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Summary

We introduced human interferon-induced, double-stranded RNA-dependent protein kinase (PKR) cDNA into tobacco plants by means of the *Agrobacterium*-mediated transformation. We were able to obtain one transgenic plant with an integrated PKR gene. Although the R1 progeny of the transgenic plant did not express a detectable level of the transgene, they showed low-level resistance to tobacco mosaic and cucumber mosaic viruses. The mechanism of the multiple virus resistance in the transgenic plants that had human PKR gene remains unclear.

Interferons which are secreted by animal cells after virus infection induce the synthesis of additional antiviral proteins, such as 2′-5′ oligoadenylate synthetase (2-5Aase) and double-stranded RNA-dependent protein kinase (PKR) (11). These mammalian antiviral systems have not been found in plant cells. Therefore, researchers have attempted to develop a mammalian antiviral system for plants. Truve *et al.* found that transgenic potato plants with rat 2-5Aase are protected from potato virus X infection under field conditions (13). We also found that transgenic tobacco plants with human 2-5Aase exhibited multiple virus resistance (2, 9). In animal cells, 2-5Aase is activated by cellular double-stranded RNA, such as replicative intermediates of RNA viruses, and the enzyme polymerizes ATP to a series of 5′-phosphorylated, 2′, 5′-linked oligoadenylates (2-

5A). 2-5A activates a latent endoribonuclease L (RNase L) which degrades viral and cellular single stranded RNAs. Since 2-5A-dependent RNase has not been detected in tobacco cells, the antiviral activity that was observed in the plants with 2-5Aase may be caused by a different mechanism from in animal cells. Mitra *et al.* (6) and Ogawa *et al.* (10) found that the transgenic tobacco plants expressing both 2-5Aase and RNase L showed high-level resistance to multiple viruses.

Although the 2-5A system in transgenic plant cells has been studied, another double-stranded RNA-dependent enzyme has not been studied to date. The interferon-induced enzyme is autophosphorylated upon binding to double-stranded RNA. Autophosphorylation of the PKR leads to phosphorylation of the alfa subunit in eukaryotic initiation factor 2 (eIF2), subsequently resulting in the inhibition of the translation initiation process (11). We generated the transgenic plants that harbored human PKR cDNA, and examined the antiviral state of the transgenic plants in this paper.

Materials and Methods

Construction of the expression vector and the transformation of plants

Human PKR cDNA was kindly provided by Dr. A.G. Hovanessian (5). The PKR-coding region was ligated into plant expression vector pBE2113 (7). The resulting plasmid, pBE2113-PK, was used to produce transgenic tobacco plants by the *Agrobacterium*-mediated transformation, as described previously (8). Mouse 2-5Aase cDNA (3), which was a gift from Dr. Y. Sokawa, was also used to transform as a control.

Polymerase chain reaction of genomic DNA

Integration of the transgene was confirmed by polymerase chain reaction (PCR) of genomic DNA, amplifying the neomycin phosphotransferase (NPT II) gene and the PKR gene. Primary transformants with both NPT II and PKR genes were self-pollinated, and the R1 progenies were selected on kanamycin medium for further tests.

Northern analysis

Total RNA was isolated from the young leaves of the R1 progeny by the acid guanidinium thiocyanate/phenol-chloroform method (1). Twenty μg of total RNA was separated on a denaturating formaldehyde gel and was blotted onto Hybond-N membrane (12). Northern blots were hybridized with the digoxigenin-labeled PKR-coding region according to the supplier (Boehringer).

Virus inoculation onto plants

Kanamycin-resistant R1 seedlings were grown in a growth room at 26°C (day)/22°C (night) under 20,000 lux illumination for a 16 h photo-period, and were inoculated with a 1 : 100 dilution of cucumber mosaic virus (CMV)-infected tobacco sap on the sixth leaves at their full expansion stage. Symptoms were recorded up to 21 days after inoculation. Tobacco mosaic virus (TMV)-resistance tests were performed by inoculating excised the ninth leaves with 20 ng/ml purified TMV. The inoculated leaves were incubated in a growth room at 23°C under 3,000 lux illumination for a 16 h photo-period. After being incubated for 4 days, the number of local necrotic lesions that had formed on the leaves were counted.

Results and Discussion

We obtained thirty-six primary transformants (R0) harboring NPT II genes. The integrated PKR genes were confirmed in only one transformant (plant number: PK1) (data not shown). Moreover, PK1 progeny did not show a detectable level of transgene (Fig. 1). As for 2-5Aase, we obtained twenty-nine R0 plants with both NPT II and 2-5Aase genes were obtained. A Northern analysis of the total RNA that was isolated from plant number ME116 showed that the transgene transcripts were the size that we predicted (Fig. 1).

Twelve PK1 R1 plants were inoculated with CMV, which is the type member of the *Cucumovirus*, and systemic symptom development was observed (Fig. 2). Wild-type plants showed systemic mosaic symptoms within 10 days after inocula-
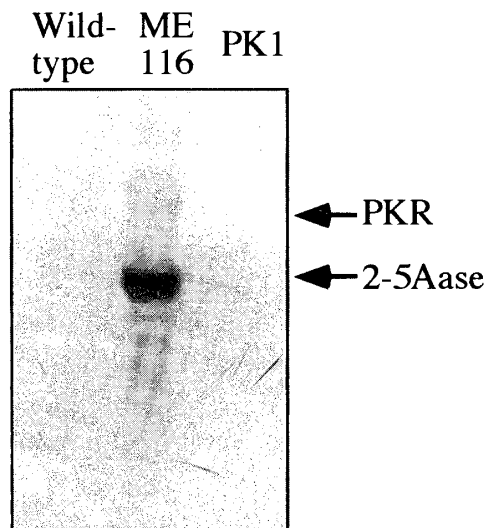

Fig. 1. Northern blot analysis of transgenic tobacco plants.
Digoxigenin-labeled PKR and 2-5Aase cDNAs were combined and used as probes.
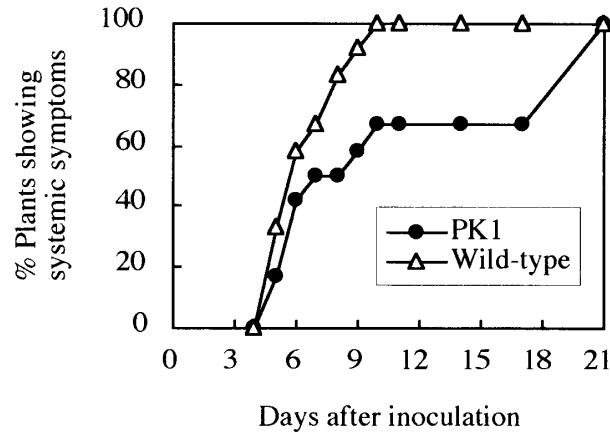Arrows indicate the predicted sizes of transgenes.

FIG. 2.   Systemic symptom development in PK1 tobacco plants after inoculation with CMV.
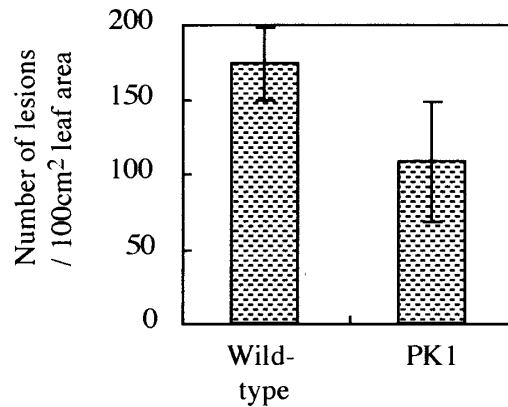

FIG. 3.   Number of necrotic local lesions formed on excised PK1 tobacco leaves inoculated with TMV.

tion, while the delay of the development of symptoms was observed in most of the PK1 plants.   Next, the PK1 plants were inoculated with other virus that belonged to a different taxonomic group, TMV, which is the type member of the *Tobamovirus*.   TMV formed local necrotic lesions on the PK1 plant leaves, but the number was significantly less than on the wild-type plants leaves (Fig. 3). Thus, the PK1 plants showed multiple resistance to CMV and TMV infections.

The only a transgenic plant was obtained by using a pBE2113-PK vector. We are unsure whether the low level of efficiency of the transformation was due to the basal transformation vector or to the PKR gene sequence.   However, the latter is more likely because transgenic plants with NPT II gene were obtained with a high levels of efficiency.

In the PK1 plants, we did not detect transgene transcripts.   This may have been caused by incorrect integration of the T-DNA region, especially the promoter region.   We have also excluded the possibility that the integrated PKR gene was 'co-suppressed' by an endogenous plant PKR gene, because plant PKR (pPKR)

was only recently characterized in barley (4). Although these results do not provide clear evidence that mammalian PKR (mPKR) confers viral resistance on plants, PKR, as well as 2-5Aase genes, may become a genetic source for the development of virus-resistant plants.

Acknowledgements

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