Aphid Transmission of a Non-Aphid-Transmissible Strain of Zucchini Yellow Mosaic Potyvirus from Transgenic Plants Expressing the Capsid Protein of Plum Pox Potyvirus

H. Lecoq[1], M. Ravelonandro[2], C. Wipf-Scheibel[1], M. Monsion[2], B. Raccah[3], and J. Dunez[2]

[1]INRA, Station de Pathologie Végétale, B.P. 94, 84143 Montfavet-cédex, France; [2]INRA, Station de Pathologie Végétale, La Grande Ferrade, B.P. 81, 33883, Villenave d'Ornon-cédex, France; [3]Department of Virology, A.R.O., The Volcani Center, P.O.B. 6, Bet Dagan 50250, Israel.

Received 11 December 1992. Accepted 16 February 1993.

Transgenic *Nicotiana benthamiana* plants expressing the coat protein of an aphid-transmissible strain of plum pox potyvirus (PPV) were infected by a non-aphid-transmissible strain of zucchini yellow mosaic potyvirus (ZYMV-NAT) in which the coat protein has a D-T-G amino acid triplet instead of the D-A-G triplet essential for aphid transmission. The aphid vector *Myzus persicae* could acquire and transmit ZYMV-NAT from these plants but not from infected *N. benthamiana* control plants that were not transformed or that were transformed but not expressing the PPV coat protein. The aphid-transmitted ZYMV subcultures were shown still to be non-aphid-transmissible from plants not expressing PPV coat protein, which indicated that their transmission was not due to RNA recombination or to reversion to the aphid-transmissible type. In immunosorbent electron microscopy experiments using the decoration technique, virus particles in the infected control plants could be coated only with ZYMV antibodies, while virus particles in the infected transgenic plants expressing the PPV coat protein could be coated not only with ZYMV antibodies but also in part with PPV antibodies. This suggests that aphid transmission of ZYMV-NAT occurred through heterologous encapsidation. These results indicate a potential risk of releasing genetically engineered plants into the environment.

Additional keyword: heteroencapsidation.

The concept of pathogen-derived resistance offers an attractive alternative to conventional plant breeding for obtaining virus-resistant plants (Sanford and Johnston 1985). Incorporation of virus coat protein (CP) genes into plant genomes has been successful in providing significant degrees of resistance to virus infections (Beachy *et al.* 1990).

MPMI, Vol. 6, No. 3, pp. 403-406
This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1993.

Usually, resistance is effective only against the virus providing the CP gene or closely related strains. However, transgenic plants expressing potyvirus CPs have been reported to present partial resistance to other potyviruses, expressed as a delay in symptom expression or a reduction in number of infected plants (Beachy *et al.* 1990).

This strategy may entail biological risks when CP-transgenic plants are exposed to infection with related or unrelated viruses. Among the potential risks are recombination between the engineered CP mRNAs and viral genomic RNAs, or heteroencapsidation of viral RNAs by the plant-produced CP (Hull 1990). This may stimulate systemic virus spread within a plant, as observed for tobacco mosaic virus (TMV) (Osbourn *et al.* 1990), or it may modify vector specificity, which is often dependent on the nature of the CP (Harrison and Robinson 1988). While such potential interactions may occur in mixed infections in nature, their occurrence may be increased in CP-transgenic crops, in which every virus infection is in essence a double infection with regard to the CP gene (De Zoeten 1991).

We report here that a non-aphid-transmissible potyvirus may be transmitted by aphids from transgenic plants expressing the CP of another potyvirus.

Transgenic *Nicotiana benthamiana* containing the full-length CP gene sequence of an aphid-transmissible strain of plum pox potyvirus (PPV) were obtained recently (Ravelonandro *et al.* 1992). Three plant lines obtained from different transformation events and expressing the PPV CP, BPC-5, -13, and -20, were used. A nontransformed line (BNT) and a transformed line not containing the CP gene (BT) were used as controls. Aphid transmission of potyviruses requires a functional CP and a helper component (Lecoq *et al.* 1991). The non-aphid-transmissible strain of zucchini yellow mosaic virus (ZYMV-NAT), produces a functional helper component (Antignus *et al.* 1989) but has a transmission-defective CP: A D-T-G triplet in the N-terminal part (Gal-On *et al.* 1992) replaces the D-A-G sequence essential for aphid transmission (Atreya *et al.* 1991).

ZYMV-NAT was mechanically inoculated to PPV CP-expressing and control plants when they had four expanded

leaves. Plants were incubated in an insect-proof greenhouse and served as virus sources for aphid transmissions.

In two independent experiments, aphid transmissions were observed from five out of the six BPC-5 transgenic plants tested but not from nontransformed plants (Table 1). The overall transmission rate of ZYMV-NAT obtained from transgenic plants with 10 viruliferous aphids per test plant was lower (4%) than that observed with an aphid-transmissible isolate of ZYMV, from melons (100%) (Lecoq *et al.* 1991) or from nontransformed *N. benthamiana* (17.5%), which is a less efficient source of ZYMV for aphids than cucurbits. In similar conditions, ZYMV-NAT was transmitted by aphids from BPC-13 and BPC-20 plants (one infected test plant out of 20 inoculated for both lines) but not from transformed plants not containing the CP gene (0 infected test plant out of 90 inoculated). This indicates that ZYMV-NAT transmission was related to the *in vivo* expression of the engineered PPV CP and not to the transformation event itself. The ZYMV subcultures thus obtained were not aphid transmissible from nontransgenic infected plants, indicating that their transmission was not due to genetic recombination with the D-A-G coding region of the PPV transgene mRNA or to reversion towards an aphid-transmissible type.

The recent finding that heteroencapsidation was involved in the transmission of ZYMV-NAT by aphids when in mixed infection with an aphid-transmissible potyvirus (Bourdin and Lecoq 1991) prompted the search for virus particles partially or totally encapsidated by the genetically engineered PPV CP.

Immunosorbent electron microscopy was conducted as previously described (Bourdin and Lecoq 1991). Grids covered with a pyroxiline membrane were coated with a

Table 1. Aphid transmission[a] of a non-aphid-transmissible isolate of zucchini yellow mosaic virus (ZYMV-NAT) from transformed *Nicotiana benthamiana* plants expressing the plum pox virus coat protein (BPC-5) and from nontransformed *N. benthamiana* plants (BNT)

Source plants[b]	Weeks after inoculation of source plants				
	2	6	9	12	Total
N. benthamiana line BPC-5	0/60[c]	6[d]/90	5/120	7/180	18/450
N. benthamiana line BNT	0/60	0/90	0/120	0/180	0/450

[a]Nonviruliferous *Myzus persicae* were allowed a 1-min acquisition access period on infected leaves before transfer to test plants (10 aphids per plant). For each source leaf (one or three per plant according to the experiment), 10 test plants (*Cucumis melo* 'Védrantais') were used. Aphids were allowed to feed on test plants for at least 2 hr before being killed by an insecticide.

[b]Two independent experiments were conducted each with three different BPC-5 or BNT source plants.

[c]Results are expressed as the cumulated number of test plants infected divided by the number of test plants inoculated. ZYMV-NAT transmission was confirmed by DAS-ELISA after a 3-wk incubation in an insect-proof greenhouse.

[d]All 18 test plants found to be infected by ZYMV were used individually as virus sources for secondary aphid transmission experiments to 10 melon plants with five *M. persicae* per plant. All secondary transmission attempts were negative, while similar experiments conducted with an aphid-transmissible ZYMV isolate led to 100% transmission (Lecoq *et al.* 1991).

mixture of ZYMV and PPV antisera, each at a dilution of 1:1,000. Plant extracts were obtained by grinding infected tissue in 4 vol (w/v) of phosphate buffer and diluting 1:30 in the same buffer. Decoration was done with antisera diluted to 1:30 in phosphate buffer and grids were negatively stained with 1% ammonium molybdate, pH 7. Observations were made with a Philips CM 10 electron microscope.

In extracts from BT and BNT control plants infected by ZYMV-NAT, no virus particles were decorated by an antiserum against PPV (Fig. 1A and Table 2), while in extracts from BNT control plants infected by PPV all particles were decorated by the PPV antiserum (Fig. 1B and Table 2).

In extracts from the three CP-transgenic lines infected by ZYMV-NAT, three types of particles were observed: Some were not decorated by the PPV antiserum, while a majority were decorated on either short or long sections, and a few appeared totally decorated (Fig. 1C and Table 2). No particles were observed in noninoculated CP-transgenic plants. These results indicated that ZYMV-NAT RNA was encapsidated, at least in part, by the endogenous PPV CP. That PPV CP occurred in few clusters along the particles instead of being distributed at random suggests that the shift from one CP type to the other in the assembly process is relatively rare and that once a CP type is involved in the encapsidation, there is a preferential affinity for CPs of its own type.

When a mixture of ZYMV and PPV antisera was used for decoration, all particles were totally decorated (Fig. 1D), confirming that particles comprised CPs from both origins. Recently, heteroencapsidated particles were also observed in transgenic potato plants expressing the potato virus Y^N (PVY^N) CP when infected by PVY^O (Farinelli *et al.* 1992).

Our study suggests that ZYMV-NAT transmission from transgenic plants expressing PPV CP occurred through heteroencapsidation. It also indicates that the PPV CP produced by transgenic *N. benthamiana* is biologically functional both for encapsidation and for aphid transmission.

Transgenic plants expressing viral CPs have been obtained for viruses belonging to seven groups and their resistance to infection under artificial and field conditions is well-documented (Beachy *et al.* 1990). Comparatively little attention has been paid to the epidemiological consequences of their cultivation on a commercial basis (De Zoeten 1991).

Heterologous encapsidation is involved in changes in vector specificity for luteoviruses and potyviruses (Rochow 1982; Bourdin and Lecoq 1991). Recently, particles were reassembled *in vitro* using cucumber mosaic cucumovirus (CMV) CP and TMV RNA. These particles were transmitted by aphids after acquisition through membranes, although TMV is normally not aphid transmissible (Chen and Francki 1990). The lack of specificity with which some viral CPs encapsidate heterologous RNAs *in vitro*, and the evidence that heteroencapsidation occurs in transgenic plants expressing viral CPs, require that the epidemiological significance of this phenomenon should be investigated carefully.

The change in transmissibility by a vector that we ob-

served here will occur only for one transmission passage. When infecting a nontransgenic plant the virus will be again encapsidated only in its own CP and recover its usual vectors. However, this event could stimulate virus spread within a transgenic crop or contribute to the introduction of the virus into a new ecological niche.

Strategies that could reduce the risk that we have shown include incorporation of CP genes with mutations or deletions in the region encoding the D-A-G triplet essential for aphid transmission, or in regions coding for amino acids interacting in particle assembly (Jagadish *et al.* 1991). Recently, the use of potyvirus CP genes with truncated termini was proved to confer high levels of resistance (Lindbo and Dougherty 1992).

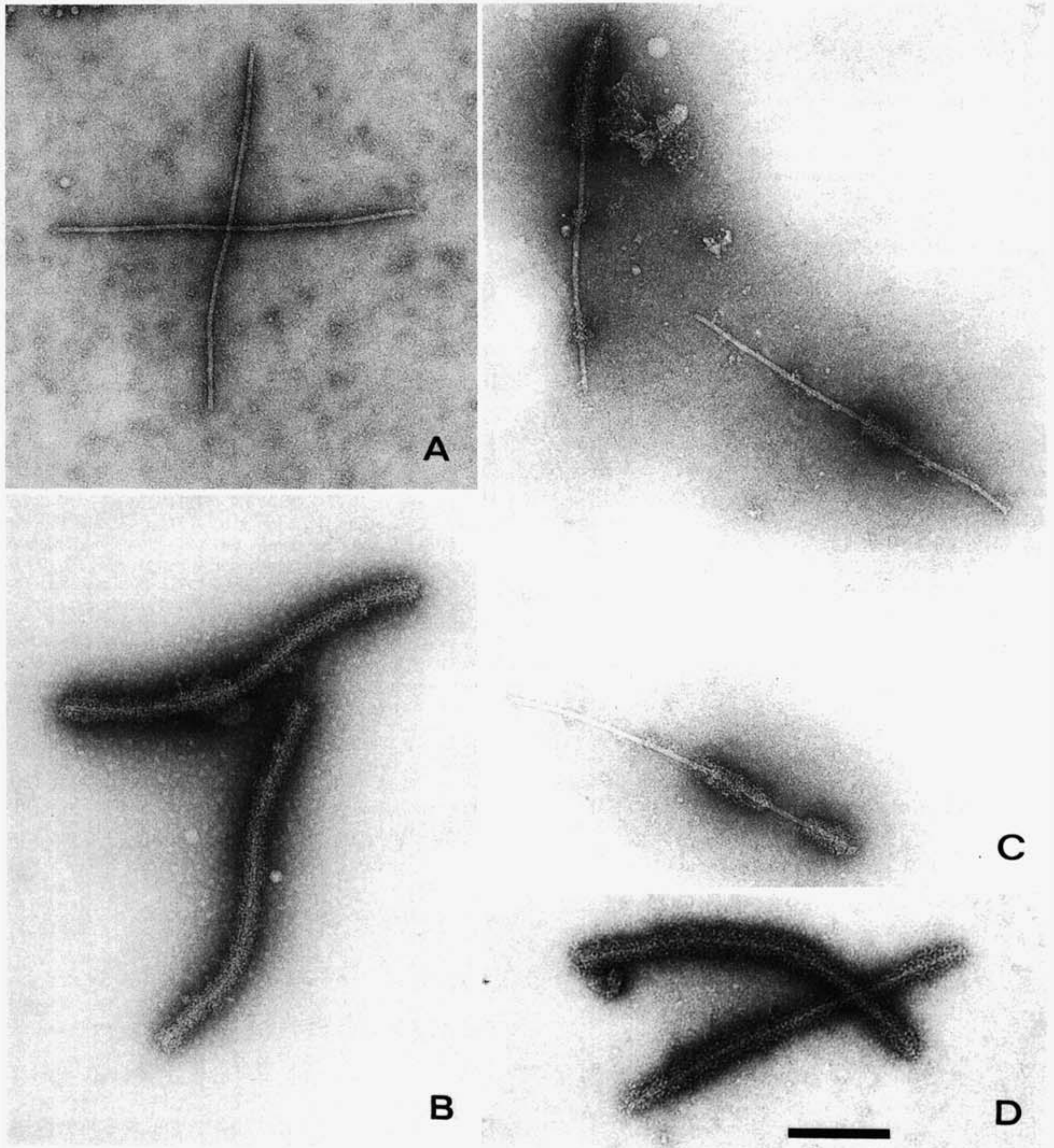

Fig. 1. Electron micrographs of virus particles in extracts from *Nicotiana benthamiana* **A, B,** not transformed or **C, D,** transformed and expressing the plum pox potyvirus (PPV) coat protein after infection by zucchini yellow mosaic virus (ZYMV-NAT) (A,C and D) or PPV (B). Particles were decorated with a PPV antiserum either alone (A, B, and C) or in mixture with an antiserum against ZYMV (D). Bar = 200 nm.

Table 2. Types of virus particles observed in infected *Nicotiana benthamiana* leaf extracts after trapping with a mixture of plum pox virus (PPV) and zucchini yellow mosaic virus (ZYMV) antibodies and decoration with PPV antibodies alone or in mixture with ZYMV antibodies

N. benthamiana line	Infecting virus	Antiserum used for decoration	Number of particles[a]		
			Totally decorated	Partially decorated	Not decorated
Nontransformed					
BNT	PPV	PPV	68 (100)[b]	0 (0)	0 (0)
	ZYMV-NAT	PPV	0 (0)	0 (0)	167 (100)
Transgenic (−CP)					
BT	ZYMV-NAT	PPV	0 (0)	0 (0)	52 (100)
Transgenic (+CP)					
BPC-5	ZYMV-NAT	PPV	2 (0.6)	273 (78.9)	71 (20.5)
	ZYMV-NAT	PPV + ZYMV	70 (100)	0 (0)	0 (0)

[a]Particles were rated as totally decorated when adsorption of IgG was detected along the entire particle length (as in Fig. 1B and D), partially decorated when adsorption of IgG was observed only on sections of the virus particle (as in Fig. 1C), and not decorated when adsorption of IgG was not detectable on electron micrographs (as in Fig. 1A). The immunosorbent electron microscopy was conducted as described in the text.

[b]Numbers of particles, followed in parentheses by the percentage distribution for each type in the treatment. Particle counts were done on micrograph prints (final magnification × 77,000). Extracts from at least four different plants were observed for each treatment. No particles were observed in noninoculated control or PPV CP-transgenic plants.

ACKNOWLEDGMENTS

We thank T. Candresse and O. Le Gall for stimulating discussions and C. Morris for reviewing the manuscript.

LITERATURE CITED

Antignus, Y., Raccah, B., Gal-on, A., and Cohen, S. 1989. Biological and serological characterization of zucchini yellow mosaic and watermelon mosaic virus 2 isolates in Israel. Phytoparasitica 17:289-298.

Atreya, P. L., Atreya, C. D., and Pirone, T. P. 1991. Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. Proc. Natl. Acad. Sci. USA 88:7887-7891.

Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474.

Bourdin, D., and Lecoq, H. 1991. Evidence that heteroencapsidation between two potyviruses is involved in aphid transmission of a non-aphid-transmissible isolate from mixed infection. Phytopathology 81:1459-1464.

Chen, B., and Francki, R. I. B. 1990. Cucumovirus transmission by the aphid *Myzus persicae* is determined solely by the viral coat protein. J. Gen. Virol. 71:939-944.

De Zoeten, G. A. 1991. Risk assessment: Do we let history repeat itself? Phytopathology 81:585-586.

Farinelli, L., Malnoë, P., and Collet G. F. 1992. Heterologous encapsidation of potato virus Y strain O (PVY^O) with the transgenic coat protein of PVY strain N (PVY^N) in *Solanum tuberosum* cv. Bintje. Bio/Technology 10:1020-1025.

Gal-on, A., Antignus, Y., Rosner, A., and Raccah, B. 1992. A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on multiplication. J. Gen. Virol. 73:2183-2187.

Harrison, B. D., and Robinson, D. J. 1988. Molecular variations in vector borne plant viruses: Epidemiological significance. Philos. Trans. R. Soc. B 321:447-462.

Hull, R. 1990. The use and misuse of viruses in cloning and expression in plants. Pages 443-457 in: Recognition and Response in Plant-Virus Interactions. R. S. S. Fraser, ed. Springer-Verlag, Berlin.

Jagadish, M. N., Ward, C. W., Gough, K. H., Tulloch, P. A., Whittaker, L. A., and Shukla, D. D. 1991. Expression of potyvirus coat protein in *Escherichia coli* and yeast and its assembly into virus-like particles. J. Gen. Virol. 72:1543-1550.

Lecoq, H., Bourdin, D., Raccah, B., Hiebert, H., and Purcifull, D. E. 1991. Characterization of a zucchini yellow mosaic virus isolate with a deficient helper component. Phytopathology 81:1087-1091.

Lindbo, J. A., and Dougherty, W. G. 1992. Pathogen-derived resistance to a potyvirus: Immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.

Osbourn, J. K., Sarkar, S., and Wilson, T. M. A. 1990. Complementation of coat protein-defective TMV mutants in transgenic tobacco plants expressing TMV coat protein. Virology 179:921-925.

Ravelonandro, M., Monsion, M., Teycheney, P. Y., Delbos, R., and Dunez, J. 1992. Construction of a chimeric viral gene expressing plum pox virus coat protein. Gene 120:167-173.

Rochow, W. F. 1982. Dependent transmission by aphids of barley yellow dwarf luteoviruses from mixed infections. Phytopathology 72:302-305.

Sanford, J. C., and Johnston, S. A. 1985. The concept of pathogen-derived resistance: Deriving resistance genes from the parasite's own genome. J. Theor. Biol. 113:395-405.