HIGH RESISTANCE AND CONTROL OF BIOLOGICAL RISKS IN TRANSGENIC PLANTS EXPRESSING MODIFIED PLUM POX VIRUS COAT PROTEIN

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Summary. – Transgenic plums transformed with the plum pox virus coat protein (PPV CP) gene displayed a resistance to the sharka disease (Ravelonandro *et al.*, 1997). However, the expression of PPV CP in transgenic plants may lead to complementation of deficient characteristic of an incoming potyvirus. Indeed, an aphid-intransmissible strain of zucchini yellow mosaic virus (ZYMV-NAT) could be transmitted when encapsidated by the engineered PPV CP (Lecoq *et al.*, 1993). To control such a risk, new PPV CP constructs were designed and introduced into *Nicotiana benthamiana* genome. In the first construct, the DAG amino acid triplet involved in the potyvirus aphid-transmission was deleted. The second construct encoded a truncated PPV CP lacking its first 140 amino acids. In the last construct, the nucleotides encoding the charged amino-acids R^{220}, Q^{221} and D^{264} localized in the core of the PPV CP were removed. A bacterial expression system was developed to show that these deletions prevent the assembly of the PPV CP subunits. For each construct, several transgenic lines were produced and first challenged with several strains of PPV. Two phenotypes of resistance were observed: recovery and immunity. Their biochemical characterization showed that the resistance was RNA-mediated and therefore can be classified as homology-dependent (Jacquet *et al.*, 1998a). Resistant lines producing high level of wild type or modified PPV CP were then inoculated with ZYMV-NAT to perform an aphid-transmission assay. Results of these experiments demonstrated that the use of modified forms of PPV CP genes in transgenic plants provide a good way to control the biological risks associated with heteroencapsidation (Jacquet *et al.*, 1998b).

Key words: plum pox virus; coat protein; transgenic plants; biological risks

Introduction

Since the first report on tobacco plants transformed with the tobacco mosaic virus (TMV) coat protein (CP) gene (Powell *et al.*, 1986), various models aiming at the virus resistance have been developed (for review see Wilson, 1993).

In parallel with the coat protein-mediated resistance, the protection could be mediated by transgene RNA alone. This RNA-mediated protection was first observed against potyviruses such as tobacco etch virus (TEV) (Lindbo and Dougherty, 1992a, 1992b) in transgenic tobaccos containing CP gene. Baulcombe (1996) defined this kind of protection as dependent on the homology between the viral trans-gene and the incoming virus. Its mechanism depends on the post-transcriptional regulation of a nuclear gene. This leads to the sense suppression of the trans-gene (for review see English *et al.*, 1996).

The sharka disease is one of the major threats to stone fruit production. Only a few natural sources of resistance have been identified to date. The exploitation of cultivars of commercial value that can have a high level of resistance takes a long time. The use of transgenic plants to control PPV has been chosen. Using transgenic *N. benthamiana* plants as models Regner *et al.* (1992) and Ravelonandro *et al.* (1993) showed the efficiency of this approach. However, Lecoq *et al.* (1993) showed that the use of viral CP may entail the biological risks. The epidemiological characteristics of an aphid-intransmissible strain, ZYMV-NAT, when infecting PPV CP transgenic plants, can be modified. The heteroencapsidation phenomenon has been shown to be the main cause of such a modification. The DAG amino acid triplet located in the

N-terminal part of CP is involved in the complementation for virus transmission. For this reason, different modified PPV CP genes have been produced to reduce the biological risks linked to the heteroencapsidation.

Materials and Methods

Modified CP gene constructs. To prevent any further reverse or random mutations, we deleted the undesired sites (Jacquet *et al.*, 1998a,b). Different CP gene constructs lacking the DAG triplet (ΔDAG), the DAG triplet plus the nucleotides encoding R^{220}, Q^{221} and D^{264} (3Δ) were produced. To address a potential involvement of the N-terminal region of CP in aphid transmission, another construct was made in which the first 420 nucleotides of the PPV CP gene were deleted (Δ140).

Plants transformed with different gene constructs. An *Agrobacterium tumefaciens* binary transformation system was used to integrate the DNA sequences of the three modified PPV CP constructs into the genome of *N. benthamiana*.

PPV detection PPV NAT strain (Maiss *et al.*, 1989) was used to characterize the PPV-resistant plants because it induced very severe symptoms on *N. benthamiana* (vein clearing, leaf curling and dwarfism). An inoculum was prepared by grinding fresh, infected *N. benthamiana* leaves in a citrate buffer (1:3). A hundred percent of the non-transgenic and control (transformed with the pBI vector) *N. benthamiana* lines showed a systemic infection 5 – 6 days post inoculation. The symptom development was observed every three days and a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was conducted every week to detect PPV in newly emerged leaves.

ZYMV-NAT inoculation and aphid transmission experiments Due to a slow migration of ZYMV in *N. benthamiana* plants, the presence of the virus was carefully verified by DAS-ELISA and Western blot analysis. The aphid transmission experiments were conducted in leaves of infected transgenic plants only. *Myzus persicae* aphids were allowed to acquire the virus from the transformed plants before transfer to melon or zucchini plants.

mRNA and CP were assayed by Northern and Western blot analyses, respectively (Jacquet *et al.*, 1998a).

Results and Discussion

Transgenic plants expressing modified forms of PPV CP

Over 30 plants were transformed with the CP gene lacking the DAG triplet, 15 with the Δ140 and 12 with the 3Δ gene construct. The levels of transgene products, mRNA and CP of the T_0 primary transformants were assayed. The steady state level of transgene mRNA was heterogeneous in the lines containing the same construct. In the majority of plants tested, the accumulation of the modified CP correlated with the steady state level of the transgene mRNA. Plants showing a high level of mRNA had a large amount of CP and *vice versa* (Fig. 1). An exception was observed in the Δ140

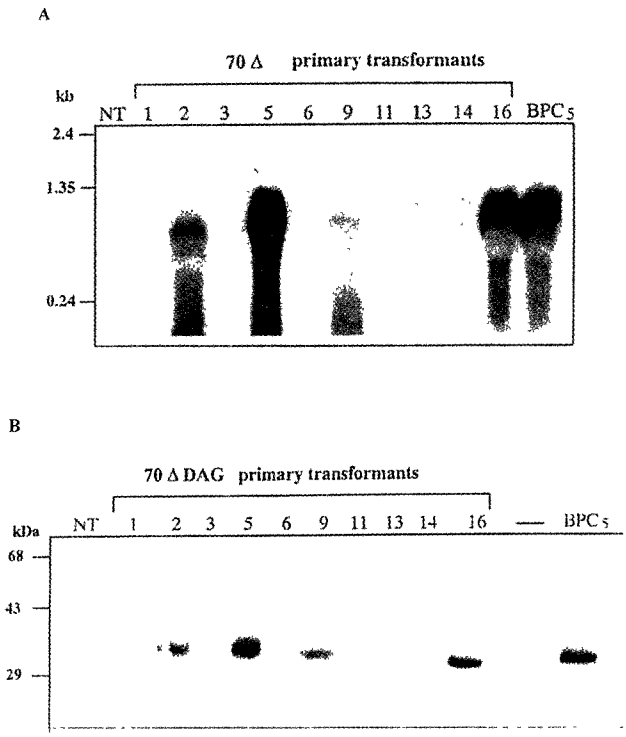

Fig. 1
Different levels of transgene products in a pool of transgenic plants expressing ΔDAG CP
mRNA transcripts (A), CP (B). Various transformants (lanes 1-16), PPV FLCP (lane BPC5), non-transformed control (lane NT).

plants, which had a high transgene mRNA level but did not accumulate CP. This may be explained by the deletion of epitopes which could be recognized by the antisera used.

Challenging assays with PPV

The PPV-NAT-inoculated plant lines showed three possible reactions: susceptibility, recovery or immunity. More than one third of the lines were fully susceptible, 60% recovered and about 10% were highly resistant. The latter phenotype is very interesting because these plants never developed symptoms and their resistance was confirmed by more sensitive detection techniques, such as the Western blot analysis or molecular hybridization, which were negative for the presence of PPV.

Risk assessment studies

They were performed with the aim to see whether the plants produced could be considered as product-safer ones or not. We chose the transgenic lines which were developing recovery reaction to PPV and were readily susceptible to ZYMV-NAT. These plants were selected with regard to their

biochemical characteristics: a high level of transgene mRNA transcripts and CP. Of course, the Δ140 plant lines selected were chosen because of high level of mRNA only. These comparative studies showed that no transmission occurred in plants expressing the modified CP and about 2 – 4% transmission rate in plants expressing normal CP gene. This rate of transmission was low because the heteroassistance involving the helper component protein of ZYMV and PPV CP probably requires some specific motifs stimulating this process. Although these results originate from an experimental model, the heteroencapsidation may be considered an important phenomenon occuring in the nature too and having a big impact especially on a system showing a frequent mixed infection: e.g transgenic cucurbits expressing ZYMV CP possibly infected with another potyvirus, papaya ringspot virus (PRSV).

Field experiments of such type would be fruitful for the assessment of the heteroencapsidation in mixed infection and possible impacts of use of native CP gene in these experiments.

Our results demonstrate that the modified PPV CP gene constructs that we developed can produce a high level of resistance to PPV and , as expected, they contribute to the reduction of the potential biological risks of use of CP gene constructs.

References

Baulcombe D (1996): Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* **10**, 1833-1844.

Beachy RN, Loesh-Fries S, Tumer NE (1990): Coat protein mediated resistance against virus infection. *Ann. Rev. Phytopathol.* **28**, 451-474.

Cassidy BG, Nelson RS (1995): Differences in protection phenotypes in tobacco plants expressing coat protein genes from peanut stripe potyvirus with or without an engineered ATG. *Mol. Plant-Microbe Interact.* **8**, 357-365.

Dougherty WG, Lindbo JL, Smith HA, Parks TD, Swaney S, Proebsting WM (1994): NA-mediated virus resistance in transgenic plants: Exploitation of a cellular pathway possibly involved in RNA degradation. *Mol. Plant-Microbe Interact.* **7**, 544-552.

English JJ, Mueller E, Baucombe DC (1996): Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**, 179-188.

Flavell RB (1994): Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl. Acad. Sci. USA* **91**, 3490-3496.

Jacquet C, Ravelonandro M, Bachelier JC, Dunez J (1998a): High resistance to plum pox virus (PPV) in transgenic plants containing modified and truncated forms of PPV coat protein gene. *Transgenic Res.* **7**, 29-39.

Jacquet C, Delecolle B, Raccah B, Lecoq H, Dunez J, Ravelonandro M (1998b): Use of modified plum pox virus coat protein genes developed to limit heteroencapsidation-associated risks in transgenic plants. *J. Gen. Virol.* **79**, 1509-1517.

Lecoq H, Ravelonandro M, Wipf-Scheibel C, Monsion M, Raccah B, Dunez J (1993): Aphid transmission of an aphid non-transmissible strain of zucchini yellow mosaic potyvirus from transgenic plants expressing the capsid protein of plum pox potyvirus. *Mol. Plant-Microbe Interact.* **6**, 403-406.

Lindbo JA, Dougherty WG (1992a): 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**, 141-153.

Lindbo JA, Dougherty WG (1992b): Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch replication in transgenic plants and protoplasts. *Virology* **189**, 725-733.

Maiss E, Timpe U, Briske A, Jelkmann W, Casper R, Himmler G, Mattanovich D, Katinger HWD (1989): The complete nucleotide sequence of plum pox virus RNA. *J. Gen. Virol.* **70**, 513-524.

Powell AP, Nelson R, de B, Hoffmann N, Rogers S, Fraley R, Beachy R (1986): Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.

Ravelonandro M, Monsion M, Delbos R, Dunez J (1993): Variable resistance to plum pox virus and potato virus Y infection in transgenic Nicotiana plants expressing plum pox virus coat protein. *Plant Sci.* **91**, 157-169.

Regner F, da Machado A, Laimer da Machado M, Steinkellner H, Mattanovitch D, Hanzer Weiss H, Katinger H (1992): Coat protein mediated resistance to plum pox virus in *Nicotiana clevelandii* and *N. benthamiana. Plant Cell Rep.* **11**, 30-33.

Silva-Rosales L, Linbo JA, Dougherty WG (1994): Analysis of transgenic tobacco plants expressing a truncated form of a potyvirus coat protein nucleotide sequence. *Plant Mol. Biol.* **24**, 929-939.

Smith HA, Swaney SL, Parks TD, Wernsman EA, Dougherty WG (1994): Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. *Plant Cell* **6**, 1441-1453.

Wilson TMA (1993): Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. *Proc. Natl. Acad Sci. USA* **90**, 3134-3141.