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Strategies to introduce resistance to prune dwarf virus in almond

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SUMMARY – Prune dwarf virus (PDV) and *Prunus* necrotic ringspot virus (PNRSV) contribute to significant losses in almond productivity, in Portugal and in other countries. The establishment of new orchards with healthy certified trees does not guarantee virus eradication for long periods. Both PDV and PNRSV can be transferred through pollen, making cross-pollination (obligatory for seed set) a common source of infection. Genetic engineering using the coat protein (CP) strategy has been a successful method to achieve plant resistance to virus diseases and might be effective to control virus diseases in almond orchards. We prepared several constructs carrying the CP PDV sequence that have been tested in *Agrobacterium*-mediated transformation of *Nicotiana benthamiana* (an easy-to-transform model plant susceptible to PDV). The transgene sequences were placed under the control of the 35S CaMV promoter, inserted in pGREEN vectors (John Innes Centre), with *npt*II (kanamycin resistance) as a selection marker. With the selected constructs we aim to understand the mechanisms underlying PDV replication in host cells.

Keywords: PDV, coat protein, transgenic plants, Ilarvirus, plant viruses, virus resistance.

RESUME – *"Stratégies pour introduire la résistance au prune dwarf virus chez l'amandier". Prune dwarf virus (PDV) et* Prunus *necrotic ringspot virus (PNRSV) contribuent à la baisse significative de productivité des amandiers (*Prunus dulcis *Mill.) au Portugal et dans d'autres pays. L'établissement de nouveaux vergers avec des arbres certifiés comme sains ne garantit pas l'éradication des virus pendant de longues périodes. Le PDV et le PNRSV peuvent être transmis par le pollen, rendant la pollinisation croisée une source d'infection. Le génie génétique employant la stratégie de l'enveloppe protéique (CP) a été une méthode efficace pour l'introduction de la résistance aux maladies virales et pourrait être efficace pour contrôler les maladies des vergers d'amandiers. Nous avons préparé plusieurs constructions avec la séquence du CP PDV testée sur* Nicotiana benthamiana *en utilisant une stratégie de transformation avec* Agrobacterium*. Les séquences transgéniques ont été placées sous contrôle du promoteur 35S du CaMV, insérées dans des vecteurs de pGREEN, avec* npt*II comme marqueur de sélection. Avec les constructions choisies nous visons à comprendre les mécanismes de réplication virale du PDV dans les cellules infectées.*

Mots-clés : *PDV, enveloppe protéique, plantes transgéniques, Ilarvirus, virus de plantes, résistance virale.*

Introduction

Ilarviruses are important pathogens of *Prunus* species worldwide. Prune dwarf virus (PDV) is one of the ilarviruses that negatively affects the productivity of almond orchards. The symptoms of PDV infection vary along the year and the severity of the affection is directly related to the orchard management. These viruses spread through pollen or seeds so the control is very difficult due cross-pollination requirements. Almond has self-incompatibility and cross-pollination is required for fruit production. The control of disease proliferation is difficult or impossible and the establishment of new orchards with healthy certified trees is not a long time solution. The re-infection of healthy orchards could occur in 2 to 3 years. Another problem is the method of analysis to ensure the healthy state. It is known that plant material considered healthy by ELISA is revealed infected when a more sensitive technique is used, namely IC/RT-PCR or Southern blotting of IC/RT-PCR products. The impact of PDV infection can be reduced with adequate orchard management, and the adequate irrigation is also an important factor. Actually, water management is a worldwide problem and the reduction of water use is necessary, and other strategies to reduce the impact of viruses in orchard production must be considered.

The lack of known resistant cultivars makes the cp-mediated cross protection approach an

attractive alternative. Engineering resistance through the introduction of the cp gene in plant genome was first demonstrated by Powell-Abel *et al.* (1986) and could be one way to obtain almond plants resistant to PDV.

Successful examples of resistance breeding against viruses from different plant virus families using the cp mediated approach have been reported (Beachy *et al.,* 1990) and this could be the strategy to successfully obtain almond plants resistant to PDV.

In the present work, we aim to study the cp mediated resistance to PDV. With the objective, we prepared different constructs carrying cp coding sequence of PDV intact or manipulated. The constructs have been inserted in a model plant (*Nicotiana benthamiana*), susceptible to PDV, by *Agrobacterium* mediated transformation. The constructs prepared were used in transformation assays of *Nicotiana benthamiana* to study: T-DNA integration patterns and levels of transgene expression; patterns of transmission to progeny; evaluation of resistance (T1 transgenic plants) and changes in genomic variability of the infecting virus; cross protection in progeny (to the ilarvirus PNRSV and ApMV)

Materials and methods

cDNA synthesis and cloning

The synthesis of cDNA from the coat protein viral RNA was performed by immunocapture/reverse transcriptional-polymerase chain reaction (IC/RT-PCR) using specific antisera (BIORAD) and specific primers for PDV, based on a published sequence (Bachman *et al*., 1994) (Raquel *et al*., 2000).

The CP-cDNA was cloned into the plasmid pCRII (TA-cloning, Invitrogen). The CP-carrying plasmids were selected by colony-PCR and the inserts were sequenced. The DNA data was analysed with DNAStar from DNASTAR Inc. USA. The nucleotide sequence of cpPDV was deposited in the NCBI genebank database with accession number AF202117.

Preparation of constructs

All recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.,* 1989).

For plant transformation, we inserted the cpPDV fragments (Sense and antisense orientation) in pGREEN vectors (Hellens *et al*., 2000) (constructs pGREENcpPDVSense and pGREENcpPDVAntisense). The cp PDV fragment without the start codon was obtained by PCR using specific primers. This amplified fragment was also inserted into pGREEN vector (construct pGREENcpPDVS/CI). To prepare the construct carrying the mutated cpPDV at R14, we used the construct carrying the cpPDV coding sequence in sense orientation and the kit Quickchange Site Directed Mutagenesis (STRATAGENE). For preparation of the construct carrying the MARs sequences, we have inserted the RB7 fragments in the pGREEN000IINOSKan35SPcpPDVSense flanking the 35SPcpPDVPolyA fragment (construct pGREENRB7senseRB7).

All these constructs were transformed into *Agrobacterium* EHA105, harbouring the transformation helper plasmid pSoup (Hellens *et al*., 2000).

Transformation of *Nicotiana benthamiana*

Leaves of 4-5 weeks old shoots of *Nicotiana benthamiana* were carefully cut with a scalpel immersed in an *Agrobacterium* EHA105 (carrying the different constructs) suspension and placed for 48 h on MS medium (Murashige and Skoog, 1962) supplemented with 1 mg/l of benzyladenine (BA) and 0.1 mg/l of naphthalene acetic acid (NAA). The leaves were then transferred to the same culture medium containing 300 mg/l cefotaxime (cef) and 50 mg/l kanamycin (kan) to regenerate kan resistant plants. The regenerated kan resistant plants were transferred to micropropagation medium containing cef (300 mg/l) and kan (100 mg/l).

T1 and T2 seedlings were germinated on a MS based medium containing kan (100-300 mg/l).

Molecular analysis of transgenic plants

The presence of *npt*II and cpPDV in the genome of kan resistant plants was detected by PCR using specific primers for each sequence. The DNA was extracted using the MiniCTAB. PCR was also used to detect the bacterial *npt*I gene using specific primers. Amplification products were analysed by gel electrophoresis on 0.8% agarose.

DNA gel blot hybridization

Total genomic DNA of *N. benthamiana* was extracted from leaves with the DNeasy®Plant Mini Kit (QIAGEN). Ten micrograms of DNA were digested with *Hin*dIII and then separated by electrophoresis on a 0.8% agarose gel. The separated DNA was transferred from gels to nylon membranes (Hybond-N+; *Amersham Pharmacia Biotech*) and hybridised according to the manufacturer's recommendations. The cDNA probe for cpPDV (630 bp) was labelled with Fl-dUTP using a *Gene Images* random prime labelling module kit (*Amersham Pharmacia Biotech*). *Gene Images* CDP-*Star* detection module (*Amersham Pharmacia Biotech*) was used to detect the fluorescein-labelled probe.

DAS-ELISA analysis

To detect the coat protein in the transgenic plants obtained from the transformation events with the constructs pGREENcpPDVSense and pGREENcpPDVMut we used the Double Antibody Sandwich – Enzyme Linked Immuno-Sorbent Assay (DAS-ELISA). PDV antisera from BIORAD was used and the procedure was as described in the protocol of the ELISA kit.

Almond genetic transformation

The constructs obtained are presently being introduced in almond using the genetic transformation protocol previously established in our lab (*Agrobacterium* EHA105-mediated transformation of leaves excised from micropropagated shoots) (Miguel and Oliveira, 1999).

Results and discussion

Nicotiana benthamiana plantlets harbouring the PGREENcpPDVSense, PGREENcpPDVAnti-Sense, PGREENcpPDVS/CI, PGREENcpPDVMut and PGREENOSKan were obtained (Table 1). The presence of *npt*II and cpPDV transgenes in the independent putative transformants was confirmed by PCR using specific primers and Southern blotting (data not shown). Accumulation of PDV coat protein was detected in plantlets harbouring PGREENcpPDVSense by DAS-ELISA and differences were observed even among plantlets with a single copy of the transgene (data not shown).

Table 1. Number of kanamycin resistant plantlets recovered from different transformation events

Constructs	No. transformed explants	Kan resistant plants
pGREENcpPDVNOSKan	17	13
PGREENcpPDVSense	136	45
PGREENcpPDVAnti-sense	107	33
PGREENcpPDVS/CI	96	20
PGREENcpPDVMut	50	6
PGREENRB7senseRB7	261	-

Aiming to evaluate the relation between the level of cp accumulation and the level of resistance, we selected two transgenic plants from PGREENcpPDVSense construct with distinct levels of cp expression (lines 5 and 35) to obtain progeny by self-fertilisation. Progenies from these lines were grown *in vitro* under selective conditions (kan) and showed Mendelian segregation for kan. These

progenies were analysed by DAS-ELISA and differences in protein synthesis were obtained (due to different transgene insertion *loci* or to homozygosity vs heterozygosity of transgene) (Fig. 1). Plantlets carrying transgene single copy from transformation with PGREENcpPDVAnti-Sense construct, were also selected for self-fertilisation. The progenies were grown *in vitro* conditions and Mendelian segregation for kan was obtained. The transgene expression is under evaluation.

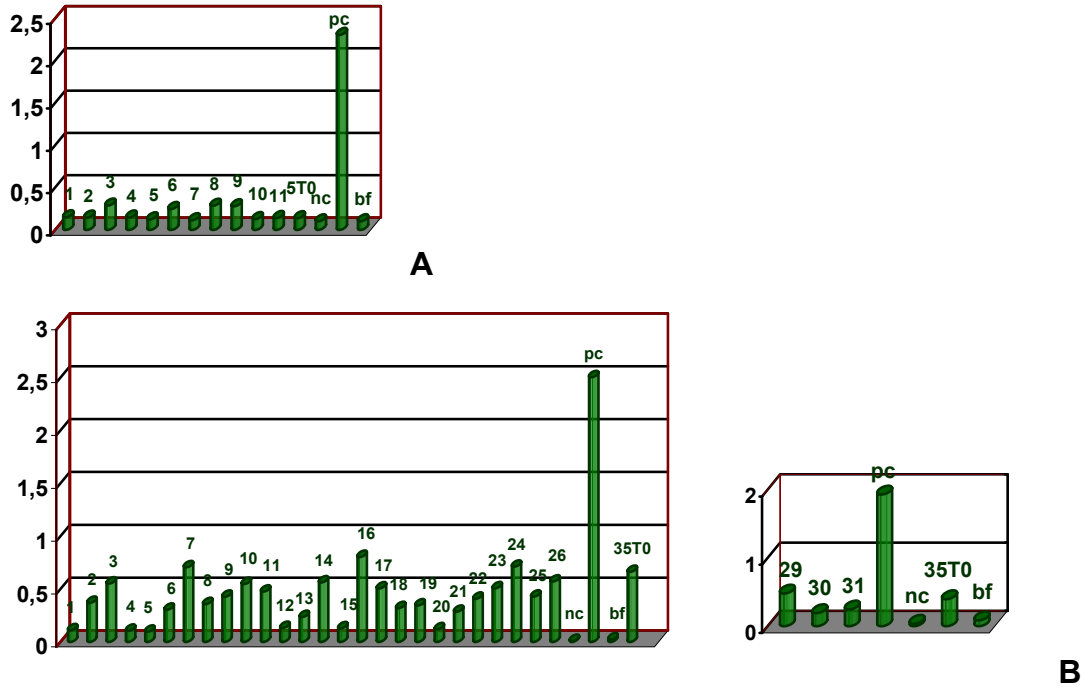

Fig. 1. DAS-ELISA analysis of transgenic lines (T1) containing cpPDV in sense orientation. A – Progeny of 5T0 plantlet; B – Progeny of 35T0 plantlet. bf –buffer; nc – negative control; numbers – transgenic lines; nt – non transgenic *Nicotiana benthamiana*; pc– positive control.

Putative transgenic lines containing the mutated and the delected cpPDV gene were also recovered and are presently being analysed for copy number and expression of transgene.

At the present moment no transgenic lines have been recovered from the transformation events with PGREENRB7senseRB7. Studies are being conducted to understand these results.

Infectivity experiments will be conducted on the transgenic *Nicotiana* progeny to understand the mechanisms of PDV resistance and to study changes in genomic variability of the infecting virus (PDV) and the cross protection in progeny (to the ilarvirus PNRSV and ApMV).

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