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Short communication

Transgenic *Nicotiana benthamiana* plants containing the P1 gene of plum pox virus are resistant to virus challenge

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Abstract

We report here the production of transgenic plants containing the P1 gene of the plum pox potyvirus genome. Two P1 constructs were engineered into *Nicotiana benthamiana* host plants via *Agrobacterium tumefaciens*. When challenged with PPV, transgenic plant lines were either immune to or recovered from infection. Biochemical studies conducted on these plant lines showed that the resistance phenotypes were correlated with the level of transgene transcript. Plant transformation with the P1 gene is a potentially useful alternative approach to create resistant plants.

Plum pox potyvirus (PPV) is the causal agent of Sharka disease that affects stone fruit trees. It infects hundreds of millions of stone-fruit trees, especially in Europe, and causes significant losses in fruit production. PPV is an aphid-transmitted virus and its control, particularly in the areas where the disease is prevalent, is very difficult. Use of resistant varieties appears to be the best approach to fight against PPV. Unfortunately, very few natural resistance sources have been identified and most are restricted to apricot (Dosba et al., 1992). Thus, the production of resistant transgenic plants by transferring viral sequences into the plant genome represents an attractive alternative (Powell-Abel et al., 1986).

Transgenic *Nicotiana benthamiana* plants expressing the PPV CP gene have already been shown to be resistant to PPV infection (Regner et al., 1992; Ravelonandro et al., 1993). The use of other potyviral CP genes also has been reported and the resulting protection has been well characterized. Resistance phenotypes often appear as immunity or recovery of the plant from infection, and are sometimes referred to as homology-dependent resistance (see for review Lindbo et al., 1993a; Baulcombe, 1996). Other studies have shown that the CP gene is not the only viral sequence able to confer resistance to virus infection. The encoding sequences for replicase NIb (Audy et al., 1994; Guo and Garcia, 1997), proteinase NIa (Maiti et al., 1993; Vardi et al., 1993), the 6kD-21kD protein (Swaney et al., 1995) and the proteinase P1 (Pehu et al., 1995) have all been successfully used.

We report here, the use of the P1 gene to product plants resistant to PPV infection. The P1 protein binds nucleic acids and functions as a proteinase and in viral amplification (Verchot et al., 1991; Brantley and Hunt, 1993; Soumounou and Laliberté, 1994; Verchot and Carrington, 1995). Other functions of this protein and the encoding gene sequence are unknown. We have transferred the PPV P1 gene into *N. benthamiana* plants and obtained transformed plants which have been assayed for PPV infectability.

Two gene constructs, designated as P1 and ΔP1, were built (Figure 1). Both were derived from the pBluescribe recombinant plasmids pPL20 and pPL27, which contain the PPV 5′-non translatable leader sequence minus the first 47 nucleotides, followed by the P1 gene. The pL27 plasmid contains a truncated form of the gene (ΔP1, from nt 48 to 929). The pL20 plasmid includes the P1 gene plus the N-terminal part of the HC gene (P1, from nt 48 to 1374). To engineer

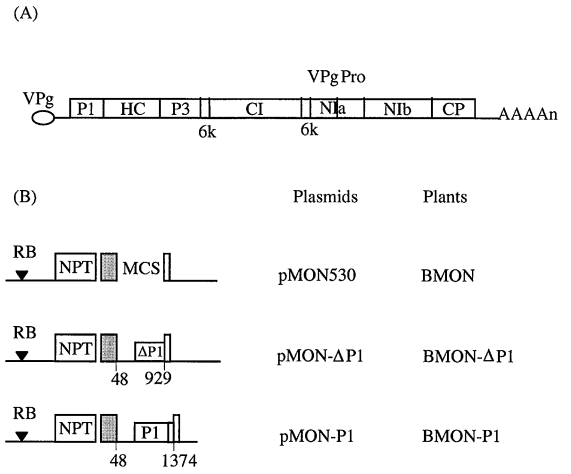

Figure 1. Schematic diagram of the two constructs ΔP1 and P1. (A) Representation of the PPV genetic map. (B) Structure of the different plant expression vectors pMON530, pMON-ΔP1 and pMON-P1. Numbers below the different figures indicate the nucleotide positions in the PPV-D genome. The NPT box represents the kanamycin resistance cassette which contains the neomycin phosphotransferase II gene that allows the selection of transformed plant cells. Foreign genes are inserted in the appropriate multiple cloning sites (MCS), which are flanked by the 35S promoter from CaMV (grey box), and the 3′-untranslatable end of the nopaline synthase gene (empty box). The MCS of pMON530 contains the sites BglII, ClaI, SmaI, KpnI, Xho1 and EcoRI. The position of the right border (RB) is indicated by a black triangle. The names of the corresponding transgenic *N. benthamiana* plants are listed beside the plasmid names.

the two constructs, the PPV sequences derived from pBluescribe recombinants, were first digested with SphI, made blunt-ended with the Klenow large fragment of DNA polymerase I, and then cut with KpnI. The subsequent fragments were subcloned, between the 35S CaMV promoter and the nopaline synthase 3′-end, into the pMON530 binary expression vector (Monsanto, St Louis, Missouri), which was treated as follows. The plasmid was first cut with XhoI, made blunt-ended with Klenow, and then digested with KpnI. The resulting plasmids were designated pMON-ΔP1 and pMON-P1. These recombinant binary plasmids were then mobilized from *E.coli* HB101 into *Agrobacterium tumefaciens* strain ASE, by triparental mating. Leaf disks of *N. benthamiana* were co-cultivated with *A. tumefaciens*, harbouring respectively pMON-ΔP1 and pMON-P1, according to the method of Horsch et al. (1985). Transformed calli were regenerated into plants on kanamycin medium.

Integrated T-DNA was examined by Southern blotting using total DNA from young leaves of the independent R_0 primary transformants, (data not shown). Fourteen transgenic plant lines positive for PPV ΔP1, designated as plants BMON-ΔP1, and four plants bearing PPV P1, designated as plants BMON-P1, were selected. The R_1 progeny, obtained by self-pollination of these primary transformants, was then grown on kanamycin medium to confirm the presence of integrated DNA. Nine BMON-ΔP1 and four BMON-P1 lines, which followed Mendelian segregation with 75% of kanamycin resistant seedlings, were self-pollinated in the greenhouse and seedlings were then tested for kanamycin resistance. The R_2 progeny of five BMON-ΔP1 lines (-1, -4, -11, -13, -14), and of four BMON-P1 lines (-4, -5, -6, -7), which exhibited 100% kanamycin resistance, were selected for further testing.

Seedlings of the R_2 homozygous BMON-ΔP1 and BMON-P1 transgenic plants were then evaluated for PPV infectability. Crude sap from PPV-D infected *N. benthamiana* leaves was mechanically inoculated onto *N. benthamiana* seedlings according to Ravelonandro et al. (1993). When compared with the control plants (transgenic BMON plants transformed with pMON530, and non-transformed plants), which were 100% susceptible, 60% of the BMON-ΔP1-13, 70-75% of the BMON-P1-6, -7 and 100% of the BMON-P1-4 and -5 plant line seedlings, did not develop any infection 49 days post inoculation. Conversely, the BMON-ΔP1-1, -4, -11, and -14 plant lines were 100% susceptible to PPV infection.

Comparative studies of one of the highly resistant BMON-P1 lines, BMON-P1-4, and the only resistant BMON-ΔP1 line, BMON-ΔP1-13 were conducted. Figure 2 shows the normalised data of three experiments performed with the two lines, when both were challenged with PPV. At least 50% of the plants displayed recovery, as previously described (Ravelonandro et al., 1993; Smith et al., 1994; Dougherty et al., 1994). Plants from the BMON-P1-4 recovered faster (between 21 and 28 d.p.i.) than those from BMON-ΔP1-13 (between 35 and 42 d.p.i). At 42 d.p.i, all the infected BMON-P1-4 plants and 50% of the infected BMON-ΔP1-13 plants had recovered. We also observed that 50% (BMON-P1-4) and 23% (BMON-ΔP1-13) of the seedlings never developed infection during the experiment, displaying the immune phenotype.

When inoculated with other strains of PPV, M and El Amar, belonging to two other phylogenetic groups than the D strain used here, (Candresse et al., 1995), the transgenic *N. benthamiana* BMON-P1-4 line always displayed a similar protection (data not shown). This is different from the results of Pehu et al. (1995)

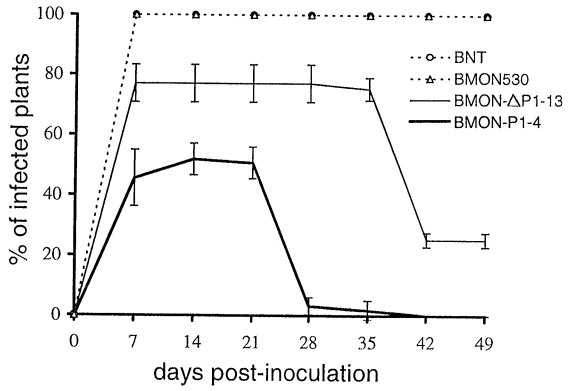

Figure 2. Recovery reaction of BMON-P1-4 and BMON-ΔP1-13 plant lines. Seedlings of R_2 BMON-P1-4 and R_2 BMON-ΔP1-13 plant lines, non-transformed *N. benthamiana* (BNT) and transformed BMON530 control plants, were inoculated with PPV-D infected plant extract, in three experiments (18 for experiment 1, 24 for exp.2, 16 for exp.3). The virus was monitored in the upper leaves by a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and by observation of the symptoms. The figure shows the percentage of seedlings which exhibited symptoms and which were positive in DAS-ELISA over 49 days. The average percentage and the standard deviation (bar) of the three experiments was calculated for each time point.

who reported that transgenic potatoes containing the PVY^0 P1 gene, displayed a resistance specific to the genome of PVY^0 and not to PVY^N. When we challenged the BMON-P1-4 plants with potato virus Y (PVY), they did not show any heterologous protection (data not shown). This specificity of the protection is also described in previous data with transgenic plants expressing PVY, TEV, and PStV CP genes (Smith et al., 1994, Lindbo and Dougherty, 1992 and 1993b, Cassidy and Nelson, 1995), the TEV 6kD-21kD encoding sequence (Swaney et al., 1995) or the PPV NIb gene (Guo and Garcia, 1997).

Our results and those published about CP transgenics, suggested the involvement of the transgene derived transcript in the protection. Thus, we analyzed the transgene transcript content of the BMON-ΔPI and -P1 lines R_2 progeny. We collected leaves of the R2 progeny of the plant lines, then extracted total RNA according to the method of Verwoerd et al. (1989). Total RNA was spotted on a nitrocellulose membrane (Hybond-C extra, Amersham) and probed with ^{32}P labeled antisense transcript of P1 RNA. Little to no P1 RNA was detected in the susceptible lines, while varying levels of RNA were found in the resistant plants (data not shown). Northern blot assays conducted on the BMON-P1-13 and P1-4 lines, showed the expected transcripts for each construct, around 900 nt for BMON-P1-13 (Figure 3A) and 1300 nt for BMON-P1-4 line 5 (Figure 3B). Both plant lines showed variable levels of the transgene derived transcripts. When these seedlings were challenged with PPV, two resistance phenotypes were displayed by these plants (Figure 3A-B). The highest levels of transcript were detected in the plants of the two lines which would display the recovery phenotype after infection. In contrast, the lowest levels of transcript were observed in the plants which would display susceptibility (line BMON-ΔP1-13) or immunity (both lines). From these northern blot assays, we observed an inverse correlation between the amount of RNA and the immune phenotype of the seedlings, except for the plantlet 7 of BMON P1-4 (Figure 3B, lane 7).

These observations are similar to those obtained by others for transgenics expressing potyviral CP sequences (Lindbo et al., 1993b, Dougherty et al., 1994, Smith et al., 1994, Cassidy and Nelson, 1995) and could be referred to as specific resistance linked to a co-suppression phenomenon (Flavell, 1994). This is supported by the lack of detection of transgene transcript RNA in BMON-P1-4 seedlings after recovery reaction (Figure 3C). The protection induced by the entire P1 sequence in transgenic plants (i.e. BMON-P1-4 and -5 lines) is greater than the protection afforded by the ΔP1 sequence (i.e. BMON-ΔP1-13 line). Our results indicate that the P1 gene of the PPV, like the NIb gene of PPV (Guo and Garcia, 1997), can be used to create transgenic plants resistant to the PPV infection. We also report, for the first time to our knowledge, some data related to the molecular characterization of the P1 protection and the possible mechanism involved. Our results suggest that the protection is possibly mediated by the transgene transcript. Further analysis, like nuclear run-off assays would be necessary to confirm that this cellular regulation is post-transcriptional (Baulcombe, 1996).

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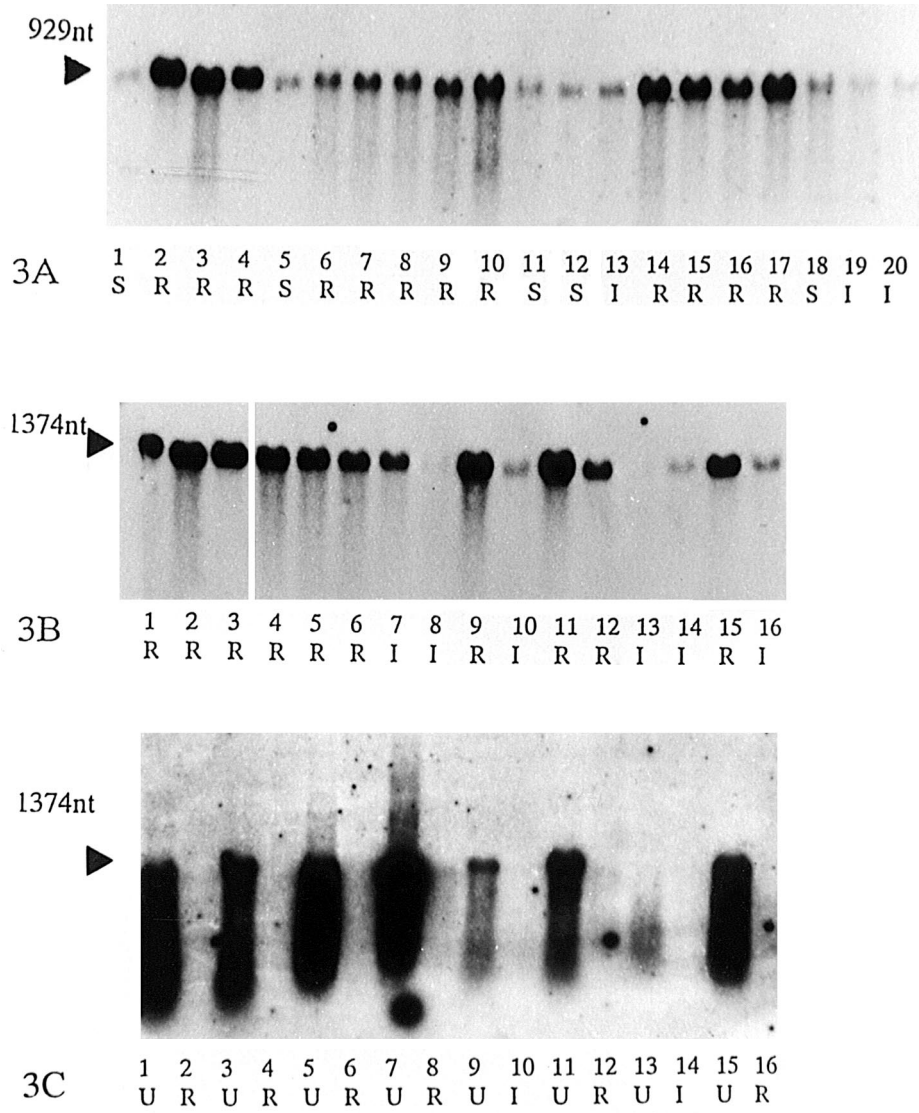

Figure 3. Northern blot analysis of BMON-P1-4 and -ΔP1-13 RNA. Five μg of *N.benthamiana* total RNA were run on formaldehyde agarose gels and transferred to a Hybond-C extra membrane. The membranes were hybridized with ^{32}P P1 probes. The autoradiograms represent (A) transgenic RNA from 20 BMON-ΔP1-13 seedlings (lanes 1 to 20); (B) transgenic RNA from 16 BMON-P1-4 seedlings (lanes 1 to 16). The transcript sizes (929 nt and 1374 nt) are noted on the left of each autoradiogram. The resistance phenotype developed by the plants after challenging with PPV, is indicated beneath each lane as recovery (R), immunity (I) and suceptibility (S); (C) transgenic RNA from 8 BMON-P1-4 seedlings before and after a challenge with PPV. The total RNA was extracted just before inoculating the plants and 2 months p.i. The lanes 1 plus 2, 3 plus 4, 5 plus 6, 7 plus 8, 9 plus10, 11 plus 12, 13 plus 14, 15 plus 16, contain paired samples from the same plants before and after inoculation by PPV (unchallenged, U, recovered, R, or immune, I).

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