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Production of Transgenic Tomato Plants with Specific TMV Resistance

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Abstract
An F_1 hybrid between *Lycopersicon esculentum and L.* peruvianum was found to be highly suitable for *Agrobacterium-mediated* gene transfer. Transgenic plants were prepared which carried an introduced chimeric TMV coat protein gene cDNA expressing the coat protein under the control of the 35S RNA gene promoter from cauliflower mosaic virus. Ltbl, a specific TMV mutant strain was used to assay the resistance conferred by the introduced coat protein gene, since the transgenic plants as well as the parent F1 plant carried the *Tm-2* gene that expresses resistance to wild type TMV strains but not to Ltbl. Coat protein was found to accumulate in fully developed fresh leaves at a level of about 2.5 µg per g fresh weight in a plant designated as 8804-150 that expressed the strongest resistance among the plants examined. The resistance was confirmed to be transmissible to the next generation. The 8804-150 plant was selected for further studies including field tests on biosafety and performance of the TMV resistance.

Discipline: Biotechnology
Additional key words: *Agrobacterium-mediated* transformation, coat protein gene, *Lycopersicon esculentum, Lycopersicon peruvianum*

Introduction

First infection with a virus generally protects the host plant from second infection with the same or closely related virus strain. More than one factor may be involved in the cross-protection. It was suggested that the viral coat protein newly synthesized after the first infection was a major factor[10]. Powell Abel et al.[8] demonstrated that transgenic tobacco plants carrying an introduced chimeric coat protein gene of TMV showed a delayed expression of mosaic symptoms when they were inoculated with TMV. Since then, a number of reports have presented evidence that transgenic plants to which coat-protein has been introduced became resistant to the same or related viruses[1,2].

Since 1984, we have attempted to transform tomato plants with a coat protein gene of TMV by *Agrobacterium*-mediated transformation. It was however found that the majority of the tomato cultivars we examined showed a low capacity to regenerate plants in tissue culture. We eventually selected an F_1 hybrid between a line of cultivated tomato and a wild relative, *Lycopersicon peruvianum*, since it was observed that leaf explants from the F_1 plant are highly suitable for *Agrobacterium*-mediated transformation and easily regenerate shoots from the transformed tissues.

Transformation of tomato with a TMV coat protein gene

An F_1 plant was grown from an *in vitro* culture of an embryo that was obtained after crossing between LA1000, a line of *Lycopersicon esculentum* (L.) Mill., and P.I. 128650, a line of *Lycopersicon peruvianum* L. Leaf-pieces excised from the F_1 plant were used for *Agrobacterium*-mediated transformation. A chimeric gene was constructed from the

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cDNA of the coat protein gene of the TMV tomato strain $L_{11}A$[6], a cauliflower mosaic virus (CaMV) 35S RNA promoter and a terminator from a Ti plasmid *tml* gene. The chimeric gene was inserted into the cloning site of pTRA410, a binary vector carrying a chimeric kanamycin resistance gene that can be expressed in plants[7]. *Agrobacterium*-mediated transformation was carried out by a method similar to that of McCormick et al.[3]. The leaf-pieces were co-cultivated with the *Agrobacterium* cells carrying the binary vector that harbored the chimeric coat protein gene. After the co-cultivation, the leaf-pieces were transferred to the culture medium containing kanamycin. From kanamycin-resistant calli grown from the leaf-pieces, many transgenic plants were regenerated. Among them, seven plants that were rooted in the medium containing kanamycin were transplanted into soil in pots, and grown in an isolated greenhouse.

The coat protein gene was detected in every DNA sample extracted from these plants by Southern hybridization. One copy of the complete sequence of the coat protein gene was estimated to be integrated in the DNA of each plant.

In order to determine whether the introduced chimeric coat protein gene expressed an inhibitory effect on the growth of TMV, five leaflets were excised from each of the seven plants, and used for the growth test of the virus. Since the original F_1 plants carried *Tm-2*, a gene conferring resistance to the wild type TMV strains, a mutant TMV strain Ltb1 that is able to grow in plants carrying the *Tm-2* gene[4] was used as inoculum for the test. After inoculation of the leaflets with Ltb1, they were incubated on moistened filter papers in petri-dishes for 7 days. After incubation, the leaflets were homogenized with phosphate buffer, and the homogenates were diluted with buffer to appropriate concentrations. Infectivity of the homogenates was assayed using leaves of Xanthi nc tobacco, which induce local lesions after inoculation of TMV. The smaller the number of local lesions induced per tobacco leaf, the stronger the resistance of the tomato leaflets to TMV, because the number of local lesions was related to the inoculum concentration. The strength of the resistance varied from plant to plant (Fig. 1). Leaf-pieces from some plants enabled the virus to grow up to about half of the amount accumulated in a non-transgenic control plant. A plant designated as

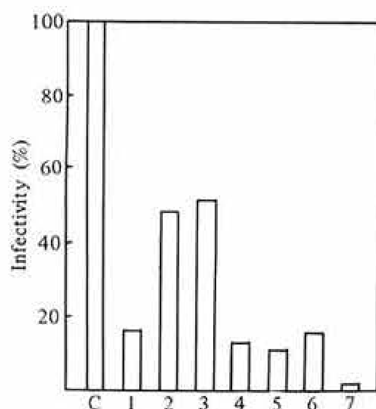

Fig. 1. Virus infectivity in 5 leaflets inoculated with TMV Ltb1 and incubated for 7 days
The infectivity in each sample was assessed by the number of local lesions produced on the Xanthi nc tobacco leaves after inoculation with TMV Ltb1 at a concentration of 10 $\mu g/ml$ in phosphate buffer. Infectivity in each sample is represented as percentage of the relative value to the infectivity in the non-transgenic control. C: Non-transgenic control, 1-7: Transgenic plants.
The 8804-150 plant in the text corresponds to the plant No. 7 in this figure.

8804-150 (No.7 in Fig. 1) showed the strongest expression of resistance. The concentration of the virus grown in the leaf pieces of this plant was about 1/40 of the amount in the control.

The coat protein synthesized was detected by western blotting using the extracts of leaves, roots and petals of the 8805-150 plant. The amount of coat protein accumulated in the fully developed fresh leaves was about 2.5 $\mu g/g$ fresh weight of tissue.

Transmission of the chimeric coat protein gene to the next generation

Like one of the parent species *L. peruvianum*, the F_1 hybrid was self-incompatible as well as cross-incompatible when it was crossed as female with the pollen of cultivated tomato. Only a few mature seeds that could germinate set when the cultivated tomato plants were fertilized with the pollen of the F_1 plant. Only three plants grew from the seeds by crossing of a cultivated variety 'Baby' with the pollen of the F_1 plant.

In order to determine whether the resistance

conferred by the coat protein gene in the 8804-150 plant was transmitted to the next generation, we used one (BC_1-1) of these three plants as female, since it was able to get seeds by cross-pollination with the pollen of the F_1 plant.

Eleven seeds obtained by the crossing were germinated, and the resulting plants were grown in soil in pots. Strength of TMV resistance was examined using Xanthi nc leaves as described before. To examine the kanamycin resistance of the plants, leaf-pieces were excised from each plant, and incubated on a nutrient agar medium containing kanamycin. Generally, if the kanamycin resistance gene is present and expressed in a leaf-piece, calli developed from the leaf-piece are likely to be kanamycin-resistant, and continue to grow in the medium containing kanamycin. As shown in Fig. 2, five plants as well as the non-transgenic control were TMV-susceptible and kanamycin-sensitive like the non-transgenic

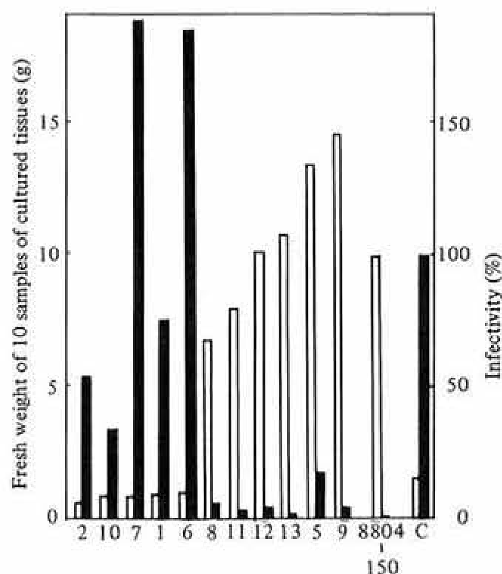

Fig. 2. Kanamycin resistance and virus infectivity in plants grown from seeds obtained by crossing between the BC_1-1 and F_1 plants
The kanamycin resistance is expressed by the fresh weight of 10 samples of leaf tissues cultured for 22 days on the medium containing kanamycin at a concentration of 100 μg/ml. Infectivity in each sample is represented as the percentage of the relative value to the infectivity in the non-transgenic control.
□ : Fresh weight of 10 samples of leaf tissues (g), ■ : Virus infectivity (%).

control, whereas six plants were as resistant to both TMV and kanamycin as the 8805-150 plant. It was thus evident that the TMV resistance conferred by the coat protein gene could be transmitted together with the kanamycin-resistance NPT II gene.

Conclusion

The F_1 plants carried the *Tm-2* gene that confers resistance to wild type TMV strains. The transgenic plants also harbored this resistance gene. It was thus necessary to use a TMV strain such as Ltb1 that was able to overcome the effect of the *Tm-2* gene for assaying the strength of the resistance conferred by the coat protein gene.

The coat protein gene may be suitable for transforming TMV-susceptible tomato varieties to resistant ones, while it may also be useful in tomato varieties carrying other TMV resistance genes such as *Tm-1*, *Tm-2* and *Tm-2*^{2}. It may protect these plants from the multiplication and spreading of mutant strains which had acquired the ability to overcome the resistance by those genes.

The 8804-150 plant has been vegetatively propagated by cutting and used to evaluate the biosafety and performance of the TMV resistance in greenhouses as well as in the field[5,9].

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