

EXPRESSION OF THE COAT PROTEIN GENES OF GRAPEVINE VIRUS A AND B IN *NICOTIANA* SPECIES AND EVALUATION OF THE RESISTANCE CONFERRED ON TRANSGENIC PLANTS

A. Minafra¹, R. Gölles², A. da Camara Machado², P. Saldarelli¹, N. Buzkan¹, V. Savino¹, G.P. Martelli¹, H. Katinger² and M. Laimer da Camara Machado²

¹ Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via G. Amendola 165/a, I-70126, Bari, Italy

² Institut für Angewandte Mikrobiologie, Universität für Bodenkultur, Wien, Austria

SUMMARY

Agrobacterium-mediated transformation of leaf discs of *N. benthamiana* and *N. occidentalis* with constructs containing the coat protein (CP) genes of grapevine virus A (GVA) or grapevine virus B (GVB), produced several lines of transgenic plants. Some of the transformant lines (GVAcp+ and GVBcp+) growing on a selective kanamycin-containing medium were assayed in R₁ generation for the expression of transgenic mRNAs and coat proteins. Some of the plants that hybridized specific digoxigenin-labeled riboprobes to both viruses, contained detectable amounts of viral CP as shown by Western blot analysis, whereas others did not. The behaviour of R₁ seedlings following challenge inoculation with homologous viruses at a concentration of 1 µg ml⁻¹ was monitored by TAS-ELISA up to 18 days post inoculation. Various levels of resistance to both viruses were observed, as expressed by reduction of virus accumulation. Some of the GVAcp+ lines also showed a 3-4 day delay in symptom appearance.

RIASSUNTO

ESPRESSIONE DEL GENE DELLA PROTEINA CAPSIDICA DEI VIRUS A E B DELLA VITE IN *NICOTIANA* E VALUTAZIONE DELLA RESISTENZA CONFERITA ALLE PIANTE TRANSGENICHE. Piante transgeniche di *N. benthamiana* e *N. occidentalis* sono state prodotte tramite la trasformazione genetica di dischi fogliari, mediata da *Agrobacterium tumefaciens*, con costrutti contenenti le proteine capsidiche, rispettivamente, di GVA e GVB. Alcune delle linee trasformate selezionate su kanamicina sono state saggiate, nella generazione R₁, per la espressione in vivo dell'RNA trascritto dal transgene e la produzione di proteina capsidica. I sementali delle linee che ibridavano le specifiche ribosonde marcate con digoxigenina potevano esprimere oppure no quantità di proteina transgeni-

ca individuabile in Western blot. La risposta alla inoculazione di sfida, effettuata sui sementali della generazione R₁ con virus omologo alla concentrazione di 1 µg ml⁻¹, è stata seguita con il saggio ELISA fino al 18° giorno successivo alla inoculazione. È stato osservato un grado variabile di resistenza alla infezione virale fra le linee poiché, nella maggior parte di esse, una sostanziale riduzione della accumulazione di virus corrispondeva ad una diversa percentuale di espressione dei sintomi.

Key words: coat protein mediated resistance, vitiviruses, *Nicotiana*, transgenic plants.

INTRODUCTION

Grapevine virus A (GVA) and B (GVB) are the putative agents of Kober stem grooving and corky bark, respectively, two diseases of the grapevine rugose wood complex of economic importance and worldwide distribution (reviewed by Boscia et al., 1997). Sources of natural resistance to GVA and GVB in grapevines are unknown, so that the control of these viruses is largely based on sanitation and certification procedures. Healthy vines, however, are exposed to reinfection in the field, both viruses being transmitted by mealybugs in a semipersistent manner (Boscia et al., 1997; La Notte et al., 1997). Ways to introduce resistance into grapevines were therefore explored, the pathogen-derived resistance approach (Sanford and Johnston, 1985) being a promising candidate, as the sequences of the coat protein (CP) genes of GVA and GVB were available (Saldarelli et al., 1996; Minafra et al., 1997a).

Prior to venturing to transform grapevines, *Nicotiana benthamiana* and *N. occidentalis*, experimental hosts of GVA and GVB, were used as models to test the behaviour and protective potential of the inserted viral genes. In this study, a preliminary account of which has been given (Minafra et al., 1997b), transcriptional cassettes containing the CP genes of GVA or GVB were prepared and transferred via *Agrobacterium tumefaciens* to *Nicotiana* hosts, and transformant lines were assayed for gene expression and virus resistance.

Corresponding author: G.P. Martelli
Fax: + 39.080.5442911
E-mail: martelli@bibagr.uniba.it

MATERIALS AND METHODS

Plasmid construction and plant transformation. The CP genes of GVA and GVB were amplified from single-stranded cDNA synthesized on purified viral RNA with specific primers (Table 1) that introduced a *Nco*I site across the start codon (CCATGG) for directional cloning in pRT 103 (Topfer et al., 1987). The *Hind*III fragment of these cassettes, which contained the 35S CaMV promoter and the nos terminator signal, was excised and inserted in the same site of a pBin19 binary vector (Bevan, 1984). These constructs were cloned in *A. tumefaciens*, strain LBA 4404, by direct CaCl_2 transformation. All molecular cloning techniques were essentially as described by Sambrook et al. (1989).

Nicotiana leaf discs were co-cultured with *Agrobacterium* cells according to Regner et al. (1992), except that 250 $\mu\text{g l}^{-1}$ cefotaxime was used as antibacterial in the selective medium. *N. benthamiana* was transformed with the GVA construct and *N. occidentalis* with the GVB construct. Single shoots were isolated on the same medium after one month, rooted, transferred to pots in a climatized glasshouse at an average temperature of 24°C and selfed as individual R_0 lines to obtain R_1 progeny seeds.

Analysis of transformed lines. Plant tissues from original transformants (R_0), either grown in vitro or in pots before selfing, were tested by molecular assays for the correct insertion of the transgenes. PCR amplification was performed on total chromosomal DNA (Della Porta et al., 1983) or reverse-transcribed total RNA.

Total nucleic acid (TNA) was extracted according to White and Kaper (1989) from R_1 seedlings, grown directly in pots and selected by dot-blot hybridization, or previously selected in vitro on half strength MS medium (Murashige and Skoog, 1962) supplemented with kanamycin (250 $\mu\text{g l}^{-1}$) (Gölles et al., 1997a). Half of the extract from 50 mg of leaf tissue, resuspended in 50 μl of water, was quickly denatured in an equal volume of 100 mM NaOH containing 5 mM EDTA (Gallitelli and Saldarelli, 1996), and immediately spotted on a positively charged nylon membrane (Hybond N+, Amersham, UK). Some TNA samples were electrophoresed in a fully-denaturing formaldehyde agarose gel and blotted overnight by capillarity with 20x SSC buffer (1x: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) on membranes of the same type.

Dot-spot or Northern blot hybridization of extracts of the transgenic lines was performed with the respective GVA and GVB digoxigenin-labelled riboprobes, consisting of fragments of CP genes transcribed from a pGEM 4z plasmid (Studier, 1991). Hybridization con-

ditions and chemiluminescent detection were as described by Gallitelli and Saldarelli (1996). The same R_1 seedlings were also analyzed in Western blots. Total proteins from about 50 mg of fully expanded leaves were extracted in 1 vol of sucrose-free extraction buffer (Goldbach et al., 1982) and separated in a standard 12% SDS-PAGE (Laemmli, 1970) before electroblotting with a semi-dry apparatus (BioRad, USA). A GVA polyclonal antiserum and GVB monoclonal antibodies (Bonavia et al., 1996), both preabsorbed with protein extracts from healthy plants, were used to detect the respective transgenic coat proteins by an alkaline phosphatase-mediated colorimetric reaction (Sambrook et al., 1989).

Virus inoculation. Challenge inoculation was done on 11 lines of *N. benthamiana* (GVAcp+) and 7 lines of *N. occidentalis* (GVBcp+). At least 10 R_1 seedlings per line, selected by hybridization for the presence of transgenic RNA (GVBcp+ lines), or screened in vitro on kanamycin (GVAcp+ lines), were mechanically inoculated at the 4-leaf stage with purified virus suspensions (1 $\mu\text{g ml}^{-1}$), and kept in a glasshouse at 24°C with a 16h-light period. Virus accumulation was monitored by TAS-ELISA using 100 mg of tissue from the 2nd and 3rd apical leaf of each inoculated plant every 3 days, from 3 to 18 days post inoculation (dpi). Readings were made between 15 and 30 min after the addition of substrate. The background given by the CP expressed in transgenic plants was repeatedly checked by ELISA, and found to have no influence on the readings for the evaluation of virus accumulation. Symptoms appearing on individual plants were recorded.

RESULTS

Selection of transgenic lines. PCR amplification on genomic DNA extracts from R_0 lines and dot-blot hybridization of TNA extracts from R_1 seedlings gave positive results for the detection of transgenic DNA and RNA, respectively, in 8 GVA lines and 5 GVB lines (not shown). Attempts to hybridize total DNA extracts, digested with restriction enzymes and separated on agarose gels before blotting, were unsuccessful.

More than 80% of individual GVAcp+ *N. benthamiana* plantlets that had been kanamycin-selected before transplanting, hybridized with the probe. However, the R_1 seedlings of some lines (e.g. lines 2 and 4) that had germinated and rooted in the presence of high kanamycin concentrations (250 $\mu\text{g l}^{-1}$), did not contain any detectable transcribed RNA perhaps because of deletion or inactivation of the CP transgene.

R_1 seeds from *N. occidentalis* lines, known to contain the GVB CP transgene as ascertained by PCR amplification of total DNA extracted from R_0 lines, did not germinate at kanamycin concentrations of 100 to 250 $\mu\text{g l}^{-1}$, customarily used for selecting transformed plants. These seeds were therefore germinated on wet paper before transplanting in soil. The percentage of transgenic seedlings of the lines expressing detectable amounts of mRNA was ca 60%, i.e. lower than that observed for GVA-transformed *N. benthamiana*, but this was likely a consequence of the lack of kanamycin selection.

Northern blots of TNA extracts from individual R_1 *N. benthamiana* and *N. occidentalis* transgenic plants revealed the presence of a mRNA transcript of about 600 nt from the inserted GVA CP and GVB CP transgenes (Fig. 1).

Protein expression in R_1 transgenic lines. A protein band of the size of GVA and GVB coat proteins (i.e. 22 kDa) was detected by Western blots in total protein extracts from individual R_1 plants of different lines (5 out of 8 tested for GVAcp+, and 2 out of 8 for GVBcp+). Fig. 2 shows detection of the viral CP in two lines each of GVAcp+ and GVBcp+. The presence of the specific protein band could be correlated with the relative quantity of mRNA from the transgene only for some lines. For instance, *N. benthamiana* GVAcp+ transgenic lines 2 and 4 that did not contain detectable amounts of mRNA did not react in Western blots (not shown). Conversely, lines 3 and 7 from GVBcp+ *N. occidentalis*, that had several strongly hybridizing seedlings, did not show the presence of the CP band in any of the singly

assayed plants (not shown). Of the GVB+cp lines, only NO2 and NOB, that expressed the mRNA, also expressed detectable CP (Fig. 2B). The 22K band was not observed in the untransformed controls.

Response to challenge inoculation. Table 1 gives the average ELISA readings of inoculated seedlings from several lines and the corresponding percentage of symptomatic plants at 18 dpi. While the increase of virus concentration was linear in inoculated untransformed plants, all of which showed typical symptoms, the virus titre in non-symptomatic plants of the transgenic lines assayed was lower than in the control. Symptom appearance was delayed by 3 to 4 days with respect to the controls in 20 to 50% of inoculated seedlings of GVAcp+ lines. In some of these lines, for instance NB4, NB6, and NB10, the average accumulation of virus was about one tenth that of the untransformed control, with a percentage of symptomatic plants from 20 to 35% (Table 1).

Delay in symptom expression was not observed in inoculated *N. occidentalis* GVBcp+ seedlings. Lines NO1, NO5, and NO9 also showed a significant containment of viral infection (about 0.1 OD at 18 dpi vs 0.9 OD in the non-transgenic control) with 15-20% of symptomatic plants vs 100%. Other lines (NOA1, NOB and NO3) showed intermediate behaviour, with a slight increase in the number of plants showing symptoms (25%) and a slightly higher range of virus accumulation (0.135-0.145 OD) (Table 1).

The presence of mRNA or its expression product (CP) did not necessarily correlate with a low level of virus infection. For example, GVA+cp lines NB1 or

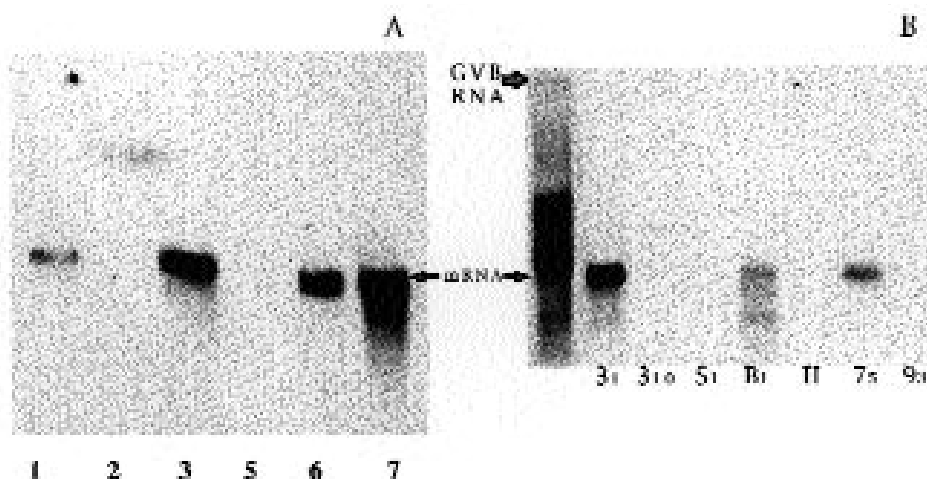


Fig. 1. Northern blot hybridization of TNA extracts from R_1 individual plants of GVA (A) and GVB (B) transgenic lines. The positions of GVB full-length genomic RNA from a naturally infected *N. occidentalis* (7600 nt) and transgenic mRNA transcripts (600 nt) are arrowed. H: TNA from an untransformed plant.

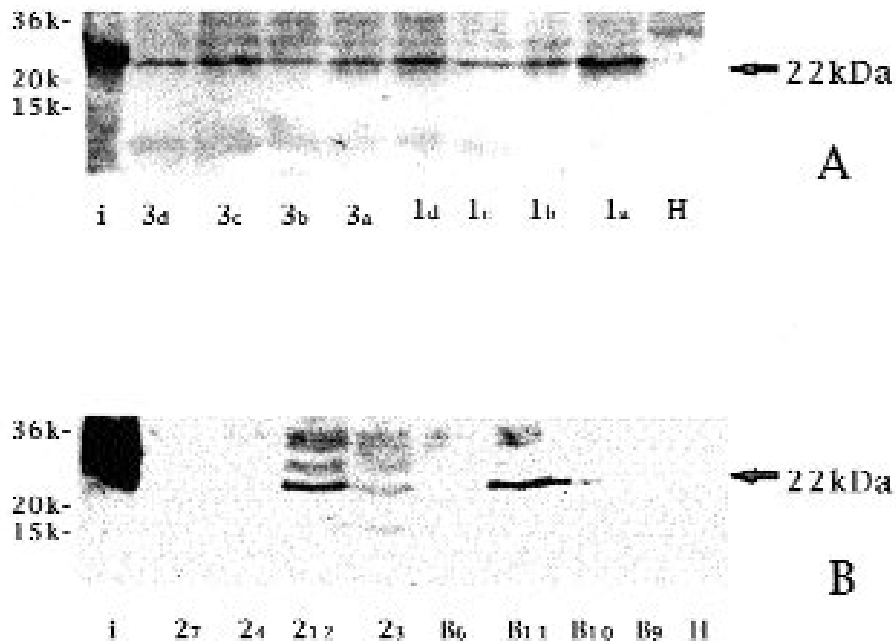


Fig. 2. Western blot of total protein extracts from R1 individual plants of transgenic lines NB1 and NB3 (A: GVA+cp *N. benthamiana*), NO2 and NOB (B: GVB+cp *N. occidentalis*). One fourth of a 1:1 (w/v) clarified protein extract from a fully-expanded young leaf (about 50 mg) was denatured and loaded. The 22 kDa coat protein is arrowed. MW markers are on the left. i: total proteins from plants naturally infected with GVA and GVB, respectively; H: total protein from healthy plants.

NB3 whose seedlings expressed both mRNA and CP, accumulated virus up to 0.3 OD, i.e. a concentration about half than that of non-transgenic control (Table 1), and the percentage of plants with symptoms was relatively low (25-40%). By contrast, line NB5, that had an undetectable level of transgene expression, showed a lower virus accumulation (barely 0.1 OD) but half of the plants showed symptoms. And further, GVBcp+ line NO3, expressing detectable amounts of mRNA but not CP, showed both lower virus titre and percentage of infected plants when compared to GVBcp+ line NO2 that did express CP. Partial reduction of virus titre over time, but not of symptoms, was observed with line NO3, while the other transgenic lines showed a continued increase in virus content, albeit lower than in the control, during the whole period of observation.

DISCUSSION

Transformation of two *Nicotiana* species with the CP genes of GVA and GVB resulted in the transcription of the respective transgenic RNAs in R₁ seedlings of 8 lines of GVAcp+ and 5 lines of GVBcp+. Translation of the mRNAs was detected in 5 and 2 of those lines, respectively. In R₁ progeny seedlings of several lines

challenge-inoculated with the respective homologous viruses at 1 µg ml⁻¹ was a clear-cut reduction of virus accumulation as compared to the controls, while a remarkable number of plants (up to 80%) did not express symptoms. Therefore, a fair level of tolerance to vitivirus infection was obtained in transgenic *Nicotiana* species. However, there was no straight forward correlation between tolerance and the amount of transgenic coat protein expressed, which agrees with the results reported by Regner et al. (1992) for plum pox virus CP-mediated resistance in herbaceous hosts, and those reviewed by Hackland et al. (1994) for several other viruses. The apparent absence of CP expression in some transgenic lines that proved tolerant to infection could indicate an effect of the mRNA in controlling virus replication (Prins and Goldbach, 1996).

Our results are in line with those recently reported by Radian-Sade et al. (1997) in Israel, where *Nicotiana* plants transformed with GVA CP showed a similar complex situation of symptomless lines supporting viral infection, lines that completely escaped infection, and lines that displayed delayed infection. The potential of CP-mediated resistance is now under study in *Vitis*, since *Agrobacterium*-mediated transformation of *V. vinifera* somatic embryos was recently obtained (Gölles et al., 1997b).

Table 1. Virus accumulation, expressed as mean OD (A_{405}), at 18 days post inoculation in symptomless and symptomatic plants of some challenge-inoculated transgenic R_1 seedlings.

Transgenic lines	Symptomatic plants (%)	Symptomless plants (A_{405})	Symptomatic plants (A_{405})
GVAcp+			
NB1	40	0.279 ± 0.011	0.705 ± 0.036
NB2	35	0.089 ± 0.055	0.736 ± 0.039
NB3	25	0.330 ± 0.073	0.700 ± 0.070
NB4	35	0.032 ± 0.008	0.486 ± 0.107
NB5	50	0.091 ± 0.020	0.663 ± 0.020
NB6	20	0.055 ± 0.004	0.227 ± 0.070
NB7	50	0.107 ± 0.036	0.541 ± 0.044
NB8	20	0.097 ± 0.024	0.480 ± 0.143
NB10	20	0.048 ± 0.008	0.740 ± 0.102
NB13	35	0.157 ± 0.076	0.710 ± 0.068
NB14	10	0.162 ± 0.046	0.750 ± 0.130
nt		–	0.630 ± 0.064
H		0.040	–
GVBcp+			
NO1	20	0.114 ± 0.0230	0.920 ± 0.153
NO2	50	0.230 ± 0.0312	0.983 ± 0.175
NO3	20	0.144 ± 0.0220	0.770 ± 0.098
NO5	15	0.136 ± 0.0290	1.190 ± 0.118
NO9	15	0.068 ± 0.0031	1.200 ± 0.148
NOA1	25	0.135 ± 0.0108	1.400 ± 0.116
NOB	25	0.145 ± 0.0720	1.213 ± 0.195
nt		–	0.890 ± 0.092
H		0.050	–

nt: inoculated untransformed control.

H: uninoculated control.

ACKNOWLEDGMENTS

This study was supported by the EU Project BIO4-CT-960773 'Risk assessment with genetically engineered woody plants expressing virus coat protein gene' and the Austrian project L763/93 supported by the Bundesministerium für Land- und Forstwirtschaft and the Niederösterreichische Landes-regierung.

REFERENCES

- Bevan M., 1984. Binary Agrobacterium vector for plant transformation. *Nucleic Acids Research* **12**: 8711-8721.
- Bonavia M., Digiaro M., Boscia D., Boari A., Bottalico G., Savino V., Martelli G.P., 1996. Studies on 'corky rugose wood' of grapevine and on the diagnosis of grapevine virus B. *Vitis* **35**: 53-58.
- Boscia D., Minafra A., Martelli G.P., 1997. Filamentous viruses of the grapevine: putative trichoviruses and capilloviruses. In: Monette P.L. (ed.) Filamentous viruses of woody plants, pp. 19-28. Research Signpost, Trivandrum, India.
- Della Porta S., Wood J., Hicks J., 1983. A plant DNA miniprep: version II. *Plant Molecular Biology Reports* **1**: 19-21.
- Gallitelli D., Saldarelli P., 1996. Molecular identification of phytopathogenic viruses. In: Clapp J.P. (ed.) *Methods in molecular biology* Vol. 50. Species diagnostic protocols: PCR and other nucleic acid methods, pp. 57-79. Humana Press, Totowa.
- Gölles R., da Camara Machado A., Minafra A., Moser R., Katinger H., Laimer da Camara Machado M., 1997b. Regeneration of *Vitis* sp. transformed with coat protein gene sequences of four different grapevine viruses. Extended Abstracts 12th Meeting ICVG, Lisbon 1997, 139.

- Gölles R., Moser R., da Camara Machado A., Katinger H., Laimer da Camara Machado M., 1997a. Viral resistance in *Nicotiana benthamiana* expressing altered forms of the coat protein gene of grapevine fanleaf virus. Extended Abstracts 12th Meeting ICVG, Lisbon 1997, 138.
- Goldbach R., Rezelman G., Zabel P., Van Kammen A., 1982. Expression of the bottom component RNA of cowpea mosaic virus: evidence that the 60-kilodalton VPg precursor is cleaved into single VPg and a 58kD polypeptide. *Journal of Virology* **42**: 630-635.
- Hackland A.F., Rybicki E.P., Thomson J.A., 1994. Coat protein-mediated resistance in transgenic plants. *Archives of Virology* **139**: 1-22.
- Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
- La Notte P., Buzkan N., Choueiri E., Minafra A., Martelli G.P., 1997. Acquisition and transmission of grapevine virus A by the mealybug *Pseudococcus longispinus*. *Journal of Plant Pathology* **78**: 79-86.
- Minafra A., Gölles R., da Camara Machado A., Saldarelli P., Savino V., Martelli G.P., Katinger H., Laimer da Camara Machado M., 1997b. Coat protein-mediated resistance against grapevine virus A and grapevine virus B in *Nicotiana benthamiana* and *Nicotiana occidentalis*. Extended Abstracts 12th Meeting ICVG, Lisbon 1997, 140.
- Minafra A., Saldarelli P., Martelli G.P., 1997a. Grapevine virus A: nucleotide sequence, genome organization, and relationship in the *Trichovirus* genus. *Archives of Virology* **142**: 417-423.
- Murashige T., Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**: 473-497.
- Prins M., Goldbach R., 1996. RNA-mediated virus resistance in transgenic plants. *Archives of Virology* **141**: 2259-2276.
- Radian-Sade S., Edelbaum O., Rubinstein Y., Gafni R., Sela I., Tanne E., 1997. Transgenic *Nicotiana benthamiana* plants resistant to grapevine virus A. Extended Abstracts 12th Meeting of ICVG, Lisbon 1997, 141-142.
- Regner F., da Camara Machado A., Laimer da Camara Machado M., Steinkellner H., Mattanovich D., Hanzer V., Weiss H., Katinger H., 1992. Coat protein mediated resistance to plum pox virus in *Nicotiana clevelandii* and *N. benthamiana*. *Plant Cell Reports* **11**: 30-33.
- Saldarelli P., Minafra A., Martelli G.P., 1996. The nucleotide sequence and genomic organization of grapevine virus B. *Journal of General Virology* **77**: 2645-2652.
- Sambrook J., Fritsch E., Maniatis T., 1989. *Molecular cloning: a laboratory manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, USA.
- Sanford J.C., Johnston S.A., 1985. The concept of parasite-derived resistance. Deriving resistance genes from the parasites' own genome. *Journal of Theoretical Biology* **113**: 395-405.
- Studier W.F., 1991. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *Journal of Molecular Biology* **219**: 37-44.
- Topfer R., Matzeit V., Gronenborn B., Schell J., Steinbiss H.H., 1987. A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Research* **15**: 5890.
- White J.L., Kaper J.M., 1989. A simple method for detection of viral satellite RNAs in small plant tissues samples. *Journal of Virological Methods* **23**: 83-94.

Received 4 May 1998

Accepted 21 August 1998