively analyzed in case of the 54-kDa transgenic plants and it appear that there are conflicting models relating resistance to either RNA and/or protein expression.

As seen before, the initial reports with both TMV and PMMoV 54-kDa transgenes showed that the resistance obtained was virus strain-specific and was expressed even if high inoculum doses of either viral RNA or intact virus were employed [20,22], which are arguments used in favor of a RNA-mediated mechanism for this type of resistance. In addition, the lack of a direct relationship between the transgene expression level in both TMV and PMMoV 54-kDa plants and the resistance observed [18,20,22] was similar to other RNA-based types of resistance [9,33].

As pointed out before, in case of PMMoV and TMV 54-kDa gene-mediated resistance, the protein encoded by the viral transgene has not been detected. However, some evidences from the early studies with the TMV 54-kDa plants suggested that expression of the 54-kDa protein was necessary for resistance, leading the authors to concluded that the transgene translation product was involved in the replicase-mediated resistance mechanism [18,21]. In these studies, where TMV resistance was also observed in a transient expression system in protoplasts, the expression of a severely truncated 54-kDa protein, only 20% the size of the wild-type protein, created by inserting a premature stop codon, did not provide any resistance to TMV [18,21]. This seemed to indicate that the full-length 54-kDa protein was involved in the 54-kDa gene-mediated resistance to TMV. In a further study, it was found that the cell-to-cell trafficking of TMV RNA by the corresponding 30-kDa movement protein was suppressed in the inoculated leaves of these resistant, TMV 54-kDa gene-expressing plants but not in control plants [34]. This observation could be interpreted as indirect evidence related to the presence of the TMV 54-kDa gene product in the transgenic plants.

In case of the transgenic plants with the frame-shifted 126/183-kDa TMV gene [24], the protein could also be the effector molecule of the resistance, competing as a dominant negative mutant with the wild-type protein. Whether the broader range of resistance found with the frame-shifted 126/183-kDa TMV transgene is a function of the transgene RNA or of the encoded defective protein product remains yet unknown, since the mechanism of this resistance has not been further analyzed.

In contrast to the TMV 54-kDa gene-mediated resistance, several experiments carried out in our laboratory with transgenic plants containing the 54-kDa gene of PMMoV, provided evidences against a protein-based mechanism of resistance. Initially, the two different types of resistance

responses exhibited by these transgenic plants [22] did complicate the analysis of the resistance mechanism. Further studies indicated that expression of the PMMoV 54-kDa protein was not essential for resistance, suggesting the involvement of the RNA product of the transgene in this system [35], although a dual RNA-protein mediated mechanism was not completely ruled out at that time. These studies cited above revealed that the wild-type, full-length 54-kDa protein was not required in mediating the resistance against PMMoV, since a truncated 54-kDa coding sequence capable of yielding a protein only one-third as long as the wild-type 54-kDa protein, provided high levels of resistance to PMMoV, similar to that provided by the unmodified 54-kDa transgene (Fig. 2) [35]. These results suggested that the resistance mediated by the PMMoV 54-kDa replicase gene was mediated by RNA rather than by the 54-kDa protein itself.

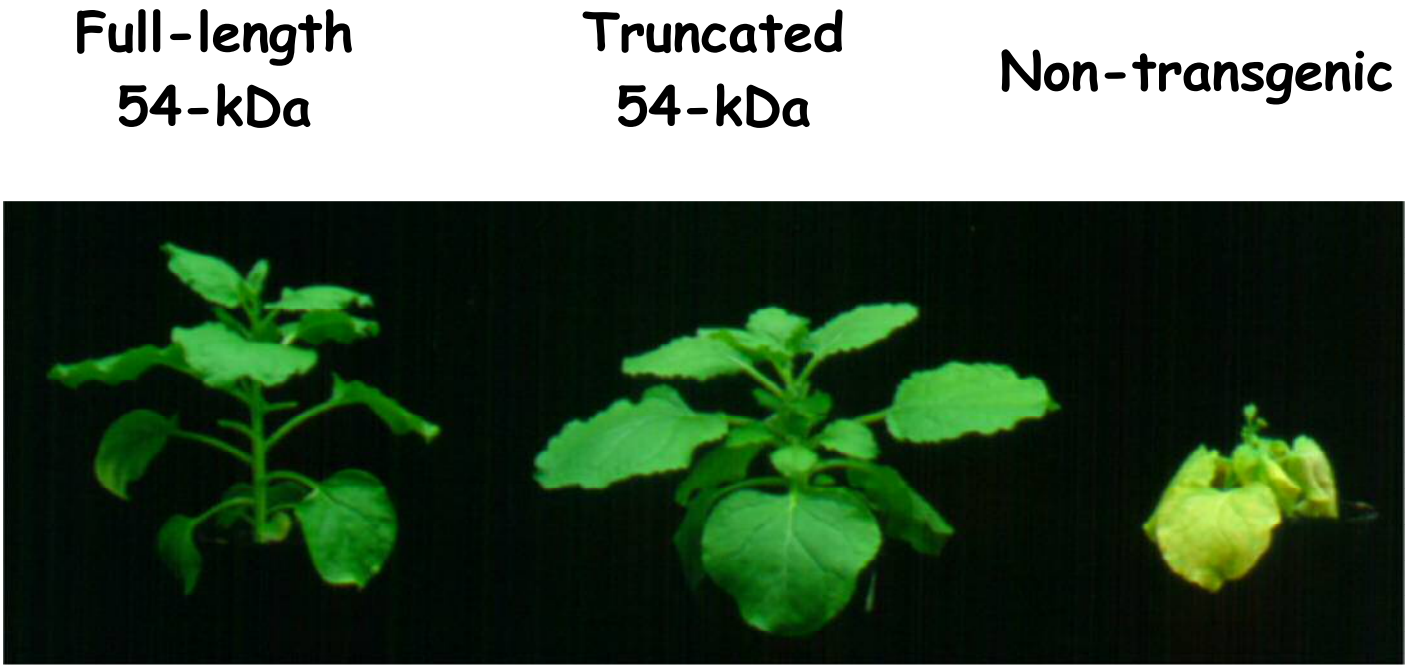

Fig. 2: Comparison of the complete resistance phenotype to PMMoV displayed by full-length 54-kDa, transgenic line 12 plants and truncated 54-kDa, transgenic line 61 plants. The susceptible phenotype displayed by a non-transgenic plant is shown at the right.

In a different study, Arce-Johnson *et al.,* [36] used transgenic plants with a construct containing the TMV 54-kDa gene sequence, similar but not identical to that reported by Golemboski *et al.,* [20]. They showed that the plants obtained with this construct exhibited moderate to high levels of resistance to TMV. Apparently, the conclusion was that the resistance they observed, on the basis of a limited number of experiments, was probably RNA-mediated rather than due to a protein-mediated effect [7]. Moreover, in a later report on transgenic plants that contained the same 54-kDa transgene developed by Golemboski *et al.* [20], Marano and Baulcombe [37] used a transient expression vector based on potato virus X (PVX) to express segments of the TMV 54-kDa gene. Plants transformed with the 54-kDa transgene were resistant to PVX

infection if the vector contained sequences of the 54-kDa gene but not if the PVX vector lacked these sequences. These results are consistent with an RNA-mediated mechanism similar to those described in a variety of systems which are due to gene silencing processess [7,10,30,31,38]. In addition, this report showed that, unlike other examples of gene silencing associated with virus resistance, the target of the resistance mechanism was the negative-strand of the 54-kDa RNA sequence and thus, the silencing was specific for the anti-sense rather than the coding strand of the target RNA. Based on these data, the authors proposed a strandspecific type of gene silencing for the resistance against TMV mediated by the 54-kDa region of the viral replicase [37].

On the other hand, it is well known that plants that are resistant via RNA-mediated, gene silencing mechanisms in general do not accumulate significant amounts of transcripts derived from the targetted transgene [33,39,40]. Again, there is conflicting evidences in this regard with the tobamovirus 54-kDa replicase-mediated resistance.

In the initial studies with the TMV 54-kDa transgenic plants [20], there was no correlation between resistance and either high or low levels of 54-kDa transgene mRNA expression. Futhermore, the resistant plants accumulated significant amounts of sense RNA transcripts [20,22], in contrast to the low level of transgene RNA observed in many other examples of homology-dependent gene silencing resistance [39,40,41,42]. Similarly, in the Marano and Baulcombe study [37], using transgenic plants with the same TMV 54-kDa transgene developed by Golemboski *et al.,* [20], the 54-kDa transgene transcripts were again easily detected, in RNA samples isolated from leaf tissue of non-infected 54-kDa transgenic plants 20 days after germination.

In the case of the transgenic plants expressing the PMMoV 54-kDa replicase sequences, preliminary analysis for transgene expression in the plants displaying complete or delayed resistance phenotypes, before and after PMMoV inoculation, showed that the two types of resistance responses appeared to be independent of the transgene transcript level [22,35] and thus, transgene silencing targetted against 54-kDa sense RNA was not observed in those plants displaying resistance [22].

Interestingly, the analysis in the work cited above [22,35], was performed with tissue samples collected from plants at an early stage of development (20 days after germination), as in the case of Marano and Baulcombe [37]. However, more recent analysis of transgene expression [43], done at a later stage of development (40 days after germination), showed that the plants displaying either the complete or the delayed resistance phenotype contained undetectable or very low levels of 54-kDa mRNA (Fig. 3). None of the plants that showed high levels of transgene mRNA at early times were resistant to virus infection [43]. When the level of transgene transcription was analyzed in nuclear run-off experiments, the resistant plants with low levels of transgene mRNA accumulation showed high levels of nascent transcripts. These results support the view that this is a type of RNA-mediated resistance and that the lack of accumulation of transgene transcripts is the result of post-trancriptional processes due to transgene silencing [35,43]. Thus, probably, PMMoV 54-kDa transcripts may be silenced to different degrees at different developmental stages. These findings are consistent with examples in which transgene silencing increases throughout plant development [42,44,45] and would explain the easy detection of transgene transcripts in TMV-resistant 54-kDa transgenic plants at an early stage of development. Alternatively, a gene

silencing mechanism directed against antisense RNA on the TMV 54-kDa transgenic plants, as proposed by Marano and Baulcombe [37], would also explain why significant amounts of sense 54-kDa transgene transcripts are detected in the plants exhibiting TMV 54-kDa homology-dependent resistance.

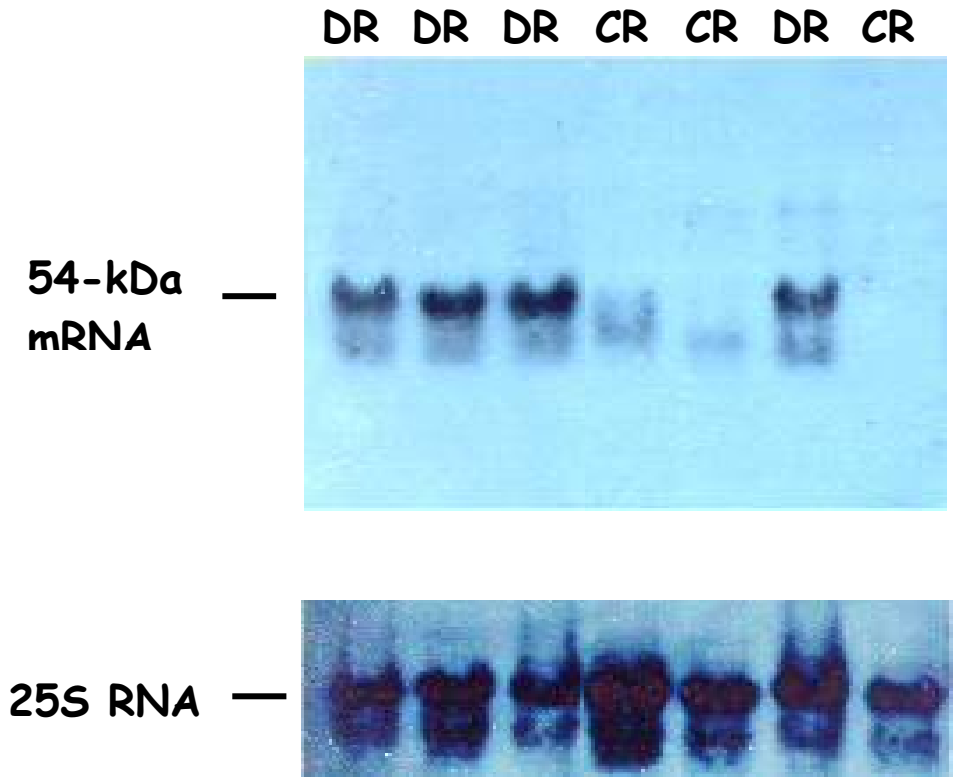

Fig. 3: Gel blot analysis of total RNA from PMMoV 54-kDa homozygous plants. Analysis of the 54-kDa mRNA accumulation levels in R2 line 12 plants before they expressed complete resistance (CR) o were initially susceptible to PMMoV infection, later expressing delayed resitance (DR). After removal of the leaf sample for RNA analysis, the plants (40 days after germination) were inoculated with PMMoV and the resistance phenotype of each plant was determined. The blot was sequentially hybridised with riboprobes homologous to PMMoV 54-kDa gene (top) and to rRNA (bottom).

Additionally, we found that in PMMoV 54-kDa transgenic plants showing complete resistance, silencing was established prior to virus infection and plants were near immune, whereas in transgenic plants showing delayed resistance, silencing was triggered only after viral infection became established. We also determined that complete resistance against PMMoV infection was strictly correlated with homozygosity of the transgene [43], a finding that introduce gene dosage as a factor in the type of resistance response observed in the PMMoV 54-kDa transgenic plant system. These and other observations suggested us that transgene homozygosity was a necessary condition to confer complete resistance to PMMoV by a gene silencing mechanism, although the exact features allowing expression of the complete resistance phenotype remain an open question. Dosage dependency of the PMMoV 54-kDa gene resistance is a feature this system shares with some other transgenic virus resistances

conferred by gene silencing phenomena [32,39,42].

Thus, in the light of these and other similar studies there is growing evidence that homology-dependent resistance mediated by the tobamovirus 54-kDa gene sequences may be related to sequence-specific gene silencing. Accordingly, this raises the possibility that the tobamovirus 54-kDa replicase resistance is mediated by RNA rather than by the 54-kDa protein, although the RNA- and protein-mediated models are not mutually exclusive.

DISEASE CONTROL AND ENVIRONMENTAL RISK OF TOBAMOVIRUS REPLICASE-MEDIATED RESISTANCE.

Some strategies for the production of virus resistant plants by genetic transformation appear to be more effective and/or useful than other strategies, as a means to control virus disease in agriculture. Transformation with replicase sequences is only one example of a number of available strategies.

This replicase-mediated resistance strategy has proved to offer important and beneficial characteristics for the potential control of tobamovirus disease, in most cases analyzed up to date. These characteristics are the complete, near immunity type of resistance that can be induced in plants transformed with tobamovirus replicase gene sequences and the fact that the plants exhibit very strong resistance to high concentrations of both virus and viral RNA inoculum [20,21,22,24,25,35]. This contrast with most other types of transgene induced resistance in which disease is only reduced or delayed and/or may be overcome with increased inoculum doses. Thus, tobamovirus replicase-mediated resistance would be particularly useful when a complete, highly resistant trait is needed.

In contrast, tobamovirus 54-kDa replicase-mediated resistant plants show a relatively narrow resistance spectrum, since the high specificity of the virus resistance is manifested only in the plants inoculated with the virus from which the transgene is derived or with its very closely related virus strains [20,22]. This homology-dependent type of resistance would mean that crop plants from fields in which different strains of a virus are present might not be protected.

One environmental effect that could be derived from the above characteristic of tobamovirus replicase-mediated resistance is the potential risk of selection of related virus strains or resistance-breaking isolates. In the case of PMMoV 54-kDa resistant plants, we have tested effective resistance against a broad range of closely related viruses as well as PMMoV strains or pathotypes and found that resistance is effective for both PMMoV pathotypes P_{12} and P_{123}, the only two pathotypes described to date in pepper crops [46,47,48,49, Tenllado and Díaz-Ruíz, unpublished data].

As with other strategies for the production of virus resistant plants by genetic transformation, another environmental concern of tobamovirus replicase-mediated resistance is the possibility of recombination between the viral mRNA produced by the transgenic plant and a more or less distantly related virus infecting the transgenic plant. However, such events seems very unlikely, since the recombinant virus would probably need a selective advantage over the wild-type challenging virus in the transgenic plant to become amplified and transmitted to other host plants.

Another potential environmental risk is the possible synergistic interaction between viral transgenes and a more or less distantly related virus infecting the transgenic plant, if any of them have the capacity to encode gene silencing suppressor

proteins [50,51,52]. In the case of tobamoviruses it has been shown that transgenic plants expressing the TMV or the PMMV 54-kDa gene did not show synergism after infection with either cucumoviruses or potyviruses [20, Tenllado and Díaz-Ruíz, unpublished data], two virus groups with members known to code for gene silencing suppressors [53,54], nor 54-kDa protein itself has suppressor activity (Tenllado and Diaz-Ruiz, unpublished result). Thus, there is currently no evidence of the involvement of tobamovirus replicase genes in synergism. In addition, such a possibility of synergistic interaction with other viruses could always readily be tested.

In any case, further investigations are important to gain a more complete understanding of the molecular mechanisms of tobamovirus replicase-mediated transgenic resistance, in order to be able to increase its efficacy reducing or eliminating the biosafety concerns related to its use in agriculture as a biotechnological tool for crop protection.

ACKNOWLEDGEMENTS

D.K., F.A.A., P.G. and D.B. are recipients of fellowships from Hungarian Academy of Sciences (Hungary), CONICET (Argentina), MEyC-PFPI and Comunidad de Madrid (Spain), respectively. F.T. is recipient of a contract from Consejo Superior de Investigaciones Científicas (Spain). Work contributing to this article was supported by project grants BIO97-0615-C02-01; BIO98-0849; BIO2000-1605-C02-02 and BIO2000-0914 from CICYT and 07B/0026/1999 from Comunidad de Madrid (Spain).

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