Plant Molecular Biology **21**: 89–97, 1993.

Genetically engineered resistance against grapevine chrome mosaic nepovirus

Véronique Brault [1], Thierry Candresse *, Olivier le Gall, René Pierre Delbos, Maryvonne Lanneau and Jean Dunez

Station de Pathologie Végétale, INRA, BP 81, 33883 Villenave d'Ornon Cédex, France (author for correspondence); [1] present address: Department of Plant Pathology, Iowa State University, Ames, Iowa 50010, USA*

Received 28 April 1992; accepted 22 August 1992

Key words: coat protein, genetically engineered protection, nepovirus, resistance, tobacco, transgenic plants

Abstract

Nepoviruses are a group of isometric plant viruses with a genome divided between two-single-stranded, positive-sense, RNA molecules. They are usually transmitted by nematodes and a number of them have significant economic impact, especially in perennial crops such as grapevine and fruit trees. Like all other picorna-like viruses, nepoviruses express their coat protein (CP) as part of a larger polyprotein which is further processed by a virus-encoded protease, a feature which poses specific problems when trying to express the viral coat protein in transgenic plants. A hybrid gene, driving the high-level expression of the CP of grapevine chrome mosaic nepovirus (GCMV) has been constructed and transferred to the genome of tobacco plants. Progeny of CP-expressing transformants show resistance against GCMV. When compared to control plants, fewer inoculated plants become infected and those that become infected accumulate reduced levels of viral RNAs. This protection was also shown to be efficient when plants are inoculated with purified viral RNA.

Introduction

Nepoviruses are an important group of phytoviruses characterized by isometric particles and by their transmission by soil-inhabiting longidorid nematodes (*Xiphinema* and *Longidorus*) [14, 21]. Their genome is divided between two separately encapsidated single-stranded genomic RNAs of positive polarity. The genomic organization and expression mechanisms of these RNAs indicate that nepoviruses should be regarded as members of the picorna-like supergroup of RNA viruses [13]. In addition to being nematode-transmitted, nepoviruses are also frequently seed-transmitted. A number of nepoviruses have a very important economic impact, mostly on perennial crops. These include nepoviruses infecting grapevine (arabis mosaic virus, grapevine fanleaf virus) and nepoviruses infecting fruit trees and small fruits (tomato ringspot virus, cherry leaf roll virus, strawberry latent ringspot virus, raspberry ringspot virus). Due to its rather limited geographic distribution, Hungarian grapevine chrome mosaic virus (GCMV), the virus used in this study, does

not have a major economic significance [20]. In regions where it is prevalent, such as several eastern European countries, it can however be locally very damaging as it induces, in infected grapevines, symptoms that are as severe as those caused by grapevine fanleaf virus. GCMV has a rather restricted host range but, in addition to grapevine, it is able to replicate efficiently in inoculated leaves of tobacco (*Nicotiana tabacum*) although it does not spread systemically in this host.

Since the original report describing the resistance to tobacco mosaic virus (TMV) of transgenic tobacco plants expressing TMV coat protein (CP) [24], similar results have been obtained with a number of other plant viruses [3]. So far, potyviruses such as soybean mosaic virus (SMV) [28] and potato virus Y (PVY) [18] are the only phytoviruses of the picorna-like supergroup of RNA viruses for which CP-mediated protection has been described. Building genes driving the expression, in transgenic plants, of the coat protein of such picorna-like viruses poses specific problems: the CP-coding sequence is deprived of upstream translation regulatory signals because the coat protein is normally expressed as the C-terminal part of a polyprotein.

The obtention of transgenic tobacco (*Nicotiana tabacum*) plants expressing the coat protein of grapevine chrome mosaic nepovirus (GCMV) is reported here. High-level expression of GCMV CP has been demonstrated in the transformed plants. CP-producing R1 and R2 plants have been obtained and shown to express resistance to GCMV infection, extending the usefulness of genetically engineered protection to nepoviruses.

Materials and methods

Virus and plants

Grapevine chrome mosaic virus [20], a gift from Dr G.P. Martelli (University of Bari, Italy) was propagated under greenhouse conditions in *Chenopodium quinoa*. The virus was purified [9] and stored at −80 °C until used for plant inoculation. Viral RNAs were purified according to [7] and kept frozen at −80 °C until needed.

Construction of the transformation vectors

All recombinant DNA techniques were performed according to standard protocols [1, 19]. A hybrid gene composed of TMV Ω′ leader [12], an AUG initiating codon and GCMV coat protein and 3′ non-coding sequences was constructed by replacing the GCMV 5′ non-coding sequence present in plasmid pC19 [6] by TMV Ω′ sequence. This hybrid gene was further subcloned in the expression cassette of pMarcel35 [27] and the construction, composed of CaMV 35S promoter, the hybrid gene and a nopaline synthase (NOS) terminator finally transferred to a pBin19 [5] derivative, pKHG4, containing kanamycin resistance, hygromycin resistance and GUS selection markers (Fig. 1; T. Candresse *et al.*, unpublished).

Transformation of tobacco

Tobacco plants (*Nicotiana tabacum* var. Xanthi) were transformed using the leaf disk procedure as described [11] and *Agrobacterium* strains LBA4404 [16] harboring the various binary vectors. In all cases, plantlet regeneration media contained kanamycin as the selective agent. Regenerated plants were finally transferred to the greenhouse and selfed to obtain R1 and R2 progeny.

Analysis of transgenic plants

Transcription of the transgene was checked either in primary transformants or in R1 and R2 progenies by northern blotting experiments using ^{32}P-labelled, strand-specific RNA probes [22]. Correct expression of the GCMV coat protein was checked using antisera raised against the purified viral particles. The GCMV CP was detected either by a DAS ELISA procedure or by western

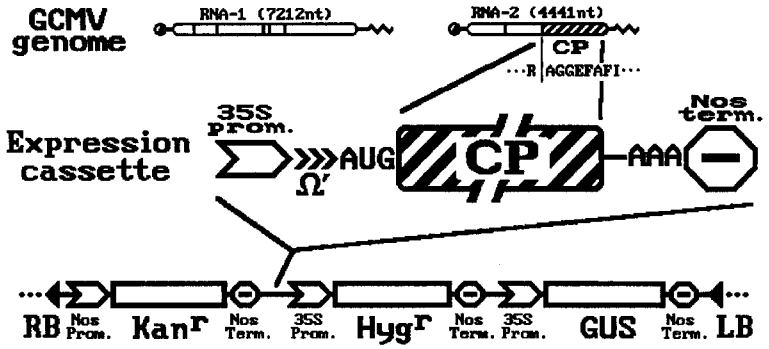

Fig. 1. Schematic representation of the T-DNA part of plasmid pKHG4 and of the hybrid gene driving the synthesis of GCMV CP. Top part: structure of the GCMV genome and position of the CP coding region (hatched). The amino acid sequence around the CP cleavage site is indicated. Middle: structure of the expression cassette containing the mutated GCMV CP-coding sequence. Ω', TMV Ω' leader; AUG, initiation codon introduced by site-specific mutagenesis; AAA, GCMV 3' non-coding region and poly(A) tail. Bottom part: structure of the T-DNA of non-recombinant pKHG4. RB and LB, right and left borders of the T-DNA; Kan^R, Hyg^R and GUS, kanamycin resistance, hygromycin resistance and β-glucuronidase transformation markers, respectively. The position of insertion of the GCMV CP expression cassette is indicated.

blotting. For western blotting experiments, plants were ground 1:4 (w/v) in AGG buffer (150 mM Tris-HCl pH 6.8, 10% SDS, 25% 2-mercaptoethanol) and 20 μl of the extracts analyzed by denaturing polyacrylamide gel electrophoresis [17], before being blotted to nitrocellulose membranes. The coat protein was finally detected using a rabbit anti-GCMV serum and peroxydase-conjugated anti-rabbit goat antibodies [8].

Analysis of protection against GCMV infection

Greenhouse-grown, four-week-old tobacco plants were dusted with carborundum (400 mesh) and inoculated with a purified GCMV suspension prepared in 50 mM Na-K phosphate buffer pH 7. A 20 μl portion of the viral suspension was applied and rubbed onto two leaves of each plant (10 μl/leaf). Two concentrations were usually used, 1 and 10 μg of virus per ml of buffer. After two weeks, the inoculated leaves were collected, weighted, and ground in two volumes of 50 mM Na-K phosphate buffer pH 7. The extracts were treated and spotted onto nitrocellulose membranes as described [7]. The viral RNAs were finally quantitated by hybridization with ^{32}P-labelled RNA probes corresponding to each of the genomic RNAs. After hybridization, the membranes were autoradiographed for 48 h at −80 °C with intensifying screens and then cut and the radioactivity retained in individual spots on the filters determined using a Kontron Betamatic scintillation counter.

Protection against viral RNA inoculation was analyzed in the same way except that plants were inoculated with purified viral RNAs in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) containing 1 mg/ml bentonite.

Results

Transformation of tobacco with the GCMV CP gene

We have previously reported the construction of a hybrid gene driving the production of GCMV CP [6]. This gene was obtained through a mutagenesis introducing an AUG codon upstream of the CP-coding sequence and linking it to the GCMV RNA2 5' non-coding sequence. Analysis of the *in vitro* translation product of this construction showed that it comigrates with authentic GCMV CP obtained from purified virions, demonstrating the correct identification of the CP cleavage site [6]. In order to try to boost the expression of CP in transgenic plants, the GCMV 5' non-coding region was replaced with the

5′ non-coding region of TMV (Ω leader), a regulatory element which has been reported to increase the translation efficiency of downstream sequences, both *in vitro* and *in vivo* [12]. Indeed, in *in vitro* translation experiments, the construct with the TMV Ω′ leader proved about twice as efficient as the construct with the GCMV leader (results not shown). The construct containing the TMV leader was cloned between a CaMV 35S promoter and a nopaline synthase (NOS) polyadenylation signal and further moved with this expression cassette to a binary plant transformation vector, pKHG4 (Fig. 1, T. Candresse *et al.*, unpublished). R1 and R2 progenies of tobacco plants (*N. tabacum*) transformed by the disarmed *Agrobacterium tumefaciens* strain LBA4404 [16] harboring either the non-recombinant vector or the vector containing the GCMV CP-coding region were obtained.

Expression of GCMV CP in transgenic tobacco plants

Genetic analysis showed most transformed plants to include a single copy of the transferred T-DNA and correct Mendelian segregation of the Kan^R and GUS transformation markers. Correct transcription of the CP transgene was confirmed by northern blotting experiments in which a single mRNA of the expected size was detected (Fig. 2). Accumulation of the GCMV coat protein could readily be detected in the transgenic plants by a sandwich-type ELISA assay (result not shown). Of the 22 transformants regenerated, 19 were shown by ELISA to express GCMV CP. The 3 plants in which no CP expression was detectable were also found to be negative for GUS activity and therefore discarded. Depending on the transgenic line used, the level of CP accumulation can be shown to be about 3–5 times lower than the levels observed during systemic infection of *Chenopodium quinoa*, the host plant routinely used for the multiplication of GCMV in our laboratory. Precise quantitations performed on a few transgenic lines show accumulation levels of up to 2–6 μg of GCMV CP per gram of fresh leaves

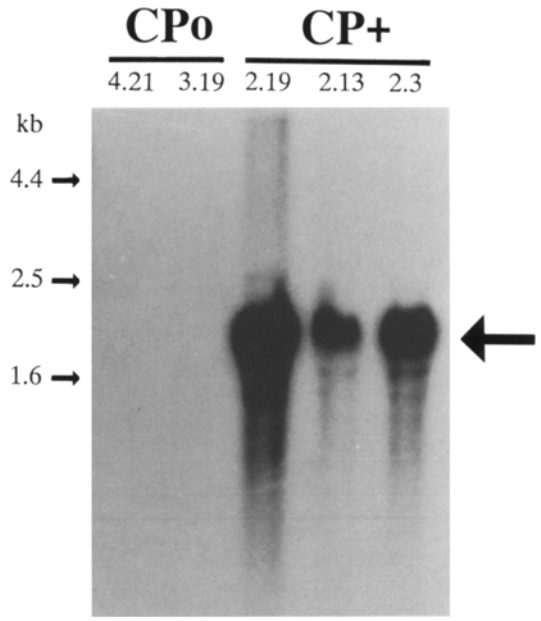

Fig. 2. Northern blotting detection of the transcription of the GCMV CP gene in transgenic *N. tabacum* plants. Ten micrograms of total RNAs extracted from two non-expressor lines (CP^0, 4.21 and 3.19) and three expressor lines (CP^+, 2.19, 2.13 and 2.3) were analyzed by denaturing agarose gel electrophoresis and blotted on a nitrocellulose membrane. The blot was then probed with a strand-specific, *in vitro* transcribed RNA probe covering the coat protein-coding region. Autoradiography was for 48 h at −80 °C, using intensifying screens.

(result not shown, see also western blotting experiment data). The first western blotting experiments performed to try to detect GCMV CP in ELISA-positive lines proved unsuccessful. Only the use of highly denaturing buffers (see Materials and methods) allowed efficient solubilization and detection of the GCMV CP. Figure 3 presents the result of an experiment in which several R2 lines were tested for GCMV CP expression. As can be seen, GCMV CP is readily detected in CP transgenic lines (noted hereafter CP^+). As expected, no expression is detected in the non-recombinant vector control line (CP^0) or in untransformed *N. tabacum* plants. Contrary to the high variability in transgene expression which is usually observed between independent transformants (e.g. [24]), all GCMV CP expressors seem to accumulate this protein to essentially similar levels. At the moment, there is no explanation for

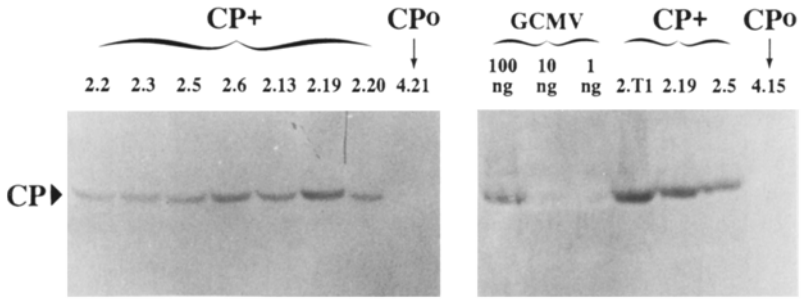

Fig. 3. Immunodetection by western blotting of GCMV CP in transgenic *N. tabacum* plants. Non-expressor lines (CP^0, 4.21 and 4.15) transformed with the non-recombinant vector were included as control, as well as 100, 10 and 1 ng of purified GCMV CP diluted in non transgenic tobacco extract (GCMV). Samples equivalent to 5 mg of fresh plant were loaded on 12% SDS-polyacrylamide gels, blotted on nitrocellulose membranes and detected using a rabbit polyclonal antiserum directed against purified GCMV particles.

this. The structure of the T-DNA transferred, with its multiplicity of genes and hence the presence of 3 CaMV 35S promoters and of their respective enhancer elements (see Fig. 1), could explain this effect.

Virus resistance of GCMV CP-expressing plants

Although *Nicotiana tabacum* is not a systemic host for GCMV, the virus will replicate in the inoculated leaves without inducing symptoms. This property was used to try to detect resistance against GCMV in the R1 and R2 progeny of CP^+ plants. Following inoculation of one-month-old plants with varying concentrations of purified GCMV, infection was scored and accumulation of the two viral RNAs was quantitated using a dot blot molecular hybridization assay [7]. The choice of this detection system was dictated by the fact that endogenous CP interferes with the ELISA assay and also because the hybridization assay allows independent quantification of the accumulation of each of the GCMV genomic RNAs. The type of results obtained is illustrated in Fig. 4 which presents an autoradiogram of a hybridization membrane on which extracts from individual CP^+ and CP^0 plants were analyzed. Fewer expressor plants became infected at the low inoculum concentration (1 μg/ml) and even at the high inoculum concentration (10 μg/ml), where most CP^+ plants became infected, they showed a strong reduction in the accumulation of the viral RNAs as compared to the CP^0 controls. Using

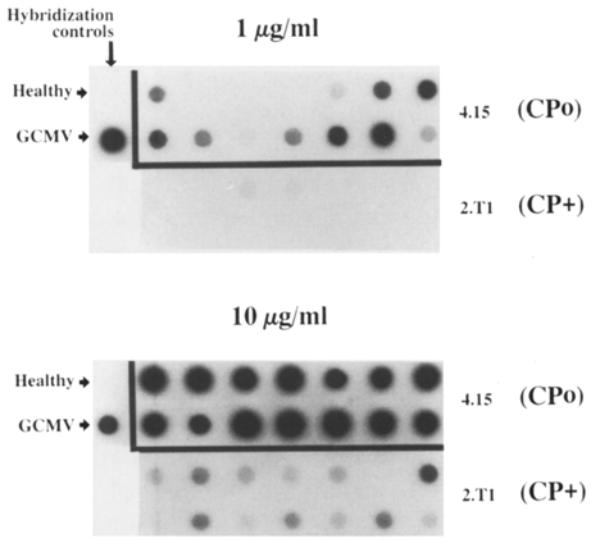

Fig. 4. Detection, by dot blot molecular hybridization, of GCMV RNA2 accumulation in the inoculated leaves of a transgenic GCMV CP expressor R2 line (CP^+, 2.T1) and of a non-expressor R2 control line transformed with the non recombinant vector (CP^0, 4.15). One-month-old plants were inoculated with 20 μl of either 1 μg/ml or 10 μg/ml purified GCMV particles. After two weeks, the inoculated leaves of each plant were harvested, weighted, ground and individually spotted on a nitrocellulose membrane. The membranes were hybridized with an *in vitro* transcribed RNA probe specific for GCMV RNA2. Positive (GCMV: GCMV-infected *Chenopodium quinoa*) and negative (uninoculated CP^+ line) hybridization controls were included on each membrane. Autoradiography was for 48 h at room temperature without intensifying screens.

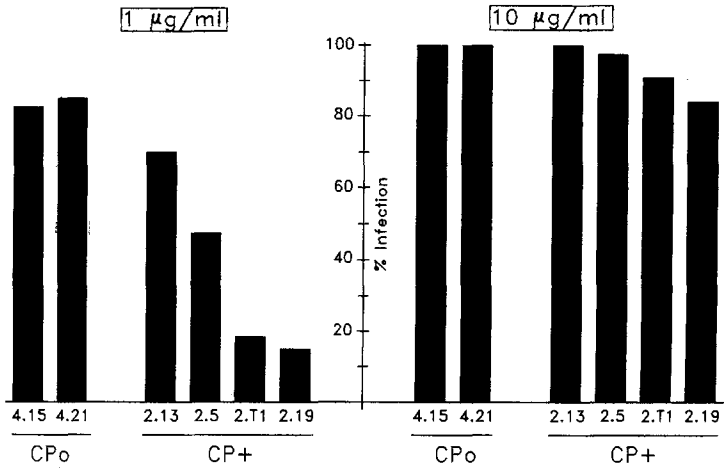

Fig. 5. Percentage of GCMV CP expressor (CP^+, lines 2.13, 2.5, 2.T1 and 2.19) or non-expressor plants (CP^0, lines 4.15 and 4.21, transformed with the non recombinant vector) infected by GCMV. One-month-old plants were inoculated with either 1 or 10 µg/ml of purified GCMV, processed as described in the legend of Fig. 4 and the number of infected plants determined by inspection of the autoradiographs. The values given are the average of several experiments involving a total number of 34 (4.15), 40 (2.13, 2.5), 54 (2.T1), 60 (4.21) and 94 (2.19) R1 or homozygous R2 progeny plants.

this strategy, 9 CP-expressing lines for which progenies had been obtained were screened and two lines (2.19 and 2.T1) showing the highest protection level were selected. The results derived from a series of experiments spanning a period of several months are synthesized as average values on Figs. 5 and 6. For the sake of clarity, only the results on two CP^0 and 4 CP^+ lines (two expressing an intermediate level of resistance (representative of the behavior of 7 of the 9 CP^+ lines tested) and the two lines expressing high resistance) are presented. Figure 5 summarizes the results expressed in terms of number of infected plants. As can be seen, at low inoculum concentration (1 µg/ml) fewer CP^+ plants become infected, specially for the two lines expressing the high resistance. At a higher inoculum concentration (10 µg/ml), this difference tends to vanish although a slight effect is still observed with the two most protected lines. Figure 6 presents the results in terms of accumulation of both viral RNAs in the infected plants, expressed as a percentage of their accumulation in the non-expressor controls. As can be seen, at both inoculum concentrations used, a strong reduction in the accumulation of both viral RNAs is observed in the CP expressors. The accumulation of the

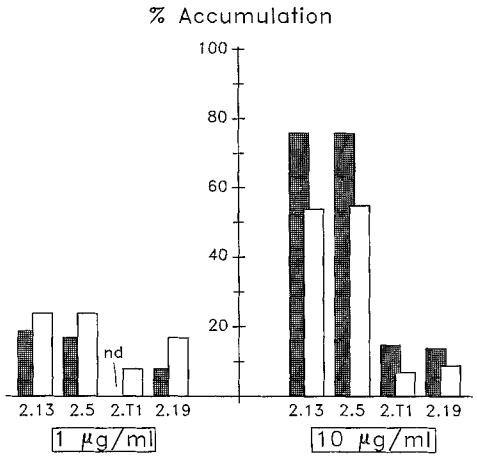

Fig. 6. Accumulation of GCMV RNA1 (empty bars) and RNA2 (hatched bars) genomic RNAs in GCMV CP expressors (CP^+, lines 2.13, 2.5, 2.T1 and 2.19), expressed as a percentage of their accumulation in non-expressor plants transformed with the non-recombinant vector (CP^0, lines 4.15 and 4.21). One-month-old plants were inoculated with either 1 or 10 µg/ml of purified GCMV, processed as described in the legend of Fig. 4 and the radioactivity retained on individual spots determined by scintillation counting of the cut membranes. The values given are the average of several experiments involving, for each line, a total number of between 20 and 94 R1 or homozygous R2 progeny plants, depending on the conditions of inoculation and of the RNA quantitated.

two GCMV genomic RNAs was affected in a similar fashion. In the two lines showing the best protection, this reduction is still close to 80–90% at the 10 μg/ml inoculum concentration, even though up to 85% of the plants become infected under these conditions (see Fig. 5). In the highly protected lines this effect persists even at inoculum concentrations of up to 50 μg/ml (result not shown). On the other hand, for the two lines showing intermediate level protection, it is clear that there is a resistance breakdown at the 10 μg/ml inoculum concentration. This is reflected by numbers of infected plants identical to those of the controls (see Fig. 5) and by an accumulation of the viral RNAs reaching about 60% of the controls.

Protection of GCMV CP-expressing plants against viral RNA inoculation

In order to further characterize the protection observed, the CP-expressing plants were challenged using viral RNA inoculation. In our case (non-systemic infection), rather high RNA concentrations have to be used in order to obtain reproducible infection of control plants. The results of a representative experiment are presented on Table 1. As can be seen, results essentially similar to those obtained upon inoculation with high concentrations of viral particles are observed. Most CP^+ plants become infected but they still show a high reduction in the accumulation of the GCMV RNAs as compared to the control plants. Thus, the expression of the CP-transgene seems to protect the plants even when they are inoculated using purified RNAs. So far these experiments have only been performed with the two best protected lines and no information is available on the behavior of lines showing only an intermediate level of resistance.

Table 1. Analysis of the protection of two CP expressor lines (CP^+) against inoculation with purified GCMV RNAs. One-month-old plants were inoculated with either 25 or 50 μg/ml of purified GCMV RNAs, processed as described in the legend of Fig. 4 and the number of infected plants determined by inspection of the autoradiographs. The radioactivity retained was determined by scintillation counting of the cut membranes. Accumulation of GCMV RNA2 is expressed as a percentage of its accumulation in non-expressor plants transformed with the non-recombinant vector (CP^0, line 4.15).

	CP^0, 4.15	CP^+, 2.T1	CP^+, 2.19
Plants infected/inoculated			
25 μg/ml	14/14	11/14	13/14
50 μg/ml	14/14	14/14	13/14
Accumulation (% of 4.15)			
25 μg/ml	100	5.6	14.3
50 μg/ml	100	16.7	41.8

Discussion

The results presented here extend the usefulness of genetically engineered protection to nepoviruses. Therefore, this strategy can be successfully applied to protect plants against viruses belonging to the two major groups of picorna-like plant RNA viruses, potyviruses and now nepoviruses. While this work was in progress, results describing the protection of transgenic *Nicotiana* plants against arabis mosaic nepovirus were published [4]. Although, due to its limited geographical distribution, GCMV is not a virus of major economic importance, it induces symptoms as severe as those caused by the two nepoviruses highly damaging to grapevine, arabis mosaic virus and grapevine fanleaf virus. Similarly, several other nepoviruses such as tomato ringspot virus have significant economic impact, specially in woody plants, and using pathogen-derived protection to obtain plants resistant to nepoviruses now seems a valuable prospect. Such a possibility is of special interest in the case of grapevine which is probably the world's most widely grown fruit crop and for which the transformation and regeneration technology is at hand [2, 23]. Given the soil-linked nature of nepovirus transmission, obtention of a few varieties of transgenic, resistant rootstock varieties could potentially suffice to protect grape varieties worldwide against nepovirus infection.

The characteristics of resistance observed in the CP-expressing lines described here are simi-

lar to those reported previously for other models [3]: resistance is characterized by the inability to infect the plants at low inoculum concentrations and by inhibition of the accumulation of virus in the infected plants. In several cases, CP protection has been reported to also include a reduced rate of systemic disease development [3]. The characteristics of our model clearly preclude any analysis of such a component of CP-induced protection. Absence of resistance to infection by TMV RNA or by partially decapsidated virions has been regarded as a sign that the primary effect of protection in TMV CP-expressing plants is at a very early event in the infection process, presumably decapsidation [26]. However, in other cases, resistance is expressed even if naked RNA is used as the inoculum [15], indicating that, at least for such viruses, resistance may be mediated by other mechanisms. In our case as well as in the case of arabis mosaic nepovirus [4] resistance is observed even if naked viral RNA is used as the inoculum. This property might thus be a general characteristic of genetically engineered protection against nepoviruses.

It is interesting to notice that, although the difference in CP expression level is low, the two lines showing the best protection are also the two lines expressing GCMV CP at the highest concentration. A similar relation between the concentration of CP accumulated in a given transgenic line and its level of resistance has been reported for essentially all viruses tested with possibly the exception of potyviruses [3, 25].

Since the demonstration that resistance against TMV is directly mediated by the coat protein and not by its messenger RNA [25], it has usually been accepted that the same is true in most cases where genetically engineered protection has been achieved using a CP gene. However, it should be stressed that in many cases no direct evidence that the protection is protein-mediated rather than RNA-mediated has been produced. In the case of nepoviruses ([4] and this work), the demonstration that the protection observed is directly mediated by the coat protein itself will require further experiments involving transgenic plants expressing mutated CP messenger RNAs.

Acknowledgements

We thank Dr T.M.A. Wilson (John Innes Institute, UK) for the gift of plasmid pIJI101 containing the TMV Ω′ leader and Dr C. Robaglia and M. Tepfer (INRA, Versailles, France) for plasmid pMarcel35. This work was supported by AIP INRA 4560 and by grants to V.B. from the Fondation Dutrenoy and the French Ministry of Research and Technology.

References

1. Ausubel FM, Brent R, Kingston RE, Moore D, Smith JA, Seidman JG, Struhl K: Current Protocols in Molecular Biology. Greene/Wiley-Interscience, New York (1987).
2. Baribault TJ, Skene KGM, Scott NS: Genetic transformation of grapevine cells. Plant Cell Rep 8: 137–140 (1989).
3. Beachy RN, Loesch-Fries S, Tumer NE: Coat protein-mediated resistance against virus infection. Annu Rev Phytopath 28: 451–474 (1990).
4. Bertioli DJ, Cooper JI, Edwards ML, Hawes WS: Arabis mosaic nepovirus coat protein in transgenic tobacco lessens disease severity and virus replication. Ann Appl Biol 120: 47–54 (1992).
5. Bevan M: Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res 12: 8711–8721 (1984).
6. Brault V, Hibrand L, Candresse T, Le Gall O, Dunez J: Nucleotide sequence and genetic organization of Hungarian grapevine chrome mosaic nepovirus RNA2. Nucl Acids Res 17: 7809–7819 (1989).
7. Bretout C, Candresse T, Le Gall O, Brault V, Ravelonandro M, Dunez J: Virus and RNA-specific molecular hybridization probes for two nepoviruses. Acta Hort 235: 231–238 (1988).
8. Candresse T, Batisti M, Renaudin J, Mouches C, Bové JM: Immunodetection of turnip yellow mosaic virus nonstructural proteins in infected chinese cabbage leaves and protoplasts. Ann Inst Pasteur (Virol) 138: 217–227 (1987).
9. Doz B, Macquaire G, Delbos RP, Dunez J: Caractéristiques et rôle du RNA3, RNA satellite du virus des anneaux noirs de la tomate. Ann Inst Pasteur (Virol) 131E: 489–499 (1980).
10. Doz B, Delbos RP, Dunez J: Prémunition: une compétition entre souches faibles et souches sévères pour une voie commune d'expression des symptômes. Les Colloques de l'INRA (INRA Ed) 11: 29–44 (1982).
11. Draper J, Scott R, Armitage P, Waleden R: Plant Genetic Transformation and Gene Expression: A Laboratory Manual. Blackwell, Oxford (1988).

12. Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TMA: The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vivo* and *in vitro*. Nucl Acids Res 15: 3257–3273 (1987).
13. Goldbach R, Wellink J: Evolution of plus strand RNA viruses. Intervirology 29: 260–267 (1988).
14. Harrison BD, Murant AF: Nepovirus group. CMI/AAB description of plant viruses, No 185 (1977).
15. Hemenway C, Fang RX, Kaniewski JJ, Chua NH, Tumer NE: Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. Embo J 7: 1273–1280 (1988).
16. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA: A binary plant vector strategy based on separation of the Vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180 (1983).
17. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685 (1970).
18. Lawson C, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer NE: Engineering resistance to mixed infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/technology 8: 127–134 (1990).
19. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
20. Martelli GP, Quacquarelli A: Grapevine chrome mosaic virus. CMI/AAB description of plant viruses No 103 (1972).
21. Martelli GP, Taylor CE: Distribution of viruses and their nematode vectors. In: Harris KF (ed) Advances in Disease Vector Research, vol. 6, pp. 151–189. Springer-Verlag, New York Inc. (1989).
22. Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR: Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmid containing a bacteriophage SP6 promoter. Nucl Acids Res 12: 7035–7056 (1984).
23. Mullins MG, Tang FCA, Faccioti D: *Agrobacterium*-mediated genetic transformation of grapevines: transgenic plants of *Virus rupestris* Scheele and buds of *Vitis vinifera* L. Bio/technology 8: 1041–1045 (1990).
24. Powell-Abel P, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN: Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232: 738–743 (1986).
25. Powell PA, Sanders RP, Tumer NE, Fraley RT, Beachy RN: Protection against tobacco mosaic virus infection in transgenic plants require accumulation of coat protein rather than coat protein RNA sequences. Virology 175: 124–130 (1990).
26. Register JC III, Beachy RN: Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology 166: 524–532 (1988).
27. Robaglia C, Vilaine F, Pautot V, Raimond F, Amselem J, Casse-Delbart F, Tepfer M: Expression vectors based on the *Agrobacterium rhizogenes* Ri plasmid transformation system. Biochemie 69: 231–237 (1987).
28. Stark DM, Beachy RN: Protection against potyvirus infection in transgenic plants: evidence for broad spectrum resistance. Bio/technology 7: 1257–1262 (1989).