Transcript Imaging and Candidate Gene Strategy for the Characterisation of *Prunus*/PPV Interactions

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Abstract

Plum pox virus (PPV), the causing agent of the sharka disease, belongs to the genus *Potyvirus* that contains the largest number of virus species infecting plants. The virus genome has been extensively characterised and sequenced. However, few data are available on its interactions with the host plant, *Prunus*. In this study, we are focusing on the cloning and characterisation of any candidate genes involved in the expression of the resistance/susceptibility trait and any polymorphic genes putatively involved in the trait variation. In order to clone candidate genes, two main approaches are currently developed: the homology cloning of genes presumed to affect the resistance/susceptibility trait and the differential screening of cDNA pools corresponding to infected and non-infected plant material. The second approach is based on the transcript imaging of the host plant response to PPV infection. Previously, it has been shown that infection by a potyvirus is associated with specific changes in host gene expression, mainly down-regulation, while the expression of some genes remained unchanged. Thereby, in the differential display approach combined to further characterisation of candidate gene expression, we aim to monitor host gene expression in response to the virus and to describe a highly regulated interaction between the *Prunus* host plant and the infecting *Plum pox virus*.

Keywords: *Plum pox virus* (PPV); sharka disease; *Prunus*; genetic resistance; tolerance; cDNA-AFLP; candidate gene

INTRODUCTION

Sharka is the most devastating disease in stone-fruit trees (*Prunus* species). It has great economic importance partly due to the severity of the damage on the fruit production and fruit quality, and partly to its wide distribution all over Europe. It has by now been reported in many countries around the Mediterranean Sea and more recently on the American continent (18th International Symposium on Virus and Virus-like Diseases of Temperate Fruit Crops).

The causal agent of the sharka disease is the *Plum pox potyvirus* (PPV). Two main serological groups of PPV isolates present in the European Community were distinguished, PPV-D for Dideron and PPV-M for Marcus. In the orchards and nurseries, the virus is efficiently propagated by grafting or by aphids. It is poorly controlled by heavy spreading of agrochemicals against the virus vector and costly eradication campaigns. Therefore, resistant cultivars with high-value added (commercial fruit qualities) are required for the control of this potyvirus in orchards and nurseries.

Interesting sources of resistance, as reported by SYRGIANNIDIS (1980), AUDERGON *et al.* (1994) and MARTINEZ-GOMEZ *et al.* (2000), do exist in the *P. armeniaca* species (apricot). North American apricot cultivars are obviously a clear source of resistant genitors among which cv. Goldrich, Stella and Stark Early Orange (SEO). In such resistant cultivars, symptoms on leaves or fruits have not been

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observed, virus particles are not detected except in two contradictory reports which could be due to differences in inoculation or evaluation methods, the isolates used or the authenticity and sanitary state of the plant material (AUDERGON *et al.* 1994; BALAN & STOIAN 1995; POLÁK *et al.* 1995; MARTINEZ-GOMEZ *et al.* 2000; FUCHS *et al.* 2001). F1 and F2 progenies were developed from crosses with several apricot genitors that carry different types of resistance. Genetic linkage maps for two apricot progenies (Polonais × SEO) (LAMBERT *et al.* 2002) and (Goldrich × Valenciano) (HURTADO *et al.* 2002) have been constructed using both co-dominant and dominant markers. The sharka resistance/susceptibility traits were mapped on Goldrich (HURTADO *et al.* 2002). The variability and genetic control of resistance are currently being analysed for the SEO source and, in the future, different resistance mechanims will be available for pyramiding in apricot.

In this study, we are focusing on the cloning and characterisation of any functional or structural candidate genes which could be linked to the resistance/susceptibility trait or at least to its expression. For this purpose, candidate genes were targeted by two ways: RNA fingerprinting and homology cloning. In the first strategy, cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) was used to display transcripts whose expression is altered during PPV infection in apricot plants. Therefore, we aim to develop useful tools for the characterisation of the resistance mechanisms known in apricot trees and the development of valuable gene markers for plant defence and resistance in *Prunus* species.

MATERIAL AND METHODS

Plant material

For RNA fingerprinting, apricot (*Prunus armeniaca* L.) cultivars, Stark Early Orange (SEO) and Goldrich, presenting an high and mild level of resistance to PPV respectively, were grafted on GF305 peach rootstocks and inoculated according to three different protocols. For Protocol 1, 10 grafted plants for each genotype were inoculated with the D-type PPV isolates by double chip-budding, while 10 other replicates were grafted with mock chip-buds (so called 'mock-inoculated plants'). Plants were pruned to promote new shoots and leaves were cut off at three designated time points on the new branches arising from the grafted plant over 3 vegetative cycles (two chilling treatments). For protocol 2, GF305 rootstocks were first inoculated and once fully infected (symptoms and ELISA positives), apricot buds were grafted. Sampling was performed over two vegetative cycles. For protocol 3, young stems where the chip-buds were applied were sampled after 1-, 5-, 10-, 20- and 30-days post-inoculation and used as starting material for RNA extraction. This last protocol appears particularly important for genotypes such as SEO where viral particles are detected only in the close vicinity of the infection point and no reaction of the host plant to PPV systemic infection is observed. For protocol 1, the inoculation procedure was checked by ELISA on scion and rootstock, twice per vegetative cycle.

Total RNA was extracted from pooled leaves (protocol 1 and 2) or stems (protocol 3) according to the method of CHANG *et al.* (1993).

RNA fingerprinting by cDNA-AFLP

For cDNA-AFLP, poly(A)+RNA was purified from 100 μg of total RNA using the Oligotex mRNA kit (Qiagen) following the manufacturer's instructions but including treatment with RQ1 Dnase I (Promega) before purification. Messenger RNA was converted directly into cDNA template using the Riboclone cDNA synthesis kit from Promega. After blunting with the T4 DNA polymerase, the resulting double stranded cDNA was phenol-extracted, ethanol-precipitated and resuspended in 10 μl of H_2O. All subsequent steps were performed as previously reported for genomic or complementary DNA AFLP (BACHEM *et al.* 1996; MONEY *et al.* 1995). Restriction enzymes used for the AFLP template production were *Mse*I and *Pst*1, both with two selective bases at the 3 prime end.

AFLP products were separated on 6% denaturing polyacrylamide gels run in 0.5 × TBE buffer at 80 W for 2 hours and stained in silver nitrate (CHALHOUB *et al.* 1997). Amplification products that differ between infected and mock-inoculated plants were excised from acrylamide gels, re-amplified and cloned in pGEM-T vector (Promega).

Candidate gene cloning

Genomic DNA from *Prunus* individuals was prepared as described in LEFORT and DOUGLAS (1999). Starting from apricot, plum or *P. davidiana*, candidate products were amplified with degenerate oligonucleotide primers designed within the respective conserved regions. PCR amplifications were performed in a 50 μl reaction volume containing 50 ng of genomic DNA, 0.2mM of each dNTP, 2.5–4.5mM $MgCl_2$, 50mM Tris-HCl

pH9, 1–3 μM of each degenerate primer pairs and 2 U of Sigma Taq DNA polymerase. The reactions were performed following either a 61–52°C or a 55–45°C touchdown program in a GeneAmp 9700 thermal cycler (Applied Biosystems).

PCR products were observed on high resolution sequencing gel as described above and purified on QiaQuick PCR purification columns (Qiagen) before cloning in the pGEM-T vector (Promega). Inserts were sequenced in duplicate from various individuals using an automated sequencing system (Genaxis, Nîmes, France). Database searches were carried out using the Advanced Blast program at the National Center for Biotechnology Information (Bethesda, Md.) (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

RNA fingerprints were generated from healthy and PPV-infected Goldrich or SEO cultivars using variable primer combinations, depending on the experimental design and the genotype (Table 1). The use of mock-inoculated plants allowed us to discriminate between a gene induction pattern due to virus infection and stress response. Apricot genotypes presenting different level of resistance or at least symptomless to PPV infection were selected in order to avoid chlorotic and cell death virus-mediated cell response. Some of the patterns are assembled in Figure 1 as a close up. Although differences in the number of bands were observed between individual primer combinations, an average of 50 to 70 bands per lane were detected (Figure 1). Bands were cut out of the high resolution sequencing gels because of their induction or repression by virus infection, thus reflecting candidate genes for *Prunus*/PPV interactions. Differential expression was checked for each individual clone by either reverse Northern, Northern or semi-quantitative RT-PCR. In the cultivar Goldrich, of 138 primer combinations tested between protocols 1 and 2, 31 fragments showed altered abundance, quantitative or qualitative, while in SEO, only few differential bands were detected. This might be reflecting different biological responses to PPV in the cultivars SEO and Goldrich, one being a true resistant and the other one, tolerant. Few if any interactions between the virus and the SEO cultivar are taking place, even in early steps after inoculation (protocol 3). Extensive analysis of the RNA fingerprinting in the case of Goldrich/PPV interactions is going on further on an automated 16 capillaries sequencer (ABI Biosystem).

Besides the RNA fingerprinting, a whole set of candidate genes were cloned, mainly in apricot, using degenerate PCR primers. Fragment sizes ranged from 300 bp to 1.2 kb. Those targeted candidate genes are involved in herbaceous plants in:

- gene-for-gene disease resistance (RGA resistance gene analogs or *R* genes from the LRR and protein kinase families) (LEISTER *et al.* 1996), similarly to the *N* gene product which belongs to the NBS-LRR family and confers resistance to *Tobacco mosaic virus*
- defence response (transcription factors, protein kinases, PR proteins) and genes coding for proteins involved in the salicylic acid pathway and hypersensitive reaction
- viral RNA silencing similar to post-transcriptional gene silencing, another type of natural defence mechanisms against viruses in plants (MATZKE *et al.* 2001).

Some other candidates were targeted, they are coding for proteins interacting with the virus and tightly implicated in the virus replication and host invasion (i.e. *RF2a*). They can be considered as candidates

Table 1. Summary of cDNA-AFLP analysis performed on apricot Goldrich and SEO cultivars

Cultivar	PPV strain for inoculation	Number of AFLP primer comb. tested	Number of bands visualised[(1)]	Differentially expressed bands quantitative or qualitative
Goldrich				
Protocol 1	PPV-D	75	3500	29
Protocol 2	PPV-M	63	3000	2 (in progress)
SEO				
Protocol 2	PPV-D, M, Turk	182	9000	9
Protocol 3	PPV-M	139	7000	6

[(1)] Average number for about 50 to 70 bands per lane

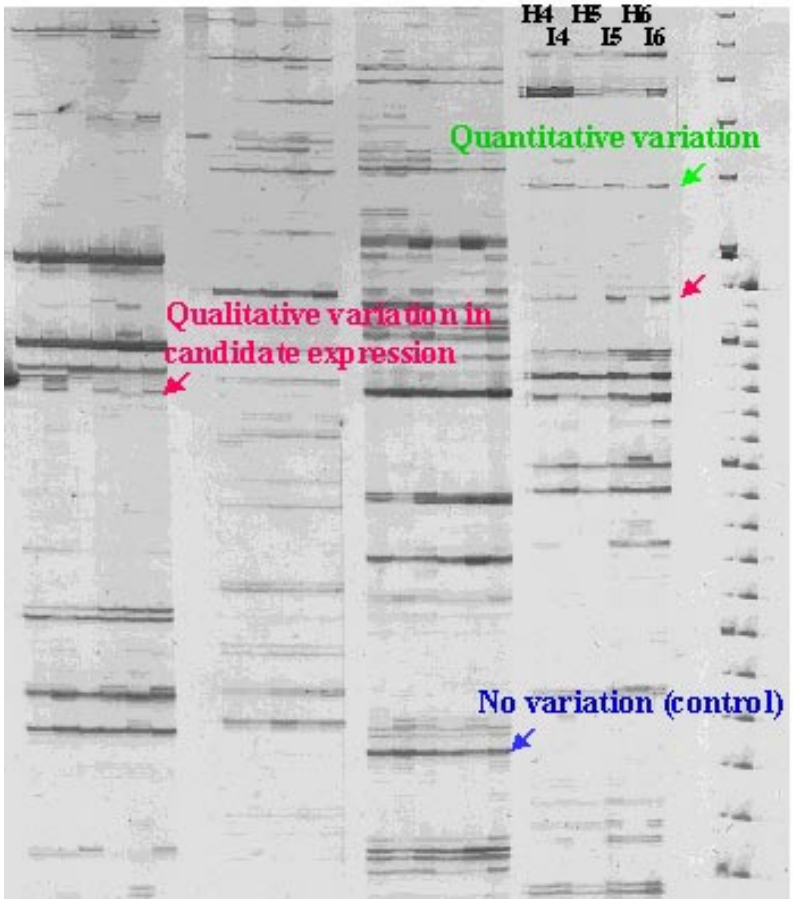

Figure 1. A typical RNA fingerprinting gel using four different *Mse*I/*Pst*I primer combinations

Twenty apricot scions (cv. Goldrich) were grafted and inoculated as described in protocol 1. *H* is referring to mock-inoculated plants while *I* is referring to plants inoculated with a D-type PPV strain. Numbers from 4 to 6 are corresponding to the three leaf samplings after the first chilling treatment (second vegetative cycle)

for susceptibility, on the contrary to resistance. All candidate genes cloned by homology will serve to identify genomic regions potentially involved in resistance/defence or susceptibility, at least clusters of active and inactive resistance genes.

Further characterisation of the expression pattern of the selected clones will be taken into account either by transcript imaging through macroarray analysis, Northern blots or by quantitative RT-PCR. We can expect that differentially expressed genes in response to PPV infection are also induced by different stresses such as other pathogen attack or wounding. For these purposes, we will test whether the candidate genes are induced by other PPV strains, other viruses infecting stone fruit trees, bacterial pathogens and water deficiency.

Candidate genes are currently being localised on the apricot (Polonais × SEO) and the interspecific peach (Summergrand × *P. davidiana*) linkage maps (J-M Audergon, J. Kervella, P. Lambert, INRA–UGAFL Avignon, data not shown). The peach BAC library from Clemson University, kindly provided by Dr B. Abbott, USA, is being used in order to align those candidate genes on the *Prunus* physical map.

In conclusion, new candidate genes whose expression is modulated by PPV infection and analogs to defence and resistance genes were cloned in *Prunus* species and correlation between their expression and the resistance to sharka disease is underway. However, the role of those candidates in defence mechanisms and *Prunus*/PPV interactions remains to be clarified.

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