

HETEROLOGOUS ENCAPSIDATION IN NON-TRANSGENIC AND TRANSGENIC *NICOTIANA* PLANTS INFECTED BY *GRAPEVINE VIRUSES A AND B*

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SUMMARY

In mixed infections of *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) in non-transgenic *Nicotiana* plants, one-sided phenotypic mixing (GVA coat protein encapsidating GVB RNA) was detected by immunocapture-RT-PCR, with a frequency ranging from 33 to 75%. Immuno-electron microscopy confirmed the presence of both capsid proteins in the same virus particles. Heteroencapsidation took also place in R1 seedlings of transgenic *Nicotiana* lines expressing GVA or GVB coat proteins, when the plants were challenge-inoculated with the heterologous virus. Finally, whole virus particles were produced when an infectious but CP-defective GVB RNA transcript was inoculated biolistically as a DNA plasmid into *Nicotiana* plants transgenically expressing GVB CP.

Key words: heteroencapsidation, transgenic plants, vitiviruses, *Nicotiana*, GMO.

INTRODUCTION

The rugose wood complex is a severe disease of the grapevine, with which *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) (genus *Vitivirus*) are associated (reviewed by Martelli, 1999). Since natural sources of resistance to GVA and GVB are unknown, the introduction of transgenic resistance was attempted by transforming constructs containing the coat protein (CP) genes of both viruses into *Nicotiana* species (Minafra *et al.*, 1998; Radian-Sade *et al.*, 2000) and grapevine somatic embryos (Gölles *et al.*, 1997). A potential risk of CP-expressing transgenes is that their expression product can coat the nucleic acid of a related incoming virus, giving complete (transcapsidation) or partial (phenotypic mixing) encapsidation of the challenging virus RNA by the transgenic CP. This could

modify the epidemiological behaviour of the incoming virus (reviewed by Timmerman-Vaughan, 1998; Hammond *et al.*, 1999).

Heteroencapsidation can take place in the presence of mixed infections in non-transgenic plants and in transgenic plants expressing a viral CP. In normal plants transcapsidation has been reported to occur between related luteoviruses (Rochow, 1972; Hu *et al.*, 1988; Creamer and Falk, 1990), potyviruses (Milne *et al.*, 1980), and vitiviruses (Milne *et al.*, 1984). Moreover, non-aphid transmissible potyvirus strains can become transmissible when phenotypic mixing develops in doubly infected plants (Hobbs and Mac Laughlin, 1990; Bourdin and Lecoq, 1991). Phenotypic mixing has also been experimentally detected in several plants expressing transgenic potyvirus CP and inoculated with a closely related virus (Farinelli *et al.*, 1992; Lecoq *et al.*, 1993; Hammond and Dienelt, 1997). Interactions between defective RNAs and CPs in transgenic plants, that abolish or complement virus assembly and/or aphid transmission have also been described (Osbourne *et al.*, 1989; Jacquet *et al.*, 1998; Varrelmann and Maiss, 2000). Transient co-expression of a functional viral gene and a mutated infectious transcript of *Potato virus X* transferred by particle bombardment has been reported to restore virus functionality (Morozov *et al.*, 1997).

This paper investigated whether heteroencapsidation occurs in non-transgenic *Nicotiana* plants doubly infected with GVA and GVB or following heterologous challenge-inoculation of transgenic CP-expressing *Nicotiana* plants, and the possible complementation of a CP-defective but infectious GVB transcript in transgenic CP-expressing *Nicotiana*.

MATERIALS AND METHODS

Mixed infections in non-transgenic *Nicotiana* plants. GVA (isolate PA3) and GVB (isolate Pastore V11) were maintained in greenhouse-grown *N. benthamiana* and *N. occidentalis*, respectively. It was known from previous experience that infection of *N. benthamiana* by GVB or *N. occidentalis* by GVA is possible

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but difficult, and that virus replication is reduced. Therefore, to establish mixed infections, GVA or GVB were first inoculated on one leaf of the heterologous host (a set of three plants at the 4-leaf stage), then on the opposite leaf with the homologous virus at different times, *i.e.* from the same day of inoculation with the heterologous virus up to the following 8 days. These experiments were repeated five times recording the percentage of infection. To verify the establishment of mixed infection after symptom appearance, systemically infected leaves were tested by ELISA using a polyclonal antiserum to GVA or monoclonal antibodies (MAbs) to GVB, according to Boscia *et al.* (1992). To test the simultaneous transmissibility of GVA and GVB, extracts from upper leaves of doubly infected plants were mechanically inoculated to healthy *Nicotiana* plants.

Immunocapture RT-PCR for heteroencapsidation analysis. Immunocapture RT-PCR was done from doubly infected plants and challenge-inoculated transgenic plants essentially as reported by Nolasco *et al.* (1993). Polypropylene microtubes (0.5 ml) were separately coated with the ascitic supernatant of MAb F5 (GVA) or purified IgG MAb B2 (GVB), both diluted 1:100 in carbonate buffer, for 2 h at 37°C. One hundred µl of leaf extract (20 mg in 1 ml of ELISA extraction buffer) were added and incubated overnight at 4°C. After thorough washing, 50 µl of cDNA reaction mix (Minafra and Gallitelli, 1995) were directly added to the tubes and incubated at 42°C for 1 h. Ten µl of the synthesized cDNA were added to 40 µl of PCR reaction (Minafra and Gallitelli, 1995) and amplified with GVA or GVB primers (Minafra and Hadidi, 1994) at an annealing temperature of 52°C. cDNA was amplified with the heterologous primer set in samples captured by a specific antibody (*i.e.* GVA primers for GVB-IgG capture and the opposite). Amplified products were electrophoresed on 1.2% agarose gels. Controls consisted of homologous and heterologous captures from single or mixed infections to verify that: (i) no direct virus binding occurred without antibody coating; (ii) there was no aspecific capture; (iii) the positive reaction was specific both in single and double infections.

Immuno-electron microscopy. Carbon-film grids were separately coated with polyclonal antisera (PABs) to GVA and GVB, diluted in phosphate buffer 1:1000 and 1:500 respectively, and floated for 30 min at 37°C on a drop of crude sap extract. The grids were then individually incubated with PABs to GVA (1:20), GVB (1:10) or with a MAb to GVA (1:100) and stained with 2% uranyl acetate. Immunogold labelling was as described by Faoro *et al.* (1991). Grids were floated on a

mixture of GVA MAb (1:1000) and GVB PABs (1:250), then on crude plant extracts. The grids were sequentially exposed to: (i) GVA MAb (1:100) for 5 min; (ii) 15 nm colloidal gold particles conjugated with antimouse antibodies; (iii) GVB PABs (1:10) for 5 min; (iv) 10 nm colloidal gold conjugated with antirabbit antibodies. Preparations treated with GVA MAb and GVB PABs followed by single immunogold labelling served as controls.

Heteroencapsidation in CP transgenic *Nicotiana* plants. GVA CP-transgenic *N. benthamiana* and GVB CP-transgenic *N. occidentalis* (Minafra *et al.*, 1998) were inoculated with GVB and GVA, respectively. R₁ seedlings of two lines (Nb 3 and 7) transformed with GVA CP and one line (No B) transformed with GVB CP were analyzed in each experiment (10 seedlings per line). The seedlings were checked for transgenic CP expression by Western blot, then were mechanically inoculated with the heterologous virus. Successful infection was detected by RT-PCR on total nucleic acids (Minafra and Gallitelli, 1995) both in symptomatic and non-symptomatic transgenic plants. Plants found to be infected with the heterologous virus were tested for possible heteroencapsidation by IC-PCR as above. Amplified DNA products were analyzed in 5% PAGE and stained with silver nitrate.

Production of a GVB mutant transcript and its inoculation in transgenic plants. The 3' end portion in pOK12 of the GVB infectious clone pCass2 FL (Saldarelli *et al.*, 2000) was modified by PCR introducing a stop codon in the CP gene, thus terminating the CP polypeptide after 50 aminoacids. The full-length clone was restored by ligation and 0.8 µg of purified DNA plasmid were coated on gold particles. These particles were shot onto the basal leaves of eight three-week-old R₁ seedlings, previously selected by Western blot, of the *N. benthamiana* line 9a, transgenic for GVB CP (unpublished information). Three weeks after bombardment, molecular hybridization with a digoxigenin-labelled riboprobe to the GVB 13k nucleic acid binding-protein gene (Saldarelli *et al.*, 1996) and ISEM with GVB MAb B2 were done respectively on total nucleic acid and crude sap extracts from systemically infected leaves.

RESULTS

Evidence of phenotypic mixing in doubly infected non-transgenic *Nicotiana* plants. A significant proportion of inoculated plants of both *Nicotiana* species proved to be mix-infected with GVA and GVB (Table 1). A higher rate of mixed infections occurred when the

Table 1. Mixed GVA and GVB infections in *Nicotiana* and IC-PCR detection of one-sided heteroencapsidation between GVA CP and GVB RNA in doubly infected non-transgenic plants.

Experiment	Inoculated plants no.	Mix-infected plants		Heteroencapsidation events	
		no.	Frequency %	no.	Frequency %
I	30	14	46	6	40
II	24	4	16	3	75
III	24	12	50	4	33
IV	36	12	33	8	60
V	24	10	42	5	50

time lag between inoculations with the two viruses was increased up to 3 days. Co-transmission of both viruses from mix-infected *Nicotiana* to healthy *Nicotiana* plants of both species was obtained by mechanical inoculation as shown by RT-PCR.

GVB primers amplified a 450 bp fragment from mix-infected *N. benthamiana* and *N. occidentalis* immunocaptured by GVA MAb (Fig. 1, lanes 10 and 12). Results were consistently negative with the opposite combination. Whereas single-virus infected plants did not yield amplification products when tested with the heterologous antibody-primer combination (Fig. 1, lanes 2 and 9), positive amplification was obtained when using homologous antibodies and primers (Fig. 1, lanes 1 and 8). This one-sided heteroencapsidation occurred in 33 to 75% of the tested plants (Table 1). No difference in this frequency was observed between the two *Nicotiana* species.

Particles with different decoration patterns were ob-

served in extracts from mix-infected plants. When exposed to GVB PABs, some particles were partially decorated (Fig. 2A, B, C), whereas others were either completely decorated or not decorated at all (not shown). Non-decorated particles had GVA CP coating as ascertained by exposure to GVA PABs (not shown). In immunogold labelling experiments, gold particles of both sizes (10 and 15 nm) were present on single virus particles (Fig. 2D), providing visual evidence of phenotypic mixing.

Phenotypic mixing in CP-transgenic plants. As observed in non-transgenic plants, inoculation of GVB to GVA CP + *N. benthamiana* plants was more difficult than the opposite combination (*i.e.*, GVA to GVB CP + *N. occidentalis*). IC-PCR again showed frequent encapsidation of GVB RNA by transgenic GVA CP (Fig. 3, lanes 1, 2, 4 and 5), whereas in the opposite combination, encapsidation of challenging GVA RNA by trans-



Fig. 1. Analysis of IC-RT-PCR products from *N. benthamiana* plants infected with GVA (430 bp fragment) and GVB (450 bp fragment). Lane 1, capture with GVA MAbs and amplification with homologous primers. No amplification products are present in lane 2 (GVB single infection, capture and amplification with GVA MAbs and primers, respectively) nor in lanes 3, 4, 5 and 6 (mixed infection, immunocapture with GVB MAbs, amplification with GVA primers) and lane 7 (healthy control). Lane 8: control (homologous MAbs and primers) from single GVB infection; lanes 10 and 12: GVB amplification from mix-infected plants (capture with GVA MAbs and amplification with GVB primers); lane 9: no amplification from GVA single infection using GVB MAbs and primers for capture and amplification, respectively. Negative controls in lanes 11 (mixed infection) and 13 (healthy) using GVA capture and GVB primers. Lane M: *I-Hind*III DNA marker.

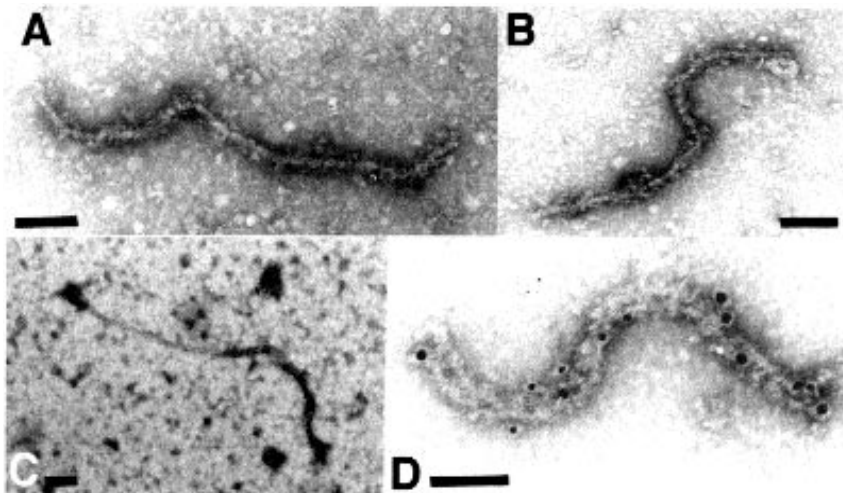


Fig. 2. Immuno-electron microscopy of heteroencapsidated virus particles in leaf dip preparations from GVA/GVB mix-infected *N. benthamiana*. Trapping was with a mixture of GVA Mabs and GVB Pabs, whereas decoration with secondary antibodies was with GVB Pabs (A, B, C) or with GVA Mabs and GVB Pabs, plus antimouse and antirabbit IgGs conjugated with gold particles of two sizes (D). Bars = 100 nm.

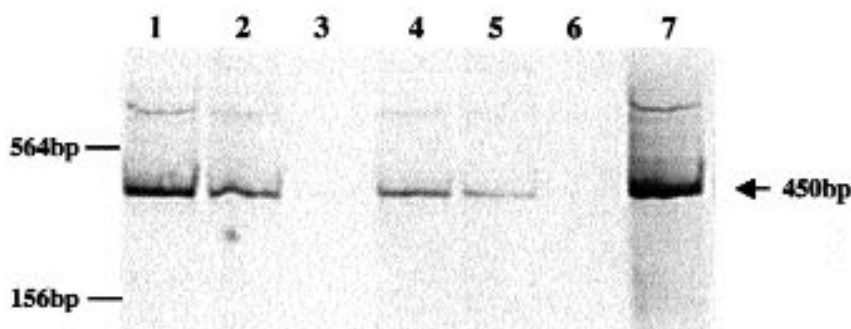


Fig. 3. Analysis of IC-RT-PCR products from GVA CP + transgenic *N. benthamiana* plants challenge-inoculated with GVB. A 450bp GVB-specific product was obtained by immunocapturing with GVA Mabs and amplifying with GVB primers (lanes 1, 2, 4 and 5). No amplification in lanes 3 and 6 (GVB infection, GVA immunocapture and amplification with GVB primers). Lane 7: positive control (GVB infection in non-transgenic *N. occidentalis*. Capture and amplification with GVB Mabs and primers, respectively).

genic GVB CP was only found in two out of 12 symptomatic plants. This result was confirmed by a second round of IC-PCR with one of the two plants a week after the first test (Fig. 4, lanes 2 and 4).

Repeated attempts to reveal phenotypic mixing by IEM in extracts from transgenic plants challenge-inoculated with heterologous viruses failed, since the particles were always fully decorated by the respective antisera.

Complementation of GVB CP-defective RNA in GVB CP-transgenic plants. Biolistic inoculation of the mutated GVB DNA clone to GVB CP-transgenic plants had an efficiency of about 80%. All inoculated seedlings contained GVB CP in amounts detectable by Western blot (Fig. 5). In two bombarded asymptomatic transgenic plants, an actively replicating GVB RNA was detected by hybridization also in the apical, uninoculated leaves. Moreover, whole virus particles were observed in leaf extracts (Fig. 6) decorated by GVB Mabs (not shown), thus providing visual evidence that complementation of the defective RNA with transgenic CP

had taken place. No virus particles were detected when the same plasmid was shot into non-transgenic *Nicotiana* plants.

DISCUSSION

Detection of heteroencapsidation in non-transgenic and transgenic *Nicotiana* plants was made possible by successful contemporary infection by GVA and GVB, following inoculation of the second virus in the same host after an interval of three days.

IC-PCR repeatedly showed amplification of GVB sequences from virions immunocaptured by specific GVA antibodies with a frequency ranging from 33 to 75%, whereas the opposite situation was not observed. Since GVA and GVB CPs share 56% identity and 67% similarity at the amino acid level (Minafra *et al.*, 1994) antibodies with no cross-reactivity were used in our experiments, to avoid recognition of common antigenic sites (Goszczyński *et al.*, 1996; Choueiri *et al.*, 1997).

Fig. 4. Analysis of IC-RT-PCR products from GVB CP + transgenic *N. occidentalis* plants challenge-inoculated with GVA. A 430 bp GVA-specific product was amplified using GVA primers after immunocapture with GVB MAbs (lanes 2 and 4). No amplification product is present in lanes 1, 3 and 5 (GVA infection, GVB immunocapture and amplification with GVA primers). GVB controls from non-transgenic *N. occidentalis*: lane 6: immunocapture-PCR; lane 7: RT-PCR from total nucleic acids; lane 8: GVA amplification from GVB CP transgenic *N. occidentalis* using GVA MAbs and primers.

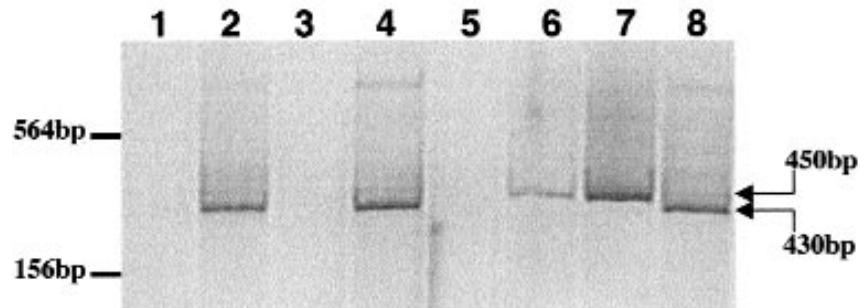


Fig. 5. Western blot of GVB CP+ transgenic *N. benthamiana* (R_1 seedlings) for detecting CP expression (22 kDa) before particle bombardment. Each lane was loaded with the equivalent amount of 10 mg of plant tissue. Lanes 1 to 7: single transgenic plants of line *No B.*; lane 8: GVB control (infected non-transgenic plant); lane 9: healthy *N. occidentalis*.

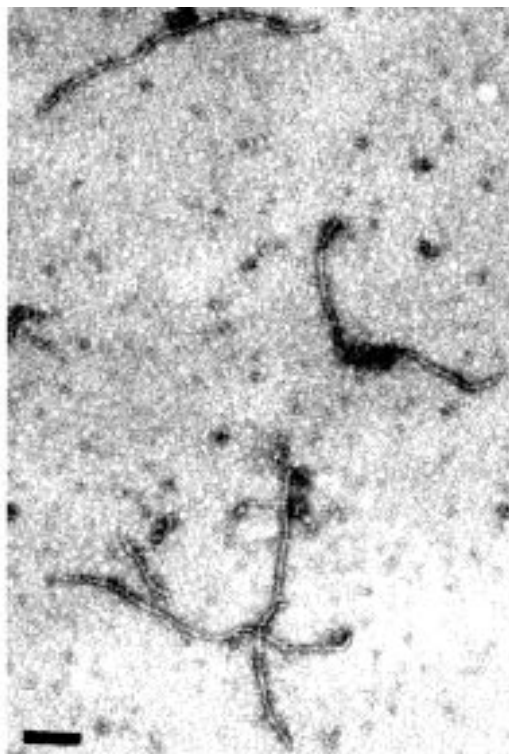
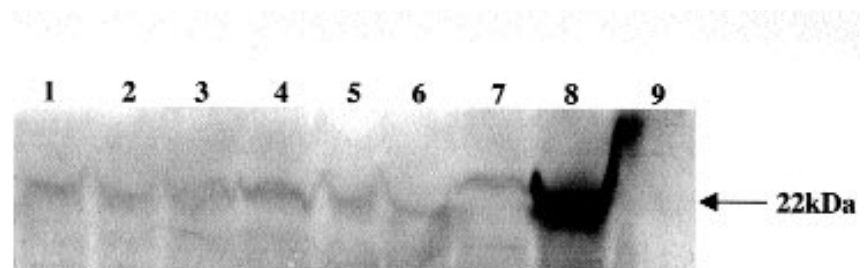


Fig. 6. Immunocaptured GVB particles from GVB CP-transgenic *N. benthamiana* shot-inoculated with a CP-defective full length GVB clone. Bar =100 nm.

Affinity between GVA CP and heterologous GVB RNA was shown by the one-way phenotypic mixing occurring in non-transgenic doubly-infected plants. We conclude that with GVA/GVB heteroencapsulation is a natural phenomenon, as with other viruses (Milne *et al.*, 1980, 1984; Hu *et al.*, 1988; Hobbs and Mac Laughlin, 1990; Bourdin and Lecoq, 1991; Creamer and Falk, 1990). Why GVB RNA was more readily encapsidated by GVA CP than the contrary could depend on the relative abundance of competitive GVA CP (due to the higher multiplication rate of GVA) or on the lower efficiency of GVB MAbs in capturing phenotypically mixed particles.

Heteroencapsulation was also recorded in *Nicotiana* plants expressing transgenic CP and it is possible that similar events take place in transgenic grapevines, even though, in these hosts, vitiviruses seem to multiply less than in *Nicotiana* species and their distribution is erratic (Boscia *et al.*, 1997). However, should heteroencapsulation occur in transgenic grapevines, it would not affect the epidemiological behaviour of GVA and GVB as both viruses have at least two mealybug vectors in common (*Planococcus ficus* and *Pseudococcus affinis*) (Boscia *et al.*, 1997).

The finding that a CP-defective GVB RNA can be coated *in planta* by the homologous CP confirms previous reports on complementation of defective viral RNA

in transgenic plants (Osbourne *et al.*, 1989; Morozov *et al.*, 1997; Jacquet *et al.*, 1998; Varrelmann and Maiss, 2000).

ACKNOWLEDGMENTS

This work was done within the project 'Risk assessment with genetically engineered woody plants expressing virus coat protein gene' (contract BIO4-CT-96-0773) supported by the European Commission.

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Received 5 September 2000

Accepted 23 November 2000