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VARIABILITY OF *PLUM POX VIRUS* POPULATIONS IN PPV-RESISTANT TRANSGENIC AND NON-TRANSGENIC PLUMS[1]

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Abstract

To determine the resistance to PPV in natural conditions, transgenic plums (*Prunus domestica*) carrying the coat protein gene of *Plum pox virus* (PPV) were established in the open field in Valencia, Spain in 1997. To assess the environmental impact that the release of transgenic plums to the field has on the dynamic and diversity of virus population, the variability of PPV populations present in transgenic vs. non-transgenic plums was compared. Serological and molecular characterisation of the PPV isolates from transgenic and non-transgenic plums was carried out. No differences in the serological or molecular variability of the PPV populations present in transgenic and non-transgenic plums was detected. The results of this work could shed light on concerns about potential biological and environmental risks associated with the liberation of genetically modified plants in the open field.

Key words: *Plum pox virus* variability, transgenic plums, ELISA-DASI, sequencing, genetic analysis

Introduction

Sharka, caused by *Plum pox virus* (PPV), is one of the most devastating diseases of stone-fruit trees, mainly affecting apricots, peaches and plums. Conventional breeding techniques focused to identify viral resistance genes and move them into economically important crops are being carried out, but limited number of such re-

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sistance genes and the long term of the experiments have promoted the use of biotechnological methods to obtain virus resistant plants. So, expressing a segment of plant viral genome within a plant can provide resistance to the virus from which the transgene was derived and in some cases to related viruses. In this way, transgenic plums carrying the coat protein (CP) gene of the PPV were obtained (Scorza et al. 1994). Five transgenic lines, named C4, C5, C6, PT-6 and PT-23, were evaluated for their engineered resistance to PPV in open field conditions. One of these lines, C5, demonstrated to be highly resistant to PPV in greenhouse (Ravelonandro et al. 1997) and in natural conditions (Scorza et al. 2004, Hily et al. 2004). The PPV resistance of the C5 can be transferred to the progeny, and therefore C5 transgenic plums could constitute an excellent source of resistance to PPV for the development of PPV resistant cultivars. However, the release of genetically modified organisms to the open field, and with more emphasis of economically important crops, has created controversies related to the environmental safety of transgenic organisms and requires an strict evaluation of risks to reduce potential environmental hazards. With this in mind, the environmental impact of transgenic plums carrying the PPV-CP on the dynamic and diversity of virus populations was assessed. Particularly, the variability of PPV populations present in transgenic and non-transgenic plums was compared by serological and molecular analysis.

Material and methods

Experimental orchards

An experimental plot (A) was established in Liria, Valencia (Spain) in 1997. 100 plums were planted in alternate five-tree rows of transgenic (*Prunus domestica* L.) and non-transgenic (*P. salicina* Lindl.) plums. Ten trees per transgenic line (C4, C5, C6, PT-6 and PT-23), making a total of 50 out of 100 trees, were assayed. The plot was surrounded by two rows of guard trees (peach × almond hybrid) that are sexually incompatible with plums. A total of 25 out of 50 non-transformed plums were graft-inoculated with a PPV-D strain (3.3 RB/GF Mp15/GF; Asensio et al. 1999) by bark chips in 1997. This PPV isolate is defective in the epitope that recognizes the monoclonal antibody 4BD7 (Cambra et al. 1994, López-Moya et al. 1994). An adjacent *P. salicina* orchard (B) was used as baseline or control plot to determine the natural variability of PPV in conventional plums in the same area.

PPV isolates

Samples were obtained from 15 non-transgenic and 22 transgenic plums (five isolates from C4, one isolate from C5, six isolates from C6, five isolates from PT-6 and five isolates from PT-23 line) from the experimental plot (A) and from 27 non-transgenic plums from the baseline plot (B). Three PPV populations were artificially defined: PPV isolates present in transgenic plums from the experimental orchard (A), PPV in non-transgenic plums from the experimental orchard (A) and

PPV in non-transgenic plums from the baseline plot (B). Samples were collected in May 2004, seven years after the establishment of the plot, and used for serological and molecular analysis. Leaves showing typical PPV symptoms were collected and grinded into individual plastic bags containing a thin net (Plant Print Diagnostics) in the presence of 1/20 (v/w) PBS buffer, pH 7.2, supplemented with 2% (w/v) polyvinylpyrrolidone and 0.2% (w/v) sodium diethyl dithiocarbamate. The same extracts were used for both serological and molecular analysis.

Serological analysis

Double-antibody sandwich indirect ELISA (DASI) using eight different monoclonal antibodies: 5B-IVIA, 4DG5, 4DG11, 4BD7, 4DB12, 1EB6 (Cambra et al. 1994), 05 (kindly provided by Dr. Milan Navratil, Czech Republic) and AL (Boscia et al. 1997) was carried out for PPV typing according to Cambra et al. (1994).

Molecular analysis

Total RNA was extracted from leaves collected from transgenic, non-transgenic and control plums by the RNeasy Plant kit from Qiagen, according to the manufacturer's instructions. For sequencing, the most hypervariable region of the potyvirus genome corresponding to the 3'end of the NIb gene and the 5'end of the coat protein gene was amplified from each analysed PPV isolate by RT-PCR. Briefly, 5 μl of total RNA extracted were incubated with 20 μl of mixture containing 1X Taq polimerase buffer (Promega), 3 mM $MgCl_2$, 250 μM dNTP, 0.3% Tritón X-100, 0.1 μM of primer 36 (5'-GAGGCAATTTGTGCWTCAATGG-3'), 0.1 μM of primer 172 (5'-TGCAGGACTGTAATGTGCCAA-3'), 1.2 U reverse transcriptase AMV (Promega) and 0.6U Taq polymerase (Promega). The reaction was carried out for 45 min at 42°C for retrotranscription and 2 min at 94°C for denaturation, followed by 40 cycles of amplification (94°C for 30 s, 60°C for 30 s and 72°C for 90 s). The amplified fragments (678 bp) were direct sequenced using the 36 and 172 primers. The nucleotide sequences were aligned by Clustal-X analysis (Thompson et al. 1997) using the Align X program from the Vector NTI package. Phylogenetic tree was built using the neighbour-joining method (NJ) of Saitou and Nei (1987). Genetic analysis were calculated using the Kimura-2 parameter algorithm in the MEGA program.

Results

Serological variability

Two different PPV serogroups were detected in transgenic plums and the same two in non-transgenic plums from the experimental plot (A). Five PPV serogroups were detected in non-transgenic plums of the baseline plot (B). The relative percentage of PPV isolates belonging to each serogroup for the tree populations is pre-

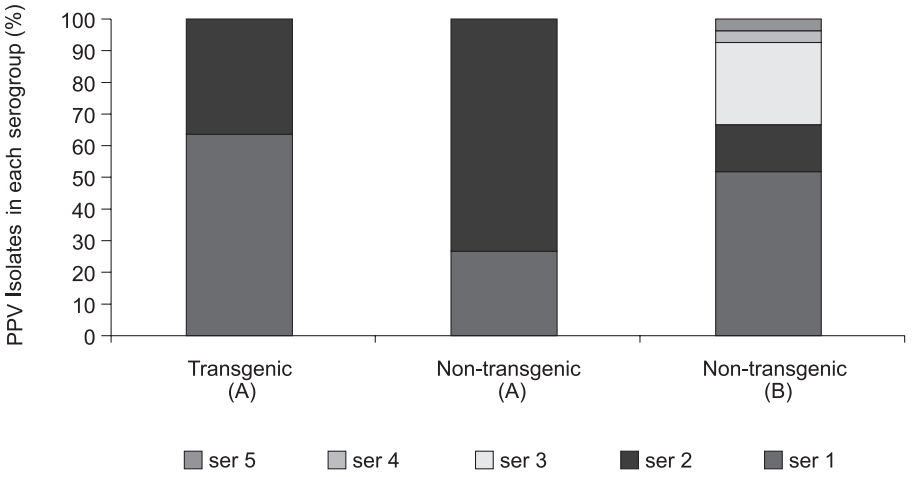

Fig. 1. Serological variability of the PPV population present in transgenic and non-transgenic plums of the experimental plot (A) and non-transgenic plums of the baseline plot (B)

sented in Figure 1. 63.6% of the PPV isolates from transgenic plums belongs to the serogroup 1 (ser 1) and 36.4% to the serogroup 2 (ser 2). In the non-transgenic plums of the experimental orchard, 26.7% of the PPV isolates belongs to ser 1 and 73.3% to ser 2. Ser 1 is the predominant serogroup detected in the non-transgenic plums of the baseline orchard (51.8% of the PPV isolates), followed by ser 3 (26%), ser 2 (14.8%) and ser 4 and 5 (3.7%). All analyzed PPV isolates were PPV-D, the only PPV strain currently present in Spain, as no PPV isolate reacted with AL Mab, specific for detection of PPV-M strain.

Molecular variability

The genetic variability among the PPV isolates in the 3' region of the virus genome is very low. The percentage of nucleic acid homologies among sequenced regions coding NIb and CP genes in the different isolates was found to range from 96.9% to 100%. The nucleotide diversity of the three PPV populations was very low: $\pi = 0.0072 \pm 0.0043$ for PPV present in transgenic plums, $\pi = 0.0054 \pm 0.0035$ for PPV in non-transgenic plums of the experimental plot (A) and $\pi = 0.0116 \pm 0.0065$ for PPV present in non-trangenic plums of the baseline plot (B). The mean nucleotide diversity between populations was 0.00163 ± 0.00058. A phylogenetic tree was constructed using the sequenced region of the PPV isolates (Fig. 2). No phylogenetic groups or clusters were defined in the PPV population.

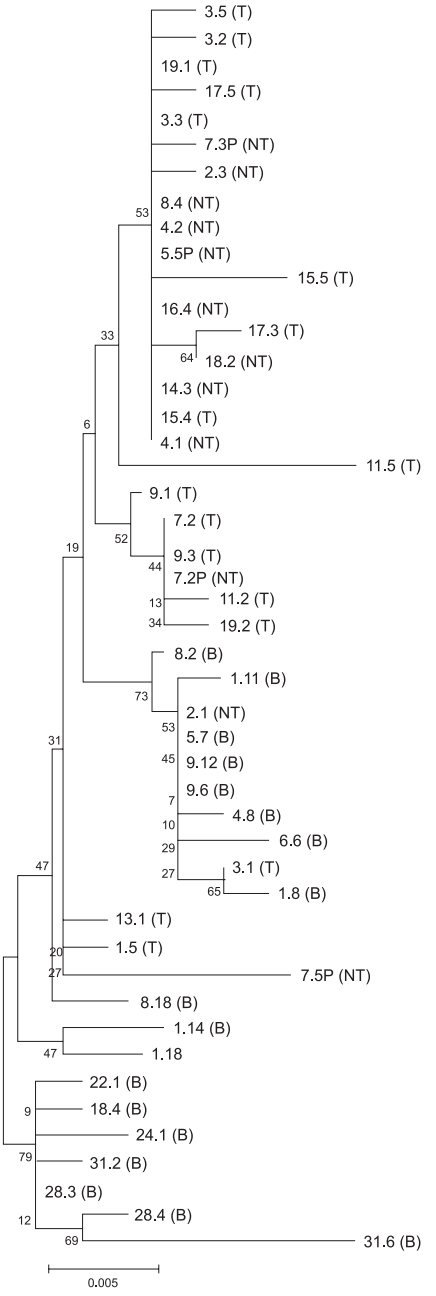

Fig. 2. Phylogenetic tree of 47 PPV isolates from the three populations: transgenic (T), non-transgenic (NT) and baseline (B), using the 3'NIb-5'CP sequence of the PPV genome. The analysis was carried out using the neighbour-joining method within Clustal-W with a bootstrap of 10 000 replicates

Discussion

One of the aims of biotechnology applied to agriculture is to obtain virus resistant plants by expressing partial or complete viral genes within the plant. However, the interaction of natural infecting viruses with the viral transcripts that express the transgenic plant can lead to changes in the dynamic and diversity of the natural virus populations. In this work, serological characterization by ELISA-DASI and molecular studies by sequencing and genetic analysis on PPV isolates infecting transgenic and non-transgenic plums, have demonstrated that there are no differences between transgenic and non-transgenic PPV populations in regard to serological and molecular variability. The same PPV serogroups were detected in transgenic and non-transgenic plums of the experimental orchard. One of these PPV serogroups (ser 2) corresponds to the 4BD7 defective isolate that was introduced, as inoculum source, in the non-transgenic plums of the experimental plot (A). It was probably transmitted from non-transgenic to transgenic plums by aphids (PPV transmission vectors). The other one (ser 1) was also detected in the adjacent baseline orchard, which is probably the source of entrance of this serogroup in the experimental plot via aphid transmission. The baseline variability of PPV in conventional plums is even higher than the detected in the experimental orchard (five serogroups), probably because this plot was established before in the field and is not protected by guard trees as is the experimental plot.

The genetic analysis of the most hypervariable region of the PPV genome has shown that the mean nucleotide diversity between populations ($\pi = 0.002$) is even lower than within populations ($\pi = 0.008$, 0.005 and 0.011 for PPV present in transgenic, non-transgenic and baseline plums, respectively). By the other hand, no phylogenetic groups or clusters were defined in the PPV populations by phylogenetic analysis. These results demonstrate that all the PPV isolates belong to a unique population and that there is no differences in the genetic diversity of the PPV populations present in transgenic vs. non-transgenic plums. We have demonstrated that the variability of PPV population is not altered when they are present in transgenic plums compared with conventional ones. Even though, a number of assays must be done prior to ensure the total absence of environmental hazard in the release of transgenic plants to the field (i.e. the possibility of recombination between the viral transgene transcript and the RNA of a field infecting virus). This kind of analysis are under investigation in our laboratory.

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