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FULL RESISTANCE TO TOBACCO MOSAIC VIRUS INFECTION CONFERRED BY THE TRANSGENIC EXPRESSION OF A RECOMBINANT ANTIBODY IN TOBACCO

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ABSTRACT

The objective of this study was to obtain transgenic tobacco (Nicotiana tabacum L. cv. Samsun NN) plants resistant to tobacco mosaic virus, TMV-vulgare strain, by the expression of a single-chain variable fragment (scFv-anti-TMV) against the TMV coat protein. The scFv-anti-TMV gene was made by combining the coding sequences of the heavy and light chain variable domains of the full antibody molecule with a sequence encoding a flexible linker. The scFv-anti-TMV was cloned under the control of the 35S promoter in the plant expression vector pBI121, and used for Agrobcterium-mediated transformation of tobacco. Integration of the scFv-anti-TMV gene into the genome of the transgenic plants and its expression were confirmed using PCR, immunoblot, and ELISA analysis. Transgenic plants expressing scFv-anti-TMV and untransformed control plants transformed with pBI121 only were inoculated with 1 μg/ml TMV. Constitutive expression of this scFv-anti-TMV resulted in complete immunity against TMV infection in transgenic plants (100% reduction of virus infection). In contrast, control plants were heavily infected by TMV. These results confirmed the applicability of antibody expression as a means of protecting plants from virus infection.

Introduction

Advances in the field of recombinant antibody technology provide an alternative means to engineer low-cost antibodies with desirable affinity and specificity by enabling one to manipulate the basic domain structure of the immunoglobulin molecule. One of the most successful approaches is to display single-chain antibodies on filamentous phage (3, 8).

The single-chain variable fragment scFv is made by linking the heavy- and light-chains of an antibody molecule with a flexible linker peptide. This linker spans the distance between the carboxyl terminus of one variable domain and the amino terminus of the other without distorting the conformation of the antigen binding site. Several linkers have been reported in the past, but the linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ has become the most popular (10).

Several scFv molecules have recently been expressed in plants for inhibition of certain physiological processes (13), the establishment of virus resistance (15, 19) or screening of mycotoxins and other natural toxins in food and agricultural products (18). In addition, because of their small

size, scFv fragments could be useful in tumor imaging and designing therapeutic strategies.

The objective of this study is the generation of tobacco mosaic virus (TMV) resistant tobacco plants via the transfer of a *scFv-anti-TMV* gene into tobacco. Here, we describe the characterization of tobacco plants expressing functional scFv molecules against the coat protein of TMV. Inoculation of transgenic plants with TMV resulted in complete immunity against this virus in transgenic plants.

Materials and Methods

1. Construction of scFv expression vector

TMV was isolated from the infected tobacco plants as described elsewhere (8). Balb/c J mice were immunized with isolated TMV coat protein and total RNA was extracted from immunized mice spleen lymphocytes. Poly(A)$^+$ mRNA was isolated through oligo(dT)-cellulose and cDNA synthesized by AMV reverse transcription according to standard procedures (14). This cDNA was used to amplify the DNA fragments encoding the variable regions of the heavy (V_H) and light (V_L) chains by polymerase chain reaction (PCR). The V_H domain was amplified using forward 5'-AGGTGCAGCTGCAGCAGTCAGG-3' and reverse 5'-GTGACCGTGGTCCCTTGGCCCC-3' primers. The V_L domain was amplified using forward 5'-TATCGAGCTCACCCAGTCTCCA-3' and the reverse 5'-GTTTTATTTCCAACTTTGTCCC-3' primers, respectively. The individually amplified V_H and V_L fragments were purified, and connected with synthetic double stranded linker DNA $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ by PCR (**Fig. 1**) using the forward 5'-CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGCAGTCG -3' and reverse 5'-GAGTCATTCTGCGGCCGCGTTTTATTTCCAACTTTGTCCC-3' primers. Amplified scFv DNA fragments were

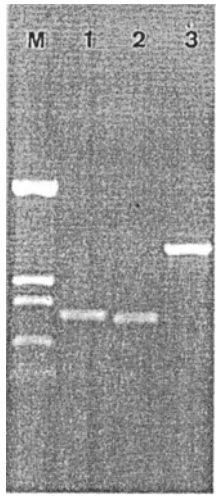

Fig. 1. VH (1) and VL (2) amplification and assembly of TMV scFv construct (3) used in this study. M-pUC19 digested with Hinf I (1417, 517, 396 and 214 bp).

digested with *Sfi* I and *Not* I restriction enzymes, purified through agarose gel electrophoresis and ligated into the phagemid vector, pCANTAB5E (Pharmacia), which had been digested with the same restriction enzymes. Recombinant phagemids were electroporated into TGI competent cells of *E. coli*. Selection of phages with the ability to bind TMV was performed by biopanning. After four rounds of biopanning, randomly selected clones were tested against TMV by phage ELISA. According to the phage ELISA result, one clone with the highest OD 405 was selected and used to construct the plant expression vector.

2. Construction of plant expression vector and transformation of tobacco

ScFv-anti-TMV was amplified from the pCANTAB5E vector by PCR using the forward 5'GATGCCATCTAGAGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGCAAGG-3' and reverse 5'-GAGGGATCCTGCGGCCGCGTTTTATTTCCAACTTTGTCCC-3' primers. Ampli-

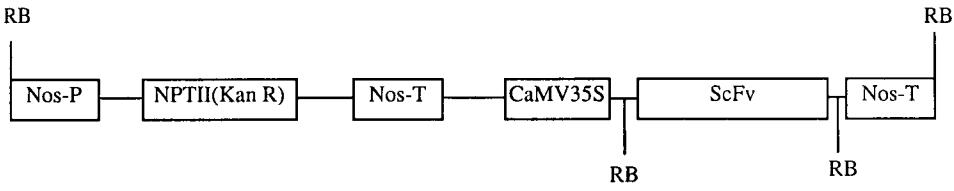

Fig. 2. The T-DNA region of binary vector pBI-121 carryng scFv construct used in the transformation experiments. scFv gene was cloned into *Xba* I (X) and *Bam* HI (B) restriction side of the pBI-121. Nos-P: Nopaline synthase (NOS) promoter, NPT II: nepmycin phosphotransferase II gene, Nos-T: NOS terminator, CaMV 35S: cauliflower mosaic virus (CaMV) 35 promoter, scFv: single chain fragment variable gene, LB and RB: left and right borders.

fied *scFv-anti-TMV* fragments were digested with *Xba* I and *Bam* HI restriction enzymes and integrated into the Xba I and Bam HI restriction sites of the pBI 121 plant expression vector between cauliflower mosaic virus (CaMV) 35S RNA promoter and nopaline synthase (NOS) terminator (**Fig. 2**). The DNA region of pBI121 also contained a gene cassette for kanamycin resistance encoded by the neomycin phosphotransferase II (*npt* II) gene (11). Binary vector carrying the *35S-scFv-anti-TMV* gene was transferred into the *Agrobacterium tumefaciens*, strain LBA 4404, by triparental mating system according to Ditta et al. (5). Transformation of tobacco (*Nicotiana tabacum* L. cv. Samsun NN) was performed by co-cultivation of leaf discs with *Agrobacterium tumefaciens* (9) and the regenerants were selected on MS media (12) containing 50 mg/l kanamycin and 500 mg/l cefotaxime.

3. Molecular analysis of transgenic plants

Genomic DNA from the leaves of primary transformants and control tobacco plants was isolated according to Walbot (17) PCR was performed using universal primers 5'-AGGTGCAGCTGCAGCAGTCAGG-'3 and 5'-GTTTTATTTCCAACTTTGTCCC-'3 which border a 750 bp fragment within *scFv-anti-TMV*. For PCR, samples were heated at 94 °C for 10min, followed by 30 cycles at 94 °C for 1 min, at 55 °C for 2 min, and at 72 °C for 2 min.

For SDS-PAGE, protein extracts from control and transgenic plants were obtained ay homogenisation of leaves in extraction buffer (50 mM Tris-HCl pH 8.O, 50 mM EDTA pH 8.0, 100 mM NaCl 2 mM PMSF, 50 μM leupeptin, 5 mM ascorbic acid). Five μl extraction buffer was used per mg tissue at 4 °C. The total soluble protein concentration was determined according to Bradford (1976) and the protein bands were identified by silver staining (1).

For immunoblot analysis of total proteins from tobacco plants, two identical gels were run in parallel. After separation, the protein bands in one gel were stained with silver nitrate, and those in the other gel were transferred to a nitrocellulose membrane. The transblotted membrane was probed with TMV antigen, then incubated first with anti-TMV from rabbits and anti-rabbit IgG-alkaline phosphatase conjugate. Finally, scFv fragments were detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate p-toluidine as described by Fischer et al. (7).

Soluble proteins extracted from transgenic and control plants were used for sandwich ELISA assay which was performed according to Fischer et al. (7). Dilutions of plant extracts were used for coating of microtiter ELISA plates, then TMV antigen was added. In addition, anti-TMV polyclonal antibody from rabbits and anti-rabbit IgG-alkaline phosphatase were supplied respectively with extensive washing

after each step, then incubated one hour at 37 °C. Extracts of tobacco plants transformed with pBI121 only were used as negative control.

4. Virus infection assays

Tobacco plants used in these experiments were placed in a growth chamber under light-dark cycles of 16 h of light and 8 h of dark with 3000 lux light intensity, 25 °C and 70 % humidity. Four randomly chosen, two month old, primary transformants and four control plants of the same age (transformed with pBI121 only), were infected with 1 μg/ml TMV-vulgare strain as described by Dietzgen (4). TMV was applied on five lower leaves of transgenic and control tobacco plants using carborandum as an abrasive. Necrotic lesions in upper leaves were counted 5 days after inoculation and the total leaf area infected was determined in order to calculate the number of necrotic lesions per cm^2 of the infected leaf.

Results and Discussion

1. Expression of TMV-specific scFv fragments in tobacco

DNA fragments encoding the variable regions of the V_H and V_L chains were amplified by PCR and joined with a linker to form a full scFv encoding DNA fragment (Fig. 1). Amplified scFv fragments were collectively isolated, purified, digested and ligated into pCANTAB5E plasmid. scFv genes were amplified from the pCANTAB5E vector by PCR, and integrated into the cloning site of the pBI121 plant expression vector (Fig. 2). Using *A. tumefaciens* mediated transformation system, binary vector carrying scFv-anti-TMV gene was transferred to tobacco and regenerants were selected on selective MS media. Individually transformed plants were transferred to soil and four randomly chosen transgenic plants were used for further analysis. All the regenerated plants appeared phenotypically normal in comparison with control plants transformed with pBI121 only.

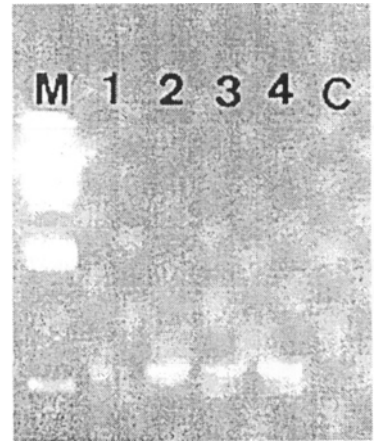

Fig. 3. PRC amplification of *scFv-anti-TMV* gene from the genome of transgenic (1, 2, 3, 4) and control (C) plants. M – Lambda DNA disested with *Hind* III (21.3, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb).

2. Characterization of transgenic plants

Four randomly selected transformants were first analyzed by PCR to confirm their transgenic status. PCR primers designed to amplify the *scFv-anti-TMV* gene were used. These analyses showed the existence of amplification products with sizes equal to the expected size of the *scFv-anti-TMV* transgene in all transformants tested (**Fig. 3**).

SDS-PAGE of total protein extracts from transgenic plants showed the presence of a 29 kDa protein on a silver-stained gel, indicating the expression of *scFv-anti-TMV* in the leaf tissue of transformed tobacco. No such protein band was visible when the total leaf proteins from control plants were examined. Expression of the transgene was further confirmed with immunoblot analysis where scFv bands were detected in total protein extracts from transgenic plants but not in total proteins extracted from control plants (**Fig. 4**).

The presence of scFv antibodies in the plant extracts taken from the transgenic plants was further detected by sandwich ELISA assay. These experiments showed that the scFv fragments were detected in

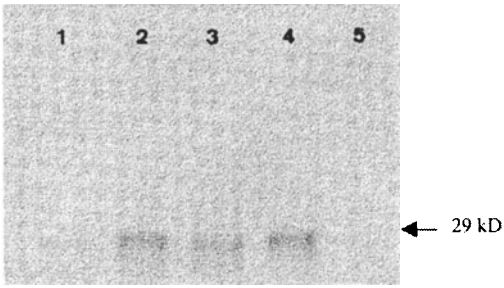

Fig. 4. Immunoblot analysis of scFv expression. Dublicate SDS-polyacrylamide gel developed with polyclonal rabbit anti-TMV and with anti-rabbit IgG-alkaline phosphatase using total protein extracts from trangenic (1, 2, 3,, 4) and control (5) plants.

tested transgenic plants but in control plants no detection of scFv fragments was obtained (data not shown).

3. Reduction of TMV induced necrotic lesions in transgenic plants

To evaluate the effectiveness of scFv-anti-TMV molecules in conferring resistance against TMV infection, inoculation experiments with TMV were carried out on control and transgenic *N. tabacum* cv. Samsun NN plants. This tobacco variety carries the N resistance gene and infection with TMV results in the induction of a hypersensitive response leading to formation of necrotic lesions. In the first experiment, four plants producing scFv antibody and four control plants were infected with 1 µg/ml TMV virus onto lower leaves of each plants. The leaves of control plants become necrotic, leading to formation of clearly visible lesions within five days after inoculation, but the leaves of transgenic plants were completely free from any lesion (**Fig. 5**). At five days after inoculation the mean number of lesions in control plants was 56 per leaf, while in transgenic plants there were no lesions. The number of necrotic lesions per cm^2 of leaf in control plants was 3.66 and these plants died within one week of inoculation. Overall, these results demonstrated that the transgenic plants were fully resistant against TMV infection. In the second experiment, seeds from transgenic plants were regenerated and T1 plants were inoculated with TMV. Transgenic T1 plants expressing scFv-anti-TMV antibodies showed 100 % protection from virus infection. In controls TMV symptoms were significantly appeared. At five days after inoculation the mean number of lesions in control T1 plants was 61 per leaf, while in transgenic plants there were no lesions.

This study describes the successful use of the TMV specific scFv gene in transgenic tobacco to prevent the infection by TMV. Previous studies have shown that production of the TMV-specific full-size rAb24 antibody led to the reduction of virus infectivity of up to 70 % in transgenic tobacco plants (16). Our results confirmed the results from previous studies on the use of antibody expression to generate virus resistant transgenic plants, however, the level of resistance in our transgenic plants was higher than those previously reported. This could be due to cytosolic expression of scFv, which resulted in a high level of resistance to TMV achieved in our transgenic plants. Similarly, Zimmermann et al. (19) found that cytosolic expression of scFv24 resulted in a significantly higher level of resistance to TMV, despite the fact that scFv levels were more than 700-fold lower in cytosol than in the apoplast. These researches recently obtained 90 % reduction

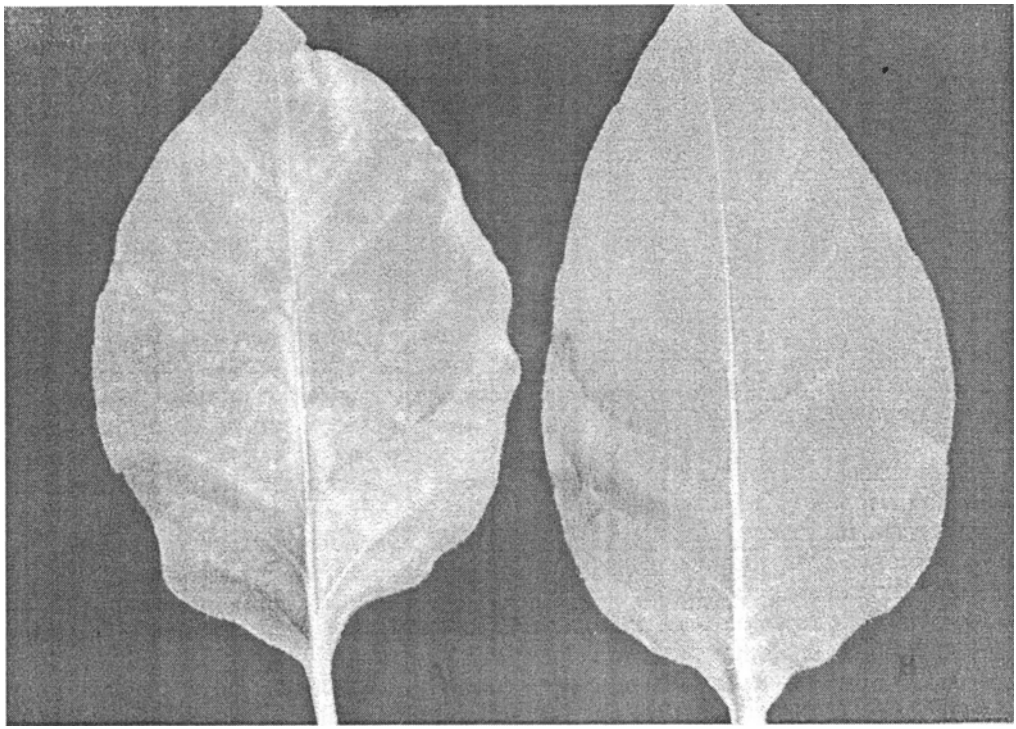

Fig. 5. Effect of TMV infection on *N. Tabacum* plants at five days after inoculation. A representative leaf from a control plant (A) and a representative leaf from a transgenic plant expressing the *scFv-anti-TMV* gene (B).

of necrotic lesions with T2 progeny by increasing the level of rAb24 expression up to 1.1 % of total soluble protein.

The construct we used to express *scFv-anti-TMV* did not contain any signal peptide. However, this did not seem to affect achievement of an expression level which is sufficient for conferring resistance. Similarly, constitutive expression of a scFv without any signal peptide successfully reduced the infection and symptom development of icosahedral tombusvirus artichoke mottled crinkle virus (AMCV) on tobacco (15). However, other studies have indicated the importance of signal peptides for high level of scFv expression in plant cells (6).

In conclusion, we have demonstrated that the accumulation of virus particles is inhibited by expression of *scFv-anti-TMV* in tobacco possibly by a mechanism which neutralizes infecting virions. As a result a high level of resistance against TMV in transgenic tobacco plants was observed. Tavladoraki et al. (15) and Zimmermann et al. (19) suggested that low levels of intracellularly expressed scFv were sufficient to neutralize invading virions either by interfering with viral uncoating or assembly of progeny virions.

Expression of scFvs specific to other important components of viruses (movement protein or replicases) in plant cells could not only be exploited to engineer new properties in transgenic crops, but also for elucidating structure-function relationships during viral pathogenesis.

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