Resistance against multiple plant viruses in plants mediated by a double stranded-RNA specific ribonuclease

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Abstract Many plant viruses have single-stranded RNAs as their genomes. During the course of replication, genomic RNAs and their complementary template RNAs are likely to form double-stranded (ds) RNA at least transiently. To attack replication intermediates of many plant RNA viruses specifically, we introduced a yeast-derived ds-RNA specific RNase gene called *pac* 1 into plants. The transformed plants showed a decrease in lesion numbers when they were challenged with tomato mosaic virus, and a delay in the appearance of symptoms when inoculated with cucumber mosaic virus or potato virus Y.

Key words: ds-RNA specific RNase; Virus resistance; Transgenic plant; Tomato mosaic virus; Cucumber mosaic virus; Potato virus Y

1. Introduction

Plant viruses cause a great deal of damage to important crops, resulting considerable losses to agronomical yields all over the world. Clearly the control of plant virus diseases is important in modern agriculture. It was originally demonstrated that expression of tobacco mosaic virus (TMV, the type member of the tobamovirus) coat protein in transgenic plants conferred resistance to virus challenge [1]. Coat protein mediated (CP-mediated) protection against plant virus has been tried for several years [2–4]. The case of replicase-mediated resistance against plant viruses was also reported [5]. These plants were highly resistent to infection by parental virus. These types of resistance, however, seem to be effective only against viral strains closely related to the source of the transgene [6].

Approaches which can endow the plants with resistance against multiple plant viruses are thus anticipated. If we could find a process common to plant viruses and attack the process using a single novel gene, it should be possible to obtain plants resistant to multiple plant viruses. Transgenic plants that expressed pokeweed antiviral protein (PAP), a ribosome-inhibiting protein, showed resistance to infection by different viruses [7]. But this protein has an adverse effect on plant physiology; plants that accumulated high level of PAP were sterile in this case [7]. We thus await new methods to make plants resistant against multiple plant viruses without significant drawbacks like sterility or dwarfness.

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Many plant viruses have single-stranded RNAs (ss-RNAs) as their genomes. It is widely accepted that these single-stranded RNA genomes are replicated through complementary template RNA made in their replicaton cycles. During the course of replication, genomic RNAs and their complementary template RNAs would be expected to form double-stranded RNA (ds-RNA), at least transiently. If we can obtain a ribonucleases (RNases) specific to ds-RNA and introduce it into plants, there should be a chance to attack replication intermediates of many plant viruses. We anticipate that expression of this transgene should have no adverse effects on normal plant growth or physiology, since this ds-RNA specific RNase would not attack plant mRNAs. If so, we should be able to develop a strategy for the suppression of multiplication of a very broad range of RNA viruses in plants. To this goal, we introduced a yeast-derived RNases gene called '*pac* 1' into plants.

2. Materials and methods

2.1. Plant materials, establishment and maintenance of pac 1 plants

Nicotiana tabacum cv. Xanthi-nc was used as a starting material to establish transgenic *pac* 1 plants. pKF1 [8] was used as a starting material to excise the DNA fragment with *pac* 1 gene; an Rsa I (1301)-Dra I (2552) fragment of 1.25 kb encompassing *pac* 1 gene was excised and replaced in place of the *gus* gene of pBI 121 plasmid (Clontech Inc. [9]), resulting in pBIpac. The plasmid was transferred to *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating with the aid of *Escherichia coli* C600 (pRK2013) [10]. The transconjugants were selected by resistance to kanamycin, rifampcin and carbenicillin at 25, 100 and 100 μg/ml, respectively. The neomycin phosphotransferase II gene confers a selectable kanamycin resistance marker on transformed plants. Leaf disks of *Nicotiana tabacum* cv. Xanthi-nc were co-cultivated with *Agrobacteria* and cultured on selective media. After a few weeks shoots appeared at almost the same frequency as with a control binary plasmid, pBI121, with the GUS gene instead of *pac* 1 sequence. About forty resistant calli were induced to regenerate shoots and roots, transferred to pots. After characterization by Southern, Northern, Western analyses (see below) three transformants (T8, H8 and H15) were selected on the basis of the expression level of the transgene. After preliminary screening of virus resistance, two R0 transformants (T8 and H8) were chosen, selfted to regenerate R1, then selfed again to generate R2 generations. Seeds from plants Nos. 5 and 6 of T8 R1 plants (we called them T8-5 and T8-6 seeds, respectively.) and those from plants Nos. 1, 8 and 10 of H8 R1 plants (we called them H8-1, H8-8, H8-10 seeds, respectively.) were found to be homozygous for kanamycin resistance. We used these seeds to propagate plants of R2 generation for later studies and called them *pac* 1 plants.

2.2. Southern, Northern and Western blot analysis

Southern blot analysis to detect integration of transgene into genomes was performed according to conventional method.

Poly(A)$^+$ mRNAs were isolated from plants by guanidine-thiocyanate method [11]. Three micrograms of mRNAs of each plant were glyoxalated, electrophoresed on a 1.5% agarose gel, and transferred to

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Hybond-N membranes (Amersham). An RsaI digested 1.0 kbp (nucleotides −128 to 895 [8]) fragment was ^{32}P-labeled using the Megaprime DNA labelling system (Amersham) and used as a probe.

To raise antisera against *pac* 1 gene product, protein that was expressed in *E. coli* was used as an antigen. A fragment of *Acc*I (residue 258 of *pac* 1 coding sequence [8]) to *Pst*I (multicloning site of pBluescript SK+) of pKF1 was blunted, ligated with an *Eco*RI linker, and inserted into *Eco*RI site of pGEX-1λT (Pharmacia) to obtain pGETpac1. *E. coli* NM522 strain was transformed with pGETpac1. The *E. coli* clone was cultured at 25°C until OD_{600} reached to 1.0. Then IPTG was added to 0.1 mM and *E. coli* was further cultured for 16 h. *pac* 1 gene products fused to glutathione *S*-transferase gene were purified with GSH-Sepharose beads (Pharmacia) according to the manufacturer's recommendation. Fifty micrograms of antigen was intradermally and subcutaneously injected into each of three separate mice six times at one-week intervals to raise antisera. Detection of antigen was performed using the antiserum and peroxidase-labeled secondary antigen (Vectastain ABC kit, Vector Laboratories, Inc.).

2.3. Virus assay

Plants were transplanted one plant per pot after one month from germination, grown for another 3–4 weeks and inoculated with viruses. Eight to twelve plants per plant line were used in one experiment; each virus assay was repeated three to four times. Tomato mosaic virus (ToMV) was inoculated onto one freshly-developed leaf per plant at 1 μg/ml in 10 mM sodium phosphate buffer (pH 7.0) with carborundum as an abrasive. Cucumber mosaic virus (CMV) was diluted to 10 μg/ml with 10 mM sodium phosphate buffer (pH 7.0) and inoculated onto two well-developed leaves of separate plants of each *pac* 1 line with carborundum as an abrasive. Potato virus Y (PVY)-T13-infected *Nicotiana sylvestris* tissue was ground in a mortar and pestle and diluted with 10 mM sodium phosphate buffer (pH 7.0), 1 mM β-mercaptoethanol and inoculated onto two well-developed leaves of separate plants of each of the *pac* 1 plants with carborundum as an abrasive. Untransformed Xanthi-nc plants, and sometimes pBI121 transformed plants, served as control plants.

3. Results

3.1. Activity of the pac 1 gene product

The *pac* 1 gene was isolated in *Schizosaccharomyces pombe* as a high-copy suppressor of temperature-sensitive meiosis defective mutant [8]. The gene sequence was determined; search in the data bank revealed the existence of a stretch of sequence well matching with RNAase III gene of *E. coli* [12,13] at the carboxyl half. Since the RNAase III is an RNAase specific to ds-RNA rather than ss-RNA, it was suspected that *pac* 1 gene product also has such an activity. The in vitro experiments using an *E. coli* lysate expressing the *pac* 1 gene product confirmed this prediction [8]. We also showed that ds-RNA carrying ToMV sequence was degraded in vitro by *E. coli* lysate expressing *pac* 1 gene product using Ribonuclease Protection Assay Kit (Ambion Inc., Texas) (Fig. 1). This result prompted us to introduce the gene into plant genome.

Table 1
Relative appearance of necrotic local lesions on untransformed and *pac* 1 Xanthi-nc plants

	Control	T8	H8
1st exp.	211 ± 19	99 ± 16	150 ± 32
2nd exp.	95 ± 42	31 ± 5	21 ± 5
3rd exp.	130 ± 33	70 ± 61	86 ± 34
Sizes of local lesions (mm)	3.96 ± 0.54	3.21 ± 0.81	3.60 ± 0.69

The average numbers of necrotic local lesions which appeared on inoculated leaves three days later are shown with standard deviations. The sizes of necrotic local lesions were observed at the first experiment with a caliper.

Fig. 1. Degradation of ds-RNA of ToMV sequences by *pac* 1 gene product produced in *E. coli*. ^{32}P labeled RNA with antisense sequence of ToMV (nucleotides 565–1449) was synthesized using T3 RNA polymerase following to the supplier's recommendation. The antisense transcript was annealed with genomic ToMV RNA and treated with RNases A and T1 to make probe ds-RNAs following to the procedure of RNase Protection Assay Kit (Ambion Inc.). Probe ds-RNA (lane 1) were mixed with control *E. coli* lysate (lanes 2, 4 and 6) or *pac* 1 expressing *E. coli* (lanes 3, 5 and 7) at the protein concentrations of 20 (lanes 2 and 3), 100 (lanes 4 and 5) or 200 μg/ml (lanes 6, 7) in the buffer of 20 mM Tris-HCl (ph 7.6), 0.1 M KCl, 10 mM $MgCl_2$ and 0.1 mM DTT. Lane 8 shows the pattern of only buffer incubation of probe ds-RNA.

3.2. Establishment of pac 1 plants

We set out to introduce this gene into tobacco plants expecting to obtain plants resistant to multiple RNA viruses as described in section 2.

From about forty kanamycin-resistant tobacco transformants, three plants were initially selected (T8, H8 and H15) by the analysis described below.

Southern blot analysis showed that transformants T8, H8, H15 have *pac* 1 transgene in their genome (not shown). Comparison with copy number controls indicated that these plants are single-copy transformants (not shown).

Northern blot analysis of the mRNA fractions isolated from T8, H8, and H15 plants confirmed the transgenes are transcribed in those plants and that the transcripts are of the expected sizes (Fig. 2a).

pac 1 gene products fused to glutathione *S*-transferase gene were expressed in *E. coli* and purified with GSH-Sepharose beads (Pharmacia). The fusion protein was injected into rabbits and antisera were raised. Western blot analysis of proteins extracted from T8, H8 and H15 plant leaves using this anti-*pac* 1 antibody showed that they are expressing the *pac* 1 gene product of the expected sizes, 41 kDa (Fig. 2b). The level of

pac 1 accumulation was estimated to 0.5–2 ng/10 μg total protein.

Since T8 and H8 plants showed higher resistance against ToMV infection than H15 plants in R0 generation, T8 and H8 plants were chosen and selfed twice to get R2 generations. Seeds from T8-5, T8-6 R1 plants and those from H-1, H-8, H-10 R1 plants were homozygous for kanamycin resistance. Arbitrarily T8-6 and H8-10 seeds were chosen, sowed and grown. Plants from those seeds (designated T8-6 and H8-10 R2 plants, respectively) and used for the assay of resistance against plant virus attack.

We could observe no detrimental effects of *pac* 1 gene expression on plant regeneration, physiology, morphology or fertility from R0 to R2 generations. All those plants looked normal.

3.3. Resistance against tomato mosaic virus

T8-6, H8-10 R2 plants were challenged with 1 μg/ml of the ToMV. ToMV cause necrotic local lesions on tobacco leaves with *N* gene, such as Xanthi-nc tobacco. ToMV formed local lesions on T8-6 and H8-10 plant leaves, but the number of the lesions was about a half to one-fourth of that on control Xanthi-nc tobacco leaves (Table 1). The *pac* 1 plants thus showed resistance to ToMV infection by local-lesion assay. The sizes of local lesion on *pac* 1 plants was slightly smaller than those on control plants. But the difference is small considering the standard deviation; we could not argue that the sizes were smaller on *pac* 1 plants (Table 1). We judged that the transgenic *pac* 1 plants showed resistance against ToMV on the basis of the decrease of the number of local lesions.

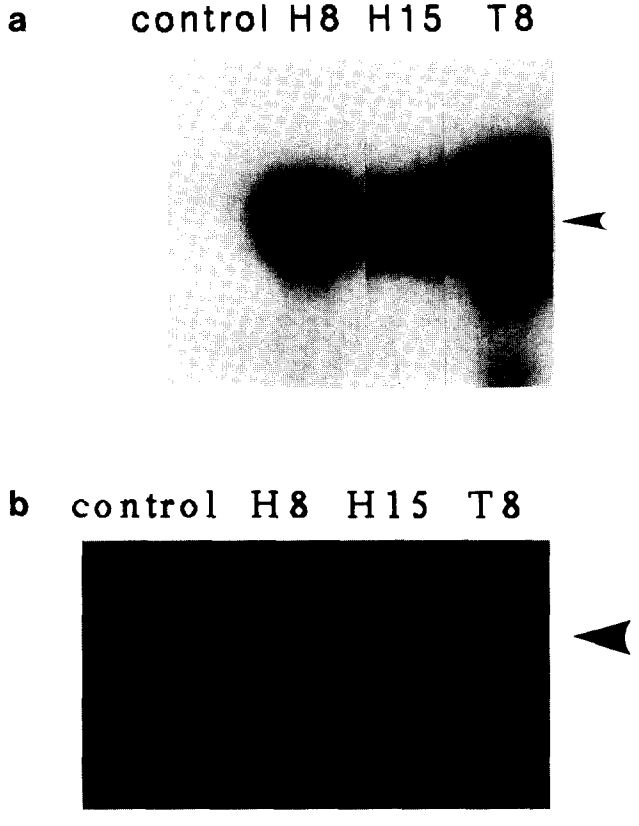

Fig. 2. (a) Northern blot analysis of expression of the *pac* 1 transgene in *pac* 1 gene plants. ^{32}P-labeled *pac* 1 cDNA was used as a probe. RNA ladders (0.24–9.5 kb, Gibco-BRL) were used to estimate the transcript size at about 1.5 kb (an arrowhead). (b) Western blot analysis of expression of the *pac* 1 transgene in *pac* 1 gene plants. An arrowhead indicates the running position of the *pac* 1 gene product (molecular weight is 41 kDa). Detection was made using Vectastain ABC kit (Vector Lab. Inc.).

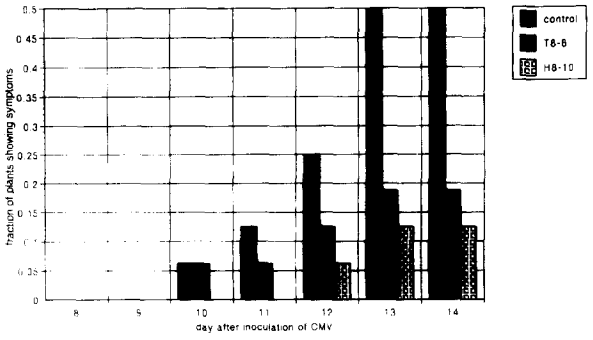

Fig. 3. Resistance against cucumber mosaic virus challenge in *pac* 1 plants. Untransformed Xanthi-nc plants served as control plants. The ratios of infected plants were scored as having positive symptoms when apical leaves demonstrated typical mosaic symptoms.

3.4. Resistance against cucumber mosaic virus and potato virus Y

Next, the *pac* 1 plants were challenged with two other viruses belonging to different taxonomic groups: CMV, which is the type member of the cucumovirus group and PVY, which is the type member of the potyvirus. CMV strain Y [14] and PVY strain T [15] were selected for this work, since they have been well characterized symptomatologically in *N. tabacum* [14,15].

CMV strain Y causes typical systemic symptoms in *N. tabacum* [14]. In one experiment, control plants began to develop characteristic mosaic symptoms by 10 days after inoculation with 10 μg/ml CMV, while most of transgenic *pac* 1 plants showed a delay in symptom development and decreased percentage of infected plants (Fig. 3). The accumulation levels of CMV in the *pac* 1 plants were analyzed by SDS-PAGE and Western blot analysis using anti-CMV coat protein antiserum, and was found to be one-third to one-fifth compared with control Xanthi-nc plants (not shown), which was consistent with the pathological assay. These results confirmed that the *pac* 1 plants also show resistance against CMV. *pac* 1 plants could not show resistance against increased levels of inoculum (50 μg/ml); a delay could hardly be seen in comparison with control plants. The results of other experiments were consistent.

PVY-T was reported to produce necrotic flecks and veinal necrotic symptoms on *N. tabacum* [15]. After challenge with PVY-T, symptoms appeared eight days after inoculation in all control plants, while all *pac* 1 plants showed a delay of about two days in symptom appearance (not shown). The accumulation level of PVY in the *pac* 1 plants was checked by Western-blot analysis using anti-PVY coat protein antiserum. *pac* 1 plants suppressed the level of coat protein accumulation by half to one-fourth compared with those of control Xanthi-nc plants (Fig. 4). The results of other experiments were also consistent.

4. Discussion

At first we checked these plants as to resistance against ToMV, a member of tobamovirus, using R0 generation. The

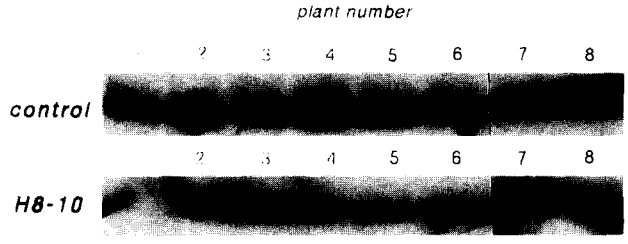

Fig. 4. Western blot analysis of accumulation of potato virus Y after challenge in *pac* 1 plants. Untransformed Xanthi-nc plants served as control plants. The leaf two leaves above the inoculated leaves was collected and ground with a mortar and a pestle. Protein samples (equivalent to about 2 mg tissue) were cast on a SDS-10% polyacrylamide gel, subjected to electrophoresis and blotted to a nitrocellulose membrane.

effect of ds RNase-mediated resistance in transformed plants was a decrease in occurrence of local necrotic lesions; possibly because of partial inhibition of virus replication in each cell. Then we selfed the plants twice to R2 generation and characterized the plants.

We confirmed that these resistance traits were stably inherited to the R2 generation. Resistance against CMV and PVY was also shown. Cucumoviruses are causal agents for virus diseases affecting a variety of important crop species [16,17]. Potyviruses also cause severe diseases in a wide spectrum of crops [17–19]. Introduction of a single gene, *pac* 1 gene, resulted in broad spectrum of resistance against multiple plant viruses. It is conceivable that this approach is effective against other plant viruses also.

H-15 transformant expresses the *pac* 1 gene product at a level comparable to T8 and H8 transformants. But the transformant did not show a significant resistance against virus attack. Thus, at present, the correlation between the expression level of the transgene and its effectiveness against virus attack is still obscure.

We have established other *pac* 1 transgenic plant lines using *N. tabacum* cv. Samsun. They showed a delay in appearance of mosaic symptom after ToMV infection. These *pac* 1 plants also showed resistance against CMV or PVY attack (not shown). We are confident that this approach is applicable to other important crops. Ds-RNases would endow plants with resistance against an even uncharacterized plant RNA viruses. This approach would be effective also to mixed infections with multiple plant viruses which will occur in the field [20,21].

Recently papers appeared which report that recombinants between transgene mRNA and viral genomic RNA/sequences will occur even at low frequencies [22,23]. Thus, there are some arguments that the wide use of transgenic plants in the field might lead to the appearance of recombinant viruses that are more severe and have an altered host range compared with the wild-type viruses [20,22–25]. We propose that other approaches like our approach also should be explored and combined with virus derived resistance strategy to minimize potential risks and maximize potential benefits of genetically engineering virus resistance.

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